The Biochemistry of Metabolic Bone Disease: Investigation and Experimental Treatment

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Institute of Orthopaedics

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Abstract

Far from being an inert support for the soft tissues, bone is one of the principal organs of calcium homeostasis, undergoing constant turnover regulated by a number of systemic hormones and paracrine factors. Any disruption to the rate or turnover or the degree to which bone formation and resorption are linked, may lead to a ‘metabolic bone disease’. This thesis examines the use of biochemical markers in the diagnosis and assessment of treatment of some of these disorders.

From basic work on the adjustment of serum total calcium to account for protein binding, the newer biomarkers of bone turnover are considered. The use of acidified urine in a collagen crosslink immunoassay is validated, as is a new immunoextraction assay for 1,25-dihydroxyvitamin D. Two osteocalcin immunoassays, directed towards different sites of the molecule, are used to indicate circulating C-terminal osteocalcin fragments. The use of serum tartrate-resistant acid phosphatase as a marker of bone resorption is shown to be promising, but limited by current assay technology.

A number of the markers were used clinically; to monitor the response to different therapies in fibrous dysplasia, and to compare privational and tumour induced osteomalacia. An atypical case of humoral hypercalcaemia of malignancy with elevated osteoblastic activity is also described. In a larger population, urinary excretion of free deoxypyridinoline and the N-telopeptide of type I collagen were used to investigate the pathogenesis of bone loss in women approaching the menopause. Bone loss during this period, apparently clinically significant in some individuals, is better indicated by the collagen telopeptide marker. The possible use of markers to target therapy in osteoporosis is examined. Serum bone-specific alkaline phosphatase was found to be the best pre-treatment indicator of response to bisphophonate therapy.

The use of bone biomarkers is discussed in the context of these experimental studies. Due to problems inherent with urinary markers, the need for a simple serum marker of bone resorption is highlighted. However it is pointed out that although several serum formation markers exist, their application lacks subtlety due to incomplete understanding of the different aspects of osteoblast function they represent.
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"The desk of a good executive should be clear; that of an investigator should be littered"

"What I have told you is subject to change without notice"

FULLER ALBRIGHT
(1900-1969)
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<tr>
<td>AHSG</td>
<td>alpha-2-HS-glycoprotein</td>
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<tr>
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<td>alkaline phosphatase</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>APD (pamidronate)</td>
<td>3-amino-1-hydroxypropyldenediphosphonate</td>
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<td>ECF</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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ELISA  enzyme-linked immunosorbant assay

Free PYD/Cr  total free pyridinoline/creatinine ratio

Gal H  galactosyl hydroxylsine
GF  glomerular filtrate
GFR  glomerular filtration rate
GGal H  glucosyl-galactosyl hydroxylsine
Gla  γ-carboxy glutamic acid

HHM  humoral hypercalcaemia of malignancy
HLA  human leukocyte antigen
HPLC  high performance liquid chromatography
HRT  hormone replacement therapy

ICTP  serum type I collagen carboxy terminal telopeptide
IGF  insulin-like growth factor
IL  interleukin
IPA  isopropyl alcohol
IRMA  immunoradiometric assay
ISE  ion-selective electrode
IU  International units
I-V  intra-venous

kD  kilo Dalton

MGP  matrix Gla protein
MRI  magnetic resonance imaging
mRNA  messenger ribonucleic acid

n  sample number
α-NP  α-naphthyl phosphate
4-NP  4-nitrophenol phosphate
NAF  nuclear accessory factor
NcAMP  nephrogenous cyclic AMP
N-terminal  amino terminal
NTx  pyridinoline cross-linked N-telopeptides

OC  osteocalcin
OHP  hydroxyproline
p  ‘p’ value for statistical significance

P  phosphate

P-C-P  phosphorus-carbon-phosphorus, the common ‘backbone’ of the bisphosphonate class of drugs

PDGF  platelet-derived growth factor

PICP  procollagen Type I carboxy terminal propeptide

PINP  procollagen Type I amino terminal propeptide

PTH  parathyroid hormone

PTHrP  parathyroid hormone-related protein

PYD  sum of both forms of Pyr and DPyr

r  regression coefficient

RIA  radioimmunoassay

RNOH(T)  Royal National Orthopaedic Hospital Trust, Stanmore, Middlesex. The site at which the clinical work towards this thesis was performed.

RRA  radioreceptor assay

SD  standard deviation

SEM  standard error of mean

t  test statistic for the Students t-test

TGF-β  transforming growth factor-β

TIO  tumour induced osteomalacia

TRAP  tartrate-resistant acid phosphatase

UV-B  solar radiation of wavelength between 290 & 315 nm

VDR  vitamin D receptor protein

VDRE  vitamin D response element

WHO  World Health Organisation

w/v  weight per volume

1,25(OH)₂D  1,25 dihydroxyvitamin D; calcitriol

25-OHD  25-hydroxyvitamin D

Système International (SI) units are used throughout unless otherwise stated.
CHAPTER I - Introduction

This thesis deals with the use of biochemical laboratory tests as an aid to the diagnosis, assessment and response to treatment of metabolic bone disease.

The physiological basis for the use of each marker in the context of normal bone metabolism will be described. Detailed discussion of the actions and interactions of the autocrine and paracrine factors involved in both normal and abnormal bone metabolism lies beyond the scope of this thesis.

Indicators of the calcium homeostatic system and more specific markers of bone turnover will then be discussed. These will include:

- Classical indicators of calcium and phosphorus metabolism.
- Hormonal indicators of the calcium homeostatic system (metabolites of vitamin D and parathyroid hormone).
- Biochemical markers of bone formation
- Biochemical markers of bone resorption
- Classical calcium and phosphorus balance studies.

The expansion of this field of study will be outlined. The changing role of such biochemical tests within the range of diagnostic facilities available to the clinician will be described.

Investigative work will assess a selection of these tests and their clinical application in a number of metabolic bone diseases.

It is stressed that this thesis is presented during a period of particularly rapid development in this field. Work in this thesis is not associated with the early development of these methods.

The Nature Of Metabolic Bone Disease.

Bone is a composite of both organic and inorganic components. A primarily collagenous inorganic matrix (the osteoid), supports an inorganic mineral complex (hydroxyapatite) formed mainly of calcium phosphate. The dominant constituent of the skeletal system, bone is a specialised connective tissue serving three major functions.

1) Mechanical
The bones of the skeletal system confer form by providing a physical framework from which the soft tissues of the organism derive support. Bones also facilitate locomotion by providing sites for muscle attachment. When these muscles contract, the bones act as levers to produce movement.

ii) Protective

Bones may be organised to provide a hard shield to protect the organs from external physical trauma (e.g. the skull and the rib-cage respectively shield the brain and organs of the chest). Similarly, the cavities of long bones (e.g. the femur) provide a safe site for the processes of blood cell production (haematopoiesis).

iii) Metabolic

Bone acts as a mineral reservoir, especially with regard to calcium and phosphorus. While the mechanical and protective roles of bone may be consistent with those of an organ, its final role is more akin to that of a metabolically active tissue, undergoing continuous turnover through resorption and consequent reformation. These processes are the result of the action of bone cells whose activity may be modified by many factors, both endogenous and exogenous. The problems which occur when such mechanisms malfunction or become unregulated form the broad spectrum of what is considered to be 'metabolic bone disease'.

The Physiological Basis Of Biochemical Markers Of Bone Turnover

Anatomy And Ultrastructure Of Bone

Bone is a highly specialised connective tissue, the fundamental constituents of which are its various cell types and the extracellular matrix upon which they are based. Unlike other connective tissues, bone has the ability to become calcified. A number of sequential steps occur during bone formation. Type I collagen is synthesised and processed within the osteoblast before secretion and further extracellular processing. Microfibrils, fibrils and fibres of collagen are then formed before extracellular maturation and mineralisation with a poorly crystalline relatively calcium deficient analogue of hydroxyapatite \( [\text{Ca}_{10}(\text{PO}_4)_{6} (\text{OH})_2] \). The final product, fully calcified bone consists of 35% matrix and 65% mineral by weight (Puzas, 1993).

Macroscopically, bones vary widely in shape, but may be roughly divided into flat bones and tubular (weight bearing) bones. The microscopic anatomy of bone may take one of two forms. Cortical bone, composed of lamellae concentrically arranged around a small central canal (an arrangement known as a "Haversian system"), constitutes over 80% of total bone mass and tends to be found towards the external part of the bone (Woolf &
Dixon, 1988). Trabecular bone (also called cancellous or sponggy bone), is the major bone form of vertebrae and the epiphyses of long bones and is also present at the iliac crest. Trabecular bone comprises a rigid three-dimensional lattice of bone plates and columns (Dequeker, 1994). Within this structure both complete struts (trabeculae) and incomplete ‘spicules’ occur.

While 80-90% of the volume of cortical bone is calcified, this figure is 15-25% in trabecular bone (Baron, 1990). Such data has led to the long standing assumption that ‘cortical bone fulfils mainly (but not exclusively) a mechanical and protective function, and the trabecular bone a metabolic function (Baron, 1990). However osteocalcin, a marker of osteoblast function, is found in cortical bone at concentrations approximately thirty times higher than in trabecular bone (Ninomiya et al., 1990). The reason for this discrepancy remains unclear.

The Extracellular Matrix

The extracellular matrix (ECM) serves as a scaffold on which tissues and organs are built. In osseous tissues the ECM acts as a support for the cells which are responsible for bone synthesis and modification. Bone is a composite of bone mineral (hydroxyapatite) deposited onto an ECM consisting primarily of a lattice of fibrillar collagen. Non-collagenous proteins of the ECM include a highly anionic ‘ground substance’, consisting mainly of glycoproteins and proteoglycans, thought to have an important role in the fixation of bone mineral to collagen fibres. While most connective tissues contain a mixture of collagen types, bone contains almost exclusively type I (90-95% of total bone matrix protein), with some type V (Broek et al., 1985; Bronkers et al., 1986). Of the 10-15% non-collagenous bone protein, around a quarter is exogenously derived. The majority of this is serum derived protein which has bound to bone mineral (Termine, 1988).

Collagen Biosynthesis

Type I collagen molecules consist of two α1(I) chains (genetic loci chromosome 13), and a structurally similar but genetically distinct α2(I) chain (chromosome 7) (Myers & Emanuel, 1987). The polypeptide chains, each a left-handed helix, intertwine to form a right-handed triple helical supercoil. Individual collagen molecules are then packaged together laterally, aggregating as larger collagen fibrils (see Fig 1.1). These constitute all the fibrous structure found in the bone extracellular space. Mineral deposition is then initialised in the gaps in the collagen fibrils (Glimcher & Krane, 1968). The formation of intra- and inter-molecular crosslinks between the fibrils aid stability and confer the high tensile strength of bone (Eyre et al., 1984).
Synthesis of type I collagen follows an extensive post-translational processing sequence. Within the osteoblastic rough endoplasmic reticulum, short N-terminal signal peptides are cleaved from the 160 kD precursor procollagen molecule (Blobel, 1977). Lysine and proline residues of procollagen then undergo enzymatic hydroxylation followed by glycosylation and galactosylation of a proportion of the resultant hydroxylysine. Such modifications occur on the chains in the single helical form (Prockop, 1979). Assembly of the triple helix (stabilised by disulphide bridges) then occurs, with resultant heterotrimeric procollagen molecules packed into the Golgi apparatus. Once triple helical procollagen molecules are exocytosed into the extracellular space (via a secretary vesicle), proteolytic processing of the procollagen occurs. Both the 25kD amino- and 35kD carboxy-terminal propeptides are cleaved by specific peptidases (Prockop et al., 1976). The non triple-helical segments that remain attached to the triple-helix of the intact collagen molecule (the telopeptides), play an important role as sites of cross link formation. Removal of the procollagen propeptide extensions appears to assist in fibril formation (Fisher et al., 1987). While a proportion may become entrapped in the bone matrix, many of these propeptide fragments escape into the serum (Krane et al., 1970).

Bone Cells

The Osteoblast

The osteoblast is the bone forming cell which produces 'authentic bone' of hydroxyapatite mineralised type I collagen. The processes of synthesis, deposition and mineralisation of the organic matrix are all undertaken by the heterogeneous family of cells known as the osteoblasts. Cells of the osteoblast lineage have common stromal cell precursors with adipocytes, reticular cells, fibroblasts and chondrocytes (Owen, 1985). The various factors involved in precursor commitment to and differentiation within the
osteoblast lineage have not been fully identified, although a number of growth regulatory factors have been implicated (Heine et al., 1987; Noda & Rodan, 1987; Tsukamoto et al., 1991). Three species of the osteoblast cell lineage are recognised, each expressing a different set of genes as a necessary prerequisite for the production of the next.

**The Pre-Osteoblast**

This committed progenitor cell occurs in a transitional state between the stromal precursor cells and true osteoblasts. Type I collagen, the earliest osteoblastic protein product, is formed during the proliferation of this cell type (Kuhn, 1987).

**The Osteoblast**

The maturation of a pre-osteoblast is a gradual process completed on the emergence of the cell at the bone surface. The synthesis of collagen continues, the mature osteoblasts being responsible for the secretion (and subsequent mineralisation) of the bone matrix. The most prominent of the enzymes associated with osteoblast differentiation is alkaline phosphatase which is maximally expressed during matrix maturation by mature osteoblasts (Rodan & Rodan, 1984). Once matrix maturation has occurred (the mechanism remains unknown, although collagen crosslinking and the removal of mineralisation inhibitors have been postulated), alkaline phosphatase levels decline as mineralisation commences (Stein et al., 1990).

Active mature osteoblasts are usually found within the matrix they have synthesised. Hydroxyapatite crystals form on the mature collagen between 5 and 50 nm from the osteoblast surface. The advancing edge of this calcification is known as the mineralisation front. During this process levels of the osteoblastic calcium-binding proteins osteocalcin and osteopontin are expressed although their roles in the mineralisation process remain unclear.

**The Osteocyte**

An osteoblast which has successfully synthesised calcified bone may become embedded within the mineralised matrix. These cells, the osteocytes, represent the fate of 10-20% of mature osteoblasts (Puzas, 1993). While osteocytes are known to produce transforming growth factor-β (TGF-β) and possibly other growth factors, the physiological significance of such production remains unclear. Osteocytes communicate with each other and bone surface cells via the remains of osteoblast dendritic processes. These canaliculi carry a bone specific fluid, separated from the extra-cellular fluid (ECF) by a 'membrane' of bone lining cells. This fluid-bone interface has been postulated to act as a buffer system for short-term variations in ECF calcium concentrations (Parfitt, 1987). However the overall function of the osteocyte remains unknown.
It is estimated that from the appearance of a pre-osteoblast population, mature osteoblasts are differentiated within a few days, and are active for up to twelve weeks before progressing into osteocytes (Kimmel & Jee, 1980; Tran Van et al., 1982).

**The Osteoclast**

Dissolution of bone mineral and the subsequent enzymatic breakdown of the organic matrix, whether during modelling (growth) or remodelling (continuous turnover of bone) is a consequence of the action of osteoclasts. The contribution of other bone cell types to bone resorption *in vivo* is insignificant. Osteoclasts are unique highly specialised multinucleated cells formed by mononuclear cell fusion rather than cell division. The invaginations and folds of the osteoclastic membrane, (an area known as the ruffled border), allow intimate contact with the bone at sites of resorption. Osteoclasts do not usually occur on normal quiescent bone surfaces.

Osteoclastic bone resorption is facilitated by the production of lysosomal enzymes. These may have a direct proteolytic action on release (e.g. cathepsin C, beta-glycerophosphatase, β-glucuronidase, etc.), or serve to generate hydrogen ions within the osteoclast (e.g. carbonic anhydrase type II)(Hakeda & Kumegawa, 1991). The H+ ions passing through the ruffled border via proton pumps cause a decreased pH within the bone resorption site (Blair et al., 1989). The increasing acidity and elevated concentrations of hydrolytic enzymes within the local environment serve to both dissolve the bone mineral and degrade the organic matrix (Baron et al., 1985; Fallon, 1984). The fact that individuals with deficient carbonic anhydrase type II gene expression have impaired bone resorption and subsequent osteopetrosis supports this theory (Sly et al., 1985). Similarly omeprazole, a proton pump inhibitor used in the treatment of gastric ulcer, has a side-effect of suppressing bone resorption, presumably via interference with the acidification process at the resorption lacunae (Mizunashi et al., 1993).

Osteoclast stimulating agents include parathyroid hormone (PTH), 1,25-dihydroxyvitamin D, interleukin-1, and β2-microglobulin. Calcitonin, γ-interferon and TGF-β (produced by osteocytes), are among the factors which inhibit osteoclastic activity. Modulation of osteoclast activity may directly affect the formation of the osteoclasts from their precursor cells or the production of osteoclastic activating factors by osteoblasts (Chambers et al., 1985). Indeed osteoclastic activation is the initial step in the bone remodelling sequence, although the mechanism by which this occurs is not well understood (Mundy, 1995).

**Normal Bone Remodelling**

Bone is a dynamic tissue undergoing constant remodelling, being continuously broken down and reformed by osteoclasts and osteoblasts. Occurring in discrete sites
throughout the skeleton, the entire remodelling cycle takes between three to four months. Understanding the regulation of the cellular functions of bone remodelling, by both systemic hormones and local factors, provides the necessary insight required for the examination of the pathophysiology of various metabolic bone diseases.

**The Sequence of Bone Remodelling**

Bone turnover is not a random mechanism. In the normal adult skeleton, bone formation follows only where bone resorption has preceded. While the exact mechanism coupling bone resorption and formation has not been elucidated, a number of locally produced cytokines (growth factors) are implicated in this process.

The sequence of events constituting bone turnover was first described by Frost (Frost, 1963; Frost, 1969). In the hypothesis resulting from these observations, remodelling is subdivided into a sequence of events at a small anatomic unit within the bone, the ‘basic multicellular unit’ (BMU) (Frost, 1973). Remodelling at each BMU follows a similar pattern; quiescent bone is activated, resorbed and then replaced by new mineralised osteoid (Figure 1.2). This process is initiated by a sequence of events leading to increased osteoclast activation. The trigger for this activation is unclear, but may involve the recognition by osteoclasts of micro-damage at the mineralised bone surface (Mundy, 1995). Osteoclast activity leads to the attraction and proliferation of osteoblast precursors and the formation of mature osteoblasts. These cells repair the resorption cavity caused by the osteoclast action. As with osteoclast activation, the mechanisms responsible for stimulating osteoblasts to lay down new bone are complex and unclear. The resorptive phase of bone turnover lasts for approximately ten days, with subsequent repair taking approximately three months. While all diseases of bone are superimposed upon this normal remodelling system, it should be noted that even in ‘normal’ adults a progressive loss of bone begins at about 35 years of age. This loss is the result of an imbalance between bone resorption and bone formation due the inability of the osteoblast to fully repair the osteoclastic resorption defect.
The remodelling of each BMU is both geographically and chronologically separate from others, emphasising local control of at least an element of the remodelling sequence. Local factors, synthesised by skeletal cells and related tissues (cartilage and blood), include the insulin-like growth factors (IGF-I and IGF-II), transforming growth factor-β (TGF-β), microglobulin, and the interleukins (Mundy, 1995). Such factors are thought to play a critical role in the coupling of bone resorption to bone formation.

The release during bone resorption of factors such as IGF-I, IGF-II and TGF-β result in osteoblast recruitment and proliferation. Indeed TGF-β, released from resorbing bone cultures, is chemotactic for bone cells (Pfeilschifter & Mundy, 1987). Other factors postulated to be involved in bone turnover include platelet-derived growth factors (PDGF), fragments of matrix proteins released on resorption, and the bone morphogenic proteins. Such regulatory factors, possibly acting in concert at different stages of osteoblastic bone formation, are primarily responsible for the coupling of bone turnover (Howard et al., 1981). The disturbance of some aspect of this coupling mechanism may lead to the osteoblastic defect found in both age-related bone loss and post-menopausal osteoporosis. Consequently a great deal of research has concentrated on identifying the
exact mechanisms by which coupling is achieved. While acknowledging the crucial future role such factors may play in the understanding, diagnosis and treatment of metabolic bone disease, these remain for the most part beyond the scope of this thesis.

**The Hormonal Regulation of Bone Remodelling.**

Systemic hormones which regulate bone cells may do so either directly or indirectly, effecting the synthesis or modulating the effects of the local factors which regulate bone remodelling. The circulating hormones acting on skeletal metabolism may be divided into two types; those under negative feedback control regulated by the serum calcium concentration (parathyroid hormones, 1,25-dihydroxyvitamin D and calcitonin), and those not under the influence of extracellular fluid calcium concentrations. This latter group includes the oestrogens and androgens, glucocorticoids, thyroid hormones, growth hormone and circulating growth regulatory factors such as IGF-I.

A brief summary of the actions of some of these hormones is given below.

**Parathyroid Hormone (PTH)**

PTH stimulates bone resorption both *in vivo* (Holtrop & King, 1977; Holtrop *et al.* 1979) and *in vitro* (Howard *et al.*, 1981; Gaillard, 1955; Gaillard *et al.*, 1979). However osteoclasts do not possess PTH receptors, and isolated osteoclasts in cell culture show no response to PTH (Chambers *et al.*, 1984; Pliam *et al.*, 1982). It is probable that the resorptive effect of PTH is mediated by osteoblasts, which are rich in PTH receptors (Silve *et al.*, 1982; Rizzoli *et al.*, 1983). *In vitro* studies show continuous PTH inhibits collagen synthesis while intermittent treatment stimulates bone formation (Canalis *et al.*, 1989). This effect has been reproduced *in vivo* in human osteoporotic subjects (Bradbeer *et al.*, 1992). The anabolic effect is apparently mediated by IGF-I, the production of which is stimulated by PTH-dependent cyclic AMP (McCarthy *et al.*, 1990). As bone formation and resorption are normally tightly coupled, a dual role for PTH via differing effects on local mediators may not be remarkable.

**Vitamin D**

1,25-dihydroxyvitamin D [1,25(OH)₂D], the active hormonal form of vitamin D, has similar actions on bone to PTH. While stimulating bone resorption, 1,25(OH)₂D has complex affects on bone formation, appearing to be necessary for normal mineralisation. However this hormone is not directly stimulatory towards bone formation (DeLuca, 1980). The production of the osteoblastic non-collagenous matrix protein osteocalcin is directly stimulated by 1,25(OH)₂D. The exact function of this protein is unknown, although it is thought to play a role in bone formation and osteoclast recruitment (Cole & Hanley, 1991).
**Calcitonin**

This 32 amino acid polypeptide has no known effect on bone formation but inhibits osteoclastic activity. However modification of bone resorption has usually been associated with pharmacological doses of calcitonin in the treatment of diseases of increased resorption (Kanis et al., 1974; Overgaard et al., 1994). Response to such treatment often proves temporary, with patients becoming resistant to calcitonin. A possible physiological role for this hormone remains more controversial.

**Sex Steroids**

Skeletal maturation and the prevention of bone loss in the mature skeleton are dependent on both oestrogens and androgens. While exogenous oestrogens have long been known to inhibit bone resorption in vivo (Henneman & Wallach, 1957), the concentrations of oestrogen receptors expressed on bone cells are low (Erikson et al., 1988; Komm et al., 1988; Oursler et al., 1991). The hypothesis that oestrogenic effects on bone cells are indirect, possibly via the inhibition of local promoters of bone resorption such as interleukin-6 (IL-6) (Jilka et al., 1992), is supported by the paucity of the effect of the hormone on bone cell cultures (Canalis & Raisz, 1978).

**Insulin**

Insulin has no effect on bone resorption, but stimulates bone matrix formation and is necessary for mineralisation. Some of its effects are direct, others possibly mediated by IGF-I (Canalis, 1993).

**Growth Hormone**

This pituitary polypeptide has been postulated to regulate bone formation via IGF production and is required for the maintenance of skeletal mass (Raisz & Rodan, 1990).

**Glucocorticoids**

This group of hormones have inhibitory effects on bone resorption (Raisz et al., 1972) but more complex anabolic actions. While long-term treatment with physiological concentrations of glucocorticoids inhibits bone collagen synthesis, short-term exposure promotes bone formation (Canalis, 1983).

**Thyroid Hormones**

Thyroid hormones stimulate bone resorption, a fact illustrated by those hyperthyroid patients who become hypercalcaemic as a result of increased resorptive activity (Mundy et al., 1976). Thyroid hormones appear to have little effect on bone matrix formation or osteoblast function.
Clearly both systemic hormones and local growth factors play a role in the regulation of normal bone remodelling. It is conceivable that while circulating hormones provide a non-specific environment to maintain normal bone tissue function, local factors provide the precise regulation of tissue growth.

**Calcium and Calcium Homeostasis**

The metallic element calcium is the most abundant mineral in the body, comprising about 1kg (25 Mol) of a 70kg individual. Ninety-nine percent of calcium is stored in bone, where it is bound to phosphate in the form of hydroxyapatite and other salts (Krane et al., 1970). These salts confer structural integrity to the framework provided by the collagenous matrix. Extra-osseous calcium, occurring in soft tissues and the extracellular fluid (ECF), accounts for only 1% of total body calcium.

Intracellular cytoplasmic fluids have lower calcium concentrations than the surrounding ECF. In both extracellular fluids and the cytosol, active (ionised) calcium concentrations are critically important in the mediation of a wide variety of cellular functions and metabolic actions. These include hormone secretion, neurotransmitter release, blood coagulation and cardiac muscle function. The tight control of extracellular calcium concentrations through the control of plasma ionised calcium (Ca^{2+}) is therefore of paramount importance to many physiological processes.

Calcium salts in the bone are metabolically active, acting as a reservoir to aid stabilisation of ionised calcium within the ECF. This is facilitated by a series of hormonal and non-hormonal mechanisms which control calcium fluxes between the ECF and the three major organs of calcium homeostasis (Fig. 1.3).

**The Organs of Calcium Homeostasis**

**The Bone**

Around 5% of total cardiac output reaches the bone (Wootton & Dore 1976), where 50% of the available plasma calcium is extracted (Shim et al., 1967; Davies et al., 1976). In all about 1% of the total skeletal calcium is freely exchangeable with the ECF (Broadus, 1993). This occurs between the ECF and a bone fluid analogous to the cerebrospinal fluid. These are separated by a 'membrane' of bone lining cells. This equilibrium, unrelated to bone formation or resorption, occurs at all bone-ECF interfaces (Levinskas & Newman, 1955; Norimatsu et al., 1979; Parfitt et al., 1989).

Calcium may be mobilised from the bone in response to a fall in plasma ionised calcium. Two hormonal factors, PTH and 1,25(OH)2D, act in concert to promote osteoclast-mediated bone resorption, the resulting calcium release replenishing the plasma pool (Garabedian et al., 1974). Remodelling occurs too slowly to play any significant role in
normal calcium homeostasis, being more important in certain pathological conditions. It is probable that the tight control of the exchangeable calcium pool at the bone-ECF interface has the primary role in rapid alterations of calcium homeostasis (Mundy, 1995).

The Gut

The average UK daily intake of calcium is usually quoted as 25mmol (1g), although there is some evidence this may be an overestimation. (Gregory et al., 1990). Between 25 and 50% of dietary calcium is absorbed from the intestinal lumen by two separate mechanisms; passive absorption dependent on the concentration gradient between the lumen and the blood, and active transcellular absorption, promoted by 1,25(OH)2D and mediated by specific binding proteins. The hydroxylation reaction which converts 25-hydroxyvitamin D (25-OHD) to 1,25(OH)2D is stimulated by PTH, which is itself produced in response to falling plasma Ca2+ (Pocotte et al., 1991). promotes 1,25(OH)2D production so increasing intestinal calcium absorption. Such control of intestinal calcium absorption plays little part in short term calcium regulation in normal individuals. Adjustments of intestinal absorption by the PTH - 1,25(OH)2D axis, maximal between 24 and 48 hours, represent a classical 'long-loop' feedback system.

The Kidney

Urinary calcium excretion is dependent on both the filtered load and reabsorption from the glomerular filtrate. The kidney filters approximately 10g of calcium per day, of which around 98% is reabsorbed (Mawer & Berry, 1995; Mundy, 1995). Reabsorption is an active process, occurring in both the proximal (influenced by sodium reabsorption), and distal tubules. This latter PTH mediated process, may be considered the immediate, 'short-loop' element of hormonal calcium homeostasis.

Activation of 25-OHD to 1,25(OH)2D occurs under the influence of PTH in the cells of the proximal tubule (Norman & Henry, 1993). Renal damage may therefore lead to decreased calcium absorption from the gut as well as reduced calcium conservation. A role for 1,25(OH)2D in the renal tubular reabsorption of calcium is not clearly established.

The Mechanism of Calcium Homeostasis - An Overview

Concentration of Ca2+ in the ECF is tightly regulated by the PTH-1,25(OH)2D integrated hormone axis. In addition to the long-loop inhibition of PTH by calcium, 1,25(OH)2D regulates PTH by a short-loop feedback, suppressing every stage from PTH gene transcription to hormone release. A further calcitropic hormone, calcitonin, is known to inhibit osteoblastic bone resorption and has a negative effect on renal tubular reabsorption of calcium (Friedman et al., 1986; Ralston et al., 1985). The precise role of this
hormone in calcium homeostasis and skeletal metabolism in humans, while not firmly established, would appear to be modest compared to that of PTH and 1,25(OH)2D (Bikle, 1993). The primary target organs of these hormones are the gut, bone and kidney. The relative roles of the kidney and bone in the short-loop maintenance of ECF Ca2+ are uncertain and complex (Mundy, 1995).

The above view of hormonally controlled calcium homeostasis is almost certainly oversimplified. The mean concentration about which the ECF calcium is controlled has been defined as the calcium set-point (Parfitt, 1987). This undergoes pressure to change (e.g. an altered dietary calcium intake). The rectification of such deviations from set-point may be considered 'error correction'. However as error correction may occur in the absence of calcitropic hormones (for example in hypoparathyroidism), it is clear that additional components participate in this process. Rapid alterations in renal calcium handling and calcium exchange at the bone-ECF interface probably provide the non-hormonal components of this mechanism.

<table>
<thead>
<tr>
<th>Bone</th>
<th>Gut</th>
<th>Kidney</th>
<th>Net</th>
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<tbody>
<tr>
<td>Ca</td>
<td>P</td>
<td>Ca</td>
<td>P</td>
</tr>
<tr>
<td>PARATHYROID HORMONE</td>
<td>↑↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1,25(OH)2D</td>
<td>↑↑</td>
<td>↑↑</td>
<td>-</td>
</tr>
<tr>
<td>CALCITONIN</td>
<td>↓↓</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.1: Target organs of the calcitropic hormones. Arrows indicate increased or decreased flow of calcium and phosphate into the blood with relation to each organ of calcium homeostasis and the overall effect.

<table>
<thead>
<tr>
<th>Parathyroid Hormone</th>
<th>1,25(OH)2D</th>
<th>Calcitonin</th>
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</thead>
<tbody>
<tr>
<td>CALCIUM</td>
<td>↓</td>
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</tr>
<tr>
<td>PHOSPHORUS</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>PARATHYROID HORMONE</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>1,25(OH)2D</td>
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Table 1.2: Effects of increases in components of the calcium homeostatic system on the production and secretion of the calcitropic hormones.
The Assessment of Calcium Status

The Separately Definable Fractions of Calcium

Serum calcium is composed of three distinct species as illustrated in Figure 1.4 (Marshall, 1976; Nordin et al., 1989):

1. Ionised calcium (the physiologically active fraction).

2. Calcium complexed to low molecular weight ionic species (principally bicarbonate, phosphate and citrate (Marshall, 1976).

3. Protein bound calcium, the only species not ultrafiltrable by the kidneys. Such binding is principally due to the interaction of Ca\(^{2+}\) with anionic carboxylated groups of serum albumin, with the majority of the remainder attached to alpha globulins.

The proportions ascribed to the fractions depends on the methods employed in their estimation. Protein-bound calcium has been estimated to be as low as 35% of the total calcium and as high as 46%, with the complexed fraction accounting for between eight and 15%. The ionised calcium fraction, (more constantly estimated as 47-50%), is confined to a narrow concentration range, both within individuals and populations (Payne et al., 1986; Pickup et al., 1977). Ultrafiltration studies indicate that variations in total serum calcium are almost entirely accounted for by variations in the amount of protein bound calcium (Moore, 1970a). This would be expected given the tight control of Ca\(^{2+}\) by the calcium homeostatic system.

The Estimation of Circulating Calcium Concentrations

Only ionised calcium is physiologically active, and is therefore the fraction of interest in clinical samples. However the lack of a reliable method for its measurement has long been a problem. Estimates of Ca\(^{2+}\) have been derived from bioassays using isolated frog hearts or rachitic cartilage (McLean & Hastings, 1934; Yendt et al., 1955) and from metal ion indicators such as murexide (Rose, 1957). While ion-selective electrodes (ISE) have been available for the measurement of ionised calcium since the late 1920’s (Fosbiner, 1929), no internationally agreed method, instrumentation or reference material exists. Despite the recognition that Ca\(^{2+}\) concentrations fall within a very narrow range \textit{in vivo}, both reference ranges and individual measurements produced on different equipment vary markedly. The inability to automate the specialised equipment required makes the measurement of Ca\(^{2+}\) by ISE even less attractive, and most laboratories use the measurement of total serum calcium as an index of Ca\(^{2+}\) status.
Figure 1.3: Calcium fluxes between the main body pools. Single arrows (and italic numbers) show unidirectional fluxes, block arrows (encircled numbers) show net fluxes. All amounts mmol/day. Represents a healthy adult in zero calcium balance with no disorder of bone turnover. While losses through sweating have been demonstrated experimentally (Consolazio et al., 1963), these are not significant in normal circumstances, especially in temperate climates.
Figure 1.4: Calcium species in human serum/plasma. Note the equilibrium between complex, ionised and protein bound fractions. Proportions are general estimated, quoted figures vary with methods of measurement.

However serum total calcium concentrations alone give an incomplete picture. Up to 50% of serum calcium may be protein bound (Berry et al., 1973). This amount is directly proportional to the serum protein concentration. Even in an individual with no disorder of calcium homeostasis, as protein concentrations increase, the total calcium level will rise as \( \text{Ca}^{2+} \) 'lost' to protein binding is replaced. Thus the assessment of \( \text{Ca}^{2+} \) using serum total calcium may spuriously indicate hypercalcaemia if the protein bound fraction is high. Conversely a true hypercalcaemia may be masked by a low protein bound calcium fraction. The adjustment of serum total calcium to account for protein binding is examined further in Chapter III of this thesis.

The reference method for the measurement of total serum calcium is atomic absorption spectrophotometry (Cali et al., 1973), but more conveniently by direct colorimetric reaction with the metal-complexing dye cresolphthalein complexone (Connerty & Briggs, 1966), a method suitable for automation (Wiener, 1980).

**Ionised Calcium**

Ionised calcium is the ideal indicator of active calcium status, especially in the new-born, those with renal disease and critically ill intensive care patients (Gray & Paterson, 1988). However since the first use of ion-selective electrodes for calcium measurement in human serum (Moore, 1969), few technological improvements have been made. Measurement of albumin-adjusted serum calcium is therefore more practical in assessing \( \text{Ca}^{2+} \) status (Iqbal et al., 1988). This is illustrated in pregnant women, in whom total serum calcium
is seen to fall during the course of gestation. Simultaneously a more substantial fall in serum albumin occurs. Only adjustment of calcium for albumin allows the identification of the increased Ca\(^{2+}\) which occurs during pregnancy (Davis et al., 1988; Payne et al., 1990), allowing alterations in 1,25(OH)\(_{2}\)D and PTH in these individuals to be explained (Davis et al., 1988; Kumar et al., 1979). Conversely Ca\(^{2+}\) measured by ISE indicated normal or decreased levels, reinforcing the fact that such measurements may be misleading.

Factors Affecting the Measurement of Ionised Calcium.

\(pH\)

The effects of pH on Ca\(^{2+}\) and its measurement have been known since the early applications of ISE technology (Moore, 1970b). A decreasing pH leads to increasing Ca\(^{2+}\) \textit{in vivo} and \textit{in vitro} (due to the loss of carbon dioxide from the sample). Attempts to avoid \textit{in vitro} changes include sample storage and transport on ice under anaerobic conditions (Ladenson & Bowers, 1973). It has been suggested that measurements in whole blood are preferred to serum as anaerobic handling is simplified (Fuchs et al., 1976), yet electrode responses to whole blood samples may be affected by haematocrit (Fogh-Andersen, 1981). Anticoagulants tend to bind calcium and are therefore unsuitable for specimens intended for Ca\(^{2+}\) measurements. Some workers attempt to overcome this by using heparin at very low concentrations (Nelson et al., 1989; Sachs et al., 1991), although this invites sample coagulation. Titration of the anticoagulant against calcium so that the negative interference of heparin is balanced by the positive contribution of haematocrit has also been attempted (Toffaletti, 1987). Alternative approaches include the tonometric alteration of the sample back to a physiological pH of 7.40 (Buckley & Russell, 1988; Thode et al., 1990) or measurement of sample pH and the application of an algorithm to express the result at the 'standard' pH of 7.40 (Braumann et al., 1983). However this correction lacks standardisation.

\textit{Temperature}

Temperature affects the analysis of Ca\(^{2+}\), and the sample electrode temperature should be controlled to inhibit variability of response (Moore, 1970b; Ladenson & Bowers, 1973). \textit{In vivo} binding of calcium by protein increases slightly with temperature (Pedersen, 1971a).

\textit{Ionic Strength}

Electrodes do not directly measure concentration. This is quantified by the multiplication of measured activity by a factor influenced by the ionic strength of the sample (Kanis & Yates, 1985; Siggaard-Andersen et al., 1983). In order to calibrate ISE apparatus
accurately, standard solutions should therefore contain physiological concentrations of the major plasma salts, principally sodium and potassium (Ladenson & Bowers, 1973). In sera of abnormal ionic strength (e.g. hypernatraemia) or mass concentration (water content, i.e. lipaemia or proteinaemia) the factor may become invalid (Ladenson & Bowers, 1973).

**Protein/Albumin Concentrations**

The Ca\(^{2+}\) concentration of serum is greater than that of its dialysate or ultrafiltrates (Ferreira & Bold, 1979; Moore, 1970a; Toffaletti et al., 1976). It was initially thought such observations represented an in vivo phenomenon known as the Donnan effect (Thode et al., 1983; Thode et al., 1987). This states that when a non-diffusible ion is present on one side of a semi-permeable membrane, it causes an unequal distribution of the diffusible ions across the membrane, i.e. a greater calcium concentration in the solution on the side containing the non-diffusible albumin. In fact the observation results from a direct effect on the measurement system due to protein denaturation by hypertonic solutions at the reference electrode liquid junction (Butler et al., 1984; Freaney et al., 1986; Payne et al., 1982). This interference may be eliminated by the use of isotonic reference electrode solutions (Payne, 1988), but liquid junctions of this type are more susceptible to influence by anionic species, rendering them no better than the established alternatives (Masters & Payne, 1993).

The relationship between serum albumin and Ca\(^{2+}\) explains the apparent increase in Ca\(^{2+}\) even on short venous stasis (Braumann et al., 1983; McEnroe et al., 1992; Payne, 1988) or on standing (Marshall et al., 1982). Albumin levels fall at the menopause, (Whitehead et al., 1994) masking Ca\(^{2+}\) increases found in post-menopausal women, making them appear too small to account for the rise in total calcium occurring at this time (Endres et al., 1987; Fogh-Andersen et al., 1984; Marshall et al., 1982; Robertson & Marshall, 1981). Similarly the apparent Ca\(^{2+}\) decrease found in oestrogen-treated post-menopausal women may be a measurement artefact resulting from the decreases in albumin this drug produces (Fogh-Andersen et al., 1984; Selby et al., 1985). Furthermore the fall in Ca\(^{2+}\) found in normal ageing adults (Linggaerde, 1972), may be an effect of decreasing albumin concentration with age. It is clear that as with total calcium, Ca\(^{2+}\) measurements should be interpreted with regard to albumin concentrations.
Table 1.3: Total, Adjusted and Ionised Calcium: Advantages And Disadvantages.

<table>
<thead>
<tr>
<th>Total Calcium</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No special sample or sample handling required</td>
<td>Abnormal plasma protein concentrations affect interpretation</td>
</tr>
<tr>
<td>Methods automated with good precision</td>
<td>May be misleading in patients with pH changes.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adjusted Calcium</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological variation less than total calcium</td>
<td>Population derived adjustment applied to individuals</td>
</tr>
<tr>
<td>Takes account of abnormal albumin concentrations</td>
<td>Adjustment may be inaccurate at very low albumin concentrations.</td>
</tr>
</tbody>
</table>

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<tr>
<th>Ionised Calcium</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Good precision</td>
<td>Anaerobic sample handling or adjustment for pH required</td>
</tr>
<tr>
<td>Measures biologically active calcium</td>
<td>Abnormal plasma protein concentrations affect measurement</td>
</tr>
<tr>
<td>Accounts for in vivo pH changes</td>
<td>Bicarbonate concentrations almost certainly affect interpretation</td>
</tr>
<tr>
<td></td>
<td>No internationally accepted standard material or analyser. Results differ according to equipment used.</td>
</tr>
</tbody>
</table>

Phosphorus

Phosphorus exists as both organic and inorganic forms in serum (Marshall, 1976). Inorganic phosphorus is a major constituent of the skeleton as the primary anionic constituent of hydroxyapatite. Organic phosphorus participates in energy transfer reactions and a variety of cellular functions as well as having a structural role as a constituent of phospholipids. While the range of phosphate concentrations in the plasma and ECF is wider than that of calcium (Morris et al., 1993), it is still maintained within a narrow range of values in normal individuals. The interactions of a variety of physiological mechanisms result in the overall control of plasma phosphate homeostasis.

Plasma phosphate concentrations are dependent primarily on renal excretion, as well as intestinal absorption and the release from soft tissues and bone (Robertson, 1976). Intestinal absorption occurs by both passive and (at lower concentrations) active transport
mechanisms. Renal excretion is a function of the glomerular filtration rate (GFR). Consequently if the GFR is low, plasma phosphate may be normal even if phosphate loss is occurring (Stamp, 1971). Renal tubular reabsorption of phosphate is decreased by PTH, calcitonin, and high dietary phosphate intake. During non-acute (over 3-4 hours) phosphate depletion, plasma phosphate is maintained by mobilisation of phosphate from bone, which contains around 85% of the total body phosphate. Decreased serum phosphate also promotes the renal activation of vitamin D. The consequent increase in intestinal phosphate absorption as well as the mobilisation of phosphate from bone serves to replenish plasma phosphate.

In healthy subjects serum phosphate concentrations exhibit a circadian variation, peaking at midnight with a nadir just before noon which reflects a peak in the circadian rhythm of PTH (Kitamura et al., 1990; Markowitz et al., 1981; Portale et al., 1987). Dietary influences on serum phosphorus levels may be removed by an overnight fast (Portale et al., 1987). About 15% of inorganic phosphate is protein bound, the remainder exists in ionic form as a mixture of monovalent and divalent ions (HPO$_4^{2-}$ and H$_2$PO$_4^-$) or complexed with sodium, calcium or magnesium. Although routine clinical laboratory methods measure only inorganic phosphorus; the terms 'phosphorus concentration' and 'phosphate concentration' are used interchangeably.

Hypophosphataemia is found in primary hyperparathyroidism, secondary hyperparathyroidism associated with vitamin D deficiency, and in tumour induced osteomalacia. Hyperphosphataemia occurs in patients with chronic renal failure and hypoparathyroidism.

**Hormones of Calcium Homeostasis**

**Vitamin D and Its Metabolites**

Vitamin D owes its classification as a vitamin to its identification as the anti-rachitic factor in cod-liver oil (McCollum et al., 1922). Strictly however, the active form of vitamin D should be regarded not as a vitamin, but as a steroid hormone. While vitamins are obligatory in the diet, synthesis of vitamin D in the skin on exposure to ultra-violet light obviates this requirement. The presence of high affinity nuclear receptors in target tissues for the active form of vitamin D further supports classification as a steroid hormone. There are three main organs of vitamin D metabolism; the skin, the liver and the kidney.

**The Organs of Vitamin D Metabolism**

**The Skin**

When solar radiation between 290 and 315nm (UV-B) is incident on the epidermis and dermis, 7-dehydrocholesterol (provitamin D$_3$) is cleaved to produce the seco-sterol pre-
vitamin D\textsubscript{3}. A time and temperature dependent isomerisation to vitamin D\textsubscript{3} follows (Holick \textit{et al.,} 1980). While the epidermal pigment melanin may limit the production of previtamin D\textsubscript{3} and consequently vitamin D\textsubscript{3} (Mawer, 1992), it is not an essential regulatory factor of vitamin D production (Holick \textit{et al.,} 1981). This is illustrated by the fact that Caucasians do not become vitamin D intoxicated at equatorial latitudes. Indeed prolonged exposure to sunlight results in the photoisomerisation of previtamin D\textsubscript{3} and the photo-degradation of vitamin D\textsubscript{3} (Webb \textit{et al.,} 1989). Thus provitamin D\textsubscript{3} synthesis is not necessarily increased, while the systemic availability of vitamin D\textsubscript{3} is limited (Webb & Holick, 1988).

Cutaneous production of vitamin D is the more important source of the provitamin. The naturally occurring dietary form of the prohormone, provitamin D\textsubscript{2} (ergocalciferol) only becomes important when sunlight exposure is limited (Holick & Adams, 1990). In such cases several countries practise fortification of foodstuffs with vitamin D\textsubscript{2} or D\textsubscript{3}.

The nomenclature of vitamin D and its metabolites may be confusing. Although the two different forms of vitamin D are equipotent with regard to biological activity, D\textsubscript{3} is more abundant and therefore usually the more important form. While this leads to many texts referring to vitamin D\textsubscript{3} alone, the presence of vitamin D\textsubscript{2} should remembered. Furthermore the term ‘vitamin D’ is commonly used to refer to the active agent of this group of metabolites. However true vitamin D exhibits little biological activity, requiring successive hydroxylations in the liver and kidney for activation. The active form of vitamin D, 1,25-dihydroxyvitamin D, is also known as calcitriol. The use of these names has become interchangeable.

\textit{The Liver}

Dietary vitamin D (ergocalciferol) is incorporated into chylomicrons and absorbed through the lymphatic system. This, together with cholecalciferol produced in the skin binds to vitamin D-binding protein (DBP) in the plasma (Haddad, 1984). The DBP-vitamin D complex is transported in the general circulation to the liver where a cytochrome P\textsubscript{450} dependent hydroxylation reaction yields 25-hydroxyvitamin D (25-OHD) (Battacharja & De Luc, 1973; Tucker \textit{et al.,} 1973). This compound, also known as calcidiol, is the major circulating form of vitamin D (Henry & Norman, 1990). The half-life of 25-OHD in the circulation is two to three weeks, and as such its concentration reflects vitamin D status from both dietary and endogenous sources (Holick \textit{et al.,} 1980).

\textit{The Kidney}

From the liver, 25-OHD is transported by the DBP to the kidney. Here a further hydroxylation reaction occurs on either carbon-1 or carbon-24 of the molecule, producing 1,25- or 24,25-dihydroxyvitamin D (Fraser & Kodicek, 1970; DeLuca, 1984). Both 25-
OHD and 24,25-dihydroxyvitamin D have limited biological activity with respect to calcium homeostasis. Conversely 1,25(OH)₂D, the major hormonal metabolite of vitamin D, is tightly regulated as part of the calcium homeostasis system. This control is affected by regulation of the renal 1α-hydroxylase enzyme which catalyses the hydroxylation of 25-OHD to 1,25(OH)₂D. While a decrease in calcium concentration alone stimulates this conversion in vitro (Fraser, 1980), this has not been convincingly demonstrated in vivo (Holick & Adams, 1990). The stimulus for increased 1,25(OH)₂D production in hypocalcaemia is actually secondary hyperparathyroidism. This cyclic AMP mediated PTH activity also results in renal tubular reabsorption of calcium and secretion of phosphate into the urine (Garabedian et al., 1972; Holick, 1987). While important in the regulation of 1,25(OH)₂D, PTH is not essential for its production. As such patients with hypoparathyroidism have low-normal rather than undetectable levels of calcitriol (DeLuca, 1984). Renal 1α-hydroxylase is also stimulated by hypophosphataemia. The mechanism controlling the production of 1,25(OH)₂D is completed by a direct negative effect of 1,25(OH)₂D on PTH production, (short-loop feedback) and a similar effect modulated via raised serum calcium levels resulting from 1,25(OH)₂D action (long-loop feedback) (Henry & Norman, 1990). Other hormones including growth hormone, oestrogen, glucocorticoids, prolactin, insulin and thyroid hormone may modulate 1,25(OH)₂D production (Iqbal, 1994).

The enzyme responsible for 1,25(OH)₂D production is not solely found in the kidney. Placental 1α-hydroxylase activity is responsible for the increased 1,25(OH)₂D levels in pregnancy (Gray et al., 1981a). In granulomatous diseases (e.g. sarcoidosis and tuberculosis) or lymphoma, excessive 1,25(OH)₂D production by macrophages leads to elevated plasma levels and subsequent hypercalcaemia (Adams et al., 1986; Davies et al., 1985). These effects usually occur after exposure to sunlight as non-renal 1α-hydroxylases are not subject to the regulatory processes affecting renal 1,25(OH)₂D production (Adams et al., 1988). The tight control of renal 1α-hydroxylase is emphasised by the markedly smaller increase in 1,25(OH)₂D than 25-OHD in vitamin D intoxication (Mawer et al., 1985).

Biological Actions of 1,25-dihydroxyvitamin D

The target cells of 1,25(OH)₂D contain a vitamin D receptor protein (VDR) which has much higher affinity for 1,25(OH)₂D than other vitamin D metabolites (Haussler et al., 1988). The presence of the hormone facilitates the binding of VDR to a nuclear accessory factor (NAF). This VDR-NAF complex results in interactions with nuclear chromatin at the vitamin D response element sites (VDRE). Transcription of the calcitriol dependent mRNA follows (Pike, 1991).
Target Tissues Of 1,25(OH)₂D

**Intestine**

1,25(OH)₂D stimulates intestinal calcium absorption by enhancing the synthesis of calcium-binding protein (CABP) (Dupret *et al.*, 1987; Thomasset & Tenenhouse, 1988). This protein is more commonly known as calbindin. Phosphate absorption from the small intestine is also promoted by 1,25(OH)₂D.

**Kidney**

In renal cells 1,25(OH)₂D down regulates 1α-hydroxylase activity, concomitantly up-regulating 24-hydroxylase activity (Henry *et al.*, 1992). As 1,25(OH)₂D is of renal origin, severe renal dysfunction may lead to an acquired vitamin D deficiency (Mawer *et al.*, 1985). As previously discussed, 1,25(OH)₂D may act in conjunction with PTH in enhancing phosphaturia and the tubular reabsorption of calcium.

**Bone**

By maintaining an adequate supply of calcium and phosphate, 1,25(OH)₂D indirectly facilitates mineralisation of bone matrix (Underwood & DeLuca, 1984). While VDR elements are not found in osteoclasts (Merke *et al.*, 1986), bone resorption is mediated by 1,25(OH)₂D, probably via an osteoblast-produced stimulating factor (Chambers, 1988). This hypothesis is supported by the requirement of osteoblastic cells for the *in vitro* activation of isolated osteoclasts (McSheedy & Chambers, 1987). Such action may be synergistic with PTH *in vivo* to increase bone resorption and so serum calcium. Calcitriol also acts as a cellular differentiation agent, inducing the production of osteoclasts from their haemopoietic precursors (Chambers, 1988; Roodman *et al.*, 1985). Osteoblasts do contain vitamin D receptors, and 1,25(OH)₂D affects osteoblast function *in vitro*, inhibiting collagen synthesis (Row & Khean, 1982). Conversely alkaline phosphatase and osteocalcin, other osteoblast products, are up-regulated by 1,25(OH)₂D (Price & Baukol, 1980; Mangolas *et al.*, 1981).

**Parathyroid Hormone**

**Action of PTH**

Parathyroid hormone (PTH) is the principal immediate regulator and determinant of the extracellular fluid (ECF) calcium concentration. The actions of PTH are co-ordinated with 1,25(OH)₂D, which is primarily responsible for the day-to-day and week-to-week calcium balance within the entire body (Habener & Potts, 1990). Each of these hormones has an influence on the production of the other.
The principal action of PTH is to increase calcium levels within the ECF, and so the blood. The primary target organs through which this is facilitated are the kidney and the skeleton. PTH has two modes of action on renal function. The first is to stimulate renal tubular reabsorption of calcium (and inhibit reabsorption of phosphate) from the glomerular filtrate. This is likely to be the most important short-term effect of PTH in the elevation of plasma calcium. The second effect of PTH on the kidney is to enhance the production of 1,25-dihydroxyvitamin D. This is achieved by stimulating renal 25-hydroxyvitamin D 1α-hydroxylase in specific cells of the proximal tubule (Suda & Kurukowa, 1983). Therefore as well as a direct effect on renal calcium conservation, PTH also acts indirectly to increase plasma calcium by increasing the intestinal absorption of calcium and phosphate.

The calcitropic activity of PTH with respect to bone is due to its action on two different cell types. PTH acts most rapidly on cells lining endosteal surfaces, ALTERING THE CALCIUM FLUX AT THE BONE-ECF INTERFACE (Kronenberg, 1993). Stimulation of osteoclast production and activity (and therefore bone resorption) then occurs (Habener & Potts, 1990). The stimulation of osteoclast activity by PTH is likely to be mediated by osteoblast produced local factors as osteoblasts, but not osteoclasts, possess PTH receptors (Garabedian et al., 1974; Reeve & Zanelli, 1986). The enhancement of osteoclastic bone resorption by PTH in vivo occurs in concert with 1,25(OH)₂D₃, and is also probably influenced by other locally produced factors (Garabedian et al., 1974; Mundy, 1995).

Osteoblastic collagen production and alkaline phosphatase activity is inhibited by PTH in vitro (Kream et al., 1980; Majeska & Rodan, 1982). However in vivo, PTH may be associated with stimulation of bone formation, especially when administered intermittently at small doses (Kronenberg 1993). Indeed, the nature of PTH secretion appears to determine its biological effect. When bone cells are exposed continually to PTH the primary effect is resorptive, intermittent exposure at lower concentrations produces anabolic effects. Although markers of bone formation are increased in hyperparathyroid patients with chronically elevated PTH levels (Minisola et al., 1994), this is probably a result of the coupling between bone resorption and formation. Normal PTH secretion occurs with a pulsatile component added to a basal activity and therefore may be considered to be anabolic in nature (Samuels et al., 1993).

**Production of PTH**

PTH is produced and secreted by the parathyroid glands which are positioned close to the thyroid. Secretion is primarily under the control of the blood calcium concentration, being stimulated by falling serum calcium and inhibited by increases (Nordin et al., 1972). Such changes are detected by highly responsive calcium ‘sensors’ present in parathyroid cells, although vitamin D appears to modulate PTH production independently.
of this process (Bradbeer et al., 1992; Brown et al., 1993; Russell et al., 1986). Encoded by a gene on chromosome 11, PTH is secreted as a single chain 94 kD protein of 84 amino acids. Biological activity resides in the amino-terminal region (Keutmann, 1987; Mawer & Berry, 1995). The molecule is actually formed as Pre-Pro-PTH, a 115 amino acid peptide. Before secretion from the parathyroid, fifteen amino acids are enzymatically cleaved from the amino-terminal to produce the prohormone Pro-PTH. Removal of a further six residues gives PTH itself. The final product is often referred to as intact PTH as further breakdown within the parathyroid gland, liver and kidney, results in a series of circulating fragments (Bringhurst et al., 1988; Segre et al., 1974). The presence of these fragments and the differing functions of the regions of the molecule from which they derive, has implications for the measurement of serum PTH.

The Circulating Heterogeneity of PTH

Intact PTH, cleared rapidly from the circulation, has a plasma half-life of approximately five minutes (Segre et al., 1974; Wood, 1992). The intact hormone is taken up mainly by the liver and to a lesser extent by the kidney and bone. Within the Kupffer cells of the liver, enzymatic cleavage in the 33-41 amino acid region leads to the production of biologically active N-terminal and inactive mid- and C-terminal fragments (Martin et al., 1979). It is thought that bone may have a preference for the uptake of bioactive 1-34 amino terminal fragments over the intact hormone (Calvo et al., 1985). While the N-terminal fragment has an extremely short half-life (approximately ninety seconds), clearance of the C-terminal fragment is much slower (Manning et al., 1981). As such in normal individuals, intact PTH represents only 5-30% of the total circulating immunoreactive PTH, the remainder being C-terminal fragments (Segre et al., 1972). Impairment of renal function leads to an even higher relative concentration of these fragments.

Assays for PTH

While sensitive biological assays for the measurement of bioactive PTH are available, these are too cumbersome for routine use (Mawer & Berry, 1995). Assays have been described for N-terminal (amino acids 1-34), mid-molecule (44-68) or C-terminal (53-84), as well as intact PTH. While C-terminal assays have the advantage of measuring the most abundant fragment, the influence of renal function raises difficulties in interpretation. Mid-molecule assays have been used as an index of PTH activity because of the longer half-life of these fragments. However the discriminatory value of such assays has been questioned (Mawer & Berry, 1995; Wood, 1992).

Of all immunoassays, those directed towards the N-terminal should best represent circulating bioactive PTH as both N-terminal fragments and intact PTH is measured. However in reality, the half-life of the N-terminal fragment is so short that only intact
PTH is measured. Furthermore problems with the sensitivity of these assays has limited their clinical use to the identification of primary hyperparathyroidism (Endres et al., 1989). Competitive radioimmunoassays for PTH fragments have now been superseded by immunoradiometric assays using antibodies directed towards two different sites on the PTH molecule (Wood, 1992). Such assays sensitively detect intact PTH even in the presence of large concentrations of C-terminal fragments (Nussbaum et al., 1987). The monitoring of patients with renal disease, where avoidance of secondary hyperparathyroidism and accompanying renal osteodystrophy is a goal, but in whom C-terminal fragments accumulate, is possible with such methods (Endres et al., 1989). Low (or in hypocalcaemia, inappropriately low) PTH concentrations are much more reliably identified using intact PTH measurements, while the precision of such assays is greatly improved over the radioimmunoassays previously used (Wood, 1992).

Factors Influencing The Interpretation Of Intact PTH Concentrations

Intact PTH levels show a circadian variation, with peak values variously reported between 2400 and 0600 hours (Kitamura et al., 1990; Logue et al., 1989). Although there appears to be no gender-related difference in PTH concentrations, a slight increase with age in both sexes occurs. However no significant increase at the time of the menopause has been demonstrated (Eastell et al., 1991; Endres et al., 1989; Sherman et al., 1990; Sokoll et al., 1988). PTH levels are reduced by dietary calcium, being significantly lower in non-fasting than fasting individuals (Morris et al., 1993; Tohme et al., 1990). During bisphosphonate infusion a rapid fall in serum calcium can cause increased PTH secretion, even when calcium concentrations remain within reference limits (Logue et al., 1989). While a short delay in the separation of blood specimens or in the freezing of separated serum has a less severe effect than originally believed (Morris et al., 1993; Wood, 1992), inappropriate sample handling may produce artifically low results (Newman & Ashby, 1988). Careful timing, collection and storage procedures for PTH samples are therefore necessary, requiring separation and freezing without delay and storage at -20°C until assay (Wood, 1992; Logue et al., 1990).

Parathyroid Hormone-Related Protein (PTHrP)

This amino acid of the parathyroid hormone family was first discovered as the humoral factor in certain tumours associated with hypercalcaemia of malignancy (Suva et al., 1987). This syndrome is characterised by hyperparathyroid-like effects despite PTH suppression. However PTHrP appears to be of more widespread physiological importance in normal individuals (Burtis, 1992). This is suggested by the wide expression of PTHrP: in foetal tissues, the central nervous system, smooth muscle cells, the uterus and placenta as well as endocrine tissues (Strewler & Nissenson, 1993). The
human PTHrP gene is found on chromosome 12, the gene product apparently consisting of three isoforms of 139, 141 and 173 amino acids. These isoforms are all homologous for PTH at the amino terminal (Strewler & Nissenson, 1993).

Although the normal physiological functions of PTHrP have yet to be elucidated, it appears this peptide may be a multi-functional molecule, acting as a calcitropic hormone and in cellular development and differentiation.

**Direct Measurement of Calcium Flux in the Individual**

**Calcium Balances**

The integrated mechanisms of calcium homeostasis dictate the overall calcium balance of the individual. This represents the mineral state of the whole organism with respect to its environment. Calcium balance may be measured by metabolic balance studies, which is compare the amount of an element entering the body to the amount leaving it.

Although simple in principle, the performance of calcium balance studies is fraught with difficulties. Calcium excretion in the faeces is both considerable and variable, necessitating collection and analysis of all stools passed during the balance period. This is unpleasant for all concerned. For convenience and to allow monitoring of response to treatment, balances are divided into four day periods. Demarcation of faecal collections with carmine dye facilitates this, allowing faecal excretion to be related to the appropriate urinary collections, which run slightly out of step. However errors in the demarcation of faecal collections are unavoidable (Parfitt, 1960). While the use of copper thiocyanate, the cationic part of which is not absorbed by the gut, has improved the reproducibility of such collections (Dick, 1969), at least two control periods on each regime remain necessary. While urinary calcium is relatively easily analysed, excretion from the skin, although probably minimal, is ignored. Calcium intake is measured by analysing the patient diet. To avoid variation this must be constant throughout the duration of the balance and for an equilibration period beforehand. This becomes tedious for the patient over a course of several weeks.

Once equilibrium is reached the balance reflects the difference between bone accretion and resorption. Normal adults, in whom bone formation and resorption are coupled, have a zero calcium balance (mineral intake equals mineral excretion). During skeletal development calcium intake exceeds calcium excretion, the positive balance being due to bone mineral accretion. A patient losing bone mineral (e.g. through osteoporosis), would be in negative calcium balance.
Over thirty-five years ago Parfitt (1960), concluded that 'in terms of information gained for outlay of time and money, calcium balances are extravagant'. The use of the technique was justified as the information gained was available in no other way. Together with isotopic studies, balance studies remain an immediate method of assessing the net difference between bone formation and resorption (Stamp et al., 1991; Thalassions et al., 1982). However biological variation, sample collection errors and analytical errors for the large number of measurements involved, all contribute to the often unacceptably high variation in calcium balance results (Fisher et al., 1976). Furthermore although they do not directly measure net mineral balance, biochemical markers of bone turnover are now commonly available. Such markers may be used in metabolic bone diseases as an aid to diagnosis and as short-term predictors of response to treatment (Riis et al., 1995). Consequently calcium balance studies have no routine use are rarely performed, even in research environments.
Biochemical Markers of Bone Formation

Alkaline Phosphatase

The Isoforms of Alkaline Phosphatase

Serum alkaline phosphatase (ALP) is the most commonly used biochemical marker of bone metabolism. While the measurement of this enzyme has long been associated with osteoblastic activity (Robinson, 1923), not all ALP activity in serum is derived solely from bone. It has been recognised for over sixty-five years that serum ALP is increased in patients with hepatobiliary disease (see Moss, 1988). Although some workers postulated a failure of the liver to excrete bone derived ALP, the application of various techniques of isoenzyme-enzyme analysis identified the liver as the source of the increased ALP activity (Hill & Sammons, 1967; Hodson et al., 1967).

There are four genetic loci for human alkaline phosphatase. Those for the placental, intestinal and germ cell isoforms are found on chromosome 2, while the gene for the tissue non-specific form is on chromosome 1 (Fishman, 1990). This gene codes for the bone, liver and kidney isoforms of ALP which result from differences in post-translational glycosylation of the protein (Crofton et al., 1982; Price, 1993; Weiss et al., 1986). The bone and liver isoforms are the most abundant in normal serum and usually account for elevated total ALP activity (Van Hoof et al., 1990). Placental or germ cell ALP may be the primary isoform in patients with cancer, while in subjects with various diseases of the digestive tract the intestinal isoenzyme predominates (Price & Thompson, 1995).

ALP is a membrane-bound enzyme. The activity of the bone isoform rises when osteoblasts are actively laying down osteoid. Overflow of osteoblastic ALP may be due to breakdown of the plasma membrane (or its vesicles), overflow of enzyme binding sites or a combination of both (Moss, 1988). The major isoforms have relatively long half-lives of about 1-3 days (approximately 1.5 days for the bone isoenzyme) (Walton et al., 1975). The dominating influence on serum levels of liver and bone ALP is therefore their rate of entry into the general circulation. Serum total ALP concentrations correlate with bone mineralisation as measured by calcium isotope studies (Charles et al., 1985; Klein et al., 1964), and have long been used as an indicator of bone formation. The major application total ALP in the field of metabolic bone disease is as an index of disease activity and response to treatment in Paget's disease (Russell et al., 1981).

The lack of specificity provided by the assay of this heterogeneous group of isoenzymes manifests as a lack of sensitivity with less marked changes in bone formation such as those found in post-menopausal osteoporosis. This situation is exacerbated by the
induction of the liver isoenzyme by numerous drugs and the variable contribution of the intestinal form (Nielsen et al., 1990a; Tohmé et al., 1991).

The Function of Osteoblastic ALP

The hereditary disorder hypophosphatasia was first described in 1948 (see Smith, 1993). In child sufferers, low ALP activity is coupled with the presence of severe rickets, suggesting an important role for the enzyme in bone mineralisation. Furthermore, inorganic pyrophosphate, a potent inhibitor of hydroxyapatite crystal formation and thus mineralisation, is markedly elevated in this condition. This led to the suggestion that the function of bone alkaline phosphatase is to hydrolyse pyrophosphate, permitting hydroxyapatite crystal growth on newly synthesised osteoid. Other possible functions of ALP include the release of the inorganic phosphate required for hydroxyapatite formation. As the enzyme is membrane bound, a transport function of some type has been postulated. At present the precise function of osteoblastic ALP remains unclear.

Measurement of Total Alkaline Phosphatase

Alkaline phosphatase is quantified by measuring the catalytic activity of the enzyme against one of its substrates. Many methods, based on differing substrates, buffer type and concentration, assay temperature and unit of measurement have been used (for review see McComb et al., 1979). Substrates utilised include β-glycerylphosphate, and 1-, or 2- napthylphosphate. King and Armstrong developed a widely adopted method using disodium phenolphosphate with units of measurement bearing their name. All of the above methods have inconveniences such as requirements for a second reaction step, pH alteration or protein precipitation. Fifty years ago Bessey published a method that was rapid to perform and is easily applicable to the autoanalyser (Bessey et al., 1946). This gained in popularity as the availability of the pure 4-nitrophenylphosphate (4-NP) substrate increased, and 4-NP has been universal in all recommended methods since 1972 (McLauchlan 1988). Recommendations on buffer type and concentration do vary however, delaying complete standardisation of ALP measurement.

Bone Specific Alkaline Phosphatase

In normal individuals, the bone and liver isoforms of ALP are present in roughly equal amounts (Van Hoof et al., 1990). Individuals with blood group types B and O exhibit elevations in intestinal ALP after fatty meals, while placental ALP increases in pregnant women. Specific measurement of the bone isoform therefore adds sensitivity to the use of ALP in metabolic bone disease. The major forms of ALP are products of the same genetic locus, differing only in the number of sialic acid and N-acetyl glucosamine residues attached to the carbohydrate side chains of the molecule (Hitz et al., 1980; Kerkhoff et al., 1968). The differentiation of the bone and liver isoforms, required in
about half of all routine requests for ALP analysis and the majority of those concerned with metabolic bone disease, is therefore a difficult process (Moss, 1988).

Methods for the Differentiation of the Isoenzymes of ALP

Heat/Chemical Inhibition

Historically, ALP isoenzyme measurement has depended on minor differences in response to chemical inhibitors and temperature which result from tissue-specific post-translational modifications of the isoforms. Heat inactivation methods are based on the differing half lives of the bone and liver isoforms at 56°C. (Moss & Whitby, 1975). This allows the proportion of each isoenzyme contributing to the total activity is indirectly calculated according to the residual activity after incubation. High concentrations of both major isoforms, or increased placental or intestinal ALP make quantitation of the relative contributions difficult. Heat inactivation methods have been more commonly adopted than alternatives utilising chemicals such as urea, phenylalanine, or neuraminidase (Gonchoroff et al., 1991). However all these methods exhibit poor resolution and offer only indirect quantitation. The requirement for sample pre-treatment and multiple analysis has made these techniques technically cumbersome, time consuming and generally unpopular.

Gel Electrophoresis

Isoenzyme separation by electrophoresis on agarose (Van Hoff et al., 1988), cellulose acetate (Rosalki & Foo, 1984), and (most commonly), polyacrylamide gels (Ramasamy, 1991) may be undertaken. Subsequent quantitation is then facilitated by densitometric scanning of the electrophoresis gel. While sample pre-treatment improves the generally poor resolution afforded by these methods, electrophoretic techniques are generally time consuming and lack sensitivity.

Lectin Precipitation

A method based on the precipitation of bone alkaline phosphatase (B-ALP) by a lectin derived from wheat germ was first described in 1984 (Rosalki & Foo, 1984). While this method was employed clinically (Behr & Barnert, 1986; Mazda & Gyure, 1988; Sorenson, 1988), between batch variability in the reactivity of lectin and the lack of a suitable control material obstructed standardisation. The introduction of a commercial version of the assay ('Iso-ALP', Boehringer Mannheim GmbH, Mannheim, Germany), lessened such problems (Rosalki et al., 1993). Although the method is both rapid and technically simple falsely elevated B-ALP levels have been noted in patients with liver disease. This may be due to a second form of the liver isoenzyme, bound to phospholipid and membrane fragments, which also interferes with electrophoretic
methods (Price, 1993). While such species may be excluded by biochemical tests of liver
disease, the non-specificity of the method is illustrated. While some have suggested
lectin precipitation lacks the quantitative accuracy of the best electrophoretic methods
(Day et al., 1992) others have found it to provide both the sensitivity and specificity
required for the precise measurement of B-ALP (Price, 1993).

Imunoassay

Improvements in the ability to differentiate B-ALP from other isoforms have resulted
from the use of monoclonal antibodies. While the first of such antibodies were two to
two times more specific for the bone isoform (Bailyes et al., 1987; Lawson et al., 1985;
Seabrook et al., 1988), Hill & Wolfert (1989) isolated a murine antibody produced by
challenge with human osteosarcoma cells which have little cross-reactivity to the liver
isoform. This led to the development of a commercially available immunoradiometric
assay (IRMA), (‘Ostase’, BM Browne Ltd, Reading UK), which has no significant
cross-reactivity to the intestinal isoform and a seven fold preference for B-ALP over liver
ALP (Panigrahi et al., 1994). This represents a cross-reactivity of about 15% (Garnero
& Delmas, 1993; Price et al., 1995). While this assay appears to have no advantage over
lectin precipitation methods in terms of assay precision (Price et al., 1995), the variability
of wheat germ lectin remains a possible problem with respect to long-term patient follow-
up. Furthermore as the IRMA measures enzyme mass rather than activity it may be used
on samples which have undergone long term storage at -20°C or multiple freeze thaw
cycles (Panigrahi et al., 1994). Recently a further commercial assay for the measurement
B-ALP has become available (‘Alkphase-B’, Metra Biosystems Ltd, Oxford, UK). This
enzyme immunoassay (EIA) is based on the selective capture of B-ALP with a
monoclonal antibody and a subsequent colorimetric reaction with 4-nitrophenol
phosphate (Gomez et al., 1994; Gomez et al., 1995). A greater specificity for the bone
isoform has been claimed by the manufacturers of this assay and others (Hata et al.,
1996), although it is possible that in the cross-reactivity experiments undertaken, sample
pre-treatment affected the immunological integrity of the liver isoform. As the EIA
quantitative step is based on enzyme activity rather than mass, sample stability is likely to
be less than that found with the IRMA method.

Clinical Use of Total and Bone-ALP

Bone-ALP undergoes a circadian variation, with peak values at 14:30 and 23:30 which
are 30% higher than the 06:30 trough. The seasonal variations exhibited by total ALP in
normal subjects (low in summer, high in winter), are likely to be due to changes in B-
ALP and correlate negatively with concentrations of 25-hydroxyvitamin D (Devgun et al.,
1991; Tohmé et al., 1991). As well as correlating with histomorphometric assessments
of bone formation, bone alkaline phosphatase (B-ALP) is found to be a better predictor of
mineralisation in normal women than total ALP (Brixen et al., 1989). Until one year of age, B-ALP is the predominant ALP isoform in human serum (Van Hoof et al., 1990). Levels peak during childhood and puberty, subsequently falling to adult levels (Stepan et al., 1985). In mature adults B-ALP levels rise with age in both sexes (Duda et al., 1988; Garnero & Delmas, 1993; Kuwana et al., 1988). As B-ALP is not excreted by the kidney, this rise is not an artefact of the age related decrease in glomerular filtration rate but consistent with the general increase in bone turnover known to be associated with ageing. Elevations in B-ALP activity have been reported in a number of disease states. These include Paget's disease (Garnero & Delmas, 1993; Deftos et al., 1990), renal osteodystrophy, (Tibi et al., 1991), primary hyperparathyroidism, (Garnero & Delmas, 1993; Gomez et al., 1995; Silverberg et al., 1991) and in patients with bone metastases (Cooper et al., 1992; Cooper & Jones, 1993). While elevated levels of ALP are also common in these conditions, the magnitude of the increase is less marked.

Osteocalcin

Osteocalcin (OC) is, together with osteonectin, one of the two the most abundant non-collagenous bone proteins and one of the ten most plentiful proteins in the entire human body (Hauschka et al., 1989). Apart from bone, this small (5.8kD, 49 amino acid) protein is also present in dentine, but not non-osseous tissues (Nishimoto & Price, 1979). The presence of three γ-glutamic acid (Gla) residues at amino acids 17, 21 and 24, led to the older, lesser used name of Bone-Gla protein (BGP) (Price et al., 1976). The Gla residues, derived from the post-translational carboxylation of glutamic acid, strongly bind calcium, so conferring the ability to bind the hydroxyapatite of mineralised bone. Such binding aids stabilisation of the tertiary protein structure (Hauschka & Carr, 1982).

Biosynthesis of Osteocalcin

Osteoblastic production of OC is stimulated by 1,25-dihydroxyvitamin D (1,25(OH)2D) (Price & Baukol, 1980; Zerwekh et al., 1985), although production does occur in the absence of this hormone (Kaplan et al., 1985). The OC gene, like that of alkaline phosphatase, is located on chromosome 1 (Puchacz et al., 1989). Osteocalcin is initially produced as a 10kD pre-pro-peptide (Pan et al., 1985), which is cleaved and carboxylated (the latter process being vitamin K dependent and stimulated by 1,25(OH)2D), before excretion into the extracellular space (Bianco et al., 1985; Hauschka et al., 1989; Skjodt et al., 1985). While the precise effect of parathyroid hormone on OC synthesis is unclear, it may serve to modulate the action of 1,25(OH)2D (Hauschka et al., 1989; Nielsen et al., 1991). Calcitonin, the other major hormone thought be involved in calcium homeostasis, appears to have no apparent effect on OC production (Beresford et al., 1984). Hormones such as oestrogen, thyroid hormone and
cyclic AMP directly affect gene transcription and mRNA levels enhancing OC protein synthesis (Hauschka et al., 1989).

While a proportion of native osteocalcin is rapidly degraded by hepatic and renal metalloenzymes (Farrugia & Melick, 1986; Price et al., 1976), 60-90% is incorporated into bone through binding to hydroxyapatite (Poser et al., 1980). Circulating intact OC is solely derived from de novo synthesis and is not released during bone resorption (Price, 1983; Taylor et al., 1990), although some workers consider that resorptive activity may produce fragments of osteocalcin (Taylor et al., 1990). The in vivo catabolism of circulating intact OC produces peptide fragments, which may react with antisera used to detect intact OC (Gundberg & Weinstein, 1986; Poser et al., 1980; Rosenquist et al., 1995; Taylor et al., 1990). As these peptide fragments are cleared by the kidney, any impairment of renal function may lead to over-estimation of OC (Delmas et al., 1983a).

Function of Osteocalcin

Although several possible roles for OC have been postulated, no precise function has been discovered (Power & Fottrell, 1991a). As levels are highest in the bone matrix following completion of the mineralisation process a role in bone maturation has been suggested (Lian et al., 1982). It is also possible OC acts as a 'messenger' for 1,25(OH)2D, facilitating bone resorption by promoting osteoclastic activity and/or osteoclast precursor differentiation (Mundy & Poser, 1983; Povolny et al., 1987; Skjodt et al., 1985). Although OC is thought to inhibit leukocyte elastase (elastin plays an important role in inter-chain cross-linking) (Hauschka, 1985), and the activity of growth factors (Povolny et al., 1987; Tsutsumi et al., 1987), the possible significance of such action is unknown.

Measurement of Osteocalcin

A fraction of de novo osteocalcin is not incorporated into the bone matrix but released into the general circulation (Hauschka et al., 1989). Measurement of circulating OC should therefore give a reliable estimate of the level of OC production and therefore osteoblastic activity. Since development of the first assay for this protein (Price & Nishimoto 1980), many immunoassays to measure serum OC have been designed (for review see Power & Fottrell, 1991a). These assays have utilised a wide range of monoclonal and polyclonal antisera (Power et al., 1989; Power & Fottrell, 1991a; Tracy et al., 1990), in both radio-isotope and enzyme-immunoassay formats (Egsmore et al., 1989; Hosoda et al., 1992; Monaghan et al., 1993). Such differences in format, as well as diversity of antibody source, epitope recognition site and matrix of calibration material have contributed to a lack of standardisation for OC assays. This effect is so marked that results from different assays are difficult to compare, even when values are normalised against those from healthy subjects (Masters et al., 1994).
Osteocalcin contains two arginine-arginine sequences at amino acids 19-20 and 43-44. These are possible sites of lesion for protease enzymes and result in a number of potential OC fragments; 1-19; 20-43; 44-49; 1-43; and 20-49 (Hosoda et al., 1992; Prigodich et al., 1985) (see Figure 1.5). Such fragments are produced by both *in vivo* and *in vitro* degradation of intact osteocalcin (Blumsohn et al., 1995a). The predominant degradation product appears to be the N-terminal midregion fragment (OC^1-43), which represents approximately 30% of the total immunoreactive osteocalcin in both normal individuals and osteoporotic patients (Gamero et al., 1994b). Assays which measure OC^1-43 as well as the intact molecule (Gamero et al., 1992), exhibit less apparent instability on storage than assays measuring intact OC alone (Blumsohn et al., 1995a; Gamero et al., 1994b). This indicates that while intact OC is highly susceptible to *in vitro* proteolytic degradation, the major OC^1-43 fragment is more stable. Furthermore the N-terminal, mid and mid-C-terminal fragments are detected in much smaller amounts than the N-terminal-mid fragments (Gamero et al., 1994b). Such findings are likely to be due to the easier accessibility of the 43-44 peptide bond to proteolytic cleavage, an effect possibly due to the conformation of the α-helix structure in the presence of calcium ions (Hauschka & Carr, 1982). Short C-terminal peptides, which may be theoretically detected by some assays are thought to undergo quick *in vivo* degradation in serum (Gamero et al., 1994b). Low molecular weight fragments of OC detected in HPLC studies with RIA quantitation (Taylor et al., 1990; Gundberg & Weinstein, 1986) have previously been speculated to be due to the release of OC fragments from bone matrix during resorption. A further study using monoclonal antibodies also detected such low molecular weight fragments (Tracy et al., 1990). The identification of such fragments and their subsequent quantitation (Gamero et al., 1994b), showed the levels of the different immunoreactive forms to be unchanged in osteoporosis compared with normal subjects.
KEY:

- Green: Human and bovine common sequence
- Blue: Human specific sequence
- G: g-carboxyglutamic acid (GLA) residues
- C: Carboxy terminal
- N: Amino terminal
- Arrow: Potential proteolytic sites

Figure 1.5: Diagrammatic representation of the osteocalcin molecule and its potential fragments.
This is strongly suggestive that such fragments are not products of bone resorption. Certainly the major 1-43 fragment is not a product of bone resorption, as serum levels of this peptide are not reduced by administration of anti-resorptive agents (Garnero et al., 1994b).

Antisera whose epitope contains one of the three Gla residues detect OC more effectively in the presence of calcium (Egsmore et al., 1989; Tanaka et al., 1986; Taylor et al., 1988). Any undercarboxylation of the glutamate residues during post-translational processing of OC would reduce their calcium-binding ability and thus the detection of OC by calcium dependent antisera. As carboxylation of OC is a vitamin K-dependent process (Hauschka et al., 1989), and many osteoporotic patients have been found to be vitamin K deficient (Hart et al., 1985), OC values measured by such assays may be artifactually low. A further group of antisera whose recognition sites are conformational may have calcium-dependent binding even if the epitope is not in a calcium-binding region (Tracy et al., 1990). The calcium-binding action of anticoagulants means that plasma is not suitable in any calcium dependent assay. In other assays, lithium heparinised plasma gives the best correlation with serum (Power & Fottrell, 1991b). Serum OC is sensitive to sample haemolysis, as erythrocyte hydrolases in the red cell lysate produce low molecular weight fragments not recognised by assay antibodies. It is postulated a similar effect may contribute to the lower OC values found in plasma (Power & Fottrell, 1991b; Tracy et al., 1990).

Polyclonal antibody based assays indicate a greater loss of OC on storage than their monoclonal counterparts, yet produce results two to three times lower than assays measuring both intact and OC1-43 (Garnero et al., 1994b). Such evidence supports the contention that conventional polyclonal based assays preferentially measure intact OC with poorer recognition of the large N-terminal midfragment. Indeed Price and Nishimoto (1980) in their original polyclonal RIA found the C-terminal region of OC necessary for antibody recognition. That polyclonal RIAs produce lower values than assays for intact OC alone is probably due to the immunochemical heterogeneity of circulating OC, for which the structural basis is unclear (Deftos et al., 1992). In order to obtain reliable results using conventional RIAs or assays specific for intact OC, sampling, separation and storage conditions need to be carefully controlled to avoid in vitro proteolytic degradation of osteocalcin. While the use of protease inhibitors in sample collection tubes has been advocated (Banfi & Daverio, 1994), such measures have not been widely adopted. It has been suggested that the higher OC values produced by monoclonal antibody assays may be partially due to reactivity with OC fractions of higher molecular weight than intact OC (Tracy et al., 1990). These forms of OC are postulated to be aggregates or protein bound OC and are apparent in normal serum (Tracy et al., 1990; Price & Nishimoto, 1980). While proteolytic cleavage of the OC propeptide is thought to be an intra-cellular process, measurable levels of this OC species has been
detected in the serum of normal children but not adults (Hosoda et al., 1993). While the reason for this is unclear it is a further indication that OC species of larger molecular weight than intact OC may appear in normal serum.

Physiological Factors Affecting Osteocalcin

Osteocalcin levels are highest in the first year of life, declining in childhood and rising again at puberty before falling to normal adult values (Blumsohn et al., 1992; Glastre et al., 1990). In adults, concentrations are higher in men than women (Duda et al., 1988; Gundberg et al., 1983; Vanderschueren et al., 1990), until female values increase at the menopause (Kelly et al., 1989; Vanderschueren et al., 1990), a rise which may be reversed by oestrogen treatment (Johansen et al., 1988). The influence of age on OC in either sex after the sixth decade is not clear, with contradictory findings reported (Power & Fottrell, 1991a). Osteocalcin exhibits a circadian variation, peaking between 2400 and 0400 with a nadir between 1000 and 1200 hours (Nielsen et al., 1991; Gundberg et al., 1985). Such changes are marked, enforcing standardised timing of blood collections for OC measurement. Seasonal variations in OC have been noted although the identification of the respective peak and trough months differ (Mosekilde et al., 1986; Thomsen et al., 1989). Day to day variations in OC concentrations make small changes difficult to interpret. Variations during the course of the menstrual cycle also occur, levels peaking during the luteal phase (Nielsen et al., 1990b). Alcohol intake decreases serum OC in a dose dependent manner (Nielsen et al., 1991c), providing a possible explanation of the association between alcohol consumption and increased incidence of osteoporosis. OC concentrations correlate well with histomorphometrically measured bone formation (but not resorption) and with isotopic measurements of bone turnover in normal individuals and in post-menopausal osteoporosis and hyperparathyroidism (Brown et al., 1984; Charles et al., 1985).

Clinical use of Osteocalcin.

Elevated serum OC levels reflect increased osteoblastic activity in hyperparathyroidism, hyperthyroidism, multiple myeloma with bone metastases, osteogenesis imperfecta and fracture healing (Borsalino et al., 1985; Power & Fottrell, 1991a). The exceptionally high levels occasionally found in patients with renal osteodystrophy reflect increased bone turnover as well as impaired renal function which leads to decreased clearance of OC and its peptides (Delmas et al., 1983a; Malluche et al., 1984). Osteocalcin values in osteoporosis and Paget's disease are examined in more detail in the discussion section of this thesis.

Underdecarboxylated_osteocalcin
Vitamin K levels are depressed in osteoporosis (Hart et al., 1985). The undercarboxylated OC produced by this deficiency has been postulated as a marker of the risk of hip fracture in elderly women (Szulc et al., 1993). Although not widely utilised, assays of undercarboxylated osteocalcin are available (Sokoll et al., 1995; Vergnaud et al., 1993). Their usefulness in the assessment of osteoporosis and other metabolic bone diseases remain uncertain.

**Procollagen Type I Carboxy-Terminal Propeptide (PICP)**

Collagens comprise a highly specialised family of glycoproteins of which there are at least sixteen genetically distinct species. Over 90% of the bone matrix consists of type I collagen, the synthesis of which is a prerequisite for bone growth (Ayad et al., 1994). The rate of type I collagen synthesis should therefore serve as an indicator for the rate of bone formation. Type I collagen is initially synthesised in the osteoblast as a large precursor procollagen molecule. The procollagen helix contains both amino- and carboxy-terminal non-helical trimeric extension domains whose function is to prevent premature association of collagen molecules into fibrils. On secretion of procollagen into the extracellular space, these domains are removed by specific endopeptidases (Peltonen et al., 1985). Both the 100kD carboxy-terminal and the 35kD aminoterminal propeptides are released into the general circulation on a one-to-one stoichiometrical basis with respect to the deposition of type I collagen (Melkko et al., 1990). The measurement of these propeptide residues has therefore been postulated to be indicative of bone formation rate. However type I collagen is not specific to bone, appearing in other tissues, principally skin and tendon (Ayad et al., 1994). This is illustrated by the markedly increased PICP levels found in interstitial fluid of surgical wounds during wound healing (Haukipuro et al., 1991). Furthermore the correlation between PICP and histomorphometrically assessed bone formation rate, although good, has been found to have a positive intercept (Parfitt et al., 1987). This is indicative of a contribution towards serum levels of PICP from non-osseous sources. Conversely patients with ovarian carcinoma, a condition producing PICP concentrations in ascites fluid twenty times higher than those in normal serum, have essentially unaltered serum PICP levels (Risteli & Risteli, 1993). On this basis, the contribution of soft tissue collagen to serum PICP concentrations would appear to be quite small.

PICP is cleared from the circulation by the mannose receptors of liver endothelial cells, a process subject to hormonal regulation (Risteli & Risteli, 1993; Smedsrød et al., 1990). PICP measurements in patients with hepatic dysfunction should therefore be interpreted with caution. Indeed raised PICP values have been found in differing stages of alcoholic liver disease, especially hepatitis and cirrhosis (Savolainen et al., 1983). A familial trait of elevated serum PICP concentrations in the absence of increased bone formation has recently been reported (Sorva et al., 1994). If this trait is found to be commonly
occurring, even with variable expressivity, the interpretation of serum PICP measurements will be greatly affected.

Assay of PICP

An assay for PICP was first produced in 1974 (Taubman et al., 1974), a RIA first categorised (incorrectly) as being directed towards the amino terminal of the PICP molecule (Risteli & Risteli, 1986). Redevelopment brought the assay into more widespread use (Melkko et al., 1990), and it became available commercially (PICP, Orion Diagnostica, Finland). This assay uses an antiserum raised against PICP from human skin fibroblast cultures treated with bacterial collagenase. The PICP produced by this method differs slightly at its amino-terminal compared to PICP produced in vivo (Melkko et al., 1990). The use of collagenase treated PICP as an assay calibrator may therefore lead to inaccurate results. A more recent assay prepares PICP by direct purification from human foetal fibroblasts, avoiding the procollagen isolation step and leading to PICP identical to that produced in vivo (Pederson & Bonde, 1994). Whether this technical improvement results in improved clinical performance of the PICP assay remains to be seen. PICP is extremely stable, withstanding multiple freeze-thaw cycles and long term storage at -20°C (Melkko et al., 1990; Pederson & Bonde, 1994).

Clinical Use of PICP

In normal individuals and patients with a range of bone diseases without mineralisation defects, PICP levels correlate significantly but not highly with measurements of bone formation as assessed by both bone histomorphometry (Parfitt et al., 1987) and calcium kinetics (Charles et al., 1994). In most metabolic bone disorders, levels of PICP correlate with other markers of bone formation such as OC and ALP (Pederson & Bonde, 1994). In general, ALP activity was assessed to be more reflective of cortical bone formation rates and PICP trabecular bone formation rates Parfitt et al., 1987). However in conditions in which matrix formation and mineralisation are uncoupled (e.g. osteomalacia), the correlation of PICP with mineralisation disappears (Risteli & Risteli, 1993).

As with many other markers of bone metabolism, PICP concentrations exhibit a diurnal variation, with a 20% increase at night, indicative of increased nocturnal bone turnover (Hassager et al., 1992a). Infusion of growth hormone and PTH increase and decrease PICP concentrations respectively (Brixen et al., 1992; Simon et al., 1988). It is possible that the circadian rhythms exhibited by these hormones produce the variations seen in PICP and other markers of bone formation.

In normal individuals, serum levels of PICP are highest during the first year of life, before falling in childhood. However PICP concentrations at puberty are not as
markedly increased over normal adult values as are osteocalcin and deoxypyridinoline (Vanderschueren et al., 1990). While Melkko et al. (1990) were unable to demonstrate PICP concentrations increasing with age in normal adult women, other workers have found age associated increases consistent with increased bone turnover in older post-menopausal women (Eastell et al., 1993; Ebeling et al., 1992; Sharp et al., 1992). Raised PICP concentrations have been reported in conditions involving increased bone formation such as Paget’s disease (Krane & Simon, 1987; Simon et al., 1984; Taubmann, et al. 1976). However Ebeling et al. (1992), failed to identify elevated levels of PICP in a number of conditions of increased bone turnover. Furthermore, other studies have failed to detect increases in post-menopausal bone turnover (Pederson & Bonde, 1994; Hassager et al., 1993). Serum PICP levels do not appear to be increased in osteoporosis (Gamero et al., 1994a; Hassager et al., 1991; Hasling et al., 1991), yet decreases have been observed on treatment with HRT or anti-resorptive therapy (Hasling et al., 1991; Hassager et al., 1991; Hassager et al., 1993). Attempts to explain such conflicting data have highlighted the differences between the PICP assay antigen and native circulating PICP (Melkko et al., 1990). Perhaps more significant however is the variability inherent in a clearance mechanism for PICP which is hormonally modulated.

**Procollagen Type I Amino-Terminal Propeptide (PINP)**

The amino terminal propeptide of type I procollagen, smaller than its C-terminal counterpart, also differs structurally and biochemically. The globular glycoprotein structure of PICP contrasts with the elongated PINP confirmation produced by a central triple helical domain. PINP also lacks the disulphide bridges which characterise PICP, leading to suggestions that following enzymatic cleavage, PINP may circulate as an intact molecule plus a number of fragments (Ebeling et al., 1992). However studies using radiolabelled PINP in rats indicate a rapid uptake of PINP from the circulation followed by intra-lysosomal degradation, rather than breakdown by extracellular proteases (Melkko et al., 1994).

PICP and PINP are cleaved from opposite ends of the same procollagen molecule on an equimolar basis. Due to their differing molecular masses, serum PINP concentrations should be three-fold higher than those of PICP. This result is found using a new RIA which utilises a trimeric human PINP liberated from the type I procollagen produced by human osteosarcoma cells (Niemi et al., 1996). An earlier described assay (Ebeling, 1992) produces concentrations of PINP one hundred times greater than those of PICP.

Assays of PINP have generally been held to be inferior to those of PICP in the assessment of type I collagen formation. However the acceptance of the hormonal influence on metabolic clearance of PICP and the newer assay of PINP has led to an upturn in interest in the amino terminal marker. In a recent study on the effects of therapy...
in post-menopausal osteoporosis, PINP was shown to exhibit a greater response to treatment than PICP (Davie et al., 1995).

**Osteonectin**

Like osteocalcin, osteonectin is a non-collagenous matrix protein which binds both calcium and collagen (Termine, 1988). While osteonectin is produced by active osteoblasts, it is not specific for bone, having a widespread tissue distribution (Tracy & Mann, 1991). This is illustrated by immunoassay measurements of osteonectin concentrations in serum and plasma. Osteonectin levels in serum are markedly higher than in plasma, indicating substantial production by active platelets. Such poor specificity means that osteonectin assays have no present clinical use in the assessment of osteoblast function.

**Alpha2-HS-Glycoprotein (AHSG)**

This 49 kD protein is not a product or by-product of bone cells, matrix production or breakdown, but is synthesised in the liver (Triffit et al., 1976). Newly synthesised bone matrix takes up circulating AHSG from the plasma, concentrating it within the bone (Pilonchery et al., 1983). Such concentration has led to a postulated role in mineralisation (Dickson et al., 1975). Assuming a constant hepatic production, changes in serum AHSG are inversely proportional to alterations in demand for AHSG by new matrix (i.e. bone formation). This has been confirmed during the increased bone turnover found in Paget's disease, in which serum AHSG concentrations are decreased in comparison to normal adults (Smith et al., 1983), and inversely correlated with ALP activity (Ashton et al., 1976). Conversely the AHSG content of Pagetic bone is markedly increased (Quelch et al., 1984). In normal females low serum AHSG levels are found during the sixth decade, presumably reflecting increased post-menopausal bone loss (Dickson et al., 1983). The ability of AHSG to stimulate bone resorption in vitro indicates a possible role in the regulation of bone turnover (Smith et al., 1983; Colclasure et al., 1988). This may limit its specificity as a marker of bone formation. At present serum AHSG measurements offer no useful addition to the panel of available markers for monitoring of bone turnover. However the relationship between AHSG phenotype and bone mineral density may have a possible clinical use in the identification of those at risk of osteoporotic fracture (Dickson, 1994).

**Free Gamma Carboxyglutamic Acid (Free Gla)**

The largest reservoir of the amino acid Gla in humans is the bone, where it occurs not only in osteocalcin, but as a non-collagenous bone protein known as matrix Gla protein (MGP). This 84 residue species contains five residues of Gla per molecule (Cancela et al., 1990). It has been suggested that the measurement of the Gla residues released from
both these species on bone breakdown may be indicative of resorptive activity (Fournier et al., 1989). Although urinary free Gla levels have been found to be increased in osteoporosis (Gundberg et al., 1983), serum free Gla levels are a poor indicator of bone resorption in early post-menopausal women, and do not significantly decrease on oestrogen treatment (Johansen et al., 1991). The poor sensitivity of this putative marker is most likely due to its presence in a number of other vitamin K-dependent proteins such as plasma proteins and coagulation factors (Petersen et al., 1979).
Biochemical Markers of Bone Resorption

Urinary Calcium

As the mineralised component of bone is essentially a calcium salt, the most obvious marker of bone resorption is the estimation of urinary calcium excretion. This assay is cheap and simple to perform, being available in all clinical laboratories. However twenty-four hour urine collections on a free diet provide limited diagnostic information. While it has been suggested that in a steady state situation urinary calcium excretion reflects intestinal calcium absorption rather than bone resorption (Eriksen et al., 1995), Nordin and Polley (1987), found twenty-four hour urinary calcium excretions not to be related to dietary intake in normal postmenopausal women.

What is undisputed is that in a fasting state, calcium excreted in the urine represents the obligatory calcium loss of the individual. Although dependent on the degree of tubular resorption, calcium excretion is also a function of the filtered calcium load. In fasting individuals the assumption was made that calcium derived from bone resorption is the primary source of that load. This led to the widespread use of fasting urinary calcium (normalised for creatinine concentration to correct for body mass) as a marker of resorptive activity. A diurnal variation of bone turnover, with increased nocturnal activity, is suggested by biochemical markers of both bone formation and resorption (Hassager et al., 1992a; McLaren et al., 1993; Nielsen et al., 1990a; Nielsen, 1991). However evidence suggests that the fasting calcium load is derived not only from resorbed bone mineral, but the PTH-mediated exchange of calcium ions which occurs at the bone-blood interface (Reeve et al., 1990). This exchange does not represent a net calcium loss, and even when the exchange rate is high due to the nocturnal peak of PTH concentrations, it is not reflective of resorptive activity. Fasting urine calcium:creatinine ratios (Ca/Cr) therefore lack specificity as a marker of bone resorption.

Raised fasting urinary calcium:creatinine ratios occur in post-menopausal osteoporosis, hyperparathyroidism, and Paget's disease. Decreased values are found in hypocalcaemic states such as osteomalacia and hypoparathyroidism as well as renal failure (Morris et al., 1993). However the intra-individual variations for fasting urine calcium:creatinine ratios are very large. The coefficient of variation of two consecutive collections has been demonstrated to be approximately 40% in osteoporotic women. Consequently the difference required for values to show a significant change (the critical difference) is very large and limits the use of Ca/Cr for monitoring bone resorption (Vasikaran et al., 1994). Similarly the interpretation of individual clinical results using population based reference ranges is limited by a large inter-individual variation (Morris et al., 1993).
It should be concluded that although cheap and easy to perform, Ca/Cr values are not a reliable indicator of bone resorptive activity.

**Urinary Phosphate**

Even in the fasting state only a small proportion of urinary phosphate excretion is reflective of bone resorption (Nordin *et al.*, 1976). Furthermore urinary phosphate excretion is poorly reproducible, especially in post-menopausal women (Morris *et al.*, 1990). Analysis of 24-hour urinary phosphate excretion is therefore of no great diagnostic value in the assessment of bone turnover.

**Hydroxyproline**

The mineral component of bone is deposited within an organic matrix, the major constituent of which is collagen. An extremely high proportion of glycine, which appears nearly every third residue, gives this protein a remarkably regular amino acid sequence. Collagen also contains two amino acids rarely observed in other proteins; hydroxyproline and hydroxylysine. Proline and lysine are the sole precursors of collagenous hydroxyproline and hydroxylysine, dietary sources of the hydroxylated residues having no role in the final composition of collagen. These post-translational hydroxylations occur only during the synthesis of mature collagen fibrils. The free amino acids are not suitable substrates. Following resorption of bone and breakdown of collagen, these amino acids are not re-utilised for collagen synthesis (Prockop *et al.*, 1979). These compounds, especially the more abundant hydroxyproline, have found a potential role as markers of bone resorption.

Eighty to ninety percent of hydroxyproline released during bone resorption circulates as free amino acid (Kivirikko, 1983), which is almost entirely reabsorbed from the renal ultra-filtrate. Oxidation and enzymatic degradation to carbon dioxide and urea occurs in the liver (Kivirikko, 1983). Hydroxyproline peptides, however, are not subject to renal tubular reabsorption and are excreted into the urine. Consequently urine contains three forms of hydroxyproline, in total comprising only 10-20% of the hydroxyproline released from bone during its resorption.

1. Free amino-acid that has escaped reabsorption (around 5% of total urinary hydroxyproline).

2. Peptides of less than approximately 5 kD (85%).

3. Peptides larger than 5 kD, the only non-dialysable form of hydroxyproline (10%).
The Specificity of Hydroxyproline as a Marker of Bone Resorption

Non-dialysable hydroxyproline, is derived from the degradation of the N-terminal fragment of the procollagen propeptide. Procollagen processing is a prerequisite step in collagen fibril formation and so bone matrix production, and therefore this fraction of hydroxyproline is actually indicative of bone formation, although it lacks the specificity to be used as a marker of this process (Haddad et al., 1970). However it is clear that despite their clinical use, total urinary hydroxyproline concentrations are not specific for bone resorption. Nor is all urinary hydroxyproline derived from bone. The C1q fraction of complement contains significant amounts of hydroxyproline, as do other collagen containing tissues such as skin, tendon, cartilage, etc. (Deacon et al., 1987). Hydroxyproline is absorbed from dietary gelatine, raising concentrations in the urine (Colwell et al., 1990). A number of attempts to overcome dietary influences have been made. The use of a collagen-free diet is effective (Gasser et al., 1979), but laborious to achieve and compliance difficult to monitor. A fast of nine hours removes dietary influences (Hodgkinson & Thompson, 1982), allowing fasting hydroxyproline concentrations to be used once corrected for creatinine concentration. While no seasonal variations in hydroxyproline excretion are found (Vanderschueren et al., 1991), diurnal variations do occur, peaking between 00:00h and 08:00h with a trough between 12:00h and 20:00h (Mautalen, 1970). Fasting urine collections are more susceptible to the diurnal variations which 24 hour collections eliminate (Eastell et al., 1988). Fasting collections at standardised times minimise both dietary and diurnal variation. Intra-individual variations in such samples are lower than in 24 hour collections, giving a more reliable indication of the hydroxyproline:creatinine ratio (Morris et al., 1990; Podenphant et al., 1986). Some workers suggest that a fasting second morning void urine is adequate for reliable estimation. (Wilson et al., 1984).

While Deacon et al. (1987), found hydroxyproline excretion to be a good indicator of radioisotope measured bone resorption, Delmas (1990), reported poor correlation with resorption as assessed by calcium kinetics and bone histomorphometry. As such hydroxyproline will eventually be replaced by more specific assays of bone resorption.

The Clinical Use of Hydroxyproline

In common with most bone markers, apart from a peak during puberty, elevated hydroxyproline concentrations in infancy decline in adulthood (Charles et al., 1985; Stepan et al., 1985). While population based reference ranges for hydroxyproline are greatly limited by large inter-individual biological variation (Morris et al., 1990), cross-sectional studies indicate increased excretion in females in their sixth decade, presumably due to increased post-menopausal bone loss (Hyldstrup et al., 1984). Some workers indicate levels rise by up to 50% within one year of the menopause (Nordin & Polley,
1987), and by a further 20% in post-menopausal women diagnosed as osteoporotic. Although hydroxyproline excretion in osteoporotic women rises, the scatter of values increases, reflecting the heterogeneity of bone turnover within this group. In such circumstances population based reference ranges become even less reliable.

Hydroxyproline has been used to monitor treatment in osteoporosis. Short term oestrogen therapy and bisphosphonate infusion both produce a significant decline in hydroxyproline:creatinine ratios (Aloia et al., 1991; Mallmin et al., 1991; Thiebaud et al., 1994). In early post-menopausal women, Podenphant et al. (1986) found fasting hydroxyproline:creatinine ratios were decreased on anti-resorptive treatment, a change not detected with 24 hour samples. Hydroxyproline excretion correlates well with forearm bone mineral content (BMC) in both adult men and women (Hyldstrup et al., 1984). Urinary hydroxyproline concentrations are significantly increased in hyperparathyroidism, correlating well with raised levels of osteocalcin (Hyldstrup et al., 1984). Significantly elevated hydroxyproline:creatinine ratios (which decrease with treatment), reflect increased bone turnover in Paget's disease (Russell, 1984; Torres et al., 1991b). Correlation with alkaline phosphatase levels in these subjects is high, and either marker may be used to assess disease activity and response to therapy.

The Measurement of Urinary Hydroxyproline

The assay for hydroxyproline is well characterised, having been first described in 1933 (Lang, 1933). Acid hydrolysis of urine at high temperature or with a cation exchange resin releases peptide-bound hydroxyproline (Goverde & Veenkamp, 1972). The hydrolysate is then oxidised with chloramine T to form a pyrrole. After removal of interfering substances, colorimetric quantification of the pyrrole is achieved by reaction with Ehrlich's reagent. The method has been successfully applied to the continuous flow analyser (Blumenkrantz & Asboe-Hansen, 1974) and the centrifugal autoanalyser (Ho & Pang, 1989). Suppression of colour development may occur in urine samples but not aqueous standards, resulting in an underestimation in sample values. This may be avoided by decolouring the urine with activated charcoal or by the use of an internal standard (Buttery et al., 1991). Increased analytical specificity may be conferred by high performance liquid chromatography, a process that with the appropriate equipment may be semi-automated (Dawson et al., 1988; Paroni et al., 1992).

Hydroxylysine Glycosides

Lysine, the other collagen amino acid which is rarely found in other tissues is also post-translationally hydroxylated. As with hydroxyproline, hydroxylysine is not re-utilised after bone resorption for new collagen synthesis. In mature collagen molecules, hydroxylysine is glycosylated, the degree of glycosylation differing according to the collagen type. The monoglycosylated form, galactosyl hydroxylysine (GalH) is most
predominant in bone, while the major form in skin collagen is the diglycosylated species, glucosyl-galactosyl hydroxylysine (GGalH). The ratio of GGalH:GalH is 1.61 in human adult skin but only 0.15 in bone. This compares favourably with the ratio of hydroxyproline present in osseous and non-osseous collagen (Segrest, 1982). GalH may therefore be regarded as a more specific index of bone resorption than hydroxyproline. GalH:creatinine ratios have been found to be significantly negatively correlated with bone mineral content, and Moro et al. (1988) suggested a possible role for this marker in the identification of osteoporotic women. Indeed it has also been suggested that GalH is as effective a marker of bone resorption as urinary pyridinoline (Bettica et al., 1992).

Analysis of GalH is currently only available using reverse phase HPLC with fluorescence detection. Such methodology requires specialised equipment, is labour intensive, technically demanding, and therefore relatively slow and expensive. Consequently the use of this marker has been limited. Evaluation against reference methods is required to assess its possible usefulness as a biochemical marker of bone resorption, while the development of a specific immunoassay would widen the availability of GalH measurements.

Collagen Crosslink Macromolecules

Pyridinium Crosslinks: Formation and Structure

Aggregation of individual collagen molecules in the extra-cellular space leads to the formation of macromolecular collagen fibrils which are initially stabilised by both electrostatic and hydrophobic forces. A time-dependent maturation then occurs due to the formation of covalent crosslinks within and between the collagen molecules. Precursor lysine and hydroxylysine aldehydes produce separate cross-linking pathways (Eyre et al., 1984). The initial products of these pathways, the borohydride-reducible keto-amines, disappear from skeletal connective tissues with age, undergoing conversion to mature non-reducible intermolecular cross-linking compounds (Eyre et al., 1988). Mature crosslinks confer stability and high tensile strength to fibrous collagen and hence are vital to the structural integrity of the skeleton (Eyre & Oguchi, 1980). These molecules, lysylpyridinoline and hydroxylysylpyridinoline have become more commonly known as deoxypyridinoline (DPyr) and pyridinoline (Pyr) respectively. While DPy r is formed exclusively in the mineralised collagens of dentine and bone, Pyr is widely distributed among many tissue types, especially cartilage (Boucek, 1981; Eyre & Oguchi, 1980, Eyre et al., 1988). Both crosslink species are absent from the skin, as post-translational glycosylation of skin collagen differs from other collagens (Pinnell et al., 1971). Pyr and DPy r occur in a molar ratio of 3.5:1 in mature bone (Eyre et al., 1988). During bone resorption these compounds are released and without being further metabolised, excreted
in the urine, mostly in their free form (see Table 1.4) (Gunja-Smith and Boucek, 1981; Fujimoto et al., 1983). As pyridinium crosslinks are formed exclusively in mature collagen, their presence in urine relates only to degradation of collagen established in the extra-cellular matrix, and not that protein which has been synthesised without being incorporated into collagen fibrils. Furthermore, pyridinoline compounds are not significantly absorbed from the diet, adding to their specificity as markers of mature collagen breakdown (Colwell et al., 1990).

<table>
<thead>
<tr>
<th>Crosslink Species In Urine</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>38</td>
</tr>
<tr>
<td>Glycosylated forms and peptides of 550 - 1000 kD</td>
<td>40</td>
</tr>
<tr>
<td>Peptides 1000 - 3500 of kD</td>
<td>15</td>
</tr>
<tr>
<td>Peptides 3500 kD and over</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 1.4: Distribution of pyridinium crosslink forms in normal adult human urine. Species of Pyr isolated by dialysis and measured by HPLC according to Seyedin et al. (1993).

The Measurement of Pyridinium Crosslinking Species

**Pyridinoline and Deoxypyridinoline**

Reference methods for analysis of mature crosslinks utilise HPLC (Black et al., 1988; Fujimoto et al., 1983; Gunja-Smith & Boucek, 1981; James et al., 1990). With minor modifications, the majority of these techniques have common steps of sample hydrolysis, pre-fractionation by partition chromatography, separation by HPLC and fluorescence detection. Such techniques contributed a great deal to the early understanding of crosslink excretion and metabolism (e.g. the presence in urine of both free and peptide-bound forms) (Fujimoto et al., 1983). Despite the introduction of an internal standard and consequent improvements in the precision of this assay (Calabresi et al., 1994), HPLC methods are limited by their technically laborious, time consuming nature and the requirement for expensive specialised equipment.

The introduction of immunoassays has increased the availability of collagen crosslink measurements. While the first such assay was described in 1982 (Robins, 1982a), only in the last few years have commercial assays been produced. The oldest, 'Pyrilinks' (Metra Biosystems Ltd, Oxford, UK) measures free Pyr and DPyr in urine with 100% cross-reactivity (Seyedin et al., 1993). Recognition of glycosylated and peptide bound forms of Pyr is minimal. While the Pyrilinks assay remains available for measurement of general collagen turnover with a possible role in the assessment of rheumatoid arthritis (Astbury et al., 1994; Seibel et al., 1989), it has been superseded by a similar enzyme immunoassay ('Pyrilinks-D' Metra Biosystems Ltd, Oxford, UK). This assay measures
free DPyr and is therefore more specific for bone resorption (Robins et al. 1994a). The monoclonal antibody used exhibits no significant interaction with peptide-bound crosslinks and less than 1% cross-reactivity with free Pyr. Both immunoassays exhibit excellent correlation with appropriate HPLC measurements (Robins et al., 1982; Seyedin et al., 1993; Robins et al., 1994a). A further commercial immunoassay for DPyr (Nichols Institute Diagnostics, Essex, UK.) utilises polyclonal antibodies to measure total DPyr by radioimmunoassay. While this assay claims to be suitable for the analysis of DPyr in serum, virtually no published data is available.

**Type I Collagen Telopeptides**

Type I collagen accounts for more than 90% of the organic matrix of bone (Ayad et al., 1994). As pyridinoline residues are primarily excreted as peptides, a reproducible fraction of cross-linked type I collagen derived pyridinolines would act as a quantitative measure of systemic bone resorption. Within bone type I collagen two pyridinoline forming sites occur; N-telopeptide to helix and C-telopeptide to helix (Eyre et al., 1984). While the C-telopeptide structure is common to all tissues in which collagen is cross-linked, the N-telopeptide interactions are characteristic of those occurring in bone type I collagen (Hanson, et al. 1992). Urinary pyridinoline cross-linked N-telopeptides (NTx) are therefore postulated to be a specific index of bone collagen resorption. The commercial version of this assay ('Osteomark', Lifescreen Ltd, Watford, UK) utilises monoclonal antibody technology in an enzyme-linked immunoassay. NTx has been found to be sensitive marker of bone resorption, correlating significantly with other markers of bone resorption in healthy individuals (Rosen et al., 1994). In early post-menopausal women, NTx excretion correlates positively with urinary DPyr and serum osteocalcin, and negatively with spinal bone mineral density (Gertz et al., 1994). Excretion of NTx alters more dramatically and for a longer duration than that of Pyr or hydroxyproline in bisphosphonate or thyroxine-induced changes of bone resorption in healthy subjects (Rosen et al., 1994).
Figure 1.6: Diagrammatic representation of inter-molecular pyridinium crosslinking between aligned quarter-staggered collagen fibrils. C-telopeptide to helix and N-telopeptide to helix (NTx assay epitope) collagen crosslinks shown. Intra-molecular crosslinking also occurs within each collagen fibril.

A more recent assay for products of bone resorption measures a C-terminal telopeptide sequence of eight amino acids specific for type I collagen ('Crosslaps', Osteometer, Denmark) (Bonde et al., 1994). It is likely that this epitope appears in urine in several forms; as a free peptide, as peptides linked to Pyr and DPyr and possibly peptides linked to the as yet unidentified cross-linking molecules of type I collagen postulated by Kuypers (Kuypers et al., 1992). It is suggested that the direction of antibodies to a linear peptide sequence will allow recognition of that sequence irrespective of the form in which it occurs. The conformational dependency exhibited by the N-telopeptide crosslink (NTx) assay would then be avoided. ‘Crosslaps’ values correlate well with urinary DPyr, Pyr and hydroxyproline, while in women reaching the menopause, the increase in crosslaps excretion, like that of NTx, is greater than the increase in other markers of bone turnover (Bonde et al., 1994; Garnero et al., 1994c).

Clinical Use Of Collagen Crosslinking Compounds

Many assessments of crosslinking compounds of mature collagen as markers of bone resorption have been made. Broadly speaking, the changes in bone resorption in a particular disease state, or a response due to treatment produce similar alterations in all of the markers discussed. What may differ is the magnitude of such changes. Unless stated, the findings discussed below refer to all of this group of markers.
The excretion of collagen-crosslink macromolecules is unaffected by diet and renal function (McLaren et al., 1993; Seibel et al., 1992a). However individuals exhibit significant variation in levels of excretion (Dawson et al., 1988; Schlemmer et al., 1992) and large circadian variations have been reported (McLaren et al., 1993). As with most biochemical markers of bone turnover, markedly elevated values in children and adolescents decrease after the pubertal growth spurt (Hanson et al., 1992). In younger adults, male and female levels do not differ (Beardsworth et al., 1990; Uebelhart et al., 1990). On reaching the menopause, levels in females rise two to three fold, increases which may be reversed by hormone replacement therapy (Uebelhart et al., 1991; Hassager et al., 1992b). In vertebral osteoporosis collagen-crosslink levels correlate with histomorphometric assessment of bone turnover (Delmas et al., 1991), and rate of bone loss as assessed by bone densitometry (Mole et al., 1992; Robins et al., 1990; Uebelhart et al., 1991). Treatment of established osteoporosis with bisphosphonates (Garnero et al., 1994a; Gertz et al., 1994; Harris et al., 1993; Rosen et al., 1994), oestrogen (Uebelhart et al., 1991), or calcitonin (Overgaard et al., 1994), reduces the excretion of crosslinking molecules. Similarly in these markers are elevated (and may be normalised on treatment) in Paget’s Disease, primary hyperparathyroidism and osteomalacia (Delmas et al., 1993; Garnero et al., 1994c; Seibel et al., 1992b; Uebelhart et al., 1990).

Cross-linking compounds of collagen are highly stable in vitro. Numerous freeze-thaw cycles and storage for several days at 4°C has little effect on sample concentration. While it has been recognised that crosslink molecules may be sensitive to ultraviolet light, (Fujimori et al., 1985; Sakura et al., 1982), the light transmission through undiluted urine is extremely poor, and the effect is most marked in aqueous standards. Blumsohn et al. (1995b), recently showed routine exposure to laboratory lighting had no significant effect on measured levels of any of the analytes discussed. Both 24 hour and fasting urine collections are commonly analysed for collagen crosslink indicators of bone resorption. However the correlations found between measurements in these samples is poor. The choice of the optimum sample will be discussed in more detail later in this thesis.

Serum Type I Collagen Carboxy-Terminal Telopeptide (ICTP)

During type I collagen degradation, ICTP is cleaved from the triple helical rod-like collagen type I and circulates in the serum as an immunologically intact fragment. These cross-linked peptides are the precursor sites for the pyridinoline crosslinks that stabilise mature collagen fibrils. Pyridinoline crosslinks bind the telopeptide region of two chains of one type collagen molecule to the telopeptide region of one chain in a second collagen molecule. Like the pyridinoline molecules which circulate and are excreted in the urine,
serum ICTP concentrations are representative of type I collagen breakdown and so serve as an indicator of bone resorption.

A radioimmunoassay for serum ICTP has been described and validated (Risteli et al., 1993) and is commercially available ('Telopeptide ICTP', Orion Diagnostica, Finland). While the ICTP antigen may be prepared from human dermis (which contains type I collagen), only negligible concentrations of ICTP are found in tissue fluids of non-osseous origin. The molecular mass of ICTP has been estimated to be between 9 and 20 kD (Price & Thompson, 1995). Peptides of this size are usually subject to glomerular filtration and some subsequent degree of reabsorption. Accordingly, serum ICTP levels would be expected to rise in subjects with renal dysfunction. Indeed in individuals in whom the glomerular filtration rate is less than two-thirds the lower limit of normal, serum ICTP concentrations are elevated (Risteli et al., 1993).

Clinical use of ICTP

Serum levels of ICTP correlate well with bone resorption as measured by both histomorphometry (Eriksen et al., 1993) and some calcium kinetic studies (Risteli & Risteli, 1993). Charles et al. (1994), while confirming such correlations in a heterogeneous group of high and low turnover metabolic bone diseases, found a poorer correlation in osteoporotic subjects and a lack of correlation in normal controls. Furthermore a correlation between ICTP and the collagen formation marker PICP could not be found in any patient group.

In normal individuals ICTP concentrations exhibit a marked circadian variation, with values at night about 20% higher than those in the afternoon (Hassager et al., 1992a). Increased ICTP levels have been found in hyperthyroidism and primary hyperparathyroidism (conditions resulting in increased bone turnover), while in myxodema low bone turnover rates are reflected by reduced circulating ICTP concentrations (Charles et al., 1994; Eriksen et al., 1993). However clinical studies using ICTP have not been encouraging. ICTP levels are a poor indicator of the activity of Paget's disease, and do not appear to be raised in late post-menopausal women (Alvarez et al., 1995; Filipponi et al., 1994; Garnero et al., 1994a). This marker also poorly reflects reductions in bone turnover induced by HRT or bisphosphonates (Garnero, et al. 1994a; Hassager et al., 1993). Furthermore levels are raised in rheumatoid arthritis (Risteli & Risteli, 1993), indicating a lack of specificity for osseous tissue. As many sufferers of post-menopausal osteoporosis have some degree of rheumatoid arthritis, the interpretation of serum ICTP concentrations in these individuals becomes difficult.
Tartrate-Resistant Acid Phosphatase (TRAP)

The acid phosphatase (AcP) enzymes catalyse the hydrolysis of monophosphate esters in an acid environment (Worthington, 1988). Ubiquitous in nature, AcPs may be differentiated according to tissue distribution, structural and immunological properties, sub-cellular location and other features (Drexler & Gignac, 1994). In humans, acid phosphatases have been found in the prostate, (prostatic AcP) red blood cells (erythrocytic AcP) and in the lysosomes of all cells (lysosomal AcP).

Function of the Acid Phosphatases

The first clinical use of AcP was in the diagnosis of prostate cancer (Gutman et al., 1936b), the diagnostic specificity of which was increased by the indirect measurement of prostatic tartrate-labile AcP activity. In 1970 it was discovered that hairy cell leukaemia cells contained large amounts of tartrate-resistant acid phosphatase (TRAP) (Cy et al., 1970). Recognition of TRAP depends upon its marked resistance to inhibition by dextrarotatory tartrate in contrast to AcP derived from the prostate or lysosomal AcP (Abul-Fadl & King, 1949). The general function of TRAP remains unknown, with no naturally occurring substrate being unequivocally identified (Bevilacqua et al., 1991). It would seem likely that TRAP has multiple functions, with characteristics depending on the cell of origin. In bone, a role in osteoclastic resorption is postulated. In vitro studies show that the resorptive activity of isolated rat osteoclasts correlates well with the activity of TRAP secreted into the incubation medium (Minkin, 1982; Moonga et al., 1990). In humans the correlation between serum TRAP activity and bone resorption (as assessed by histomorphometry), strongly implies a role for the isoenzyme in that process (Moss, 1992). Furthermore the ruffled border of osteoclasts is involved in the release and activation of TRAP (Miller et al., 1985), while inhibition of osteoclastic TRAP by molybdate ions or antibodies leads to a reduction in bone resorption (Zaidi et al., 1989). Similarly bisphosphonate inhibition of bone resorption inhibits the activity of osteoclastic TRAP (Felix et al., 1976).

However osteoclastic TRAP is not itself sufficient to facilitate bone resorption. Adding aluminium fluoride, cholera toxin, or dibutyryl cyclic AMP to culture media inhibits bone resorption, while increasing TRAP activity (Moonga et al., 1990). An in vivo uncoupling of TRAP activity and bone resorptive activity may be found in that form of osteopetrosis (marble bone disease) associated with deficiency of the osteoclastic lysosomal enzyme carbonic anhydrase type II. While this disorder prevents normal osteoclastic resorptive function, TRAP levels are markedly increased, reflecting the abnormal activity of the defective osteoclasts (Smith, 1993). While a reduction in bone resorption may therefore be dissociated from a reduction in TRAP activity, the overall evidence for an essential role for osteoclastic TRAP in the resorptive process is
compelling. As such many workers have advocated the use of TRAP as a biochemical marker of bone resorption.

**Isolation and Identification of AcP Isoforms**

The separation of human AcP isoforms has been facilitated by polyacrylamide gel electrophoresis, with subsequent identification made on the basis of electrophoretic mobility (Heyden et al., 1977). TRAP is the fifth band to appear on such gels, and as such is often characterised as band 5, isoenzyme 5 or human type 5 TRAP. This band consists of two components, denoted bands 5a and 5b, which have the same substrate specificity and inhibitor sensitivities and may be regarded as isoforms of the same enzyme (Janckila et al., 1992). Osteoclast TRAP is indistinguishable from band 5b (Lam et al., 1980). Human TRAP from the erythrocyte is anionic and does not appear on the acidic acrylamide columns. While its existence is therefore not properly recognised when band 5 AcP is characterised as TRAP, erythrocytic AcP has different substrate specificity, cross-reactivity to antisera, molecular weight, and electrophoretic mobility from other TRAP isoforms (Echtebu et al., 1987; Lam et al., 1982; Whitaker et al., 1989). Molecular biology techniques have revealed only a single human TRAP gene, indicating differing TRAPs arise from translational and post-translational processing of the same mRNA transcript (Ling & Roberts, 1993)

**Analysis of TRAP**

TRAP may be measured in a number of different ways. Best characterised and utilised are methods based on the catalytic activity of the enzyme. A variety of substrates have been used, most notably 4-nitrophenylphosphate (4-NP) and α-napthylphosphate (α-NP). Measured concentrations are substrate dependent, with the faster hydrolysis of 4-NP leading to higher values than are given using α-NP (Drexler & Gignac, 1994). Indeed α-NP is the preferred substrate for the prostatic fraction (Babson et al., 1959). Tartrate inhibition of enzyme activity is more marked towards the hydrolysis of 4-NP both in electrophoretic studies and patient samples (Lam et al., 1978a; Lam et al., 1978b). Schiele et al. (1988), compared both substrates in two patient groups; children aged 4-9 years, and adults in their third decade. Levels of both total AcP and TRAP decreased with age using both methods, however with 4-NP the decrease in TRAP contributed to 85% of the total, while this figure was only 70% using α-NP, supporting the contention that α-NP is less specific for TRAP than 4-NP.

As such 4-NP has been the preferred substrate of many workers. Lau et al. (1987), described a serum assay based on the reaction with 4-NP, despite the fact that erythrocyte rupture and consequent increase in erythrocyte TRAP are unavoidable in serum preparation. Diluted serum requires pre-treatment at 37°C in order to remove the reactivity of this isoform towards 4-NP and to lessen the effects of a non-competitive
inhibitor of TRAP found in serum. Such treatment does not inhibit the activity of platelet TRAP released during clotting, which leads to 5-10% greater values in serum than plasma (Chen et al., 1979).

Conversely erythrocytic TRAP lacks activity towards α-NP (Lam et al., 1978a; Small & McNutt, 1984), while isoenzyme studies using α-NP as substrate have found platelet AcP to be tartrate sensitive (Chen et al., 1979). According to immuno-cytochemical analysis only cells of the mononuclear phagocyte system, osteoclasts and alveolar macrophages express TRAP under normal conditions, while AcP is present in all leukocytes (Drexler & Gignac, 1994). Thus while cells in serum contribute to background AcP levels, the presence of tartrate with α-NP as substrate reduces their interference. Plasma samples are unsuitable for α-NP assays; a slow precipitation of fibrinogen resulting in large apparent increases in TRAP activity (Small & McNutt, 1984). While the α-NP method does display positive interference by bilirubin, its easy adaptation to automation, with no sample pre-treatment, affords analytical simplicity. However the low catalytic activity exhibited by TRAP to this substrate limits the sensitivity of the assay. This is compounded by a non-specific pseudo-catalytic activity of the Fast Red TR chromagen which occurs in the absence of substrate (Cooper et al., 1982).

A further problem in the analysis of TRAP based on its activity is the inherent instability of the enzyme itself. Storage at -70°C seems to have no significant effect on the levels of TRAP activity, but -20°C is less favourable, Lau et al. (1987), reporting a significant decline in activity with length of storage at this temperature. As -70°C storage may not be available to all, a range of recommendations for sample storage at -20°C are available. Some workers advocate analysis immediately or within two hours (Schiele et al., 1988, Stepan et al., 1983). Others allow storage; for one week, two weeks or 'until analysis' (Lam et al., 1978a; Scarnecchia et al., 1991; Torres et al., 1991b). Sample acidification, recommended by the manufacturers of commercial assay kits, may lead to an increase in storage time without loss of activity (Cooper et al., 1982).

Clinical Use of TRAP

In children and adolescents TRAP activity is greater than in normal young adults (Monti et al., 1990; Panteghini & Pagani, 1989). Indeed TRAP accounts for around 90% of total AcP activity in children aged up to twelve years. TRAP is elevated in Paget's disease, primary hyperparathyroidism, chronic renal failure and multiple myeloma with lytic bone lesions (Kraenzlin et al., 1990; Scarnecchia et al., 1991; Stepan et al., 1983; Monti et al., 1990; Torres et al., 1991b). Decreased TRAP concentrations in patients with the hereditary defect osteogenesis imperfecta suggest a reduction in bone turnover in this condition (Rico et al., 1991).
In females TRAP levels increase in both naturally and surgically induced menopause (Revilla et al., 1992; Rico & Villa, 1993; Stepan et al., 1987). Raised levels have also been found in post-menopausal osteoporosis (Monti, et al. 1990; Panteghini & Pagani, 1989; Schiele et al., 1988). Furthermore TRAP activity has been shown to correlate negatively with bone mineral content and bone mineral density (de la Piedra et al., 1989). Unfortunately this correlation cannot be converted into a significantly accurate regression equation for predictive use. In conditions of increased bone resorption, TRAP activity returns to normal on treatment with anti-resorptive agents, (Rico & Villa, 1993; Stepan et al., 1989) or in the case of primary hyperparathyroidism, surgical removal of the parathyroid adenoma (Scarnecchia et al., 1991).

Urinary hydroxyproline:creatinine ratios, (Lam et al., 1980), levels of ALP (Revilla et al., 1992; Torres et al., 1991b), B-ALP (Kraenzlin et al., 1990), osteocalcin, (Torres et al., 1991b) and the bone derived growth factor β2-microglobulin (Rico & Villa, 1993), all correlate well with TRAP activity in a number of conditions of elevated bone resorption. However findings in groups with normal or moderately increased bone turnover are more equivocal. While Panteghini et al. (1989), found a good correlation between TRAP and B-ALP in children, this did not hold in adults over 20 years of age, while Schiele et al. (1988), found the correlation between TRAP and total ALP highly significant in the case of women and children, but not so in men. This may reflect a degree of non-specificity due to interference from the higher levels of prostatic AcP found in men. At typical substrate and tartrate concentrations, inhibition of the prostatic fraction is approximately 95% (Whitaker et al., 1989). As the background of tartrate-sensitive isoenzymes increases, the effect of incomplete inhibition becomes predominant, and a significant over-estimation of TRAP may ensue. Such effects, marked in spectrophotometric assays, are minimised by immunoassay techniques.

Many different reference limits have been published for TRAP. Differences may be accounted for by method (immunoassay or spectrophotometric), substrate (4-NP or α-NP), buffer (citrate or acetate), pH, or the presence of transphosphorylating agents such as 1,5 pentandiol. Within the group of assays based on catalytic activity, disagreements on the choice of substrates continue.

It would seem that immunoassay technology offers the best hope for the routine use of TRAP as a biochemical marker of bone resorption. Considerable efforts have been directed to this task over the last 10-15 years (Kraenzlin et al., 1990; Whitaker et al., 1989). Despite this, no validated assay for osteoclastic or type 5 TRAP is currently available for commercial use, although very recently immunoassays claiming to be specific for osteoclastic TRAP have been described (Chamberlain et al., 1995; Cheung et al., 1995).
Non-Biochemical Diagnosis and Assessment of Metabolic Bone Disease.

Biochemical indicators of calcium homeostasis and bone turnover may aid the diagnosis, assessment and understanding of the metabolic bone diseases. However these assays are only part of a range of investigations available to the clinician in this field.

Bone Mineral Content and Bone Mineral Density

Even within the context of ever accelerating medical technologies, a thorough physical examination and medical history remain the cornerstone of accurate diagnosis (Whyte, 1993). Historically the only non-invasive methods available for the direct evaluation of bone mineral have been radiographic. However 30-50% of bone may be lost without alteration in radiographic appearance, severely limiting the sensitivity of such techniques (Andran, 1951; Urist et al., 1962). Attempted improvements have included the photodensitometry of X-ray films, the grading of changes in the trabecular pattern in the upper end of the femur (the Singh Index), and radiographic morphometry, the measurement of cortical bone thickness in various long bones (Anderson et al., 1966; Barnett & Nordin, 1960; Singh et al., 1972) While such techniques now have little application in the developed world, their cheapness and easy availability means a continued role in countries where resources are limited. Qualitatively, the role of radiography in the diagnosis of fracture, (especially spinal crush fractures, which may be asymptomatic), remains assured.

Today measurement of bone mineral density (BMD) may be undertaken with the accuracy and precision required to detect relatively small changes in bone density within individuals. The principle of densitometric techniques is broadly similar to that of conventional radiography in that the incident energy absorbed by the bone is proportional to its density. Initial applications of this technology used monochromatic photons from a radionuclide source (single photon absorptiometry), to measure bone at peripheral skeletal sites. From the 1960’s onwards the use of a dual photon sources allowed compensation for soft tissue thickness and accurate though not particularly precise measurement of bone mineral content (BMC) and subsequently BMD (Cameron & Sorenson, 1963; Reed, 1966). Replacement of the isotopic energy with low energy X-rays led to the development of dual-energy X-ray absorptiometry (DXA or DEXA), and a consequent improvement in measurement precision (Cullum et al., 1989). Although alternative methodologies such as broad band ultrasound attenuation (Langton et al., 1984) and quantitative computed tomography (Isherwood et al., 1976) are available, DEXA is currently the method for the assessment of BMD best combining affordability, accuracy, precision and low radiation dose.
Bone Biopsy and Histomorphometry

The concomitant study of both bone cell activity and matrix turnover is only achievable by examination of sections of the bone itself. The method by which these sections are obtained is known as bone biopsy. Quantitative examination of bone obtained in this manner has become known as histomorphometry. Introduced in the 1960's, histomorphometry has contributed much to the understanding of bone physiology in both normal bone and in patients suffering from metabolic bone disease (Recker, 1993).

The standard site of bone biopsy is the anterior iliac crest, due to the easy accessibility of bone at this site. Furthermore trabecular, cortical, periosteal and subcortical bone are all present in such sections (Christiansen et al., 1993). A specialised instrument, a bone trephine, is used to obtain a bone core of 5-10 mm in diameter (Lalor et al., 1986). Undecalcified sections allow the measurement of matrix mineralisation. Dynamic measurements of turnover may be made by giving the patient two courses of tetracycline antibiotics (which stain the bone), at known intervals before the biopsy procedure (Frost, 1969).

Histomorphometry has a number of inherent limitations. Interpretations on which many measurements are based are subjective, leading to inter-observer variation in results. The procedure is invasive, leading to a finite if small risk for the patient and an absence of reference data based on large populations. Furthermore the site specific nature of the sampling may mean the measurements produced may not reflect the skeleton as a whole. Access to the technical laboratory expertise required may also be limited.

In certain clinical conditions such as osteomalacia, bone biopsy remains the only reliable means of diagnosis and assessment of disease severity. It has previously been postulated that bone biopsy may be used to distinguish the heterogeneity of bone turnover in osteoporosis in order to target and assess appropriate treatments. However biopsy of osteopenic bone is fraught with the possibility of crushing the core due to the nature of the specimen. It is likely that combinations of bone densitometry and biochemistry may afford a more convenient approach to targeting and monitoring of therapy in osteoporosis.

Radioisotope Techniques

The use of radioisotope tracers with bone avidity such as $^{47}$Ca, $^{18}$F, $^{85}$Sr and $^{99}$Tc have been used in conjunction with balance studies and quantitative scanning methods to assess bone turnover and skeletal blood flow (Reeve, 1990). Isotopic balance studies suffer from similar problems to those of conventional calcium balance studies, coupled with the need to give a radioactive dose to the patient. The estimation of bone blood-flow is useful in the identification of sites of activity ("hot-spots") in Paget's disease. While
estimates of bone turnover by isotopic scanning provide dynamic information on bone metabolism, such procedures tend to remain the preserve of specialised research departments.
CHAPTER II - Methods

Assay Performance Characteristics

Accuracy

The clinical usefulness of any analytical method is dependent on pre-analytical, analytical and post-analytical factors (Marshall & Bangert 1995). These include for example, biological factors relating to the patient, analytical data identified during the validation of a method, and the predictive value of the test when the results are interpreted in a clinical environment.

In general a method should be validated considering its reproducibility, its accuracy, i.e. the ability of an assay to produce results that reflects the true values, its specificity and its analytical sensitivity which indicates the lowest value for a biological sample that can be distinguished from zero. In modern laboratory practice where commercial kits are often used, the experiments to test the specificity and cross-reactivity in particular may be difficult and not cost effective to perform. However, the reproducibility, the clinical significance of small changes in the value of an analyte in relation to the sensitivity of a method, as well as the possible interference of a related analyte, e.g. cross-reactivity of different isoforms of alkaline phosphatase, are of prime importance in the interpretation of results in clinical practice.

For routine methodologies the estimation of accuracy is relatively easy. Commercially available control sera containing known concentrations of a number of common analytes is widely available. By circulating batches of such serum to a large number of laboratories, method based consensus values are generated. Control sera used for routine analysis in these studies ('Seronorm' and 'Pathonorm', Nycomed Ltd, Birmingham, UK) is available at both 'normal' and abnormally high concentrations of analytes to indicate accuracy over a wide analytical range. Participation in an independent quality assurance scheme (Randox International Quality Assurance Scheme, Randox Laboratories, Co. Antrim, UK) in which control sera are analysed 'blind' allows independent assessment of methodological bias as well as performance comparison with other laboratories. This laboratory performed acceptably in this scheme throughout the course of the studies which comprise this thesis.

Accuracy for less commonly performed specialist immunoassays is more difficult to gauge. As relatively few laboratories perform the immunoassays used in this thesis, no external quality assurance schemes exist. Accuracy is therefore determined using the control materials (usually two concentrations) provided by the assay manufacturer.
Controls are repeated at the beginning and end of each assay in order to monitor intra-assay drift.

**Precision**

All immunoassays and radioreceptor assays were performed in duplicate. Performance characteristics detailing assay precision should ideally be investigated in the users laboratory. This is accomplished by repeated measurements of a number of specimens both within a single assay run (intra-assay variation) and over a number of assay runs (inter-assay variation). While this was easily achieved for routine colorimetric methods, financial constraints and the limited number of assay runs performed prevented such action with the (markedly more expensive) immunoassays used. In an attempt to indicate the precision of such assays, relevant information provided by the manufactures has been summarised and included with the description of the assay methodology. For more detailed information, the package inserts/assay instructions provided by the manufacturer of each assay are included in the appendix to this thesis as is the extracted data in tabulated form.

**Serum Calcium**

Measurement of total serum calcium is based on the reference method for atomic absorption spectrophotometry as described by Cali et al. (1973).

**Apparatus**

Varian AA-20-ABQ double-beam atomic absorption spectrophotometer (Varian Ltd, Walton-On-Thames, UK)

Variable Diluter (Hook & Tucker Instruments Ltd, UK)

**Reagents**

‘Spectra-pure’ calcium carbonate (Johnson & Matthay, UK)

Lanthanum chloride solution 10% w/v (AAS grade, Fisons Scientific Equipment, Loughborough, UK)

**Reagent Preparation**

Stock blank solution (diluent for preparation of standards):

8.182 g sodium chloride (NaCl)

372.8 mg potassium chloride (KCl)
Make up to 1 litre with distilled water. This gives a solution of 140 mmol/L sodium and 5 mmol/L potassium.

**Stock standard:** 50 mmol/L

Calcium carbonate should be heated at 110°C for 24 hours to ensure an anhydrous sample.

Weigh 1.2512 g of the dried CaCO₃ into a 250 ml volumetric flask.

Add approximately 50 ml distilled water. Then add sufficient concentrated HCl to ensure the CaCO₃ is in solution before making up to volume with distilled H₂O.

**Working standards**

Using ‘A’ grade volumetric glassware, pipette respectively 4.0, 5.0 and 6.0 ml stock standard into 100 ml volumetric flasks.

Make up to volume with stock blank solution. This gives working standard concentrations of 2.00, 2.50 and 3.00 mmol/L.

**Sample diluent**

10ml AAS. Grade lanthanum chloride
10ml concentrated HCl
Dilute to 1 litre with distilled water

**Sample Preparation:**

Samples, standards and quality control samples are prepared in the same manner.

0.1 ml sample, standard or control.
5.0 ml acidified lanthanum sample diluent.
Vortex thoroughly.

All dilutions are performed in duplicate.

**Instrument Conditions**

Wavelength: 422.7 nm
Slit Width: 0.5 nm

Air-acetylene stoichiometric flame: Compressed air flow 3.0 ml/min; acetylene flow 1.5 ml/min.

"Hi-vac" nebuliser.

**Procedural Notes**
1. All glassware, including test tubes for sample dilution should be soaked overnight in a solution of 1.0 mol/litre HCl. Several washes with distilled water follow.

2. Working standards are prepared in the stock blank solution containing 140 mmol sodium and 5 mmol potassium. This ensures the standards are analogous to the samples in containing physiological quantities of these elements.

3. The sample diluent contains acidified lanthanum chloride. Lanthanum removes the inhibition produced by poorly volatile salts of phosphate and sulphate. Pybus et al. (1970), found a pH below 2.5 and a 10 mmol/L lanthanum concentration would adequately suppress interference from up to 16 mmol/L phosphate. Although our lanthanum concentration is slightly less than this (7.2 mmol/L), the concentrations of phosphates in serum or urine at the dilutions used are very much lower than 16 mmol/L. The magnitude of the dilution also serves to remove the variable interference conferred by serum proteins.

4. On asking the instrument to 'read', a five second programmed delay occurs during which sample solution is aspirated but no absorbance reading taken. This allows the flame to stabilise. Two, three-second integrations of the absorbance measurements are then made, and the CV between them reported. Solutions with CVs greater than 2.5% between the two integrations were re-sampled.

**Performance Characteristics**

Duplicate concentrations should be within 0.03 mmol/L. If this target was not reached a further dilution was made. At the lower end of the standard curve 2.00 ± 0.03 mmol represents a CV between samples of 1.1%. The standard deviation falls further as the ± 0.03 mmol/L tolerance is applied to higher concentrations. The inter-assay precision equals 1.3% at both 2.60 and 3.06 mmol/l (n=25).

**Albumin**

Determination of serum albumin is performed by a spectrophotometric method based on the binding to albumin of bromocresol green (BGC). The method is applied to the Cobas Bio autoanalyser with absorbance at 600 nm measured after the standard reaction time of two minutes (short BCG) as according to the method of Doumas et al. (1971).

**Apparatus:**
Cobas Bio Autoanalyser (Roche Diagnostic Systems, Welwyn. UK)

**Reagents:**
BCG Albumin Reagent (Randox Laboratories Ltd, Co. Antrim. UK)
The absorption maxima for the BCG-Albumin complex is 630 nm. The Cobas Bio analyser used in this study is only equipped with a spectrophotometric filter to measure 600 nm. However this change makes no significant difference to the albumin values obtained (Hill, 1985).

The intra-assay coefficient of variation equals 1.5% at 43 g/L (n=25). Inter-assay CV is 2.3% at 34 g/L and 3.0% at 45 g/L (n=10).

**Phosphate**

Although organic and inorganic forms are present in serum, only inorganic phosphate is ordinarily measured. The measurement is based on the reaction of phosphate ions with ammonium molybdate to form a spectrophotometrically measurable phosphomolybdate complex.

**Apparatus:**
Cobas Bio Autoanalyser (Roche Diagnostic Systems, Welwyn. UK).
**Reagents:**
Unimate 7 PHOS (Roche Diagnostic Systems, Welwyn. UK).

Assay characteristics: intra-assay CV=1.4% at 2.22 mmol/l (n=25); inter-assay CV=6.0% at 1.35 mmol/l, 3.9% at 2.72 mmol/l (n=10).

**Alkaline Phosphatase**

ALP activity was analysed using a commercial assay kit (ALP opt., Randox Laboratories, Co. Antrim UK) adapted by the manufacturer to the Cobas Bio autoanalyser.

\[
p\text{-nitrophenylphosphate } + \text{H}_2\text{O} \xrightarrow{\text{ALP}} \text{phosphate } + \text{p-nitrophenol}
\]

The p-nitrophenol reaction product is measured spectrophotometrically at 405 nm.

Intra-assay CV; 1.6% at 537 IU/L (n=25). Inter-assay CV 4.7% at 221 IU/L; 4.4% at 586 IU/L (n=10).

ALP activity in patient sera and the assessment of analytical CV using control sera are affected by storage conditions. While serum ALP activity increases with storage at room temperature or when refrigerated (2% per day), activity in frozen sera is thought to decrease, slowly recovering on thawing (Moss & Henderson 1994). A similar increase in activity has been noted in the reconstituted lyophilised preparations used as control sera. As the analytical variation of this assay is calculated using repeat measurements of such material it was decided to investigate this effect experimentally.
Changes in ALP Activity on Thawing of Frozen, Previously Lyophilised Control Material.

Method

Lyophilised control sera both 'normal' and 'elevated' ('Seronorm' and 'Pathonorm', Nycomed Ltd, Birmingham, UK) were reconstituted according to the manufacturers instructions. Aliquots of the serum were then frozen for several days at -20°C. Aliquots of serum from normal volunteers (n=5) were stored in the same way. On the day of analysis a single aliquot of each sample was removed from the freezer and placed at room temperature. Exactly one hour later, further aliquots were removed from storage. This process continued for a total of eight samples. The final sample was at room temperature for only that time required to enable it to thaw from frozen. This 'thawing time' was disregarded and the final sample designated t=0. Previous samples were identified according to the time spent thawed at room temperature 'post-thaw'. ALP activity for each sample was then analysed in duplicate in a single batch.

Results

The increase in activity relative to the sample with the shortest time at room temperature after thawing (t=0) is shown in Table and Figure 2.1.

<table>
<thead>
<tr>
<th>Thaw time (Hrs at room temp)</th>
<th>'Normal' Control (IU/L)</th>
<th>'Normal' Control (% of t=0)</th>
<th>'High' Control (IU/L)</th>
<th>'High' Control (% of t=0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>128.1</td>
<td>100.00</td>
<td>289.7</td>
<td>100.00</td>
</tr>
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<td>1</td>
<td>142.4</td>
<td>111.16</td>
<td>296.2</td>
<td>102.24</td>
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<td>2</td>
<td>153.3</td>
<td>119.67</td>
<td>313.8</td>
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<td>131.54</td>
<td>330.4</td>
<td>114.05</td>
</tr>
<tr>
<td>5</td>
<td>174.4</td>
<td>136.14</td>
<td>342.1</td>
<td>118.09</td>
</tr>
<tr>
<td>6</td>
<td>175.6</td>
<td>137.08</td>
<td>342.2</td>
<td>118.12</td>
</tr>
<tr>
<td>7</td>
<td>180.6</td>
<td>140.98</td>
<td>348.0</td>
<td>120.12</td>
</tr>
</tbody>
</table>

Table 2.1: Increase in ALP activity on thawing of frozen reconstituted lyophilised control material. Samples from five normal human volunteers demonstrated no increase in activity during this period.

Discussion

The data indicates a time dependent increase in ALP activity on thawing reconstituted previously lyophilised control sera. The rate of increase decreases with time, being most
It is clear that inter-assay variation assessed by using repeated measurements of thawed previously lyophilised control sera may be artifactually increased unless the time such samples spend at room temperature is standardised. The negligible increase in ALP activity in previously frozen patient sera is similar to that reported in the literature (Moss & Henderson 1994) and is of little clinical significance. In practice this laboratory removes all samples for ALP analysis from frozen storage at 0900 hours with sample analysis at 1600 hours.

**Urinary Hydroxyproline**

Hydroxyproline is quantified using a two stage process. Chloramine T is used to oxidise hydroxyproline to a pyrrole which is subsequently incubated with p-dimethylaminobenzaldehyde (Ehrlich’s reagent). The reaction product is then measured by colorimetry. As only free hydroxyproline reacts with the chloramine T, samples are subjected to acid hydrolysis in order to release peptide bound hydroxyproline. Urines are decolourised with activated charcoal, and the addition of a cation exchange resin and a citrate/acetate buffer brings the test sample to the appropriate pH. The final quantification step is automated using a Technicon autoanalyser. This is a combination and modification of several methods (Lang, 1933; Blumenkrantz & Asboe-Hansen, 1974).
Urinary Hydroxyproline

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A diagrammatic representation of the autoanalyser flow method is shown in Figure 2.1

Equipment

Continuous flow autoanalyser (Technicon Ltd, UK).

Reagents

Citrate / Acetate Buffer
50 g citric acid monohydrate (Sigma Chemical, Poole, Dorset, UK).
72 g anhydrous sodium acetate (Fisons Scientific Equipment, Leics, UK).
34 g sodium hydroxide AR grade (Fisons Scientific Equipment, Leics, UK).
12 ml glacial acetic acid AR grade (Fisons Scientific Equipment, Leics, UK).

Make up to 1 litre with distilled water, adjust pH to 6.0 with acetic acid or sodium hydroxide.

Oxidising Reagent
0.4986 g chloramine T (Sigma Chemical, Poole, Dorset, UK).
30 ml distilled H2O
50 ml citrate/acetate buffer pH 6.0
20 ml propan-2-ol (Fisons Scientific Equipment, Leics, UK).
Final chloramine T concentration: 17.7 mmol/L.

Ehrlich’s Reagent
7.0 g p-dimethylaminobenzaldehyde (Sigma Chemical, Poole, Dorset, UK).
Dissolve in 30 ml 60% perchloric acid (Fisons Scientific Equipment, Leics, UK).
Make up to 100 ml with propan-2-ol.
Final reagent concentration 0.5 mmol/L.
Resin/Charcoal Powder
40 g of anion exchange resin (Dowex 1 x 8-400, Sigma Chemical, Poole, Dorset, UK)
20 g activated charcoal (Sigma Chemical, Poole, Dorset, UK)
Mix well
Wash mixture several times in a Buchner funnel with 6N HCl.
Rinse with distilled H_2O
Dry with ethanol and diethyl ether.

Standard
1.3113 g of L-hydroxyproline (Fisons Scientific Equipment, Leics, UK), made up to
100 ml with 1M HCl.
This gives a stock hydroxyproline standard of 0.1 M from which working standards of
0.002, 0.004, 0.006, 0.01, 0.015, and 0.02 M are prepared.

Sample Preparation
To 0.5 ml urine add 0.5 ml concentrated HCl, in a stoppered quickfit tube. Heat to 98°C
overnight to complete the hydrolysis.
Add 9.0 ml of citrate/acetate buffer to the hydrolysate, together with a small amount of
the charcoal/resin mixture. This solution is then allowed to settle or is separated by
centrifugation, and the supernatant placed on the autoanalyser.

Assay Characteristics
Repeat analysis of commercially available control sera ('Lyphocheck Urine Control',
Bio-Rad Ltd, Hemel Hempstead, UK) show intra-assay CVs of 6.9% and 1.7%, and
inter-assay CVs of 3.1% and 13% at 0.22 and 1.06 mmol/l.
Figure 2.2: Diagram of autoanalyser analysis of hydroxyproline
Creatinine

Creatinine measurement is based on the Jaffe reaction, first described in 1886 (Jaffe, 1886). In this reaction, creatinine produces a red-orange adduct in the presence of picrate ions in an alkaline medium.

Apparatus:
Cobas Bio Autoanalyser (Roche Diagnostic Systems, Welwyn, UK).

Reagents:
Unimate 7 CREAT (Roche Diagnostic Systems, Welwyn, UK).

Urine samples require appropriate dilution (1:20 or 1:50) in distilled water before analysis. Inter-assay CV: 4.6% at 201 nmol/l; 3.8% at 512 nmol/l (n=6).

Tartrate-Resistant Acid Phosphatase

Automated methods using α-NP as substrate

Boehringer Mannheim ACP (Ref. No. MPR1)
Intra-assay CV is 6.7% at 2.04 IU/L (n=28); inter-assay CV 7.9% at 2.21 IU/L (n=20).

Randox Acid Phosphatase (AC1012 Randox Laboratories, Co. Antrim, UK.)
Intra-assay CV is 7.1% at 2.41 IU/L (n=20); inter-assay CV 6.9% at 2.65 IU/L (n=20).

In both of these methods TRAP activity is measured with an α-NP substrate using a modification of the method of Hillman (1971). The rate of reaction is measured by coupling the α-napthol released from hydrolysis of the substrate with Fast Red TR salt. This gives an azo complex whose formation is monitored spectrophotometrically at a wavelength of 405 nm. The main difference between these two assays is the inclusion of the transphosphorylating agent pentane-diol in the BM system.

\[ \text{α-napthylphosphate} + \text{H}_2\text{O} \rightarrow \text{TRAP} \rightarrow \text{phosphate} + \text{α-napthol} \]

\[ \text{α-napthol} + 4 \text{chloro-2-methylphenyl diazonium salt}^{*} \rightarrow \text{azo dye} \]

*(Fast Red TR Salt)
<table>
<thead>
<tr>
<th>Parameter Listing</th>
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<th>BM</th>
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<tbody>
<tr>
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<tr>
<td>Buffer pH</td>
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<tr>
<td>1-naphthyl phosphate (mmol/L)</td>
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<td>Sodium tartrate (mmol/L)</td>
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</tr>
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<td>Acetic acid stabiliser (mmol/L)</td>
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<td>3</td>
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<tr>
<td>Pentane diol (mmol/L)</td>
<td>-</td>
<td>220</td>
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</tbody>
</table>

Table 2.2: Automated analysis of TRAP using two commercial kits employing α-napthyl phosphate as a substrate. Final reagent concentrations in the reaction cuvette.

<table>
<thead>
<tr>
<th>Parameter Listing</th>
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<td>Blanking Mode</td>
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<td>1</td>
</tr>
<tr>
<td>Printout Mode</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.3: Parameter listings for TRAP analysis on the Cobas Bio autoanalyser. The assays were calibrated using a factor based on the molar extinction coefficient of the reaction product.
1,25-Dihydroxyvitamin D

In-House Method (HPLC-RRA)

Measurement of 1,25-dihydroxyvitamin D was measured using HPLC and radio-receptor assay (modifications of Eisman et al., 1976; Reinhardt et al., 1984, Chen et al., 1990).

Outline

Vitamin D metabolites, extracted from their serum binding protein using acetonitrile, are separated and partially purified from interfering lipids using reverse-phase pre-packed column chromatography. 1,25(OH)₂D has more hydroxyl groups (three) than 25-OHD (two), resulting in a difference in polarity between the compounds. This is used as the basis of a HPLC separation procedure. The 1,25(OH)₂D fraction is then quantified using a competitive protein binding assay with charcoal separation.

Apparatus

- Temperature Controlled Ultracentrifuge
- Phillips P8700 UV/Visible Scanning Spectrophotometer
- Oxygen Free Nitrogen (BOC Ltd, UK)
- Bond Elut C18 columns + VacElut system (Analytichem International, UK)
- Waters HPLC System with fraction collector
- Refrigerated centrifuge
- Liquid Scintillation Counter

Preparation of Calf Thymus Receptor for 1,25 (OH)₂D Assay

Thymus from a 12 week old calf slaughtered for other purposes was obtained as a gift from the Rowett Research Institute, Aberdeen, UK. The age of the calf is important as older animals express less receptor per weight of thymus. However the slaughter of animals of this age is relatively rare, making calf thymus gland both expensive and difficult to obtain.

Homogenisation buffer

- 50 mM K₂HPO₄ (trihydrate) 5.71g
- 5 mM dithiothreitol 0.39g
- 1 mM EDTA 0.19g
- 400 mM KCl 14.91g
- 400 ml ice-cold dH₂O

Adjust to pH 7.5 and make up to 500 ml with dH₂O. Store at 4°C.
Thymus glands were homogenised (25% w/v) in ice-cold homogenisation buffer in short (15-20 second) bursts. Homogenates were cold centrifuged for one hour at 100,000g and the cytosol (minus the pellet and floating lipid layer) fractionated by the slow addition of solid (NH₄)₂SO₄ to 35% saturation (calculated using the nomogram of Dixon, 1953). The cytosol was then stirred slowly for one hour and centrifuged at 20,000 g for 20 minutes. The supernatant was discarded and the pellets were re-suspended in a volume of ice cold buffer equivalent to the discarded supernatant. Aliquots (3 ml) were stored in stoppered tubes at -70°C.

The frozen receptor pellet should be re-suspended in an appropriate amount of assay buffer before use. This should be by magnetic stirring, as vortex mixing produces foaming which may signify protein denaturation. The resulting receptor solution is extremely thermo-labile and should be kept on ice at all times. The dilution factor for the receptor pellet is pre-determined by a titre test. In this test differing dilutions of receptor solution are used to determine non-specific binding (NSB) and zero binding (B₀). The test is otherwise performed exactly as the normal radioreceptor assay. Typical findings are illustrated below:

<table>
<thead>
<tr>
<th>Volume of assay buffer (ml) added per ml of receptor pellet</th>
<th>Mean Zero Binding (%)</th>
<th>Mean NSB Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>5.6</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>2.8</td>
</tr>
<tr>
<td>8</td>
<td>34</td>
<td>3.6</td>
</tr>
<tr>
<td>10</td>
<td>27</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Table 2.4: Titre test to determine appropriate dilution for calf thymus vitamin D receptor pellet for 1,25(OH)₂D radioreceptor assay.

The choice of receptor dilution is a compromise of the following criteria:

- The lowest possible amount of NSB. The extraordinarily high concentration of 1,25(OH)₂D in the NSB standard should fill all available receptor sites making any residual assay label binding non-specific.
- Zero binding of 25-35% of added tritiated 1,25(OH)₂D (Chen et al., 1990).

The ideal dilution would therefore appear to be the addition of 6ml buffer to each 1ml of receptor pellet. However the difficulty with which the receptor pellets are obtained (both technically and with respect to availability) necessitates that the volume of receptor
solution produced be considered. As such it was decided to reconstitute using 8ml of assay buffer per 1ml of receptor pellet.

Standards

Pure solid 1,25(OH)2D3 obtained as a gift (approximately 1.0 mg 1α,25-dihydroxycholecalciferol from Roche Pharmaceuticals, Welwyn Garden City, UK) was dissolved in 100 ml absolute ethanol (‘SpectroSol’ Grade, BDH Laboratories, Merck Ltd, Poole, UK). This was designated the primary standard and stored under nitrogen in the dark (foil covered) at -70°C.

Determination of Primary Standard Concentration

The concentration of the primary standard was calculated from absorbance measurements and the molecular extinction coefficient.

1,25(OH)2D3  Molar extinction coefficient = 18 200 M⁻¹cm⁻¹ at 264 nm (γmax).
Molecular formula = C₂₇H₄₄O₃
Molecular weight = 416.64

Absorbance of primary standard at 264 nm = 0.429

\[
\frac{0.415}{18200} = 2.280 \times 10^5 \text{ M}^{-1} \text{ l}^{-1} = 22.802 \mu \text{M} \text{ l}^{-1}
\]

\[
22.802 \times 416.64 = 9500 \mu \text{g l}^{-1} = 9.500 \mu \text{g ml}^{-1}
\]

The purity of these stock standards may be assessed spectrophotometrically using the following criteria:

• On scanning, pure compounds exhibit a valley in the spectra (γmin) at 228 nm. An upfield shift in γmin indicates degradation or contamination.

• The ratio γmax:γmin should be above 1.5 in pure metabolites

The primary standard is checked in this manner every six months.

Stock Standard (1 μg ml⁻¹)

As 9.50 μg primary standard = 1.00 ml  
1μg = \frac{1000}{9.50} = 105.3 μl

105.3 μl primary standard plus 894.7 μl absolute ethanol gives a stock standard of 1 μg ml⁻¹.

Working Standards
Non-Specific Binding (NSB) Standard

To 200\(\mu\)l stock standard (200ng) add 1050 \(\mu\)l of absolute ethanol.

This gives a concentration of
\[
50 \times \frac{200}{1250} = 8.0 \text{ ng/50\(\mu\)l}
\]

Assay Standards

Take 10 \(\mu\)l of the NSB standard (1.6 ng) and make up to 2.0 ml with absolute ethanol.

This gives a concentration of
\[
50 \times \frac{1.6}{2000} = 0.04 \text{ ng/50\(\mu\)l (40 pg/50\(\mu\)l)}
\]

This is the top assay standard. Serial dilutions of this standard (made by adding 200\(\mu\)l to 200\(\mu\)l absolute ethanol) gives standards of 40, 20, 10, 5, 2.5, and 1.25 pg per 50\(\mu\)l.

Radiolabelled 1,25(OH)\(_2\)D\(_3\)

High specific activity (\(^3\)H)-1,25(OH)\(_2\)D\(_3\) was purchased from Amersham International, Amersham, UK.

Assay Tracer

The working concentration for assay label in the 1,25(OH)\(_2\)D\(_3\) assay is around 2500 cpm/20 \(\mu\)l of solution.

Recovery Tracer

Approximately 800-1000 counts per 20 \(\mu\)l.

Assay Buffer

\[
\begin{align*}
450 \text{ mM KCl} & \quad 33.56\text{g} \\
1.5 \text{ mM EDTA} & \quad 0.56\text{g} \\
50 \text{ mM Tris-HCl ('Aristar' Grade)} & \quad 7.88\text{g} \\
10 \text{ mM Na}_2\text{MoO}_4 & \quad 2.42\text{g} \\
0.1\% (w/v) \text{ NaN}_3 & \quad 1.0\text{g} \\
0.1\% (w/v) \text{ Gelatine} & \quad 1.0\text{g}
\end{align*}
\]

Dissolve the gelatine by heating in approximately 400 ml distilled water (dH\(_2\)O).

After cooling, add the remaining constituents, make up to 900ml with ice-cold dH\(_2\)O and adjust to pH 7.5 at 4\(^\circ\)C, bringing the final volume to 1 litre.

Store at 4\(^\circ\)C. Stable for 2-3 months.

Immediately before use add 0.077g dithiothreitol per 100ml buffer.
Dextran-Coated Charcoal Suspension (DCC)

a. 0.1 M boric acid buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>6.18 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.50 g</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.20 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>900 ml</td>
</tr>
</tbody>
</table>

Adjusting pH to 8.6 and make up volume to 1 litre. Store at 4°C for not more than two months.

b. Norit A charcoal 6.0 g

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran T-70</td>
<td>0.6 g</td>
</tr>
</tbody>
</table>

Suspend the charcoal and dextran in 100 ml of 0.1M boric acid buffer and stir overnight at 4°C. Centrifuge the suspension at 2500 rpm for 20 minutes. Discard the supernatant and re-suspend the pellet in 500 ml of 0.1M boric acid buffer. Stir the suspension at 4°C for 15 minutes prior to use in the assay. Make fresh every six to eight weeks.

**Extraction:**

Pipette 1 ml aliquots of patient serum into 12 x 75 mm thin-walled borosilicate glass tubes.

Add 20 μl of recovery label to each sample.

Pipette 20 μl of each label into four counting vials (total recovery counts).

Vortex the samples and leave for 10 minutes.

Add 2 ml of acetonitrile to each sample, Centrifuge for 10 minutes at 1500 rpm.

**Purification using Bond Elut C₁₈ Columns**

Before use the columns are washed through sequentially with 2 x 2.5 ml washes of hexane, chloroform, methanol and water.

Load delipidated sample.

Wash through the columns with 2 x 2.5 ml of methanol:water (70%) to remove polar lipids.

Wash through the columns with 6 ml methanol.

Dry the acetonitrile eluate under N₂ and re-suspend in 100μl of hexane:isopropanol.

**HPLC Separation**
A mobile phase of hexane:isopropanol elutes through a silica column solid phase. Both the polarity and flow rate of the solvent will determine the elution time of the compound of interest and its separation from interfering metabolites. This laboratory found a combination of 88% hexane:12% isopropanol (by volume) and a flow rate of 1.0 ml/minute afforded excellent separation of 25-OHD and 1,25(OH)₂D. However it is recommended that each laboratory work to optimise the HPLC separation stage. As only one sample may be purified at any time, it is desirable to minimise the elution time of the 1,25(OH)₂D. However analysts should be aware of the dangers of co-elution of interfering metabolites. Conversely, extended elution times are not only laborious but may result in an unwanted separation of 1,25(OH)₂D₂ and 1,25(OH)₂D₃.

Calibration of HPLC Columns

Before sample purification, the HPLC column must be 'calibrated' to identify the elution time of 1,25-dihydroxyvitamin D. This is accomplished with tritiated 1,25(OH)₂D₃ which is injected onto the column and thirty second fractions of eluate collected. The activity of each fraction is measured and the elution time of 1,25(OH)₂D₃ determined. Tritiated 1,25(OH)₂D₂ was unavailable, and assumed to co-elute with 1,25(OH)₂D₃ under the conditions described. A typical elution profile showing the separation of radiolabelled 25-OHD and 1,25(OH)₂D₃ is shown in figure 3.6 as part of the comparison of methods for 1,25-dihydroxyvitamin D analysis.

Patient samples, reconstituted in HPLC solvent are then injected on to the HPLC column. The fraction corresponding to the 1,25(OH)₂D₃ peak, plus the adjacent fractions are collected. The purified sample of 1,25(OH)₂D is now dried under N₂ and reconstituted in 200 µl ice-cold absolute ethanol.

Recovery

Pipette 50 µl of the reconstituted sample into a counting vial and add 5 ml of scintillant. Shake vigorously to mix thoroughly and count for 10 minutes. Also count the total recovery count vials from day one. Calculate recovery as follows:

\[
1,25 \text{ (OH)}_2 \text{D recovery (\%)} = \frac{\text{cpm in 50 \mu l}}{\text{total recovery counts}}
\]

Dihydroxyvitamin D Radioreceptor Assay

1. Set up labelled 12 x 75 mm borosilicate glass tubes, in duplicate, for the standard curve, NSB, B₀, unknowns, and controls.

2. Keep all reagents on ice and set up the assay in an ice/water bath.
3. Add 50μl of ethanol to B tubes.
4. Add 50μl of NSB standard to NSB tubes.
5. Add 50μl of NSB standard to standard tubes.
6. Add 50μl of sample to assay tubes. When performed in duplicate this represents only one-half (100μl/200μl) of the reconstituted extract. However 50μl of extracted sample or control has previously been used for recovery estimation and in practice little more than 150μl may be reproducibly recovered in total. This division of sample between recovery tubes and assay appears to be the best compromise available and was determined empirically over several assays.
7. Add 500 μl of receptor solution to all tubes.
8. Mix well and incubate in a water bath at 25°C for one hour.
9. Place assay tubes in an ice-water bath for five minutes.
10. Add 20 μl of assay tracer solution to all tubes, as well as to two scintillation vials containing 5 ml of scintillation cocktail (total count tubes).
11. Mix well and incubate in a 25°C water bath for one hour.
12. Place assay tubes in an ice-water bath for five minutes
13. Add 200 μl of cold dextran coated charcoal suspension to each tube (DCC should be mixed thoroughly prior to and during addition).
14. Mix well and incubate on ice for thirty minutes.
15. Centrifuge at 3000 rpm for fifteen minutes at 4°C.
16. Carefully pour the supernatant into scintillation vials containing 5 ml of scintillation cocktail. Touch the rim of the tube against the surface of the cocktail to ensure transfer of all of the supernatant. Avoid the accidental transfer of charcoal particles into the scintillation vial.

The final step involves the addition of relatively large volumes of supernatent to the scintillant. Samples should be mixed very thoroughly before counting while the performance characteristics of the scintillation fluid should be considered to ensure maximum counting efficiency.

Calculations
% B/B₀ = \frac{(CPM \text{ of standard or unknown} - CPM \text{ of NSB})}{CPM \text{ of B₀ tube} - CPM \text{ of NSB}} \times 100

\text{Recovery} = \frac{\text{Sample recovery CPM}}{\text{Total recovery counts CPM}} \times 4

\text{Sample Concentration} = \frac{\text{Tube concentration}}{\text{Recovery}} \times 4

B₀ = \text{zero binding (blank); NSB = non-specific binding (control)}

\text{Figure 2.3: Illustration of standard curve for 1,25(OH)₂D radioreceptor assay. Curve displayed uses mean values for eight consecutive assays.}

\text{Assay Characteristics}

\text{Sample Volume}

A major problem with this assay is the low concentrations of 1,25(OH)₂D in the final assay tubes due to cumulative losses in the chromatographic steps. Even though recovery estimations are performed, assay variability is high at low analyte concentrations. To counteract this, sample volumes greater than 1ml were used whenever possible. It is also desirable to achieve as high a recovery as possible in order to avoid reliance on the multiplication of a low assay result by a large multiplication factor. Assays should therefore be repeated if recoveries are less than 50%. Consequently the sample volume used in this assay is a compromise between the desire to maximise the concentration of 1,25(OH)₂D in the final assay tube and the possible need to repeat the assay due to poor recovery.
**Blank Measurements**

The possibility of contamination of solvents and chromatographic materials necessitates monitoring for possible interferences. This is accomplished by treating a water or saline blank identically to specimens. This laboratory has also used lyophilised control material (UK External Quality Assessment Scheme, Birmingham UK) for this purpose as it appears to contain no 1,25(OH)$_2$D. Undetectable levels of vitamin D in such samples indicate the absence of positive interference.

**Recovery**

Attempts to investigate recovery of spiked samples in control sera led to unacceptably high intra-assay variability, possibly due to a lack of vitamin D binding protein in such material. Such experiments should only be performed on pooled human sera.

Pooled sera (total volume 32 ml) spiked with 50µl NSB standard (diluted 10X)

\[
\text{Analysed pooled sera (n=4) mean}=29.1 \text{ pg/ml}
\]

Theoretical value of spiked sera = (29.1 + 24.96) = 54.06 pg/ml

\[
\text{Analysed spiked sera (n=4) mean}=51.7 \text{ pg/ml}
\]

\[
\text{Mean Recovery} = \frac{51.7}{54.06} = 96\%
\]

Such data was the only available indication of the accuracy of the method apart from a small sample of a QC pool provided as a gift by the Department of Molecular Endocrinology, University College & Middlesex School of Medicine. This material, previously analysed by a dual column technique at 37 pg/ml, was found to be 34.3 pg/ml by this laboratory (mean of two samples analysed in a single batch).

**Assay Precision**

Intra-assay precision: (all n=4), 6.3% at 72.9 pg/ml; 5.1% at 53.4 pg/ml; 10.8% at 38.1 pg/ml; 10.1% at 25.4 pg/ml and 19.4% at 15.6 pg/ml.

Inter-assay precision: 13.7% for replicate analysis of a control pool of mean concentration 39.1 pg/ml over seven analytical runs.
DEPROTEINISATION

1 ml serum + \[^{3}H\text{-}1,25(\text{OH})_{2}D_{3}\]

1 ml acetonitrile
Vortex and centrifuge

Add supernatent to
1ml K2HPO4 buffer

COLUMN CHROMATOGRAPHY

Apply to prewashed C18 column

Add to cartridge: 2 x 2.5 ml Methanol:Water (70:30)

DISCARD

6 ml Methanol
COLLECT ELUATE

Dry under Nitrogen and reconstitute in 100μl HPLC solvent

HPLC

Load sample onto 'calibrated' silica HPLC column
Mobile phase hexane:isopropanol (88:12)

Collect samples corresponding to elution time of \[^{3}H\text{-}1,25(\text{OH})_{2}D_{3}\]

NON-EQUILIBRIUM RADIORECEPTOR ASSAY

Dry under Nitrogen
Reconstitute in 200μl ice-cold ethanol

2 x 50μl for assay 50μl for recovery estimation

Incubate 1 hr at 25°C with 500μl of vitamin D receptor solution (from calf thymus)

Add assay tracer:20μl \[^{3}H\text{-}1,25(\text{OH})_{2}D_{3}\] of approx. 2500 cpm
Incubate for 1 hour at 25°C

Cool in ice bath (5 minutes) before addition of dextran-coated charcoal (DCC). Incubate for 30 min at 4°C to remove unbound tracer

Centrifuge and decant supernatent into scintillation fluid for counting, subsequent data manipulation and recovery correction

Figure 2.4: Flow diagram outlining the determination of 1,25-dihydroxyvitamin D using column extraction, HPLC purification and subsequent radioreceptor assay.
**Single Column Extraction Method:**

The need for specialised equipment and the technical difficulties inherent with HPLC led to the desire to eliminate this step from the purification procedure. This was achieved by the use of dual column solid phase extraction and chromatography (Reinhardt *et al.*, 1984). In this method alkaline buffered deproteinised serum is undergoes chromatography on a reverse phase octadecysilanol (C18) column. Removal of salts and polar lipids with water and 70% methanol is followed by elution of the vitamin D metabolites. The eluate is dried, reconstituted and metabolites separated by normal phase chromatography on a silica minicolumn. This is facilitated by the use of increasingly polar hexane:isopropanol mixtures.

The method was further modified by Hollis (1986), by removing the silica cartridge and ‘phase-switching’ solvents on a single C18(OH) column. Sample loaded onto pre-washed columns undergoes removal of salts and polar lipid with water and 70% methanol before elution of the vitamin D metabolites. 25-OHD is eluted with 10% methylene chloride in hexane, the majority of 24,25(OH)2D by 1% isopropanol (IPA) in hexane, and 1,25(OH)2D by 3% IPA in hexane.

**Commercial Method:** (Gamma-B 1,25-dihydroxyvitamin D, IDS Ltd, Tyne & Wear, UK)

A commercial radioimmunoassay for the measurement of 1,25-dihydroxyvitamin D was also used. This method is based on immunoextraction of interfering metabolites before radioimmunoassay. The assay begins with the delipidation of 500μl of sample (serum or plasma) with a small volume of dextran sulphate/magnesium chloride. This is half the sample volume typically required by methods utilising chromatographic extraction. After centrifugation these samples are ready for the immunoextraction procedure.

Sample immunoextraction and all subsequent steps are performed in duplicate. The immunoextraction device comprises of a small plastic capsule containing a solid-phase bound monoclonal antibody specific for 1,25-dihydroxyvitamin D. Delipidated samples (100μl) are added and the capsules rotated for three hours at room temperature to allow maximal binding to occur. The capsules are then placed in disposable plastic tubes, and washed to remove non-bound substances. The wash solution is forced through the sample by centrifugation.

The solid phase is retained within the immunoextraction capsule by a plastic frit. This laboratory found that in early batches of this assay, the frit was not secure in some capsules (on average one or two per kit). The resulting loss of the solid phase was not apparent until washing was attempted, resulting in complete loss of the sample. The
Manufacturers have since addressed this problem, altering the assembly process of the immunoextraction capsule.

The capsule is then transferred to a glass (assay) tube, and the 1,25-dihydroxyvitamin D removed with 'elution reagent'. After three elutions, the total volume (450µl) is evaporated by placing the tubes in a heating block (30°C) under a stream of nitrogen. Samples are then reconstituted in 100µl assay buffer and are ready for immunoassay. Claimed immunoextraction efficiency is 100%, removing the need for the recovery estimations required when using other extraction methods.

The immunoassay step for 1,25-dihydroxyvitamin D is a simple $^{125}$I-labelled radioimmunoassay (RIA). The primary antibody (sheep monoclonal anti-1,25 D) is added to the sample and incubated overnight at 2-8°C. This antibody exhibits 100% cross-reactivity towards 1,25(OH)$_2$D$_3$ and 80% towards 1,25(OH)$_2$D$_2$. Radiolabelled antigen is then added and a further incubation of two hours at room temperature undertaken. After this time a separation antibody (solid phase bound anti-sheep) is added to the reaction mixture. After 30 minutes at room temperature the solid phase is compacted by centrifugation and the supernatant discarded. The amount of radiolabelled antigen captured by the solid-phase antibody (determined by gamma-counting) is inversely proportional to the amount of antigen present in the sample. Quantitation is facilitated by the inclusion of six calibrators of known concentration in the immunoassay. Control samples provided with the assay kit undergo all stages of the procedure including delipidation.

Assay characteristics as quoted by the manufacturer: Intra-assay CV=8% at 13.2 pg/ml (n=10); inter-assay CV=10% at 11.5 pg/ml. The assay sensitivity is 2.1 pg/ml. In this laboratory inter-assay variability equals 14% at 24 pg/ml and 9% at 59 pg/ml (seventeen assay runs).

Other commercially available methods used without deviation from the manufacturers instructions were:

**Parathyroid hormone**: (Intact-PTH Parathyroid Hormone, Nichols Institute Diagnostics, Essex, UK). This two-site radioimmunoassay measuring only biologically active intact PTH is based on the work on Nussbaum *et al.* (1987). Two polyclonal goat antibodies (purified by affinity chromatography) are directed to different parts of the PTH molecule. One antibody, immobilised onto plastic beads, binds PTH only in the 39-84 amino acid region (mid- and C-terminal). The second antibody, which is $^{125}$I-labelled, binds only in the 1-34 amino acid (N-terminal) region. Both antibodies are incubated simultaneously with the test serum for 22± 2 hours at room temperature. Although mid-region and C-terminal fragments are bound by the immobilised anti-PTH antibody, only intact PTH forms a radiolabelled 'sandwich' complex which may be detected by gamma-
using QC samples. The repeat analysis of an in-house serum pool is a further method of estimating such precision, however in less frequently performed assays, analyte stability becomes a confusing factor.

![Radiolabelled Anti-PTH (1-34)](image)

**Figure 2.5:** Two site immunoradiometric assay for intact PTH. Only intact PTH (1-84) forms the radiolabelled ‘sandwich’ complex necessary for detection. The high density of anti-PTH (39-84) on the bead ensures no interference even at high C-terminal fragment concentrations. Other fragments exhibit little interference due to their short half-life in vivo.

**Cyclic adenosine monophosphate (cAMP):** (Biotrak cAMP Dual range enzymeimmunoassay system, Amersham Life Sciences, Amersham, Bucks).

This enzyme immunoassay is based on the competition between unlabelled sample cAMP and peroxidase labelled cAMP for a specific antibody (rabbit anti-cAMP). As the amount of antibody and labelled cAMP are fixed, the binding of the peroxidase-cAMP conjugate will be inversely proportional to the concentration of the sample cAMP. The solid phase (microtitre plate well) is coated with a second (donkey anti-rabbit) antibody, allowing capture of antibody-antigen complexes. Unbound ligand is then washed away, and the amount of peroxidase bound to the solid phase determined by its reaction with a tetramethylbenzidine/hydrogen peroxide substrate. When the reaction is halted by acidification, the reaction product is spectrophotometrically quantified at 450nm. Acetylation of standards and unknowns increases assay sensitivity, allowing the measurement of unknowns over two different concentration ranges.

Assay characteristics: acetylation protocol, sensitivity 14 pg/ml, intra-assay CV 5.7% (mean CV of three concentrations analysed 14 times each); non-acetylation protocol:
will be inversely proportional to the concentration of the sample cAMP. The solid phase (microtire plate well) is coated with a second (donkey anti-rabbit) antibody, allowing capture of antibody-antigen complexes. Unbound ligand is then washed away, and the amount of peroxidase bound to the solid phase determined by its reaction with a tetramethylbenzidine/hydrogen peroxide substrate. When the reaction is halted by acidification, the reaction product is spectrophotometrically quantified at 450nm. Acetylation of standards and unknowns increases assay sensitivity, allowing the measurement of unknowns over two different concentration ranges.

Assay characteristics: acetylation protocol, sensitivity 14 pg/ml, intra-assay CV 5.7% (mean CV of three concentrations analysed 14 times each); non-acetylation protocol: sensitivity 38.4 pg/ml, intra-assay CV 9.6%. As this assay was only used once for a particular case study, inter-assay precision is not relevant.

**25-Hydroxyvitamin D:** (INCSTAR 25-OHD, Incstar Ltd, Berks., UK). This two step procedure involves extraction of vitamin D metabolites with acetonitrile. The subsequent RIA uses an antibody specific to 25-OHD followed by a second antibody precipitating complex.

This assay exhibits 100% cross-reactivity between 25-OHD$_2$ and 25-OHD$_3$. Intra-assay precision 6.2% at 18.9 ng/ml (n=20); inter-assay CV 15.6% at 27.0 ng/ml (n=20).

**Bone-specific alkaline phosphatase (B-ALP):** (Ostase, BM Browne Ltd, Reading, UK). The assay for B-ALP, an isotopically-labelled solid phase two-site IRMA, is formatted exactly as that for PTH. Samples containing skeletal ALP react with two mouse-monoclonal antibodies (solid phase and an $^{125}$I-labelled) directed towards different antigenic sites on the B-ALP molecule. Washing the beads after an overnight incubation at 2-8°C leaves only the solid-phase/B-ALP/$^{125}$I-labelled antibody complex to be measured by gamma counting. The radioactivity measured is directly proportional to the B-ALP present in the test sample (serum only for patient samples). The standard curve is based on the inclusion of six calibrators of differing concentration. Exhibiting a seven-fold preference for B-ALP over the liver isoenzyme, the assay measures enzyme mass rather than activity. Results are therefore expressed in units of µg/litre with a minimum detection limit of $2.0\,\mu g/litre$.

Assay precision: manufacturer's quoted mean CV over a range of concentrations (n=3, range 11.7 to 77.4 µg/l) = 4.9% (intra-assay, n=21) and 7.4% (inter-assay, n= 20 runs).

**Carboxy-terminal propeptide of type I procollagen (PICP):** (PICP, Orion Diagnostica, Finland). This radioimmunoassay is based on the work of Melkko et al. (1990). In this assay 100 µl of sample serum or PICP standard is incubated for two hours at 37°C with rabbit anti-PICP antiserum and $^{125}$I-labelled PICP. During this time
competition for binding sites on the antibody occurs. A second (anti-rabbit) antibody, covalently bound to solid particles is then added. After subsequent centrifugation the supernatant containing free antigen is then discarded, the sediment containing the precipitated antibody-antigen complex. As the labelled and unlabelled antigens were allowed to freely compete for limited number of binding sites on the PICP antibody, the amount of radioactive antigen (measured by gamma-counting) is inversely proportional to the amount of unlabelled antigen in the sample or standard. The inclusion of five known standards allows the calculation of a calibration curve and estimation of unknowns.

Assay precision: manufacturer’s quoted mean CV over a range of concentrations (n=4, range 54 to 451 μg/l) = 2.8% (intra-assay, n=16) and 5.1% (inter-assay, n= 8 runs). Minimum detection limit 1.2 μg/l.

**Osteocalcin:** (‘OSCAtest’, Shield Diagnostics, Dundee, UK). This RIA, claimed to measure only intact osteocalcin, is described in detail and compared to another OC method in Chapter III of this thesis; (‘The Comparison of Two Immunoassays for Osteocalcin’).

Minimum detection limit 1.8 ng/ml. Manufacturer’s quoted assay precision (CV) at 10 ng/ml = 5% (intra-assay n=10) and 6% (inter-assay, n=10 runs).

**Urinary total free pyridinolines:** (Pyrilinks, Metra Biosystems, Oxford, UK). Minimum detection limit 15 nM pyridinoline. Assay precision: manufacturer’s quoted mean CV over a range of concentrations (n=3, range 66 to 407 nM) = 7.8% (intra-assay, n=52) and 7.0% (inter-assay, n=8 runs).

**Urinary free deoxypyridinoline:** (Pyrilinks-D, Metra Biosystems, Oxford, UK). Minimum detection limit 3 mM free deoxypyridinoline. Assay precision: manufacturer’s quoted mean CV using six control pools (range 11.9 to 191.1 nM) is 3.2% (intra-assay, n=52) and 4.1% (inter-assay, six replicates repeated over nine runs). In this laboratory inter-assay variability (single control sample of 93 nM repeated over nine assays) was 14%.

The Metra Biosystems assays for total free pyridinoline and free deoxypyridinoline are formatted in exactly the same way, differing only in the epitope recognised by the solid phase (microplate well-bound) mouse monoclonal antibody. In these enzyme immunoassays (EIA), antigen in the sample competes with an antigen-alkaline phosphatase conjugate for the anti-pyridinoline antibody coated within each microwell. After washing away unbound sample antigen and conjugate, an alkaline phosphate substrate (p-nitrophenol phosphate) is allowed to react for one hour at room temperature
Urinary collagen N-telopeptide crosslinks (NTx) (‘Osteomark’, Lifescreen Ltd., Watford, UK). This enzyme-linked immunosorbent assay (ELISA) is specific for the N-telopeptide cross-linking domain of osseous collagen. The assay is performed using an human antigen-coated microwell plate (the solid phase) to which urine samples, standards or controls are added. A mouse monoclonal antibody-horseradish peroxidase conjugate is subsequently added to all wells. During a 90 minute incubation at room temperature antigens in the sample and solid phase compete to bind the antibody conjugate. After washing the plates to remove unbound material, a buffered substrate-chromogen reagent (a substrate for the horseradish peroxidase) is added. During a final incubation at room temperature (15 min) a blue colour develops due to the presence of solid phase antigen-antibody conjugate complex in the well. As such the colour intensity is inversely proportional to the amount antigen in the test sample. Stopping the reaction with sulphuric acid results in a colour change from blue to yellow. This may be measured spectrophotometrically in an appropriate microplate reader at 450 nm.

A standard curve from which unknowns may be estimated is derived from the inclusion of six levels of calibrator. Assay values are standardised to equivalent amounts of bone collagen. The bone collagen equivalent (BCE) values are then normalised for urine volume (24h collections) or urinary creatinine concentration (single collections). Final values are therefore expressed as nM BCE/day or nM BCE/mmol creatinine.

Assay precision: manufacturer’s quoted mean intra-assay CV over a range of concentrations from 26 to 2640 nM BCE equalled 7.6% (n=40). Inter-assay variability, tested using three urine samples (79 to 1167 nM BCE) analysed in duplicate over 20 assay runs, had a mean CV of 4.0%. The minimum detection limit of the assay is 20 nM BCE. In this laboratory inter-assay variability (single control sample of 1654 nM repeated over nine assays) was 9%.

Reference Intervals

An observed value may only be reliably interpreted by comparison with reference data. A reference value may be defined as ‘a value obtained by observation or measurement of a particular type of quantity on a reference individual’ (International Federation of Clinical Chemistry, 1987a). From a number of such reference values a reference interval, bounded by a pair of reference limits may be calculated. The reference interval obtained will depend on the population of reference individuals (with respect to state of health or disease, age, sex etc.). The definition of a reference interval is purposely vague in order to ensure the composition of the reference population used is fully detailed. The concept of reference limits has replaced the now obsolete terms ‘normal range’ and ‘normal limits’. The use of such nomenclature is ambiguous as it may refer to normality in a statistical, epidemiological, or clinical sense.
Recommendations for the selection of reference populations have been well detailed (International Federation of Clinical Chemistry, 1987b). As analytical considerations (method, equipment etc.) also effect the production of reference intervals, it is proper for each laboratory to define its own values. However this is often precluded by resource limitations, especially in the case of commercially purchased immunoassays. This is the case for many of the analysis used in this thesis. Reproduced below are the reference ranges (supplied by the manufacturer for immunoassays) with any details of the reference population as quoted. It should be noted that many larger clinical studies published in the literature detail their own reference limits for a number of these analytes.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Reference Population</th>
<th>No.</th>
<th>Reference Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact PTH</td>
<td>‘Healthy’ fasting adults</td>
<td>253</td>
<td>1.0-6.5 pmol/l</td>
</tr>
<tr>
<td>1,25(OH)₂D</td>
<td>‘Normal’ adults</td>
<td>132</td>
<td>20-46 pg/ml</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Men</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21-30 years</td>
<td>79</td>
<td>6-20 ng/ml</td>
</tr>
<tr>
<td></td>
<td>31-40 years</td>
<td>76</td>
<td>4-15 ng/ml</td>
</tr>
<tr>
<td></td>
<td>41-50 years</td>
<td>81</td>
<td>4-12 ng/ml</td>
</tr>
<tr>
<td></td>
<td>51-60 years</td>
<td>32</td>
<td>4-12 ng/ml</td>
</tr>
<tr>
<td></td>
<td>61-70 years</td>
<td>18</td>
<td>5-12 ng/ml</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21-30 years</td>
<td>82</td>
<td>4-20 ng/ml</td>
</tr>
<tr>
<td></td>
<td>31-40 years</td>
<td>61</td>
<td>3.5-15 ng/ml</td>
</tr>
<tr>
<td></td>
<td>41-50 years</td>
<td>113</td>
<td>4-12 ng/ml</td>
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<tr>
<td></td>
<td>51-60 years</td>
<td>96</td>
<td>4-12 ng/ml</td>
</tr>
<tr>
<td></td>
<td>61-70 years</td>
<td>40</td>
<td>4-12 ng/ml</td>
</tr>
<tr>
<td>B-ALP</td>
<td>Men</td>
<td>217</td>
<td>20.1 µg/l</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>261</td>
<td>21.3 µg/l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Upper reference limits)</td>
</tr>
<tr>
<td>PICP</td>
<td>Men</td>
<td>33</td>
<td>38-202 µg/l</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>41</td>
<td>50-170 µg/l</td>
</tr>
<tr>
<td>Urinary calcium (24 hr)</td>
<td>Adults</td>
<td></td>
<td>2.5-7.5 mmol</td>
</tr>
<tr>
<td></td>
<td>Average Diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary calcium (Fasting)</td>
<td>Upper limit</td>
<td>0.25</td>
<td>mM Ca mM Cr</td>
</tr>
<tr>
<td>Hydroxyproline (24 hr)</td>
<td>Adults</td>
<td></td>
<td>0.05-0.53 mmol</td>
</tr>
<tr>
<td>Analyte</td>
<td>Reference Population</td>
<td>No.</td>
<td>Reference Limits</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------</td>
<td>-----</td>
<td>--------------------</td>
</tr>
<tr>
<td>Urinary OHP</td>
<td>Pre-menopausal</td>
<td></td>
<td>0.09-0.24 mM Cr</td>
</tr>
<tr>
<td>(Fasting)</td>
<td>Post-menopausal</td>
<td></td>
<td>0.09-0.31 mM Cr</td>
</tr>
<tr>
<td></td>
<td>Post-menopausal</td>
<td></td>
<td>0.09-0.31 mM Cr</td>
</tr>
<tr>
<td></td>
<td>Total Pyr Men</td>
<td>118</td>
<td>13-26 nM Pyr mM Cr</td>
</tr>
<tr>
<td></td>
<td>‘Normals’ (25-44 yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>301</td>
<td>16-37 nM Pyr mM Cr</td>
</tr>
<tr>
<td></td>
<td>‘Normals’ (25-55 yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Free DPyr Men</td>
<td>58</td>
<td>2.0-5.0 nM DPyr mM Cr</td>
</tr>
<tr>
<td></td>
<td>‘Normals’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>55</td>
<td>2.0-6.0 nM DPyr mM Cr</td>
</tr>
<tr>
<td></td>
<td>Abnormal bone turnover</td>
<td></td>
<td>&gt;7.4 nM DPyr mM Cr</td>
</tr>
<tr>
<td></td>
<td>NTx Women Pre-menopausal</td>
<td>258</td>
<td>65 nM BCE mM Cr</td>
</tr>
<tr>
<td></td>
<td>(Upper reference limit)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NTx Women Post-menopausal</td>
<td>248</td>
<td>131 nM BCE mM Cr</td>
</tr>
<tr>
<td></td>
<td>(Upper reference limit)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5: Analyte reference limits
CHAPTER III - The Assessment And Validation Of Markers Of Calcium Homeostasis And Bone Turnover.

The ‘Correction’ of Serum Calcium Values

Background

Alterations in serum protein (and therefore protein-bound calcium) concentrations may arise from venous occlusion during venepuncture (Philpot, 1958), disease processes (Prasad & Flink, 1958) or a change in posture (Pedersen, 1972). To account for such changes total serum calcium is ‘corrected’ by a factor designed to represent the amount of protein-bound calcium, (Dent, 1962) so making it more indicative of the ionised calcium concentration.

The earliest correction factors used plasma specific gravity to estimate total protein concentration (Dent, 1962). However glucose, urea and lipid levels all contribute to specific gravity, while globulins contribute to both specific gravity and total protein measurements, yet bind little calcium. Correction factors based on such measurements give unacceptably wide reference limits (Payne et al., 1973). The linear relationship between total calcium and serum albumin (McLean & Hastings, 1935) has subsequently been used as a basis for correction (Orrell, 1971). The majority of protein-bound calcium is bound to albumin, and alterations in the concentrations of other calcium-binding protein species are generally considered of minimal importance (Berry et al., 1973). While correlations between calcium and specific gravity or total protein are similar, that of calcium on albumin is much stronger (Payne et al., 1973). Almost all laboratories report albumin corrected as well as total calcium concentrations, and many correction factors have been published in the literature (Duncan, 1995; Kanis & Yates, 1985; Orrell, 1971; Pain et al., 1975; Payne et al., 1979). The methods employed for calcium and albumin estimation, the mean value of albumin used for correction and the correction factor used, all influence the final ‘corrected’ calcium reference range. It is therefore recommended such ranges should be established by each laboratory rather than taken from published data (Anonymous, 1977).

The nature of the in-patient population of the Royal National Orthopaedic Hospital allows us to determine the correction factor and reference ranges for our laboratory in fasting, resting individuals with no medical illness prior to elective surgery. Such a patient group could be expected to provide the best possible estimate of ‘true’ calcium status. During this study it was decided to check the conventional wisdom which suggests the avoidance of tourniquet use when taking blood samples for serum calcium estimation.
Methods

Patient Selection

The Royal National Orthopaedic Hospital, Stanmore, Middlesex undertakes many routine elective orthopaedic operations. Patients admitted for such procedures are ideal subjects in the study for the establishment of reference ranges. Selected individuals bled preoperatively would be both resting and fasting overnight, so minimising the effects of dietary calcium intake and posture induced changes in serum albumin and so total calcium concentrations.

Patients between the ages of 18 and 85 were selected randomly from operating theatre lists. Those using thiazide diuretics or steroid compounds were excluded, as were individuals with primary or secondary bone disease, prolonged immobilisation, or an acute medical condition. Thus the test population comprised individuals with no disorder of calcium homeostasis or acute illness which would influence the concentrations of serum albumin and total calcium (Prasad & Flink, 1958). Ethical committee approval was obtained and all participating patients gave informed consent.

Eighty-two patients gave samples for estimation of reference ranges. These samples were taken without venous occlusion whenever possible. On the occasions this proved difficult a tourniquet was applied but released for ten seconds after insertion of the syringe needle. This was facilitated by the use of syringe type collection/separation tubes (Monovette, Sarstedt Ltd, Leicestershire, UK) rather than vacuum tubes. Of the 82 patients, 19 participated in the study on the effects of venous occlusion on the measurement of total serum calcium concentration. A pre-stasis sample was taken, then with the needle indwelling, occlusion was achieved by the inflation of a sphygmomanometer cuff to 90mm Hg. After two minutes occlusion a further sample was taken.

Serum Calcium

Measurement of total serum calcium was based on the reference method for atomic absorption spectrophotometry as described by Cali et al. (Cali, 1973) and detailed in Chapter II of this thesis. For this study all samples were analysed in duplicate.

Serum Albumin

Serum albumin was estimated by a standard spectrophotometric method using bromocresol green as detailed in Chapter II of this thesis. For this study all samples were analysed in duplicate.
Statistical Analysis

Statistical analyses were performed using both JMP and Statview statistical packages for the Apple Macintosh computer. Sample distributions were checked by a normal probability plot in which normal, (i.e. Gaussian) distributions appear as a straight line, and by the Shapiro-Wilk W-test for normality (‘normal’ distributions give a probability for the test statistic of p < 0.05). All populations were tested for normality before the application of appropriate parametric or non-parametric statistical tests.

Results

Reference range estimation

Visual inspection of the results presented as a histogram is an easy and reliable method for the identification of possible outliers (International Federation of Clinical Chemistry, 1987c). The relevant histograms are displayed as Figs 3.1 and 3.2. Samples with unexpectedly high serum total calcium values were assayed for parathyroid hormone. Of this group one patient was diagnosed as having primary hyperparathyroidism and excluded from further study. One patient had a markedly low albumin of 31.0 g/L, a value 3.90 standard deviations below the all patient mean. No other result was greater than 2.50 standard deviations from the mean in either direction. No reason could be found for the hypoalbuminaemia. Correction of the serum calcium for this albumin concentration (using a literature derived factor) gave a frankly hypercalcaemic value of 2.72 mmol/L. No signs or symptoms of hypercalcaemia were noted. The patient failed to show similar hypoalbuminaemia on recall. It was decided that the evidence available was indicative of an artifactually low albumin concentration. The data from this patient was then excluded from further analysis. One further individual with low albumin was excluded. The patient had suffered a motorcycle accident two weeks prior to venepuncture. Records indicated this to be more serious than was understood at the time of inclusion in the study. As trauma is known to decrease patient albumin for several weeks the patient no longer fulfilled the inclusion criteria and was excluded from further analysis.
Figure 3.1: Histogram of serum calcium concentrations in all patients participating in the study. The patient with marked hypercalcaemia was found to have primary hyperparathyroidism and excluded from the study.

Figure 3.2: Histogram showing serum albumin concentrations in the remaining 81 patients. Two further patients were excluded from data analysis at this time (see text).
**Serum Albumin**

Measured albumin values fitted a Gaussian (normal) distribution as assessed by percentile plot and the Shapiro-Wilk W test for normality. As such 95% reference intervals were calculated using $\pm 1.96$ standard deviations from the mean. This gave a reference range for in-patient albumin concentrations of 39 - 50 g/L around a mean value of 44 g/L.

**Serum Total Calcium**

Total calcium values did not fit a Gaussian distribution. Reference values were therefore calculated by using the 95 percent (i.e. the 2.5% and 97.5%) values around the median. This gave a reference range for in-patient total calcium concentrations of 2.21 - 2.62 mmol/L. The sex specific reference ranges were as follows.

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>-1.96 SD</th>
<th>Mean</th>
<th>+1.96 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male total calcium (mmol/L)</td>
<td>32</td>
<td>2.17</td>
<td>2.384</td>
<td>2.60</td>
</tr>
<tr>
<td>Female total calcium (mmol/L)</td>
<td>47</td>
<td>2.18</td>
<td>2.356</td>
<td>2.53</td>
</tr>
</tbody>
</table>

**Table 3.1** Sex-specific reference limits for total serum calcium in a hospital in-patient population with no known disorders of calcium homeostasis.

As age is thought to affect total calcium status, the age distributions of each population was investigated (Figure 3.3 and 3.4).

**Calculation of correction factor for the variation of total calcium with albumin.**

The correlation between calcium and albumin was investigated in the remaining 79 patients. (See Figure 3.5)
FIGURE 3.3: Age distribution of the in-patient reference female population of 47 female subjects used in the establishment of reference limits of serum total calcium and serum albumin.

Figure 3.4: Age distribution of the in-patient reference population of 32 male subjects used in the establishment of reference limits of serum total calcium and serum albumin.
Figure 3.5: Scatterplot and corresponding regression line showing the variation of serum total calcium with serum albumin in seventy-nine resting fasting in-patients with no known disorders of calcium metabolism. Regression equation: Calcium = 0.021*Albumin+1.477 (r=0.62 p<0.0001).

The slope of this equation (0.021) represents the change in calcium concentration (in mmol/L) induced by a 1.0 g/L alteration in serum albumin. Thus 0.02 mmol/L should be added to the measured total calcium for every 1 g/L the serum albumin is below the albumin population mean of 44 g/L. For albumin concentrations above 44 g/L, a similar subtraction should be made from the measured calcium value. The intercept of the regression equation represents the total calcium concentration at zero albumin, i.e. the plasma ultrafiltrable (ionised plus complexed) calcium. The value of 1.45 mmol/L agrees well with that of Payne et al. (1973) of 1.43 mmol/L and is of a similar magnitude to others (Moore, 1970a; Morris et al., 1990; Nordin et al., 1989; Toffaletti et al., 1976).

The correction factor was then applied to all 79 results. This gave a normally distributed population of corrected calcium values, and a new reference interval was calculated using ± 1.96 standard deviations (SD) from the mean.

**Corrected Calcium reference range (mmol/L)**

n = 79, mean = 2.37, SD = 0.078
mean ± 1.96 SD = 2.22 - 2.52 mmol/L

Application of the correction factor confers normality on the distribution and markedly narrows the reference limits. The scatter of the values, as represented by the standard deviation of the mean is also reduced (Table 3.2). This range is narrower than reference limits for total calcium as found in this and other studies (Nordin, 1989; Whitehead, 1994; Sinton, 1986). The correction factor was also applied to the sex specific reference ranges.

<table>
<thead>
<tr>
<th></th>
<th>-1.96 SD</th>
<th>Mean</th>
<th>+1.96 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male corrected calcium (mmol/L)</td>
<td>2.19</td>
<td>2.366</td>
<td>2.54</td>
</tr>
<tr>
<td>Female corrected calcium (mmol/L)</td>
<td>2.23</td>
<td>2.367</td>
<td>2.50</td>
</tr>
</tbody>
</table>

**Table 3.2:** Sex-specific reference limits, total serum calcium 'corrected' for serum albumin concentration.

Serum calcium concentrations were also examined within the female group to assess the effect of menopausal status on serum calcium levels. Post-menopausal women taking hormone replacement therapy were excluded as it is known that such treatment affects serum albumin and calcium concentrations (Fogh-Andersen *et al.*, 1984; Briggs & Briggs, 1979; Young *et al.*, 1968).

<table>
<thead>
<tr>
<th>No.</th>
<th>Mean Total Ca (mmol/L)</th>
<th>Std. Deviation</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-menopausal</td>
<td>26</td>
<td>2.353</td>
<td>0.092</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>15</td>
<td>2.374</td>
<td>0.090</td>
</tr>
</tbody>
</table>

**Table 3.3:** Comparison of serum total calcium concentrations in pre- and post-menopausal women.

The Mann-Whitney U test for unpaired means showed no significant difference between these groups (*t* = 0.707 df=39  *p*=0.48). However on application of the adjustment factor the results become:

<table>
<thead>
<tr>
<th>No.</th>
<th>Mean Adjusted Ca (mmol/L)</th>
<th>Std. Deviation</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-menopausal</td>
<td>26</td>
<td>2.352</td>
<td>0.065</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>15</td>
<td>2.396</td>
<td>0.069</td>
</tr>
</tbody>
</table>

**Table 3.4:** Comparison of albumin-corrected serum total calcium concentrations in pre- and post-menopausal women.
In this case the Mann-Whitney U test shows a significant difference between the means. ($t=1.998$ df $=39$ $p=0.05$). Thus the adjusted calcium identifies the increase in Ca$^{2+}$ found in post-menopausal women (Marshall et al., 1982; Robertson & Marshall, 1981), a phenomenon not indicated in this study by serum total calcium values alone.

**Venous stasis study**

Data from the 19 patients who agree to participate in the study of the effects of venous stasis on calcium and albumin was also collected

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (pre-stasis, g/L)</td>
<td>44.497</td>
<td>2.937</td>
<td>0.674</td>
</tr>
<tr>
<td>Albumin (post-stasis, g/L)</td>
<td>45.776</td>
<td>3.674</td>
<td>0.843</td>
</tr>
<tr>
<td>Calcium (pre-stasis, mmol/L)</td>
<td>2.375</td>
<td>0.113</td>
<td>0.026</td>
</tr>
<tr>
<td>Calcium (post-stasis, mmol/L)</td>
<td>2.398</td>
<td>0.097</td>
<td>0.022</td>
</tr>
</tbody>
</table>

**Table 3.5:** The effect of venous stasis on serum albumin and calcium concentrations. Samples taken with no venous stasis are compared with those taken after blocking venous return for two minutes at 90 mm Hg.

It is important to note than in studies of this type, it is the distribution of the differences between the groups, not that of the groups themselves that dictate whether parametric or non-parametric statistics should be used. The differences in pre- and post-stasis concentration for both albumin and total calcium were normally distributed as assessed by the normal quantile plot and Shapiro-Wilk test for normality (albumin: $W=0.941$ $p=0.29$; calcium: $W=0.95$ $p=0.37$).

<table>
<thead>
<tr>
<th></th>
<th>Mean Difference (pre - post)</th>
<th>DF</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>-1.279</td>
<td>18</td>
<td>-3.248</td>
<td>0.0045</td>
</tr>
<tr>
<td>Calcium</td>
<td>- 0.023</td>
<td>18</td>
<td>-2.890</td>
<td>0.0097</td>
</tr>
</tbody>
</table>

**Table 3.6:** Paired t-test for comparison of means of pre- and post-venous stasis total calcium and albumin.

Both albumin and calcium concentrations are statistically significantly higher in post-stasis samples. The ratio of the mean calcium and albumin differences, an alternative method of estimating the correction factor for the variation of total calcium with albumin, is 0.018.
The results were re-examined after adjusting the total calcium with the population derived factor for the variation of calcium with albumin:

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>S.D.</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected calcium (pre-stasis, mmol/L)</td>
<td>2.375</td>
<td>0.113</td>
<td>0.026</td>
</tr>
<tr>
<td>Corrected calcium (post-stasis, mmol/L)</td>
<td>2.398</td>
<td>0.097</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Table 3.7: Comparison of the mean values of pre- and post venous stasis corrected calcium.

Distribution of differences remained normal (Shapiro-Wilk W=0.96 p=0.64) and a paired t-test applied for comparison of the means was carried out.

<table>
<thead>
<tr>
<th></th>
<th>Mean Difference (pre - post)</th>
<th>DF</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected calcium</td>
<td>0.004</td>
<td>18</td>
<td>0.392</td>
<td>0.6994</td>
</tr>
</tbody>
</table>

Table 3.8: Paired t-test for comparison of the mean values of pre and post venous stasis corrected calcium.

Not only does adjusting the calcium results for albumin bring down the spread of post-stasis values as expressed by the standard deviation, but the statistically significant difference between the pre and post stasis mean concentrations disappears.

Discussion

Reference Intervals

It has long been recognised that all laboratories should establish their own reference limits. Although the sample size in this study is smaller than the preferred minimum recommended by the International Federation of Clinical Chemistry (1987c), it is over the forty values needed to calculate a reference range based on 95% reference limits (0.025 and 0.975 fractiles). In normally distributed populations, these limits may be represented by ± 1.96 standard deviations from the mean value. Although the distribution of serum total calcium results for the whole population was not normal, distributions for each sex fitted a Gaussian pattern. This indicates a possible difference in the distribution for each of the sexes. Statistical analysis of the means (Students t-test for unpaired means) indicates no significant difference between males and females (t = -1.219, df = 77, p = 0.2267). However the 95% reference limits of the two populations appear to be slightly different, with higher male values at the mean and upper limit. A summation of these two
differently (but normally) distributed sub-groups could result in the tendency towards bi-modality observed in the population as a whole.

Data to support the proposition of differing total calcium concentrations in young male and female populations does exist (Sinton et al., 1986; Whitehead et al., 1994). There is also a well documented increase in total calcium values in females at the menopause, (Marshall et al., 1982; Nordin et al., 1989; Sinton et al., 1986; Whitehead et al., 1994; Young & Nordin, 1967), while values in men tend to decrease with age. This leads to higher mean and reference limits in post-menopausal women compared to age matched male subjects (Nordin et al., 1989; Whitehead et al., 1994). The differences observed in this study could therefore arise from a population loaded with young subjects in whom male total calcium reference limits are higher than in females. Examination of age distributions reveals this is not the case. Furthermore the mean ages of the male (45.0 yrs) and female (46.8 yrs) populations show no significant difference (Mann-Whitney U test, Z=-0.310, p=0.76). The male total calcium reference limits found in this study should be interpreted with caution. The distribution barely passes the Shapiro-Wilk test for normality at p = 0.09, and an extended tail is conferred on the distribution by two values above 2.60 mmol/L (data not shown). In a study this size, this could account for the higher upper reference limit found in men. It is possible that differences in serum total calcium are due entirely to differences in serum albumin concentrations. Male corrected calcium values are greater at the upper limit, and smaller at the lower limit. As the mean values do not differ, this is simply a function of the greater standard deviation of the male population mean.

Increased hydrostatic pressure on change of posture from supine to upright results in a reduced plasma volume and increased serum albumin concentration. This effect causes higher serum total calcium concentrations in out-patients (Sinton et al., 1986), a difference which disappears on correction of calcium for albumin (Dent, 1962; Orrell, 1971). The design of this study attempts to minimise the natural haemoconcentration occurring due to upright posture and muscle action. It is possible that within out-patient groups, male subjects have higher albumin and so total calcium levels due to their generally larger body size and muscle mass. Muscle mass tends to decrease with age explaining the age-related decrease in serum albumin, and (effects of the menopause apart) total calcium found in outpatients of both sexes. The presence of this effect in younger subjects (i.e. from mid 20's) onwards, (Sinton et al., 1986; Whitehead et al., 1994) indicates the underlying cause is unlikely to be a pathological defect of albumin metabolism.

Studies investigating a change of Ca^{2+} with sex, age or posture are susceptible to measurement artefact in that ionised calcium analysers show positive interference to serum albumin (Freaney et al., 1986; Thode et al., 1983). It is difficult to obtain subjects
in a truly recumbent state. Within any population, demonstration of higher Ca\(^{2+}\) concentrations in a sub-group would only be conclusive in truly supine subjects matched for age and serum albumin concentrations.

**Calculation of Correction Factor**

It is appropriate at this point to consider the phrase 'corrected calcium'. The use of this term has two inherent problems. Firstly it may be confused with a medical or surgical intervention designed to return (active ionised) calcium to normal. Second, and perhaps more importantly, it implies that the total calcium as measured is an inaccurate value. This is not so. The total calcium is simply altered to become more indicative of the amount of Ca\(^{2+}\) present. The term 'adjusted calcium' is therefore more appropriate.

As previously discussed, the relationship between total calcium and protein is principally due to the binding of Ca\(^{2+}\) to albumin (Pedersen, 1971a). Adjustments made for total protein concentrations are therefore less accurate than those for serum albumin, especially when the distribution of serum proteins is abnormal (Dent & Watson, 1968; Payne, *et al.* 1973). In hypergammaglobulinaemia, the principal hypoalbuminaemic abnormality, increased gamma globulins contribute to total protein concentration, while binding a negligible amount of calcium (Pedersen, 1971b).

Adjusted calcium measurements are more 'powerful' that those of total calcium alone, being essential for the identification of both spurious hypercalcaemia and apparently normocalcaemic individuals who are not actually so (Anonymous, 1977). Iqbal *et al.* (1988), found the clinical management of around 8% of patients might have differed had calcium results not been adjusted for albumin. Calcium concentrations of siblings fall within a narrow sub-band of the reference range, (Payne *et al.*, 1986) indicating a small intra-individual variation of serum calcium, and allowing the possibility of pathological change within the population reference interval. This phenomenon is only apparent on the adjustment of total calcium for albumin. Similarly the use of adjusted rather than total calcium concentrations increases the power of data generated in this study, i.e., the comparison of calcium status in pre- and post-menopausal women.

Conflicting approaches on the calculation of adjustment factors were taken. While Berry *et al.* (1973) derived a mean regression factor from a number of individuals in whom venous return was blocked for fifteen minutes, Payne *et al.*, (1973) adjusted calcium values to the mean of the normal range in a population with no disorders of calcium homeostasis, but the widest range of protein concentrations available. These markedly different experimental approaches produced similar results (0.02 and 0.025 mmol/L calcium per gram of albumin respectively).
The correlation coefficient of calcium on albumin found in this study \((r=0.61)\) is lower than that of Payne \((r=0.867)\), probably due to our use of 'normal' albumin values. The correlation is still markedly higher than that quoted by Payne for normal patients \((r=<0.4)\).

Pain et al. (1975), finding posture experiments, tourniquet studies and multiple measurements gave a wide range of correction factors, criticised the use of average regression coefficients suggesting the calculation of a regression coefficient for each individual. As well as proposing an inconvenient approach, this study was heavily criticised on statistical grounds and for the inclusion of acutely and chronically ill patients (Hodkinson, 1976; Payne et al., 1976; Phillips & Pain, 1977; Ramsay & Shelton, 1976).

Our study was designed to eradicate such criticism, yet a similarly wide range of individual regression factors were found using venous occlusion. It should be noted that changes produced by postural, tourniquet and sequential measurement methods are small compared with the total amount measured. Errors are relatively large and compounded by the need to make four (two calcium and two albumin) measurements in total. A common regression coefficient derived from patients with normal and abnormal albumin concentrations but with no clinical suspicion of disordered calcium homeostasis is the most suitable for calculation of the adjustment factor.

It has been suggested that absolute correction factors, however derived, give inaccurate values at abnormal concentrations of \(\text{Ca}^{2+}\) (Hodkinson, 1974; Parfitt, 1974). The use of average factors while crude, is better than not adjusting the calcium (Anonymous 1977). While the slope of regression of calcium versus albumin does lead to over-compensation at each end of the scale, corrections with absolute factors lead to effective indications of abnormal charges in \(\text{Ca}^{2+}\). The fact that 68% of hypocalcaemic patients may be attributed to low albumin concentrations alone supports this position (Payne et al., 1979). Furthermore there is a reduction in the in-patient variability of serum calcium once albumin concentrations are taken into account.

The use of bromocresol green (BCG) has been criticised as resulting in the overestimation of albumin, especially at low albumin concentrations, due to binding by other plasma proteins (Hill, 1985). However, the alternative autoanalyser method using bromocresol purple (BCP), is not perfect. Albumin concentrations in serum of paediatric haemodialysis patients and adults with renal insufficiency are systematically underestimated by this method (Maguire & Price, 1986; Wells et al., 1985). Furthermore comparisons of overall correlations with reference methods show little difference between BCG and BCP methods, especially when BCG is analysed with short reaction times (McGinlay & Payne, 1988).
It has been suggested that below 30 g/L albumin, different adjustment factors be used to account for increased binding of calcium to globulins (McEnroe et al., 1992). However, it may be argued that the non-specific dye binding of proteins other than albumin to BCG reflects the contribution towards calcium binding of the serum globulins at low albumin concentrations. Certainly BCG-measured albumin gives slightly better correlations with total calcium than does albumin assayed by other methods (McGinlay & Payne, 1988).

**The Use of The Tourniquet In Venepuncture for Serum Calcium Estimation**

It has long been considered that a single regression coefficient may not make adjustment for the variable changes that follow venous stasis, and therefore it is important to take blood samples with no venous stasis. This was contested by McMullan et al. (1990), who found Ca^{2+} unaltered by ten minutes of tourniquet application, and increases in total calcium disappearing on adjustment for albumin. In the same study the increase in total calcium induced by one minute of tourniquet application was negligible even without albumin correction. Our studies confirm this. Two minutes of venous occlusion was found to give significantly different mean values for total calcium and albumin. However, the magnitude of the mean difference is small (0.02 mmol/L for serum total calcium) and disappears on adjustment of the total calcium for albumin. Furthermore, the largest difference in total calcium induced was 0.08 mmol/L. This value is smaller than the difference in successive measurements required to reach significance at the 95% probability level. This required change, the 'critical difference', is 0.18 mmol/L for total calcium (Morris et al., 1990).

Binding of calcium to albumin is not only proportional to the total calcium concentration but influenced by ligands in plasma including globulins, bicarbonate and lactate. Binding may also be influenced by plasma pH. Nordin et al. (1989) provided numerical equations for the estimate of Ca^{2+} based on some of these factors, but perhaps the most important consequence of this information is more qualitative. Adjustments of total calcium may only be applied to patients with no abnormality of blood pH (Walker & Payne, 1979). As a combination of venous occlusion and forearm exercise produces a variable increase in lactate and decrease in pH the variable changes in calcium and albumin found by many venous occlusion studies may be explained (Berry et al., 1973; Phillips & Pain, 1977).
The Measurement of 1,25-dihydroxyvitamin D: Comparison of a New Commercial Immunoassay with a Reference Method

Background

The major circulating metabolite of vitamin D, (25-hydroxyvitamin D; calcidiol) is present in normal individuals in ng/ml concentrations. The concentration of the primary hormonal metabolite, 1,25-dihydroxyvitamin (1,25(OH)₂D; calcitriol) in normal serum is in the order of pg/ml. Consequently, assays for 1,25(OH)₂D have characteristically concentrated on the specificity of measurement of the metabolite of interest in the presence of its more abundant precursor.

Apart from techniques such as bioassay (Stem et al., 1978) and isotope-dilution mass fragmentography (Bjorkhein et al., 1979), both of which are unsuitable for routine use, assays of 1,25(OH)₂D may be broken down into three principal steps:

1) The extraction of 1,25(OH)₂D from the sample (serum or plasma may be used)

2) Separation of the 1,25(OH)₂D from other vitamin D metabolites

3) Quantitation of the 1,25(OH)₂D

Quantitation steps fall into two different categories, these determining the separation techniques required. Methods using antibodies in radioimmunoassays (RIA), classically require preparative separation of the metabolites by high performance liquid chromatography (HPLC). Naturally occurring receptors to 1,25(OH)₂D exhibiting greater specificity than most antisera are utilised in assays analogous in format to RIA (Mawer & Berry, 1995). In these radio-receptor assays (RRA), extensive sample purification is still necessary, although HPLC is not essential.

Since the first RRA was described (Brumbaugh et al., 1974), most efforts have been directed towards the simplification of the separation procedures involved and the improvement of assay sensitivity. Change of the receptor source from chick intestine (Eisman et al., 1976) to calf thymus (Reinhardt et al., 1984) saw a marked improvement in assay sensitivity. Calf thymus binding-protein exhibits a relative insensitivity to lipid interference and a higher affinity for 1,25(OH)₂D, coupled with poorer recognition of other vitamin D metabolites. This improvement allowed some workers to advocate the abandonment of HPLC purification, first for a dual cartridge method using silica and C₁₈ micro-columns (Reinhardt et al., 1984) and later a single cartridge extraction and purification procedure with ‘phase switching’ of solvents (Hollis, 1986). Both of these methods have been used as the basis for commercially available assays which perform acceptably in comparison with HPLC-RRA reference methods (Bertelloni et al., 1993).
Although expensive in kit form, the calf thymus required is not otherwise easily available. Furthermore variation between batches and poorer stability than antibodies have been reported (Mawer & Berry, 1995), while some workers prefer the security of HPLC separation to remove possible interference from other vitamin D metabolites.

The superior long term reproducibility and stability of antibodies have led to their use by some laboratories. However the polyclonal antisera used have usually been directed against 1,25(OH)2D3 and have shown poor cross-reactivity with 1,25(OH)2D2 (Bouillon et al., 1980; Gray et al., 1981b). An assay using monoclonal antibodies exhibiting equipotency for the two 1,25-dihydroxylated metabolites has been described (Mawer et al., 1990). This assay is therefore thought to exhibit the stability and reproducibility of antibodies together with the accuracy for total 1,25(OH)2D shown by receptor assays.

Recently a commercial assay (‘Gamma-B 1,25-dihydroxyvitamin D’, IDS Ltd, Tyne & Wear, UK), utilising a new purification format has become available. This method is based on sample immunopurification with a monoclonal capture antibody specific for 1,25(OH)2D. Subsequent quantitation utilises an RIA format incorporating a 125\(^{-}\)I labelled tracer. The assay antibody exhibits 100% cross-reactivity for 1,25(OH)2D3 and 80% for 1,25(OH)2D2. As these two metabolites are biologically equipotent in humans (Holick & Adams, 1990), this leads to a marginal under-estimation in total 1,25(OH)2D. Such a discrepancy is most likely to be important in those relying on vitamin D supplementation. While the consumption of such supplements is commonplace in the USA, this is unusual in the UK (Horst, 1984).

Our ‘in-house’ assay for 1,25(OH)2D of RRA following HPLC purification may be considered a reference method for the measurement of this hormone. However calf thymus receptor protein can prove difficult to obtain, is relatively unstable and exhibits variability between batches. Furthermore HPLC purification requires specialised equipment, is laborious and technically demanding. Factors such as these combine to produce what has been described as ‘this notoriously difficult assay’ (Mawer & Berry, 1995) It was therefore decided to investigate aspects of the alternative methodologies available for the measurement of 1,25(OH)2D.

Three methods were considered:

I) In-house HPLC-RRA method (modified from Reinhardt et al. 1984)

II) Single C\(_{18}(O)\) Column Extraction Method (Hollis, 1986)

III) ‘Gamma-B 1,25(OH)\(_2\)D’ assay (IDS Ltd)

The methodologies and performance characteristics of these assays may be found in the appropriate section of this thesis (Chapter II).
Initially the separation of 25-OHD and 1,25(OH)\textsubscript{2}D by methods I and II was considered.

**Results**

The separation of the tritiated metabolites of vitamin D by methods I and II are shown in figures 3.6, 3.7 and 3.8.

**Figure 3.6:** Separation of tritiated 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D by HPLC. Mobile phase of hexane:propan-2-ol (88:12) run on a silica column. Using a fraction collector, eluate is collected at 30 second intervals. The first peak represents 25-OHD, the latter 1,25(OH)\textsubscript{2}D. The figure indicates the excellent separation of these two metabolites afforded by this technique.
Figure 3.7: Elution profile of tritiated 25-hydroxyvitamin D from Bond-Elut C18-OH columns. Each solvent was added according to the method of Hollis (1986) except as five separate aliquots of 1ml. This figure shows the complete elution of 25-OHD by 10% methylene chloride in hexane.

Figure 3.8: Bar chart indicating the concentration of final eluant required for the removal of 1,25(OH)_{2}D from Bond-Elut C18-OH columns. Increasingly polar solvent mixtures were added to the column in 5 ml aliquots.

Considered together, figures 3.7 and 3.8 demonstrate that 25-OHD and 1,25(OH)_{2}D will not co-elute from C_{18}-OH columns using the solvents described. Comparison of in-house HPLC-RRA method with IDS Gamma 1,25(OH)_{2}D method was then undertaken.
in thirteen clinical samples with a wide range of 1,25(OH)_{2}D concentrations. While the samples had been measured by HPLC-RRA in different assay runs, they were determined in a single batch by the candidate method. The results are illustrated in table 3.9 and figures 3.7 and 3.8.

<table>
<thead>
<tr>
<th></th>
<th>HPLC-RRA</th>
<th>IDS Gamma-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Samples</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Mean 1,25(OH)_{2}D (pg/ml)</td>
<td>67.8</td>
<td>72.3</td>
</tr>
<tr>
<td>Std Error</td>
<td>13.7</td>
<td>15.4</td>
</tr>
</tbody>
</table>

Table 3.9: Comparison of two methods of measuring 1,25(OH)_{2}D. In-house HPLC-radio receptor assay quantitation compared with commercial assay (IDS) using immunological separation and radio-immunoassay measurement.

Figure 3.9: Regression analysis for the comparison of two methods for the measurement of serum 1,25(OH)_{2}D. In-house method using HPLC separation and quantitation by radio-receptor assay (x) versus a commercial method ('Gamma-B', IDS Ltd) employing immunological separation and subsequent radioimmunoassay (y).

The difference between the two sets of mean values, compared using Wilcoxon's signed rank test showed no significant difference between the means (Z=-0.978, p=0.33). The actual figure for the mean difference (y-x) of 4.5 pg/ml over such a wide concentration range illustrates the lack of bias between the two methods.
Discussion

Although HPLC is the more powerful method of separating vitamin D metabolites, separation of tritiated vitamin D metabolites was achieved by single C18-OH columns. All 25-OHD appears to elute in 10% methylene chloride:hexane, while the majority of the 1,25(OH)2D required 6% propan-2-ol (IPA) to elute.

It should be noted however that while showing the elution profile of 25-OHD clearly, addition of each solvent as five 1ml aliquots will result in a more efficient extraction than the single 5ml wash described by Hollis (1986). Furthermore Figs 3.7 and 3.8 show the radioactivity in each fraction as a percentage of the total recovered. Comparison of counts recovered with those added show losses of approximately 20%. Recovery determinations are therefore essential in both HPLC and column chromatographic methods. The assay result then requires a manual correction to account for the estimated sample loss. A further disadvantage of the C18-OH column method is the multiple step separation procedure. Apart from being labour intensive (although not compared to HPLC) this method uses a number of toxic and flammable solvents.

As the elution experiments described above were performed, samples of calf thymus receptor became unavailable due to a freezer malfunction. While commercial versions of the C18-OH assay are available, the development and marketing of a non-chromatographic antibody-based method for 1,25(OH)2D made further assessment of the C18-OH assay unattractive. The results of initial investigations using single column methods are included for completeness.
It was therefore decided to compare the IDS-Gamma-B assay system with our ‘in-house’ procedure. The IDS method offers the attraction of markedly reduced ‘hands-on’ laboratory time, higher sample throughput and a reduced sample volume requirement. The use of a highly efficient solid-phase bound immunoextraction antibody obviates the need for recovery determinations, while the iodinated RIA format results in greater counting efficiency and reduced counting times.

Figure 3.9 shows the IDS method to be highly and significantly correlated to the ‘reference’ HPLC-RRA method. The small sample used for comparison is accepted as a limitation. This is due to the HPLC-RRA method being ‘sample-hungry’ requiring repeat analysis due to poor recoveries. However the correlation of the IDS method with the HPLC-RRA supports the recent findings of other authors (Durham et al., 1995; Laurie et al., 1995). No significant difference in the sample means was found by the Wilcoxon signed rank test. The Bland-Altman plot suggests the differences between the two methods are random rather than due to systematic bias. The large variability of that bias, represented by the degree of scatter around the zero mean difference point on the y axis in Fig 3.10, is most likely due to the large inter-assay coefficient of variation exhibited by the reference method.

**Comparison of Two Immunoassays for Osteocalcin**

Few assays suffer from a lack of standardisation as severely as those for osteocalcin (OC). Differences in assay format, species matrix of standard and antibody recognition site all contribute to this problem. It was decided to compare the assay used in our clinical studies (‘OSCAtest’, Henning GmbH, Berlin, Germany), with an alternative method using a different assay format (‘NovoCalcin’, Metra Biosystems, Oxford, UK).

**Methods**

Forty serum samples from individuals with a variety of metabolic bone diseases were assayed for serum osteocalcin by each method. Samples were analysed in duplicate in a single batch.

**OSCAtest**

This competitive radioimmunoassay (RIA) is based on the work of Catherwood (Catherwood et al., 1985). Sheep polyclonal antibodies directed towards the 37-49 amino acid sequence of osteocalcin are immobilised on the assay tube. Sample OC competes for the antibody binding sites with a radiolabelled osteocalcin fragment analogue (38-49) As such the assay is C-terminal specific. This is represented in Figure 3.11.
The NovoCalcin assay kit is formatted as an enzyme-linked immunosorbent assay (ELISA) based on the work of Kelm (Kelm et al., 1992). The assay is based upon the competition between sample OC and immobilised human OC standards for a fluid-phase mouse monoclonal antibody raised against bovine osteocalcin (see Fig 3.10).

Results

Data produced is shown in Table 3.10.

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>NovoCalcin ELISA</th>
<th>OSCAtest RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (ng/mL)</td>
<td>10.8</td>
<td>13.1</td>
</tr>
<tr>
<td>Minimum (ng/mL)</td>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Maximum (ng/mL)</td>
<td>32.4</td>
<td>59.0</td>
</tr>
<tr>
<td>Std. deviation (ng/mL)</td>
<td>6.6</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Table 3.10: Osteocalcin concentrations measured by two different methods in forty patient samples.
Osteocalcin coated micro-titre well

Figure 3.12: Assay of serum osteocalcin using NovoCalc ELISA format. After incubation sample and excess antibody are washed from the system. Subsequent incubation with a second antibody conjugated to alkaline phosphatase allows colorimetric determination using para-nitrophenol phosphate substrate. Intensity of colour developed (measured at 405 nm) is inversely proportional to amount of sample OC.

The difference between the method means was assessed using Wilcoxon’s signed rank test. This showed that the mean value of the OSCAtest RIA was significantly higher than that of the ELISA with which it was compared (Z= -4.008, p= <0.0001). The level of agreement between the two sets of results may also be assessed by a scatterplot of the raw data and the fitting of an appropriate regression line (Figure 3.13).

Although the Spearman coefficient shows the values obtained to be highly correlated, one would expect this from two methods for measuring the same analyte, especially over such a wide concentration range. Method comparisons are more properly carried out using the Bland-Altman difference plot. This is illustrated in Figure 3.14.
Figure 3.13: Regression analysis for two methods for the measurement of osteocalcin. Radioimmunoassay utilising polyclonal antibodies (x) versus monoclonal based enzyme-linked immunosorbent assay.

Figure 3.14 Bland-Altman difference plot comparing a RIA method (x) with an ELISA (y) method for the measurement of serum osteocalcin. The mean bias x-y = 2.3 ng/mL.
Discussion

It is clear from the summary data table that the mean OC values given by RIA are higher than those obtained by the ELISA method. The mean difference between the methods is 2.3 ng/mL, a difference confirmed as statistically significant by the Wilcoxon signed rank test. It would be wrong to draw conclusions based on mean data alone however. Inspection of the Bland-Altman plot reveals a more complex picture in which the between-method differences only become apparent with increasing osteocalcin concentration. If the Wilcoxon signed rank test is applied exclusively to results within the reference range (the RIA kit has the most comprehensive data; quoted range 4-13 ng/mL) the difference between the two methods is still statistically significant (n=26; z=-3.02; p=0.003). However the mean difference has practically disappeared and has no clinical significance whatsoever (ELISA mean = 7.2 ng/mL; RIA mean 7.9 ng/mL; mean difference 0.7 ng/mL). Thus the numerical data confirms the visual information of the Bland-Altman plot in that there is no bias between the methods within the analyte reference range. The reason behind the greater RIA values at higher concentrations was then investigated. Firstly the two samples with the highest OC values by RIA were diluted and re-analysed by the same method. Confirmation of the original results suggests that the high OC concentrations obtained by RIA were not due to the flattening of the original RIA curve at higher concentrations. Samples giving the largest difference in OC values were found to differ in the disease states from which they were drawn (Paget’s disease, Engelmann’s disease, privational osteomalacia), the only similarity being increased bone turnover.

As the discrepancy in the OC values must be due to relative over-estimation by the RIA method or under-estimation by the ELISA method, the specificity of the two methods for measuring intact osteocalcin was examined. The structure of osteocalcin and its potential proteolytic fragments are discussed and represented diagrammatically in Chapter I of this thesis. Initial overview of the methodologies would tend to indicate an under-estimation by the ELISA method to be unlikely. In competition assays most ‘interferences’ manifest as positive effects, (i.e. a decrease in binding of the primary antibody to the well-coated osteocalcin would result in an increased final OC result). A negative interference in an ELISA format of this type would have to be due to a reduction in recognition of sample OC by the antibody. It is theoretically possible that in renal failure such an effect may occur due to a non-enzymatic alteration of the circulating OC molecule. Such a change could affect the conformation of the molecule and reduce its recognition by a conformationally-dependent antibody such as the one used in the NovoCalcin ELISA. However none of the patients in this study showed any renal insufficiency as assessed by serum creatinine concentrations. As such it is more probable that the higher values given by the RIA are due to the recognition of one or more of the fragments of OC by the OSCAtest antibody but not the ELISA monoclonal antibody. While Taylor et al. (1990)
found such a fragment apparently specific to Paget’s disease, in this study the RIA gave increased OC values in other conditions of increased bone turnover.

The Use of Acidified Urine In the Collagen Crosslinks Immunoassay

Introduction

An evaluation of the ‘Pyrilinks’ assay for free pyridinolines (Metra Biosystems (UK) Ltd, Oxford, UK) has recently appeared in the literature (Hata & Miura, 1994). Their findings, indicating the usefulness of this method in monitoring urinary total free pyridinoline excretion as a marker of bone resorption, are consistent with those of others workers (Delmas et al., 1993; Seyedin et al., 1993).

This assay measures free pyridinolines (i.e. pyridinoline and deoxypyridinoline) with 100% cross-reactivity using a monoclonal anti-pyridinoline antibody in an ELISA format. Recognition of the various peptide bound forms of both crosslinks is negligible.

The manufacturer, Hata and Miura (1994), and other authors (Delmas et al., 1993; Seyedin et al., 1993) use the abbreviation ‘PYD’ to represent the sum of both forms of pyridinium crosslink as measured by this method. The use of ‘PYD’ in this manner leads to a potential for confusion. It is possible this term may be misinterpreted as indicating either total or free pyridinoline. Furthermore although pyridinoline is the more abundant of the two forms, the contribution from free deoxypyridinoline to the final result is not recognised by the use of ‘PYD’. Although more unwieldy, the term ‘total free pyridinolines’ is a more accurate description of the compounds measured by this assay.

The prospective use of this assay in the screening and monitoring of patients with osteoporosis and other metabolic bone diseases led us to consider its possible use with ‘routine’ urine samples.

It is common in this and many other laboratories to receive urine containing hydrochloric acid (HCl) as a preservative. This is used for its bacteriostatic action and to encourage the dissociation of calcium salts in samples for urinary calcium estimation. However the ‘Pyrilinks’ assay protocol insists on the use of unpreserved urine. Discussions with the kit manufacturer led to a suggestion that acidified urine may be used on adjustment of the sample pH to that of normal urine. However this is a cumbersome process involving alteration of the sample volume. It was therefore decided to investigate the use of this ELISA with acidified urine samples.
Methods

All analyses were performed in duplicate according to the manufacturers suggested protocol. This is described in detail in the methods chapter of this thesis.

The following paired samples were analysed: acidified versus unacidified (n=20), and acidified versus ‘pH corrected’ (n=10). Urine samples were taken from normal subjects and patients suffering from a variety of metabolic bone diseases. Both 24 hour and two hour fasting collections were used.

The concentration and volume of HCl used for sample acidification varies with laboratory. In this study samples were acidified with 10 ml of concentrated HCl irrespective of volume. This ensured the most acidic environment likely to occur in clinical samples.

Urine samples for pH correction were adjusted to the pH of their unacidified pair using concentrated sodium hydroxide. A volume correction for the addition of alkali was then made.

Results

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>No.</th>
<th>Mean</th>
<th>Std Error</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidified</td>
<td>10</td>
<td>594</td>
<td>262</td>
<td>35</td>
<td>1962</td>
</tr>
<tr>
<td>pH corrected</td>
<td>10</td>
<td>578</td>
<td>245</td>
<td>79</td>
<td>1862</td>
</tr>
<tr>
<td>Acidified</td>
<td>20</td>
<td>482</td>
<td>111</td>
<td>47</td>
<td>2585</td>
</tr>
<tr>
<td>Unacidified</td>
<td>20</td>
<td>490</td>
<td>118</td>
<td>49</td>
<td>2532</td>
</tr>
</tbody>
</table>

Table 3.11: Comparison of acidified with pH corrected and unacidified urine in the Metra Biosystems ‘Pyrilinks’ assay for total free pyridinolines. All results nmol/l.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Number of pairs</th>
<th>Z-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidified</td>
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<td>pH corrected</td>
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<td></td>
<td></td>
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<tr>
<td>Acidified</td>
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<td>-.845</td>
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<tr>
<td>Unacidified</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.12: Statistical comparison of acidified with pH corrected and unacidified urine in the Metra Biosystems ‘Pyrilinks’ assay for total free pyridinolines. Analysis of sample means performed using Wilcoxon’s signed rank test.
No significant difference was found between the means of either of the paired groups.

The data was subsequently plotted to investigate the correlation between the two groups, as shown in Figure 3.15.

![Graph showing regression analysis for the comparison of sample type used in Pyrilinks ELISA assay.](image)

**Figure 3.15** Regression analysis for the comparison of sample type used in Pyrilinks ELISA assay. Total free pyridinolines in acidified urine plotted against total free pyridinolines in unacidified (open circles) and in pH corrected urine (closed circles). The regression line is $y=0.984x + 8.3$. The Spearman rank correlation coefficient =0.961

However, method comparison is more appropriately carried out using the difference plot described by Bland and Altman (1986). This is shown as Figure 3.16.

Visual inspection of Figure 3.16 shows no obvious relationship between the difference in the results and their mean. The ‘outlier’ shown in the Bland-Altman plot (acidified and unacidified values differ by more than 500 nmol/L) represents a 24 hour collection from a patient with active Paget’s disease. The lower (acidified) pre-treatment result represents a value fifteen times the upper limit of the manufacturers quoted reference range as expressed relative to urine creatinine concentration. As such the difference between these results is unlikely to be clinically significant. As a post-treatment sample from the same patient showed excellent agreement between acidified and non-acidified samples, it is probable that this discrepancy is due to an analytical error.
Figure 3.16: Difference plot for Pyrilinks ELISA sample type. Comparison of unacidified (x, open circles) and pH corrected (x, closed circles) with acidified urine (y). The lack of bias shown by using acidified urine is illustrated by the mean difference (y-x) which equals -0.1 nM.

Discussion

While this study confirms the suitability of use of acidified urines in the Pyrilinks assay, a more recent assay, measuring free deoxypyridinoline alone, is now available ('Pyrilinks-D', Metra Biosystems UK). Deoxypyridinoline is primarily located in type I collagen of bone, while pyridinoline is also found in cartilage and other soft tissues (Eyre et al., 1984). As such the 'Pyrilinks-D' assay represents a more specific indicator of bone resorption than its predecessor (Robins et al., 1994a). As the newer assay involves a ten-fold dilution of urine sample in a phosphate buffer before analysis, it would seem improbable that sample acidification would affect the assay.

While it has been superseded in the measurement of bone-specific collagen turnover, the 'Pyrilinks' assay remains available as a general indicator of bone resorption. It may also have applications in other connective tissue disorders, especially conditions such as the arthritic diseases, in which the ratios of urinary pyridinoline and deoxypyridinoline become altered (Astbury et al., 1994; MacDonald et al., 1994).

This study, while indicating the suitability of acidified urine samples in this assay, has not attempted to address the desirability of their use. Urine measurements are commonly
corrected for creatinine to account for urine concentration and body size (as muscle mass). The most common method of creatinine estimation is reaction with alkaline picrate, the Jaffé reaction, is pH dependent with sample acidification suppressing colour development (McLauchlan, 1988). Appropriately acidified aqueous standards are commonly used to overcome this problem. The argument for sample acidification is strengthened by the increase it produces in the stability of pyridinolines on exposure to UV light (Blumsohn et al., 1995b).

The relatively poor specificity of Pyr as a marker of bone turnover is illustrated by the highest increase of any of the crosslink markers in patients with hyperparathyroidism (in which connective tissues other than bone contribute to collagen turnover) (Seibel et al., 1992b). However Pyr may still be used as an indicator of general collagen turnover. It has been postulated that the relative excretion of Pyr and DPyr may be of some value as markers of disease activity in osteo- and rheumatoid arthritis (Seibel et al., 1992a). However while Astbury et al. (1994), found raised values in both these markers as compared with healthy controls, no correlation with clinical indices of disease severity could be found.

The reproducibility of total free pyridinoline measurements in patients with metabolic bone disease.

The marked diurnal variation in the urinary excretion of pyridinium crosslinks has been reported previously (Schlemmer et al., 1992). Despite this spot urine collections are still suggested by assay manufacturers as suitable for the analysis of pyridinium crosslinks. In an attempt to minimise both cost and intra-individual variation, total free pyridinolines were analysed on a pool of two 24h samples (i.e., a 48 hour collection), rather than 24 hour, timed fasting or random samples.

During the course of collating data for this thesis, it became apparent that in several patients, 48 hour pooled urine samples had been analysed for PYD on several consecutive days before commencement of treatment. The examination of this data gives some information on intra-individual variation of PYD excretion.

Method

Nine patients with various metabolic bone diseases had 24 hour urine samples collected before treatment. These were subsequently pooled into 48 hour samples for cost convenience. Samples from several consecutive 48 hour pools (minimum 4, maximum 7, mean 4.4), were analysed for PYD:creatinine ratio.
Results

The mean intra-individual coefficient of variation in the nine patients studied was 30% (minimum 7%; maximum 70%). The PYD/creatinine ratios of the selected patients ranged from 21 to 340 mmol/mmol, with a mean value of 124 nmol/mmol. There was no apparent correlation between concentration and coefficient of variation.

Discussion

This study confirms the large intra-individual variation found in urinary PYD measurements, a phenomenon common to many or all urinary markers of bone resorption. The magnitude of the variation differs markedly between individuals, making assessments of what constitutes a significant change between measurements difficult. Although matched fasting samples from these patients were unavailable, comparison with other studies (Colwell et al., 1993; Seyedin et al., 1993) using other samples types (but similar patient numbers), indicate coefficients of variation of similar magnitude. This suggests that analysis of 48 hour urine pools may not improve intra-individual variation over 24 hour collections. However even if this is the case, the pure cost benefits of such actions are undeniable.

Comparison of Twenty-Four Hour and Fasting Urine Collections For the Analysis Of Free Deoxypyridinoline.

Although the manufacturer of the ‘Pyrilinks-D’ immunoassay (Metra Biosystems, Oxford, UK) states that either 24 hour, fasting or random urine specimens may be analysed, there is some disagreement on the optimum specimen to use for the analysis of this metabolite. It was decided to take advantage of the in-patient status of patients to be treated at the Royal National Orthopaedic Hospital to compare free DPyr levels in 24 hour and fasting urine collections.

Method

Thirty-two patients with a variety of metabolic bone diseases admitted for in-patient treatment at the RNOHT gave urine specimens before the commencement of treatment. Urine samples were collected for a 22 hour period, from 0800 to 0600 hours. At this point a further collection was started and any urine produced until 0800 hours saved separately. This was designated the two hour fasting urine sample. A small aliquot was taken for analysis and the remainder added to the 22 hour sample to make a twenty-four hour collection.
Free DPyr was analysed by ELISA. For comparison other biochemical markers of bone metabolism (total- and bone-specific alkaline phosphatase, osteocalcin, the carboxy-terminal propeptide of type I collagen) were also analysed.

**Results**

The following values were obtained:

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Mean DPyr/Cr (nmol/mmol)</th>
<th>Std Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hour collection</td>
<td>32</td>
<td>8.9</td>
<td>5.6</td>
</tr>
<tr>
<td>2 hour fasting collection</td>
<td>32</td>
<td>5.6</td>
<td>14.0</td>
</tr>
</tbody>
</table>

Table 3.13: Comparison of urinary free deoxypyridinoline:creatinine ratios in timed fasting and twenty-four hour urine collections.

The two data sets were further analysed using correlation analysis and Wilcoxon's test for paired non-normally distributed populations. This showed that the two sets of data were not strongly correlated ($r=0.52 \ p=0.002$) and that the difference between the two groups was highly significant ($z=-3.4 \ p=0.001$).

Each sample type was also compared to other biochemical markers of bone metabolism:

<table>
<thead>
<tr>
<th></th>
<th>Total ALP (n=25)</th>
<th>Bone-ALP (n=22)</th>
<th>PICP (n=20)</th>
<th>Osteocalcin (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hour DPyr/Cr</td>
<td>.586</td>
<td>.485</td>
<td>.466</td>
<td>.796</td>
</tr>
<tr>
<td>2 hr fasting DPyr/Cr</td>
<td>.293</td>
<td>.111</td>
<td>.315</td>
<td>.610</td>
</tr>
</tbody>
</table>

Table 3.14: Comparison of free DPyr/Cr ratio in fasting and twenty-four hour urine collections with other markers of bone turnover. Values indicate correlation ($r$).

**Discussion**

This study indicates a poor correlation between free deoxypyridinoline concentrations in fasting and twenty-four hour urine collections, a finding supported by others (Colwell *et al.*, 1993; Uebelhart *et al.*, 1991). The higher levels in fasting samples, representative of increased nocturnal bone loss, were found by these workers to be a better indicator of bone loss. However in this study values from twenty-four hour collections were better correlated with all other markers of bone metabolism studied. It is possible that the variability of free DPyr in fasting urine collections may be higher than that in day long collections. Furthermore the use of creatinine values to correct for muscle mass (and
therefore indirectly skeletal mass) may not be advised in patients receiving drugs affecting muscle mass such as corticosteroids.

The findings of this study together with the need to measure free DPyr in patients with steroid-induced osteoporosis led to the use of 24 hour urine collections in the relevant section of this thesis (see Chapter IV). In such studies free DPyr will be expressed both relative to creatinine and for comparison, as a daily excretion.

**Comparison of Two Commercial Spectrophotometric Methods for the Analysis of Serum Tartrate-Resistant Acid Phosphatase (TRAP).**

**Introduction**

The measurement of TRAP is accepted as having potential as a marker of bone resorption (Price & Thompson, 1995). However as no commercial immunoassay is currently available, spectrophotometric methods predominate. The most common of these, using a nitrophenol substrate is cumbersome to use involving a number of pre-treatment steps and the need for immediate blood processing (Lau et al., 1987). However an alternative method using α-napthyl phosphate as a substrate, claimed to be specific for osteoclastic TRAP, has the attraction of being directly applicable to the autoanalyser. Two commercial kits which use this method to measure TRAP (as part of the indirect measurement of prostatic acid phosphatase), were tested for their use in assessing bone resorption.

**Methods**

As part of a different study on the assessment of serum calcium reference ranges patients with no known disorders of calcium metabolism had fasting morning blood samples taken. The menopausal status, and use of oral contraceptives or hormone replacement therapy (HRT) was recorded as was the patients age. This group constituted the normal population. Patients attending the RNOH for treatment for osteoporosis and Paget's disease also had measurements of TRAP activity made as an adjunct to other blood tests. Serum samples were allowed to clot for ten minutes before refrigerated centrifugation. One millilitre aliquots of serum were stored at -20°C after the addition of one drop (approximately 10μl) of a stabiliser solution (acetic acid) included with the BM kit.

Serum TRAP was measured by commercially available methods ('Randox Acid Phosphatase AC1012', Randox Laboratories, Co. Antrim UK, and 'BM ACP MPR1' Boehringer Mannheim Ltd UK). These methods are similar except for the inclusion of an alcohol accelerator in the latter. For full details refer to the methods section of this thesis.
Results

The summary data for the different populations is presented in Table 3.15 and illustrated in Figure 3.17.

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>No.</th>
<th>TRAP (Randox) IU/L</th>
<th>SD</th>
<th>TRAP (BM) IU/L</th>
<th>SD</th>
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</thead>
<tbody>
<tr>
<td>Pre-menopausal females</td>
<td>35</td>
<td>2.34</td>
<td>0.49</td>
<td>1.51</td>
<td>0.33</td>
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<tr>
<td>Post-menopausal females</td>
<td>37</td>
<td>3.01</td>
<td>0.52</td>
<td>2.04</td>
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<tr>
<td>Users of oral contraceptives</td>
<td>10</td>
<td>2.36</td>
<td>0.77</td>
<td>1.55</td>
<td>0.36</td>
</tr>
<tr>
<td>Post-menopausal on HRT</td>
<td>9</td>
<td>2.30</td>
<td>0.25</td>
<td>1.61</td>
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<tr>
<td>Paget's disease</td>
<td>19</td>
<td>4.41</td>
<td>1.46</td>
<td>2.93</td>
<td>1.28</td>
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</table>

Table 3.15: Levels of TRAP in normal females and patients with Paget's disease.

Figure 3.17: Mean TRAP levels (± one standard deviation) in normal females and Pagetic patients of both sexes.
Comparison of pre- and post menopausal groups found TRAP as measured by both methods to be significantly higher in post-menopausal women compared to pre-menopausal controls (Mann-Whitney U test p<0.0001, both methods). This difference disappears in those post-menopausal women taking HRT. Simple comparisons of pre- and post-menopausal groups do not take into account the compounding effect of age. However multiple regression analysis confirms that it is the menopause which accounts for the difference in TRAP levels in these two groups (Randox p=0.001, BM p=0.006).

The effect of age on TRAP levels in males and females is presented in Figures 3.18-3.21, while correlation’s with other markers of bone metabolism in a sub-groups of patients with untreated osteoporosis are detailed in Table 3.18.
**Figure 3.18:** Relationship between TRAP and male age. Commercial colorimetric method (Randox Laboratories) using α-naphthylphosphate as a substrate. Linear regression equation $y = 0.03x + 2.654$, $r = 0.07$, $p = 0.68$

**Figure 3.19:** Relationship between TRAP and male age. Commercial colorimetric method (Boehringer Mannheim) using α-naphthylphosphate as a substrate. Linear regression equation $y = 0.01x + 1.423$, $r = 0.30$, $p = 0.07$
Figure 3.20: Relationship of TRAP to age in normal females. TRAP measured by a commercial colorimetric method (Randox Laboratories) using α-napthylphosphate as a substrate.

Figure 3.21: Relationship of TRAP to age in normal females. TRAP measured by a commercial colorimetric method (Boehringer Mannheim) using α-napthylphosphate as a substrate.
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<th>Median</th>
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Table 3.16: Summary data for TRAP (Randox method) response to APD in sixteen patients with osteoporosis and nine patients with Paget’s disease.

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<th>No.</th>
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<td>2</td>
<td>61.4</td>
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</table>

Table 3.17: Summary data for TRAP response to APD (BM method) in sixteen patients with osteoporosis and nine patients with Paget’s disease.
Figure 3.22: TRAP response to APD infusion. Sixteen patients with osteoporosis (upper graphs) and nine patients with Paget’s disease (lower graphs). TRAP measured using both Randox (black) and BM methods (red).
Figure 3.23: Median changes in TRAP on administration of APD in sixteen patients with osteoporosis and nine with Paget's disease. Curves weighted for sample numbers at each time point. Probability of regression effect being better fit than overall mean equals ‘p’ value.

<table>
<thead>
<tr>
<th>Marker</th>
<th>No.</th>
<th>TRAP (Randox) r</th>
<th>p</th>
<th>TRAP (BM) r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total alkaline phosphatase</td>
<td>40</td>
<td>.29</td>
<td>.07</td>
<td>.31</td>
<td>.05</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>14</td>
<td>.26</td>
<td>-</td>
<td>.04</td>
<td>-</td>
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<td>PICP</td>
<td>14</td>
<td>.37</td>
<td>-</td>
<td>.34</td>
<td>-</td>
</tr>
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<td>Bone specific ALP</td>
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<td>.25</td>
<td>-</td>
<td>.45</td>
<td>-</td>
</tr>
<tr>
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<td>.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TRAP (Randox method)</td>
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<td>-</td>
<td>-</td>
<td>.93</td>
<td>.001</td>
</tr>
<tr>
<td>Free DPyr</td>
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<td>.002</td>
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<td>.002</td>
</tr>
<tr>
<td>Hydroxyproline/Cr</td>
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<td>.02</td>
<td>.52</td>
<td>.002</td>
</tr>
<tr>
<td>Fasting urinary Calcium/Cr</td>
<td>31</td>
<td>.41</td>
<td>.08</td>
<td>.53</td>
<td>.002</td>
</tr>
</tbody>
</table>

Table 3.18: Comparison of serum TRAP with other indicators of bone turnover in a subgroup of patients about to receive treatment for established osteoporosis.
Discussion

Both TRAP methods identified the increase in bone turnover known to occur at the menopause and in Paget's disease. The increases given by the BM method were higher in both cases (35% vs. 29% increase due to menopause, 94% vs. 88% due to Paget's). The increase due to the menopause is higher than that found in studies using this substrate or p-nitrophenylphosphate (Revilla *et al.*, 1992; Scarsechhia *et al.*, 1991) and similar to that of de la Piedra *et al.* (1989). In men the BM but not the Randox method reflects age associated increase in bone turnover. Furthermore post-menopausal patients on HRT did not have higher TRAP levels than pre-menopausal controls while in TRAP activity decreases on bisphosphonate administration. Such data indicates TRAP levels are reflecting bone resorption.

As one would expect, correlation between the two methods is high. In a sub-group of patients with untreated osteoporosis, correlations significant correlations with other resorption markers but not are found. As an increase of bone resorption over bone formation may be expected in this patient group, one would not expect markers of the two processes to correlate well. This picture is also found in women undergoing artificial menopause (Stephan *et al.*, 1989).
CHAPTER IV - The Clinical Use of Biochemical Markers Of Bone Turnover

Biochemical Markers of Bone and Mineral Metabolism To Compare Two Modes of Treatment In A Single Patient With Fibrous Dysplasia.

Introduction

Sometimes known as fibro-osseous dysplasia, fibrous dysplasia is a rare condition characterised by expanding fibrous lesions of bone forming mesenchyme. The variable amount of bone and connective tissue at affected sites may lead to the typical ‘ground-glass’ appearance on radiography. These lesions of collagenous fibrous tissue within the interior of the affected bone result from a primary defect of fibroblast proliferation and maturation in chondrocytes and osteocytes (Lateur, 1994). While this condition presents in both sexes, the polyostotic form (typically identified before ten years of age) effects more females than males (Jaffe, 1958; Harris et al., 1962). In the more common monostotic form (typically developing during the second and third decade), clinical complaints tend to be mild. In severe polyostotic fibrous dysplasia, pain, pathologic fracture and gross deformity may result. Fibrous dysplasia may be associated with endocrine disorders such as sexual precocity, acromegaly, hyperparathyroidism, Cushings syndrome, hyperthyroidism, goitre, accelerated skeletal growth and maturation and gynecomastia (Perlman et al., 1987). The triad of polyostotic fibrous dysplasia, skin pigmentation (café au lait spots of irregular outline) and endocrine disturbances is known as the McCune-Albright syndrome. In such patients an activating mutation of the alpha-subunit of the G-protein which stimulates adenylyl cyclase has been found in osteoblastic progenitor cells (Shenker et al., 1994). This mutation may cause the increased osteoblastic proliferation and abnormal differentiation which leads to the lesions of fibrous dysplasia.

While serum alkaline phosphatase activity may be elevated, calcium and phosphate levels are typically normal. However in rare individuals with widespread bony lesions, renal phosphate wasting causes hypophosphataemic osteomalacia similar to that found in tumour-induced osteomalacia.

Case History

APD treatment

A 57 year old male was referred to the RNOH in 1991 with swelling and pain in the right hand. Previous medical history indicated five fractures of the upper humerus between the
ages of 12 and 52. Radiography and isotope bone scanning showed characteristic features of multi-focal fibrous dysplasia. Despite the long standing nature of his condition, no previous medical treatment had been attempted.

Fibrous dysplasia has traditionally offered few treatment options to the clinician. Surgical treatment such as curettage or subtotal resection with subsequent bone grafting is recommended only in symptomatic patients with areas liable to pathological fracture (Perlman et al., 1987). Furthermore such treatment is directed towards symptoms rather than the underlying condition. Although the pathogenesis of fibrous dysplasia is poorly understood, similarities with Paget's disease (i.e. increased rate of bone turnover), suggest the use of anti-resorptive agents may be of some benefit. However calcitonin treatment produces neither radiological or clinical change, and no reduction in bone turnover as assessed by serum alkaline phosphatase and urinary hydroxyproline (Bell et al., 1970; Hjelmstedt & Ljunghall 1979). It was therefore decided to experimentally treat this patient (informed consent given), with the more potent anti-resorptive agent pamidronate (APD). An initial 60 mg infusion was repeated after one month and then bi-monthly for almost two years.

Markers of bone and mineral metabolism are shown in Table 4.1 and Figs. 4.1 and 4.2.

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>1,25(OH)₂D (pg/ml)</th>
<th>PTH (pmol/l)</th>
<th>Osteocalcin (ng/ml)</th>
<th>ALP (IU/L)</th>
<th>Adjusted Ca (mmol/l)</th>
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Pre-treatment free deoxypyridinoline/creatinine ratio (fasting urine) was 8.5 nmol/mmol (reference range 2.0-5.0).

Table 4.1: Markers of bone formation and calcium homeostasis in APD-treated polyostotic fibrous dysplasia.

Clinically the patient responded well to treatment, with no-side effects and a reported decrease in pain. Follow-up after one year of treatment revealed no obvious radiological change in affected sites on plain X-ray, while isotope bone scanning showed no new foci of uptake. At this time no analgesia had been required since commencement of treatment. As such this treatment was deemed successful to this point.

The patient complained of returning pain after 15 months, and three months later calcitonin added to the treatment regime. Although pain desisted for a further few months, the patient complained of the usual side-effects associated with calcitonin (flushing, headaches, nausea and vomiting) and all treatment was stopped after a total of
22 months. On withdrawal of treatment osteocalcin and alkaline phosphatase values were still markedly elevated (23.8 ng/ml and 506 IU/L respectively).

Figure 4.1: The short term response to APD treatment in a single patient with polyostotic fibrous dysplasia. Shaded areas are indicative of population based reference ranges. Total free pyridinolines ratios were performed on fasting samples.
Figure 4.2: Biochemical follow-up to APD treatment in a single patient with polyostotic fibrous dysplasia. Shaded areas are indicative of population based reference ranges.
Calcitriol treatment

The patient was kept under review and complained of steadily worsening pain. One year after cessation of APD treatment a further fracture of the humerus occurred. As the previous treatment had not been deemed successful, alternatives were considered.

Calcitriol (1,25-dihydroxyvitamin D) is known to have anti-proliferative and differentiation-inducing effects on osteoclast and osteoblast-like cells in vitro (Thavarajah et al., 1993; Van den Bemd et al., 1995). As the primary defect in fibrous dysplasia appears related to proliferation and differentiation in the bone-forming mesenchyme, it was reasoned that calcitriol therapy may prove beneficial. Again the patient gave informed consent before commencement of treatment in October 1994. Calcitriol (12 µg per day) was given for nine days during hospital admission. As this represents a massive dose of calcitriol, serum calcium was monitored on a daily basis while a low calcium diet helped prevent hypercalcaemia. The patient was then discharged on 0.5 µg calcitriol per day, persisting with this treatment for approximately one year, but feeling little benefit, withdrew treatment himself. Unfortunately he was also unwilling to have radiography and isotope bone scans at this time. Changes in marker of bone and mineral homeostasis induced by this treatment are shown in Table 4.2 and Figures 4.3, 4.4 and 4.5.

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</table>

Table 4.2: Markers of bone formation and calcium homeostasis in calcitriol-treated polyostotic fibrous dysplasia.
Figure 4.3: Biochemical follow-up to calcitriol treatment (12ug/day) in a single patient with polyostotic fibrous dysplasia. Markers of calcium homeostasis and bone formation. Shaded areas are indicative of population based reference ranges.
Figure 4.4: Biochemical follow-up to calcitriol treatment (12ug/day) in a single patient with polyostotic fibrous dysplasia. Markers of calcium homeostasis and bone formation. Results expressed as percentage change from pre-treatment values.
Figure 4.5: Short-term biochemical follow-up to calcitriol treatment (12ug/day) in a single patient with polyostotic fibrous dysplasia. Markers of bone resorption. Shaded areas are indicative of population based reference ranges.
Discussion

Urinary markers of bone resorption were examined pre-treatment and during short-term follow-up. Pre-treatment raised urinary hydroxyproline and total free pyridinolines (PYD) confirmed increased bone resorption. Urine calcium excretion was not increased in either 24-hour collections or fasting samples corrected for creatinine. Urine calcium collections within the normal population reference range indicate their poor specificity as markers of increased bone turnover.

During the short follow-up period after APD infusion urinary calcium (fasting and 24-hour) values fall to below pre-treatment levels. APD has an immediate anti-resorptive effect, but delayed inhibition of bone formation, so decreasing the plasma calcium pool. This results in the conservation of urinary calcium. Hydroxyproline and total free pyridinolines (performed on fasting samples) do not decrease following treatment. This is discussed further in Chapter V.

Serum ALP showed a significant decrease on APD treatment (22% over the follow-up period in Fig. 4.1). However serum ALP never dropped below 488 IU/L during the whole course of treatment, and had risen marginally when treatment was abandoned. Such changes were not mirrored by osteocalcin which fell only marginally during APD treatment.

Since this patient was lost to follow-up, a report has appeared in the literature of pamidronate treatment (180 mg every six months) in nine patients with symptomatic polyostotic fibrous dysplasia (Liens et al., 1994). The treatment was deemed successful, primarily due to decreased bone pain in 12 of 14 affected sites (not returning during a mean follow-up period of 26 months). The mean decrease in ALP produced by three months of treatment was 26%. These changes are of similar magnitude and pattern obtained in our patient.

The effects of calcitriol treatment are more difficult to interpret. The restricted calcium diet helped the patient maintain normocalcaemia despite increased serum 1,25(OH)2D and the compensatory decrease in PTH. Pharmacological doses of calcitriol have long been characterised as potent stimulator of bone resorption (Peacock et al., 1974), although it has been postulated that this may be modulated by dietary calcium intakes (Gram et al., 1996). Although in our patient hydroxyproline and TRAP values are equivocal, increases in free deoxypyridinoline and urinary calcium (while on a constantly low calcium diet) suggest an increase in bone resorption. The response of markers of bone formation to short-term calcitriol is discussed in detail in Chapter V.

Both calcitriol and APD treatment are successful in decreasing ALP levels to a certain degree in fibrous dysplasia. However vastly different mechanisms underlie this finding.
While APD decreases bone turnover, high-dose calcitriol induces matrix formation and (during calcium restriction) bone resorption. As our patient felt no clinical benefit from the calcitriol treatment, this would argue against the use of ALP as an indicator of disease progress in this condition. Furthermore when on APD treatment, the patient complained of returning pain despite no significant increase in ALP values.

**Differences in Biochemical Response to Treatment in Privational and Tumour-Induced Osteomalacia.**

**Introduction**

The group of disorders characterised by delayed mineralisation of newly formed bone matrix is encompassed by the terms rickets and osteomalacia (Peacock, 1978). Rickets affects children and adolescents, with a characteristic delay in calcification of the epiphyseal cartilage most obviously manifesting as abnormal bone growth. In adults the disease affects remodelling bone and is termed osteomalacia.

The main features of both rickets and osteomalacia are bone pain and tenderness, skeletal deformity and muscle weakness, occasionally with signs of tetany from associated hypocalcaemia. Localised pain may be associated with an underlying pseudofracture (Looser zone), a characteristic ribbon-like zone of decalcification visible on X-ray. The definitive diagnosis of osteomalacia is given by bone biopsy and subsequent histomorphometry. The establishment of defective mineralisation is greatly aided by prior administration of oral tetracycline, a fluorescent compound which binds bone at sites of active mineralisation (Freemont, 1995).

Osteomalacia was first differentiated from osteoporosis and osteitis fibrosa in the late 19th century on the basis of histopathology (Pommer, 1885). Radiographic and biochemical delineation of these conditions was carried out from the first half of the 20th century onward (Stamp, 1992). While originally considered a condition of vitamin D deficiency, the osteomalacic syndromes have come to represent any condition characterised by defective mineralisation. Any form of rickets or osteomalacia, unless due to a combination of inadequate dietary consumption (and/or malabsorption) and poor endogenous production of vitamin D may be classified as 'metabolic'. (Stamp, 1994). Over 50 such forms of rickets have been described. For a comprehensive list of these conditions the reader is referred elsewhere (Parfitt, 1990; Stamp, 1992; Stamp, 1994).
Tumour Induced Osteomalacia (TIO).

Apart from a prevalence in certain populations immigrant to Northern latitudes, nutritional rickets and osteomalacia have become rare in the developed world (Stamp, 1994; Stamp & Tovey, 1995). However metabolic rickets, of which the inherited hypophosphataemic syndromes are the commonest, continue to be a problem (Evans et al., 1980). Osteomalacia is also commonly found secondary to intestinal malabsorption syndromes, renal tubular acidosis and chronic renal insufficiency. While the diagnosis and treatment of such patients is usually relatively simple and effective, a small number of patients with late onset osteomalacia have no family history of rickets, and none of the primary conditions known to induce defective mineralisation. On closer examination and follow-up the osteomalacia is often associated with the presence of some kind of tumour. While the tumours are typically benign and of mesenchymal origin, osteomalacia associated with malignancy has been reported in a rare number of cases (Harvey et al., 1992).

Since the first recognition of TIO (also known as tumour-associated or oncogenic osteomalacia) by Prader et al. in 1957 approximately 80 cases have been recognised, over 40% of these in the last decade (Drezner 1993). As the tumour causes the osteomalacia and not the reverse, the term oncogenic osteomalacia is less appropriate than the other forms of nomenclature.

Case Histories

This study compares the biochemical response to treatment in four osteomalacic patients, two with privational osteomalacia, two with TIO:

Patient 1
IP, a forty year-old female of Asian origin was referred to the RNOH where osteomalacia was confirmed by the presence of Looser’s zones on X-ray. An excellent therapeutic response to vitamin D (Figure 4.6) treatment precluded osteomalacia of a metabolic origin. After a course of treatment lasting nine months, the radiological lesions were completely healed and treatment deemed successful.

Patient 2
JS, a 61 year-old female vegetarian of Asian origin was referred to the RNOH with a painful left hip. DEXA indicated a decreased bone mineral density while X-ray indicated Looser’s zones in the region of the left femoral neck. Over a 15 month treatment period the symptoms and biochemical abnormalities of osteomalacia disappeared (Figures 4.7 - 4.8), and the patient was discharged from care on continued vitamin D treatment.
Patient 3  MC. This 62 year-old woman was referred with a long history of hip and back pain. Radiographic examination showed Looser's zones. Dietary history indicated adequate in calcium and vitamin D intake and no underlying cause for the osteomalacia could be found. A thorough examination of the patient coupled with an MRI scan revealed a residual hemangiopericytoma on the sole of the left foot. This connective tissue variant of a mesenchymal tumour is the tumour most commonly associated with TIO. Removal of the tumour alone initiated healing (Figures 4.9 - 4.10) and the patient was discharged on calcitriol and mineral supplements in an attempt to heal the skeleton as quickly as possible. The dose of calcitriol was decreased with time, and halted 13 months after tumour removal. At this point serum ALP was normal and the skeleton judged to be fully remineralised. At the latest follow-up, three-and-a-half years after surgical intervention, neither the tumour or symptoms had recurred.

Patient 4  JD, a 59 year-old Asian female was admitted for treatment of osteomalacia which had been confirmed radiographically and by bone biopsy but had not responded to treatment with 1α-hydroxycholecalciferol. The patient was treated for privational osteomalacia with very large doses of calcitriol. However no marked improvement was noted. Eventually the patient admitted to a lump in the labia. This was excised and histologically identified as a hemangiopericytoma. The osteomalacia improved as assessed clinically, radiographically and biochemically (Figures 4.11 - 4.13) and all treatment was stopped.
Figure 4.6: The biochemical response to treatment of privational osteomalacia. **Patient IP, markers of calcium and phosphate homeostasis.** Shaded areas are indicative of reference ranges (for full list see Chapter II of this thesis).
**Patient JS, markers of mineral homeostasis and bone formation.** Shaded areas are indicative of reference ranges. Ca & Vit D tablets contain 2.4 mmol calcium, 440U ergocalciferol. Ossopan contains 4.4 mmol Ca, 2.5 mmol P per tablet. Vit A and D capsules each contain 400U vitamin D.
Pre-treatment results not shown: free deoxypyridinoline (reference range 2.0-6.0 nmol per mmol creatinine): 20.8 (fasting sample); 22.1 (twenty-four hour collection).

Figure 4.8: The biochemical response to treatment of privational osteomalacia. Patient JS, urine biochemistry. Shaded areas are indicative of reference ranges (for full list see Chapter II of this thesis). Ca & Vit D tablets contain 2.4 mmol calcium, 440U ergocalciferol. Ossopan contains 4.4 mmol Ca, 2.5 mmol P per tablet. Vit A and D capsules each contain 400U vitamin D.
Figure 4.9: The biochemical response to treatment of tumour-induced osteomalacia. Patient MC, markers of bone formation and mineral homeostasis. Shaded areas are indicative of reference ranges (for full list see Chapter II of this thesis). Ca & Vit D tablets contain 2.4 mmol calcium, 440U ergocalciferol. Ossopan contains 4.4 mmol Ca, 2.5 mmol P per tablet.
Figure 4.10: The biochemical response to treatment of tumour-induced osteomalacia. **Patient MC, urine biochemistry.** Shaded areas are indicative of reference ranges (for full list see Chapter II of this thesis). Ca & Vit D tablets contain 2.4 mmol calcium, 440U ergocalciferol. Ossopan contains 4.4 mmol Ca, 2.5 mmol P per tablet. Phosphate Sandoz contains 16 mmol P per tablet.
Figure 4.11: The biochemical response to treatment of tumour-induced osteomalacia. **Patient JD, markers of calcium and phosphate homeostasis.** Shaded areas are indicative of reference ranges (for full list see Chapter II of this thesis). Ca & Vit D tablets contain 2.4 mmol calcium, 440U ergocalciferol. Ossopan contains 4.4 mmol Ca, 2.5 mmol P per tablet. Phosphate Sandoz contains 16 mmol P per tablet.
Figure 4.12: The biochemical response to treatment of tumour-induced osteomalacia. **Patient JD, markers of bone formation.** Shaded areas are indicative of reference ranges (for full list see Chapter II of this thesis). Ca & Vit D tablets contain 2.4 mmol calcium, 440U ergocalciferol. Ossopan contains 4.4 mmol Ca, 2.5 mmol P per tablet. Phosphate Sandoz contains 16 mmol P per tablet.
Figure 4.13: The biochemical response to biochemical treatment of tumour-induced osteomalacia. *Patient JD, urine biochemistry.* Shaded areas are indicative of reference ranges (for full list see Chapter II of this thesis). Ca & Vit D tablets contain 2.4 mmol calcium, 440U ergocalciferol. Ossopan contains 4.4 mmol Ca, 2.5 mmol P per tablet. Phosphate Sandoz contains 16 mmol P per tablet.
Discussion

The case studies described above exhibit the biochemical contrasts between healing privational osteomalacia, calcitriol-treated TIO, and the excision of the causative tumour in TIO.

Both patients with privational osteomalacia exhibit characteristic pre-treatment biochemistry with abnormally low 25-OHD, markedly elevated ALP and hypocalcaemia associated with gross secondary hyperparathyroidism. Both patients had normal rather than suppressed 1,25(OH)2D, which though surprising has been reported previously (Eastwood et al., 1979; Peacock et al., 1979). It is possible that any vitamin D osteomalacic patients do receive is immediately metabolised to 1,25(OH)2D. In this way 1,25(OH)2D may appear normal in the presence of a low 25-OHD. The logical conclusion of this proposed mechanism is that supranormal concentrations of 1,25(OH)2D are required to heal established osteomalacia.

Both patients showed increased B-ALP and osteocalcin, while only one patient exhibited an elevation of PICP. Urinary markers of bone resorption were available for one patient with privational osteomalacia. Both fasting and twenty-four hour collections indicated increased hydroxyproline and free deoxypyridinoline excretion. However fasting urine calcium did not exhibit the marked hypocaliuria expected in this condition.

Vitamin D treatment produced a slow normalisation of 25-OHD and a massive increase in 1,25(OH)2D, synchronous with restoration of normocalcaemia. Also striking is the prolonged duration of secondary hyperparathyroidism, despite normocalcaemia and elevated 1,25(OH)2D concentrations. Healing osteomalacia is indicated by gradually decreasing ALP, following a characteristic ‘flare’ in values. Both of these features are shown more rapidly and sensitively by the bone fraction alone (Patient JS, Figure 4.7). While the small number of estimations for PICP and OC precludes comment on changes in these formation markers in the first patient, in the second patient more data is available. During the phase of increasing calcitriol concentrations, OC levels increase (after a small flare) while those of PICP fall. Levels of both markers then fall back to normal as healing occurs.

TIO shares classical biochemical and radiological features with a number of the hypophosphataemic osteomalacias. As such diagnosis is dependant upon identification of the causative tumour by examination or MRI. Striking features pre-treatment include: severe hypophosphatasia but no elevation of PTH; normal serum and urine calcium; low 1,25(OH)2D in the presence of normal 25-OHD (not shown in the final patient due to previous 1α-hydroxycholecalciferol treatment). This is despite hypophosphataemia, which in normal individuals increases 1,25(OH)2D production by stimulating renal 25-hydroxyvitamin D-1α-hydroxylase.
Tumour removal in both patients resulted in the dramatic correction of hypophosphataemia and a massive rise in 1,25(OH)2D. As this occurs a fall in serum calcium is accompanied by a rise in PTH. On post-resection follow-up, markers of bone formation increase transiently, returning towards normal as healing occurs. It is unclear whether the trigger for this response is the hypercalcitriolaemia or the normalisation of hypophosphatasia.

While successful removal of the causative tumour results in clinical and biochemical cure of TIO, complete and permanent resection may not always be possible (Drezner 1993). In such cases patients appear to respond well to calcitriol in combination with phosphate supplementation (Leicht et al., 1990). Patient JD (Figs. 4.11 - 4.13) was treated in this manner as her tumour went unnoticed. Particularly striking is the prolonged massive calcitriol dosage needed to initiate healing without producing hypercalcaemia or supranormal calcitriol concentrations. Despite such doses concomitant with phosphate supplementation, hypophosphataemia persists. However during such treatment markers of bone formation increased in a smaller but similar manner to the increases produced by tumour resection in patient MC (Figure 4.9).

While urine phosphate concentrations are within the wide reference limits ascribed, all patients with TIO have a reduced renal tubular reabsorption of phosphate (not performed in these patients). As such it has long been proposed that a humoral factor produced by the tumour is responsible for both the renal phosphate leak and the inhibition of the conversion of 25-OHD to 1,25(OH)2D. Indeed inhibition of 25-hydroxyvitamin D-1α-hydroxylase, the enzyme responsible for this conversion, by tumour extracts has been accomplished in vitro (Miyauchi et al., 1988). Recently a -heat-labile factor of between eight and twenty-five kilodaltons that inhibits renal tubular reabsorption of phosphate has been identified from the tumour of patient with TIO (Cai et al., 1994).

It has been proposed that the disturbances of phosphate metabolism in TIO may be due to an unregulated over-production of a substance which has a role in normal physiological processes (Econs & Drezner, 1994). The rationale for the normal existence of this hormone is supported by the fact that changes in phosphate homeostasis are often ascribed to changes in PTH secretion. This is despite the fact PTH exerts opposite effects on serum phosphate concentration and the mobilisation of phosphate from bone while increasing urinary phosphate excretion. The factor identified by Cai et al., while promoting the latter by decreasing renal tubular phosphate reabsorption, does not appear to directly influence bone remodelling or mobilisation from calcium or phosphate from bone.

Despite the characteristic features of privational osteomalacia described earlier, the condition is a biochemically heterogeneous one, resulting in serum PTH and urinary...
calcium excretion being poor diagnostic tools in the detection of this condition (Bingham & Fitzpatrick, 1993). However in most patients serum ALP, especially its bone-specific form, are useful in diagnosis and monitoring the response to treatment.

The Diagnosis and Management of Humoral Hypercalcaemia of Malignancy

Introduction

Hypercalcaemia is one of the commonest complications of malignancy, an association long recognised by clinicians (Gutman et al., 1936). However the pathophysiology of such hypercalcaemia is heterogeneous. While almost all tumour-induced hypercalcaemia is associated with osteolysis in areas of widespread bony metastases, other less common forms occur (Fisken et al., 1980). A further sub-group of haematological malignancies (e.g. multiple myeloma) cause hypercalcaemia, those affected patients having evidence of bone involvement (Grill & Martin, 1993).

Hypercalcaemia may also be caused by a systemic factor secreted by a tumour with little or no skeletal involvement (Stewart, 1993). Patients affected by this syndrome, known as humoral hypercalcaemia of malignancy (HHM), exhibit enhanced osteoclastic activity, and markedly increased urine calcium excretion (Martin & Atkins 1979, Stewart et al., 1980). For many years the nature of the humoral factor remained uncertain. Although many similarities with primary hyperparathyroidism were noted, PTH concentrations did not appear to be elevated in HHM (Stewart et al., 1980). However elevated nephrogenous cAMP excretion in these patients indicate an interaction of the humoral factor with proximal tubular PTH receptors. The cDNA of the PTH-like factor responsible, eventually isolated from a cancer cell line established from a patient with HHM, was designated parathyroid hormone related protein (PTHrP) (Suva et al., 1987).

Where possible the reduction of the tumour burden will aid the resolution of HHM. When such therapy is limited or hypercalcaemia requires rapid resolution, the reduction of osteoclastic bone resorption and augmentation of renal calcium clearance are attempted (Stewart, 1993). This is commonly attempted by a combination of bisphosphonate treatment and the restoration of intravascular volume (Kanis et al., 1991).
**Case History**

NW, a 49 year-old male, was in-patient at the RNOH for a left hemi-pelvic replacement when routine biochemistry revealed severe hypercalcaemia and hypophosphatasia. Despite this, serum PTH levels were undetectable using a two-site immunoassay for the intact molecule. It was decided to treat the severe hypercalcaemia using APD and hydration with normal saline. Biochemical follow-up is indicated in Figures 4.14 and 4.15.

Other biochemical parameters were measured only at pre-treatment. Results (with appropriate reference limits) were:

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While the response to APD was both moderate and disappointingly temporary, surgeons proceeded with the hemi-pelvectomy. During the next two months two further APD infusions of increasing dose (90 and 120 mg) were required to control the persistent hypercalcaemia. At this point histology on biopsy tissue taken during the surgical procedure revealed the presence of a metastatic endocrine tumour of the pancreas. This is unusual in that carcinoid tumours are not usually associated with HHM. Literature searches revealed a patient with a malignant pancreatic endocrine tumour secreting PTHrP who was successfully treated using the long acting somatostatin analogue ocreotide (Wynick *et al.*, 1990). This treatment rapidly decreased serum calcium and suppressed PTHrP secretion, and was tried in our patient who was proving refractory to ever increasing doses of APD. Unfortunately such treatment (250 µg subcutaneous injections daily for six days) appeared to have no effect (Figure 4.16) and was abandoned. After this time further APD infusions of increasing doses were required, and the patient was discharged requiring continuous oral clodronate to control his hypercalcaemia.
Figure 4.14: The treatment of humoral hypercalcaemia of malignancy (HHM) with APD. Evidence for increased bone formation in the presence of elevated PTHrP excretion. Shaded areas represent reference ranges. Other reference limits: PTHrP <0.7 to 2.6 pmol/L; NcAMP 0.5 to 2.5 nmol per 100 ml glomerular filtrate.
Figure 4.15: The treatment of humoral hypercalcaemia of malignancy (HHM) with APD. Hypercalcaemia and increased bone resorption. Shaded areas represent reference ranges.
Figure 4.16: The treatment of humoral hypercalcaemia of malignancy (HHM) with the somatostatin analogue ocreotide 250 μg subcutaneous injections daily for six days. Shaded areas represent reference ranges.

**Discussion**

A patient with atypical HHM is presented. Hypercalcaemia associated with pancreatic endocrine tumours are most commonly found due to primary hyperparathyroidism as part of the syndrome multiple endocrine neoplasia type I. However in this case PTHrP levels are abnormally high while PTH cannot be detected. Few other such cases can be found in the literature (Drucker et al., 1989; Mao et al., 1995). Notable in this case is the failure of treatment of the ocreotide to restore normocalcaemia, despite successful results using somatostatin analogues in other patients with neuroendocrine tumours (Anthony et al., 1995; Wynick et al., 1990).
The use of APD to inhibit bone resorption in HHM is illustrated by post-infusion decreases in serum TRAP and urine hydroxyproline and total free pyridinoline. Despite this therapy the patient remained hypercalcaemic, indicting the renal component of HHM. Furthermore production of PTHrP is unaffected by APD treatment, and can only be regulated by reduction of the tumour burden.

Exceptional in this patient are the elevated levels of bone formation markers. Although PTHrP fragments stimulate bone formation in vitro (Murray et al., 1989; Stewart, 1993), bone histomorphometric and biochemical studies in HHM indicate a profound uncoupling of bone turnover, with markedly reduced osteoblastic activity (Stewart et al., 1982; Nakayama et al., 1996). This is obviously not the case in our patient, suggesting the action of other humoral factors. Interestingly, in vitro studies with a human pancreatic cancer cell line produced from a patient with HHM indicate the production of transforming growth factors (TGF) as well as PTHrP (Nagata et al., 1989). While transforming growth factors are dependent on interactions with other cytokines in vivo, studies in animal models and in vitro have demonstrated stimulation of osteoblastic activity and down-regulation of the receptor for PTHrP (Ignotz & Massague, 1986; Noda & Camilliere, 1989; Jongen et al., 1995).

The Assessment of Experimental Therapy In Osteoporosis

What is Osteoporosis?

Osteoporosis is a generic term used to describe a specific form of osteopenia, which may be defined as a deficiency of calcified bone (Bauer, 1960). Osteopenia is not a recently recognised phenomenon. Almost 150 years ago it was noted that 'the bones of aged people not infrequently become extremely light and spongy, readily break, and from the diminished amount of compact tissues may in the case of the flat bones such as the pelvis, be indented by firm pressure with the finger' (Tomes & De Morgan, 1853). However osteopenia requires further differentiation for effective diagnosis and treatment. Despite first being differentiated over 100 years ago (Pommer, 1885), the two most common adult osteopenic conditions, osteoporosis and osteomalacia are commonly confused. While osteoporosis is characterised by a decreased density of normally mineralised matrix, osteomalacia is a condition of insufficient mineralised bone matrix (see Fig 4.16 & 4.17).

The classical definition of osteoporosis was made in 1948 by Albright and Reifenstein who described the condition as of one of 'too little bone in the bone'. While osteoporosis is therefore characterised by low bone density, the composition of the remaining bone is normal. The current World Health Organisation (WHO) definition of osteoporosis is: 'A
disease characterised by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk' (WHO Study Group, 1994). The concept of deteriorating bone micro-architecture is an important one.

While osteoporosis is primarily a disease of loss of bone mineral density, this alone is often not enough to explain the fractures which occur. However while the assessment of such micro-architectural damage is unable to be assessed, measurements of bone mineral density may be readily made.

The WHO sub-divides osteoporosis into two classes depending on the severity of osteopenia. When assessed in terms of bone mineral density (BMD) or bone mineral content (BMC) individuals with values between 1.0 and 2.5 standard deviations (SD) below the sex-matched young adult mean are described as having osteopenia, while those having BMD and BMC values 2.5 SD or more below the young adult mean are considered to be suffering from osteoporosis (Barlow, 1994). It has also been proposed that a further category, that of severe osteoporosis be established for those individuals

Figure 4.17: The differentiation of osteopenia. Comparison of bone mass and degree of bone mineralisation in osteoporotic and osteomalacic bone.

below the sex-matched young adult mean are described as having osteopenia, while those having BMD and BMC values 2.5 SD or more below the young adult mean are considered to be suffering from osteoporosis (Barlow, 1994). It has also been proposed that a further category, that of severe osteoporosis be established for those individuals
with a BMD or BMC more than 2.5 SD below the young adult mean value in the presence of one or more fragility fractures (Kanis et al., 1994). The risk of fragility fractures increases with declining BMD (Hui et al., 1988; Melton et al., 1993), with an estimated 1.5 to 3-fold increase in fracture risk for each decrease in BMD representing one standard deviation (SD) of the mean population value (Kanis et al., 1994). Comparing the measured BMD with the sex-matched young adult mean BMD gives a value known as the 'T-score', which has replaced comparisons with sex and age-matched reference means (Z-score). This is because even though fracture risk increases with age in response to decreasing BMD, the overall incidence of osteoporosis as identified by Z-score remains unchanged. Using definitions based on T-scores means the prevalence of osteoporosis in females increases almost exponentially with age after 50 years, a pattern which more accurately reflects the clinical presentation of these patients (Kanis et al., 1994).

**The Epidemiology of Osteoporosis**

Bone fractures are most common in the young and the elderly. While long bone fractures predominate in the young, in those above the age of 45 years fractures of the hip, spine and wrist predominate (Melton, 1988). These are the classic fractures of osteoporosis. The characteristics of these fractures were recognised over a century ago (Cooper, 1824). Fracture incidence increases with age, is higher in females and may be associated with only moderate trauma at sites containing large amounts of trabecular bone.

The estimation of the number of individuals suffering from osteoporosis is difficult, and it is not possible to estimate the total number of whole body fractures that are caused by osteoporosis (Barlow, 1994). Problems in identifying the frequency of vertebral fracture and its attendant morbidity have also been reported (Kanis & McCloskey, 1992; Kanis & the WHO Study Group 1994; Melton et al., 1993). However, the estimated cost of hip fractures, (the most severe fracture characteristic of osteoporosis) in England alone was £742 million in 1992/93 (Cooper & Jones, 1993). Estimates of the incidence of osteoporosis in the US have varied widely, ranging between 15 and 20 million (Benger et al., 1988; Melton et al., 1989; Norris, 1992), with a current annual cost to the US healthcare system of $10 billion (Mundy, 1995). The most recent estimates of osteoporosis using the WHO definitions suggest a likely incidence of 30% in UK and US Caucasian women (Kanis et al., 1994; Melton et al., 1989). The degree of morbidity and mortality associated with osteoporotic fracture is not disputed (Silverman, 1992). Demographic changes will cause a further increase in the incidence of these fractures over the next sixty years (Barlow, 1994).

Women are at particular risk of fracture due to lower peak bone mass, accelerated bone loss at the menopause, a greater likelihood of falls and a significantly greater longevity
than men (Hansen et al., 1991). These factors combine to mean that women account for 75% of osteoporotic fractures in most Western counties. At the menopause Caucasian women face a remaining lifetime fracture risk of between 30 and 40% (Kanis & the WHO Study Group, 1994).

The use of BMD measurements to aid the understanding of osteoporosis led to the concept of the "fracture threshold". This represents a theoretical point below which patients will suffer an osteoporotic fracture. Such approaches suffered from the arbitrary setting of the threshold which lacks either sensitivity or specificity, depending on the value selected. Furthermore, the imposition of a single cut-off limit ignores the fact that risk of fracture increases continuously with decreasing BMD and does not alter in a quantum fashion (Kanis et al., 1994).

**Why does Osteoporosis Occur?**

Fractures occur due to a mechanical imbalance between bone strength and any force applied to it. Bone mass and therefore bone density in later life, depend on the peak bone mass achieved on cessation of linear growth and the subsequent bone loss.

Environmental factors such as calcium intake and physical activity interact with genetic influences to determine the peak bone mass of an individual (Johnston et al., 1992). In girls, the age of onset of puberty is also thought to play a role (Johnson & Slemenda, 1994). In pre-menopausal females, the gonadal hormone oestrogen is known to be a major factor in the maintenance of bone mass. Indeed the most established treatment (and prophylactic therapy), against osteoporosis related to artificial or natural menopause is the replacement of lost oestrogen. This is known as hormone replacement therapy (HRT).

A number of risk factors for osteoporosis have been identified. These include: family history, white race, small stature, female gender, early menopause, late menarche, low dietary calcium, sedentary lifestyle, smoking, drug-use. Another major cause of osteoporosis, especially in younger subjects is related to the use of corticosteroid drugs. Steroids inhibit bone formation while reducing intestinal calcium absorption and increasing urinary calcium reabsorption. Compensatory (secondary) hyperparathyroidism and increased bone resorption follow (Hahn, 1993). While reduction of corticosteroid dose removes the cause of the osteoporosis, this may not be possible in all cases. In such situations the use of anti-resorptive agents such as the bisphosphonates have proved effective (Reid et al., 1988).

Although of much lower incidence than in women, osteoporosis does occur in men, often related to hypogonadism. Once all other causes (endocrinopathies, osteomalacia, drug-induced etc.), have been excluded, a diagnosis of idiopathic osteoporosis may be made (Jackson & Kleerekoper, 1990). While secondary osteoporosis is most readily combated
by the treatment of the underlying disorder, the treatment of idiopathic male osteoporosis is not well established. This may be due to a possible heterogeneity of bone metabolism in this condition, with sub-groups of low bone formation and increased bone turnover (Perry et al., 1984). The possibility of two such distinct groups has also been postulated to occur within post-menopausal osteoporosis (Arlot et al., 1990). This supposition is supported by wide variations in biochemical markers of bone turnover such as osteocalcin, which has been variously reported as low, normal and high in post-menopausal osteoporosis (Brown et al., 1984; Duda et al., 1988; Ismail et al., 1986). This heterogeneity occurs within post-menopausal osteoporosis (Type I osteoporosis) and should not be confused with the delineation of this condition from age-related (type II osteoporosis) (Riggs & Melton, 1983). While biochemical markers of bone metabolism have been successfully used in predicting bone loss and response to treatment (Christiansen et al., 1987; Gamero et al., 1994a), it may be that different sub-groups and types of osteoporosis may exhibit a range of responses to different modes of therapy. The assessment of bone turnover, possibly using biochemical markers of bone metabolism may have a role in choosing the optimal therapy in an individual patient.

The Assessment of Experimental Therapy in Osteoporosis

This study aims to investigate the usefulness of biochemical markers of bone in predicting response to treatment of osteoporosis with an experimental anti-resorptive agent. While HRT is well established in the prevention of post-menopausal osteoporosis, the increases in BMD produced in the treatment of established osteoporosis are small. The anti-resorptive agent etidronate ('Didronel', Proctor & Gamble Ltd) not only reduces but reverses bone loss. However the second and third generation of bisphosphonate drugs are more potent inhibitors of bone resorption, and unlike etidronate, have no anti-mineralisation effect. Consequently the use of such drugs is being studied for the treatment of established osteoporosis. At the RNOHT, trials with 3-amino-1-hydroxypropilidene (APD or pamidronate) have been undertaken.

Methods

Patient Group

Twenty-seven patients (steroid induced n=7, male n=10, female post-menopausal n=10) with established osteoporosis were admitted to receive I-V treatment with APD. This treatment was given as part of a larger study investigating the use of this drug in the treatment of established osteoporosis. It should be noted that this treatment was given in response to perceived clinical need. Such patients had particularly severe osteoporosis, or had proven refractory to, or unsuitable for, other therapies. While the initial dose varied to some degree, patients then received 45-60mg infusions every 4-6 weeks.
Figure 4.18: Bone loss in osteoporosis. Thinned broken-trabeculae with micro-architectural damage (top) compared with normal trabecular bone (bottom)
The lack of control and the variations in treatment regimens are noted and accepted as limitations of this study. However the effect of differing dose is limited by the variability in bioavailability of the drug in individuals, an effect likely to be related to skeletal size and renal clearance (Daley-Yates et al., 1991; Leyvraz et al., 1992).

**Measurements**

BMD was measured at both spine and hip using a Hologic QDR-100 bone densitometer (Vertec Scientific Reading, UK). Values were normalised to the initial value (100%) and the rate of change calculated by the slope of a linear regression fit on subsequent measurements over the treatment period (minimum 6, maximum 24, mean 19 months).

Biochemical measurements were made according to the methods section of this thesis (Chapter II).

Serum measurements made were:
- serum total alkaline phosphatase (ALP);
- adjusted calcium (Adjusted Ca);
- serum phosphate (P);
- 1,25-dihydroxyvitamin D (1,25);
- parathyroid hormone (PTH);
- short term increase in PTH on initial treatment (ΔPTH);
- osteocalcin (OC);
- carboxy-terminal of type I procollagen (PICP);
- bone-specific alkaline phosphatase (B-ALP);
- tartrate-resistant acid phosphatase by Randox method (TRAP\textsubscript{Randox});
- tartrate-resistant acid phosphatase by Boehringer Mannheim method (TRAP\textsubscript{BM}).

Twenty-four hour urinary measurements made were:
- Free deoxypyridinoline/creatinine ratio (Free DPyr/Cr);
- free deoxypyridinoline excretion per day (Free DPyr Excn.);
- hydroxyproline/creatinine ratio (OHP:Cr);
- hydroxyproline excretion (OHP Excn.);
- total free pyridinoline/creatinine ratio (Free PYD/Cr);
- calcium excretion per day (Ca Excn).

Also in a small sub-group of patients improvement in calcium balance (ΔBal) and initial calcium balance (Bal t=0) were measured.
Results

Patient outcomes varied markedly but were generally very favourable. While it is not the primary purpose of this study to compare APD with other therapies for osteoporosis, the mean increases in BMD are worth consideration (Table 4.2).

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<td>No.</td>
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<tr>
<td>Post-menopausal</td>
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<tr>
<td>Male</td>
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<tr>
<td>Steroid-induced</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
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</table>

Table 4.3: Increase in bone mineral density produced by treatment of established osteoporosis with APD.

These increases are greater than those produced by conventional therapies such as HRT or the first-generation bisphosphonate, etidronate. (Lombardi & Santora, 1993; Hillard et al., 1994). Consequently the short term changes induced by treatment with pamidronate were compared with those in four patients treated initially with etidronate (Figs. 4.19 and 4.20) in whom the mean spinal BMD increase was 3.2% (SD 0.9%).

The correlation between the pre-treatment levels of markers of bone turnover and the increase in spinal and hip BMD was also examined. These are detailed in Table 4.4.
Figure 4.19 Short term biochemical response to a single APD infusion in twenty-seven osteoporotic patients. As doses varied, and response is dose dependent, mean values are not indicated.
Figure 4.20: Short term response to treatment in four patients with I-V etidronate. Mean values not plotted due to different treatment regimens.
### Table 4.4: Correlation between pre-treatment indicators of calcium homeostasis and bone metabolism and response to treatment with the anti-resorptive agent APD. Correlation values are Pearson product moment coefficient (r) for normal distributions unless indicated with an asterisk. In such cases, the Spearman rank correlation ($r_s$) for non-Gaussian distributions is used.

**Discussion**

While not designed to compare differing treatments, this study is indicative that larger increases in spinal BMD are achieved by treatment with APD than with HRT or etidronate. The small increase in BMD at the hip reinforces the concept of cortical bone not responding to anti-resorptive treatment as well as the more metabolically active trabecular bone (Akesson *et al.*, 1993).
While the mechanism of action of the bisphosphonates is not fully established, their relative potency in the inhibition of bone resorption known to vary, being a function of the side-chain substitutions on the P-C-P backbone (see Appendix). The greater efficacy of APD over etidronate may not be directly due to the inhibition of bone resorption alone. BMD continues to respond to APD, even though the suppression of bone turnover is maximal and non-progressive after one year of treatment (Valkema et al., 1989). It would therefore appear that APD exerts an anabolic effect on bone. This may lie in the stimulation of cytokines such as IL-I and IL-6, both of which are implicated in the acute phase response observed clinically with APD but not etidronate (Geddes et al., 1994). However examination of the short-term response to treatment with these drugs (Figures 4.18 and 4.19) offers a further possible cause. While a reduction in markers of bone resorption on APD treatment is well established (Mallmin et al., 1991), fewer studies have examined the short-term response to treatment as an indicator of the mode of action of the drug. The anti-resorptive effect of APD is immediate but inhibition of formation lags behind, resulting in a depletion of the plasma calcium pool. Although urinary calcium excretion is reduced as a response (not shown), a fall in serum calcium ensues. This stimulates a compensatory increase in serum PTH, even when the patients remain normocalcaemic with respect to population based reference ranges. PTH acts to increase the synthesis of 1,25-dihydroxyvitamin D (long-loop calcium homeostasis) and reduces the renal tubular reabsorption of phosphate, so reducing serum phosphate levels. Etidronate does not produce such a hypocalcaemic response, presumably due to the concurrent inhibition of bone formation with resorption (McCloskey et al., 1987). Indeed etidronate induces hyperphosphataemia due to enhanced renal tubular reabsorption of phosphate (McCloskey et al., 1988).

In the group of twenty-seven patients pre-treatment ‘routine’ biochemistry was unremarkable while ‘bone’ biochemistry showed the variance expected from the three different sub-groups represented. However this study intends not to compare the aetiology of these conditions, but to use the diversity of bone turnover rates to assess the potential of biochemical tests to predict response to anti-resorptive treatment.

Levels of PICP appear negatively correlated with the small changes produced by treatment at the less metabolically active cortical site (hip). However all other biochemical markers of calcium homeostasis and bone turnover were not related to response to treatment at this site.

Increase in BMD at the spine was not significantly correlated with the formation markers OC and PICP. While total ALP was significantly correlated with response to treatment, the significance and strength of association was markedly increased by measurement of the bone isoenzyme alone.
The findings with the resorption markers are a little surprising, with urinary hydroxyproline/creatinine ratio more significantly correlated with response to treatment than the more specific free deoxypyridinoline. As well as these matrix resorption markers, a correlation with the osteoclastic enzyme TRAP was found.

Figure 4.21: Response to APD treatment of established osteoporosis as a function of pre-treatment serum bone-alkaline phosphatase concentration.

Figure 4.22: The use of appropriate correlation analysis. Response to APD treatment of established osteoporosis as a function of pre-treatment urine hydroxyproline/creatinine ratio. LEFT: Scatterplot of all 24 results available. Distribution of OHP/Cr non-Gaussian with an obvious outlier. Inappropriate use of Pearson correlation coefficient gives \( r \) of 0.15 (non-significant). Spearman correlation coefficient \( r_s = 0.66 \), \( p = 0.0005 \). RIGHT: Scatterplot of results after removal of outlier. Pearson correlation is now 0.54.
The importance of using appropriate correlation analysis in such studies is demonstrated in Fig 2.22. The inclusion of a single outlier destroys the otherwise strong correlation between hydroxyproline/creatinine ratio and response to treatment. The Pearson correlation, not be used for non-Gaussian distributions, is particularly sensitive to interference from outliers (Campbell & Machin, 1994). In such cases the Spearman correlation should be used.

The correlations of markers of bone turnover and response to anti-resorptive treatment in osteoporosis are discussed in more detail in Chapter V of this thesis.

**Estimation of bone turnover at the pre-, peri- and postmenopause - A longitudinal study.**

**Introduction - Female Gonadal Function and the Menopause**

Germ cell production in the female is characterised by a monthly cycle under the control of both pituitary and ovarian hormones (Ur, 1992). The monthly process of ovulation is chiefly promoted by the pituitary gonadotrophins; follicle stimulating hormone (FSH), and luteinising hormone (LH). The ovary itself secretes oestrogens, androgens and progesterone. The decline in the number of primordial follicles that occurs during reproductive life becomes much steeper at approximately forty years of age, until eventually the oocyte store is exhausted (Richardson et al., 1987). It is this ovarian failure which characterises the menopause.

The menopause is correctly defined as the cessation of menstruation, after which time no more menses occur (Brockie, 1992). The transitional phase during which ovarian function diminishes is known as the climacteric or perimenopause. The sequence of hormonal events that occurs during the transition from normal premenopausal menstrual cycles to the irregular and often anovulatory cycles of the perimenopause and subsequent amenorrhoea is not completely resolved (MacNaughton et al., 1992). However the gross endocrine changes which occur have been characterised. A gradual reduction in ovarian responsiveness to gonadotrophin stimulation during the perimenopause, coupled with the depletion in oocyte number, results in a reduction in oestrogen production during this period (Vagenakis, 1989). Eventually oestrogen production falls below a critical threshold, and amenorrhoea results. The woman may now be considered to be postmenopausal. The postmenopausal woman is not totally oestrogen deficient. Despite the cessation of production of the primary ovarian hormone oestradiol, the peripheral conversion of ovarian and adrenal androgens to oestrone in the liver and peripheral tissues (principally fat) provides oestrogenic activity (Ur, 1992).
The realisation that the menopause is a major risk factor for osteoporosis was first proposed in 1940 by Albright (Albright et al., 1940). Indeed the term 'postmenopausal osteoporosis' was coined at this time to describe the fractures commonly found in women following natural cessation of ovarian function. The connection between such bone loss, the menopause and the positive effect of oestrogen treatment led to the recognition that the loss of oestrogenic activity is of central importance to the development of postmenopausal bone loss.

The morbidity, mortality and healthcare costs of osteoporotic fractures have been well documented (Barlow, 1994). In an ageing population these are likely to increase. Postmenopausal osteoporosis may be prevented by exogenous oestrogens (hormone replacement therapy; HRT), provided that patients at risk are identified and treated before a clinically significant degree of bone loss occurs. To identify the optimum time for such treatment, much research has focused on the pattern of bone loss throughout female life. An increase in the rate of bone remodelling in response to a loss of oestrogen is central to the proposed mechanism of postmenopausal bone loss. However bone turnover in the transmenopausal period has been poorly studied longitudinally. This study aims to use biochemical markers of bone metabolism to investigate bone turnover in the peri- and early postmenopausal period. Bone resorptive activity was assessed by measurement of urinary type I collagen crosslinked N-telopeptides (NTx) and free deoxypyridinoline (DPyr), bone formation by serum total alkaline phosphatase.

**Methods**

**Patient Group**

As part of larger studies on climacteric symptoms and bone mineral density in middle aged women (Cox et al., 1991; Khan et al., 1994), subjects of appropriate age were contacted through a general practitioner list in Beaconsfield, Buckinghamshire. Of those responding, those with previous artificial or natural menopause, thyroid or hyperparathyroid disorders, Cushing's syndrome or steroid use were excluded. Of the remainder, seventy-seven agreed to be biochemically assessed thorough the menopausal period. Fasting urine samples were used to assess rates of bone resorption by commercially available methods. Follow-up of the women was attempted on an annual basis, although the success was variable. However all women completed at least one follow up visit (minimum number of samples 2, maximum 5, mean 4.2). This explains the difference in numbers at each time point. Protocol errors resulted in fewer women having alkaline phosphatase measurements than resorption markers estimations.
Biochemical Measurements

Type I collagen crosslinked N-telopeptides (NTx) and free deoxypyridinoline (DPyr) was also measured by immunoassay (see Chapter II). Urinary excretions of both resorption markers were expressed relative to the urinary creatinine concentration. Serum total alkaline phosphatase was measured by a standard colorimetric method.

Statistical Analysis

Data reduction, statistical analysis and sample size weighted curve fits were performed using Statview and JMP statistical software packages for the Apple Macintosh computer.

Results

Mean data for each biochemical marker is expressed in Tables 4.5, 4.6 and 4.7.

The longitudinal changes for each individual are represented in:

Figures 4.23 (free deoxypyridinoline), 4.25 (N-telopeptide of type I collagen), and 4.27 (serum total alkaline phosphatase). The mean values for each time point and a line of best fit (second order polynomial curve fit) for those means are also represented on these figures. The mean values with a curve fit weighted for sample size at each time point are represented in Figures 4.24, 4.26, and 4.28.

The percentage increase in bone turnover marker from a baseline of four years before the menopause is shown in Figure 4.29.

Figure 4.30, shows percentile plots used to illustrate the population distribution of each marker.
<table>
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<th>Table 4.5</th>
<th>Time from Menopause (±0.5 Years)</th>
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Tables 4.5, 4.6 and 4.7: Longitudinal changes in biochemical markers of bone metabolism through the menopause. Mean values of urinary free deoxypyridinoline (i), urinary N-telopeptide of type 1 collagen (ii), and serum total alkaline phosphatase (iii). Time period defined by self-diagnosis of menopause as date of last menstrual period. Change expressed as percentage of initial mean value (menopause-4).
Figures 4.23 and 4.24: Longitudinal changes of urinary free deoxypyridinoline: creatinine ratio in the perimenopause. (i) Individual changes and sample population mean over time in 77 patients. (ii) Sample population mean weighted for sample size showing 95% confidence limits for the curve fit (red) and the population (blue).
Figures 4.25 and 4.26: Longitudinal changes of urinary N-telopeptide of type I collagen:creatinine ratio in the perimenopause. (i) Individual changes and sample population mean over time in 77 patients. (ii) Sample population mean weighted for sample size showing 95% confidence limits for the curve fit (red) and the population (blue).
Figures 4.27 and 4.28: Longitudinal changes of serum total alkaline phosphatase in the perimenopause. (i) Individual changes and sample population mean over time in 55 patients. (ii) Sample population mean weighted for sample size showing 95% confidence limits for the curve fit (red) and the population (blue).
Figure 4.29 Longitudinal changes in biochemical markers of bone metabolism in the perimenopause. Mean values of urinary free deoxypyridinoline (red), urinary N-telopeptide of type I collagen (blue), and serum total alkaline phosphatase (green). To enable comparison between the markers, yearly values are expressed as a percentage of the initial, 'menopause - 4' population mean. Curve fits not weighted for sample size.
Figure 4.30: Percentile plots indicating distributions of biochemical markers at one year after menopause (m+1). Successive horizontal lines indicate marker values at 10, 25, 50, 75 and 90 percentile.
Discussion

Comparison of Resorption Markers

The increase of both free DPyr and NTx in the premenopausal to postmenopausal period is similar to that given by cross-sectional studies comparing these groups (Robins et al., 1994b; Seibel et al., 1993; Sone et al., 1995). While some studies indicate a larger elevation in resorption markers than demonstrated here, it should be noted that in our study even those individuals of the longest premenopausal duration may be suffering some oestrogenic deficit. While the values for percentage increase with time should be interpreted with caution due to the small number of samples on which the 100% level is based, this approach allows broad comparison of the two resorption markers. This shows the magnitude of the increases to be much greater for NTx than free DPyr.

While Robins et al. (1994b) found similar levels of bone resorption (as expressed by DPyr) in peri- and post-menopausal women, the definition of peri-menopausal as ‘within two years of menstruation’ is somewhat unusual. Indeed the variability of the definition of pre-, peri-, and post-menopausal makes the comparison of many bone density as well as biochemical studies difficult. Such data should always be carefully reviewed.

The Pattern Of Menopausal Bone Loss.

There are two major determinants of risk for osteoporotic fracture. These are the peak bone mass of the individual, and the subsequent bone loss undergone (Seeman, 1994). The importance of oestrogen deficiency in the development of post-menopausal bone loss is well recognised, and local mediators of bone turnover may be involved, but the absolute pathogenesis is still poorly defined (Cohen-Solal et al., 1995; Romagnoli et al., 1993).

Disagreement exists on the effect of oestrogen loss at the menopause on bone loss at different skeletal sites. It has been argued that trabecular bone is especially sensitive to the loss of oestrogen. Despite some indication that the menopause has little effect on the loss of cortical bone (Riggs et al., 1981; Riggs et al., 1986), there is broad agreement in the concept of increased trabecular bone loss at the menopause. However the starting point, severity and duration of this loss are of some debate (Baran, 1994). Many studies have indicated that the greatest bone loss in vertebrae (and those regions of the femur with high trabecular bone content), occurs during the two to three years immediately after the menopause (Falch & Sandvik, 1990; Hansen et al., 1995; Pouilles et al., 1993). However bone loss in the early perimenopause has also been demonstrated, with some studies indicating a higher rate of bone loss in perimenopausal than early postmenopausal women (Elders et al., 1988; Hagino et al., 1992). Most workers agree that the rate of trabecular bone loss slows with the length of the postmenopausal period. A
comprehensive longitudinal study by Hansen *et al.* (1995), found all the significant vertebral bone loss which occurred did so within eight years of the menopause. The pattern of loss for cortical bone is more uncertain. However the effectiveness of exogenous oestrogens in reducing bone loss at all skeletal sites indicates oestrogen deficiency-related bone loss is a generalised phenomenon (Godfriedsen *et al.*, 1986).

It is clear that oestrogenic activity declines before the menopause. Decreased oestrogen concentrations have been found in perimenopausal women with regular cycles, as well as those with menstrual irregularities (MacNaughton *et al.*, 1992; McCarthy *et al.*, 1990; Sherman *et al.*, 1976). During the last decade of menstrual life, the rate of follicular depletion increases dramatically, decreasing oestrogenic potential. (Richardson *et al.*, 1987). Indeed significantly higher oestrogen concentrations have been noted in early compared to late perimenopausal women (Slemenda *et al.*, 1987). Bone loss ascribed to loss of oestrogenic activity in perimenopausal women has previously been noted (Prior, 1990). This study suggests a rise in urinary markers of bone resorption before the cessation of menses, a pattern mirrored by serum total alkaline phosphatase (ALP). It is reasonable to conclude that as the menopause approaches, decreasing oestrogen concentrations herald the onset of increased bone turnover (and so bone loss) in perimenopausal women. It would seem likely that the pattern of endocrine changes and attendant bone loss described would vary markedly between individuals.

For bone loss to occur there must be an imbalance between bone resorption and bone formation. This study suggests that the osteoblastic response to decreasing oestrogen concentrations may be not as rapid as that of osteoclasts, resulting in a disproportionate increase of resorption over formation which is maximal immediately after the menopause. It is accepted that ALP is not a sensitive marker of bone formation, and that its increase at the menopause may be due in part to the liver isoenzyme (Schiele *et al.*, 1983). However any contribution to total ALP activity from this source would act to minimise not enhance the apparent difference between bone formation and resorption.

Identification of 'At-Risk' Individuals

While a recent review concluded that premenopausal bone loss is of no clinical significance in premenopausal women (Riis, 1994), this study supports the view that women suffer bone loss before the cessation of menses. Indeed some women are likely to suffer a high rate of loss for several years before the menopause. This would be clinically significant. Furthermore approximately one in three postmenopausal women is said to belong to a sub-population of 'fast bone losers' (Christiansen *et al.*, 1987; Christiansen *et al.*, 1990).

Although no appropriate longitudinal studies have been conducted to ascertain whether a group of sustained fast losers exist (Christiansen, 1994), fast losers at the menopause
have low bone mass when reassessed twelve years postmenopausally (Hansen et al., 1991). Even if ‘fast-loss’ is not sustained, it has been suggested that even relatively short (2-3 years) periods of rapid bone loss may contribute to the perforation of trabeculae and so increased fracture incidence (Johnson & Slemenda, 1994). This is supported by Takagi et al. (1995) who found that compared to premenopausal controls, early postmenopausal women had a significantly inferior bone quality as assessed by the number of trabecular perforations. Attempts to identify individuals ‘at-risk’ of fracture due to rapid bone loss or low bone density are therefore proposed. However achievement of this is not straightforward.

The assessment of risk factor status alone has not proved useful in the identification of perimenopausal women with low bone mass. (Elders et al., 1989). As bone loss is oestrogen dependent it has been suggested that hormone replacement therapy might be most effective in those with lower endogenous oestrogen concentrations. However the correlation between oestrogen levels and bone mass or rates of bone loss are not strong enough to be predictive (Adami et al., 1994; Slemenda et al., 1987). While bone mass measurement at the menopause attempts to identify those most at risk from postmenopausal bone loss (Lindsay, 1994), its predictive value in the identification of future fracture risk is not established (Raisz, 1994). Bone densitometry techniques require sequential measurements to identify fast losing individuals, possibly delaying identification at a time when a significant proportion of vertebral bone loss may be occurring (Christiansen, 1994). Indeed it has been estimated a woman waiting five years for a follow-up measurement could lose 15-20% of the total bone mass in this period (Raisz, 1994).

It is known that the rate of bone turnover in postmenopausal osteoporosis is proportional to the rate of bone loss (Citivelli et al., 1988), and that the menopause is associated with sharp increases in biochemical markers of such activity (Nilas & Christiansen, 1987). The combined measurement of bone mineral density and biochemical markers of bone turnover shortly after the menopause is effective in predicting rates of bone loss during a twelve year follow-up (Hansen et al., 1991). The potential role of biochemical markers in the identification of ‘fast-losing’ individuals is thus emphasised.

Figure 4.30 shows percentile plots of the biochemical markers one year after menopause. Using histograms the nature of the population distribution may be markedly altered by the selection of the number of groups and the data range each group represents. The increase in the slope at around the eightieth percentile indicates a skewed or bimodally distributed population. This data is supportive of the presence of a sub-population of fast bone losers.
Serum ALP in the Comparison of Two Treatment Regimens in Paget's Disease

Paget's Disease

The classic monograph first describing this condition was the description of a chronic inflammation of bone in a single patient by Sir James Paget almost 120 years ago (Paget 1877). Although originally called osteitis deformans by Paget, this condition now universally bears the authors name. The bone deformities that result from this condition allow its identification in skeletal remains, confirming the antiquity of this condition (Hutchinson, 1889; Wells & Woodhouse, 1975).

Paget's disease is characterised as a condition in which osteoclasts possessing abnormally numerous nuclei increase in both number and activity, resulting in excessive bone resorption. Normal physiological coupling of bone turnover is preserved, and a compensatory increase in bone formation follows. However although the osteoblasts themselves are normal, new bone is produced in a disordered, woven pattern (Singer 1994). Paget's disease, usually presenting due to pain, can affect any bone in the skeleton, at a single site (monostotic) or several sites (polyostotic).

Clinical expression of the disorder varies from almost asymptomatic (probably 90% of patients) to crippling, depending on the localisation, activity and extent of the disease process (Krane & Simon, 1964; Siris, 1995). Skull involvement may lead to deafness and impingement on the cranial nerve. Pagetic bones are brittle and have a tendency to spontaneous fracture (DeDeuxchaisnes, 1994). More serious complications of this condition include the possibility of high cardiac output failure in subjects with over one-third of the skeleton affected, and a prevalence of osteosarcoma thirty times higher than that of the normal population. The prevalence of Paget's disease varies greatly between and within countries, occurring most commonly in the UK, Western Europe and Australasia. In Europe, prevalence diminishes with distance from the UK, with the disease virtually unknown in Scandinavia (Detheridge et al., 1982). Within the UK, a striking prevalence in Manchester/Central Lancashire makes this region the "world capital" of this disease (Barker et al., 1980). The prevalence of this disease for the entire UK has been estimated at around 5% (Detheridge et al., 1982).

Paget's disease is often detected due to the elevated levels of serum alkaline phosphatase which are a consequence of the marked increase in bone turnover at affected sites. Radiography and isotope bone scans also have a role in the differential diagnosis while bone biopsy has no routine role in this condition (Mundy 1995).
The pathogenesis of Paget's disease remains unknown, although a primary disorder of bone metabolism and/or its hormonal regulatory processes appear unlikely. Paget's original observation of an inflammatory process suggested the involvement of an infectious agent. Inclusion bodies, a characteristic of persistent viral infection appear in the nuclei of Pagetic osteoclasts, implicating a slow viral infection, a RNA virus of the paramyxovirus family being the most likely candidate (Rebel et al., 1964; Mills & Singer, 1976). Of this group canine distemper virus has been proposed due to a putative increase in disease incidence in dog downers (Holdaway et al., 1990; O'DriscoU & Anderson, 1985). However such an association was not found in other studies (Siris et al., 1990, Stamp et al., 1986), while the low incidence of Paget's disease in veterinarians does little to support the proposed involvement of any animal virus (Holt, 1985). The slow virus theory therefore remains a hypothesis, with characterisation and isolation the proposed infective agent required before the riddle is solved. Indeed the process may be multifactorial, with heredity, possibly linked to the HLA locus having some role (Gordon et al., 1994, Siris 1994).

Anti-pagetic therapy is focused on the suppression of the over-active osteoclasts characteristic of the condition. The first universally available treatment for Paget's disease was calcitonin. Although associated with pain relief, maintenance of bone turnover (as assessed by biochemical parameters) to 50% of pre-treatment value requires indefinite treatment, the so-called plateau phenomenon (Siris, 1993) and on cessation of treatment a brisk 'rebound effect' occurs (Kanis et al., 1974). Unpleasant side-effects (nausea, flushing, sweating, diarrhoea) also ensue. Consequently treatment of Paget's with calcitonin continues to be superseded by the use of bisphosphonates. The first generation bisphosphonate disodium-1,1-bisphosphonate (etidronate) suffers from variable bioavailability and a therapeutic dose close to that which causes a mineralisation defect (Boyce et al., 1984). The second and third generation of these drugs exhibit increased potency with regard to anti-resorptive activity without producing mineralisation defects (Vignery & Baron, 1980). Biochemical remission with a short course of APD is more effective and of longer duration than may be achieved with etidronate. However despite the use of APD in Paget's disease for approximately ten years, for the majority of this time no standardised scheme of administration has existed (DeDeuxchaisnes, 1994). This drug is administered intra-venously due to variable gastrointestinal availability and increased incidence of erosive esophagitis with oral preparations (Lufkin et al., 1994). During this period of uncertainty it was decided to compare the treatment regime at the RNOHT, Stanmore with that of another clinical site (Addenbrookes Hospital, Cambridge).
Methods

Consecutive patients with radiologically proven Paget's disease were included in the study. Group 1 (Cambridge, n=19) were given a single 105 mg intra-venous infusion of APD, Group 2 (Stanmore, n=19) a multiple regimen (30 mg initially, then three further infusions of 45-60 mg over one month). Patients were followed until relapse, defined by recurrence of symptoms or biochemical relapse (ALP greater than twice the minimum value achieved on treatment).

Serum alkaline phosphatase was assessed at both centres using standard methods (see chapter II of this thesis for the method in use in this laboratory).

Results

The treatment regimens are properly compared using survival analysis. (Fig 4.31). This takes account of retreatment undertaken on the basis of recurring symptoms and increased ALP levels. The probability of remaining relapse-free is higher for longer in Group 2. This is confirmed to be statistically significant by the logrank test (p=0.003). The median ALP values for the two groups with time (Table 4.8, Fig. 4.32), indicates median ALP values fall more quickly and to a greater extent than those in Group 1. The difference between the nadir value in the two groups is statistically significant (Mann-Whitney test Z= -2.61, p= 0.009).

Figure 4.31: Survival curve for the assessment of differing APD treatment regimens in Paget's disease. Group 1, single infusion, compared with a multi-dose regimen (Group 2).
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Table 4.8: Serum alkaline phosphatase values in the response to APD treatment of Paget's disease. Median values for each treatment group (normalised to the pre-treatment value), are given as a function of time after treatment. Group 1, single infusion, compared with a multi-dose regimen (Group 2).

![Graph showing serum alkaline phosphatase values over time for Group 1 and Group 2.](image)

Figure 4.32: Serum alkaline phosphatase values in the response to APD treatment of Paget's disease. Median values for each treatment group (normalised to the pre-treatment value), are given as a function of time after treatment. Group 1, single infusion, compared with a multi-dose regimen (Group 2).
Discussion

The main objective of the overall study was the comparison of two differing regimens for the treatment of Paget's disease with APD. This study confirms our interim report (Ismail et al., 1994) that the multi-dose regimen is more effective than a single infusion in the treatment. This conclusion may have been reached using survival analysis alone without recourse to biochemical data other than for the assessment of relapse. However the relative decreases in ALP values for each treatment reinforces the survival curve data and confirms the usefulness of this marker in the assessment of efficacy of treatment in Paget's disease.

Although abnormal remodelling in Paget's disease is focal in nature, the magnitude of the increase is such that in many cases a clear elevation in most biochemical markers of bone turnover may be noted (Alvarez et al., 1995). Despite the markedly increased turnover, calcium balance in affected subjects is close to zero, indicating markers of formation and resorption should increase by a similar magnitude (Krane & Simon 1987). Indeed this is the case with total alkaline phosphatase (ALP) and urinary hydroxyproline (Harnick et al., 1986). However many of the newer markers of bone metabolism do not appear to be as useful as total ALP as a marker of disease activity or response to treatment (Delmas 1986, Filipponi 1994, Alvarez 1995).

The bone specific isoform of alkaline phosphatase (B-ALP) is marginally more sensitive than total ALP in the assessment of Paget's disease. However the low cost and easy availability of ALP measurements make it currently the best, most cost-effective approach in assessment of disease activity. Indeed trials of therapeutic regimens continue to include biochemical remission and relapse as assessed by ALP as an end-point indicator of drug efficacy (Patel 1993). As the potency of anti-resorptive agents used in the treatment of this condition continues, specific markers of bone resorption may replace ALP or B-ALP as the primary indicator in disease management (Russell 1994).
CHAPTER V - DISCUSSION

Bone, a composite of both inorganic and organic components, undergoes constant turnover due to osteoblastic and osteoclastic activity. The enzymes and proteins released by these cells and the products of matrix formation and breakdown may be utilised as biochemical markers of bone metabolism.

Calcium Homeostasis

Bone is a major organ of calcium homeostasis, and the estimation of serum calcium has long held a central role in the assessment of bone mineral homeostasis. However the simple accurate measurement of (physiologically active) ionised calcium remains an unachieved ideal. Lack of international standardisation for reference and control material leads to analysers showing massive variation in results (Uldall et al., 1985). Although the measurement of ionised calcium is available in over half of general hospitals in the USA (Kost, 1993), a recent survey indicated that this figure was only 7% for hospital laboratories in a typical UK health region (Duncan, 1995).

Total serum calcium levels only become meaningful on their adjustment to account for binding of calcium to different anionic species (including proteins). Adjustment is best accomplished by derivation of correction factors based on the binding of calcium to albumin as measured by the short BCG method. This is demonstrated in Chapter III of this thesis. Adjustment factors should be derived from the regression of calcium on albumin in a number of patients with no disorders of calcium metabolism. Tourniquet studies produce mean regression coefficients from a very wide spread of individual regression coefficients. This spread is a function of the small changes in total calcium produced by venous stasis. Such studies are inappropriate for the calculation of calcium adjustment factors.

The inaccuracy of the accepted wisdom of avoiding venous occlusion when taking blood samples for serum total calcium is also demonstrated. Stasis need not be avoided as long as values are adjusted for albumin concentrations. However combinations of venous occlusion and forearm exercise should be avoided as variable changes in calcium binding to albumin are produced.

It would seem reasonable to conclude that the accurate measurement of Ca$^{2+}$ represents the future of assessment of calcium activity, especially in laboratories serving specialised units or clinics. For routine purposes, the measurement of total calcium adjusted for serum albumin seems to be the easiest, most cost-effective and reliable way to assess serum calcium status. However despite the fact this measurement has been available for many years, a recent study found only 14% of laboratories surveyed to have formally derived or validated their adjustment algorithm (Duncan & Muller, 1995). Furthermore it
would appear ‘mythology’ holds more importance than fact as regards sample collection procedures.

Estimations of calcitropic hormones are of interest in a number of metabolic bone diseases. The analysis of 1,25(OH)₂D, the active form of vitamin D, has previously been limited by laborious and technically difficult assay procedures. These are detailed in Chapter II. A new immunoassay (IDS-Gamma-B) has recently become available which avoids the extensive sample preparation and separation obligatory in older methods. Its use and comparison with a reference method is undertaken in Chapter III. This study supports the new assay system as a suitable convenient substitute for HPLC-RRA measurements of 1,25(OH)₂D. The reduced hands-on analysis time, convenience, lower sample volume requirement, better analytical precision and lack of need for recovery estimation all support the use of this method. The small number of samples used in the comparison is accepted as a weakness. In the HPLC-RRA assay, the large volume of serum required, coupled with the regular repetition of analysis due to poor sample recovery, resulted in limited sample numbers for measurement by the candidate method. Furthermore the high cost of the candidate method makes more extensive method comparison unattractive. For financial reasons alone, precision studies were not undertaken with this new assay. However such cost implications, coupled with the rarity of laboratories performing the HPLC-RRA method means this comparison, however small, is likely to remain one of very few (especially independently of the assay manufacturer) to compare the Gamma-B IDS assay kit with the ‘reference’ HPLC-RRA method.

The author of a solid-phase cartridge extraction-RRA method has recently criticised the IDS Gamma-B assay, claiming positive interference from other 1α-hydroxylated metabolites of vitamin D (Hollis, 1995). This is feasible due to the nature of the immunoextraction procedure, the solid-phase antibody being directed towards the 1α-hydroxylated A-ring of 1,25(OH)₂D. Literature evidence for the presence of the interfering metabolite (1,25(OH)₂D₃-26,23-lactone) in normal sera is sketchy, appearing only in abstracts (Ishizuka et al., 1988; Ishizuka et al., 1992). Hollis (1995) appears to show almost 100% co-recognition of the 1,25(OH)₂D and the lactone in experiments in which the latter is exogenously added. However the only study reporting the occurrence of this lactone in normal serum estimates its concentration at approximately 130 pg/ml (Ishizuka et al., 1992). It seems reasonable to surmise that this compound cannot be circulating at these concentrations while cross-reacting with the IDS-Gamma-B assay to the degree suggested by Hollis. Preliminary evidence suggests interference from other circulating 1α-hydroxylated vitamin D metabolites does not occur in normal circumstances (Berry et al., 1996). Furthermore any such interference would make the
degree of correlation with other established methods difficult to achieve (Durham et al., 1995; Laurie et al., 1995, this thesis).  

Clinical Studies

The clinical use of biochemical markers of bone turnover is explored in Chapter IV. Their application in patient studies allows consideration of the relative utility of these markers, while putting into context some of the assessment studies undertaken in Chapter III of this thesis.

Fibrous Dysplasia

Two modes of treatment (APD, Figs 4.1-4.2; & calcitriol, Figs 4.3-4.5) are examined in a single patient with fibrous dysplasia. While fibrous dysplasia is likely to be primarily a defect of the bone-forming mesenchyme, biochemical markers indicate a condition of raised bone turnover. While results from a single patient should be interpreted with caution, it is noted that pre-treatment values of hydroxyproline and free pyridinoline are elevated to a much greater extent than (the bone-specific) deoxypyridinoline. It is possible this may reflect the poor intra-molecular cross-linking found on ultrastructural examination of affected bone (Mikhailova & Osipenkova-Vichtomova, 1993).

Notable in the follow-up to APD treatment is the failure of PYD to indicate a decrease in bone resorption. Many studies have shown bisphosphonate induced decreases in urinary markers of bone resorption in this time scale (Rosen et al., 1994; Pedrazzoni et al., 1995), and this thesis has demonstrated a similar short-term decrease in serum TRAP (Figure 3.22). While increases in fasting urinary pyridinolines have been reported one week after APD infusion (Mallmin et al., 1991), these were even more marked in a comparable placebo group. Such findings are indicative of the large day-to-day variation found with these markers (Popp-Snijders et al., 1996).

A short flare in OC, preceding 1,25(OH)2D elevation, is noted on APD treatment. This may result from the APD binding to hydroxyapatite, so producing a reduction in OC-hydroxyapatite binding, and a consequent increase in circulating OC levels (Fleisch, 1983; Papapoulos et al., 1987).

Calcitriol administration increases both PICP and OC (Figure 4.3 & 4.4), as found in other studies (Zerwekh et al., 1985; Gram et al., 1991; Gram et al., 1996). Despite the fact both are markers of osteoblast function, an increase of OC before that of PICP is clearly visible. The short-term increase in B-ALP is minimal. Such findings may be explained by examining the life span of the osteoblast and its products. Markers of osteoblastic activity are produced under different developmental phases of osteoblastic lineage and function in vitro (Owen et al., 1990). Type I collagen, the earliest
osteoblastic protein product, is produced during the proliferation of pre-osteoblasts (Kuhn, 1987). ALP is maximally expressed during matrix maturation by mature osteoblasts, declining once maturation has occurred and mineralisation commences (Rodan & Rodan, 1984; Stein et al., 1990). During mineralisation osteocalcin is expressed. It is estimated that from the appearance of a pre-osteoblast population, mature osteoblasts are differentiated within a few days, and are active for up to twelve weeks before progressing into osteocytes (Kimmel & Jee, 1980; Tran Van et al., 1982).

The rapid increase in OC represents a \(1,25(\text{OH})_2\text{D}\)-dependent stimulation of already present mature osteoblasts rather than the differentiation and recruitment of new osteoblasts. The increase in PICP will lag behind as the process of osteoblast differentiation occurs and new collagen matrix is formed. The lack of B-ALP response supports this, indicating a paucity of the matrix maturation process in the short-term. In the longer term patient calcitriol treatment actually decreases B-ALP. This phenomenon has been noted to a lesser extent in normal male volunteers (Gram et al., 1996). As B-ALP decreases during mineralisation, it is possible that the rapid calcitriol-induced increase in osteocalcin is representative of a mineralisation burst linked to suppression of osteoblastic ALP activity.

While both treatments decreased markers of bone formation in this patient over one month follow-up, only APD improved the clinical outcome of the patient in relation to pain. This argues against the use of these markers in monitoring response to treatment in fibrous dysplasia.

**Privational and Tumour Induced Osteomalacia**

The biochemical contrasts between healing privational osteomalacia, calcitriol-treated TIO, and the excision of the causative tumour in TIO are examined in Chapter IV (Figs 4.6 - 4.13).

Elevated ALP activities are found in rickets and osteomalacia (Demiaux et al., 1992; McComb et al., 1979), conditions characterised by the osteoblastic over-production of poorly mineralised osteoid (Stamp, 1994). This indicates that ALP reflects osteoblastic activity but is not necessarily representative of mineralisation, the latter being regulated by other factors such as levels of calcium and/or phosphate. The characteristic flare in ALP values seen on treatment of vitamin D deficient osteomalacia may represent the response of the 'accumulated' enzyme to a treatment-induced influx of its substrate (Stamp, 1994). Over several months of treatment, ALP activity declines as normal bone formation resumes, while osteoblast activity and mineralisation become 're-coupled'. Indeed ALP concentrations are positively correlated with matrix formation but negatively correlated with mineral apposition rate in this condition (Demiaux et al., 1992).
The uncoupling of bone turnover in the osteomalacic syndromes gives further information on markers of bone formation. Although Charles et al. (1992), found OC a reliable indicator of organ level mineralisation rate, (with reduced values in osteomalacia), this is not the general view. As OC binds to bone via the calcium of hydroxyapatite, incorporation is slowed due to the primary mineralisation defect. Consequently OC levels better reflect osteoid formation in this condition (Demiaux et al., 1992). The correlation of PICP with mineralisation also disappears (Risteli and Risteli, 1993).

Our studies in the treatment of TIO and privational osteomalacia raise a number of questions. Is the transient hyperphosphataemia which occurs in treated privational osteomalacia, 'valuable' to enhance skeletal remineralisation? Furthermore the causal relationship between this effect, delayed resolution of hypocalcaemia (presumably due to a predominant uptake of calcium by the skeleton), and the changes in calcitriol require further clarification. If the hypercalcitriolaemia expedites bone remineralisation, it may be this situation should be sought post-operatively in TIO. Further studies of TIO and other genetic disorders such as X-linked hypophosphataemic rickets are likely to aid the identification of a hormone primarily responsible for the regulation of renal phosphate reabsorption. The site of the production of this factor, its regulatory mechanisms and precise role in normal mineral homeostasis remain to be elucidated.

**Humoral Hypercalcaemia of Malignancy**

Bone histomorphometric and biochemical studies in HHM usually indicate a profound uncoupling of bone turnover, with markedly reduced osteoblastic activity (Stewart et al., 1982; Nakayama et al., 1996). The markedly increased bone turnover in our case study (Figure 4.14 - 4.16), is completely atypical of HHM. This lends support to the concept of HHM associated with malignant neuroendocrine tumours as a special pathological entity. In such cases co-secretion of other peptide cytokines is postulated to modify the clinical expression of the condition. While the rarity of this condition and the poor understanding of the processes involved mean this syndrome cannot be separately categorised at present (Mao et al., 1995), our findings represent a small but significant step forward in this process.

**The Assessment of Experimental Therapy in Osteoporosis**

Each remodelling sequence in osteoporosis involves bone loss. Inhibition of resorption, (and consequently formation due to coupling), reduces bone turnover, and therefore bone loss. This is the basis of treatment with bisphosphonate drugs.

While differences in anti-resorptive potency explain differing responses to etidronate and APD, the hypocalcaemia, increased 1,25(OH)2D and secondary hyperparathyroidism induced by APD (Figs 4.19 & 4.20) may contribute to the continued response to long
term treatment. This may be due to the anabolic effect of intermittent PTH (Canalis et al., 1989), which is enhanced by concomitant administration of calcitriol (Bradbeer et al., 1992; Slovik et al., 1986).

With the probable introduction of anabolic agents, therapies for established osteoporosis of all types will become more refined. When this occurs, assessment of bone turnover will prove important in choosing the optimal therapy. The examination of the relationship between markers of calcium homeostasis/bone turnover and the response to APD treatment was therefore undertaken.

**Osteocalcin**

Contrary to other studies, osteocalcin was found to be a poor indicator of identifying those likely to respond to therapy (Citivelli et al., 1988; Garnero et al., 1994a). However there is conflicting data on the utility of OC. Values have been reported as low (Canigia et al., 1986; Ismail et al., 1986), high (Delmas et al., 1983b; Epstein et al., 1984), or normal (Brown et al., 1984) in post-menopausal osteoporosis. While a highly specific indicator of osteoblast function, a number of technical problems with the assay of OC have been encountered. While pre-analytical factors could partially explain such discrepancies, this is compounded by variation in immunoassay specificity.

The heterogeneity of immunoreactive osteocalcin is of much recent interest. The presence (but not structure) of immunoreactive fragments has been reported in chronic renal failure (Gundberg & Weinstein, 1986), Paget’s disease (Taylor et al., 1990) and normal individuals (Tracy et al., 1990). Of all the fragments potentially generated by the proteolytic cleavage at amino acids 19-20 and/or 43-44 (see Fig 1.5), only the short C-terminal fragment (43-49) has not been detected as circulating in vivo. (Garnero et al., 1994b).

The C-terminal specificity of the antibody in the OSCAtest assay system used in this thesis has led to the manufacturer claiming specificity for intact osteocalcin. Certainly OSCAtest measured OC exhibits poor in vitro stability (Blumsohn et al., 1995a) and gives lower OC values than IRMA assays measuring both intact and 1-43 OC (Masters et al., 1994), suggesting little or no recognition of 1-43 OC. More likely is recognition of C-terminal fragments, especially the mid-C-terminal fragment which represents 13-14% of the value of intact OC in normal and osteoporotic patients (Garnero et al., 1994b).

It is not certain that the proportions of the differing OC fragments remain unchanged in conditions of altered bone turnover. Preliminary data indicate such changes in postmenopausal osteoporosis (Diaz Diego & de la Piedra, 1996), while Garnero et al. (1994b) indicated that the N-terminal midfragment increased from 83% of the intact OC value in normal subjects to 91% in Pagetic serum. In a comparison of eight OC assays
(including intact plus N-terminal midfragment measuring assays), Masters et al. (1994) found increased OC values in Paget’s patients to be most marked using the OSCA test. This suggests an increased production of C-terminal fragments. The same study found all assays indicating decreased values in pregnancy. The probable physiological mechanism behind this is the placental-dependent enzymatic degradation of intact OC (Rodin et al., 1989). The decrease was least marked using OSCA test indicating some recognition of one of the fragments produced.

In the study comparing OSCA test with an alternative method (Chapter III), samples with markedly raised OC values showing good inter-method agreement came from patients with different conditions to those in whom elevated OC values were discrepant. This indicates that increased circulating C-terminal fragments (both absolutely and as a proportion of total OC) may vary in different diseases.

Intact OC levels have long been known to respond oestrogen or vitamin K administration in post-menopausal women (Podenphant et al., 1984; Knapen et al., 1989). However a recent study indicated intact-plus-N-terminal OC significantly decreased on oestrogen treatment while intact OC alone did not. Conversely intact OC values increased slightly over the course of the study (Rosenquist et al., 1995). This difference is most likely to be a reflection of the study design. As all OC measurements were made on completion of the study, the poor stability of intact OC rather than its poor response to treatment is highlighted. This criticism is supported by similar increases in late post-menopausal osteoporotic women over pre-menopausal controls for assays measuring either intact OC or intact-plus-N-terminal OC (Garnero et al., 1994b; Rosenquist et al., 1995).

OC concentrations in post-menopausal women have also been found to be significantly related to loss of bone mineral on follow-up (Johansen et al., 1988). Although the circadian rhythm of OC appears to be unaffected (Pietschmann et al., 1990), values obtained many studies show large overlap with normal controls, reflecting the heterogeneous nature of post-menopausal bone loss and limiting the diagnostic use of OC measurements in individuals.

While mindful of the possibility of varying proportions of immunoreactive fragments in patients with differing pathologies, the recognition of the need to differentiate between the various immunoreactive species should lead to improvements in the clinical application of OC. In future the assessment of undercarboxylated OC may have a clinical application.

**Bone-Alkaline Phosphatase**

While pre-treatment ALP is positively correlated with response to bisphosphonate treatment, the correlation increases in strength and significance on measurement of the bone specific isoform (Table 4.4). There is no doubt that the measurement of B-ALP is
more sensitive than that of ALP. At the menopause B-ALP increases by a significantly higher degree than ALP, while response of the bone-specific isoenzyme is more marked on treatment of Paget's disease (Gamero & Delmas, 1993).

Pre-immunoassay methods of measuring B-ALP gave conflicting information, some workers discounting sex-related differences in normal adults (Price, 1993; Schoneau et al., 1986), others describing higher values in men (Moss, 1966; Onica et al., 1986; Sorenson, 1988). This difference, reversed as females pass through the menopause, is confirmed by immunoassay (Price, 1993), as are increases with age in the older female reference population (Price et al., 1995). Detection of subtle alterations in bone metabolism confirm the sensitivity of the immunoassay methods for B-ALP.

Although both markers of bone formation, B-ALP but not OC appears correlated strongly with treatment induced increases in spinal BMD. Apart from the analytical and pre-analytical considerations discussed above, it should be reinforced these markers do not measure the same aspects of bone formation (see sections on fibrous dysplasia and osteomalacia). A lack of correlation between OC and ALP has been found in healthy control and postmenopausal osteoporotic women (Diaz-Diego et al., 1995). Furthermore unlike OC, B-ALP is unaffected by age related decreases in glomerular filtration rate (Delmas et al., 1983a)

This study indicates B-ALP to be the most useful pre-treatment indicator of response to APD. This parallels findings of Garnero et al. (1994a), who identified changes in B-ALP as the best single predictive biochemical index of response to bisphosphonate therapy. These findings indicate that patients with increased bone turnover but little osteoblastic deficit may benefit most from anti-resorptive therapy.

Procollagen Type I Carboxy-Terminal Propeptide

No significant correlation between PICP and response to APD treatment was found. While PICP has logical potential as a bone formation marker, it appears less sensitive than other such markers, failing to recognise increased bone turnover at the menopause or in post-menopausal osteoporosis (Hasling et al., 1991; Hassager et al., 1991; Hassager et al., 1993; Gamero et al., 1994a; Pederson & Bonde, 1994). The contribution to serum PICP concentrations from non-osseous type I collagen biosynthesis, questionable immunoassay antigenic specificity and an inherently variable metabolic clearance all detract from the use of PICP at this time.

Urinary Markers of Bone Resorption
Twenty-four hour urine calcium excretion correlates poorly with response to treatment, supporting the view that although cheap and easy to perform, this measurement is not a reliable indicator of bone resorption (Eriksen et al., 1995).

Hydroxyproline excretion is always influenced by diet, renal and hepatic function, complement activity and the turnover of non-osseous collagen. Despite these major biological limitations on its specificity, urinary hydroxyproline was the marker of bone resorption best correlated with response to treatment. The stronger correlation of OHP than the more bone specific DPyr seems surprising. It is appropriate at this point to consider the clinical utility of such markers as a function of their reproducibility.

The usefulness of any biomarker depends on the summation of biological and assay related variability (Morris et al., 1990). On direct comparison, intra-individual variation for hydroxyproline has been found to be lower than that for deoxypyridinoline (Gertz et al., 1994). Furthermore while within-patient variation of around 30% for two consecutive fasting OHP:creatinine ratios has been reported (Vasikaran et al., 1994), a recent review details studies reporting intra-individual variations of up to 63% for urinary collagen crosslinks (James et al., 1996). These variations are compounded by higher analytical variations for free DPyr than OHP as found both in this thesis and in the literature (Randall et al., 1996).

It has been observed that the necessary analytical precision as derived from the biological variation is not easily achieved with current methods of DPyr estimation. This leads to the discovery that consecutive measurements on an individual need to be approximately 50% different to be statistically significant (Panteghini & Pagani, 1996). It is reasonable to conclude that in the rush to apply measurements of pyridinium crosslinks, the consequences of large combined analytical and biological variations have been widely overlooked. Further confusion arises from the various use of first morning, two-hour fasting and 24 hour urine collections. This is despite the very large (up to 100%) circadian variation in crosslink excretion which changes most rapidly in the early morning period and has considerable heterogeneity between individuals (Blumsohn et al., 1994b; James et al., 1996). This heterogeneity, reflected by lower between-subject variations in 24 hour collections (Panteghini & Pagani, 1995), may lead to the lack of strong correlation found between paired two- and twenty-four hour samples and the higher standard deviation of mean values for two hour samples (Figs 3.13 & 3.14).

Recently it has been suggested that within-subject variation may be markedly reduced simply by pooling of consecutive samples (Popp-Snijders et al., 1996). A study in a small group of patients (Chapter III) suggests an average intra-individual variation of 30% for 48 hour urine pools, little improved over that for fasting or 24 hour collections. However the cost benefits of such actions are obvious.
Further complications arise on deciding whether to express urinary analytes as concentrations, excretions per unit time or 'corrected' for creatinine to allow for a combination of urine volume and patient body size (as lean muscle mass). Such correction makes urinary markers more predictive of treatment response (Table 4.4), despite possible effects on muscle mass in the steroid-induced patients. Other studies have also indicated increased clinical utility due to creatinine correction (Colwell et al., 1993; Hassager et al., 1992b). This is likely to be a consequence of a reduction in biological variance which is irrespective of sample type (Panteghini & Pagani, 1996).

The lack of information regarding the possible use of acidified urines in the free pyridinoline immunoassay (established in Table 3.12) reinforces the impression that newer markers of bone resorption have entered clinical use with a number of physiological and analytical variables unaddressed.

**Tartrate-Resistant Acid Phosphatase**

Although catalytic measurements of TRAP are generally deemed to be of limited usefulness, TRAP correlated with treatment response (Table 4.4). Furthermore this enzyme showed a gradual increase with age in both sexes, and a marked increase at menopause in females (Figs 3.19-3.21). This pattern is analogous to that previously detailed for OC and B-ALP (Duda et al., 1988). Furthermore the increase in bone turnover in Paget's disease, and at the menopause (and its reversal by HRT) are identified by TRAP measurements (Fig 3.17). Decreases in TRAP activity are also noted in groups of bisphosphonate treated Pagetic and osteoporotic patients (Fig 3.22 & Fig 3.23), and in a single case of HHM.

TRAP methods used in this thesis, based on catalytic activity towards α-NP, seem at least as useful as other current methods. Their easy automation and lack of reactivity towards erythrocytic or platelet TRAP led Rico & Villa (1993), to suggest α-NP as the preferred spectrophotometric substrate for TRAP. Although the low catalytic activity of TRAP towards α-NP is a barrier to sensitivity, it seems paradoxical that the 4-NP based method of Lau et al. (1987) requires the incubation of diluted serum at 37°C for one hour as a pre-treatment step, despite evidence that TRAP experiences a time and temperature dependent loss of activity.

TRAP estimations may have proved more useful in this thesis than in other studies due to the small specialist unit in which the author is based. This allows the carefully controlled sample preparation and storage not possible in larger institutions. Immunoassays specific for osteoclastic TRAP offer the best hope for the routine use of TRAP as a marker of bone resorption. Assays claiming such specificity have recently been described but have yet to undergo thorough clinical assessment (Chamberlain et al., 1995; Cheung et al., 1995).
Changes In Bone Turnover During The Perimenopause

While bone loss in the perimenopausal period has been previously suggested (Elders et al., 1989; Johnson et al., 1985; Steinberg et al., 1989), little is known of its pathogenesis. This longitudinal study aids the evaluation of the pattern of bone loss in the peri-menopausal period. Although usually lasting two to three years, this may predate the menopause by 8-10 years in some women (Whitehead & Godfree, 1992). The perimenopause may therefore be associated with clinically significant bone loss.

This study suggests that the accelerated phase of bone loss begins several years before the clinical menopause. A rise in urinary markers of bone resorption before the cessation of menses is mirrored to a lesser degree by ALP (Fig 4.29). It is possible this is simply due to the poorer sensitivity of ALP. However a model in which oestrogen reduction results in a osteoblastic response less rapid than that of osteoclasts, resulting in a disproportionate increase of resorption over formation, is supported by other studies. In oophrectomised women, DPyr peaks one year after surgery and osteocalcin one year later still (Hashimoto et al., 1995). A recent cross-sectional study (Garnero et al., 1996) indicates greater increases in resorption markers than formation markers in perimenopausal women.

That resorption markers (Figs 4.23 - 4.26) plateau for several years after the menopause while formation markers continue to rise (Figs 4.27 & 4.28), mirrors changes found in artificial menopause (Stepan et al., 1987; Stepan et al., 1989). Cross-sectional data also indicates no postmenopausal increase in resorptive indices (Schlemmer et al., 1993; Sone et al., 1995; Tohme et al., 1990), while increases in osteoblastic markers continue (Delmas et al., 1983c; Duda et al., 1988; Schiele et al., 1983; Schiele et al., 1988). Such data supports the hypothesised reduction in the degree of uncoupling (and consequent decrease in the rate of bone loss) as the postmenopausal period progresses (Reeve et al., 1995).

While intermittently low oestrogen levels are the most probable cause of perimenopausal bone loss (Slemenda et al., 1987), such changes may be associated with minimal disturbances in the menstrual cycle. This study supports the view that oestrogen prophylaxis or other intervention should be considered before the cessation of menses in some women (Cooper, 1993). While attempts to enhance peak bone mass may be undertaken almost universally, prevention of bone loss during the climacteric would allow an individual to reach the menopause with a better bone 'reserve' (Gambacciani et al., 1994). It should be noted that although female fecundity declines with age, as long as ovulation continues pregnancy is possible (Federation CECOS, 1982; Guillebaud, 1985). There is some evidence to suggest that due to a number of factors (social, demographic etc.), coital frequency in older women may be increasing (Harper, 1992).
In suitable subjects (fit, normotensive, non-smoking, cumulative oral oestrogen use less than 20 years), the use of a low dose combined pill may be considered suitable contraception. Although this would mask actual menopause, such exogenous oestradiol would provide protection against perimenopausal bone loss.

This study also allows a direct comparison of NTx and free DPyr as markers of bone resorption. The increase in response to oestrogenic deficit is more rapid and pronounced for NTx (Fig 4.29). This may explain the seemingly contradictory findings that NTx excretion is strongly correlated with free DPyr in normal individuals, but only weakly so in early postmenopausal women. (Gertz et al., 1994; Rosen et al., 1994). The combination of the (claimed) higher specificity, lower intra-individual variability, and lower analytical variability of NTx measurements suggest it as the more useful marker of bone resorption (Gertz et al., 1994; Hanson et al., 1992; James et al., 1996).

**Paget’s Disease**

While OC is elevated in Paget’s disease, the increase is not of the same magnitude as that of ALP which better reflects histomorphometrically assessed bone formation (Delmas et al., 1986; Duda et al., 1988). Some workers have suggested this discrepancy may be a function of increased OC-hydroxyapatite binding (Torres et al., 1991a), yet as circulating OC appears to be normally carboxylated (Merle & Delmas, 1990), the reason for the difference is unclear. It is possible that the rapid bone turnover inherent in this condition leads to an increase in OC trapped in the bone matrix.

NTx is a better discriminator of disease activity and response to treatment than PYD and free DPyr (Blumsohn et al., 1995c; Randall et al., 1996). However there is little evidence to suggest these markers may be applied to greater clinical benefit than ALP (Alvarez et al., 1995). In our Paget’s study (Fig 4.31 & 4.32), ALP correctly distinguishes the difference between two modes of treatment established by survival analysis of re-treatment data.
Conclusions

Less than twenty years ago the only readily available biochemical means of assessing bone turnover was the measurement of serum total alkaline phosphatase, urinary calcium and hydroxyproline. The range of markers now available is a measure of developments in not only assay technology, but the understanding of the biochemistry and physiology of bone, and the recognition of osteoporosis as a major healthcare problem.

The attraction of non-invasive measurements of bone turnover, which respond quickly to physiological change and may be repeated at relatively little cost is great indeed. Furthermore, unlike physical methods, biochemical markers are reflective of changes in the entire skeleton. This has led to a number of roles for biochemical markers of bone turnover:

As Diagnostic Tools:

Biochemical markers are currently developing a role as an adjunct to physical measurements of bone density. While markers of bone metabolism are not diagnostic in osteoporosis due to the heterogeneous nature of the condition, biochemical markers will identify the degree to which decreased osteoblastic or increased osteoclastic activity is responsible.

In post-menopausal osteoporosis, it is generally accepted that the rate of bone turnover is strongly correlated with bone loss. Identification of 'fast-losers' would allow early therapeutic intervention in those adjudged to be most at risk of fracture in later life. It has been claimed that almost 80% of both 'fast' and 'slow bone losers' may be identified using only fasting urine calcium, hydroxyproline and serum ALP (Christiansen et al., 1987). The specificity and sensitivity of such identifications should be enhanced using newer, more bone specific markers.

The wide spectrum of bone turnover in osteoporotic patients results in a range of responses to anti-resorptive treatment. It may be that those with low turnover osteoporosis would be better served by a form of anabolic treatment. It seems probable that as therapies become more sophisticated, bone markers will be of use in assigning patients to appropriate treatments (Overgaard et al., 1994). The early stages of this process are addressed in this thesis.

Markers may not only be applied to osteoporosis; the presence of osteopenia is currently one of the indicators for surgery as treatment for primary hyperparathyroidism. Biochemical markers may identify those most likely to respond to such treatment with increasing bone mass.
Influence On Patient Compliance With Therapy

A further role in enforcing compliance to treatment may be possible. Bone mass measurement has been demonstrated to have a positive impact on the rate of compliance with HRT (Ryan et al., 1992). A reduction in a bone turnover demonstrated by a biochemical marker may have a similar effect, especially in the early stages of treatment when a bone density measurement will show little or no change.

Monitoring Response to Therapy

A well recognised use for biomarkers of bone turnover is in monitoring response to treatment (Blumsohn et al., 1995c; Garnero et al., 1994; Harris et al., 1993; Riis et al., 1995; Stepan et al., 1989). However it has been demonstrated that markers of bone resorption differ markedly in their capacity to reflect bisphosphonate induced decreases in resorptive activity (Pedrazzoni et al., 1995).

The total precision (analytical plus biological) of urinary resorption markers tends to be lower than that of serum bone formation markers. The magnitude of any treatment-induced changes in such resorption markers must therefore be higher than in the serum formation markers before a true response may be assumed (Azria & Russell, 1992).

Summary

Various markers of bone formation or resorption are used to represent the cellular functions and turnover of bone. However numerous discrepancies occur. Many are demonstrated in this thesis and are supported by the most cursory review of the literature. Analytical specificity, the differing contributions of non-bone tissues, and the reflection of different components of bone resorption or formation should all be considered in the application of these markers.

Of all the markers of bone metabolism, no single markers of formation or resorption may be said to be 'the best'. Different markers are elevated to different degrees in each clinical situation, a reflection of the pathological processes occurring at the cellular level. As suggested by Deftos (1991), it would appear a more sophisticated application of the use of ALP and OC in clinical studies depends upon a fuller understanding of the differences between the two measurements. While many studies have indicated osteocalcin to be the most sensitive marker of bone formation, it was of little use in the study addressing prediction of the response to treatment of established osteoporosis. While this may be reflective of assay technology rather than in vivo changes in intact osteocalcin, B-ALP appears to be a better indicator of bone formation in these studies.
Generally the performance of free deoxypyridinoline as a marker of bone resorption has been disappointing in studies comprising this thesis. The N-telopeptide of collagen appears a more sensitive indicator of resorptive activity.

**Future Developments**

With many laboratories unwilling or unable to analyse total deoxypyridinoline by HPLC, immunoassays for free deoxypyridinoline markedly increased the availability of sensitive and specific biochemical measurements of bone resorption. That the proportions of free and peptide-bound crosslinks are independent of type and severity of disease is important in determining the utility of free crosslink immunoassays. While initial reports indicated this was the case (Robins *et al.*, 1994a), the proportion of free DPyr has been found to vary between disease states and on anti-resorptive treatment (Gamero *et al.*, 1995). It is possible that the greater responsiveness of NTx to alterations in the rate of bone resorption is related to this finding.

A recent model has proposed that collagen fragments containing deoxypyridinoline are sequentially degraded to free pyridinoline, and that this sequence contains a rate limiting step between the peptide forms (measured as NTx) and the free forms (measured by Pyrilinks-D) (Randall *et al.*, 1996). As such NTx may be an overestimate of bone resorption, free DPyr an underestimate, or a mixture of both. It is clear that an effective comparison of urinary bone resorption markers relies on further elucidation of the pathways of bone collagen breakdown.

One of the biggest disadvantages in the use of biochemical markers of bone turnover is the difficulty in comparing bone formation (serum markers) with bone resorption (various urinary collagen degradation markers differing only subtly in their target antigen). The need for a reliable, readily available serum resorption marker is therefore emphasised. Furthermore serum markers are easier to collect and display lower biological and analytical variation than urinary markers (Blumsohn *et al.*, 1994a). Serum ICTP has potential as a marker of type I collagen breakdown and therefore as an indicator of bone resorption. However the few studies to assess the clinical use of this marker to date have yielded disappointing results. Crosslinking compounds circulate in serum, but at present are only measurable by HPLC.

This thesis examines a possible role for serum TRAP as a marker of osteoclastic activity. It would appear that the usefulness of this marker is limited by assay technology rather than physiology. Spectrophotometric measurements of TRAP are of limited value due to the poor catalytic activity of the enzyme towards the different substrates available. While the relative merits of markers of different aspects of bone resorption require further evaluation, immunoassays specific for osteoclastic TRAP may prove a valuable marker of bone resorption.
In the longer term the future lies with the understanding of the complex and overlapping effects of the large number of cytokines and growth factors involved in the regulation of bone turnover. However the rush towards ever more sophisticated and expensive markers of bone metabolism should not obscure the utility of older markers in certain circumstances (Christiansen et al., 1987; Patel et al., 1993). Their cheapness and easy availability means a continued role, especially in parts of the world where severe financial constraints apply.

Suggestions for Further Work

The study on pre- and peri-menopausal bone loss offers some intriguing possibilities for future work. As collagen crosslinks are remarkably stable in urine, the sensitivity of other indicators of bone resorption (total and free deoxypyridinoline by HPLC, 'Crosslaps' ELISA etc.) may be studied. A long term follow up of this patient group may also prove useful. It is possible that the rate at which bone is lost contributes to micro-architectural damage in bone. This damage, an important factor in fracture risk, cannot be assessed by measurement of bone mineral density. The examination of fracture incidence in the 'fast-losers' compared to the 'normal' individuals may be indicative of the pathogenesis and risk involved in rapid peri-menopausal bone loss. The use of bone markers in the prediction of response to treatment and the targeting of therapy deserves further attention. A serum marker of bone resorption such as TRAP may improve the predictive value, as would a combination of markers.

Bone turnover is regulated by a variety of systemic and locally synthesised factors. The established indicators of calcium homeostasis may have a limited future use, unless PTH or calcitriol become established as therapies for osteoporosis. Increasing analysis of the local factors regulating bone remodelling, especially those involved in the coupling of formation and resorption is likely.
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Appendix

The chemical structure of the bisphosphonates. Relative potency etidronate=1, pamidronate (APD)=100, alendronate=500-1000.
Appendix 2: Analytical variation for assays used in this thesis.

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<th>ANALYTE (analyte name)</th>
<th>Intra-assay variation</th>
<th>Inter-assay variation</th>
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<tr>
<td>OH-P (mmol/l)</td>
<td>0.22</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1.06</td>
<td>20</td>
</tr>
<tr>
<td>Creatinine (nmol/l)</td>
<td>N/A</td>
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<tr>
<td>TRAPBM (IU/L)</td>
<td>2.04</td>
<td>28</td>
</tr>
<tr>
<td>TRAPRandomx (IU/L)</td>
<td>2.41</td>
<td>20</td>
</tr>
<tr>
<td>1,25(OH) D2 HPLC (pg/ml)</td>
<td></td>
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<tr>
<td>1,25(OH) D2 Kit (pg/ml)</td>
<td>13.2</td>
<td>10</td>
</tr>
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<td>PTH (pmol/l)</td>
<td>4.0</td>
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<td></td>
<td>26.6</td>
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<tr>
<td>Bone-ALP (ug/l)</td>
<td>13.2</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>48.6</td>
<td>21</td>
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</tbody>
</table>
P21. Differences in biochemical response to treatment between privational and tumour induced osteomalacia
PE Fox and TCB Stamp
Department of Bone and Mineral Metabolism, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex

A 61 year old patient of Asian origin presented with classical vitamin D deficiency osteomalacia. In addition to characteristic routine biochemistry, serum levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D were abnormally low and intact PTH was excessive. A dose of 1600 units vitamin D restored normocalcaemia and slowly raised serum 25-hydroxyvitamin D. However, levels of 1,25-dihydroxyvitamin D rose rapidly to exceed the normal upper limit for over six months, with raised PTH levels persisting for a similar period.

A 59 year old female with an eight year history of hypophosphataemic osteomalacia was subsequently shown to have a tumour of the perineum which was incompletely resected. Histology revealed a hemangiopericytoma. Biochemical investigations showed hypophosphataemia associated with normocalcaemia and a persistently low 1,25-dihydroxyvitamin D despite normal PTH and 25-hydroxyvitamin D levels. Massive phosphate supplementation lowered serum calcium within normal boundaries, PTH rose to abnormally high levels but no change in 1,25-dihydroxyvitamin D ensued. The addition of calcitriol restored normal 1,25-dihydroxyvitamin D levels and steadily healed the disease but did not affect serum phosphorus concentrations. Tumour resection, even though incomplete rapidly restored normophosphataemia: 1,25-dihydroxyvitamin D levels rose further but remained within the normal range.

The above results underline basic differences in vitamin D metabolism and endocrinology in the two differing forms of osteomalacia.
<table>
<thead>
<tr>
<th>ANALYTE</th>
<th>Intra-assay variation</th>
<th>Inter-assay variation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Conc</td>
<td>Sample No.</td>
</tr>
<tr>
<td>PICP (ug/l)</td>
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<td>16</td>
</tr>
<tr>
<td></td>
<td>451</td>
<td>16</td>
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<tr>
<td>Osteocalcin (ng/ml)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total Free PYD (nmol/l)</td>
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<td>52</td>
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<td></td>
<td>398.9</td>
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<tr>
<td>Free DPyr (nmol/l)</td>
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<td>NTx (nmol BCE)</td>
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<td></td>
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<td>2640</td>
<td>40</td>
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</table>

For further details refer to Chapter Two (Methods) and/or the assay instructions/package inserts provided by the manufacturer which are bound with this thesis.
Immunoassay of collagen crosslinks in acidified urine

P Fox and T C B Stamp

From the Department of Bone and Mineral Metabolism, Institute of Orthopaedics, University College Medical School, Royal National Orthopaedic Hospital Trust, Stanmore, Middlesex HA7 4LP, UK

Additional key phrases: pyridinoline; deoxypyridinoline; bone resorption; pyridinoline/deoxypyridinoline ratio

We read with interest the evaluation of the Metra Biosystems 'Pyrilinks' assay for free pyridinolines (Metra Biosystems (UK) Ltd, Oxford, UK. Product number 8010) by Hata and Miura. Their findings are indeed consistent with those of Seyedin, Delmas and others' in showing the usefulness of this method in monitoring urinary total free pyridinoline excretion as a marker of bone resorption.

This assay measures free pyridinolines (i.e. pyridinoline and deoxypyridinoline) with 100% cross-reactivity. Recognition of peptide bound forms of both crosslinks is negligible.

The manufacturer, Hata and Miura, and other authors use the abbreviation PYD to represent the sum of both forms of pyridinium crosslink as measured by this method. We feel the use of PYD in this manner leads to a potential for confusion. It is possible this term may be misinterpreted as indicating either total or free pyridinoline, with no contribution from free deoxypyridinoline to the final result. Although more unwieldy, the term 'total free pyridinolines' is a more accurate description of the compounds measured by this assay.

The prospective use of this assay in the screening and monitoring of patients with osteoporosis and other metabolic bone diseases led us to consider its possible use with 'routine' urine samples.

While it is common in this and many other laboratories to receive urine containing hydrochloric acid (HCl) as a preservative, the assay protocol insists on the use of unpreserved urine. The kit manufacturer has also suggested that acidified urine be 'pH corrected' before assay. We therefore decided to investigate the use of this ELISA with acidified urine samples.

METHODS

All analyses were performed in duplicate according to the manufacturers suggested protocol.

The following paired samples were analysed: acidified versus unacidified (n = 20); and acidified versus 'pH corrected' (n = 10).

Urine samples were taken from normal subjects and patients suffering from a variety of metabolic bone diseases. Both 24 h and 2 h fasting collections were used.

The concentration and volume of HCl used for sample acidification varies with laboratory. In this study samples were acidified with 10 mL conc HCl irrespective of volume. This ensured the most acidic conditions likely to occur in clinical samples.

Urine samples for pH correction were adjusted to the pH of their unacidified pair using concentrated sodium hydroxide. A volume correction for the addition of alkali was then made.

RESULTS AND DISCUSSION

Results were analysed using Wilcoxon's signed rank test. No significant difference was found between either of the paired groups (Table 1).

The data was subsequently plotted to investigate the correlation between the two groups, as shown in Fig. 1. However, method comparison is more appropriately carried out using the difference plot described by Bland and Altman (Fig. 2).

<table>
<thead>
<tr>
<th>Table 1. Analysis of results using the Wilcoxon's signed rank test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of</strong></td>
</tr>
<tr>
<td><strong>pairs</strong></td>
</tr>
<tr>
<td>Acidified</td>
</tr>
<tr>
<td>pH corrected</td>
</tr>
<tr>
<td>Unacidified</td>
</tr>
</tbody>
</table>
Immunooassay of collagen crosslinks

such, the difference between these results is unlikely to be clinically significant.

As a post-treatment sample from the same patient showed excellent agreement between acidified and non-acidified samples, it is probable that this discrepancy is due to an analytical error.

CONCLUSION

A more recent assay, measuring free deoxypyridinoline alone, is now available from the same manufacturer ('Pyrilinks-D', Metra Biosystems, UK). Deoxypyridinoline is primarily located in bone collagen, while pyridinoline is also found in cartilage and other soft tissues. The new assay represents a more specific indicator of bone resorption than its predecessor. As the Pyrilinks-D assay involves a 10-fold dilution of urine sample in a phosphate buffer before analysis, it would seem improbable that sample acidification would affect the assay.

The 'Pyrilinks' assay remains available as a general indicator of bone resorption. It may also have applications in other connective tissue disorders, especially those conditions in which the urinary pyridinoline/deoxypyridinoline ratio becomes altered.

We conclude that acidified urine samples are suitable for use in this assay.

REFERENCES


Accepted for publication 3 November 1994
TREATMENT OF PAGET'S DISEASE WITH I-V PAMIDRONATE (APD) - FOUR YEAR FOLLOW UP
A.A Ismail, P. Fox, A.J Goldstein, T.C.B Stamp
Metabolic Unit, Royal National Orthopaedic Hospital, Stanmore, Middlesex.

Intravenous APD has been shown to be effective treatment in symptomatic Paget's disease(1). This study is an extended follow up of patients treated with I-V APD for assessing both symptomatic and/or biochemical relapse of Paget's disease.

**Patients/Protocol** 31 patients (M=17,F=14, mean age=71yrs, range 49-83yrs) were treated with either standard regime of 45-60 mg I-V APD in 4 infusions over 2 months (n=18), or a varying regimen, some patients receiving daily infusions over 8 days, others weekly then fortnightly infusions over 26 weeks (n=13). Patients were assessed weekly during treatment, then at 3-6 monthly intervals. At the end of four years 22 patients remained under follow up.

**Results** Serum alkaline phosphatase (sAP) reached its nadir in the first 6 months (average of 26% of pretreatment level) and thereafter rising slowly (32% of pre-treatment levels at 1 yr, 36% at 2yr, 38% at 3yrs. and 43% at 4yrs). Urinary hydroxyproline/creatinine ratio fell by an average of 53% at 2 months and decreased further to 60% at 1 yr. Only two patients required re-treatment due to recurrence of symptoms or biochemical relapse (≥ double minimum value of sAP) by 2yrs (7%), a further 7 patients between 2-3 yrs. and 3 more patients by 4yrs (52% of patients still in remission at 4 yrs.). Patients were withdrawn after re-treatment and not included thereafter. No significant difference in results was observed in patients given the standard regime or varying dose regimens.

**Conclusion** 1 This study confirms both short and longterm efficacy of I-V APD, and is the longest follow up study in such patients.
2 Short term treatment regimens appear as effective as more prolonged regimens.

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NOTE Leaves 268-275 are appendices
NOTE Photocopies of 1 published paper and 2 abstracts of published papers bound in between leaves 271-275 9 pamphlets in end pocket
SUBJECT Medicine (Board of Studies)
MARC s1997 en W 00001 eng bam
INTENDED USE
The Tandem-R Ostase immunoradiometric assay is an in vitro device indicated for the quantitative measurement of serum skeletal alkaline phosphatase (skeletal ALP) in patients with metabolic bone disorders.

SUMMARY AND EXPLANATION OF THE TEST
Human alkaline phosphatases (ALP) are a group of similar enzymes coded for by at least four different gene loci that catalyze the hydrolysis of phosphate esters at an alkaline pH. They occur in different tissues and are usually named after the tissue in which they are found. The designations for the ALP isozymes are: • Tissue non-specific (bone, liver, kidney). • Intestinal • Placental • Placental-like. ALP is a membrane-bound enzyme that is released into the serum by a variety of mechanisms in both normal and disease processes. Normally the skeletal and liver ALP isozymes are the predominant forms detected in serum. The skeletal isoenzyme of ALP is a transmembrane glycoprotein found on the cell surface of osteoblasts. Osteoblasts are the cells responsible for the formation of new bone. The function of skeletal ALP is not clearly understood although it may play a role in skeletal mineralization. The membrane-bound tetrameric skeletal ALP is released into the circulation as a dimer by phospholipase cleavage of a membrane-anchoring phosphatidylglycan moiety that is attached to the carboxyl-terminal of the protein. Skeletal ALP shares a common protein structure with liver ALP, the other predominant ALP isoenzyme found in serum. The skeletal and liver isoenzymes, however, show subtle differences in a number of properties including electrophoretic mobility, chemical inactivation, lectin reactivity, thermostability and neuraminidase susceptibility. These differences may be related to the reported tissue-specific post translational differences in the skeletal and liver isoenzymes, although a direct correlation has yet to be established. Methods to differentiate and quantitate the skeletal and liver ALP isoenzymes in serum have been based on exploiting the differential susceptibilities of the isoenzymes to the treatments mentioned above. However, the methodologies employed generally exhibit poor resolution, require sample pretreatment, offer indirect quantitation, are technically cumbersome and are not suitable for routine laboratory use. Consequently, measurement of total serum ALP activity is frequently used for assessing bone. Unfortunately this approach lacks the sensitivity and specificity required for reliable clinical assessment of metabolic bone diseases.

Measurement of serum levels of skeletal ALP has been shown to be useful in evaluating patients with Paget’s disease, osteomalacia, primary hyperparathyroidism, renal osteodystrophy, osteoporosis and skeletal metastases. Paget’s disease is a chronic skeletal disease of unknown origin which is a local proliferation of the normal cellular components of bone. Paget’s disease is more prevalent than once thought with the incidence rate in certain populations at 3-5% in middle-aged patients and 10-15% in the elderly. This disease does not affect young individuals. The majority of patients with Paget’s disease do not have symptoms and often go undiagnosed unless an abnormal X-ray or serum ALP level is found in the course of a medical evaluation for unrelated reasons. The most common complaints in symptomatic patients are pain and deformity.

For the Tandem-R Ostase assay, hybridoma technology was employed to identify and produce two monoclonal antibodies that demonstrate specificity to the skeletal isoenzyme of ALP in serum. These monoclonal antibodies, when used in the dual monoclonal antibody Tandem R format, allow for simple, convenient, reproducible and direct quantitation of serum skeletal ALP.

PRINCIPLE OF THE PROCEDURE
The Tandem-R Ostase assay is a solid phase, two-site immunoradiometric assay. Samples containing skeletal ALP are reacted with a plastic bead (solid phase) coated with a monoclonal antibody directed toward a site on the skeletal ALP molecule and, simultaneously, with a radiolabeled monoclonal antibody directed against a different antigenic site on the same skeletal ALP molecule. Following the formation of the solid phase/skeletal ALP/antibody sandwich, the bead is washed to remove unbound labeled antibody. The radioactivity bound to the solid phase is measured in a gamma counter. The amount of radioactivity measured is directly proportional to the concentration of skeletal ALP present in the test sample, which is determined from a standard curve. The standard curve is based on the concurrent testing of the Tandem-R Ostase Calibrators which contains 0 to 120 µg skeletal ALP/L.
MATERIALS REQUIRED BUT NOT PROVIDED
Plastic test tubes, round bottom, 12 x 75 mm (glass tubes must not be used)
Test tube rack
Gamma counter
Repeating precision pipettes: 100 µL (± 1%)
Repeating pipette: 2 mL
Disposable tip precision pipette: 100 µL (± 1%)
Aspiration device (e.g., Hybriflase™) or decant rack
Parallel™ or equivalent for covering tubes
Distilled water
Forceps
Container for storage of wash solution
Refrigemation unit maintained at 2°C to 8°C

MATERIALS AVAILABLE FROM HYBRITECH
Decant Rack Cat. # 2015
Test tube rack Cat. # 2016
Hybritech Bead Gun™ Cat. # 2036
Hybriflase™ Bead Washing System Cat. # 2038
Hybritech Bead Handling System Cat. # 2058
Gamma counter Cat. # 2011

PREPARATION OF REAGENTS
- Bring all reagents to room temperature (18°C to 25°C) prior to use.
- All Calibrators, Controls, and patient specimens should be tested in duplicate at the same time.
- The assay procedure is as follows:
  1. Label test tubes appropriately (glass tubes must not be used).
  2. Pipette 100 µL of Zero Diluent/Calibrator, Calibrators, Controls and specimens into each tube as labeled.
  3. Pipette 100 µL of tracer antibody into each tube.
  4. Shake the test tube rack by hand for 15 seconds to mix reagents. Do not vortex.
  5. Introduce one bead into each tube after blotting the residual droplet which remains on the bead after removal from the container. Do not permit the beads to dry.
  6. Cover and shake the test tube rack by hand for 15 seconds to mix reagents.

ASSAY PROCEDURE
- Serum specimens and kit components should be mixed well prior to use.
- All Calibrators, Controls, and patient specimens should be tested in duplicate at the same time.
- The assay procedure is as follows:
  1. Label test tubes appropriately (glass tubes must not be used).
  2. Pipette 100 µL of Zero Diluent/Calibrator, Calibrator, Controls and specimens into each tube as labeled.
  3. Pipette 100 µL of tracer antibody into each tube.
  4. Shake the test tube rack by hand for 15 seconds to mix reagents. Do not vortex.
  5. Introduce one bead into each tube after blotting the residual droplet which remains on the bead after removal from the container. Do not permit the beads to dry.
  6. Cover and shake the test tube rack by hand for 15 seconds to mix reagents.

NOTES ON BEAD WASHING: Immunoradiometric assays require efficient washing to avoid cross-contamination.
To prepare the wash solution, add the contents of 1 bottle (18 mL) of the Wash Concentrate to 500 mL of distilled water and mix.

ASSAY PROCEDURE
- Serum specimens and kit components should be mixed well prior to use.
- All Calibrators, Controls, and patient specimens should be tested in duplicate at the same time.
- The assay procedure is as follows:
  1. Label test tubes appropriately (glass tubes must not be used).
  2. Pipette 100 µL of Zero Diluent/Calibrator, Calibrators, Controls and specimens into each tube as labeled.
  3. Pipette 100 µL of tracer antibody into each tube.
  4. Shake the test tube rack by hand for 15 seconds to mix reagents. Do not vortex.
  5. Introduce one bead into each tube after blotting the residual droplet which remains on the bead after removal from the container. Do not permit the beads to dry.
  6. Cover and shake the test tube rack by hand for 15 seconds to mix reagents.

TESTING PROCEDURE:
- Do not vortex.
  7. Incubate overnight (19 hours +/- 2 hrs) in a refrigerator or cold room at 2°C to 8°C.
  8. After incubating, remove the rack to room temperature and wash the beads three times by:
   a. Dispensing 2 mL of wash solution into the tubes. Pipette the solution down the side of each tube with enough force to lift the bead off the bottom of the tube.
   b. Aspirating the liquid from each tube. Alternatively, the wash solution may be removed by using a decant rack and recap the wash solution after each wash.
   c. Aspirating or decanting the tubes one extra time after the final wash.

NOTES:
- Do not let the beads stand in wash solution longer than 15 minutes total time during the three washes (Refer to Procedure Note 2 for details).
- Do not permit the beads to dry between washes.

9. Count each tube in a gamma counter and record the counts per minute.
10. Calculate the results as described in "Calculation of Results".

PROCEDURAL NOTES
1. If a specimen was found to contain greater than 120 pg skeletal ALP/L, the specimen may be diluted with the Zero Diluent/Calibrator (A) and assayed according to the Assay Procedure. The dilution factor must be incorporated into the Calculation of Results. Each specimen should be mixed thoroughly prior to testing. It is desirable to dilute the serum samples that contain more than 120 µg skeletal ALP/L so that the diluted sample reads greater than 5 µg/L on the standard curve.
2. NOTE ON BEAD WASHING - Immunoradiometric assays require efficient washing to remove the unbound radiolabeled antibody. Therefore, it is very important to wash each tube and bead efficiently, removing the last droplets of the wash solution to achieve optimal results. A squeezeable, plastic wash bottle, Conical-type syringe or pipette may be used to wash the walls of the tube and the bead. Dispense the wash solution into each tube with sufficient force to "float" the bead at least 2.5 cm from the bottom of the test tube and up into the wash solution. The wash solution should be removed thoroughly by either an aspirating device or a decant rack. The inverted decant rack should be blotted on absorbent paper after the final wash.
3. For convenience, repeating pipettes may be used for pipetting Tracer antibody and wash solution. Pipettes with disposable tips are recommended for pipetting the Calibrators, Controls and specimens. The pipette tip should be changed after each specimen is pipetted to avoid potential sample carry-over and contamination of the reagents or samples.
4. To ensure consistency of timing of the assay, the size of the assay should be limited to the number of samples that can be pipetted in 20 minutes.
5. Do not use glass test tubes.
6. The temperature at which the overnight incubation is conducted should be controlled to ensure that it does not exceed 8°C.
7. Maximum binding in the Tandem-R Ostase assay is observed when the overnight incubation is performed between 2°C and 4°C.

CALCULATION OF RESULTS
The Tandem-R Ostase assay results may be calculated by using computer-assisted methods or manually on linear graph paper.

Computer-Assisted Method
Computer-assisted data reduction may be used to calculate results for the Tandem-R Ostase Assay. A point-to-point curve fit is recommended. The point-to-point software connects a straight line between the means of Calibrator replicates - including the 0 µg skeletal ALP/L and the Calibrator - and provides good results with the procedures and calibration methods described.
For additional information on computer-assisted data reduction, or the use of software developed for competitive binding assays, consult your Hybritech Sales Representative.

Manual Method
The Tandem-R-Ostase calibration curve may be constructed manually on linear graph paper by plotting the counts per minute (CPM) for each Calibrator replicate on the y-axis versus the concentration of skeletal ALP on the x-axis. Connect a straight line between each point. Do not force the curve to a straight line.
To determine the concentration of skeletal ALP in a patient specimen, extend a horizontal line from the mean CPM value for the test sample. At the point of intersection of the horizontal line and the calibration curve, drop a vertical line to the x-axis and read the concentration in µg of skeletal ALP/L.
If the sample was diluted prior to assay ing, the observed skeletal ALP concentration must be multiplied by the dilution factor.

Example data

<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
<th>CPMs</th>
<th>Mean CPMs</th>
<th>µg skeletal ALP/L</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Zero Calibrator (A)</td>
<td>180</td>
<td>213</td>
<td></td>
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<tr>
<td>2</td>
<td></td>
<td>245</td>
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<td>3</td>
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<td>4</td>
<td>Calibrator (C)</td>
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<tr>
<td>16</td>
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</tbody>
</table>

QUALITY CONTROL
Good laboratory practices include the use of control specimens throughout an assay run to ensure that all reagents and protocols are performing properly. The Tandem-R Ostase assay provides two control materials: the Low Control contains 20 µg skeletal ALP/L and the High Control contains approximately 90 µg skeletal ALP/L. Refer to the insert card provided in the kit for the control range values.

This example is given for information only.

![Example curve](image)

Example curve

This example is given for information only.

![Example curve](image)

Example curve

This example is given for information only.

![Example curve](image)

Example curve

This example is given for information only.
PERFORMANCE CHARACTERISTICS

Within-RUN Precision
Within-run precision was determined by assaying serum pools containing various concentrations of skeletal ALP. Each serum pool was assayed in triplicate. The data are presented below.

Serum Pool
Number of Replicates 1 2 3
Mean μg skeletal ALP / L 13.2 26.7 48.6
Standard Deviation 0.89 1.13 1.79
Coefficient of Variation % 6.7 4.2 3.7

Between-RUN Precision
Between-run precision was determined by duplicate measurements of three serum pools over a series of 20 individually calibrated runs.

Serum Pool
Number of Assays 2 2 2
Mean μg skeletal ALP / L 11.7 40.6 77.4
Standard Deviation 0.95 2.93 5.42
Coefficient of Variation % 8.1 7.2 7.6

Recovery and Dilution
Various quantities of a serum sample containing elevated levels of skeletal ALP were added to human sera containing endogenous skeletal ALP and the samples were assayed in triplicate.

Expected Concentration μg skeletal ALP / L

Endogenous Concentration μg skeletal ALP / L

Observed Concentration μg skeletal ALP / L

% Recovery *

10.8 14.6 28.4 112
52.5 14.6 72.1 107
78.3 14.6 95.7 103

* % recovery equals the observed concentration divided by the expected concentration plus the endogenous concentration times 100.

A serum sample containing an elevated skeletal ALP concentration was diluted with the Zero Diluent/Calibrator (A) and assayed in triplicate at multiple dilutions.

Dilution

Expected Concentration µg skeletal ALP/L

Observed Concentration µg skeletal ALP/L

% Recovery *

neat 77.0
1:1.5 51.3 55.4 108
1:2 38.5 38.6 100
1:3 25.7 28.8 112
1:4 19.3 20.0 104
1:5 15.4 15.3 99

% recovery equals the observed concentration divided by the expected concentration times 100.

Interference by Drugs

Various concentrations of drugs were added to three separate serum pools containing approximately 25, 50 and 75 μg skeletal ALP/L, and assayed in duplicate. The drugs and the highest concentrations tested were:

- acetaminophen, 350 μg/mL
- aspirin, 350 μg/mL
- calcium, 500 μg/mL
- calcitonin-human, 80 μg/mL
- calcitonin-salmon, 60 IU/mL
- estrogen, 100 μg/mL
- etidronate, 350 μg/mL
- ibuprofen, 150 μg/mL
- norethindrone/norethynestradiol mixture (oral contraceptive), 3.0 mg/mL
- vitamin D, 400 IU/mL

These drugs did not interfere with the recovery of skeletal ALP from the serum pools in the Tandem-R Ostase assay.

Minimum Detectable Concentration

The minimum detectable concentration is estimated to be 2.0 μg of skeletal ALP/L. The minimum detectable concentration is defined as the concentration of skeletal ALP that corresponds to the CPM that are two standard deviations greater than the mean CPM of 20 replicate determinations of the Zero Diluent/Calibrator (A) using Tracer Antibody at expiration of the kit.
Pour déterminer la quantité de la phosphatase alcaline d'origine osseuse (PAL osseuse) dans le sérum.

DOMAINE D'APPLICATION
La méthode immunoradiométrique Tandem-R Ostase est un système de dosage in vitro permettant de déterminer la concentration de la PAL osseuse dans le sérum à partir de prélèvements sanguins. Elle est utilisée pour le diagnostic, le suivi et l'évaluation des troubles osseux.

PRINCIPES DE LA MÉTHODE
Le Tandem-R Ostase est une méthode de dosage immunoradioimmunométrique (RIA) qui permet de mesurer la PAL osseuse dans le sérum. Elle est basée sur une technique de dosage immuno-radiométrique sur phase solide (PRIS) et utilisée dans de nombreux laboratoires de biochimie pour le diagnostic et le suivi des troubles osseux.

PRÉCAUTIONS D'EMPLOI
- Prêter attention aux éclaboussures de solution radioactive, à l'azoture de sodium et au détergent approprié. Le matériel contaminé doit être joint aux déchets radioactifs.
- Respecter les consignes de sécurité et les consignes de manipulation du kit.
- S'assurer que les échantillons sont prêts à l'emploi et que les solutions sont correctement préparées.
- Ne pas utiliser le kit pour le dosage de la PAL osseuse dans des échantillons de sang contenant des anticorps spécifiques de la PAL osseuse.

CONSERVATION ET STABILITÉ
- Les réactifs de la trousse Tandem-R Ostase doivent être conservés entre 2° et 8°C, à l'abri de la lumière, et dans un emballage hermétique.
- Les échantillons de PAL osseuse doivent être conservés à une température de -20°C ou à une température inférieure.

OBTENTION DES ECHANTILLONS ET PREPARATION
- Les échantillons de PAL osseuse doivent être prélevés dans les 12 premières heures après le réveil.
- Les échantillons de PAL osseuse doivent être conservés à une température de -20°C ou à une température inférieure.
- Il est recommandé de manipuler les échantillons de PAL osseuse de manière aseptique.

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- Il est recommandé de manipuler les échantillons de PAL osseuse de manière aseptique.
**MATERIAUX REQUIS MAIS NON FOURNIS**
Tubes à essai en polystyrène, fond rond 12 X 75 mm (pas de tubes en verre).
Portoir de tubes à essai.
Pipettes de précision à embout jetable: 100 μL (+/-1%)
Système de lavage des billes (Système de manipulation des billes)
Compteur gamma
Eau distillée
Parafilm* ou équivalent pour couvrir les tubes.

**Pipettes**
Conteneur pour la conservation de la solution de lavage
Parallél ou équivalent pour couvrir les tubes.

---

**MATERIAUX DISPONIBLES CHEZ HYBRITECH**

- Portoir de décantation
- Portoir de tubes
- Hybriwash®: Bead Washing System,
- (Système de lavage des billes)
- Hybrihandling System,
- (Système de manipulation des billes)
- Compteur gamma

---

**PRÉPARATION DES RÉACTIFS**

- Amener tous les réactifs à température ambiante (18° à 25°C) avant utilisation.
- Bien homogénéiser les réactifs avant leur utilisation par agitation modérée ou par rotation.
- Utiliser un nouveau embout de pipette pour chaque échantillon et éliminer afin d'éviter des contaminations croisées.
- Ajouter 1 flacon (18 mL) de la solution de lavage concentrée à 500 mL d'eau distillée et mélanger.

---

**MODE OPERATOIRE**

- Amener tous les échantillons sériques et les réactifs de la trousse à température ambiante et mélanger convenablement avant utilisation.
- Tout les échantillons et les prélèvements de patients doivent être testés en double exemplaire.

**Le mode opératoire est le suivant :**

1. Numéroté les tubes d'essai (en polystyrène) de façon appropriée.
2. Pipeter 100 μL de chaque étalon (A-G), de chaque contrôle (1-2) et de l'échantillon dans chaque tube numéroté à cet effet.
3. Pipeter 100 μL d'anticorps marqué dans chaque tube.
4. Agiter le portoir de tubes à la main pendant 15 secondes pour mélanger les réactifs. Ne pas vortexer.

**Exemple de résultats**

<table>
<thead>
<tr>
<th>Tube Description</th>
<th>CPMs</th>
<th>Mean CPMs</th>
<th>μg PAL osseuse/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etalon zéro (A)</td>
<td>180</td>
<td>213</td>
<td></td>
</tr>
<tr>
<td>Etalon (B)</td>
<td>245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etalon (C)</td>
<td>4255</td>
<td>4242</td>
<td></td>
</tr>
<tr>
<td>Etalon (D)</td>
<td>4229</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etalon (E)</td>
<td>8036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etalon (F)</td>
<td>11275</td>
<td>10971</td>
<td></td>
</tr>
<tr>
<td>Contrôle bas (1)</td>
<td>5237</td>
<td>5523</td>
<td>19,8</td>
</tr>
<tr>
<td>Contrôle haut (2)</td>
<td>24627</td>
<td>24933</td>
<td></td>
</tr>
<tr>
<td>Echantillon patient</td>
<td>23539</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Attention :**

- Ne pas laisser tremper les billes dans la solution de lavage plus de 15 minutes en tout pendant les trois lavages (voir "Remarques à propos du mode opératoire", Point 2).
- Ne pas laisser sécher les billes entre les lavages.
- Ne pas tarder les tubes dans la solution de lavage plus de 15 minutes en tout pendant les trois lavages (voir "Remarques à propos du mode opératoire", Point 2).

**Calcul des résultats**

- Exemple de résultats

**Courbe exemple**

- CPMs vs μg PAL osseuse/L

---

**Remarques à propos du mode opératoire**

1. Si l'échantillon contient plus de 120 μg de PAL osseuse/L, il doit être dilué avec l'étalon zéro (A) pour les échantillons et être analysé selon le mode opératoire. Le facteur de dilution doit être pris en compte dans le calcul des résultats. Chaque échantillon doit être mélangé convenablement avant analyse. Il est recommandé de diviser les échantillons équivalents contenant plus de 120 μg de PAL osseuse/L de façon à ce que les valeurs des échantillons dilués soient supérieures à 5 μg/L sur la courbe d'étalonnage.

2. **Remarque concernant le lavage des billes :** Les analyses immunoradiométriques exigent un lavage efficace afin d'éliminer les anticorps marqués non liés. De ce fait, il est très important d'effectuer des lavages efficaces des billes et des tubes et d'éliminer les dernières gouttes de solution de lavage afin d'obtenir les meilleurs résultats. Un facteur de lavage en plastique comprimable, un seringue (type Cornwall) ou une pipette à répétition peut être utilisé pour taver les parois des tubes ainsi que les billes. Ajouter la solution de lavage dans chaque tube avec une pression suffisante pour permettre de faire "fondre" la billes dans la solution à une distance d'eau à moins de 2,5 cm du fond du tube d'analyse. La solution de lavage doit être complètement éliminée, soit en utilisant un moyen d'aspiration, soit par décantation à l'aide d'un portoir de décantation. Ce dernier, retourné, doit être épongé sur du papier absorbant après le lavage final.

**Exemple de résultats**

<table>
<thead>
<tr>
<th>Tube Description</th>
<th>CPMs</th>
<th>Mean CPMs</th>
<th>μg PAL osseuse/L</th>
</tr>
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<tbody>
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<td>180</td>
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</tr>
<tr>
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<td>24627</td>
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<td></td>
</tr>
<tr>
<td>Echantillon patient</td>
<td>23539</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**CET exemple type est donné à titre informatif.**
CONTRÔLE DE QUALITE
Les bonnes pratiques de laboratoire recommandent l'utilisation d'échantillons de contrôle tout au long de l'analyse afin de s'assurer du bon déroulement du protocole. Deux contrôles sont fournis avec la trousse Tandem-R Ostase: le contrôle bas contient environ 20 pg de PAL osseuse/L et le contrôle haut contient environ 90 pg de PAL osseuse/L.

LIMITES
La reproductibilité de la PAL hépatique avec la méthode de dosage Tandem-R Ostase est approximativement de 15% de la réactivité de la PAL osseuse. Des échantillons sériques contenant des concentrations élevées en PAL osseuse peuvent donner des résultats faussés élevés avec la méthode de dosage Tandem-R Ostase.

VALEURS ATTENDUS
Le dosage Tandem-R Ostase a été évalué au cours de deux études de populations d'individus apparemment sains (âgés de 20 à 79 ans). La moyenne des concentrations de PAL osseuse et leur distribution sont présentées dans le tableau et les figures suivants :

<table>
<thead>
<tr>
<th>Sexe</th>
<th>n</th>
<th>Concentration moyenne (µg PAL/L)</th>
<th>Déviation standard</th>
<th>95è Percentille</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masculin</td>
<td>217</td>
<td>12,3</td>
<td>4,3</td>
<td>20,1</td>
</tr>
<tr>
<td>Féminin</td>
<td>261</td>
<td>11,5</td>
<td>4,3</td>
<td>21,3</td>
</tr>
<tr>
<td>Total</td>
<td>478</td>
<td>11,8</td>
<td>4,3</td>
<td>20,5</td>
</tr>
</tbody>
</table>

Distribution normale de la PAL osseuse

Les résultats des observations mentionnés ci-dessus ne sont représentatives que des études rapportées et ne reflètent pas nécessairement celles qui seront observées dans chaque laboratoire clinique. Chaque laboratoire devra établir ses propres valeurs usuelles.

CARACTERISTIQUES DU DOSAGE

Reproductibilité intra-série
La reproductibilité intra-série a été évaluée en mesurant des sérum contenant différentes concentrations de PAL osseuse. Chaque échantillon a été mesuré 21 fois dans une série. Les résultats obtenus sont présentés ci-dessous :

<table>
<thead>
<tr>
<th>Echantillon sérique</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nombre d'échantillons</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Concentration moyenne (µg PAL osseuse/L)</td>
<td>13,2</td>
<td>26,7</td>
<td>48,6</td>
</tr>
<tr>
<td>Déviation standard</td>
<td>0,89</td>
<td>1,13</td>
<td>1,79</td>
</tr>
<tr>
<td>Coefficient de variation %</td>
<td>6,7</td>
<td>4,2</td>
<td>3,7</td>
</tr>
</tbody>
</table>

Reproductibilité inter-séries
La reproductibilité inter-séries a été évaluée en mesurant 3 sérum de PAL osseuse différents dans 20 séries de dosages effectués individuellement.

<table>
<thead>
<tr>
<th>Echantillon sérique</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nombre d'échantillons</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Concentration moyenne (µg PAL osseuse/L)</td>
<td>11,7</td>
<td>40,6</td>
<td>77,4</td>
</tr>
<tr>
<td>Déviation standard</td>
<td>0,96</td>
<td>2,93</td>
<td>5,42</td>
</tr>
<tr>
<td>Coefficient de variation %</td>
<td>8,1</td>
<td>7,2</td>
<td>7,0</td>
</tr>
</tbody>
</table>

RECUPERATION ET DILUTION

Des concentrations d'échantillon sérique contenant de la PAL osseuse a été additionnées à des sérum humains contenant de la PAL osseuse endogène et les échantillons ont ensuite été mesurés en triple.

<table>
<thead>
<tr>
<th>Concentration attendue en µg PAL osseuse/L</th>
<th>Concentration endogène en µg PAL osseuse/L</th>
<th>Concentration mesurée en µg PAL osseuse/L</th>
<th>% de récupération *</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,8</td>
<td>14,6</td>
<td>29,4</td>
<td>112</td>
</tr>
<tr>
<td>52,5</td>
<td>14,6</td>
<td>72,1</td>
<td>107</td>
</tr>
<tr>
<td>78,5</td>
<td>14,6</td>
<td>95,7</td>
<td>103</td>
</tr>
</tbody>
</table>

* % de récupération est égal à la concentration mesurée divisée par la somme des concentrations attendues et endogène, multipliée par 100.

Un échantillon sérique ayant une concentration élevée de PAL osseuse a été dilué en série avec l'étalon Ostase 0 pg/L et mesuré en triple aux différentes dilutions.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Concentration attendue en µg PAL osseuse/L</th>
<th>Concentration mesurée en µg PAL osseuse/L</th>
<th>% de récupération *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1,5</td>
<td>51,3</td>
<td>55,4</td>
<td>108</td>
</tr>
<tr>
<td>1:2</td>
<td>38,5</td>
<td>38,6</td>
<td>100</td>
</tr>
<tr>
<td>1:3</td>
<td>23,7</td>
<td>28,8</td>
<td>112</td>
</tr>
<tr>
<td>1:4</td>
<td>19,3</td>
<td>20,0</td>
<td>104</td>
</tr>
<tr>
<td>1:5</td>
<td>15,4</td>
<td>15,3</td>
<td>99</td>
</tr>
</tbody>
</table>

* % de récupération est égal à la concentration mesurée divisée par la concentration attendue et multipliée par 100.

INTERFERENCES

L'hémoglobine et la bilirubine ont été testées à des concentrations atteignant respectivement 500 mg/dL et 25 mg/dL. Elles n'interfèrent pas dans le test Tandem-R Ostase.

Les résultats obtenus sont les suivants :

- 100 U/L de PAL intestinale donne un résultat de 2,2 µg/L avec le test Tandem-R Ostase.
- 100 U/L d'activité de PAL osseuse donne approximativement 38,4 +/- 8,1 µg/L avec le dosage Tandem-R Ostase.
- 100 U/L d'activité de PAL hépatique donne approximativement 7,7 +/- 3,0 µg/L avec le dosage Tandem-R Ostase.

INTERFERENCES PHARMACOLOGIQUES

Diverses concentrations de substances médicamenteuses ont été additionnées à des échantillons sériques contenant 25, 50 et 75 µg de PAL osseuse/L. Les échantillons ont ensuite été mesurés en double. Les produits ajoutés et leurs valeurs de concentration sont les suivants :

- acétaminophène, 350 µg/mL;
- aspirine, 350 µg/mL;
- calcium, 500 µg/mL;
- calcitonine (hormone), 80 µg/mL;
- calcitonine (saumon), 60 UI/mL;
- oestrogène, 100 µg/mL;
- eldrolactone, 350 µg/mL;
- ibuprofène, 150 µg/mL;
- norethidrone / ethinylestradiol (contraception orale), 3,0 mg/mL;
- vitamine D, 400 UI/mL.

Ces substances médicamenteuses n'ont eu d'influence sur la récupération de la PAL osseuse dans les échantillons sériques, dans le dosage Tandem-R Ostase.

Concentration minimale détectable

La concentration minimale détectable de PAL osseuse est estimée à 2,0 µg/L. La concentration minimale détectable est définie comme la concentration de PAL osseuse correspondant à l'activité endogène de PAL et dosée par la méthode Tandem-R Ostase.

Les protéines totales ont été testées à des concentrations atteignant environ 500 mg/dL et 25 mg/dL. Elles n'interfèrent pas dans le test Tandem-R Ostase.

Les résultats obtenus sont les suivants :

- 100 U/L d'activité de PAL osseuse donne approximativement 38,4 +/- 8,1 µg/L avec le dosage Tandem-R Ostase.
- 100 U/L d'activité de PAL hépatique donne approximativement 7,7 +/- 3,0 µg/L avec le dosage Tandem-R Ostase.

Remarques: Tous les articles ne sont pas disponibles dans chaque pays; vous pouvez vous adresser à votre fournisseur local pour obtenir des informations complémentaires.

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Tél: 32 - 41 - 87 70 00

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ZUSAMMENFASSUNG UND TESTPRINZIP


RESULTATSBERECHNUNG


HINWEIS: Die Kugeln dürfen während der drei Waschzyklen nicht länger als 5 Minuten in der Waschlösung inkubiert. Die Temperatur darf 8 °C NICHT überschreiten.

Die Testdurchführung ist wie folgt:

1. Teströhrchen angemessen beschreiben (Keine Glasröhrchen benutzen)
2. 100 µL Standards (A-H), Kontrollen (1-2) und Proben gemäß Beschriftung in die Teströhrchen pipettieren.
3. 100 µL Tracer-Antikörper in jedes Teströhrchen pipettieren.
4. Im Teströhrchenfänger von Hand 15 Sekunden schütten, um die Reagenzien zu mischen. Die Lösung muss die richtige Tracer-Konzentration aufweisen, die sich an die Kugel befestigt, aufgesättigt werden, wo die Tracer-Antikörper jedoch nicht völlig trocken werden darf.
5. Teströhrchenfänger von Hand 15 Sekunden schütten, um die Reagenzien zu mischen. Die Lösung muss die richtige Tracer-Konzentration aufweisen, die sich an die Kugel befestigt, aufgesättigt werden, wo die Tracer-Antikörper jedoch nicht völlig trocken werden darf.
6. Teströhrchenfänger von Hand 15 Sekunden schütten, um die Reagenzien zu mischen. Die Lösung muss die richtige Tracer-Konzentration aufweisen, die sich an die Kugel befestigt, aufgesättigt werden, wo die Tracer-Antikörper jedoch nicht völlig trocken werden darf.
10. Die Resultate werden wie unter "RESULTATSBERECHNUNG" beschrieben ermittelt.


RESULTATSBERECHNUNG


Manuelle Auswertung


RESULTATSBERECHNUNG


Manuelle Auswertung

Die Reaktivität der Leber-AP im Serum wurde analytisch für den Tandem-R Ostase-Test mit

Acetaminophen, 350 pg/mL;
Aspirin*, 350 pg/mL;
Calcium (Salm), 60 lU/mL;
Calcitonin (human), 80 pg/mL;
Calcitonin (Salm), 60 lU/mL;
Etidronat, 250 pg/mL; Ibuprofen, 150 pg/mL;
Norethindron/Ethinylestradiol-Mischung (orales Kontrazeptivum), 3.0 mg/mL;
Calcitonin (Salm), 60 lU/mL;
Calcitonin (Salm), 60 lU/mL;
Calcium (Salm), 60 lU/mL;
Östrogen, 150 pg/mL;
Etidronat, 250 pg/mL;
Ibuprofen, 150 pg/mL;
Norethindron/Ethinylestradiol-Mischung (orales Kontrazeptivum), 3.0 mg/mL;
Vitamin D, 400 lU/mL;
Diese Chemotherapeutika hatten keinen Einfluss auf die Wiederfindung von alkalischer Skelett-Phosphatase in den Serumproben.

Nachweisgrenze

** Der Kauf dieses Kits berechtigt zur Verwendung unter U.S. Patent Nr. 4 375 110, 4 486 530

** Tandem ®, Ostase™, Bead Gun™ und Hybriwash® sind ein Warenzeichen von Hybritech Incorporated.

Für weitere Informationen setzen Sie sich bitte mit Hybritech in Verbindung.
Tel.: 32 41 67 79 00

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NovoCalcin Kit
96 assays for Osteocalcin

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Store at 2° - 8° C

Metra Biosystems, Inc.
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Phone: 415-813-6132 Fax: 415-494-7148
1-800-524-6318 (U.S. only)

Made in U.S.A.
For Research Use Only. Not For Use In Diagnostic Procedures.

Read the entire product insert thoroughly before beginning the assay. The NovoCalcin kit should be stored at 2 to 8°C until use.

Intended Use
The NovoCalcin immunoassay measures "de novo", newly synthesized osteocalcin in serum or tissue culture medium (serum free) to quantify bone turnover.

Background on Osteocalcin
Osteocalcin or BGP (bone gla protein) is found exclusively in bone tissue. It is a 5800 molecular weight extra-hepatic vitamin K dependent protein produced by osteoblasts. It contains three gamma-carboxyglutamic acid residues which are thought to be involved in calcium ion and hydroxyapatite binding. It accounts for 10 - 20% of the non-collagenous protein in bone. While the in vivo function of osteocalcin is unknown, its affinity for bone mineral constituents implies a role in bone formation.

The NovoCalcin Assay
The NovoCalcin assay is a competitive, enzyme-linked immunosorbent assay (ELISA). The assay uses Osteocalcin Coated Strip Wells, a Monoclonal anti-Osteocalcin Antibody, and an anti-Mouse Alkaline Phosphatase (AP) Conjugate to quantify osteocalcin in serum or tissue culture medium (serum free).

The osteocalcin in the sample competes with the osteocalcin on the strip well for the Monoclonal Antibody. A second antibody-enzyme conjugate (anti-Mouse IgG AP Conjugate) is then added to the strip well to bind to the anti-Osteocalcin Monoclonal Antibody. A substrate, p-Nitrophenyl Phosphate (pNPP), is added to produce a yellow color which is then read at 405 nm.

Materials Provided
Sufficient materials are provided to analyze 40 samples in duplicate, plus standards and controls.
### Kit Contents

<table>
<thead>
<tr>
<th>Item</th>
<th>Qty/Vol.</th>
<th>Part #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Tablets, 20 mg each</td>
<td>3 x 20 mg</td>
<td>0012</td>
</tr>
<tr>
<td>(p-Nitrophenyl Phosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 N NaOH</td>
<td>1 x 10 mL</td>
<td>4029</td>
</tr>
<tr>
<td>Anti-Osteocalcin</td>
<td>1 x 15 mL</td>
<td>4089</td>
</tr>
<tr>
<td>(Monoclonal anti-Osteocalcin Antibody)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NovoCalcin Standard A</td>
<td>1 each</td>
<td>4168</td>
</tr>
<tr>
<td>NovoCalcin Standard B</td>
<td>1 each</td>
<td>4169</td>
</tr>
<tr>
<td>NovoCalcin Standard C</td>
<td>1 each</td>
<td>4170</td>
</tr>
<tr>
<td>NovoCalcin Standard D</td>
<td>1 each</td>
<td>4171</td>
</tr>
<tr>
<td>NovoCalcin Standard E</td>
<td>1 each</td>
<td>4172</td>
</tr>
<tr>
<td>NovoCalcin Standard F</td>
<td>1 each</td>
<td>4173</td>
</tr>
<tr>
<td>NovoCalcin Control, Low</td>
<td>1 each</td>
<td>4174</td>
</tr>
<tr>
<td>NovoCalcin Control, High</td>
<td>1 each</td>
<td>4175</td>
</tr>
<tr>
<td>Tris Wash Buffer</td>
<td>2 x 30 mL</td>
<td>4176</td>
</tr>
<tr>
<td>Concentrate (10X)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate Buffer</td>
<td>3 x 10 mL</td>
<td>4177</td>
</tr>
<tr>
<td>Osteocalcin Coated strip</td>
<td>6 each</td>
<td>4178</td>
</tr>
<tr>
<td>Frame, Stripwell</td>
<td>1 each</td>
<td>4179</td>
</tr>
<tr>
<td>Enzyme Conjugate (lyophilized),</td>
<td>3 each</td>
<td>4180</td>
</tr>
<tr>
<td>Goat anti-Mouse IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Materials Required BUT NOT Provided

- Micropipettes
- Plate Reader
- Deionized Water
- 8 or 12 channel Micropipette
- Container for wash buffer dilution
- Software capable of calculating 4-parameter curve fit

### Specimen Collection and Storage

Osteocalcin in serum has been reported to be sensitive to proteolysis. It is recommended that blood be kept at 2-8°C immediately after collection and during processing. Serum should be aliquoted and frozen at ≤20°C within 4 hours of collection. If collection and processing is performed at ambient temperature, serum must be processed and tested or frozen (≤20°C) within 2 hours of collection. Sample collection tubes containing EDTA must not be used as the EDTA chelates Ca^{2+} and the Monoclonal anti-Osteocalcin Antibody is Ca^{2+} dependent.
NOVOCALCIN ASSAY PROTOCOL
WE STRONGLY RECOMMEND THAT ALL RE-
AGENTS BE BROUGHT TO ROOM TEMPERATURE
BEFORE BEGINNING THE ASSAY. (2 HOURS TO
OVERNIGHT RECOMMENDED.)

Determine amount of each reagent required for the
number of strips desired.

<table>
<thead>
<tr>
<th>vials of:</th>
<th># of Strips</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Conjugate</td>
<td>2 3 4 6</td>
</tr>
<tr>
<td>Substrate</td>
<td></td>
</tr>
<tr>
<td>mL of 1x Wash Buffer</td>
<td>100 150 200 300</td>
</tr>
</tbody>
</table>

Reagent Preparation
1. Prepare required amount of 1X Wash Buffer
   (see table) by diluting 10X Tris Wash Buffer
   Concentrate 1 to 10 with deionized (DI) water.

2. Reconstitute each required vial of Enzyme
   Conjugate (see table) with 10 mL of 1X Wash
   Buffer. Allow the pellet to completely dissolve.

3. Reconstitute Standards and Controls with 0.5mL
   of 1x Wash Buffer. Allow the pellet to completely
dissolve.

4. Place desired number of Osteocalcin Strips in the
   Stripwell Frame.

Sample/anti-Osteocalcin Incubation
1. Using a micropipettor, add 25 μL of sample,
   NovoCalcin Standards or Controls to each well of
   the Osteocalcin Coated strips. (Duplicates
   recommended.) Freeze unused portions of
   Standards and Controls at ≤-20°C. Do not
   freeze/thaw more than 4 times.

2. Add 125 μL of anti-Osteocalcin to each well and
   incubate for 2 hours (± 10 minutes) at room
   temperature (18 - 28°C).
Enzyme Conjugate Incubation
1. Manually invert/empty strips. Add 300μL of 1X Wash Buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash.

2. Add 150 μL of the reconstituted Enzyme Conjugate to each well.

3. Incubate for 60 minutes (± 5 minutes) at room temperature (18 - 28°C).

4. Prepare Working Substrate Solution by putting 1 Substrate Tablet into each required bottle of Substrate Buffer (see table). Allow 30 - 60 minutes for the tablet to dissolve, invert to completely mix.

Substrate Incubation
1. Manually invert/empty strips. Add 300μL of 1X Wash Buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash.

2. Add 150 μL of Working Substrate Solution to each well.

3. Incubate for 30 minutes (± 5 minutes) at room temperature (18 - 28°C).

Stop/Read
1. Add 50 μL of 3 N NaOH to each well to stop the reaction.

2. Read the Optical Density (O.D.) at 405 nm. Assure that no large bubbles are present in wells and that the bottom of the strips are clean. Strips should be read within 30 minutes of 3 N NaOH addition.

3. Quantitation software with a 4-parameter curve fitting equation must be used to analyze the NovoCalcin assay.
Representative Standard Curve
Standard Osteocalcin levels: 0, 2, 4, 8, 16, 32 ng/mL

NOVOCALCIN STANDARD CURVE

NovoCalcin Assay Performance Characteristics
The Monoclonal anti-Osteocalcin Antibody has been raised against bovine osteocalcin. Because of the high homology of bovine osteocalcin with human osteocalcin, there is 100% cross-reactivity in this assay between these two species. This antibody is believed to be a "conformationally dependent" antibody and thus will recognize only intact "de novo" osteocalcin and not fragments from resorbed bone tissue. The strips are coated with human osteocalcin, and the standards are human osteocalcin prepared from bone.

Performance characteristics of the assay are:

- Within run C.V. $\leq 10\%$
- Between run C.V. $\leq 15\%$

Sensitivity
The NovoCalcin Assay has a minimum detection limit of 2.0 ng/mL.
Special Instructions

1. It is recommended that blood be kept at 2-8°C immediately after collection and during processing. Serum should be aliquoted and frozen at ≤20°C within 4 hours of collection. If collection and processing is performed at ambient temperature, serum must be processed and tested or frozen (≤-20°C) within 2 hours of collection.

2. Each determination is preferably performed in duplicate.

3. The Certificate of Analysis included in this kit is to be used to verify that the results obtained are similar to those obtained at Metra Biosystems, Inc. The data provided are lot-specific. The O.D. values for the standard points for osteocalcin are provided and are to be used as a guideline only. The results obtained may differ from those obtained at Metra Biosystems, Inc. depending on your laboratory conditions.

An acceptable range is provided for the control values. The control values are provided to verify the validity of the curve and sample results. If the control values are NOT within the acceptable range, the assay results should be considered questionable and if possible, the samples should be repeated.

4. If the O.D. of the NovoCalcin Standard “A” is less than 0.8 or greater than 2.0, the results should be considered questionable and if possible, the samples should be repeated.

Limitations

1. Sample collection tubes containing EDTA must not be used as the EDTA chelates Ca+^2 and the Monoclonal anti-Osteocalcin Antibody is Ca+^2 dependent.

2. A standard curve must be performed with each assay.

3. All reagents supplied should be used as an integral unit prior to the expiration date indicated on the package label.
Limitations...

4. Assay reagents should be stored as indicated.

5. For human use only. Osteocalcin values from other species have not been established with the NovoCalcin Assay.

6. Do not use Osteocalcin coated strip if foil bag is punctured.

7. This assay is validated for manual washing only.

Warnings and Precautions

1. All Serum samples should be treated as potentially biohazardous material, unless known to be free of infectious disease agents.

2. Sodium azide is used as a preservative. It may be fatal if swallowed or absorbed through the skin. Do not mix with acids. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with large volume of water to prevent azide build-up.

3. 3 N NaOH is poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing. If contact is made, wash with water. If ingested, call a physician.

4. Test kits and components should be disposed of in a manner consistent with relevant regulations.
References


Notes
Gamma-B 1,25-Dihydroxy Vitamin D

Radioimmunoassay for the quantitative determination of 1,25-dihydroxy vitamin D in human serum or plasma

For Research Use Only

CodeAA-54F1 - 20 Patient Samples
Shelf Life and Storage of Reagents

This kit is stable until the stated expiry date if stored as specified. Upon receipt, store all reagents at 2-8°C.

Reconstituted calibrators and controls are stable at -20°C until the expiry of the kit.

Materials Provided with the Kit

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Code</th>
<th>AA-54F1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrators (A-F)</td>
<td>AA-5401</td>
<td>1 set of 6</td>
</tr>
<tr>
<td>Primary Antibody</td>
<td>AA-5402</td>
<td>1</td>
</tr>
<tr>
<td>125I-1,25D</td>
<td>AA-5403</td>
<td>1</td>
</tr>
<tr>
<td>Sac-CeH</td>
<td>AA-5404</td>
<td>1</td>
</tr>
<tr>
<td>Controls</td>
<td>AA-5405</td>
<td>1 set of 2</td>
</tr>
<tr>
<td>Delipidation Reagent</td>
<td>AA-5407</td>
<td>1</td>
</tr>
<tr>
<td>Immunocapsules</td>
<td>AA-5406</td>
<td>40</td>
</tr>
<tr>
<td>Elution Reagent</td>
<td>AA-5408</td>
<td>1</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>AA-5409</td>
<td>1</td>
</tr>
<tr>
<td>Wash Concentrate</td>
<td>AC-WASH</td>
<td>1</td>
</tr>
</tbody>
</table>

Reagents

1. Delipidation Reagent:
   A solution of dextran sulphate and magnesium chloride, 1.2mL per bottle. Store at 2-8°C.

2. Immunocapsules:
   40 capsules containing monoclonal antibody to 1,25D linked to solid phase particles in suspension with vitamin D binding protein inhibitor. Store at 2-8°C.

3. Elution Reagent:
   Proprietary formulation for elution of 1,25D from immunocapsules, 20mL per bottle. Store at 2-8°C.

4. Calibrators:
   6 bottles each containing lyophilised 1,25D in 1 mL BSA-phosphate buffer with 0.09% sodium azide. The exact value of each calibrator is printed on the bottle label. Store at 2-8°C.

5. 125I-1,25D-Dihydroxy Vitamin D:
   125I-1,25D in BSA-phosphate buffer with 0.09% sodium azide and proprietary stabilisers. Radioactive content <111 kBq (3pCi) per bottle, 15mL per bottle. Store at 2-8°C.

6. Primary Antibody:
   Sheep anti-1,25D in BSA-phosphate buffer containing 0.09% sodium azide, 15mL per bottle. Store at 2-8°C.

7. Sac-CeH:
   Anti-sheep IgG coupled to cellulose suspended in buffer containing 0.09% sodium azide, 10mL per bottle. Store at 2-8°C.

8. Assay Buffer:
   BSA-phosphate buffer containing 0.09% sodium azide, 10mL per bottle. Store at 2-8°C.

9. Wash Concentrate:
   Phosphate buffered saline containing Tween, 25mL per bottle. Store at 2-8°C.

10. Controls:
    2 Bottles each containing 1.2 mL of lyophilised human serum with 0.09% sodium azide. Store at 2-8°C.

Materials Required but not Provided

1. Disposable 12 x 75 mm borosilicate glass tubes
2. Disposable 12 x 75 mm polystyrene tubes (optional)
3. Precision pipetting devices to deliver 50μL, 100μL, 150μL, 200μL, 300μL, 500μL and 4mL
4. Vortex mixer
5. End-over-end or roller mixer
6. Heating block or water bath at 30°C
7. Nitrogen supply and manifold
8. Centrifuge capable of attaining 2000g.
9. Gamma counter capable of counting 125I.

Preparation of Reagents

Calibrators are supplied in lyophilised form. Reconstitute immediately before use. Add 1mL distilled or deionised water to each bottle. Replace stopper and leave to reconstitute, inverting several times to ensure complete reconstitution.

Controls are supplied in lyophilised form. Reconstitute immediately before use. Add 1.2mL distilled or deionised water to each bottle. Replace stopper and leave 15 - 20 minutes to reconstitute, inverting several times to ensure complete reconstitution.

If calibrators or controls are to be used more than once, they must be frozen (-20°C) within 1 hour of reconstitution. When re-using frozen calibrators or controls, thaw at room temperature, mix well and use within 1 hour.

Wash solution: prepare by diluting the contents of each bottle of Wash Concentrate with 475 mL distilled water.

All other reagents are supplied ready for use. Reagents should be mixed by repeated inversion prior to use in the assay.

Sample Preparation

1. Label glass or plastic tubes, one per sample.
2. Add 500μL of sample to appropriate tubes.
3. Add 50μL of Delipidation Reagent to all tubes. Vortex all tubes.
4. Centrifuge at 2 000 g for 30 minutes.

Note: Take care not to disturb the pellet when handling delipidated samples. If pellet becomes suspended or if sample is not clear, then repeat centrifugation.

Alternative Sample Preparation:
Suitable for samples where volume available is less than 500μL.

1. Label conical-bottom plastic tubes or microcentrifuge tubes, one per sample.
2. Add sample (e.g. 250μL) to appropriate tubes.
3. Add 0.1 X sample volume of Delipidation Reagent (e.g. 25μL) to appropriate tubes. Vortex all tubes.
4. Centrifuge at 2 000 g for 30 minutes, or at 10 000 g for 10 min. (microcentrifuge).

Immunoextraction Procedure

1. Label immunocapsules, two for each sample.
2. Vortex immunocapsules and allow solid phase to settle. Stand capsules upright in foam rack for 3-5 minutes.
3. Remove top screw caps from immunocapsules. Add 100μL of delipidated sample to immunocapsules in duplicate. Replace caps securely.
4. Place immunocapsules in foam rack and rotate end-over-end at 5-20 revolutions per minute for 3 hours at room temperature (18-25°C). Foam racks can be easily attached to a blood tube rotator by means of cut-out slot. Alternatively, foam rack may be wedged inside a suitable plastic beaker and rotated on a bottle roller.
5. Stand capsules upright in foam rack for 3-5 minutes to allow gel to settle. Tap to dislodge any gel adhering to the screw caps. Allow gel to settle for a further 1-2 minutes. Remove screw cap and bottom cap from capsules and place each capsule in a plastic (or glass) tube. Centrifuge at low speed (500-750g) for approximately 1 minute to remove sample.
6. Add 500μL of diluted wash solution to each capsule. Add carefully to avoid solid phase splashing out of the capsule. Centrifuge at low speed (500-750g) for approximately 1 minute to wash immunoextraction gel.
7. Repeat the above wash step.
8. Label glass assay tubes, one for each immunocapsule, and transfer capsules to the glass tubes.

9. Add 150μL of Elution Reagent to all capsules. Allow reagent to soak into solid phase for 1 to 2 minutes. Centrifuge at low speed (500-750g) for approximately 1 minute to collect eluate.

10. Repeat above step a further two times. The total elution volume collected is therefore 450μL for each sample.

11. Discard immunocapsules and place tubes in a heating block or water bath set to 30°C. Evaporate the eluates under a gentle flow of nitrogen. Evaporation should take 20 - 30 minutes and leave a white residue of buffer salts inside the tubes.

12. Add 100μL of Assay Buffer to each tube and vortex to dissolve residues. The immunopurified samples are now ready for assay.

Assay Procedure

Allow all assay reagents to come to room temperature and mix gently before use in the assay.

Prepare labelled glass tubes in duplicate for calibrators and non-specific binding (NSB) tubes.

1. Add 100μL of each calibrator to appropriately labelled tubes. Add 300μL of Assay Buffer to NSB tubes.

2. Assemble sample extract tubes from step 12 above.

3. Add 200μL Primary Antibody to all tubes except NSB tubes.

4. Vortex gently without foaming and incubate overnight (16-24hrs) at 2-8°C.

5. Add 200μL 125I-labeled 1,25-Dihydroxy Vitamin D to all tubes including two additional tubes to be set aside as total counts (TC). Vortex gently without foaming and incubate for 2 hours at room temperature (18-25°C).

6. Add 100μL Sac-Cel (mix well to resuspend immediately before use) to all tubes except TC tubes. Vortex gently without foaming and incubate at room temperature (18-25°C) for 30 minutes.

7. Add 4mL diluted wash solution to all tubes except TC tubes and centrifuge at 2000g for 20 minutes.

8. Decant supernatants and allow inverted tubes to drain on a pad of absorbent tissue. Blot rims to remove remaining drops of liquid.

9. Count all tubes in a suitable gamma counter for at least 1 minute.

Calculation of Results

Calculate the percent binding (B/Bo%) of each calibrator, control and unknown as follows:

\[ B/Bo\% = \frac{\text{mean counts} - \text{mean NSB counts}}{\text{mean counts for '0' - mean NSB}} \times 100 \]

Prepare a standard curve on semi-log paper by plotting B/Bo% on the ordinate against concentration of 1,25D on the abscissa. Calculate B/Bo% for each unknown and read values off curve in pmol/L

Conversion of Units

For conversion of 1,25D results,

\[ \text{pmol/L} \times 0.42 \rightarrow \text{pg/mL} \]

\[ \text{pg/mL} \times 2.4 \rightarrow \text{pmol/L} \]

Sample Assay Data

This information is for reference only and should not be used for the calculation of any patient result. The exact value for each calibrator is printed on the bottle label.

<table>
<thead>
<tr>
<th>Tube</th>
<th>cpm</th>
<th>Mean cpm</th>
<th>B/Bo%</th>
<th>Result pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>40381</td>
<td>40217</td>
<td>40399</td>
<td></td>
</tr>
<tr>
<td>3, 4</td>
<td>793</td>
<td>727</td>
<td>760</td>
<td></td>
</tr>
<tr>
<td>5, 6</td>
<td>10114</td>
<td>9989</td>
<td>9292</td>
<td>100%</td>
</tr>
<tr>
<td>7, 8</td>
<td>9349</td>
<td>9479</td>
<td>8655</td>
<td>93.1%</td>
</tr>
<tr>
<td>9, 10</td>
<td>8579</td>
<td>8539</td>
<td>7799</td>
<td>83.9%</td>
</tr>
<tr>
<td>11, 12</td>
<td>6532</td>
<td>6541</td>
<td>5777</td>
<td>62.2%</td>
</tr>
<tr>
<td>13, 14</td>
<td>4805</td>
<td>4744</td>
<td>4015</td>
<td>43.1%</td>
</tr>
<tr>
<td>15, 16</td>
<td>3412</td>
<td>3562</td>
<td>2726</td>
<td>29.3%</td>
</tr>
<tr>
<td>17, 18</td>
<td>6085</td>
<td>6227</td>
<td>5396</td>
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<tr>
<td>19, 20</td>
<td>4768</td>
<td>4779</td>
<td>4023</td>
<td>43.3%</td>
</tr>
</tbody>
</table>

Typical Calibration Curve

Sensitivity

The sensitivity, defined as the concentration corresponding to the mean minus 2 standard deviations of 10 replicates of the zero calibrator, is 5 pmol/L (2.1 pg/mL).

Precision

<table>
<thead>
<tr>
<th>Intra-assay variation (n=10)</th>
<th>Inter-assay variation (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol/L</td>
<td>CV</td>
</tr>
<tr>
<td>31.0</td>
<td>8%</td>
</tr>
<tr>
<td>118</td>
<td>5%</td>
</tr>
<tr>
<td>195</td>
<td>6%</td>
</tr>
<tr>
<td>27.3</td>
<td>10%</td>
</tr>
</tbody>
</table>

Specificity

The specificity of the Gamma-B 1,25D kit has been assessed with the following analytes at 50% displacement of tracer.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-Dihydroxyvitamin D3</td>
<td>100.0%</td>
</tr>
<tr>
<td>1,25-Dihydroxyvitamin D2</td>
<td>80.0%</td>
</tr>
<tr>
<td>24,25-Dihydroxyvitamin D3</td>
<td>&lt;0.0015%</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D3</td>
<td>0.0013%</td>
</tr>
</tbody>
</table>
### Recovery

Samples spiked with 38.6 pmol/L 1,25D<sub>3</sub>, immunoextracted, and assayed in duplicate.

<table>
<thead>
<tr>
<th>Serum conc. (pmol/L)</th>
<th>Spiked sample (pmol/L)</th>
<th>Measured spike (pmol/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.6</td>
<td>47.4</td>
<td>37.8</td>
</tr>
<tr>
<td>2</td>
<td>14.8</td>
<td>55.8</td>
<td>41.1</td>
</tr>
<tr>
<td>3</td>
<td>84.4</td>
<td>128.4</td>
<td>44.0</td>
</tr>
<tr>
<td>4</td>
<td>2.6</td>
<td>35.5</td>
<td>32.9</td>
</tr>
<tr>
<td>5</td>
<td>8.6</td>
<td>40.8</td>
<td>32.2</td>
</tr>
<tr>
<td>6</td>
<td>59.5</td>
<td>103.1</td>
<td>43.6</td>
</tr>
<tr>
<td>7</td>
<td>62.8</td>
<td>104.4</td>
<td>41.6</td>
</tr>
<tr>
<td>8</td>
<td>91.7</td>
<td>129.3</td>
<td>37.6</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
<td><strong>93%</strong></td>
</tr>
</tbody>
</table>

### Linearity

Samples diluted prior to extraction and assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Observed conc. (pmol/L)</th>
<th>Expected conc. (pmol/L)</th>
<th>Obs./Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>24.7</td>
<td>-</td>
<td>123%</td>
</tr>
<tr>
<td>A/2</td>
<td>15.2</td>
<td>12.4</td>
<td>123%</td>
</tr>
<tr>
<td>A/4</td>
<td>6.5</td>
<td>6.2</td>
<td>104%</td>
</tr>
<tr>
<td>B</td>
<td>30.1</td>
<td>-</td>
<td>110%</td>
</tr>
<tr>
<td>B/2</td>
<td>13.9</td>
<td>15.1</td>
<td>93%</td>
</tr>
<tr>
<td>B/4</td>
<td>8.3</td>
<td>7.5</td>
<td>110%</td>
</tr>
<tr>
<td>C</td>
<td>132</td>
<td>-</td>
<td>107%</td>
</tr>
<tr>
<td>C/2</td>
<td>63.0</td>
<td>66.0</td>
<td>95%</td>
</tr>
<tr>
<td>C/4</td>
<td>29.9</td>
<td>33.0</td>
<td>90%</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
<td><strong>102%</strong></td>
</tr>
</tbody>
</table>

### Expected Values

The following range data have been determined using the Gamma-B 1,25-Dihydroxyvitamin D kit and are provided for guidance only. Each laboratory should determine ranges for their local population.

- **Normal Adults**: 48 - 110 pmol/L (n=132)
- **Primary Hyperparathyroid**: 47 - 223 pmol/L (n=14)
- **Chronic Renal Failure**: <5 - 32 pmol/L (n=16)

### Reference

Gamma-B 1,25-Dihydroxy Vitamin D RIA Kit

Intended Use

For Research Use Only

The IDS Gamma-B kit is a complete assay system for the purification of 1,25-dihydroxy vitamin D (1,25D) in human serum or plasma by immunoreaction followed by quantitation by 125I radioimmunoassay.

Clinical Significance

Vitamin D is a commonly used term for the family of closely-related molecules derived from naturally occurring 7-dehydrocholesterol (pro-vitamin D3). Pro-vitamin D3, primarily of dietary origin, undergoes photochemical conversion in the skin to ‘parent’ vitamin D$_3$ (cholecalciferol) upon exposure to sunlight. This compound is biologically inactive, but enters the circulation and is hydroxylated in the liver to active 25-hydroxy vitamin D (25D$_3$). A small proportion of this becomes further hydroxylated in the kidney to the highly potent calcitriol hormone 1,25D$_3$.

Biosynthetic vitamin D$_3$ (vitamin D$_3$ or ergocalciferol) is widely used as a food supplement (e.g. margarine) and follows an identical hydroxylation pathway to form bioactive 1,25D$_3$.

As a result, Vitamin D status in many subjects will be determined by the presence of both 1,25D$_3$ and 1,25D$_2$. It is therefore essential to measure both forms of active vitamin D for maximum diagnostic utility. 1,25D$_3$ is largely bound to Vitamin D Binding Protein and albumin in the circulation.

1,25D$_3$ is one of the major regulators of calcium (and phosphate) metabolism, stimulating intestinal calcium absorption and increasing bone resorption. It also inhibits parathyroid hormone (PTH) production both by direct action on the parathyroid glands and indirectly by raising serum calcium levels. 1,25D$_3$ production is itself stimulated by parathyroid hormone (PTH), thus providing an effective control loop.

Hypovitaminosis D is commonly associated with dietary insufficiency, most frequently with vegetarianism, and is also associated with low exposure to sunlight (e.g. the elderly and institutionalised) and skin pigmentation.

1,25D$_3$ production appears to be impaired in early renal failure though this may not be a renal effect. In late-stage renal failure, 1alpha-hydroxylation may be impaired, with low 1,25D$_3$ levels at a result.

In hereditary vitamin D resistant rickets there is end-organ resistance to 1,25D$_3$ and therefore 1,25D$_3$ levels are raised.

The role of 1,25D$_3$ in osteoporosis has not been firmly established. The success of hormone replacement therapy and of the bisphosphonates in increasing bone density in patients with osteoporosis is well established, but 1,25D$_3$ involvement remains poorly understood.

Method Description

The IDS Gamma-B kit is a complete assay system for the purification of 1,25D$_3$ in patient samples by immunoreaction followed by quantitation by 125I RIA. Patient samples are delipidated and 1,25D$_3$ extracted from potential cross-reactants by incubation for 3 hours with a highly specific solid phase monoclonal anti-1,25D$_3$. The immunoreaction gel is then washed and purified 1,25D eluted directly into glass assay tubes. Reconstituted eluates and calibrators are incubated overnight with a highly specific 1,25D sheep anti-1,25D$_3$. 125I-1,25D$_3$ is added and incubation continued for 2 hrs. Separation of bound from free is achieved by a short incubation with Sac-Cel followed by centrifugation, decantation and counting. Bound radioactivity is inversely proportional to the concentration of 1,25D$_3$.

Precautions for Users

The Gamma-B 1,25-Dihydroxy Vitamin D kit is for in vitro research use only and is not for internal use in humans or animals. This product must be used strictly in accordance with the instructions set out in the Product Insert. IDS Limited will not be held responsible for any loss or damage (except as required by statute) however caused, arising out of non-compliance with the instructions provided.

Handling of Radioactive Materials

1. This kit contains radioactive material (Iodine 125). Appropriate precautions and good laboratory practices must be used in storage, handling and disposal of material. Radioactive material must be received, acquired, possessed and used only by physicians, clinical laboratories or hospitals, and only for in vitro laboratory tests. Its receipt, acquisition, possession, use and transfer are subject to local regulations.

2. Store radioactive materials in the original container, in a specifically designated, properly labelled area. Access to radioactive materials must be limited to authorised personnel only.

3. Do not eat, drink, smoke or apply cosmetics in areas where radioactive materials are stored or handled. Do not pipette radioactive material by mouth.

4. Always wear a protective laboratory coat and disposable gloves when handling radioactive materials. Wash hands thoroughly afterwards.

5. Areas where spills occur should be wiped immediately with suitable absorbent material, which should then be disposed of as radioactive waste. The contaminated area should then be washed using an alkaline detergent or radiological decontamination solution.

6. Disposal of radioactive material should be in accordance with local regulations.

7. Persons under 18 should not be permitted to handle radioactive material or enter radioactive areas.

8. Radioactive areas must be kept clean. Use disposable or easily decontaminated laboratory ware and absorbent covers on laboratory bench surfaces to minimise contamination.

Elution Reagent

Highly Flammable (flashpoint 13°C). Keep container tightly closed. Keep away from sources of ignition - No Smoking.

Sodium Azide

Some reagents in this kit contain sodium azide as a preservative, which may react with lead, copper or brass plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent azide build-up. For further information consult the local regulations.

Human Serum

All human serum based materials were negative when tested for HIV (1&2) antibody, hepatitis-B surface antigen and hepatitis-C antibody, but should be handled as if capable of transmitting disease.

Limitations of Use

1. Samples suspected of containing analyte concentrations in excess of the highest standard should be assayed in dilution.

2. Samples that contain detectable background radioactivity should not be used. Any suspect samples should be screened for radioactivity before performing the assay. The sample should be held until the radioactivity has decayed, or a further sample obtained.

Quality Control

The regular use of control samples at several analyte levels is advised to ensure day-to-day validity of results. The controls should be tested as unknowns. Quality Control charts should be maintained to follow the assay performance.

To ensure good precision it is most important that the detectors in multi-well counters are well matched and not contaminated.

Specimens Collection and Storage

The assay should be performed using serum or plasma specimens. Specimens should be separated as soon as possible after collection, and assayed within 12 hours. For longer term storage, freeze at -20°C. Avoid repeated freeze/thaw cycles.

Grossly haemolysed, lipaemic or turbid specimens must not be used.
## Gamma-B 1,25-Dihydroxy Vitamin D Flow Diagram

### Sample Preparation

<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
<th>Sample</th>
<th>DELIPIDATION REAGENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample 1</td>
<td>500µL</td>
<td>30µL</td>
</tr>
<tr>
<td>2</td>
<td>Sample 2, etc</td>
<td>500µL</td>
<td>30µL</td>
</tr>
</tbody>
</table>

Centrifuge tubes for 30 minutes at 2000 g. Supernatants are now ready for immunoextraction.

### Immunoextraction

<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
<th>IMMUNOCAPSULES</th>
<th>Sample</th>
<th>WASH</th>
<th>ELUTION REAGENT</th>
<th>ASSAY BUFFER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample 1</td>
<td>Vortex</td>
<td>100µL</td>
<td>Caps</td>
<td>Centrifuge caps</td>
<td>Discard</td>
</tr>
<tr>
<td>2</td>
<td>Sample 2</td>
<td>immunocapsules</td>
<td>100µL</td>
<td>Caps</td>
<td>Centrifuge caps</td>
<td>100µL</td>
</tr>
<tr>
<td>3</td>
<td>Sample 3</td>
<td>and allow solid</td>
<td>100µL</td>
<td>Add 500µL wash</td>
<td>assay</td>
<td>100µL</td>
</tr>
<tr>
<td>4</td>
<td>Sample 4</td>
<td>phase to active</td>
<td>100µL</td>
<td>temp</td>
<td>solution and centrifuge</td>
<td>100µL</td>
</tr>
</tbody>
</table>

Soak for 1-2 mins or centrifuge for 1 min, 500-750 g. Centrifuge tubes are now ready for assay.

### Immunoassay

<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
<th>Sample/Calibrator</th>
<th>PRIMARY ANTIBODY</th>
<th>(125) 1,25-D</th>
<th>SAC-CEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total</td>
<td>-</td>
<td>-</td>
<td>200µL</td>
<td>100µL</td>
</tr>
<tr>
<td>3</td>
<td>NSB</td>
<td>300µL</td>
<td>-</td>
<td>200µL</td>
<td>100µL</td>
</tr>
<tr>
<td>5</td>
<td>Calibrator A</td>
<td>100µL</td>
<td>Vortex and</td>
<td>200µL</td>
<td>Vortex and</td>
</tr>
<tr>
<td>7</td>
<td>Calibrator B</td>
<td>100µL</td>
<td>Vortex and</td>
<td>200µL</td>
<td>for 30 minutes</td>
</tr>
<tr>
<td>9</td>
<td>Calibrator C</td>
<td>100µL</td>
<td>Overnight</td>
<td>200µL</td>
<td>200µL</td>
</tr>
<tr>
<td>11</td>
<td>Calibrator D</td>
<td>100µL</td>
<td>1-2°C</td>
<td>200µL</td>
<td>200µL</td>
</tr>
<tr>
<td>13</td>
<td>Calibrator E</td>
<td>100µL</td>
<td>at room temperature</td>
<td>200µL</td>
<td>200µL</td>
</tr>
<tr>
<td>15, 16</td>
<td>Calibrator F</td>
<td>100µL</td>
<td>Centrifuge tubes for</td>
<td>200µL</td>
<td></td>
</tr>
<tr>
<td>17, 18*</td>
<td>Sample 1</td>
<td>100µL</td>
<td>200µL</td>
<td>200µL</td>
<td>20 minutes at 2000 g</td>
</tr>
<tr>
<td>19, 20</td>
<td>Sample 2</td>
<td>100µL</td>
<td>200µL</td>
<td>200µL</td>
<td>Decant, blow and count tubes</td>
</tr>
</tbody>
</table>

* Set aside until counting. ** Assay Buffer. # Sample extract.
An Enzyme-linked Immunosorbent Assay for the Measurement of Cross-linked N-telopeptides of Type I Collagen (NTx) in Human Urine

INSTRUCTIONS FOR USE

For in vitro diagnostic use only.
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Intended Use of Osteomark®

Osteomark® is a urinary assay that provides a quantitative measure of the excretion of cross-linked N-telopeptides of type I collagen (NTx) as an indicator of human bone resorption.

Summary and Explanation of the Test

Mammalian bone is continuously remodeled through a coupled process of bone resorption by osteoclasts followed by bone formation by osteoblasts. This process is necessary for normal development and maintenance of the skeleton. Abnormalities in this tightly coupled process often result in changes in skeletal mass and shape. The measurement of specific degradation products of bone matrix provide analytical data of the rate of bone metabolism.

Approximately 90% of the organic matrix of bone tissue is type I collagen. Type I collagen, a helical protein that is cross-linked at the N-terminal and C-terminal ends of the molecule, forms the basic fabric and tensile strength of bone tissue. Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are the predominant cross-linking amino acids of bone collagen. Total pyridinoline cross-links are excreted in the urine in free and peptide-bound forms, and have been utilized for research of human bone resorption by high-performance liquid chromatography techniques.
The discovery of urinary cross-linked N-telopeptides of type I collagen (NTx) has provided a specific biochemical marker of human bone resorption which can be analyzed by immunoassay. The NTx molecule is specific to bone due to the unique amino acid sequences and orientation of the cross-linked alpha-2 (I) N-telopeptide. Generation of the NTx molecule is mediated by osteoclasts on bone, and is found in the urine as a stable end-product of degradation.

Assay Principles

The Osteomark® assay is a competitive-inhibition enzyme-linked immunosorbent assay (ELISA) that utilizes microwells as the solid phase onto which NTx has been adsorbed. NTx in the specimen competes with the solid phase NTx for binding sites of a monoclonal antibody labeled with horseradish peroxidase. The amount of antibody bound to the solid phase is therefore indirectly proportional to the amount of NTx in the specimen. Quantitation of the NTx concentration in the specimen is determined spectrophotometrically and calculated from a standard calibration curve. Assay values are corrected for urinary dilution by urinary creatinine analysis and expressed in nanomoles bone collagen equivalents per liter (nM BCE) per millimole creatinine per liter (mM creatinine).
Kit Components

Supplied Materials Sufficient for 96 wells

Instructions for Use .................................................. 1 booklet
Antigen Coated 96-Well Plate, 12x8 well strips .......... 1 plate
Calibrators:
  1 nM BCE .......................................................... 0.4 mL
  30 nM BCE ........................................................ 0.4 mL
  100 nM BCE ....................................................... 0.4 mL
  300 nM BCE ....................................................... 0.4 mL
  1000 nM BCE ..................................................... 0.4 mL
  3000 nM BCE ..................................................... 0.4 mL
Level I Urine Control .................................................. 0.4 mL
Level II Urine Control ................................................ 0.4 mL
Antibody Conjugate Concentrate ................................. 0.4 mL
Antibody Conjugate Diluent ........................................ 30 mL
30X Wash Concentrate ............................................... 125 mL
Buffered Substrate .................................................. 30 mL
Chromogen Reagent .................................................. 0.9 mL
Stopping Reagent ................................................... 25 mL
Plate Sealers ................................................................ 1 pad

Reagent Descriptions

Antigen Coated 96-Well Plate, 12x8 well strips.
  Purified NTx adsorbed onto microwell strips.

Assay Calibrators: 1, 30, 100, 300, 1000, 3000 nM BCE,
  1 vial each. Purified NTx in buffered diluent. ProClin™
  300 (0.05%) included as a preservative.

Level I and Level II Urine Controls, 1 vial each.
  Human urine with known NTx concentration. ProClin™
  300 (0.05%) included as a preservative.
Antibody Conjugate Concentrate, 1 vial.
  Purified murine monoclonal antibody directed against NTx and conjugated to horseradish peroxidase.
  Thimerosal (0.0001%) is included as a preservative.
  Supplied as a 100X concentrated reagent.

Antibody Conjugate Diluent, 1 bottle.
  Buffered reagent with protein stabilizers, into which Antibody Conjugate Concentrate is diluted. ProClin™
  300 (0.05%) included as a preservative.

Chromogen Reagent, 1 vial.
  3,3',5,5' - tetramethylbenzidine (TMB) in dimethylsulfoxide (DMSO). Supplied as a 100X concentrated reagent.

30X Wash Concentrate, 1 bottle.
  Ionic detergent solution. Supplied as 30X concentrate.

Buffered Substrate, 1 bottle.
  Buffered hydrogen peroxide.

Stopping Reagent, 1 bottle.
  1N sulfuric acid.

Storage of Reagents
  Reagents must be stored at 2 - 8°C when not in use.
  Reagents must be brought to room temperature before use.
  Do not expose reagents to temperatures greater than 30°C or less than 2°C. Diluted wash solution may be stored at room temperature for up to one month.
Materials Required but Not Supplied

- Single and multichannel pipettors capable of delivering 25 µL, 100 µL and 200 µL volumes.
- Disposable pipet tips.
- Automated microwell washer.
- Microwell or microstrip spectrophotometric reader. The reader must read at 450 nm with a 630 nm reference filter and detect absorbances from 0 to 3.000 optical density units.
- Software capable of calculating a 4-parameter curve fit is recommended.
- Deionized water.

Urine Specimen Collection and Storage

- Collect a second morning void (spot) urine specimen or a 24 hour urine specimen in an appropriate collection device with a tight fitting lid.
- **DO NOT ADD PRESERVATIVE TO URINE SPECIMEN.**
- Specimens that are obviously contaminated with whole blood or have extensive hemolysis may interfere with the assay and should be discarded. Recollection of these specimens is recommended.
- Store refrigerated (2 - 8°C) for up to 72 hours. Store frozen (-20°C or below) for longer term storage.
- **For monitoring therapy, baseline samples should be collected prior to initiation of therapy.** Subsequent specimens for comparison should be collected at the same time of day as the baseline specimen.
Warnings and Precautions

- For in vitro diagnostic use only

- The Antigen Coated 96-Well Plate, Calibrators, and Urine Controls contain human urine and/or processed antigen from human bone tissue and therefore should be handled as potentially infectious materials and disposed of appropriately.

- The Stopping Reagent contains 1N sulfuric acid. Avoid contact with skin or eyes. If exposed, flush area with water for 15 minutes. If eyes are exposed, obtain medical attention.

- The Chromogen Reagent contains 3,3',5,5'- tetramethyl-benzidine (TMB) and dimethylsulfoxide (DMSO). Dimethylsulfoxide is readily absorbed through the skin. If exposed, flush area with water for 15 minutes. If eyes are exposed, obtain additional medical attention.

- Urine specimens may contain infectious agents and should be disposed of properly. Decontamination is most effectively accomplished with a 0.5% solution of sodium hypochlorite (1:10 dilution of household bleach) or autoclave one hour at 121°C. Do not autoclave solutions containing sodium hypochlorite. Do not add sodium hypochlorite solution to acid.

- Never pipette reagents or clinical specimens by mouth.

- Wear protective gloves and clothing when handling specimens and reagents, washing hands thoroughly after use.

- Do not use reagents beyond their expiration dates.

- Do not mix components from different lots of Osteomark® kits.

- Microwell strips must be kept desiccated. Reseal unused microwell strips in the pouch containing desiccant.

- Do not reuse microwells. Dispose of properly after use.
Assay Procedure

Preparatory Steps

1. Allow all specimens and reagents to equilibrate to room temperature (18 - 28°C) before performing the assay. Frozen urine specimens may be thawed at 37°C, in either a water bath or an incubator, then brought to room temperature prior to use in the assay. The Chromogen Reagent contains dimethylsulfoxide (DMSO), which may solidify when refrigerated but is liquid at room temperature.

2. Prepare the working strength wash solution. Dilute 30X Wash Concentrate 1:30 with deionized water (1 part 30X Wash Concentrate to 29 parts deionized water) and mix for a minimum of five (5) minutes. This solution is stable for one (1) month at room temperature.

3. Create a plate map. Run each Calibrator, Control, and specimen in duplicate. An example of a plate map is provided below for an Osteomark® run with 4 specimens:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 nM BCE Calibrator</td>
<td>1000 nM BCE Calibrator</td>
<td>Specimen #1</td>
</tr>
<tr>
<td>B</td>
<td>1 nM BCE Calibrator</td>
<td>1000 nM BCE Calibrator</td>
<td>Specimen #1</td>
</tr>
<tr>
<td>C</td>
<td>30 nM BCE Calibrator</td>
<td>3000 nM BCE Calibrator</td>
<td>Specimen #2</td>
</tr>
<tr>
<td>D</td>
<td>30 nM BCE Calibrator</td>
<td>3000 nM BCE Calibrator</td>
<td>Specimen #2</td>
</tr>
<tr>
<td>E</td>
<td>100 nM BCE Calibrator</td>
<td>Level I Urine Control</td>
<td>Specimen #3</td>
</tr>
<tr>
<td>F</td>
<td>100 nM BCE Calibrator</td>
<td>Level I Urine Control</td>
<td>Specimen #3</td>
</tr>
<tr>
<td>G</td>
<td>300 nM BCE Calibrator</td>
<td>Level II Urine Control</td>
<td>Specimen #4</td>
</tr>
<tr>
<td>H</td>
<td>300 nM BCE Calibrator</td>
<td>Level II Urine Control</td>
<td>Specimen #4</td>
</tr>
</tbody>
</table>
4. Using a clean disposable plastic container, dilute the Antibody Conjugate Concentrate 1:101 using the Antibody Conjugate Diluent. Determine the volume of each reagent required to provide sufficient working strength conjugate solution for the assay according to the table below. Mix gently by inversion only. **Do not vortex or use a magnetic stir bar.** Avoid foaming. Use the working strength conjugate solution within one hour of preparation. Do not reuse the container.

<table>
<thead>
<tr>
<th>Total Number of Strips</th>
<th>Antibody Conjugate Concentrate (µL)</th>
<th>Antibody Conjugate Diluent (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>70</td>
<td>7</td>
</tr>
<tr>
<td>4-6</td>
<td>120</td>
<td>12</td>
</tr>
<tr>
<td>7-9</td>
<td>180</td>
<td>18</td>
</tr>
<tr>
<td>10-12</td>
<td>240</td>
<td>24</td>
</tr>
</tbody>
</table>

5. Prior to pipetting, gently mix the Calibrators, Controls and specimens. Avoid foaming. Allow cloudy or turbid specimens to settle 5 to 10 minutes prior to pipetting. Specimens containing particulates may be centrifuged before use.

6. Remove the appropriate number of microwell strips from the sealed foil pouch. Place any unused strips back in the pouch, resealing the pouch along the zipper. Do not remove the desiccant pillow from the foil pouch.
Specimen and Antibody Incubation

Once the assay has been started, complete it without interruption.

7. Following the plate map created in Step 3, pipette 25 μL of each Calibrator, Control, or specimen into the bottom of duplicate wells. Use a calibrated pipettor and new pipette tips for each Calibrator, Control, or urine specimen.

8. Using a multichannel pipettor, deliver 200 μL of the working strength conjugate solution into each microwell. Apply a plate sealer and swirl the plate gently on a flat surface for 5-10 seconds to ensure mixing.

9. Incubate the plate at room temperature (18 - 28°C) for 90 ± 5 minutes.

10. Prepare the Chromogen/Buffered Substrate solution during the last 10 minutes of incubation by making a 1:101 dilution of the Chromogen Reagent into the Buffered Substrate. Determine the volume of each reagent required to provide sufficient Chromogen/Buffered Substrate solution for the assay according to the table below. Pipette the Buffered Substrate into a clean plastic disposable container. Thoroughly mix the Chromogen Reagent prior to pipetting. Add the Chromogen Reagent to the Buffered Substrate Reagent and invert gently to mix. Do not vortex or shake vigorously or use a magnetic stir bar to mix. Use the Chromogen/Buffered Substrate solution within 30 minutes of preparation. The Chromogen/Buffered Substrate solution should be colorless when mixed. A blue color indicates that the reagent has been contaminated and must be discarded.
<table>
<thead>
<tr>
<th>Total Number of Strips</th>
<th>Chromogen Reagent (µL)</th>
<th>Buffered Substrate (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>70</td>
<td>7</td>
</tr>
<tr>
<td>4-6</td>
<td>120</td>
<td>12</td>
</tr>
<tr>
<td>7-9</td>
<td>180</td>
<td>18</td>
</tr>
<tr>
<td>10-12</td>
<td>240</td>
<td>24</td>
</tr>
</tbody>
</table>

11. At the end of the incubation period, carefully remove and discard the plate sealer. Aspirate the liquid from each well and wash the plate five (5) times with the working strength wash solution using an automated plate washer. Dispense at least 350 µL of working strength wash solution per well. Aspirate the wells after each wash. When five washes are complete, grasp the plate frame at the center of each side and invert the plate, blotting on absorbent paper towel. Immediately add the prepared Chromogen/Buffered Substrate solution as described below.

**Color Development and Measurement**

12. Pipet 200 µL of the Chromogen/Buffered Substrate solution prepared in step 10 into each well using a multichannel pipettor. Cover the plate with a new plate sealer.

13. Incubate at room temperature for 15 ± 1 minutes. A blue color will develop in wells containing bound antibody-horseradish peroxidase conjugate.

14. Following incubation, carefully remove and discard the plate sealer. Using a multichannel pipettor, add 100 µl of Stopping Reagent to each well. Wells which have developed a blue color will now turn yellow.
15. Swirl the plate gently on a flat surface for 5-10 seconds to ensure mixing. Allow the plate to sit at room temperature for 5 minutes before reading absorbance values.

16. Within 30 minutes of adding the Stopping Reagent, read the absorbance of the Calibrators, Controls, and urine specimens. Use a microwell plate reader at 450 nm with a reference filter of 630 nm. The reader must have a maximum optical density reading of 3.000.

Analysis of Results

1. Determine concentration values (nM BCE) of Controls and specimens from the calibration curve. The most accurate results are obtained using a 4-parameter curve fitting equation.

EXAMPLE:

CALIBRATION CURVE
2. Assay results are valid if the following criteria are met:
   • The mean absorbance value of the 1 nM BCE Calibrator must be $\geq 1.500$.
   • The span of the calibrator curve (difference between absorbance values of the 1 nM BCE and 3000 nM BCE Calibrators) should be $\geq 1.300$ absorbance value.

3. The recommended coefficient of variation (% CV) between specimen duplicates is $\leq 20\%$. Specimens with $>20\%$ CV should be rerun.

4. The Level I Urine Control has been manufactured to be within the range of 320 to 480 nM BCE. The Level II Urine Control has been manufactured to be within the range of 1120 to 1680 nM BCE. Actual control ranges should be established in your laboratory.

5. The lower limit of detection is 20 nM BCE.

6. Specimens that exceed 3000 nM BCE may be diluted 1:5 in a urine specimen or pool of urine with known nM BCE <50, and retested. When using urine as a diluent, the nM BCE of the urine diluent should be confirmed by testing it as a specimen in the same plate as the diluted unknown specimen. The dilution factor and background (the diluent nM BCE) should be incorporated into the final calculation.

Example: 840 nM BCE assay value derived from a 1:5 dilution of a 4000 nM BCE specimen using a urine diluent of known Osteomark® value (50 nM BCE)

$$\begin{align*}
840 \text{ nM BCE} - (0.8 \times 50 \text{ nM BCE}) &= 800 \text{ nM BCE} \\
800 \text{ nM BCE} \times 5 \text{ (dilution factor)} &= 4000 \text{ nM BCE}
\end{align*}$$

Note: 1:5 dilutions represent 80% diluent (0.8), 20% specimen contribution.
7. Report the concentration values for urine specimens as nM BCE/mM creatinine, as shown in the following example:

\[ \text{Assay value} = 360 \text{ nM BCE} \]
\[ \text{Urinary creatinine} = \frac{60 \text{ mg/dL creatinine}}{11.3^*} \]
\[ = 5.3 \text{ mM creatinine} \]

\[ \frac{360 \text{ nM BCE}}{5.3 \text{ mM creatinine}} = 68 \text{ nM BCE/mM creatinine} \]

*Note: Conversion factor used to convert mg creatinine per dL to millimole creatinine per liter.

**Limitations of the Procedure**

While Osteomark® is used as an indicator of bone resorption, use of this test has not been established to predict development of osteoporosis or future fracture risk. Use of this test has not been established in menopause, Paget’s disease of bone, primary hyperparathyroidism or hyperthyroidism. When using Osteomark® to monitor therapy, results may be confounded in patients afflicted with clinical conditions known to affect bone resorption, e.g., metastases to bone. Osteomark® results should be interpreted in conjunction with clinical findings and other diagnostic results.

**Interfering Substances**

Various urine components and microorganisms were evaluated for an interfering effect in the Osteomark® assay. The organisms tested, *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *C. albicans* (ATCC 14053), did not interfere with assay performance. Human albumin, bilirubin, glucose, and
vitamin C did not interfere with assay performance. Specimens obviously contaminated with whole blood or that have extensive hemolysis may interfere with assay performance. These specimens should be discarded, and a specimen recollected.

**Expected Values**

Multi-center, cross-sectional studies were conducted to determine the reference range for normal premenopausal and postmenopausal women. The comparative results between the two groups are presented in Table 1 below. Mean, standard deviation, and the upper limit of the 95% confidence interval (95th percentile) are presented. These data are representative of the subjects tested in the two studies; each laboratory should establish its own normal range.

**Table 1 - Expected Osteomark® Values**

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Mean*</th>
<th>Std Dev</th>
<th>95th Percentile*</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-menopausal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>35</td>
<td>15</td>
<td>65</td>
<td>258</td>
</tr>
<tr>
<td>(mean age 36 years, range 25-49)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-menopausal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>57</td>
<td>39</td>
<td>131</td>
<td>248</td>
</tr>
<tr>
<td>(&lt;3 years postmenopause, mean age 51 years, range 40-58)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*expressed as nanomoles BCE/millimole creatinine

The expected within-subject variability was determined from urine specimens from eight healthy subjects collected every 2-3 days over approximately 2 months. The average of the individual within-subject longitudinal variation was 19.3%. The average between-subject longitudinal variation was 38.3%.
Performance Characteristics

Reproducibility and Precision

Intra-assay variability was assessed using eight urine specimens tested in replicates of 10 by each of four operators. Results are provided below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (nM BCE)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>B</td>
<td>111</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>172</td>
<td>8</td>
</tr>
<tr>
<td>D</td>
<td>417</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>694</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>1113</td>
<td>5</td>
</tr>
<tr>
<td>G</td>
<td>1768</td>
<td>6</td>
</tr>
<tr>
<td>H</td>
<td>2640</td>
<td>5</td>
</tr>
</tbody>
</table>

Inter-assay variability was assessed using three urine specimens tested in duplicate by one operator over 20 separate assay runs. Results are provided below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (nM BCE)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>79</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>412</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>1167</td>
<td>4</td>
</tr>
</tbody>
</table>
Total assay precision was evaluated by testing the Level I Urine Control and the Level II Urine Control at three clinical laboratory sites over a 30 day period. Results are provided below:

<table>
<thead>
<tr>
<th>Urine Control</th>
<th>Mean (nM BCE)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level I</td>
<td>439</td>
<td>10</td>
</tr>
<tr>
<td>Level II</td>
<td>1537</td>
<td>7</td>
</tr>
</tbody>
</table>

**Antigen Recovery**

Antigen recovery was evaluated by adding known amounts of NTx to each of three urine specimens of known NTx concentration. Recovery represented the observed assay value of the “spiked” specimens, calculated as a percent of the expected urine value (baseline urine value plus added antigen (NTx) value). The results demonstrated an average antigen recovery of 105% across the assay range.

**Dilutional Linearity**

Dilutional linearity was evaluated by performing serial dilutions of four urine specimens with high nM BCE values into a urine specimen with a low nM BCE value. Results demonstrated correlation coefficients of $r = 0.999$ to $r = 1.000$ across the assay range.
Use of Normal and Abnormal Specimens

A multi-center, non-randomized, prospective longitudinal clinical trial was conducted to determine the ability of the Osteomark® assay to monitor the effect of estrogen suppressing therapy on bone resorption in premenopausal women. Figure 1 represents the mean (± SEM) Osteomark® values obtained throughout the trial along with the corresponding mean (± SEM) estradiol values for each timepoint. The mean Osteomark® value at baseline was 44 (range 8 - 199) nM BCE/mM creatinine. The mean Osteomark® value while the subjects were estrogen suppressed was 68 nM BCE/mM creatinine, a 68% increase from baseline. The mean serum estradiol level was 21 pg/mL during this time period. These mean values while estrogen suppressed were concordant with postmenopausal values for the two analytes.

Figure 2 provides a graph of the Osteomark® percent change from baseline throughout the trial for each subject. Sixty three percent (55/88) of the subjects had a mean percent change from baseline of 30% or greater (p = 0.025). Subjects exhibiting <30% change (33/88 or 37%) had a mean baseline Osteomark® value that was higher (59.97 nM BCE/mM creatinine) than those with a ≥30% change (35.47 nM BCE/mM creatinine), accounting for the lesser percent change in Osteomark® in these individuals. The average on-therapy Osteomark® value for the <30% change group was lower (50.99 nM BCE/mM creatinine) than the ≥30% group (66.22 nM BCE/mM creatinine). The <30% change group also tended to lose less bone at the spine than those who had a ≥30% change.
The mean 68% increase from baseline in Osteomark® correlated to a mean percent decrease at six months of -3.7% in lumbar spine (L1-L4) bone mineral density (BMD), as measured by dual energy x-ray absorptiometry \((r = -0.46, p<0.01)\). Three months after cessation of estrogen suppression therapy, the mean Osteomark® value returned to baseline \((44 \text{ nM BCE/mM creatinine})\) as serum estradiol levels returned to normal premenopausal levels. Bone density measurements for the spine remained below baseline \((-2.4\%)\) at the 3 month post estrogen suppression therapy timepoint.

The results of the clinical trial demonstrate that Osteomark® provides a quantitative measure of the excretion of cross-linked N-telopeptides as an indicator of human bone resorption, and can monitor the effect of therapy. Those subjects in whom Osteomark® values do not change 30% of the baseline value, should be followed with additional clinical and laboratory data to properly assess the response to treatment.

**Figure 1: Osteomark® Values and Serum Estradiol Levels (± SEM) During and After Estrogen Suppression Therapy**

![Graph showing Osteomark® values and serum estradiol levels during and after estrogen suppression therapy.](image)
Figure 2: Osteomark® Percent Change From Baseline During and After Estrogen Suppressing Therapy
Osteomark® (PN 9006) Quick Reference

1. Thoroughly read the Assay Procedure before you begin.
2. Bring kit components and specimens to room temperature.
3. Dilute the Antibody Conjugate Concentrate into the Antibody Conjugate Diluent, using a 1:101 ratio. Use the working strength conjugate solution within one hour of preparation.
4. Pipette 25 μL samples of each Calibrator, Control, and specimen into duplicate microwells.
5. Pipette 200 μL of the working strength conjugate solution into each microwell. Gently swirl to mix. Incubate the plate at room temperature for 90 ± 5 minutes.
6. Prepare Chromogen Reagent/Buffered Substrate solution during the last 10 minutes of incubation. Dilute the Chromogen Reagent into the Buffered Substrate using a 1:101 ratio. Mix gently BY INVERSION ONLY. Do not vortex or mix with a magnetic stir bar. Use the Chromogen/Buffered Substrate within 30 minutes of preparation.
7. Wash microwells five (5) times with the working strength wash solution at 350 μL per well, and blot on absorbent paper towel.
8. Add 200 μL of Chromogen/Buffered Substrate to each microwell, and incubate at room temperature for 15 ± 1 minutes.
9. Add 100 μL of Stopping Reagent to each microwell. Gently swirl the plate to mix.
10. Incubate at room temperature for 5 minutes and read the absorbance of each microwell at 450 nm - 630 nm. Calculate the results using the standard curve.
References:


Technical Information

For further information or technical assistance, contact Ostex Technical Services at:
1-206-292-8082.
1-800-99-OSTEX.

Warranty Statement

These products are warranted to perform as described in their labeling and the Ostex International, Inc. literature when used in accordance with their instructions. There are no warranties which extend beyond this express warranty and Ostex International, Inc. disclaims any implied warranty of merchantability or warranty of fitness for particular purpose. Ostex International, Inc.'s sole obligation and purchaser's exclusive remedy for breach of this warranty shall be, at the option of Ostex International, Inc. to repair or replace the products. In no event shall Ostex International, Inc. be liable for any proximate, incidental or consequential damages in connection with the products.

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(206) 292-8625 FAX
1-800-99-OSTEX.

Made in U.S.A. 7002-A
rev 6/95
PROCOLLAGEN,
PICP [^{125}I] 
Radioimmunoassay Kit

January 1993

Kit Instructions

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substance. The labelled and unlabelled antigens are then allowed to compete for the limited number of high affinity binding sites of the antibody. The amount of radioactive antigen in the antigen-antibody complex is reversely proportional to the amount of unlabelled antigen in the reaction mixture. After washing away the free antigen, the residual radioactivity is counted and the actual concentration is calculated with the aid of a standard curve based on known amounts of unlabelled antigen analyzed in parallel with the unknown.

Specifically in this kit, 100 µl of sample serum is mixed with 200 µl of PICP antiserum and 200 µl of ¹²⁵I-labelled PICP. After a 2-hour incubation at 37°C, separation reagent is added, tubes are allowed to stand for a short while and then centrifuged. The supernatant is then removed and the sediment containing the precipitated antibody-antigen complex is counted in a gamma counter.

A standard curve is produced by calculating the binding of five standards as a percentage of the maximum possible binding and plotting these values. The CPM results for unknowns are then expressed in terms of bound radioactivity. PICP values may then be read directly from the standard curve. As an alternative to the manual calculation of results, the data may be analyzed using a computer program allowing, for example, spline function curve fitting.
INTENDED USE

The ORION DIAGNOSTICA Procollagen PICP radioimmunoassay is a quantitative test designed for in vitro measurement of the concentration of carboxyterminal propeptide of type I procollagen (PICP) in human serum and other biological fluids (8).

CLINICAL INFORMATION ON PICP

Type I collagen is the most abundant collagen type in the body and the only collagen type found in bones and tendons. It accounts for more than 90% of the organic matrix of bone (1). In addition, type I collagen is found in loose connective tissues together with other collagen types such as III, V and VI. In these locations the proportion of type I collagen is the largest.

Type I collagen is derived from a larger protein, type I procollagen, which has propeptide extensions at both ends of the molecule (2). These propeptides are removed by specific proteinases before the collagen molecules are assembled into fibres. The sequence removed from the carboxyterminal end of the molecule, known as the carboxyterminal propeptide of type I procollagen (PICP), can be found in blood, its level reflecting the synthesis of type I collagen.

Changes in the concentration of PICP are found, for example, during growth (3). In addition, synthesis of type I collagen can be altered during the pathogenesis of many kinds of disease. Because bone is a metabolically active tissue throughout life, an indicator of type I collagen turnover is particularly useful as a biochemical marker in
metabolic bone disease (4-7).

In the Orion Diagnostica PICP RIA, cross-reaction with the carboxyterminal propeptide of type III procollagen (PIIICP) has been minimized by careful purification of the antigen and selection of antiserum.

The assay may be of use in diagnosis and/or follow-up of the following areas:

1. Growth induction in children (e.g. suffering from a chronic disease that has adverse effects on growth).

2. A rapid bone loser state after the menopause and its treatment by estrogens or other drugs that slow down the metabolic rate of bone.

3. Local measurement of the rate of type I collagen synthesis in wound fluid, cerebrospinal fluid, broncho-alveolar lavage fluid etc.

The reference interval for men and women is 38 - 202 µg/l; higher concentrations (related to growth velocity) are found in infants and children. After the menopause, higher levels are found due to increased bone turnover.

PRINCIPLES OF TEST

The PICP assay kit is based on the widely used radioimmunoassay technique. A sample containing an unknown amount of the substance to be assayed is mixed with a standard amount of a radioactively labelled derivative of the same
REAGENTS

Materials provided

PICP $^{125}$I REAGENT, 1 vial
A ready to use solution of $^{125}$I-labelled PICP in PBS buffer with BSA, a red colour additive and 0.05% sodium azide as a preservative. The volume supplied is 22 ml and the radioactivity is less than 200 kBq.

PICP ANTISERUM, 1 vial
A ready to use solution of PICP antiserum (rabbit) in PBS buffer with BSA, a blue colour additive and 0.05% sodium azide as a preservative. The volume supplied is 22 ml.

PICP STANDARDS, 6 vials
Ready to use standards in PBS buffer with BSA and 0.05% sodium azide as a preservative. Volumes supplied are 0.75 ml and concentrations are 0, 25, 50, 100, 200 and 500 µg/l.

PICP CONTROLS, 2 vials
Two lyophilized human serum controls with different PICP concentrations containing 0.1% sodium azide as a preservative. Expected values are indicated on the separate result sheet provided with the kit. Volumes after reconstitution are 1.0 ml.

PICP SEPARATION REAGENT, 1 bottle
A ready to use suspension of a second antibody covalently bound to solid particles with 0.1% sodium azide as a preservative. The volume supplied is 60 ml.

Warnings and precautions

FOR IN VITRO DIAGNOSTIC USE

Not for internal or external use in humans or animals.

5
Do not mix or use the reagents from one test unit with those of another test unit which has a different lot number.

Do not use reagents after the expiration date stated on each reagent container's label.

Persons who receive, acquire, possess or use $^{125}$I by authority of section 31.11 USNRC regulations or equivalent agreement state regulations are authorized by regulations to possess at any one time at any one location of storage or use a maximum of 200 microcuries of $^{125}$I in individual units not exceeding 10 microcuries per unit. The radioactive material must be stored, until used, in the original shipping container or a container which provides equivalent radiation protection. Transfer to non-licensed persons is prohibited. Any transfer of materials must be in the unopened, labelled shipping container as received from the supplier.

The following precautions should be observed when handling radioactive materials:

1. All radioactive materials should be stored in specifically designated areas.

2. Handling of radioactive materials should be conducted only in authorized areas.

3. There should be no pipetting by mouth.

4. Eating, drinking and smoking while handling radioactive materials should be prohibited.

5. Hands should be protected by disposable gloves and thoroughly washed after handling radioactive materials.
6. Spills of radioactive materials should be wiped up immediately and the contaminated cleaning materials transferred to the radioactive waste receptacle. Surfaces involved should be washed thoroughly with an appropriate decontaminant. (Cleanup can be facilitated by covering the work surface with disposable absorbent material.)

7. All liquid or dispersible materials in this kit may be discarded into the sanitary sewerage system with copious flushing with water.

8. Solid or dry radioactive waste material may be discarded in the usual manner after labelling is defaced or removed.

Should any questions arise regarding the proper handling and disposal of radioactive material, please contact your Orion Diagnostica representative.

Handle all patient specimens as potentially infectious.

CAUTION 1
Source material of human blood used in the preparation of this kit has been found to be non-reactive for HBsAg and HIV antibodies when tested with licenced reagents. However, since no known test can offer complete assurance, kit reagents should nevertheless be treated as though they are capable of transmitting infectious diseases.

CAUTION 2
Reagents contain sodium azide (NaN₃) as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up. Please refer to decontamination procedures as outlined by U.S. Centers for Disease Control (Atlanta, Ga., USA).
Reconstitution of reagents

1. Bring all reagents to room temperature.

2. Accurately add 1.0 ml distilled water to the PICP control vials. Cap and mix well by gentle swirling or inversion to avoid foaming. Allow to stand for 30 minutes before use.

3. Record the applicable expiration date (following reconstitution) on each vial.

4. Other reagents are ready to use.

Storage

The kit should be stored at 2 - 8°C. Reagents are stable until the expiration dates stated on reagent containers’ labels. The stability of reconstituted controls is indicated on the separate result sheet provided with the kit.

Physical or chemical indications of instability

Alterations in the physical appearance of the reagents or in the slope of the standard curve, or values of control sera outside the manufacturer’s range may be an indication of reagent instability.

INSTRUMENTS

Any scintillation counter capable of measuring $^{125}$I may be used. Maximum counting efficiency should be ensured. Consult the operations manual supplied by the instrument manufacturer for details of installation, use and maintenance.

Centrifugation capacity of 2000 g minimum is required for the procedure. Table 1 illustrates the relationship between centrifuge speed and rotating radius necessary to attain this centrifugal force.
Consult the operations manual supplied by the centrifuge manufacturer for a detailed nomogram for computation of the required speed setting for a particular centrifuge rotor and for information concerning the installation, use and maintenance of the centrifuge.

Table 1  Centrifuge parameters necessary to attain 2000 g centrifugal force

<table>
<thead>
<tr>
<th>Rotating radius (cm)</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed (RPM)</td>
<td>4500</td>
<td>3500</td>
<td>3000</td>
<td>2500</td>
</tr>
<tr>
<td>Centrifugal force (g)</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
</tr>
</tbody>
</table>

**SPECIMEN COLLECTION AND PREPARATION**

No special preparation of the patient is required. A venous blood sample is collected aseptically and the serum is separated using a normal procedure.

Information on the use of the kit with other biological fluids, and on the recommended methods for collection of these specimens is available from Orion Diagnostica.

**Storage**

Samples may be stored at 4°C for up to 5 days. It is recommended that storage for longer periods should be at temperatures of at least -20°C. Repeated freezing and thawing should be avoided.

**Dilution**

Samples with high PICP concentrations may be diluted at least up to 1:40 with zero-standard. Saline can also be used for dilutions but may cause a small change in the final result.
PROCEDURE

Materials required but not provided

MICROPIPETTE for accurately dispensing 100 μl
SEMAUTOMATIC PIPETTES for accurately dispensing 200 μl and 500 μl
TEST TUBES (eg 12 x 75 mm)
ABSORBENT PAPER
PARAFILM
VORTEX MIXER
ASPIRATOR SYSTEM (eg water suction pump with attached Pasteur pipette)
CENTRIFUGE capable of at least 2000 g
GAMMA COUNTER, manual or automatic

Details of the procedure

1. Bring all reagents, controls and samples to room temperature.

2. Set up and label a series of test tubes in duplicate for NSB (non-specific binding), standards, controls and unknown samples and totals.

3. After mixing, pipette 100 μl of the appropriate standard, control or patient sample into each tube. Any patient sample may be pipetted into the NSB tubes.

4. Pipette 200 μl of PICP [(125I)] reagent (red) into all tubes.

5. Pipette 200 μl of PICP antiserum solution (blue) into all tubes except NSB and total. Pipette 200 μl of distilled water into NSB tubes.
6. Mix well, cap or cover tubes and incubate at 37°C for 2 hours.

7. Mix the separation reagent thoroughly by gentle inversion and add 500 μl to all tubes except total.

8. Thoroughly mix the tube contents (eg with a vortex mixer) and leave to stand for 30 minutes at room temperature.

9. Centrifuge tubes for 15 minutes at 2000 x g (minimum) at between 4°C and 20°C (4°C is recommended).

10. Aspirate or place the tubes carefully into decantation racks and decant the supernatant. Dry the mouth of the tube by tapping against absorbent paper (see note below).

11. Count the antibody-bound radioactivity remaining in each tube, by counting for at least 1 minute/tube.

12. Determine the final PICP concentration of samples by interpolation from the standard curve.

**Note**
When decanting, the use of a decantation rack is recommended. The tubes should be inverted with a smooth action. They should be kept inverted and allowed to drain briefly against several layers of absorbent paper. Finally, the rims should be blotted with firm tapping motions against the absorbent paper. Do not re-invert the tubes once they have been turned upright.
Table 2 Assay procedure - summary
(all volumes given in $\mu$l - any patient sample can be used for NSB)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total</th>
<th>NSB</th>
<th>Standard</th>
<th>Control and Unkn.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette sample</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Pipette PICP $\left(\text{I}^{125}\text{i}\right)$ (red)</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Pipette PICP antiserum (blue)</td>
<td></td>
<td></td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Pipette distilled water</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vortex</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Incubate 2h/37°C</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Pipette separation agent</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Vortex</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Incubate 30 min/RT</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Centrifuge 15 min/2000g</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Aspirate or decant</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Count for 1 min or 10000 counts</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
CALCULATION OF RESULTS

If automatic result processing is to be used with results, spline function curve fitting is recommended. Below we present a method of deriving results using a manually produced standard curve drawn on semi-log graph paper. (See Table 3 and Figure 1.)

The method of calculation described involves the expression of radioactivity of samples as a percentage of the maximum binding.

1. Calculate the mean of counts from NSB tubes and subtract this value from all standard and unknown tube counts.

2. Calculate the mean of the NSB-corrected counts for the zero-standard tubes.

3. Calculate the B/B₀ % from:

\[
B/B₀ \% = \frac{(\text{standard or sample count} - \text{NSB})}{(\text{mean zero-standard count} - \text{NSB})} \times 100
\]

4. Draw a standard curve on semi-log graph paper with B/B₀ % on the ordinate and PICP concentrations (μg/l) of the standards on the abscissa.

5. Read the PICP concentrations of the unknowns from the standard curve and average the duplicate values.
Table 3 Calculation of results using typical data

<table>
<thead>
<tr>
<th>Tube</th>
<th>cpm</th>
<th>% B/B₀</th>
<th>PICP (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>52235.8</td>
<td>52628.6</td>
<td>52447.2</td>
</tr>
<tr>
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<td>190.7</td>
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</table>
EXPECTED VALUES

Reference values for serum PICP were calculated for healthy adults, based on samples from 75 blood donors (Figure 2). In men between 20 and 60 years, the PICP concentration was inversely related to age, whereas in women no such relationship could be found. In both sexes, the distribution of the concentrations was somewhat skewed. The reference intervals (mean \( \pm 2SD \)) were 50-170 \( \mu g \) and 38-202 \( \mu g/l \) for women and men, respectively.

Samples, both as serum and as plasma, from 8 healthy volunteers were tested. Mean PICP concentrations were 124 (SD 49) \( \mu g/l \) for serum and 119 (SD 42) \( \mu g/l \) for plasma. The Pearson correlation coefficient was 0.997 (\( P < 0.001 \)).
Figure 2 PICP concentrations in healthy Finnish blood donors, 41 women and 33 men. In the plots of PICP against age, for women, $r = -0.133$ (not significant) and for men, $r = -0.546$, $P < 0.001$. In the frequency graphs, the groupings are based on SD's.
PERFORMANCE CHARACTERISTICS

Recovery

Two serum samples with different concentrations of PICP were mixed in different ratios and analyzed. The mean recovery was 99.2% (SD 5.7%, n = 15) within the concentration range 90 to 315 μg/l. No interference resulting from the use of hemolyzed, lipemic or icteric sera was detected.

Precision

Intra- and inter-assay variations were tested using serum samples containing four different concentrations of the PICP antigen. Results were as shown in Tables 4 and 5

Table 4 Intra-assay precision

<table>
<thead>
<tr>
<th>Concentration μg/l (n = 16)</th>
<th>CV %</th>
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<tr>
<td>54</td>
<td>3.1</td>
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<tr>
<td>103</td>
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<tr>
<td>451</td>
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Table 5 Inter-assay precision

<table>
<thead>
<tr>
<th>Concentration μg/l (n = 8)</th>
<th>CV %</th>
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<tbody>
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<td>52</td>
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<td>216</td>
<td>6.6</td>
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<tr>
<td>435</td>
<td>4.0</td>
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</table>
Sensitivity

The sensitivity of the method, defined as the detectable concentration equivalent to twice the standard deviation of the zero binding value is 1.2 μg/l.

REFERENCES


**Kit Contents**
(KIT SUFFICIENT FOR 100 TUBES)
- PICP $[^{125}]$ inbuffer solution
- PICP antiserum, ready to use
- PICP standards, ready to use
- PICP controls LYOPH.
- PICP separation agent

**Assay procedure - summary**
(all volumes given in $\mu$l - any patient sample for NSB)

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>NSB</th>
<th>Standard</th>
<th>Control and Unkn.</th>
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<td>100</td>
<td>100</td>
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<tr>
<td>Pipette PICP $[^{125}]$ (red)</td>
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<td>200</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Pipette PICP antiserum (blue)</td>
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<tr>
<td>Pipette distilled water</td>
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<td>200</td>
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<tr>
<td>Vortex</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Incubate 2h/37°C</td>
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<td>x</td>
<td>x</td>
<td>x</td>
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<tr>
<td>Pipette separation agent</td>
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<tr>
<td>Vortex</td>
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<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Incubate 30 min/RT</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Centrifuge 15 min/2000g</td>
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<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Aspirate or decant</td>
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<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Count for 1 min or 10000 counts</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
INTACT PTH — PARATHYROID HORMONE

DIRECTIONAL INSERT
GEBRAUCHSANLEITUNG
NOTICE D’EMPLOI

Nichols Institute Diagnostics
INTACT PTH
For the quantitative determination of human intact parathyroid hormone in serum

INTACT PTH Parathyroid Hormone 100T Kit - Catalog No. 40-2170

1 Vial Reagent A (PTH Antibody Coated Beads)
2 Vials Reagent B (3H PTH Antibody Solution)
<10 μCi/vial =<370 kBq/vial
6 Vials Reagent C, D-H (Standards)
1 Vial Reagent D (Optional Standard)
1 Vial Reagent E (Wash Solution Concentrate)
2 Vials Reagent J-K (Controls 1&2)

For In Vitro Diagnostic Use
Store at 2-8°

SUMMARY AND BACKGROUND

The chemical structure of human parathyroid hormone has been established over the last decade. Biologically active and intact human parathyroid hormone (PTH) is an 84 amino acid polypeptide. It is initially biosynthesized in the parathyroid gland as a larger molecular precursor consisting of 115 amino acids, referred to as pre-proparathyroid hormone. Preproparathyroid hormone is converted to an intermediate, or proparathyroid hormone, consisting of 90 amino acids. Proparathyroid hormone is then converted by additional proteolytic modification to parathyroid hormone.

Considerable evidence suggests that there is proteolytic modification of the hormone intraglandularly: during hypercalcemia and reduced secretion of parathyroid hormone there is further extensive intracellular degradation of the intact polypeptide to smaller fragments. In addition to the formation of fragments within the gland, there is peripheral proteolytic modification of the intact (1-84) amino acid polypeptide. Upon release from the gland into the general circulation, it is metabolized by the liver and the kidney. This peripheral metabolism of PTH results in the production of peptide fragments comprised chiefly of the middle and carboxyl portions of the molecule. Thus, human serum contains a mixture of PTH molecules including the intact hormone and much greater concentrations of fragments comprising the middle and carboxyl portion of the hormone.

Only very low concentrations of the N-fragment(s), if present in the serum at all, contribute to the circulating pool of immunoreactive parathyroid hormone.

The major PTH peptides found in the circulation have different metabolic half-lives. The native or intact peptide has a half-life measured in minutes, whereas the fragments comprising the middle and carboxyl terminal portions of the molecule have half-lives that are 10 to 20-fold longer than the native hormone. For this reason, the concentration of biologically inactive fragments representing the middle and carboxyl regions of the molecule is high relative to that of the biologically active intact hormone. This ratio varies from one normal individual to another and in patients with hyperparathyroidism, and is predictably increased in patients with chronic renal failure.

Nichols Institute Diagnostics now makes available an Intact PTH Immunoassay which accurately qualifies intact human PTH in the normal range and in elevated and suppressed ranges. The system has a sensitivity of 1pg/mL of PTH 1-84, and is highly specific for only the biologically active and intact PTH molecule. This assay has been validated by extensive clinical studies in well defined populations of various parathyroid states.
(Data on file.)
**CLINICAL SIGNIFICANCE**

PTH plays an important role in calcium homeostasis by maintaining the concentration of ionized calcium within the precise limits necessary to achieve metabolic and neuroregulatory functions of this essential mineral. At low levels of serum calcium, secondary to an inadequate assimilation of dietary calcium, parathyroid glands increase secretion of the hormone. This results in mobilization of calcium from the large skeletal stores into the extra-cellular fluid, increased absorption of dietary calcium, and decreased renal clearance of urinary calcium. When serum calcium levels increase, smaller quantities of parathyroid hormone are secreted into the bloodstream.

The measurement of PTH is an important aid in the assessment of calcium metabolism disorders. When evaluated along with calcium levels, the quantitation of PTH values has proven useful in distinguishing normal individuals from patients with primary hyperparathyroidism, hypoparathyroidism and patients with tumor hypercalcaemia. The high and variable concentrations of biologically inactive PTH fragments have interfered with the use of C-terminal and mid-region PTH assays to assess parathyroid function in patients with compromised renal function. Intact and N-terminal specific assays, which measure only the biologically active hormone, provide a more precise assessment of parathyroid function in these patients, including those with renal disease as well as those with age-related diseases in glomerular filtration.

**TEST PRINCIPLE**

The Intact PTH Immunoassay is a two-site immunoradiometric assay (IRM A) for the measurement of the biologically intact 84 amino acid chain of PTH. Two different goat polyclonal antibodies to human PTH have been purified by affinity chromatography to be specific for well defined regions on the PTH molecule. One antibody is prepared to bind only the mid-region and C-terminal PTH 38-84 and this antibody is immobilized onto plastic beads. The other antibody is prepared to bind only the N-terminal PTH 1-34 and this antibody is radiolabeled for detection.

The sample containing PTH is incubated simultaneously with an antibody-coated bead and 125I-labeled antibody. Intact PTH present in the sample is bound by both the immobilized and labeled antibodies to form a “sandwich” complex:

\[
\text{Bead Anti-PTH (39-84) — Intact PTH (1-84) — }^{125}\text{I Anti-PTH (1-34)}
\]

Although mid-region and C-terminal fragments are bound by the antibody coated bead, only the intact PTH 1-84 forms the sandwich complex necessary for detection. The capacity of the immobilized antibody has been adjusted to exhibit no interference by inactive fragments, even at very elevated levels.

At the end of the assay incubation, the bead is washed to remove unbound components and the radioactivity bound to the solid phase is measured in a gamma counter. Since the formation of a sandwich complex occurs only in the presence of an intact PTH molecule, the radioactivity of the bead bound complex is directly proportional to the amount of intact PTH in the sample.

A dose response curve of radioactivity vs. concentration is generated using results obtained from standards which are assayed concurrently with the unknowns. Concentrations of intact PTH present in the controls and patient samples are determined directly from this curve.
PRECAUTIONS

This radioactive material may be received, acquired, possessed and used by physicians, clinical laboratories or hospitals, and only for in vitro laboratory tests not involving internal or external administration of the material, or the radiations therefrom, to human beings or animals. Its receipt, acquisition, possession, use, and transfer are subject to the regulations and a general license of the U.S. Nuclear Regulatory Commission, or a state with which the commission has entered into an agreement for the exercise of regulatory authority.

1. Radioactive material should be stored in a special designated area in the original container. This storage area should be as far from the work area as practical.
2. Radioactive material should be used only in designated work areas.
3. **Radioactive materials should not be pipetted by mouth.**
4. Spills should be wiped up with a suitable absorbent material, and the involved surfaces washed with an alkaline detergent. The contaminated absorbent material should be placed in a suitable container for disposal.
5. Unused radioactive material from this kit should be disposed of in accordance with the recommendations of local regulatory agencies.
6. Do not eat or drink in the designated areas. Food handling and storage, as well as smoking and other similarly potentially hazardous activities, are prohibited in these areas.
7. Practice good hygiene by avoiding contact with radioactive materials through use of disposable gloves, laboratory coats and other protective devices. Wash thoroughly after handling radioactive materials.
8. Persons under 18 should not be permitted to handle radioactive material or enter radioactive areas.
9. Radioactive areas must be kept exceptionally clean. Contamination of the laboratory and equipment can usually be prevented by careful work procedures, disposable or easily decontaminated laboratory ware, and use of absorbent covers on lab bench surfaces.

Users are expected to adhere to these precautions which are in accord with "California Radiation and Control Regulations", Section 30192(a) (3), and other similar regulations.

**For In Vitro Diagnostic Use**

**CAUTION: Potential Biohazardous Material**

HANDLE ASSAY REAGENTS AS IF CAPABLE OF TRANSMITTING AN INFECTIOUS AGENT.

The human source material used in the preparation of this product has been tested by an FDA approved method for the presence of the antibody to Human Immunodeficiency Virus (HIV) as well as for hepatitis B surface antigen, HVC and found to be negative. Because no test method can offer complete assurance that HIV, hepatitis B virus, HVC or other infectious agents are absent, these reagents should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health Manual "Biosafety in Microbiological and Biomedical Laboratories," 1984.

**NOTE:** Reagents in this assay contain sodium azide as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush drains with generous amounts of cold water to prevent azide buildup. In addition, consult the manual guideline "Safety Management No. CDC-22, Decontamination of Laboratory Sink Drains to Remove Azide Salts" (Centers for Disease Control, Atlanta, Georgia, April 30,1976).
SPECIMEN COLLECTION AND PREPARATION

The determination of intact PTH should be performed on serum. EDTA plasma has also been shown to be an acceptable sample. To assay the specimen in singlicate, 200 µL of serum is required (duplicate 400 µL). For the most accurate comparison with normal values, a fasting morning serum sample should be obtained. Collect blood sample in a red-top venipuncture tube (no additives) and allow blood to clot. Centrifuge the sample, preferably in a refrigerated centrifuge, and separate serum from cells.

FREEZE SAMPLE IMMEDIATELY (-20°C or below) or store as outlined in the stability table below.

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<thead>
<tr>
<th>STORAGE CONDITION</th>
<th>TIME</th>
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<tbody>
<tr>
<td>At room temperature after collection</td>
<td>2 hours</td>
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<tr>
<td>Refrigerated at 4°C</td>
<td>8 hours</td>
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<tr>
<td>Frozen at - 20°C</td>
<td>4 months</td>
</tr>
<tr>
<td>Frozen at - 70°C</td>
<td>11 months</td>
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MATERIALS

Materials Provided:

1. **PTH Antibody Coated Beads** (30-0510) - Reagent A
   1 container of 100 anti-PTH 39-84 (goat) coated polystyrene beads - 8 mm diameter.

2. **¹²⁵I-PTH Antibody Solution** (30-0511) - Reagent B
   2 vials each containing 5.5 mL of ¹²⁵I-labeled anti-PTH 1-34 (goat) in phosphate buffered saline with (protein stabilizers) and 0.1% sodium azide. Each vial contains less than 10 µCi (370 kBq) of radioactivity.

3. **Intact PTH Zero Standard** (30-0512) - Reagent C
   1 vial containing lyophilized buffered human serum with 0.1% sodium azide.

4. **Intact PTH Standards** (30-0513 to 0517) - Reagents D-H
   5 vials each containing human PTH 1-84 lyophilized in buffered human serum with 0.1% sodium azide; refer to vial labels for exact concentrations.

5. **Wash Solution Concentrate** (30-0010) - Reagent I
   1 vial containing 50 mL of a surfactant in phosphate buffered saline with 0.2% sodium azide.

6. **Intact PTH Controls** (30-0518, 0519) Reagents J-K
   2 vials each containing human PTH 1-84 lyophilized in buffered human serum with 0.1% sodium azide; refer to the enclosed Technical Data Sheet for control ranges.

(Optional)

7. **Intact PTH Standard** (30-0509) Reagent D
   1 vial containing human PTH 1-84 lyophilized in buffered human serum with 0.1% sodium azide; refer to vial label for exact concentration.
Materials Required But Not Provided:

1. Polystyrene or polypropylene test tubes, round bottom, 12 x 75 mm.
2. Standard test tube rack or Automated Bead Wash Station assay rack (catalog #35-0015).
3. Precision pipettors: 100 µL and 200 µL.
4. Repeating dispenser: 2.0 mL.
5. Parafilm or equivalent for covering tubes.
6. Bead dispenser (catalog #39-8193) or multi-bead dispenser (catalog #35-0048) for use with Automated Wash Station assay rack.
7. Decanting System (catalog #39-8190) or Nichols Institute Diagnostics System Washer or equivalent.
8. Container for storage of Wash Solution.
9. Gamma counter.
10. Distilled or deionized water.
11. Vortex mixer.
12. Timer.
13. Repeating syringes capable of precisely dispensing 100 µL (optional).
14. 2.0 mL and 4.0 mL volumetric pipets for reconstituting standards and controls.

REAGENT PREPARATION AND STORAGE

1. Reagent C: Intact PTH Zero Standard
   Reconstitute the vial with 4.0 mL of distilled or deionized water. Allow the vial to stand 5 minutes then mix thoroughly by gentle inversion to insure complete reconstitution.
2. Reagents D<sub>1</sub>(optional), D<sub>2</sub>-H: Intact PTH Standards
   Reconstitute each vial with 2.0 mL of distilled or deionized water. Allow the vial to stand 5 minutes then mix thoroughly by gentle inversion to insure complete reconstitution.
3. Working Reagent I: Wash Solution
   Mix contents of Wash Solution concentrate thoroughly. Add Wash Solution concentrate (50 mL) to 450 mL of distilled or deionized water and mix.
4. Reagents J & K: Intact PTH Controls
   Reconstitute each vial with 2.0 mL of distilled or deionized water. Allow the vial to stand 5 minutes then mix thoroughly by gentle inversion to insure complete reconstitution.
5. Completely mix all reagents before use, by gentle agitation or swirling.
6. Store all kit components at 2-8°C upon receipt prior to use.
7. Use standards and controls immediately upon reconstitution. Freeze (-20°C) the remaining standards and controls immediately after use. Standards and controls are stable -20°C for 6 weeks after reconstitution with up to 3 freeze thaw cycles when handled as recommended in "procedural notes" section. Store other components at 2-8°C. Wash Solution upon dilution may be stored at room temperature.

ASSAY PROCEDURE

1. Pipet 200 µL of standard (Reagents C-H), control (Reagents J-K) or patient sample into appropriately labeled tubes.
2. Pipet 100 µL of the 125I-PTH Antibody Solution (Reagent B) into all tubes.
3. Vortex all tubes.
4. Using Bead Dispenser add one bead (Reagent A) to each test tube. If using forceps, tilt test tube rack to approximately a 30 degree angle and add one bead to each tube. Cover test tube rack with Parafilm® or equivalent.
5. Incubate test tubes at room temperature for 22 ± 2 hours.
6. Wash beads twice by dispensing 2.0 mL working Wash Solution into each tube and completely, decanting or aspirating the liquid from each tube. Alternatively, use an automated bead wash station programmed to aspirate the reaction mixture and then wash twice with 2.0 mL working Wash Solution.
7. Count each test tube in gamma counter for one minute and record count.
## INTACT PTH Immunoassay Flow Chart

<table>
<thead>
<tr>
<th>TUBE NUMBER</th>
<th>TUBE LABEL</th>
<th>STANDARDS CONTROLS/PATIENTS</th>
<th>¹²⁵I-PTH ANTIBODY SOLUTION REAGENT B</th>
<th>ANTIBODY COATED BEAD REAGENT A</th>
<th>INCUBATE AT ROOM TEMPERATURE</th>
<th>WASH SOLUTION WORKING REAGENT I</th>
<th>MEASURE RADIOACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>ZERO STD REAGENT C</td>
<td>200 µL</td>
<td>100 µL</td>
<td>One Bead</td>
<td>2.0 mL</td>
<td>Wash Twice Aspirate or Decant</td>
<td>Count One Minute</td>
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</tbody>
</table>

If data reduction requires total count tubes, label duplicate tubes appropriately, pipet 100 µL of ¹²⁵I-PTH Antibody Solution (Reagent B) into each tube and cap.
PROCEDURAL NOTES

1. Intact PTH 1-84 is a very labile molecule. Set up the assay immediately upon the reconstitución or the thawing of all standards, controls, and samples. Since the key factors in maintaining the integrity of the intact hormone are control of the time and temperature, it is strongly recommended that the standards, controls, patient samples, and assay tubes be kept on ice while setting up the assay.

2. It is recommended that all standards and controls be assayed in duplicate. The average counts per minute of duplicate sets should then be used for reduction of data and the calculation of results.

3. The sample and the $^{125}$I-PTH Antibody Solution (Reagent B) should be carefully pipetted into the bottom one-fourth of the test tube.

4. The Bead Dispenser or forceps should be used for transferring beads to the test tubes. Do not use fingers to transfer beads. When using forceps, tilting the test tube rack prior to adding the beads allows the beads to roll into the reaction solution without excessive splashing.

5. The washing step is an important part of the total assay procedure. Add the Wash Solution with sufficient force to raise the bead from the bottom of the test tube. Thorough and complete aspiration or decanting of the Wash Solution is essential.

6. Patient samples with values greater than the highest standard (Reagent H) may be diluted with zero standard (Reagent C) and reassayed. Multiply the result by the dilution factor.

7. Each component used in any one assay should be of the same lot number and stored under identical conditions.

8. In order to properly evaluate the performance of any immunoassay it is necessary to incorporate adequate controls. Nichols Institute Diagnostics strongly recommends that all laboratories include properly aliquoted and stored in-house patient pools, in addition to those provided with the product, when performing the assay.

CALCULATIONS

Standard Curve:

The standard curve is generated using prepared intact PTH standards. Refer to individual vial labels for exact concentrations.

Generate the curve as follows:*  
1. Calculate the average CPM for each pair of assay tubes.
2. Subtract the average CPM of the Zero Standard tubes from all other average counts to obtain corrected CPM.
3. The standard curve is prepared by plotting the corrected CPM of each standard level on the ordinate against the standard concentration on the abcissa using log-log graph paper.

*Computer assisted data reduction programs for IRMA assays may also be used for calculations of the Intact PTH Immunoassay. The four parameter logistics program per Rodbard is one of these methods.

Standard Calculation Example:

<table>
<thead>
<tr>
<th>Description</th>
<th>CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average CPM of Zero Standard</td>
<td>1023</td>
</tr>
<tr>
<td>Average CPM of 7.5 pg/mL Standard</td>
<td>1561</td>
</tr>
<tr>
<td>Corrected CPM</td>
<td></td>
</tr>
<tr>
<td>CPM (7.5 pg/mL) - CPM (0 pg/mL)</td>
<td>538</td>
</tr>
</tbody>
</table>
Values Less Than Standard $D_2$:

NOTE: If optional Standard $D_1$ (7.5 pg/mL) is used in the assay, substitute Standard $D_1$ for Standard $D_2$ in the calculation below.

Value of unknown = \[
\frac{\text{Corrected CPM (unknown)}}{\text{Corrected CPM (Std } D_2)} \times \text{Value of Std } D_2
\]

Intact PTH Immunoassay
Sample Standard Curve

The data is presented as an example only and should not be used in lieu of a standard curve prepared with each assay.
## Sample Data: Intact PTH Immunoassay

<table>
<thead>
<tr>
<th>TUBE NUMBER</th>
<th>TUBE LABEL</th>
<th>CPM</th>
<th>AVERAGE CPM</th>
<th>CORRECTED CPM</th>
<th>RESULT pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>STD C 0 pg/mL</td>
<td>1144</td>
<td>901</td>
<td>1023</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>STD D 7.5 pg/mL</td>
<td>1558</td>
<td>1563</td>
<td>1561</td>
<td>538</td>
</tr>
<tr>
<td>3</td>
<td>STD D 17 pg/mL</td>
<td>2347</td>
<td>2298</td>
<td>2323</td>
<td>1300</td>
</tr>
<tr>
<td>4</td>
<td>STD E 58 pg/mL</td>
<td>5240</td>
<td>5273</td>
<td>5257</td>
<td>4243</td>
</tr>
<tr>
<td>5</td>
<td>STD F 185 pg/mL</td>
<td>14695</td>
<td>15067</td>
<td>14881</td>
<td>13858</td>
</tr>
<tr>
<td>6</td>
<td>STD G 580 pg/mL</td>
<td>40121</td>
<td>38920</td>
<td>39521</td>
<td>38498</td>
</tr>
<tr>
<td>7</td>
<td>STD H 1750 pg/mL</td>
<td>78455</td>
<td>77121</td>
<td>77788</td>
<td>76765</td>
</tr>
<tr>
<td>8</td>
<td>Control J</td>
<td>3724</td>
<td>3808</td>
<td>3724</td>
<td>2701</td>
</tr>
<tr>
<td>9</td>
<td>Control K</td>
<td>23034</td>
<td>23342</td>
<td>23188</td>
<td>22165</td>
</tr>
<tr>
<td>10</td>
<td>Control K</td>
<td>23034</td>
<td>23342</td>
<td>23188</td>
<td>22165</td>
</tr>
</tbody>
</table>


QUALITY CONTROL

The reproducibility of standard curve parameters and control sera values should be within established limits of laboratory acceptability. Commonly used measures of variability are discussed by Rodbard, et al. If the precision of the assay does not comply with the established limits and repetition excludes errors in technique, check the following areas:

1. Pipetting and timing devices.
2. Gamma counter calibration (including crystal placement).
3. Expiration date on reagent package and prepared reagents.
4. Storage and incubation conditions.
5. Cleanliness of all system components.
6. Purity of water.
7. Thoroughness of washing step.

LIMITATIONS

The highest concentration of intact PTH measurable without dilution is the value of the highest standard (Reagent H) and the lowest measurable concentration is 1.0 pg/mL (assay sensitivity).

Variations in protein concentrations of ± 25% have little or no effect on the value of intact PTH obtained.

The Intact PTH Immunoassay has been designed so that the high dose "hook effect" is not a problem for samples with elevated PTH values. Samples with intact PTH levels with between the highest standard (Reagent H) and 100,000 pg/mL will read greater than the highest standard and should be diluted and reassayed for correct values.

Like any analyte used as a diagnostic adjunct, intact PTH results must be interpreted carefully with the overall clinical presentations and other supportive diagnostic tests.

Immunoassays are optimized and calibrated for the determination of antigens in their intact and unaltered state. Genetic variations or degradation of antigens into subunits or other fragments may alter antibody binding characteristics and affect final results. Such samples may exhibit discordant results between different assays as the effect of such altered states are particular to each defined antibody assay.

EXPECTED VALUES

Nichols Institute Diagnostics recommends that each laboratory establish its own range of expected values. The levels of intact PTH were determined in sera from two hundred fifty three (253) apparently healthy, fasting adults using the Intact PTH Immunoassay. The geometric mean ±2 standard deviations were calculated to be 10 to 65 pg/mL. See Figure 2.

Eighty-eight (88) patients with surgically confirmed primary hyperparathyroidism were evaluated. Intact PTH values ranged from 53 to 1160 pg/mL. Total calcium values ranged from 10.5 to 16.3 mg/dL.

Values of intact PTH found in sixty-three (63) patients with hypercalcemia of malignancy (tumor hypercalcemia) ranged from undetectable (<1 pg/mL) to 22 pg/mL. Fifty-nine (59) of the sixty-three (63) samples were below the lower limit of the normal range. Total calcium values ranged from 10.5 to 17.6 mg/dL.

Eight (8) patients diagnosed as having hypoparathyroidism had values of intact PTH ranging from undetectable (<1 pg/mL) to 21 pg/mL. Total calcium values ranged from 6.3 to 8.5 mg/dL.

PTH values in one hundred twenty-three (123) patients with chronic renal failure were measured and analyzed with respect to results from quantitative bone histomorphometry. The intact PTH levels in the 34 patients with advanced osteitis fibrosa were higher showing a mean of 1033±103 pg/mL (±SE) than in those with the early form of osteitis fibrosa (mean of 362±56 pg/mL). All PTH levels in those patients with osteitis fibrosa by biopsy were higher than normal, except for two patients with early disease. PTH
levels in those with advanced osteitis were higher than those of any patient with osteomalacia and higher than 90% of those with aplastic bone disease.

Interpretation of intact PTH results should take into account the serum calcium concentrations and the physiological interplay between these two elements in the various PTH/calcium disorders. See Figure 2. Consistent with good clinical practice, it is recommended that intact PTH results, even when used in conjunction with calcium values, should be interpreted with caution and with consideration of the overall clinical manifestations.

Results presented in this study represent a limited number of samples. It should be noted that some overlap of intact PTH values from patients with disorders of parathyroid functions does exist. See Figure 2.

Additionally, it is important to assure proper handling and storage of serum samples. Improper storage of samples may result in loss of assayable intact PTH.

Figure 1: Bone histomorphometry and Intact PTH (IRMA) values in chronic renal disease.
Figure 2: Intact PTH (IRMA) values and total calcium in various disease states.
SPECIFIC PERFORMANCE CHARACTERISTICS

Precision and Reproducibility

The precision (intra-assay variance) of the Intact PTH Immunoassay was calculated from replicate determinations on each of two quality control sera in a single assay (n=20). The reproducibility (inter-assay variance) was calculated from data obtained during a four week period (n=20).

<table>
<thead>
<tr>
<th>INTRA-ASSAY VARIATION</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Value (pg/mL)</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>3.4%</td>
</tr>
<tr>
<td>266</td>
<td>1.8%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>INTER-ASSAY VARIATION</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Value (pg/mL)</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>5.6%</td>
</tr>
<tr>
<td>277</td>
<td>6.1%</td>
</tr>
</tbody>
</table>

Accuracy

The Intact PTH Immunoassay was compared to the Nichols Institute Diagnostics INS-PTH Radioimunoassay. A population of 143 samples from both normal and abnormal individuals was assayed by each method. Least squares regression analysis was performed on the comparative data; a correlation coefficient (r) = 0.98 was obtained.

Sensitivity

The sensitivity of this assay is defined as the smallest single value which can be distinguished from zero at the 95% confidence limit. The Intact PTH Immunoassay has a calculated sensitivity of 1 pg/mL.

Specificity and Cross-Reactivity

The antibodies used in the Intact PTH Immunoassay were purified by an affinity chromatography to be specific for well defined regions on the PTH molecule. The $^{125}$I-labeled antibody recognizes only the N-terminal 1-34 amino acid sequence of PTH and has zero cross-reactivity with the 39-84 segment. The antibody immobilized on the bead recognizes only the mid and C-terminal 39-84 amino acid sequence and has zero cross-reactivity with the 1-34 segment. When these antibodies are employed together in a two-site immunoradiometric assay, only intact PTH is measured.

To further validate the fact that PTH fragments do not cross-react or interfere with results obtained using the Intact PTH Immunoassay, various PTH fragments representing highly elevated physiological concentrations were studied. Human PTH 1-34 at a concentration of 300 pg/mL and human PTH fragments 39-68, 53-84, 44-68 and 39-84 each at concentrations of 100,000 pg/mL were spiked into the zero standard (Reagent C) and into the mid-range standard (Reagent F). The results showed zero crossreactivity and zero interference from any of the fragments assayed in this manner.
Recovery

Various amounts PTH (1-84) were added to three different sera to determine the recovery. The results are described in the following table:

<table>
<thead>
<tr>
<th>Serum Sample</th>
<th>Endogenous PTH (pg/mL)</th>
<th>PTH Added (pg/mL)</th>
<th>Expected Value (pg/mL)</th>
<th>PTH Recovered (pg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>147</td>
<td>153</td>
<td>160</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>219</td>
<td>225</td>
<td>224</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>290</td>
<td>236</td>
<td>307</td>
<td>104</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>176</td>
<td>203</td>
<td>209</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>258</td>
<td>285</td>
<td>290</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>339</td>
<td>366</td>
<td>372</td>
<td>102</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>256</td>
<td>282</td>
<td>265</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>378</td>
<td>404</td>
<td>376</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>498</td>
<td>524</td>
<td>503</td>
<td>96</td>
</tr>
</tbody>
</table>

Parallelism

Three patients sera were diluted with Zero Standard. Results in pg/mL are shown below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Measured</th>
<th>Results Corrected with Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Undiluted</td>
<td>759</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>387</td>
<td>774</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>194</td>
<td>776</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>94</td>
<td>752</td>
</tr>
<tr>
<td>B</td>
<td>Undiluted</td>
<td>501</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>233</td>
<td>466</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>113</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>61</td>
<td>488</td>
</tr>
<tr>
<td>C</td>
<td>Undiluted</td>
<td>468</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>221</td>
<td>442</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>109</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>57</td>
<td>456</td>
</tr>
</tbody>
</table>

BIBLIOGRAPHY


**Intact PTH Animal References**

**Dogs**


**Dogs**

INTACT PTH
Immmunoassay für die quantitative Bestimmung von intaktem humanen Parathormon in EDTA-Plasma oder Serum

Kit für 100 Bestimmungen - Cat No. 40-2170

1 Dose Reagenz A (PTH Antikörper beschichtete Kugeln)
2 Fläschchen Reagenz B (125JPTH Antikörperlösung <10 μCi/Flasche oder <370 kBq/Flasche)
6 Fläschchen Reagenz C, D, H (Standards)
1 Fläschchen Reagenz D, (optional)
1 Fläschchen Reagenz I (Wasserlösungskonzentrat)
2 Fläschchen Reagenz J-K (Kontrollen 1 & 2)

Achtung: Radioaktives Material
Nicht zum inneren oder äußeren Gebrauch bei Menschen oder Tieren

Nur für in vitro Diagnostik
Beider Testdurchführung Gebrauchsanleitung beachten! Lagerung bei 2-8°C

ZUSAMMENFASSUNG UND HINTERGRUND


Nur eine sehr geringe Konzentration des N-terminalen Fragments, falls überhaupt vorhanden, trägt zum Gesamtpool des immunoreaktiven Parathormones bei.


KLINISCHE BEDEUTUNG

Das PTH spielt eine wichtige Rolle bei der Kalziumhomöostasis durch Aufrechterhaltung der ionisierten Kalziumkonzentration innerhalb ganz bestimmter Grenzen, wichtig um die metabolische neuroregulatorische Funktion dieses lebensnotwendigen Minerals zu erzielen. Im Falle von niedrigen

Die Messung des PTH ist eine wichtige Hilfe bei der Feststellung von Kalziumstoffwechsel-Störungen. Bezieht man die Kalziumwerte bei der Auswertung mit ein, erweist sich die Quantifizierung der PTH Werte als nützlich, um Gesunde von Patienten mit primären Hyperparathyreoidismus, Hypoparathyreoidismus und Patienten mit Tumorhypercalcämie (3,9,12,25,27) zu unterscheiden.


TESTPRINZIP


Die PTH enthaltende Probe wird gleichzeitig mit einer Antikörper beschichteten Kugel und 125I markierten Antikörper inkubiert. Das in der Probe vorhandene PTH wird von beiden, dem fixierten wie auch dem markierten Antikörper, zu einem "Sandwich-Komplex" gebunden.

Kugel Anti-PTH (39-84) — Intaktes PTH (1-84) — 125I Anti-PTH (1-34)

Obwohl Mittelregion und C-terminale Fragmente von dem Antikörper überzogenen Kugeln gebunden werden, bildet jedoch nur das intakte PTH 1-84 den "Sandwich-Komplex" der für den Nachweis notwendig ist. Die Kapazität des fixierten Antikörpers wurde so angeglichen, daß keine Beeinträchtigung durch inaktive Fragmente, auch bei sehr hohen Werten, auftritt. Am Ende der Assayinkubation wird die Kugel gewaschen, um die nicht gebundenen Komponenten zu entfernen. Die auf der festen Phase gebundene Radioaktivität wird in einem Gammazähler gemessen. Da die Formation eines "Sandwich-Komplexes" nur in Gegenwart eines intakten PTH Moleküles stattfindet, steht die Radioaktivität des kugelgebundenen Komplexes in direktem Verhältnis zu der in der Probe vorhandenen Menge an intaktem PTH.

Mit den gemessenen Counts der Standards wird eine Eichkurve erstellt. Die Konzentration der intakten PTH Kontroll- und Patientenproben werden direkt aus dieser Kurve ermittelt.

VORSICHTSMAßREGELN


2) Mit radioaktivem Material darf nur in gekennzeichneten Arbeitsbereichen gearbeitet werden.

3) Radioaktive Substanzen dürfen nicht mit dem Mund pipettiert werden.
4) Spritzer sollten mit geeignetem absorbierendem Material aufgewischt und die betreffende Oberfläche mit alkalischem Reinigungsmittel gereinigt werden. Das kontamierte absorbierende Material muß in einen dafür vorgesehenen Abfallbehälter geworfen werden.

5) Nicht genutzte radioaktive Substanzen dieses Besteckes müssen gemäß den Bestimmungen der lokalen Aufsichtsämter entsorgt werden.

6) Essen oder Trinken ist in diesen Bereichen verboten, ebenso wie Nahrungsmittellagerung, Rauchen oder ähnliche Aktivitäten.


8) Personen unter 18 Jahren ist es nicht gestattet mit radioaktivem Material umzugehen oder radioaktive Bereiche zu betreten.


Von den Anwendern wird erwartet, daß sie sich an die Vorsichtsmaßregeln gemäß der Strahlenschutz-Verordnung und anderen entsprechenden Bestimmungen halten.

**Nur zum diagnostischen in vitro Gebrauch**

**Achtung: Potentiell gefährliches Biomaterial**


**PROBENSAMMLUNG UND VORBEREITUNG**


Frieren Sie die Probe sofort ein (-20°C oder niedriger) und lagern sie wie in der folgenden Tabelle angegeben:

<table>
<thead>
<tr>
<th>Lagerbedingungen</th>
<th>Zeit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bei Raumtemperatur nach Abnahme</td>
<td>2 Stunden</td>
</tr>
<tr>
<td>gekühlt bei 4°C</td>
<td>8 Stunden</td>
</tr>
<tr>
<td>gefroren bei -20°C</td>
<td>4 Monate</td>
</tr>
<tr>
<td>gefroren bei -70°C</td>
<td>11 Monate</td>
</tr>
</tbody>
</table>
1. **PTH Antikörper beschichtete Kugeln - Reagenz A**
   Behälter mit 100 anti-PTH 39-84 Ziegenantikörper beschichtete Polystyren Kugeln 8 mm Durchmesser

2. **125I-J-PTH Antikörper Lösung - Reagenz B**
   2 Flaschen mit je 5,5 ml 125I-markiertes anti-PTH 1-34 Ziegenantikörper in Phosphat gepufferter Kochsalzlösung mit Proteinstabilisatoren und 0.1% Natriumazid. Jede Flasche enthält weniger als 10 μCi (370 kBq) Radioaktivität

3. **Intakt PTH Null Standard - Reagenz C**
   1 Flasche mit lyophilisiertem Humanserum mit 0.1 % Natriumazid.

4. **Intakt PTH Standards - Reagenz D - H**
   5 Flaschen mit human PTH 1-84 lyophilisiert in Serum mit 0.1%, Natriumazid; die exakte Konzentration finden Sie auf dem Flaschenetikett.

5. **Waschlösungskonzentrat - Reagenz I**
   1 Fläschchen mit 50 ml eines Surfactant in Phosphat gepufferter Kochsalzlösung mit 0.2 % Natriumazid.

6. **Intakt PTH Kontrollen - Reagenz J - K**
   2 Fläschchen mit PTH 1-84 lyophilisiertem Humanserum mit 0.1% Natriumazid; die Kontrollbereiche finden Sie in dem beiliegenden Qualitätskontrollblatt.

7. **Intakt PTH Standard - Reagenz D, (optional)**
   1 Fläschchen mit human PTH 1-84 lyophilisiert in Serum mit 0,1% Natriumazid, die exakte Konzentration finden sie auf dem Flaschenetikett.

Zusätzlich erforderliches Zubehör:

1. Polystyren oder Polypropylen Teströhrchen, mit abgerundetem Boden, 12 x 75 mm
2. Reagenzglasständere
3. Pipetten: 100 μl und 200 μl
4. Mehrfach-Dispensor: 2.0 ml
5. Parafilm oder etwas gleichwertiges zur Abdeckung der Röhrchen
6. Kugel Dispensor (Katalog #8193) oder Pinzetten zum Transfer der Kugeln
7. Dekantiersystem (Katalog #8190) oder Aspirationsgerät
8. Flasche zur Aufbewahrung der Waschlösung
9. Gammazählern
10. Destilliertes oder entmineralisiertes Wasser
11. Vortexer
12. Stoppuhr
13. Multipipette für 100 μl (mikro)
14. 2.0 ml und 0.4 ml volumetrische Pipetten zur Rekonstitution der Standards und Kontrollen

**REAGENZVORBEREITUNG UND LAGERUNG**

1. Reagenz C: Intakt PTH Null-Standard
   Rekonstituieren Sie die Flasche mit 4.0 ml destilliertem oder entmineralisiertem Wasser. Lassen Sie die Flasche für 5 Minuten stehen und vermischen sie die Substanzen anschließend durch sanfte Drehung, um komplette Rekonstitution zu gewährleisten.

2. Reagenz D, (optional), D₂-H: Intakt PTH-Standard
   Rekonstituieren Sie jede Flasche mit 2.0 ml destilliertem oder entmineralisiertem Wasser. Lassen Sie jede die Flaschen für 5 Minuten stehen und vermischen Sie die Substanzen anschließend durch sanfte Drehung, um komplette Rekonstitution zu gewährleisten.

3. Arbeitsreagenz I: Waschlösung
   Mischen Sie das Waschlösungskonzentrat gründlich. Fügen Sie das Konzentrat (50 ml) zu 450 ml destilliertem Wasser hinzu und mischen es.

4. Reagenz J & K: Intakt PTH Kontrollen
   Rekonstituieren Sie jede Flasche mit 2.0 ml destilliertem Wasser. Lassen Sie sie für 5 Minuten.
# Intakt PTH Immunoassay Pipettierschema

<table>
<thead>
<tr>
<th>Röhrchen Nummer</th>
<th>Röhrchen Bezeichnung</th>
<th>Standards/ Kontrollen/ Patienten</th>
<th>125J-PTH-Antikörperlösung Reagenz B</th>
<th>Antikörper beschichtete Kugel Reagenz A</th>
<th>Inkubieren bei Raumtemperatur</th>
<th>Arbeits Waschlösung Reagenz I</th>
<th>Meßung der Radioaktivität</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>Null Std. Reagenz C</td>
<td>200 µl</td>
<td>100 µl</td>
<td>Eine Kugel</td>
<td></td>
<td>2.0 mL</td>
<td></td>
</tr>
<tr>
<td>3,4</td>
<td>Standard D₁ (optional)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,6</td>
<td>Standard D₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,8</td>
<td>Standard E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9,10</td>
<td>Standard F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11,12</td>
<td>Standard G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13,14</td>
<td>Standard H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15,16</td>
<td>Kontrolle 1 (J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17,18</td>
<td>Kontrolle 2 (K)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Patient 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Patient 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>etc.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inkubieren bei Raumtemperatur: 20 ± 2 Stunden
Zweimal waschen, Flüssigkeit dekantieren oder absaugen
Eine Minute

Meßung der Radioaktivität: Eine Minute
stehen und mischen sie anschließend gründlich durch leichte Inversion, um vollständige Rekonstitution zu gewährleisten.
5. Mischen Sie alle Reagenzien komplett vor Gebrauch durch leichtes Schütteln oder kreisende Bewegungen.
6. Lagern Sie alle Besteckkomponenten bei 2-8°C nach Erhalt bis zum Gebrauch.

ASSAY ARBEITSANLEITUNG
2. Pipettieren Sie 100 µl der Antikörperlösung (Reagenz B) in alle Röhrchen.
3. Vortexen Sie alle Röhrchen.
5. Inkubieren Sie die Teströhrchen bei Raumtemperatur für 22±2 Stunden.
6. Waschen Sie die Kugeln zweimal in dem Sie 2.0 ml Washlösung in die Röhrchen geben und die Flüssigkeit aus jedem Röhrchen komplett absaugen oder dekantieren.
7. Zählen Sie jedes Teströhrchen im Gammazählern für eine 1 Minute und zeichnen Sie die Impulsraten auf.

BEMERKUNGEN ZUR ARBEITSANLEITUNG
2. Es wird empfohlen, alle Standards und Kontrollen zweifach zu bestimmen. Die Durchschnittsimpulse pro Minute von den Doppelbestimmungen werden dann zur Ergebniskalkulation genutzt.
3. Die Probe und die 125 J-PTH Antikörperlösung (Reagenz B) muß vorsichtig in das untere Viertel des Teströhrchens pipettiert werden.
4. Sollten Sie Pinzetten benutzen, können Sie die Beads durch vorheriges Neigen des Reagenzglasständers, ohne übermäßiges Spritzen in die Reaktionslösung rollen lassen. Um die Beads in das Teströhrchen zu transferrieren, sollten Sie den Kugel Dispenser oder Pinzetten benutzen, nicht Ihre Finger.
6. Sollte der Counter Total-Counts erfordern, markieren Sie die Duplikatröhrchen entsprechend und pipettieren Sie 100 µl 125 J-PTH Antikörperlösung (Reagenz B) in jedes Röhrchen und decken sie ab.
8. Jede Komponente, die in jedem einzelnen Assay benutzt wird, sollte von der gleichen Charge sein und unter gleichen Bedingungen gelagert werden.
**BERECHNUNGEN**

**Standardkurve:**
Die Standardkurve wird unter Verwendung gebrauchsfertiger PTH Standards erstellt. Die genaue Konzentration finden Sie auf den einzelnen Flaschenetiketten. Erstellen Sie die Kurve wie folgt:

2. Sie erhalten die korrekten CPM durch Subtraktion der Mittel-CPM der Null Standard Röhrchen von allen anderen Durchschnittsimpulsen.
3. Die Standardkurve wird durch Plotten der korrekten CPM jedes Standardwertes auf der Ordinate gegen die Standardkonzentration auf der Abzisse des doppelt logarithmischen Millimeterpapiers vorbereitet.


**STANDARD BERECHNUNGSBEISPIEL:**

Durchschnittliche CPM
- des Null Standards = 1023
- des 7,5 pg/ml Standard = 1561

Korrigierte CPM
- CPM (7,5 pg/ml) - CPM (0 pg/ml) = 1561 - 1023 = 538

Werte kleiner als Standard Dg:
ACHTUNG: Bei Verwendung des optionalen Standard D2 (7,5 pg/ml) ersetzt man den Standard Dg in der nachfolgenden Berechnungsformel durch den Standard D2.

Werte die kleiner als der Standard D2 aber größer als 1 pg/ml (Assay-Empfindlichkeit) sind, können nach folgender Formel berechnet werden:

\[
\text{Konzentration der Probe} = \frac{\text{korrigierte CPM (unbekannt)}}{\text{korrigierte CPM (Std. D2)}} \times \text{Konzentration von Std. D2}
\]

Intakt PTH Immunoassay
Beispiel einer Standard Kurve
<table>
<thead>
<tr>
<th>Röhrchen Nummer</th>
<th>Röhrchen Beschreibung</th>
<th>CPM</th>
<th>Mittelwert CPM</th>
<th>Korrigierte CPM</th>
<th>Ergebnis pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Std. C 0 pg/ml</td>
<td>1144</td>
<td>901</td>
<td>1023</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>901</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Std. D₁ 7.5 pg/ml</td>
<td>1558</td>
<td>1563</td>
<td>1561</td>
<td>538</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1563</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Std. D₂ 17 pg/ml</td>
<td>2347</td>
<td>2298</td>
<td>2323</td>
<td>1300</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>2298</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Std. E 58 pg/ml</td>
<td>5240</td>
<td>5273</td>
<td>5257</td>
<td>4243</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>5273</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Std. F 185 pg/ml</td>
<td>14695</td>
<td>15067</td>
<td>14881</td>
<td>13858</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>15067</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Std. G 580 pg/ml</td>
<td>40121</td>
<td>38920</td>
<td>39521</td>
<td>38498</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>38920</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Std. H 1750 pg/ml</td>
<td>78455</td>
<td>77121</td>
<td>77788</td>
<td>76765</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>77121</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Kontrolle J</td>
<td>3724</td>
<td>3808</td>
<td>3724</td>
<td>2701</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>3808</td>
<td></td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>17</td>
<td>Kontrolle K</td>
<td>23034</td>
<td>23342</td>
<td>23188</td>
<td>22165</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>23342</td>
<td></td>
<td></td>
<td>300</td>
</tr>
</tbody>
</table>
QUALITÄTSKONTROLLE

Die Reproduzierbarkeit der Standardkurven Parameter und Kontrollserenwerte sollte innerhalb der bereits vom Labor festgelegten Akzeptanzgrenzen liegen. Die im allgemeinen benutzten Variabilitätsmaße werden von Rodbard et al. erörtert. Sollte die Präzision des Assays sich nicht an die bereits ermittelten Grenzen halten und eine Wiederholung technische Fehler ausschließt, empfiehlt es sich folgende mögliche Fehlerquellen zu überprüfen:

1. Pipettier- und Meßgeräte
2. Eichung des Gammazählers (inklusive Kristallplazierung)
3. Verfalldatum auf der Reagenzpackung und gebrauchsfertiger Reagenzien
4. Lager- und Inkubationsbedingung
5. Reinheit aller Systemkomponenten
6. Reinheit des Wassers
7. Gründlichkeit des Waschschrittes

GRENZEN DES VERFAHRENS

Die höchste Konzentration des Intakt PTH, messbar ohne Verdünnung entspricht dem Wert des höchsten Standards (Reagenz H) und der niedrigsten messbaren Konzentration mit 1.0 pg/ml (Assay Empfindlichkeit).

Abweichungen der Proteinkonzentrationen von ±25 % haben nur geringen bis keinen Einfluß auf die erzielten Intakt PTH Werte. Das Intakt PTH Immunoassay System wurde so entwickelt, daß der "Hook Effekt" bei Proben mit erhöhten PTH Werten kein Problem darstellt. Proben mit intakten PTH Werten zwischen dem höchsten Standard (Reagenz H) und 100.000 pg/ml werden größer sein als der höchste Standard und sollten deshalb, um korrekte Werte hervorzubringen, verdünnt und nochmals neu bestimmt werden.

Wie jede Analyse, die zu diagnostischen Zwecken genutzt wird, muß man die Intakt PTH Ergebnisse unter Berücksichtigung der gesamten klinischen Darstellungen und anderer unterstützenden diagnostischen Tests beurteilen.

ERWARTETE WERTE


Die Werte von 8 Patienten mit der Diagnose Hypoparathyreoidismus lagen zwischen nicht nachweisbar (<1 pg/ml) und 21 pg/ml. Die Gesamtkalziumwerte lagen zwischen 6,3 und 8,5 mg/dl.


Es ist außerdem wichtig auf die richtige Handhabung und Lagerung der Serumproben zu achten. Unsachgemäße Lagerung der Proben kann zu niederigen PTH Werten führen, aufgrund des Zerfalls des PTH-Moleküls.

Abbildung 2: Intakt PTH Werte in Serum und Gesamt-Kalzium in verschiedenen Krankheitsstadien.
Abbildung 1: Knochenhistomorphometrie und Intakt PTH (IRMA) Werte in chronischen Nierenerkrankungen

**TEST-CHARAKTERISTIKA**

Präzision und Reproduzierbarkeit


<table>
<thead>
<tr>
<th>Intra-Assay Varianz</th>
<th>Mittelwert (pg/mL)</th>
<th>Variationskoeffizient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40</td>
<td>3.4 %</td>
</tr>
<tr>
<td></td>
<td>266</td>
<td>1.8 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inter-Assay Varianz</th>
<th>Mittelwert (pg/mL)</th>
<th>Variationskoeffizient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>38</td>
<td>5.6 %</td>
</tr>
<tr>
<td></td>
<td>277</td>
<td>6.1 %</td>
</tr>
</tbody>
</table>
RICHTIGKEIT

Das Intakt PTH Immunoassay System wurde verglichen mit dem INS-PTH Radioimmunoassay von Nichols Institute Diagnostica. 143 Proben wurden sowohl von den normalen als auch den nicht-normalen Personen mit beiden Methoden geprüft. Es wurde eine Analyse der Vergleichsdaten durchgeführt; man erhielt einen Korrelations-Koeffizient \( r = 0.98 \).

SENSITIVITÄT

Die Sensitivität dieses Assays ist als der kleinste Einzelwert definiert, den man bei einem Vertrauensbereich von 95 % noch von Null unterscheiden kann. Das Intakt PTH Immunoassay System hat eine berechnete Sensitivität von 1 pg/ml.

SPEZIFITÄT UND KREUZREAKTIVITÄT


Um die Tatsache noch weiter zu bestätigen, daß PTH Fragmente nicht kreuz reagieren oder die durch das Intakt PTH Immunoassay System erhaltenen Werte nicht beeinflussen, hat man mit verschiedenen PTH Fragmenten mit erhöhter physiologischer Konzentration Studien durchgeführt. Human PTH 1-43 in einer Konzentration von 300 pg/ml und Human PTH Fragmente 39-68, 53-84, 44-68 und 39-84 je in Konzentrationen von 100,000 pg/ml wurden in den Null-Standard (Reagenz C) und in einen Standard mittlerer Konzentration (Reagenz F) gegeben. Die Ergebnisse zeigten keine Kreuzaktivität und keine Störungen der in dieser Art und Weise bestimmten Fragmente.

WIEDERFINDUNG

Drei verschiedene Seren wurden mit unterschiedlichen Mengen PTH (1-84) versetzt, um die Wiederfindung zu bestimmen. Die erzielten Resultate sind in der folgenden Tabelle beschrieben:

<table>
<thead>
<tr>
<th>Serum Probe</th>
<th>Endogenes PTH (pg/ml)</th>
<th>Zugefügt. PTH (pg/ml)</th>
<th>Erwarteter Wert (pg/ml)</th>
<th>Wiedergef. PTH (pg/ml)</th>
<th>% Wiederfindung</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>147</td>
<td>153</td>
<td>160</td>
<td>105%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>219</td>
<td>225</td>
<td>224</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>290</td>
<td>296</td>
<td>307</td>
<td>104%</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>176</td>
<td>203</td>
<td>209</td>
<td>103%</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>258</td>
<td>285</td>
<td>290</td>
<td>102%</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>339</td>
<td>366</td>
<td>372</td>
<td>102%</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>256</td>
<td>282</td>
<td>265</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>378</td>
<td>404</td>
<td>376</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>498</td>
<td>524</td>
<td>503</td>
<td>96%</td>
</tr>
</tbody>
</table>
Drei Patientenseren wurden mit Null-Standard verdünnt. Die Resultate in pg/ml sind unten aufgeführt:

<table>
<thead>
<tr>
<th>Probe</th>
<th>Verdünnung</th>
<th>gemessen</th>
<th>Ergebnisse um den Verdünnungsfaktor korrigiert</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>unverdünnt</td>
<td>759</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>387</td>
<td>774</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>194</td>
<td>776</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>94</td>
<td>752</td>
</tr>
<tr>
<td>B</td>
<td>unverdünnt</td>
<td>501</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>233</td>
<td>466</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>113</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>61</td>
<td>488</td>
</tr>
<tr>
<td>C</td>
<td>unverdünnt</td>
<td>468</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>221</td>
<td>442</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>109</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>57</td>
<td>456</td>
</tr>
</tbody>
</table>

**LITERATUR**


Literaturliste zu Intakt PTH in Tierspezies

Hunde

**INTRODUCTION**

La structure chimique de l'hormone parathyroïdienne humaine a été établie durant les dix dernières années. La Parathormone (PTH) humaine intacte biologiquement active est un polypeptide de 84 acides aminés.

La biosynthèse débute dans la glande parathyroïde sous forme d'un précurseur moléculaire, constitué de 115 acides aminés: la Prépro-parathormone. Celle-ci est transformée en une molécule intermédiaire de 90 acides aminés. Cette préproparathormone, sous l'effet d'une protéolyse supplémentaire, est elle-même convertie en parathormone.

La modification protéolytique de l'hormone semble être intraglandulaire; en cas d'hypercalcémie et de réduction de la sécrétion de PTH, il y a une dégradation intracellulaire plus importante du polypeptide intact en de plus petits fragments. Associée à la formation de ces fragments dans la glande, il y a une modification protéolytique périphérique du polypeptide intact. Une fois relâché par la glande dans la circulation générale, le polypeptide est métabolisé dans le foie et le rein. Le métabolisme périphérique de la PTH aboutit à la production de fragments polypeptidiques comprenant principalement les parties moyennes et carboxy-terminales de la molécule. Ainsi, le sérum humain contient un mélange de molécules: l'hormone intacte et des fragments carboxy-terminaux de l'hormone à des concentrations élevées. Des concentrations très faibles des fragments N-terminaux, s'ils sont présents dans le sérum, participent au pool circulant de la PTH immuno-réactive.

Les principaux peptides de PTH trouvés dans la circulation ont des demi-vies métaboliques différentes. La demi-vie du peptide intact ou natif se mesure en minutes, alors que les fragments contenant les portions moyennes ou carboxy-terminales ont des demi-vies 10 à 20 fois plus longues que celle de l'hormone native. Ainsi, la concentration des fragments biologiquement inactifs, représentés par les portions moyennes et carboxy-terminales de la molécule, est élevée par rapport à la concentration moyenne de l'hormone intacte biologiquement active. Ce ratio varie d'un sujet normal à l'autre et chez des malades ayant un hyperparathyroïdisme; il est élevé de façon prévisible chez les insuffisants rénaux chroniques.

L'Institut Nichols présente un dosage radioimmunologique de la PTH intacte, qui quantifie avec précision la PTH humaine. Le dosage a une sensibilité de 1 pg/ml et permet de mesurer spécifiquement la molécule intacte et biologiquement active de PTH. Cette technique de dosage a été validée par de très nombreuses études cliniques, portant sur des populations bien définies et présentant des états divers de la fonction parathyroïdienne.
INTÉRÊT CLINIQUE

La PTH joue un rôle important dans l’homéostasie du calcium, en maintenant la concentration du calcium ionisé dans les limites précises nécessaires aux fonctions métaboliques et neurogénératrices de cet élément essentiel. En cas de calcémie basse, secondaire à une mauvaise assimilation du calcium alimentaire, les glandes parathyroïdiennes augmentent leur sécrétion de l’hormone. Il en résulte une mobilisation du calcium stocké dans le squelette vers les liquides extracellulaires, une augmentation de l’absorption du calcium alimentaire et une diminution de la clairance rénale du calcium. Lorsque la calcémie augmente, de plus faibles quantités de PTH sont libérées dans le courant sanguin.

La mesure du taux de PTH est un élément important dans l’évaluation des troubles du métabolisme calcique. Lorsqu’il est mesuré avec la calcémie, il permet de distinguer les sujets normaux des sujets ayant une hyperparathyroïdie primaire, une hypoparathyroïdie ou une hypercalcémie tumorale. Des concentrations élevées et variables des fragments de PTH biologiquement inactifs sont gênantes pour le dosage des parties moyennes et C-terminales de la PTH, quand on évalue la fonction parathyroïdienne chez des malades ayant des troubles de la fonction rénale. Les dosages spécifiques de la molécule intacte et de la partie N-terminale, qui mesurent seulement l’hormone biologiquement active, permettent une évaluation plus précise de la fonction parathyroïdienne chez ces malades, incluant aussi bien ceux ayant une maladie rénale que ceux ayant des troubles de la filtration glomérulaire liés à l’âge.

PRINCIPE DU DOSAGE

Le dosage immunologique de la PTH intacte est un dosage immunoradiométrique à 2 sites, qui mesure la chaîne biologiquement intacte à 84 acides amines de la PTH. Deux types différents d’anticorps polyclonaux de chèvre anti-PTH humaine ont été purifiés par chromatographie d’affinité, afin de leur donner une spécificité pour des zones bien définies de la molécule de PTH. Le premier se lie uniquement aux parties moyennes et C-terminales (PTH 39-84), et est fixé sur des billes de polystyrène. Le deuxième se lie à la région N-terminale (PTH 1-34), et est radiomarqué.

L’échantillon contenant la PTH est incubé simultanément avec une bille recouverte du premier anticorps et avec le deuxième anticorps marqué à l’iode-125. La PTH intacte contenue dans l’échantillon se lie à la fois à l’anticorps fixé et à l’anticorps marqué, et va former ainsi un complexe "sandwich":

Bille anti-PTH (39-84) — PTH intacte (1-84) — I125 anti-PTH (1-34)

Bien que les fragments correspondant aux parties moyennes et C-terminales soient liés par la bille recouverte d’anticorps, seule la PTH intacte 1-84 permet de former le complexe-sandwich nécessaire à la détection. La spécificité de l’anticorps a été affinée pour qu’il n’y ait pas d’interférence avec des taux, même élevés, de fragments inactifs.

Après incubation, la bille est lavée pour éliminer les fragments non liés. La radioactivité liée à la phase solide est mesurée dans un compteur gamma. Comme la formation du complexe sandwich survient uniquement en présence d’une molécule de PTH intacte, la radioactivité du complexe lié à la bille est directement proportionnelle à la quantité de PTH intacte contenue dans l’échantillon sanguin. Une courbe d’étalonnage (radioactivité/concentration) est tracée en utilisant les résultats obtenus à partir de standards, qui sont mesurés en même temps que les échantillons à tester. Les concentrations de PTH intacte des tubes de contrôle et des échantillons des malades sont déterminées directement à partir de cette courbe.
RÈGLES DE BASE DE RADIOPROTECTION

Ce matériel radioactif peut être reçu, acquis, possédé, utilisé par des médecins dans les hôpitaux, des laboratoires, uniquement pour l’analyse in vitro qui n’implique pas d’administration interne ou externe des réactifs à des êtres humains ou des animaux. Sa réception, son acquisition, sa possession, son utilisation et son transfert sont soumis aux lois en cours et à la réglementation nationale en vigueur.

1. Le matériel radioactif doit être gardé dans le container d’origine, dans un endroit spécialement prévu.
2. Le matériel radioactif doit être utilisé seulement dans des zones de travail bien définies.
3. Les réactifs de cette trousse contenant de l’azide de sodium, éviter toute contamination de la peau et des muqueuses. Les substances radioactives ne doivent pas être pipetées à la bouche.
4. Tout liquide renversé doit être essuyé avec un matériel absorbant adéquat et les zones contaminées nettoyées avec un détergent alcalin. Le matériel absorbant contaminé doit être placé dans un container adapté.
5. Il faut se débarrasser du matériel radioactif inutilisé en suivant les recommandations des autorités compétentes locales et la réglementation en vigueur.
6. Ne pas boire ni manger dans la zone contrôlée. Il est interdit d’y amener ou d’y garder de la nourriture, de fumer ou d’avoir des activités semblables qui pourraient être potentiellement dangereuses.
7. Suivre les directives concernant les règles d’hygiène: Eviter le contact avec les substances radioactives en utilisant des gants jetables, des tenues de laboratoire et tout autre matériel de protection. Se laver soigneusement après avoir manié les substances radioactives.
8. Les sujets de moins de 18 ans ne sont pas autorisés à manier les substances radioactives ni à pénétrer dans les zones contrôlées.

Le matériel d’origine humaine utilisé dans la préparation de ce produit a été testé selon une méthode approuvée par la FDA pour la recherche d’anticorps anti-HIV, anti-HCV et d’antigènes HBs qui s’est révélée négative. Sachant qu’aucune méthode ne permet d’assurer l’absence totale des virus de l’hépatite B, des virus HIV et HCV ou d’autres agents infectieux, il est recommandé de manipuler ces réactifs suivant les précautions d’usage observées pour les sérums humains ou dérivés sanguins potentiellement infectieux.

PRÉPARATION ET CONSERVATION DES ÉCHANTILLONS

La recherche de PTH intacte doit être faite sur le sérum. (On peut également utiliser du plasma EDTA.) Pour faire une détermination de l’échantillon, 200 µl de sérum sont nécessaires (400 µl pour un dosage en double). Afin d’avoir une comparaison aussi précise que possible avec les valeurs normales, faire le prélèvement à jeun. Recueillir le sang dans un tube, sans additif, et laisser coaguler. Puis centrifuger l’échantillon, de préférence dans une centrifugeuse réfrigérée, et séparer le sérum des cellules. CONGELER IMMEDIATEMENT L’ÉCHANTILLON (≤ -20°C) ou stocker selon les indications qui figurent dans le tableau ci-dessous:

<table>
<thead>
<tr>
<th>CONDITIONS DE STOCKAGE</th>
<th>DURÉE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A température ambiante après le prélèvement</td>
<td>2 heures</td>
</tr>
<tr>
<td>À 4°C</td>
<td>8 heures</td>
</tr>
<tr>
<td>Congelé à - 20°C</td>
<td>4 mois</td>
</tr>
<tr>
<td>Congelé à - 70°C</td>
<td>11 mois</td>
</tr>
</tbody>
</table>
Trousse pour 100 dosages

1. **Réactif A: Billes recouvertes d’anticorps anti-PTH (30-0510)**
   - 1 boîte contenant 100 billes de polystyrène recouvertes d’anticorps anti-PTH 39-84 (chèvre)
   - Ø 8 mm.

2. **Réactif B: Solution d’anticorps anti-PTH marqué à l’iode 125 (30-0511)**
   - 2 flacons contenant chacun 5,5 ml d’anticorps anti-PTH 1-34 (chèvre) marqué à l’iode 125, dans une solution de tampon phosphate, contenant un stabilisant protéique et 0,1% d’azide de sodium.

3. **Réactif C: Standard zéro de PTH Intacte (30-0512)**
   - 1 flacon de sérum humain lyophilisé avec 0,1% d’azide de sodium.

4. **Réactifs D₁ à H: Standards de PTH Intacte (30-0513 à 0517)**
   - 5 flacons de standards PTH intacte humaine 1-84 dans du sérum humain avec 0,1% d’azide de sodium. Les concentrations respectives sont indiquées sur chaque étiquette.

5. **Réactif I: Solution de lavage concentrée (30-0010)**
   - 1 flacon contenant 50 ml de tensioactif, dans du tampon phosphate, avec 0,2% d’azide de sodium.

6. **Réactifs J à K: Sérums de contrôle (30-0518, 0519)**
   - 2 flacons contenant chacun de la PTH humaine 1-84 lyophilisée dans du sérum humain, avec 0,1% d’azide de sodium. Se reporter à la fiche de contrôle pour les concentrations respectives.

7. **Réactif D₂: Standard de PTH Intacte (30-0509)**
   - 1 flacon de standard PTH intacte humaine 1-84 dans du sérum humain contenant 0,1% d’azide de sodium. La concentration exacte est indiquée sur l’étiquette.

---

**MATÉRIEL NÉCESSAIRE**

- Tubes de polystyrène ou de polyéthylène à fond arrondi 12x75 mm
- Portoir de tubes
- Pipettes de précision : 100 µl - 200 µl
- Dispenseur: 2 ml - 4 ml
- Film de recouvrement des tubes, type parafilm
- Dispenseur de billes
- Portoir de décantation (cat. N 39-8190) ou Système de lavage Nichols ou équivalent
- Récipient pour stocker la solution de lavage
- Compteur à scintillations gamma
- Eau distillée ou désionisée
- Agitateur type Vortex

---

**PRÉPARATION ET CONSERVATION DES RÉACTIFS**

1. **Réactif C: Standard Zéro de PTH Intacte**
   - Reconstituer le flacon avec 4 ml d’eau distillée ou désionisée. Maintenir le flacon droit 5 minutes et mélanger parfaitement en le renversant doucement pour permettre une reconstitution complète.

2. **Réactifs D₁ (facultatif), D₂ - H: Standards de PTH Intacte**
   - Reconstituer le flacon avec 2 ml d’eau distillée ou désionisée. Maintenir le flacon droit 5 minutes et mélanger parfaitement en le renversant doucement pour permettre une reconstitution complète.

3. **Réactifs I: Solution de lavage concentrée**
   - Mélanger parfaitement le contenu du concentré de solution de lavage. Ajouter 50 ml de cette solution à 450 ml d’eau distillée ou désionisée et mélanger.

4. **Réactifs J et K: Sérums de contrôle de PTH Intacte**
   - Reconstituer chaque flacon avec 2 ml d’eau distillée ou désionisée. Maintenir le flacon droit 5 minutes et mélanger parfaitement pour permettre une reconstitution complète.

5. Mélanger complètement tous les réactifs avant de les utiliser.

6. Conserver tous les composants du kit à 2-8°C dès leur réception avant de les utiliser.

7. **Utiliser les standards et les sérums de contrôle immédiatement après reconstitution. Congeler (-20°C) les standards et les sérums de contrôle restants, immédiatement après leur utilisation. Stocker les autres composants à 2-8°C. La solution de lavage, une fois diluée, peut être conservée à température ambiante.**
PROTOCOLE DU DOSAGE

1. Pipeter 200 µl de solution standard (réactifs C à H), de sérum de contrôle (J et K) ou d'échantillon du malade, dans les tubes appropriés.
2. Pipeter 100 µl de la solution d'anticorps anti-PTH marqué à l'I-125 (réactif B) dans tous les tubes.
3. Agiter tous les tubes au Vortex.
4. Prendre le distributeur de billes et ajouter une bille (réactif A) dans chaque tube (si des pinces sont utilisées, incliner le portoir de tubes selon un angle d'environ 30° et ajouter une bille dans chaque tube). Recouvrir le portoir avec un parafilm.
5. Laisser incuber les tubes à température ambiante pendant 22 ± 2 heures.
6. Laver deux fois les billes en versant 2 ml de solution de lavage dans chaque tube. Puis aspirer complètement ou décanter le liquide de chaque tube.
7. Faire le comptage de chaque tube à essai dans un compteur gamma et noter les résultats.
**SCHÉMA DU DOSAGE**

<table>
<thead>
<tr>
<th>NO TUBE</th>
<th>RÉFÉRENCE DU TUBE</th>
<th>STANDARDS/CONTROLES/PATIENTS</th>
<th>(^{125})I PTH ANTISÉRUM REACTIF B</th>
<th>BILLES RECOUVERTES D'ANTICORPS REACTIF A</th>
<th>INCUBATION À TA</th>
<th>SOLUTION DE LAVAGE REACTIF I</th>
<th>MESURE DE LA RADIOactivité</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>ZERO STANDARD</td>
<td>200 µL</td>
<td>100 µL</td>
<td>1 Bille</td>
<td>22±2h</td>
<td>2.0 mL</td>
<td>Comptage sur 1 minute</td>
</tr>
<tr>
<td>1,2</td>
<td>REACTIF C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4</td>
<td>STANDARD D,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,6</td>
<td>STANDARD D,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,8</td>
<td>STANDARD E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9,10</td>
<td>STANDARD F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11,12</td>
<td>STANDARD G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13,14</td>
<td>STANDARD H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15,16</td>
<td>CONTROLE J</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17,18</td>
<td>CONTROLE K</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>PATIENT 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>PATIENT 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>ETC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Si les calculs nécessitent une mesure de l'activité totale, pipeter 100 µL de la solution d'anticorps marqué à l'iode 125 (réactif B) dans 2 tubes et les fermer.*
PRÉCAUTIONS D’EMPLOI

1. La molécule de PTH intacte 1-84 est très labile. Débuter l’essai dès la reconstitution ou la décongélation de tous les réactifs: standards, sérums de contrôle et échantillons. Dans la mesure où le temps et la température sont des facteurs critiques pour le maintien de l’intégrité de la molécule de PTH intacte, il est fortement recommandé de conserver les standards, les contrôles, les échantillons de malade et les tubes à essai dans la glace pendant la mise en place du dosage.

2. Il est conseillé de faire une double détermination des standards et des sérums de contrôle. La moyenne des coups par minute est utilisée pour calculer les résultats.

3. L’échantillon et la solution d’anticorps marqué à l’iode-125 (réactif B) doivent être pipetés avec précaution dans le quart inférieur du tube à essai.

4. En cas d’utilisation de pinces, le fait d’incliner le portoir avant d’ajouter les billes permet à celles-ci de rouler dans la solution de réactif sans éclabousser. Les pinces ou le distributeur de billes doivent être utilisés pour transférer les billes dans les tubes à essai. Ne pas les toucher avec les doigts.

5. L’étape du lavage est une phase importante de déroulement du dosage. Ajouter la solution de lavage avec assez de force pour soulever la bille du fond du tube à essai. Une aspiration complète et parfaite ou une décantation de la solution de lavage est capitale.


7. Les échantillons de malade qui ont un taux plus élevé que le niveau supérieur des réactifs standards (réactif H) peuvent être dilués avec le standard zéro (réactif C) et être à nouveau dosés. Multiplier le résultat par le facteur de dilution.

8. Chaque composant utilisé dans un dosage doit avoir le même numéro de lot et être conservé dans des conditions identiques.


EXPRESSION DES RÉSULTATS

La courbe standard est obtenue en utilisant des standards de PTH intacte préparés à l’avance. Se référer aux étiquettes de chaque flacon pour connaître les concentrations exactes. Construire la courbe comme suit:

- Calculer les CPM moyens pour chaque paire de tubes à essai.
- Soustraire les CPM moyens des tubes de standard zéro de tous les autres comptages moyens pour obtenir les CPM corrigés.
- La courbe standard se construit en portant en ordonnée les CPM corrigés et, en abscisse, la concentration de chaque standard. Utilisez un papier graphique log-log.

Nota: Les programmes assistés d’ordinateur pour des dosages IRMA peuvent être aussi utilisés pour les calculs du dosage radioimmunologique de la PTH intacte. La fonction logistique à quatre paramètres de Rodbard est une de ces méthodes.

Exemple de Calculs

- Moyenne des CPM du standard zéro : 1023
- Moyenne des CPM du standard 7,5 pg/ml : 1516
- Moyenne corrigée :
  CPM (7,5 pg/ml) - CPM (0 pg/ml) = 1561 - 1023 = 538
**Valeur inférieure au standard D₂**

Remarque : Si on utilise le standard D₃ (7,5 pg/mL) dans le dosage, remplacer le standard D₂ par le standard D₁ dans le calcul ci-dessous :

\[
\text{Valeur inconnue} = \frac{\text{CPM corrigés (inconnue) \times concentration du standard D₂}}{\text{CPM corrigés (Standard D₂)}}
\]

**Intact PTH Courbe Standard**

Les résultats ici présentés ne sont que des exemples et ne peuvent en aucun cas être utilisés comme courbe standard.
## EXEMPLE DE RÉSULTATS

<table>
<thead>
<tr>
<th>TUBE N°</th>
<th>RÉFÉRENCE DU TUBE</th>
<th>CPM</th>
<th>MOYENNE CPM</th>
<th>CPM CORRIGÉS</th>
<th>RESULTAT pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>STD C</td>
<td>1144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0 pg/mL</td>
<td>901</td>
<td>1023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>STD D</td>
<td>1558</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7.5 pg/mL</td>
<td>1563</td>
<td>1561</td>
<td>538</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>STD D</td>
<td>2347</td>
<td></td>
<td>2323</td>
<td>1300</td>
</tr>
<tr>
<td>6</td>
<td>17 pg/mL</td>
<td>2298</td>
<td>2323</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>STD E</td>
<td>5240</td>
<td></td>
<td></td>
<td>4243</td>
</tr>
<tr>
<td>8</td>
<td>58 pg/mL</td>
<td>5273</td>
<td>5257</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>STD F</td>
<td>14695</td>
<td></td>
<td>14881</td>
<td>13858</td>
</tr>
<tr>
<td>10</td>
<td>185 pg/mL</td>
<td>15067</td>
<td>15067</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>STD G</td>
<td>40121</td>
<td></td>
<td>39521</td>
<td>38498</td>
</tr>
<tr>
<td>12</td>
<td>580 pg/mL</td>
<td>36920</td>
<td>3724</td>
<td>38498</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>STD H</td>
<td>78455</td>
<td></td>
<td>77788</td>
<td>176765</td>
</tr>
<tr>
<td>14</td>
<td>1750 pg/mL</td>
<td>77121</td>
<td>77121</td>
<td>176765</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Contrôle J</td>
<td>3724</td>
<td></td>
<td>3724</td>
<td>2701</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>3808</td>
<td>3724</td>
<td>2701</td>
<td>38</td>
</tr>
<tr>
<td>17</td>
<td>Contrôle K</td>
<td>23034</td>
<td></td>
<td>23188</td>
<td>22165</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>23342</td>
<td>23188</td>
<td>22165</td>
<td>300</td>
</tr>
</tbody>
</table>
CONTRÔLE DE QUALITÉ

La reproductibilité des valeurs de la courbe standard et des valeurs des sérums de contrôle doit se situer dans les limites d'acceptabilité du laboratoire. Les mesures habituellement utilisées pour la variabilité sont exposées par Rodbard et Coll. Si la précision de l'essai ne correspond pas aux limites établies et si une erreur technique est éliminée en répétant le dosage, vérifier les points suivants:

- Pipettes - minuteurs.
- La calibration du compteur gamma (y compris la position du cristal).
- La date d'expiration des réactifs et de la trousse.
- Les conditions d'incubation et de stockage.
- La propreté de tous les composants du système.
- La pureté de l'eau.
- Le soin apporté aux étapes du lavage.

LIMITES DU DOSAGE

La plus forte concentration de PTH intacte mesurable sans aucune dilution est la valeur du plus haut standard (réactif H); la concentration la plus basse mesurable est de 1 pg/ml (sensibilité de l'essai).

Des variations de concentrations en protéine de l'ordre de ±25% n'ont peu ou pas d'effet sur la valeur obtenue de PTH intacte.

Le dosage radioimmunologique de la PTH intacte a été conçu pour que l'effet 'crochet' survenant pour des concentrations particulièrement élevées de PTH soit minimisé. Les échantillons ayant des taux de PTH compris entre le plus élevé des standards (réactif H) et 100 000 pg/ml seront appréciés comme supérieurs aux taux du standard H et devront être dilués et redosés pour avoir des valeurs correctes.

Comme tout paramètre utilisé comme complément diagnostique, les résultats de la PTH intacte doivent être interprétés avec précaution en tenant compte du contexte clinique et des autres tests diagnostiques complémentaires.

VALEURS ATTENDUES

L'Institut Nichols recommande à chaque laboratoire d'établir sa propre gamme de valeurs. On a dosé la PTH intacte dans le sérum de 253 sujets apparemment sains et à jeûn, en utilisant le dosage de l'INTACT PTH. La moyenne géométrique des valeurs obtenues ±2 écarts-types est comprise entre 10 et 65 pg/ml (voir Figure 2).

Chez 88 malades atteints d'hyperparathyroïdie primaire confirmée chirurgicale, on a trouvé des taux qui variaient entre 53 et 1180 pg/ml pour des taux de calcium total variant entre 10,5 et 16,3 mg/dl.

Chez 63 malades souffrant d'hypercalcémie d'origine maligne, les valeurs de la PTH intacte s'échelonnaient entre des taux indécelables (<1 pg/ml) et 22 pg/ml. Sur les 63 échantillons étudiés, 59 se situaient en dessous de la limite inférieure de la normale. Les taux de calcium total variaient entre 10,5 et 17,6 mg/dl.

Chez 8 malades souffrant d'hypoparathyroïdie, les valeurs de la PTH intacte s'échelonnaient entre des taux indétectables (<1 pg/ml) et 21 pg/ml. Les taux de calcium total variaient entre 6,3 et 8,5 mg/dl.

Chez 123 malades atteints de néphropathies chroniques, on a comparé les taux de PTH intacte et les résultats d'une histomorphométrie quantitative des os: 34 malades atteints d'ostéite fibrokystique aiguë avaient des taux moyens égaux à 1033±103 pg/ml (±DS), ces taux sont plus élevés que dans les formes récentes (moyenne 362±56 pg/ml).
Tous les taux de PTH chez ces malades atteints d'ostéite fibreuse confirmée par biopsie étaient supérieurs à la normale, à l'exception de 2 malades à un stade précoce.

Les taux de PTH chez les malades atteints d'ostéite à un stade avancé étaient plus élevés que les taux des malades atteints d'ostéomalacie et dans 90% des cas plus élevés que les taux des malades atteints d'aplasie osseuse.

L'interprétation des résultats de la PTH intacte doit prendre en compte les concentrations de la calcémie et la relation physiologique de ces deux éléments dans les différents troubles PTH/calcium (voir Figure 2). En accord avec les bonnes pratiques cliniques, il est recommandé que les résultats de PTH intacte, même combinés avec ceux de la calcémie, soient interprétés avec prudence en tenant compte du contexte clinique.

Les résultats donnés dans cette étude représentent un nombre limité d'échantillons. Il faut noter qu'il existe un certain niveau de chevauchement des valeurs de la PTH chez les malades ayant un dysfonctionnement parathyroidien (voir Figure 2).

De plus, il est important de s'assurer que le maniement et le stockage des échantillons sanguins sont corrects. Un stockage incorrect des échantillons peut occasionner une perte de PHT intacte détectable.

**Figure 1:** Histomorphométrie des os et taux de PTH intacte (IRMA) dans les insuffisances rénales aiguës.
Figure 2: Taux de PTH intacte (IRMA) et calcium total dans différentes pathologies.

• Précision et Reproductibilité

La reproductibilité intra-essai du dosage de l’INTACT PTH a été calculée à partir de déterminations répétées faites au cours d’un même dosage sur deux séums servant au contrôle de qualité (n = 20). La reproductibilité inter-essais a été calculée à partir de résultats obtenus sur une période de 4 semaines (n = 20).

<table>
<thead>
<tr>
<th>Intra-Essai</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moyenne (pg/mL)</td>
<td>CV%</td>
</tr>
<tr>
<td>40</td>
<td>3,4%</td>
</tr>
<tr>
<td>266</td>
<td>1,8%</td>
</tr>
</tbody>
</table>
• Exactitude

L’INTACT PTH a été comparé au dosage radioimmunologique INS PTH de l’Institut Nichols. Un ensemble de 143 échantillons provenant de sujets sains et malades a été testé par chacune des deux méthodes. Une analyse de régression par la méthode des moindres carrés a été faite sur les données comparées; un coefficient de corrélation (r) de 0,98 a été obtenu.

• Sensibilité

La sensibilité du dosage est définie comme étant la plus petite valeur qui est différente de zéro avec un intervalle de confiance de 95%. L’INTACT PTH a une sensibilité calculée à 1 pg/ml.

• Spécificité et Réactions Croisées

Les anticorps employés dans l’INTACT PTH ont été purifiés par chromatographie d’affinité pour être spécifiques de régions bien définies de la molécule de PTH. Les anticorps marqués à l’iode-125 reconnaissent uniquement la séquence d’acides aminés 1-34 de la partie N-terminale, et n’ont pas de réaction croisée avec le segment 39-84. Les anticorps immobilisés sur les billes reconnaissent uniquement la séquence d’acides aminés 39-84 des régions moyennes et C-terminales, et n’ont pas de réaction croisée avec le segment 1-34. Quand ces deux anticorps sont employés simultanément pour un essai immunoradiométrique à deux sites, seule la PTH intacte est mesurée.

Pour confirmer le fait que les fragments de PTH n’interfèrent pas ou n’ont pas de réaction croisée avec les résultats obtenus avec l’INTACT PTH, des fragments variés de PTH représentant des concentrations physiologiques très élevées ont été étudiés. De la PTH humaine 1-34 à une concentration de 300 pg/ml et des fragments de PTH humaine 39-68, 44-68, et 39-84, chacun à une concentration de 100 000 pg/ml, ont été testés dans le standard zéro (réactif C) et dans un standard de valeur moyenne (réactif F). Les résultats montrent qu’il n’y a ni interférence ni réaction croisée avec chacun des fragments de cette façon.

• Test de Surcharge

Des quantités différentes de PTH (1-84) ont été ajoutées à 3 sérums de concentrations différentes, pour établir le pourcentage de récupération (voir les résultats ci-dessous).

<table>
<thead>
<tr>
<th>Sérum</th>
<th>PTH (pg/mL)</th>
<th>PTH Ajoutée (pg/mL)</th>
<th>Valeurs Théoriques (pg/mL)</th>
<th>Valeurs Trouvées (pg/mL)</th>
<th>Récupération %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>147</td>
<td>153</td>
<td>160</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>219</td>
<td>225</td>
<td>224</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>290</td>
<td>296</td>
<td>307</td>
<td>104</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>176</td>
<td>203</td>
<td>209</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>258</td>
<td>285</td>
<td>290</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>339</td>
<td>366</td>
<td>372</td>
<td>102</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>256</td>
<td>282</td>
<td>265</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>378</td>
<td>404</td>
<td>376</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>498</td>
<td>524</td>
<td>503</td>
<td>96</td>
</tr>
</tbody>
</table>
Parallélisme

3 séums de malades sont dilués avec le standard zéro (voir les résultats ci-dessous en pg/mL)

<table>
<thead>
<tr>
<th>Echantillon</th>
<th>Dilution</th>
<th>Valeur Mesurée</th>
<th>Valeur Corrigée</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Non dilué</td>
<td>759</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>387</td>
<td>774</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>194</td>
<td>776</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>94</td>
<td>752</td>
</tr>
<tr>
<td>B</td>
<td>Non dilué</td>
<td>501</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>233</td>
<td>466</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>113</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>61</td>
<td>488</td>
</tr>
<tr>
<td>C</td>
<td>Non dilué</td>
<td>468</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>221</td>
<td>442</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>109</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>57</td>
<td>456</td>
</tr>
</tbody>
</table>

**BIBLIOGRAPHIE**


Fabricant:
Nichols Institute Diagnostics
San Juan Capistrano
CA 92675 U.S.A.

Distributeur:
Mallinckrodt Medical
7, avenue du
General De Gaulle
BP 50
91002 EVRY cedex
PYRILINKS-D

Kit

96 assays for Deoxypyridinoline Crosslinks

Store at 2-8°C

Metra Biosystems, Inc.
3181 Porter Drive
Palo Alto CA 94304-1213
USA
phone 415-813-6132  fax 415-494-7148
800-524-6318 (US only)
Made in U.S.A.

METRA BIOSYSTEMS
Read the entire product insert thoroughly before beginning the assay. The Pyrilinks-D kit should be stored at 2-8°C until use.

INTENDED USE
The Pyrilinks-D assay measures free deoxypyridinoline (DPD) crosslinks in urine. Deoxypyridinoline has been shown to be a biochemical indicator of bone resorption.

BACKGROUND ON DEOXYPYRIDINOLINE
Deoxypyridinoline is found mainly in Type I collagen of bone. After the collagen matrix is formed, the enzymatic action of lysyl oxidase on the amino acid lysine causes the formation of the covalent crosslink DPD. This crosslink, DPD, plays an important part in the structural role of collagen. In the process of bone degradation, DPD is released into the circulation and cleared by the kidney.

PRINCIPLES OF THE ASSAY
The Pyrilinks-D assay is a competitive enzyme immunoassay in a microtiter plate format utilizing a monoclonal anti-deoxypyridinoline antibody coated on the plate to capture DPD. DPD in the sample competes with conjugated DPD-alkaline phosphatase for the antibody and the reaction is detected with a pNPP substrate. Pyrilinks-D results are corrected for urinary concentration by creatinine.
### MATERIALS

<table>
<thead>
<tr>
<th>Kit Contents</th>
<th>Qty/Vol</th>
<th>Part No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Tablet, 20 mg (p-Nitrophenyl Phosphate)</td>
<td>3 each</td>
<td>0012</td>
</tr>
<tr>
<td>Plate Cover</td>
<td>3 each</td>
<td>0047</td>
</tr>
<tr>
<td>Stripwell Frame</td>
<td>1 each</td>
<td>4179</td>
</tr>
<tr>
<td>Substrate Buffer</td>
<td>3 x 10mL</td>
<td>4198</td>
</tr>
<tr>
<td>10X Wash Buffer</td>
<td>1 x 30mL</td>
<td>4199</td>
</tr>
<tr>
<td>Stop Solution (1N NaOH)</td>
<td>1 x 15mL</td>
<td>4200</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>1 x 60mL</td>
<td>4201</td>
</tr>
<tr>
<td>Enzyme Conjugate (lyophilized)</td>
<td>3 each</td>
<td>4202</td>
</tr>
<tr>
<td>(DPD-alkaline phosphatase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD Standard A (0nM)</td>
<td>0.3mL</td>
<td>4203</td>
</tr>
<tr>
<td>DPD Standard B (3nM)</td>
<td>0.3mL</td>
<td>4204</td>
</tr>
<tr>
<td>DPD Standard C (10nM)</td>
<td>0.3mL</td>
<td>4205</td>
</tr>
<tr>
<td>DPD Standard D (30nM)</td>
<td>0.3mL</td>
<td>4206</td>
</tr>
<tr>
<td>DPD Standard E (100nM)</td>
<td>0.3mL</td>
<td>4207</td>
</tr>
<tr>
<td>DPD Standard F (300nM)</td>
<td>0.3mL</td>
<td>4208</td>
</tr>
<tr>
<td>DPD &amp; Creatinine Control, Low</td>
<td>1.5mL</td>
<td>4209</td>
</tr>
<tr>
<td>DPD &amp; Creatinine Control, High</td>
<td>1.5mL</td>
<td>4210</td>
</tr>
<tr>
<td>Anti-DPD Coated Strip (Monoclonal mouse anti-DPD)</td>
<td>6 each</td>
<td>4211</td>
</tr>
</tbody>
</table>
Materials Required BUT NOT Provided
Micropipettes
Plate Reader
Deionized or Distilled Water
Container for wash buffer dilution
Tubes for dilution of samples, standards and controls
Software capable of calculating 4-parameter curve fit
Creatinine Assay

SPECIMEN COLLECTION & STORAGE
The Pyrilinks-D assay can be carried out using preservative free First Morning Void (FMV), twenty four hour, Second Morning Void (2 hour fasting) or random urine samples, if the deoxypyrindinoline levels are greater than 3 nM. Keep the urine sample refrigerated (2-8°C) for storage of less than 7 days, or freeze the sample at ≤−20°C for longer storage. Do not subject sample to more than 5 freeze/thaw cycles. Avoid prolonged exposure to light, especially direct sunlight.

TECHNIQUES FOR OPTIMAL PERFORMANCE
1. The Enzyme Conjugate, DPD Standards and Controls are light sensitive. Avoid prolonged light exposure, especially direct sunlight. Store reagents in the dark when not in use.

2. The Substrate Buffer must be brought to room temperature (20-28°C) before beginning the assay. (2 hours to overnight recommended.)
TECHNIQUES FOR OPTIMAL PERFORMANCE...

3. The Pyrilinks-D assay is sensitive to washing conditions. The wash step should be completed within 2 minutes. If the wash step CANNOT be completed within 2 minutes, follow the Special Washing Instructions located throughout the assay protocol.

PYRILINKS-D ASSAY PROTOCOL

Determine amount of each reagent required for the number of strips to be used.

<table>
<thead>
<tr>
<th># of Strips</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Samples</td>
<td>8</td>
<td>16</td>
<td>24</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>1</th>
<th>2*</th>
<th>2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Conjugate (vial)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate (bottle)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1X Wash Buffer (mL)</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td>300</td>
</tr>
</tbody>
</table>

* When more than 1 bottle or vial is to be used, combine the containers of that reagent, and mix prior to use.
Reagent Preparation
1. Prepare required amount of 1X Wash Buffer (see table) by diluting 10X Wash Buffer Concentrate 1 to 10 with deionized (DI) water. Store at room temperature (20-28°C)

Special Washing Instructions: Prepare 1X Wash Buffer as above and store at 2-8°C until immediately prior to use.

Sample/Enzyme Conjugate Incubation
1. Dilute samples, Standards and Controls 1:10 with Assay Buffer.
   (e.g. 50µL sample + 450µL Assay Buffer)

2. Reconstitute each required vial of Enzyme Conjugate (see Table) with 7mL of Assay Buffer.

   Store reconstituted Enzyme Conjugate at 2-8°C until immediately prior to use.

3. Place desired number of Anti-DPD Coated Strips in the Stripwell Frame. Label strips in case of accidental removal from Stripwell Frame.

4. Add 50µL diluted Standard, Control or sample to each well of the Anti-DPD Coated Strips.
Sample/Enzyme Conjugate Incubation...
5. Add 100μL of reconstituted Enzyme Conjugate to each well. Cover plate with Plate Cover provided. Incubate for 2 hours (±5 minutes) at 2-8°C. This incubation should be carried out in the dark.

6. Prepare Working Substrate Solution by putting 1 Substrate Tablet into each required bottle of room temperature Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

Substrate Incubation
1. Manually invert/empty strips. Add 300μL of 1X Wash Buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash. While plate is inverted, carefully wipe bottom of strips with a lint-free paper towel to ensure that the bottom of the strips are clean.
   Special Washing Instructions: Perform wash step as above, using cold (2-8°C) 1X Wash Buffer. Allow plate to drain for 5-10 minutes before adding substrate.

2. Add 150μL of Working Substrate Solution to each well.

3. Incubate for 60 minutes (± 5 minutes) at room temperature (20-28°C). **See next page.
** Read Special Instructions page 15 if you cannot maintain 20-28°C room temperature.

**Stop/Read**

1. Add 100μL of Stop Solution to each well. Add Stop Solution in the same pattern and time intervals as the Substrate Solution addition.

2. Read the Optical Density (OD) at 405 nm. Assure that no large bubbles are present in wells and that the bottom of the strips are clean. Strips should be read within **30 minutes** of Stop Solution addition.

3. Quantitation software with a 4-parameter curve fitting equation **must** be used to analyze the Pyrilinks-D assay.

**Representative Standard Curve**

Standard DPD levels: 0, 3, 10, 30, 100, 300 nM
INTERPRETATION OF RESULTS
Results obtained from the Pyrilinks-D assay must be corrected for variations in urine concentration by dividing the DPD value (nM) by the creatinine value (mM) of each sample. The final results will be expressed as DPD nM/Creatinine mM. The Dpd & Creatinine control values should be confirmed in your creatinine assay to ensure correlation with values generated at Metra Biosystems, Inc.

QUICK GUIDE TO ASSAY STEPS
1. Dilute Standards, Controls and samples 1:10
2. Add 50µL diluted Standards, Controls and samples
3. Add 100µL Enzyme Conjugate
4. Incubate 2 hours at 2-8°C in the dark
5. Wash 3 times
6. Add 150µL Substrate Solution
7. Incubate 60 ± 5 minutes at room temperature
8. Add 100µL Stop Solution and read OD at 405nm
PERFORMANCE CHARACTERISTICS

Antibody Specificity
The Deoxypyridinoline antibody has selective, high affinity for free deoxypyridinoline and negligible binding to DPD peptides and free or peptide bound pyridinoline (PYD).

<table>
<thead>
<tr>
<th>% Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free DPD</td>
</tr>
<tr>
<td>PYD</td>
</tr>
<tr>
<td>PYD/DPD peptides</td>
</tr>
<tr>
<td>≥1000 MW</td>
</tr>
<tr>
<td>≥3500 MW</td>
</tr>
</tbody>
</table>

Sensitivity
The minimum detection limit of the Pyrilinks-D Assay is 3nM.

Recovery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous (nM)</th>
<th>Added (nM)</th>
<th>Observed (nM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.60</td>
<td>49.19</td>
<td>74.99</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>46.96</td>
<td>51.61</td>
<td>95.93</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>59.56</td>
<td>47.16</td>
<td>106.30</td>
<td>99</td>
</tr>
</tbody>
</table>
### Linearity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution Factor</th>
<th>Observed (nM)</th>
<th>Expected (nM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>27.34</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>13.67</td>
<td>13.67</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>6.54</td>
<td>6.84</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>3.40</td>
<td>3.42</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>50.68</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>23.16</td>
<td>25.34</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>12.54</td>
<td>12.67</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>6.75</td>
<td>6.34</td>
<td>106</td>
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<td>3</td>
<td>-</td>
<td>72.68</td>
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<td>-</td>
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<td>1:2</td>
<td>36.57</td>
<td>36.34</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>18.23</td>
<td>18.17</td>
<td>100</td>
</tr>
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<td></td>
<td>1:8</td>
<td>9.21</td>
<td>9.09</td>
<td>101</td>
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</tbody>
</table>

### Precision

Within-run and between-run precision was determined by assaying 6 urine samples in 10 different runs.

<table>
<thead>
<tr>
<th>Pyrilinks-D</th>
<th>Within-run$^1$</th>
<th>Between-run$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPD(nM)</td>
<td>C.V. %</td>
<td>C.V. %</td>
</tr>
<tr>
<td>11.9</td>
<td>9.5</td>
<td>8.1</td>
</tr>
<tr>
<td>16.5</td>
<td>6.2</td>
<td>8.0</td>
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<tr>
<td>31.7</td>
<td>4.8</td>
<td>7.6</td>
</tr>
<tr>
<td>77.5</td>
<td>5.6</td>
<td>6.3</td>
</tr>
<tr>
<td>158.4</td>
<td>6.9</td>
<td>10.3</td>
</tr>
<tr>
<td>191.1</td>
<td>3.6</td>
<td>8.7</td>
</tr>
</tbody>
</table>

1 n=40       2 n=10 runs
Accuracy
Pyrilinks-D values correlate well with total DPD measured by HPLC.

\[ y = -1.9 + 0.50x \]
\[ r = 0.93 \]
\[ n = 56 \]
Pyrilinks-D correlates well with collagen crosslinks measured by Pyrilinks™ (Metra Biosystems, Inc.).

\[ y = 5.3 + 0.1x \]

\[ r = 0.96 \]

\[ n = 40 \]
SPECIAL INSTRUCTIONS

1. The Deoxypyridinoline Standards, Controls and Enzyme Conjugate are light sensitive. Avoid prolonged light exposure, especially direct sunlight. Store reagents in the dark when not in use.

2. Each determination is preferably performed in duplicate.

3. The Certificate of Analysis included in this kit is to be used to verify that the results obtained are similar to those obtained at Metra Biosystems, Inc. The data provided are lot-specific. The OD values for the standard points for DPD are provided and are to be used as a guideline only. The results obtained may differ from those obtained at Metra Biosystems, Inc.

Quality control ranges are provided. The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits, and most laboratories utilize a ± 2 to 3 standard deviation (SD) rule for control samples. If the control values are NOT within your laboratory's acceptable range, the assay results should be considered questionable and if possible, the samples should be repeated.
SPECIAL INSTRUCTIONS...

4. If the OD of the Pyrilinks-D Standard "A" is less than 0.8 the results should be considered questionable and if possible, the samples should be repeated. If room temperature cannot be maintained between 20-28°C and an absorbance of >2.0 is not compatible with your plate reader, monitor the development of substrate in the Standard "A" wells; stop the reaction when the OD reaches 1.2-1.5; then read the plate.

LIMITATIONS

1. A standard curve must be performed with each assay.

2. All reagents supplied should be used as an integral unit prior to the expiration date indicated on the package label.

3. Assay reagents should be stored as indicated.

4. DPD values from non-human species have not been evaluated with the Pyrilinks-D Assay.

5. This assay is validated for manual washing only.

6. Do not use Anti-DPD Coated Strip if foil bag is punctured.
WARNINGS AND PRECAUTIONS

1. All urine samples should be treated as potentially biohazardous material.

2. 1N NaOH is poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing. If contact is made, wash with water. If ingested, call a physician.

3. Sodium azide is used as a preservative. It may be fatal if swallowed or absorbed through the skin. Do not mix with acids. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.

4. Test kits and components should be disposed of in a manner consistent with relevant regulations.
REFERENCES


REFERENCES


PYRILINKS®

96 Assays for Pyridinium Crosslinks
For In Vitro Diagnostic Use

Store at 2-8°C

Metra Biosystems, Inc.
265 North Whisman Road
Mountain View, CA 94043
USA
Phone 415-903-9400  Fax 415-903-9500
800-524-6318 (US only)
Made in USA

0315 C rev 10/95
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Read the entire product insert thoroughly before beginning the assay. The Pyrilinks® kit should be stored at 2-8°C until use.

**INTENDED USE**

Pyrilinks is a urinary assay that provides a quantitative measure of the excretion of pyridinium crosslinks as an indicator of type I collagen resorption, especially bone collagen.

**SUMMARY AND EXPLANATION**

Type I collagen, a triple helical protein, is the major structural protein in the skeleton and other connective tissues, with bone representing the greatest mass of these tissues. Approximately 90% of the organic matrix of bone is type I collagen\(^9\). Type I collagen of bone is crosslinked by specific molecules which provide rigidity and strength. Crosslinks of mature type I collagen in bone are the pyridinium crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpd)\(^1\). Pyd and Dpd are formed by the enzymatic action of lysyl oxidase on the amino acids lysine and hydroxylysine.

Bone is constantly undergoing a metabolic process called remodeling. This includes a degradation process, bone resorption, mediated by the action of osteoclasts, and a building process, bone formation, mediated by the action of osteoblasts\(^1\). Remodeling is required for the maintenance and overall health of bone and is tightly coupled; that is, resorption and formation are in balance.
SUMMARY AND EXPLANATION...

In abnormal states of bone metabolism this process becomes uncoupled and resorption often exceeds formation which results in a net loss of bone. An accurate assessment of bone metabolism is critical for determining the severity of metabolic bone disease and response to therapy. The measurement of specific degradation products of bone collagen resorption provides analytical data of the rate of bone metabolism. Pyd and Dpd are released into the circulation during the resorption process and are excreted unmetabolized in urine and their levels in urine are unaffected by diet, making them suitable for assessing resorption.

For the Pyrilinks assay, antibody technology was employed to produce monoclonal antibodies that demonstrate specificity for pyridinium crosslinks. The specificity of the monoclonal antibodies used in the Pyrilinks assay allow for simple, convenient, reproducible and direct quantitation of pyridinium crosslinks in urine.

PRINCIPLE OF THE PROCEDURE

The Pyrilinks assay is a competitive enzyme immunoassay in a microtiter strip format utilizing a monoclonal anti-pyridinoline antibody to measure Pyd and Dpd in urine. The Pyd and Dpd in the sample compete for the antibody with Pyd coated on the strip. The reaction is detected with a pNPP substrate. Pyrilinks results are corrected for urinary concentration by creatinine.
**REAGENTS AND MATERIALS**

Pyrilinks part number 8010

<table>
<thead>
<tr>
<th>Kit Contents</th>
<th>Qty/Vol</th>
<th>Part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Tablet</td>
<td>3 each</td>
<td>0012</td>
</tr>
<tr>
<td>Tape Cover</td>
<td>3 each</td>
<td>0047</td>
</tr>
<tr>
<td>Stripwell Frame</td>
<td>1 each</td>
<td>4179</td>
</tr>
<tr>
<td>Enzyme Conjugate (lyophilized)</td>
<td>3 each</td>
<td>4250</td>
</tr>
<tr>
<td>Pyd Standard A (0nM)</td>
<td>0.3mL</td>
<td>4251</td>
</tr>
<tr>
<td>Pyd Standard B (15nM)</td>
<td>0.3mL</td>
<td>4252</td>
</tr>
<tr>
<td>Pyd Standard C (40nM)</td>
<td>0.3mL</td>
<td>4253</td>
</tr>
<tr>
<td>Pyd Standard D (100nM)</td>
<td>0.3mL</td>
<td>4254</td>
</tr>
<tr>
<td>Pyd Standard E (250nM)</td>
<td>0.3mL</td>
<td>4255</td>
</tr>
<tr>
<td>Pyd Standard F (750nM)</td>
<td>0.3mL</td>
<td>4256</td>
</tr>
<tr>
<td>Control, Low</td>
<td>0.3mL</td>
<td>4257</td>
</tr>
<tr>
<td>Control, High</td>
<td>0.3mL</td>
<td>4258</td>
</tr>
<tr>
<td>Pyd Coated Strip</td>
<td>6 each</td>
<td>4261</td>
</tr>
<tr>
<td>10X Wash Buffer</td>
<td>30mL</td>
<td>4313</td>
</tr>
<tr>
<td>Substrate Buffer (3)</td>
<td>10mL</td>
<td>4314</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>15mL</td>
<td>4315</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>60mL</td>
<td>4316</td>
</tr>
</tbody>
</table>

**Reagent Descriptions**

**Pyridinoline Coated Strips, 2X8 wells**
Pyd purified from bovine bone adsorbed onto stripwells.

**Pyridinoline Standards:**
0, 15, 40, 100, 250, 750 nM Pyd
Pyd purified from human urine in 10mM phosphoric acid containing sodium azide (0.05%) as a preservative.
REAGENTS AND MATERIALS...
Reagent Descriptions...

Low/High Controls
Purified Pyd from human urine in 10mM phosphoric acid containing sodium azide (0.05%) as a preservative.

Enzyme Conjugate
Lyophilized murine monoclonal anti-Pyd antibody conjugated to alkaline phosphatase containing buffer salts, and stabilizers.

10X Wash Buffer
Ionic detergent in a buffered solution containing sodium azide (0.05%) as a preservative.

Assay Buffer
Ionic detergent in a buffered solution containing sodium azide (0.05%) as a preservative.

Substrate Buffer
A diethanolamine and magnesium chloride solution containing sodium azide (0.05%) as a preservative.

Substrate Tablets
p-Nitrophenyl phosphate (20mg each)

Stop Solution
1N NaOH
REAGENTS AND MATERIALS...

Materials Required BUT NOT Provided

- Micropipets to deliver 50-300µL
- Items suitable for liquid measurement of 7-300mL
- Container for wash buffer dilution
- Deionized or distilled water
- Tubes for dilution of samples, standards and controls
- Plate reader capable of reading at 405nm
- 4-parameter calibration curve fitting software
- Creatinine values (mM) for urine samples

WARNINGS

1. For *In Vitro* Diagnostic Use.

2. All urine samples should be treated as potentially biohazardous material.

3. The Standards and Controls contain Pyd purified from human urine and should be treated as potentially biohazardous material.

4. Standards and Controls are in 10mM phosphoric acid. Avoid contact with skin, eyes or clothing. Do not ingest. If contact is made, wash with water. If ingested, call a physician.

5. 1N NaOH is poisonous and can cause severe burns. Do not ingest. Avoid contact with skin, eyes or clothing. If contact is made, wash with water. If ingested, call a physician.
WARNINGS...

6. Sodium azide is used as a preservative. It may be fatal if swallowed or absorbed through the skin. Do not mix with acids. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal of reagents, flush with a large volume of water to prevent azide build-up.

7. Test kits and components should be disposed of in a manner consistent with relevant regulations.

PRECAUTIONS

1. The Pyridinoline Standards, Controls, Pyd Coated Strips, and urine samples are light sensitive. Avoid prolonged exposure to light, especially direct or indirect sunlight. Store reagents in the dark when not in use. Samples and reagents are not significantly affected by normal, artificial laboratory lighting when handled as directed in the Assay Procedure.

2. All reagents supplied should be used as an integral unit prior to the expiration date indicated on the package label.

3. Assay reagents should be stored as indicated.

4. Do not use Pyd Coated Strip if foil bag is punctured.

5. Samples greater than 750nM should be further diluted in Assay Buffer and retested. Be sure to include the dilution factor in the final calculation.
PRECAUTIONS...
6. Each sample should be tested in duplicate.

7. A standard curve must be performed with each assay.

8. A 4-parameter calibration curve fit must be used for accurate results. Equation: \( y = \frac{(A-D)}{(1+(x/C)^B)} + D \)

9. This assay has been validated for manual washing.

10. The Certificate of Analysis included in this kit is lot specific and is to be used to verify that the results obtained by your laboratory are similar to those obtained at Metra Biosystems, Inc. The OD values are to be used as a guideline only. The results obtained by your laboratory may differ.

   Quality control ranges are provided. The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits. If the control values are NOT within your laboratory's acceptance limits, the assay results should be considered questionable and the samples should be repeated.

11. If the OD of the Pyrilinks Standard "A" is less than 0.8, the results should be considered questionable and the samples should be repeated.
PRECAUTIONS...
12. If room temperature cannot be maintained between 20-28°C and an absorbance of >2.0 is not compatible with your plate reader, monitor the development of substrate in the Standard "A" wells; stop the reaction when the OD reaches 1.2-1.5, then read the strip(s).

13. Use of multi-channel pipets or repeat pipetors is recommended to ensure timely addition of reagents.

14. For accurate measurement of samples the addition of samples and standards must be precise. Pipet carefully using only calibrated equipment.

REAGENT PREPARATION AND STORAGE
1. Wash Buffer - See Procedural Note page 11
Prepare required amount of 1X Wash Buffer (see table on page 11) by diluting 10X Wash Buffer 1:10 with deionized water. Store at room temperature (20-28°C). Special Washing Instructions: Prepare 1X Wash Buffer as above and store at 2-8°C until use. Use 1X Wash Buffer within 24 hours.

2. Enzyme Conjugate
Prepare Enzyme Conjugate within 2 hours of use. Reconstitute each required vial of Enzyme Conjugate (see table on page 11) with 7 mL of cold (2-8°C) Assay Buffer. Store reconstituted Enzyme Conjugate at 2-8°C until use.
3. Working Substrate Solution
The Substrate Buffer must be brought to room temperature (20-28°C) before beginning the assay. (Two hours to overnight recommended.) Prepare Working Substrate Solution within 1 hour of use. Put one Substrate Tablet into each required bottle of room temperature Substrate Buffer (see table on page 11). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.

SPECIMEN COLLECTION AND STORAGE
The Pyrilinks assay can be carried out using a preservative free First Morning Void (FMV) urine collection. Longitudinal collections (eg. when assessing changes in resorption) should be collected at approximately the same time each day. Keep the urine sample refrigerated (2-8°C) for storage of less than 4 days, or freeze the sample at ≤ -20°C for longer storage. Do not subject sample to more than 3 freeze/thaw cycles. Avoid prolonged exposure to light, especially direct sunlight. Samples are not affected by normal, artificial laboratory lighting.
## PYRILINKS ASSAY PROCEDURE

**PROCEDURAL NOTE:** The Pyrilinks assay is sensitive to washing conditions. The entire wash step should be completed within 2 minutes. If the wash step CANNOT be completed within 2 minutes, follow the *Special Washing Instructions* located in the Reagent Preparation and Substrate Incubation sections.

Determine amount of each reagent required for the number of strips to be used.

<table>
<thead>
<tr>
<th># of Strips</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Samples</td>
<td>8</td>
<td>16</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>(tested in duplicate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Enzyme Conjugate (vial)**
  - 1
  - 1
  - 2*
  - 2*

- **Substrate Buffer (bottle)**
  - 1
  - 1
  - 2*
  - 2*

- **1X Wash Buffer (mL)**
  - 100
  - 150
  - 200
  - 300

* When more than one bottle or vial is to be used, combine the contents of that reagent, and mix prior to use.
PYRILINKS ASSAY PROCEDURE...

Sample/Enzyme Conjugate Incubation

1. Dilute samples, Standards and Controls 1:10 with Assay Buffer (e.g. 50μL sample + 450μL Assay Buffer).

2. Place desired number of Pyd Coated Strips in the Stripwell Frame just prior to use. Label strips to prevent mix-up in case of accidental removal from Stripwell Frame.

3. Add 50μL diluted Standard, Control or sample to each well of the Pyd Coated Strips. This procedure should be completed within 30 minutes.

4. Add 100μL of reconstituted Enzyme Conjugate to each well. Cover plate with Tape Cover provided. Incubate for 3 hours (±10 minutes) at 2-8°C. This incubation should be carried out in the dark.

5. Prepare Working Substrate Solution within 1 hour of use. Put one Substrate Tablet into each required bottle of room temperature Substrate Buffer (see table). Allow 30-60 minutes for tablet(s) to dissolve. Vigorously shake bottle(s) to completely mix.
PYRILINKS ASSAY PROCEDURE...

Substrate Incubation

1. Manually invert/empty strips. Add at least 250μL of 1X Wash Buffer to each well and manually invert/empty strips. Repeat two more times for a total of three washes. Vigorously blot the strips dry on paper towels after the last wash. While strips are inverted, carefully wipe bottom of strips with a lint-free paper towel to ensure that the bottom of the strips are clean.

Special Washing Instructions: Perform wash step as above, using cold (2-8°C) 1X Wash Buffer. After last wash, allow strips to drain on paper towels for 5-10 minutes to equilibrate to room temperature before adding substrate.

2. Add 150μL of Working Substrate Solution to each well.

3. Incubate for 60 minutes (± 5 minutes) at room temperature (20-28°C).

Stop/Read

1. Add 100μL of Stop Solution to each well. Add Stop Solution in the same pattern and time intervals as the Substrate Solution addition.

2. Read the Optical Density (OD) at 405 nm. Assure that no large bubbles are present in the wells and that the bottom of the strips are clean. Strips should be read within 15 minutes of Stop Solution addition.
PYRILINKS ASSAY PROCEDURE...

Stop/Read...

3. Quantitation software with a 4-parameter calibration curve fitting equation must be used to analyze the Pyrilinks assay results.

4. Determine concentration of samples and Controls from the Standard curve.

5. Control values should be within the range specified in the Certificate of Analysis supplied with the kit.

Representative Standard Curve

Standard Pyd levels: 0, 15, 40, 100, 250, 750 nM
QUICK GUIDE TO ASSAY STEPS

1. Dilute Standards, Controls and samples 1:10

2. Add 50µL diluted Standards, Controls and samples

3. Add 100µL cold Enzyme Conjugate

4. Incubate 3 hours ±10 minutes at 2-8°C in the dark

5. Wash 3 times

6. Add 150µL room temperature Substrate Solution

7. Incubate 60±5 minutes at room temperature

8. Add 100µL Stop Solution and read OD at 405nm
ANALYSIS OF RESULTS
Results obtained from the Pyrilinks assay must be corrected for variations in urine concentration by dividing the pyridinium crosslinks value (nM) by the creatinine value (mM) of each sample (creatinine mg/dL x 0.088 = mM). The final Pyrilinks results will be expressed as nM Pyd&Dpd / mM creatinine.

LIMITATIONS
1. While Pyrilinks is used as an indicator of type I collagen resorption, especially bone collagen, use of this test has not been established to predict development of osteoporosis or future fracture risk. Use of this test has not been established in menopause, Paget's disease of bone, primary hyperparathyroidism or hyperthyroidism. Results may be confounded in patients afflicted with clinical conditions known to affect bone collagen resorption, e.g. bone metastases in addition to diseases and conditions listed above. Pyrilinks results should be interpreted in conjunction with clinical findings and other diagnostic results.
PYRILINKS EXPECTED VALUES

Pyrilinks reference ranges have been established for healthy males (n=118) and healthy premenopausal females (n=301) over 25 years of age. For the purposes of establishing reference ranges, normal subjects were defined as:

- Basically healthy, no bone, endocrine or chronic disorders.
- Regular menstrual cycles (females).
- Not pregnant or breast feeding (females).
- Not currently taking any medication known to influence bone metabolism (e.g. corticosteroids, GnRH analogs, anticonvulsants, heparin, thyroid medication).

Values may be influenced by such factors as low estrogen production, low calcium intake, low physical activity, or diseases known to affect bone metabolism such as osteoporosis, Paget's disease, hyperparathyroidism, hyperthyroidism and bone metastases. Estrogen deficiency in postmenopausal women can result in elevated bone resorption. It is suggested that the premenopausal reference range be used to interpret results in postmenopausal women. Each laboratory should establish its own normal reference range. The results are expressed as nonparametric reference intervals (90%CI).

<table>
<thead>
<tr>
<th>Females (25-44 yrs):</th>
<th>Males (25-55 yrs):</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.0 - 37.0 nM/mM</td>
<td>12.8 - 25.6 nM/mM</td>
</tr>
</tbody>
</table>
**PYRILINKS EXPECTED VALUES.**
The expected within-subject variability was determined from urine specimens from 49 healthy subjects (26 premenopausal females and 23 males) collected for five nonconsecutive days over 2 weeks. The average of the individual within-subject longitudinal variation was 15%. Between-subject variability is reflected in the nonparametric reference intervals shown above.

**PERFORMANCE CHARACTERISTICS**

**Antibody Specifications**
The Pyridinoline antibody demonstrates selective, high affinity for free pyridinoline and deoxypyridinoline and negligible binding to Pyd and Dpd peptides.

<table>
<thead>
<tr>
<th>% Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Pyd</td>
</tr>
<tr>
<td>Free Dpd</td>
</tr>
<tr>
<td>Pyd/Dpd peptides</td>
</tr>
</tbody>
</table>

**Sensitivity**
The minimum detection limit of the Pyrilinks assay is 7.5 nM, determined by the upper 3 SD limit in a zero standard study.
PERFORMANCE CHARACTERISTICS...

Recovery - Spike Recovery
Spike recovery was determined by adding a known quantity of purified Pyd to urine samples with different levels of endogenous Pyd. Typical results are provided below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous (nM)</th>
<th>Added (nM)</th>
<th>Observed (nM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.7</td>
<td>68.2</td>
<td>84.1</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>71.0</td>
<td>68.2</td>
<td>140.8</td>
<td>102</td>
</tr>
<tr>
<td>3</td>
<td>141.0</td>
<td>68.2</td>
<td>215.5</td>
<td>109</td>
</tr>
</tbody>
</table>

Recovery - Linearity
Linearity was determined by serially diluting samples and comparing observed values with expected values. Typical results are provided below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution Factor</th>
<th>Observed (nM)</th>
<th>Expected (nM)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>neat</td>
<td>261.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1:2</td>
<td>127.8</td>
<td>130.8</td>
<td>98</td>
</tr>
<tr>
<td>1</td>
<td>1:4</td>
<td>59.9</td>
<td>65.4</td>
<td>92</td>
</tr>
<tr>
<td>1</td>
<td>1:8</td>
<td>31.1</td>
<td>32.7</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>neat</td>
<td>382.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1:2</td>
<td>183.2</td>
<td>191.0</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>1:4</td>
<td>90.4</td>
<td>95.5</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>1:8</td>
<td>57.9</td>
<td>57.1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>neat</td>
<td>412.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>1:2</td>
<td>199.0</td>
<td>206.2</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>1:4</td>
<td>98.2</td>
<td>103.1</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>1:8</td>
<td>47.8</td>
<td>51.5</td>
<td>88</td>
</tr>
</tbody>
</table>
PERFORMANCE CHARACTERISTICS...

Precision
Within-run and between-run precision were determined by assaying 3 urine samples in 8 different runs. Samples shown below represent a range of nM values. For a female with a creatinine level of 5mM, samples 1 through 3 represent low normal, high normal and elevated resorption (13.2 nM/mM, 32.0 nM/mM, and 81.4 nM/mM, respectively).

<table>
<thead>
<tr>
<th>Pyrilinks (nM Pyd/Dpd)</th>
<th>Within-run¹ C.V. (%)</th>
<th>Between-run² C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>9.9</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>7.0</td>
</tr>
<tr>
<td>3</td>
<td>407</td>
<td>6.6</td>
</tr>
</tbody>
</table>

¹n=52 ²n=8 runs

Clinical Studies
Clinical studies were performed to evaluate urine pyridinium crosslink levels obtained using the Pyrilinks assay. The first study was conducted at clinical investigation sites using 52 samples from healthy volunteers and 138 samples from patients with known bone disorders (osteoporosis, drug-induced osteoporosis, Paget's disease, hyperparathyroidism and hyperthyroidism). These diseases often involve elevated bone collagen resorption, although not all subjects would be expected to have elevated resorption at the time of sample collection. One hundred and one of the 138 patients diagnosed with a disorder did not have elevated pyridinoline values as measured by HPLC. The Pyrilinks pyridinium crosslink values in healthy subjects ranged from 13.7 to 49.4 nM/mM and in patients ranged from 9.8 to 135.9 nM/mM.
PERFORMANCE CHARACTERISTICS...
Clinical Studies...
In the study, the Pyrilinks assay was compared to a research HPLC method\textsuperscript{11} for measuring pyridinoline. The HPLC threshold was determined, in a study of 84 healthy adult subjects, to be 50 nM/mM for males and 60 nM/mM for females (95\% confidence interval upper limit for each gender).

Using elevated pyridinoline determined by HPLC as the classification method, the receiver operating characteristic (ROC) technique was used to define an optimal relative sensitivity and specificity in the described population. Relative sensitivity and specificity are presented in Table I. A two-by-two contingency table showing the number of subjects in each classification is shown in Figure I.

Table I

| Pyrilinks | relative sens. = 84\%, spec. = 82\% |

Figure I

<table>
<thead>
<tr>
<th>HPLC Pyridinoline</th>
<th>Elevated</th>
<th>Not Elevated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pyrilinks +</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td>Pyrilinks -</td>
<td>7</td>
<td>119</td>
</tr>
</tbody>
</table>
PERFORMANCE CHARACTERISTICS...
Clinical Studies...
In a second study, the Pyrilinks assay results were compared in a mixed population of 39 samples from healthy subjects and 99 samples from Paget's disease patients. Although Paget's disease represents a model for identifying active bone collagen resorption, some of the patients in this study are undergoing treatment or may be in remission, and may not have elevated resorption at the time of sample collection. In this study, healthy subjects ranged from 12.8 to 33.2 nM/mM. Paget's disease patients ranged from 14.4 to 667.6 nM/mM.

Using the diagnosis of Paget's disease as the classification method, the ROC technique was used to define an optimal relative sensitivity and specificity in this population. Relative sensitivity and specificity are shown in Table II. A two-by-two contingency table is shown in Figure II.

Table II

<table>
<thead>
<tr>
<th>Pyrilinks</th>
<th>relative sens. = 89%, spec. = 95%</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Paget's Diagnosis</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrilinks +</td>
<td>88</td>
<td>2</td>
</tr>
<tr>
<td>-</td>
<td>11</td>
<td>37</td>
</tr>
</tbody>
</table>


OSCAtest®
Osteocalcin (BGP)

Coated Tube System

Radioimmunoassay for the determination of intact osteocalcin (BGP) in human serum

Instruction manual
English: page 29–56

HENNING BERLIN GMBH
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Precautions – please read carefully!

This kit is for in vitro use only.

The expiry dates stated on the main container label and all vial labels have to be observed.

All reagents should be stored at 4–8°C in their original shipping container before use.

The reagents contain sodium azide as a preservative and must not be swallowed or allowed to come into contact with the skin or mucous membranes.

The following precautions should be observed in handling radioactive material:

- Do not pipette by mouth.
- Do not smoke, eat, or drink while handling radioactive materials.
- Always use protective gloves while handling radioactive materials.
- Spills should be wiped up quickly and thoroughly, and the contaminated materials should be treated as radioactive waste.
- Solid and liquid radioactive waste is to be treated according to local regulations.

We are permitted to transfer the radioactive material in this kit only to laboratories and persons holding a valid handling license for radioactive material!

Please follow the working instructions carefully.

Berlin, December 1991
Osteocalcin: biochemistry, physiology

Osteocalcin is a calcium-binding protein of bone with 49 amino acids (in humans) and a molecular weight of about 5,800 Da\(^1\). It comprises 1–2% of total bone protein\(^2\) and contains in humans two gamma-carboxyglutamic acid (GLA) residues\(^1\), hence “bone GLA protein” (BGP).

The synthesis of osteocalcin is vitamin K-dependent and is markedly stimulated by 1,25-dihydroxyvitamin D\(_3\). The precise function of this protein in bone metabolism has not yet been clarified. Suggested functions include the formation of mineralized bone matrix and/or mediator for bone resorption\(^2,1\).

Osteocalcin is solely synthesized by the osteoblasts and is incorporated into the extracellular matrix of bone. However, a fraction of newly synthesized intact osteocalcin (about 15%) is released into the circulation, where it can be measured by radioimmunoassay\(^1\). Due to the good correlation between serum osteocalcin levels and histomorphometric indices of bone formation\(^4,5\), the serum level directly reflects the activity of the osteoblasts.

But osteocalcin levels seem also to be dependent on the intensity of mineralization\(^6\). If hydroxyapatite formation is increased, eg. in Paget's disease of bone, the newly synthesized osteocalcin is bound (or trapped) by hydroxyapatite, thus preventing an increase in serum level of osteocalcin\(^6\).

Thus, with the exception of states of excessive hydroxyapatite formation, osteocalcin is a specific and sensitive marker of bone formation rate.
Clinical significance of osteocalcin determinations

Postmenopausal osteoporosis

In women with untreated postmenopausal osteoporosis, there is a wide range of individual osteocalcin values, reflecting the heterogeneity of the disease. Serum osteocalcin is in the normal range or decreased in patients with low osteoblastic activity ("low turnover" osteoporosis) and is increased in the one third of patients having a high bone turnover.

Recognizing this variable level of bone turnover might be important for choosing the optimal osteoporosis therapy. Indeed, a study by Civitelli et al.\(^7\) has shown that the subgroup of osteoporotic patients with high turnover (characterized by an increase of whole-body retention of technetium-labelled diphosphonates and by increased serum osteocalcin and urinary hydroxyproline levels) showed an increase of spinal bone mineral density after one year of calcitonin therapy. In contrast, those with low turnover had no increase of bone mass despite the same therapy\(^7\).

Sodium fluoride, which has been shown to stimulate the formation of mineralized tissue, is also used to treat osteoporosis. In patients treated with fluoride, the increase in serum osteocalcin parallels the increase in bone mineral density of the spine\(^8\).

Furthermore, it has been reported that serum osteocalcin is elevated in response to therapeutic calcitriol \([1,25(\text{OH})_2\text{D}_3]\) in osteoporotic women\(^9,10\). The response in osteoporotic women was nearly double the response in controls\(^11\).

Due to two independent studies\(^12,13\), in untreated postmenopausal women followed for 2 to 4 years, serum osteocalcin is the best
single biochemical marker reflecting the spontaneous rate of bone loss as assessed by repeated measurements of the bone mineral content of the radius and of the spine\textsuperscript{12,13}. Estrogen\textsuperscript{14,15} as well as cyclic estrogen-gestagen\textsuperscript{37} therapy as it is used for prophylaxis or treatment in peri- and early postmenopausal women leads to a decrease in osteocalcin levels. Thus, osteocalcin is a valuable parameter for the decision about the adequate osteoporosis therapy for the individual patient and for the follow-up of treatment response.

**Corticosteroid treatment**

Long-term corticosteroid treatment results in a marked inhibition of osteoblastic activity, which is reflected by subnormal serum levels of osteocalcin\textsuperscript{18,19}. The decline of serum osteocalcin occurs within a few days after initiation of corticosteroid therapy\textsuperscript{17,18}, and the decrease is unaffected by an exercise program\textsuperscript{20}. Gradual reduction of the steroid dosage\textsuperscript{18} or stop of therapy\textsuperscript{17} results in an increase of osteocalcin to pretreatment levels. During therapy of corticosteroid-induced osteoporosis with nandrolone decanoate an increase of bone density was paralleled by an increase in serum osteocalcin\textsuperscript{21}. Thus, osteocalcin is helpful for monitoring bone turnover in corticosteroid therapy.

**Renal osteodystrophy**

As osteocalcin has been found to be cleared by the kidney in the rat\textsuperscript{22}, renal failure could theoretically increase serum osteocalcin
levels independent of the level of bone turnover. Actually, two studies have shown that serum osteocalcin is decreased only in patients with a glomerular filtration rate below 20-40 ml/min\textsuperscript{23,24}. Furthermore, surprisingly in patients on chronic maintenance dialysis various groups have reported a good correlation between the biochemical marker osteocalcin and histomorphometric indices of bone formation\textsuperscript{5,25}.

In hemodialysis patients serum osteocalcin is much higher in those with high turnover and prevailing hyperparathyroid bone disease than in those with low turnover\textsuperscript{25,26,5}. A discrimination between patients with or without osteomalacia within the the low turnover group cannot be achieved on the basis of serum osteocalcin levels alone\textsuperscript{5,25}, but may be possible taking into account serum levels of intact PTH, osteocalcin and aluminium\textsuperscript{25}.

Thus, serum osteocalcin is a valuable marker for evaluating bone turnover in hemodialyzed patients and also assessing the effects of treatment altering bone turnover.

**Other disorders of bone turnover**

While serum osteocalcin levels are in the lower range of normal or subnormal in hypoparathyroidism\textsuperscript{27,28}, they are in the higher range of normal or increased in patients with primary hyperparathyroidism\textsuperscript{29,27} and hyperthyroidism\textsuperscript{27}. Osteocalcin levels correlate with bone disease activity\textsuperscript{30}.

Serum osteocalcin levels are decreased in children with growth hormone (GH) deficiency\textsuperscript{31} but normal in GH-deficient adults\textsuperscript{32}. 
Treatment with growth hormone leads to an increase in osteocalcin levels in children and adults\textsuperscript{31,32} and may thus be a helpful marker for monitoring therapy, though it was looked upon only as a slow indicator of therapeutic effect in one study\textsuperscript{33}.

**Paget's disease**

In Paget's disease the mean value of serum osteocalcin is increased, but individual values can be normal in up to 50% of the patients, despite clinical and biochemical evidence of active disease, especially increased serum levels of alkaline phosphatase\textsuperscript{34,27,35}. As mineralization is often excessively increased in Paget's disease of bone, the relatively low osteocalcin levels probably reflect an enhanced binding (or trapping) of the molecule into the woven bone, thus reducing the fraction of newly synthesized osteocalcin released into the circulation\textsuperscript{36}. Another explanation for the low osteocalcin levels would be dysregulation of osteocalcin synthesis by pagetic and less mature osteoblasts. Thus, the determination of osteocalcin is of little help in Paget's disease.
Specimen collection

Studies by HENNING BERLIN GMBH (unpublished) have shown that osteocalcin is degraded in blood samples (serum and plasma). But this degradation differs widely among individuals, the half-life period ranging from a few hours to several days.

In order to obtain reliable results when measuring osteocalcin, it is crucial to process blood samples (centrifuge, freeze) immediately after taking blood. A time of 3/4 of an hour should not be exceeded between taking blood and freezing the sample. It is also recommended that the samples be thawed only shortly before use in the assay.

This degradation of osteocalcin in blood samples is independent of the test system used and can be observed in all osteocalcin kits.

With OSCAtest® Osteocalcin (BGP) the measurement of osteocalcin can be performed in serum, EDTA-, citrate-, and heparin plasma.
Principle of the procedure

OSCAtest® Osteocalcin (BGP) for the determination of intact osteocalcin in human serum (or plasma) is a competitive radio-immunoassay, using the coated tube technique.

During incubation osteocalcin from human serum samples or standards competes with a fragment analogue acting as tracer \([^{125}\text{I}]-\text{osteocalcin (38–49)}\) for the antigen binding sites of a highly specific antibody immobilized on the inner surface of the tube. The concentration of the tracer and the concentration of the antibody are constant in all the tubes within one assay. Thus, the only variable parameter is the concentration of unlabelled osteocalcin in the sample.

A high concentration of osteocalcin in the sample leads to reduced binding of \(^{125}\text{I}\)-labelled osteocalcin to the antibody. Finally, the amount of radioactivity bound to the tube is reversely proportional to the osteocalcin concentration of the tested sample.

After the incubation period the tracer that has not bound to the tube is removed by aspirating the liquid phase. The radioactivity remaining in the tube is measured.

The antibody was raised against intact human osteocalcin. As preliminary studies show that this C-terminal fragment does not exist in vivo, the antibody solely recognizes **intact osteocalcin in human serum.** Since intact human osteocalcin is employed in the standards as well, OSCAtest® Osteocalcin (BGP) is based on a homologous system.
Contents of the kit

This kit provides sufficient reagents for 100 determinations:

A  Tracer (purified by HPLC) *
   \(^{125}\text{I}-\text{osteocalcin } (38-49),\)
   1 vial for 27 ml, red coloured, lyophilized;
   approx. 44 kBq per vial, 
   before use to be reconstituted with buffer B

B  Buffer
   1 vial containing 27 ml, ready for use.
   Before using Tracer A reconstitute with Buffer B 
   and mark vial B as radioactive.

C  Coated tubes
   coated with anti-\(h\)-osteocalcin (38-49)-antibody 
   (polyclonal, sheep)
   2 x 50 coated tubes, ready for use.

D  Washing solution **
   1 vial containing 10 ml, concentrate.
   The concentrate should be filled up to 500 ml with 
   destilled water.

G  Zero-serum
   1 vial à 6 ml, ready for use, 
   for reconstitution of standards and controls

* If less than 100 determinations are carried out per assay, all reconstituted reagents 
  must be frozen immediately after pipetting.

** The washing solution is also adapted to Dynotest\textsuperscript{®} TSH, Dynotest\textsuperscript{®} FT\(_4\), Dynotest\textsuperscript{®} Tg, 
  Dynotest\textsuperscript{®} anti-TPO, DYNOtest\textsuperscript{®} ACTH and THYRAK-ASSAY\textsuperscript{®}. 
Osteocalcin zero-standard *
1 vial for 0.5 ml; lyophilized,
before use reconstitute with 0.5 ml zero-serum.

Osteocalcin standards *
5 vials for 0.5 ml; lyophilized,
before use reconstitute with 0.5 ml zero-serum.
Concentrations: 0.3; 0.8; 2.1; 5.2; 13.1 nmol/l
2.0; 5.0; 12.5; 31.2; 78 ng/ml

Control sera I and II *
2 vials for 0.5 ml; lyophilized,
before use reconstitute with 0.5 ml zero-serum.
For further details see leaflet enclosed.

Note
All lyophilized reagents should be reconstituted
approximately a quarter of an hour before use.
Duration of reconstitution: at least 15 minutes.

Stability
Please refer to expiry date on kit.

Storage
Reconstituted reagents must be kept frozen. Store all
other reagents at 4–8 °C.

Accessories
3 sheets of adhesive foil

*If less than 100 determinations are carried out per assay, all reconstituted reagents
must be frozen immediately after pipetting.
Upon request
1 rack for 50 tubes

Additional equipment
- Micro-pipettes (50 μl, 250 μl)
- or equivalent dispenser-diluter systems
- Pipette/dispenser (1 ml)
- Sample mixer (e.g. Vortex)
- Gamma-counter.
### Incubation scheme

**OSCAtest® Osteocalcin (BGP)**

<table>
<thead>
<tr>
<th>1</th>
<th>Number the coated tubes (a, b)</th>
<th>T</th>
<th>0</th>
<th>1–5</th>
<th>6 etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Pipette zero-standard µl</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>standards µl</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>samples µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>Pipette tracer µl</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>Incubate</td>
<td>over night (20–24 h) at 4–8°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pipette washing solution ml</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Decant</td>
<td>the liquid completely Wash and repeat steps 5 and 6 once</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Measure radioactivity</td>
<td>recommended counting time: 1 min.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

→ Calculation of results
Test procedure
OSCAtest® Osteocalcin (BGP)

Note
The following note generally applies to measurements of osteocalcin and should be observed regardless of the test system used (cf. "specimen collection", p. 38).

If samples are not analyzed within 45 minutes after blood has been taken, they must be stored at $-20 \degree C$!

After thawing serum samples must be processed at once and ought not to be left standing. Likewise, they must be frozen immediately after use.

If high osteocalcin levels are expected, dilutions should be performed using osteocalcin zero-serum.

Hemolytic and lipemic sera may lead to falsely low osteocalcin values.

Agitate standards and serum samples gently before use (avoid foam formation) and pipette, as all other reagents, at room temperature.

1. Number the tubes serially (a, b for duplicates), label for total radioactivity tubes: Ta,b.

2. Into the tubes 0 a, b ... 5 a, b pipette 50 μl of osteocalcin standards with increasing concentrations and into the tubes 6 a, b etc. pipette 50 μl of each sample.

3. Into each tube – including T a, b – pipette 250 μl of tracer. The tubes T a, b are now kept separately until the radioactivity is measured (cf. point 7).
4. Agitate all tubes shortly on a vortex, then cover the tubes with the adhesive foil enclosed and incubate for 20–24 hours at 4–8 °C.

5. Wash the coated tubes with 2 ml of diluted washing solution and decant.

6. Repeat step 5 and 6 once and leave the tubes upside down for 5–10 minutes on blotting paper.

**Note** This can be carried out by using a wash comb washing 5 tubes simultaneously (available from Henning Berlin GmbH).

7. Measure the radioactivity of each tube, including T a, b. Recommended counting time: 1 minute.
Calculation of results

In case of computer-assisted analysis of OSCAtest® Osteocalcin (BGP), a special program suitable for radioimmunoassay and adapted to the counter-computer combination should be used.

When calculating the results of OSCAtest® Osteocalcin (BGP) without assistance of a computer, it is recommended that the mean count rate of each sample (B) should be related to the mean count rate of the zero-standard (B₀) and the results be expressed as percent B/B₀.

In case of the zero-standard \( B/B₀ = 100\% \)

Using semilogarithmic paper the mean percent values \( B/B₀ \) of each standard (ordinate, linear) are plotted against the corresponding osteocalcin concentration (abscissa, logarithmic) to obtain a standard curve. The mean percent values \( B/B₀ \) of the unknown samples are then used to determine the corresponding osteocalcin concentrations directly in nmol/l (ng/ml).

**Conversion factor: ng/ml = nmol/l x 5.97**

To check the binding capacity of the assay \( B₀/T \) is calculated:

\[
\frac{B₀}{T} \quad (\%) = \frac{\text{mean count rate } 0 \ a, b}{\text{mean count rate } T \ a, b} \times 100
\]

Under the assay conditions described \( B₀/T \) is normally in the range of 40–50\%. 
## Calculation example

<table>
<thead>
<tr>
<th>Test tube</th>
<th>cpm (a)</th>
<th>cpm (b)</th>
<th>$\bar{x}$</th>
<th>$B/B_0$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity T</td>
<td>51 280</td>
<td>51 182</td>
<td>51 231</td>
<td>–</td>
</tr>
<tr>
<td>Zero-standard</td>
<td>22 910</td>
<td>23 198</td>
<td>23 054</td>
<td>100</td>
</tr>
<tr>
<td>Standard 1 0.3 nmol/l</td>
<td>18 628</td>
<td>18 796</td>
<td>18 712</td>
<td>81</td>
</tr>
<tr>
<td>Standard 2 0.8 nmol/l</td>
<td>14 085</td>
<td>14 307</td>
<td>14 196</td>
<td>62</td>
</tr>
<tr>
<td>Standard 3 2.1 nmol/l</td>
<td>9 440</td>
<td>9 320</td>
<td>9 380</td>
<td>41</td>
</tr>
<tr>
<td>Standard 4 5.2 nmol/l</td>
<td>4 972</td>
<td>5 220</td>
<td>5 096</td>
<td>22</td>
</tr>
<tr>
<td>Standard 5 13.1 nmol/l</td>
<td>3 131</td>
<td>2 943</td>
<td>3 037</td>
<td>13</td>
</tr>
<tr>
<td>Sample 6</td>
<td>13 760</td>
<td>13 900</td>
<td>13 830</td>
<td>60</td>
</tr>
</tbody>
</table>

### Calculating the binding capacity:

\[
\frac{B_0}{T} = \frac{23 054}{51 231} \times 100 = 45\%
\]

### Calculating the osteocalcin concentration of serum sample 6:

\[
\frac{B_6}{B_0} = \frac{13 830}{32 054} \times 100 = 60\% = 5.5 \text{ nmol/l}
\]
Standard curve OSCAtest® Osteocalcin (BGP)

B/B₀ (%)

0 20 40 60 80 100

0 2.0 5 12.5 31 78

ng/ml

0.3 0.8 2.1 5.2 13.1

nmol/l

osteocalcin concentration
Assay characteristics

1. Dilution

<table>
<thead>
<tr>
<th>Dilution</th>
<th>measured OC-conc. (pg/ml)</th>
<th>calculated OC-conc. (pg/ml)</th>
<th>recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>orig.</td>
<td>13.5</td>
<td>6.8</td>
<td>107</td>
</tr>
<tr>
<td>1: 2</td>
<td>7.2</td>
<td>6.8</td>
<td>107</td>
</tr>
<tr>
<td>1: 4</td>
<td>3.6</td>
<td>3.4</td>
<td>107</td>
</tr>
<tr>
<td>1: 6</td>
<td>2.2</td>
<td>2.3</td>
<td>98</td>
</tr>
<tr>
<td>1: 8</td>
<td>1.6</td>
<td>1.7</td>
<td>95</td>
</tr>
<tr>
<td>1:10</td>
<td>1.6</td>
<td>1.4</td>
<td>119</td>
</tr>
</tbody>
</table>

All the coefficients of variation of the patient sera depicted in the fig. are based on 10fold determinations. Based on these results the functional assay sensitivity – lowest concentration in which intra-assay CV is < 10% and inter-assay CV is < 20% – of the OSCAtest® Osteocalcin (BGP) is about 1.8 ng/ml (0.3 nmol/l)
Normal values

Most of the up to now published studies examining the time course of serum osteocalcin levels found a circadian rhythm (e.g. 36). Due to these studies serum osteocalcin levels reach a peak at night and a nadir in the morning at 8-11 a.m. Thus, it is recommended to take blood for osteocalcin determinations during this time.

On the basis that blood sampling was done according to the precautions mentioned beforehand (cf. p. 36) the determination of osteocalcin in a group of normal persons without overt bone disease yielded data which indicate dependency of osteocalcin concentration on age and sex, as it has also been reported in the literature.

Osteocalcin values of men and women over the age of 40 have only been accepted as normal, if values of bone mineral density measured (BMD) by dual photon absorptiometry (DPA) have been within normal limits (±2 s).

For women over the age of 40 normal values were regarded to be the same as for men. It was assumed that there is indeed an increase in bone turnover – despite still normal BMD values – in the women with elevated osteocalcin values (above 12 ng/ml), as it has been observed (e.g. 36) in women after a decrease in estrogen levels (e.g. postmenopausal, after oophorectomy).

It is recommended that each laboratory establishes its own normal and pathological ranges of serum osteocalcin levels as it is usually done of other laboratory parameters. This will enable the kit manufacturer to be checked and will also indicate any particular variations in serum osteocalcin levels as well as any special prevalences of certain pathophysiological situations. In addition, it will provide an assessment of the quality of the results obtained with the kit.

Therefore the following data only provide a guide to values which might be expected.
Osteocalcin normal values in women

Osteocalcin normal values in men
Osteocalcin normal values in relation to age and sex

**Children and adolescents**

<table>
<thead>
<tr>
<th>age group (years)</th>
<th>1-10</th>
<th>11-15</th>
<th>16-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal range (nmol/l)</td>
<td>1.7-6.7</td>
<td>1.7-13.4*</td>
<td>1.7-8.4*</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>10-40</td>
<td>10-80*</td>
<td>10-50*</td>
</tr>
<tr>
<td>number (n)</td>
<td>21</td>
<td>36</td>
<td>19</td>
</tr>
</tbody>
</table>

*high osteocalcin levels are to be expected during periods of accelerated growth

**Women**

<table>
<thead>
<tr>
<th>age group (years)</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
<th>51-60</th>
<th>61-70</th>
<th>71-80</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal range (nmol/l)</td>
<td>0.7-3.4</td>
<td>0.6-2.5</td>
<td>0.7-2.0</td>
<td>0.7-2.0</td>
<td>0.7-2.0</td>
<td>(0.7-2.0)</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>4-20</td>
<td>3.5-15</td>
<td>4-12</td>
<td>4-12</td>
<td>4-12</td>
<td>(4-12)</td>
</tr>
<tr>
<td>number (n)</td>
<td>82</td>
<td>61</td>
<td>113</td>
<td>96</td>
<td>40</td>
<td>3</td>
</tr>
</tbody>
</table>

**Men**

<table>
<thead>
<tr>
<th>age group (years)</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
<th>51-60</th>
<th>61-70</th>
<th>71-80</th>
<th>81-90</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal range (nmol/l)</td>
<td>1.0-3.4</td>
<td>0.7-2.5</td>
<td>0.7-2.0</td>
<td>0.7-2.0</td>
<td>0.7-2.0</td>
<td>0.8-2.0</td>
<td>(0.7-2.0)</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>6-20</td>
<td>4-15</td>
<td>4-12</td>
<td>4-12</td>
<td>4-12</td>
<td>5-12</td>
<td>(4-12)</td>
</tr>
<tr>
<td>number (n)</td>
<td>79</td>
<td>76</td>
<td>81</td>
<td>32</td>
<td>18</td>
<td>18</td>
<td>7</td>
</tr>
</tbody>
</table>
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HENNING BERLIN GMBH

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Radioimmunoassay for the determination of intact osteocalcin (BGP) in human serum

Arbeitsanleitung
Deutsch: Seite 1–27

Instruction manual
English: page 29–56
Inhaltsverzeichnis

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Wichtige Hinweise – bitte sorgfältig lesen!

Dieser Reagenziensatz ist **nur zum In-vitro-Gebrauch** bestimmt!

Die auf der äußeren Umhüllung und den Etiketten dieses Reagenziensatzes angegebenen Verfallsdaten sind unbedingt zu beachten. Die Reagenzien sind bis zur Verwendung im **Versandbehälter bel 4–8°C zu lagern.**

Die Reagenzien enthalten als Konservierungsmittel Natriumazid. Verschlucken, Berührung mit der Haut oder den Schleimhäuten ist zu vermeiden!

Bezüglich der Lagerung und des Umgangs mit radioaktiven Stoffen wird auf die Vorschriften der Strahlenschutzverordnung (Bundesgesetzblatt 1, S. 1321, 1989) hingewiesen.

**Folgende Vorsichtsmaßnahmen sind unbedingt einzuhalten:**

- Beim Umgang mit radioaktiven Stoffen nicht essen, trinken oder rauchen.

- Radioaktives Material niemals mit dem Mund pipettieren.

- Hände mit Gummihandschuhen schützen.

- Verschüttetes radioaktives Material sofort wegwischen, kontaminierte Geräte mit geeigneten Detergentien dekontaminieren, ggf. wie radioaktiven Abfall behandeln.

Aufgrund der gesetzlichen Bestimmungen dürfen wir radioaktive Reagenzien nur an Personen abgeben, die im Besitz einer gültigen Umgangsgenehmigung sind.
Es wird empfohlen, mittels laborinterner und/oder im Kit mitgeliefertem Kontrollseren Qualitätskontrollen entsprechend der Eichordnung (Bundesgesetzblatt I, S. 1657, 1988) und den Richtlinien der Bundesärztekammer zur Qualitätssicherung in medizinischen Laboratorien (Dt. Ärzteblatt 85, Heft 11, S. 699, 1988) durchzuführen.

In diesem Reagenziensatz ist Humanserum enthalten. Es werden nur Seren verwendet, die HBsAg-negativ und HIV-I-Antikörper-negativ sind. Trotzdem müssen bei der Handhabung Vorsichtsmaßregeln wie bei Patientenproben eingehalten werden.

**Die folgende Arbeitsanleitung ist strikt einzuhalten!**

Berlin, Dezember 1991
Osteocalcin: Biochemie und Physiologie

Osteocalcin ist ein Calcium-bindendes Knochenprotein mit 49 Aminosäuren (beim Menschen) und einem Molekulargewicht von ca. 5800 Da\(^1\). Osteocalcin macht etwa 1–2% des gesamten Knochenproteins aus\(^2\) und enthält beim Menschen zwei Gamma-Carboxyglutaminsäurereste (GLA)\(^1\), die zu der parallel verwendeten Bezeichnung „bone GLA protein“ (BGP) führten.

Die Synthese von Osteocalcin ist Vitamin K-abhängig und wird sehr stark durch 1,25-Dihydroxyvitamin D\(_3\) stimuliert\(^3\). Die genaue Rolle dieses Proteins im Knochenstoffwechsel ist bislang noch ungeklärt. Diskutiert werden Funktionen bei der Knochenmineralisation bzw. als Mediator für die Knochenresorption\(^2,1\).

Osteocalcin wird ausschließlich von Osteoblasten synthetisiert und anschließend in die Knochenmatrix eingebaut. Ein Teil des neu synthetisierten Osteocalcins (ca. 15%) wird aber in das Blut abgegeben, wo es mittels Radioimmunoassay gemessen werden kann\(^1\). Aufgrund guter Korrelationen zwischen Osteocalcin-Konzentration im Serum und histomorphometrischen Befunden\(^4,5\) ist davon auszugehen, daß Serum-Osteocalcinspiegel direkt die Aktivität der Osteoblasten widerspiegeln.

Die Serumspiegel von Osteocalcin sind darüberhinaus aber auch von der Intensität der Mineralisation abhängig. Bei gesteigerter Hydroxyapatitbildung, wie z. B. beim Morbus Paget, wird ein Großteil des neu gebildeten Osteocalcins an Hydroxyapatit gebunden („weggefangen“), so daß die Serumspiegel trotz erhöhter Osteoblasten-Aktivität nicht ansteigen\(^6\).

Mit der Ausnahme von Erkrankungen mit exzessiver Hydroxyapatit-Neubildung gilt Osteocalcin daher als ein spezifischer und sensitiver Marker für den Knochenaufbau.
Klinische Bedeutung der Osteocalcin-Bestimmung

Postmenopausale Osteoporose


Weiterhin ist berichtet worden, daß Serum-Osteocalcin bei Frauen mit postmenopausaler Osteoporose nach Gabe von


In zwei unabhängig voneinander durchgeführten Studien an über zwei bis vier Jahre untersuchten postmenopausalen Frauen stellte sich heraus, daß unter den biochemischen Markern Serum-Osteocalcin beim Vergleich mit Knochendichtemessungen am Radius und an der Wirbelsäule am genauesten die Höhe des spontanen Knochenverlustes anzeigte¹²,¹³.

Insgesamt erscheint Osteocalcin als ein wertvoller Parameter für die Entscheidung über die adäquate Osteoporose-Therapie für den einzelnen Patienten und für die Beurteilung des Therapieerfolges.

**Behandlung mit Kortikosteroiden**

Langandauernde Kortikosteroid-Behandlung führt zu einer deutlichen Hemmung der Aktivität der Osteoblasten, was sich in subnormalen Serum-Osteocalcin-Konzentrationen widerspiegelt¹⁸,¹⁹. Der Abfall des Serum-Osteocalcins tritt schon wenige Tage nach Beginn der Kortikosteroid-Therapie auf¹⁷,¹⁸ und läßt

Nach diesen Ergebnissen ist die Bestimmung von Osteocalcin hilfreich für die Beurteilung des Knochenstoffwechsels unter Kortikosteroid-Therapie.

**Renale Osteopathie**


Bei Hämodialysepatienten mit erhöhtem Knochenstoffwechsel und deutlichem sekundärem Hyperparathyreoidismus finden sich sehr viel höhere Osteocalcin-Werte als bei denen mit erniedrigtem
Knochenstoffwechsel\textsuperscript{25,26,5}. Allein aufgrund des Serum-Osteocalcin-Spiegels ist bei den Patienten mit erniedrigtem Knochenstoffwechsel keine Unterscheidung zwischen denen mit und denen ohne Osteomalazie möglich\textsuperscript{5,25}. Dies könnte jedoch erreichbar sein, wenn man zusätzlich die Serumspiegel von intaktem PTH und Aluminium berücksichtigt\textsuperscript{25}.

**Andere Erkrankungen mit Knochenbeteiligung**

Bei Patienten mit Hypoparathyreoidismus werden niedrig normale bzw. erniedrigte Serum-Osteocalcin-Konzentrationen beobachtet\textsuperscript{27,28}, bei Patienten mit primärem Hyperparathyreoidismus\textsuperscript{29,27} und Hyperthyreose\textsuperscript{27} hochnormale bzw. erhöhte Werte. Osteocalcin-Spiegel korrelieren, zumindest bei primärem Hyperparathyreoidismus, mit dem Ausmaß der Knochenbeteiligung\textsuperscript{30}.

Bei Wachstumshormonmangel bei Kindern sind die Serum-Osteocalcin-Spiegel erniedrigt\textsuperscript{31}, bei Erwachsenen dagegen normal\textsuperscript{32}. Unter Therapie mit Wachstumshormon steigen die Osteocalcin-Werte aber in beiden Gruppen an\textsuperscript{31,32}. Osteocalcin erscheint als hilfreicher Parameter für die Beurteilung des Therapieerfolges, obwohl es in einer Studie nur den Therapieerfolg erst recht spät angezeigt hat\textsuperscript{33}.

**Morbus Paget**

In einer Vielzahl von Studien wurden bei Patienten mit Morbus Paget in bis zu 50\% der Fälle normale Osteocalcin-Werte gefun-
den, obwohl klinische und biochemische Parameter, v. a. erhöhte Serumspiegel von alkalischer Phosphatase, für eine aktive Erkran-
kung sprachen\textsuperscript{\textdegree}\textsuperscript{\textdegree}\textsuperscript{\textdegree}. Dies läßt sich vermutlich dadurch erklären, 
daß beim Morbus Paget die Intensität der Mineralisation stark 
erhöht ist, so daß ein Großteil des neugebildeten Osteocalcins an 
Hydroxyapatit gebunden („weggefangen“) wird, so daß die 
Serum-Osteocalcin-Konzentrationen trotz erhöhter Osteoblasten-
Aktivität nicht ansteigen\textsuperscript{\textdegree}. Möglicherweise liegt beim Morbus 
Paget auch eine Dysregulation der Osteocalcin-Synthese bei den 
Osteoblasten vor, die den für den Paget typischen Knochen 
bilden.

Die Osteocalcin-Bestimmung ist infolgedessen für die Beurteilung 
des Morbus Paget wenig hilfreich.
Probenbehandlung

Untersuchungen der Fa. HENNING BERLIN GMBH (unveröffentlicht) haben gezeigt, daß Osteocalcin in Blutproben (Serum bzw. Plasma) abgebaut wird. Dieser Abbau variiert allerdings individuell sehr stark, mit Halbwertszeiten zwischen wenigen Stunden und mehreren Tagen.

Um verlässliche Meßergebnisse bei der Bestimmung von Osteocalcin zu erhalten, ist es daher unbedingt erforderlich, die Proben möglichst sofort, spätestens aber nach einer 3/4 Stunde, nach der Blutentnahme weiterzuverarbeiten (Zentrifugieren, Einfrieren). Aus demselben Grund dürfen die Proben erst kurz vor der Assaydurchführung aufgetaut werden.

Die Bestimmung von Osteocalcin mittels des OSCAtest® Osteocalcin (BGP) kann in Serum und in EDTA-, Zitrat- und Heparin-Plasma durchgeführt werden.

Zum Meßprinzip des OSCAtest® Osteocalcin (BGP)

Der OSCAtest® Osteocalcin (BGP) ist ein kompetitiver Radioimmunoassay zur Bestimmung von intaktem Osteocalcin in menschlichem Serum (oder Plasma) mittels Coated-tube-Technik.

Osteocalcin aus menschlichem Serum bzw. humanes Osteocalcin aus den Standards konkurriert während der Inkubationsphase mit dem Tracer \[^{125}\text{I}-\text{Osteocalcin (38–49)}\] um die Antigenbindungsstelle des an der Röhrchenwand fixierten hochspezifischen Antikörpers gegen Osteocalcin (37–49). Eine hohe Konzentration von Osteocalcin in der Probe führt zu einer verringerten Bindung von \[^{125}\text{I}-\text{markiertem Osteocalcin an den Antikörper. Die Höhe der an der Röhrchenwand gebundenen Radioaktivität ist also umgekehrt proportional zur gesuchten Osteocalcin-Konzentration in der jeweiligen Probe.}

Nach Reaktionsende wird der nicht an der Röhrchenwand gebundene Traceranteil durch Dekantieren der flüssigen Phase beseitigt und die im Röhrchen verbleibende Radioaktivität in einem Gamma-Counter gemessen.

Der Antikörper erkennt in humanem Serum ausschließlich intaktes Osteocalcin, da das C-terminale Fragment nach bisherigen Untersuchungen in vivo nicht existiert. Als Standardmaterial wird intaktes humanes Osteocalcin verwendet. Somit handelt es sich beim OSCAtest® Osteocalcin (BGP) um ein homologes System.
Inhalt des Reagenziensatzes

Der Reagenziensatz OSCAtest® Osteocalcin (BGP) enthält folgende Komponenten in Mengen ausreichend für 100 Bestimmungen:

A  **Tracer (HPLC gereinigt)** *
\(^{125}\text{I}-\text{Osteocalcin (38–49)},
1 Flasche für 27 ml, lyophilisiert,
Aktivität ca. 44 kBq, – rot eingefärbt –
Vor Gebrauch mit 27 ml Puffer B aufnehmen.

B  **Puffer**
1 Flasche mit 27 ml, gebrauchsfertig,
zum Rekonstituieren des Tracers A.
Flasche B als radioaktiv kennzeichnen,
(Radioaktivitätsaufkleber auf Wunsch erhältlich).

C  **Coated Tubes** (Teströhrchen)
beschichtet mit Anti-Osteocalcin (37–49)-Antiserum
(polyklonal, Schaf)
2 x 50 Stück, gebrauchsfertig.

D  **Waschlösung** **
1 Flasche à 10 ml, Konzentrat,
für 500 ml dest. Wasser.

G  **Osteocalcin-Nullserum** (Humanserum)
1 Flasche à 6 ml, gebrauchsfertig,
zum Rekonstituieren der Standards und Kontrollen.

* Werden in einem Assaylauf weniger als 100 Bestimmungen durchgeführt, müssen die rekonstituierten Reagenzien sofort nach Ansetzen des Assays eingefroren werden.

** Die Waschlösung ist auch für DYNOtest® TSH, DYNOtest® FT₄, DYNOtest® Tg,
DYNOtest® anti-TPO, DYNOtest® ACTH und THYRAK-Assay® verwendbar.
0 Osteocalcin-Nullstandard* 
1 Fläschchen für 0,5 ml, lyophilisiert; 
zur Rekonstitution mit 0,5 ml Nullserum

1–5 Osteocalcin-Standards* 
5 Fläschchen für 0,5 ml, lyophilisiert; 
zur Rekonstitution mit je 0,5 ml Nullserum 
Konzentrationen: 0,3; 0,8; 2,1; 5,2; 13,1 nmol/l
2,0; 5,0; 12,5; 31; 78 ng/ml

I, ii Kontrollseren I und II* 
2 Fläschchen für 0,5 ml, lyophilisiert; 
zur Rekonstitution mit je 0,5 ml Nullserum 
Nähere Angaben siehe Beipackzettel

Haltbarkeit 
Beachten Sie die Haltbarkeit auf dem Kit! 
Die Haltbarkeit des Reagenziensatzes wird von der 
Laufzeit des radioaktiven Tracers bestimmt und beträgt 
maximal sechs Wochen in ungeöffnetem Zustand.

Lagerung Sämtliche Reagenzien und die Teströhrchen bei 
4–8 °C lagern!

Zubehör 3 Bögen Klebefolie 
Auf 
1 Inkubationsgestell für 50 Teströhrchen 
Wunsch 1 Absaugvorrichtung 
1 Dispenser 1 ml 
Erforderliche 
Mikroliter-Pipetten (50 µl, 250 µl) 
Vibrationsmischer 
Gamma-Counter

*Werden in einem Assaylauf weniger als 100 Bestimmungen durchgeführt, müssen die 
rekonstituierten Reagenzien sofort nach Ansetzen des Assays eingefroren werden.
**Inkubationsschema**  
**OSCAtest® Osteocalcin (BGP)**

<table>
<thead>
<tr>
<th>Schritt</th>
<th>Beschreibung</th>
<th>Einheiten/a</th>
<th>T</th>
<th>0</th>
<th>1-5</th>
<th>6 etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Numerieren der Teströhrchen (a, b)</td>
<td></td>
<td>T</td>
<td>0</td>
<td>1-5</td>
<td>6 etc.</td>
</tr>
<tr>
<td>2</td>
<td>Pipettieren Nullstandard µl</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Standards µl</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Patientenseren µl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Pipettieren Tracer µl</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Inkubieren über Nacht (20-24 h) bei 4-8 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pipettieren Waschlösung ml</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Dekantieren Röhrchen kurz auf Zellstoff abklopfen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waschen</td>
<td>Schritt 5 und 6 einmal wiederholen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Messen der Radioaktivität empfohlene Meßzeit:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 Minute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

→ Auswertung
**Hinweis**

Der folgende Hinweis gilt ganz allgemein für Osteocalcin-Bestimmungen und ist unabhängig vom Testsystem (s. „Probenbehandlung“, S. 10).

Proben, die nicht innerhalb einer 3/4 Stunde nach Blutentnahme im Assay eingesetzt werden, müssen eingefroren und bei −20 °C gelagert werden.

Nach dem Auftauen müssen die Proben sofort weiterverarbeitet und dürfen nicht stehengelassen werden. Entsprechend sind sie sofort nach Gebrauch wieder einzufrieren.

Die Verdünnung von Seren mit hoher Osteocalcin-Konzentration sollte mit dem Nullserum erfolgen.

Hämolytische oder lipämische Seren können zu falsch niedrigen Osteocalcin-Werten führen!

Alle Reagenzien, Standards und Patientenserener vor Gebrauch durchmischen (Schaumbildung vermeiden und bei Raumtemperatur pipettieren.

1. Die Teströhrchen werden fortlaufend numeriert (a, b für Doppelbestimmungen). Die Teströhrchen T a, b dienen der Messung der Totalaktivität.

2. in die Teströhrchen 0 (a, b) – 5 (a, b) werden jeweils 50 µl Osteocalcin-Standard mit steigender Konzentration pipettiert, in die Teströhrchen 6 a, b etc. jeweils 50 µl Patientenserum.
3. In jedes Teströhrchen werden 250 µl Tracer pipettiert. Die Teströhrchen T a, b werden bis zur Messung der Radioaktivität (s. Punkt 7) der weiteren Bearbeitung entzogen.


5. Die Coated tubes werden gewaschen. Dazu wird in jedes Coated tube (nicht T a, b) 2 ml der gebrauchsfertigen Waschlösung pipettiert und anschließend dekantiert.


Hinweis Zur einfachen Durchführung des Waschens empfiehlt sich die Verwendung eines Waschkammes mit dem fünf Coated tubes gleichzeitig bearbeitet werden können (erhältlich bei HENNING BERLIN GMBH).

7. Die Radioaktivität eines jeden Teströhrchens, einschließlich T a, b, wird in einem Gamma-Counter gemessen. Empfohlene Meßzeit: 1 Minute.
Auswertung

Bei computergestützter Auswertung des OСCAtest® Osteocalcin (BGP) ist ein auf die Rechner-Counter-Kombination abgestimmtes Auswerteprogramm anzuwählen, das für Radioimmunoassays (RIA) gebräuchlich ist.

Bei Auswertung ohne Computer wird die mittlere Impulsrate eines jeden Teströhrchens (B) auf die mittlere Impulsrate des Nullstandards (B₀) bezogen und in Prozent ausgedrückt.

Im Falle des Nullstandards gilt: \( B/B₀ = 100 \% \)

Auf semilogarithmischem Millimeterpapier wird aus den errechneten Prozentwerten \( B/B₀ \) der Standards (Ordinate, linear) und den zugehörigen Osteocalcin-Konzentrationen (Abszisse, logarithmisch) eine Standardkurve gezeichnet. Der Osteocalcin gehalt unbekannter Seren wird anhand der jeweiligen Prozentwerte \( B/B₀ \) über die Standardkurve direkt in nmol/l (bzw. ng/ml) abgelesen.

Umrechnungsfaktor: \( \text{ng/ml} = \text{nmol/l} \times 5,97 \)

Zur Kontrolle der Bindungsfähigkeit des Assays wird \( B₀/T \) berechnet:

\[
\frac{B₀}{T} (\%) = \frac{\text{mittlere Impulsrate } 0 \text{ a,b}}{\text{mittlere Impulsrate } T \text{ a,b}} \times 100
\]

Unter den angegebenen Assaybedingungen sollte \( B₀/T \) bei 40–50\% liegen.
**Berechnungsbeispiel**

<table>
<thead>
<tr>
<th>Teströhrchen</th>
<th>IpM (a)</th>
<th>IpM (b)</th>
<th>MW</th>
<th>B/B₀ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Totalaktivität T</td>
<td>51 280</td>
<td>51 182</td>
<td>51 231</td>
<td>-</td>
</tr>
<tr>
<td>Nullstandard</td>
<td>22 910</td>
<td>23 198</td>
<td>23 054</td>
<td>100</td>
</tr>
<tr>
<td>Standard 1 0,3 nmol/l</td>
<td>18 628</td>
<td>18 796</td>
<td>18 712</td>
<td>81</td>
</tr>
<tr>
<td>Standard 2 0,8 nmol/l</td>
<td>14 085</td>
<td>14 307</td>
<td>14 196</td>
<td>62</td>
</tr>
<tr>
<td>Standard 3 2,1 nmol/l</td>
<td>9 440</td>
<td>9 320</td>
<td>9 380</td>
<td>41</td>
</tr>
<tr>
<td>Standard 4 5,2 nmol/l</td>
<td>4 972</td>
<td>5 220</td>
<td>5 096</td>
<td>22</td>
</tr>
<tr>
<td>Standard 5 13,1 nmol/l</td>
<td>3 131</td>
<td>2 943</td>
<td>3 037</td>
<td>13</td>
</tr>
<tr>
<td>Patientenserum 6</td>
<td>13 760</td>
<td>13 900</td>
<td>13 830</td>
<td>60</td>
</tr>
</tbody>
</table>

**Kontrolle der Bindungsfähigkeit:**

\[
\frac{B_0}{T} = \frac{23 054}{51 231} \times 100 = 45\%
\]

**Berechnung der Osteocalcin-Konzentration des Patientenserums 6:**

\[
\frac{B_6}{B_0} = \frac{13 830}{32 054} \times 100 = 60\% = 5,5 \text{ nmol/l}
\]
Standardkurve OSCAtest® Osteocalcin (BGP)

B/B₀ (%)

Osteocalcin-Konzentration

ng/ml

nmol/l

0,3 0,8 2,1 5,2 13,1
Assaycharakteristika

1. Verdünnung

<table>
<thead>
<tr>
<th>Verdünnung</th>
<th>gemessene OC-Konz. (pg/ml)</th>
<th>errechnete OC-Konz. (pg/ml)</th>
<th>Wiederfindung (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>orig.</td>
<td>13,5</td>
<td>13,5</td>
<td>100</td>
</tr>
<tr>
<td>1: 2</td>
<td>7,2</td>
<td>6,8</td>
<td>107</td>
</tr>
<tr>
<td>1: 4</td>
<td>3,6</td>
<td>3,4</td>
<td>107</td>
</tr>
<tr>
<td>1: 6</td>
<td>2,2</td>
<td>2,3</td>
<td>98</td>
</tr>
<tr>
<td>1: 8</td>
<td>1,6</td>
<td>1,7</td>
<td>95</td>
</tr>
<tr>
<td>1:10</td>
<td>1,6</td>
<td>1,4</td>
<td>119</td>
</tr>
<tr>
<td>orig. (180)</td>
<td>(180)</td>
<td>(180)</td>
<td></td>
</tr>
<tr>
<td>1: 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: 4</td>
<td>45,0</td>
<td>45,0</td>
<td>100</td>
</tr>
<tr>
<td>1: 6</td>
<td>29,2</td>
<td>30,0</td>
<td>97</td>
</tr>
<tr>
<td>1: 8</td>
<td>23,9</td>
<td>22,5</td>
<td>106</td>
</tr>
<tr>
<td>1:10</td>
<td>18,5</td>
<td>18,5</td>
<td>103</td>
</tr>
</tbody>
</table>

2. Präzision

Sämtliche in der Abb. dargestellten Variationskoeffizienten von Patientenserien wurden jeweils in 10fach-Bestimmung ermittelt. Danach ergibt sich für den OSCAtest® Osteocalcin (BGP) eine funktionale Assaysensitivität – niedrigste Konzentration, in der der Intra-Assay-Variationskoeffizient < 10% und der Inter-Assay-Variationskoeffizient < 20% ist – von ca. 1,8 ng/ml (0,3 nmol/l)
Normalwerte

In den meisten vorliegenden Studien, die sich mit der Frage tageszeitabhängiger Serum-Osteocalcin-Spiegel beschäftigten, wurde eine circadiane Rhythmik beobachtet (z. B. 36). Danach sind die Serum-Osteocalcin-Spiegel am höchsten nachts und am niedrigsten morgens zwischen 8 und 11 Uhr. Es ist deshalb zu empfehlen, die Blutentnahme in diese Zeit zu legen.


Von den Frauen und Männern über 40 Jahre wurden Osteocalcin-Werte nur in den Fällen zur Bestimmung des Normbereiches herangezogen, in denen gleichzeitig normale Werte bei der Knochendichtemessung mittels Dualphotonen-Absorptiometrie (DPA) erhoben wurden.


Auf diese Weise sollen regionale Besonderheiten, Prävalenzen bestimmter Knochenstoffwechselerkrankungen, aber auch die methodische Qualität des einzelnen Labors bzw. der jeweils verwendeten Testsätze entsprechend Berücksichtigung finden. Insofern haben die nachfolgend für den OSCAtest® Osteocalcin (BGP) aufgeführten Daten lediglich orientierenden Charakter.
Osteocalcin-Normalwerte bei Frauen

Alter (Jahre)

Osteocalcin (ng/ml)

Osteocalcin-Normalwerte bei Männern

Alter (Jahre)

Osteocalcin (ng/ml)
### Osteocalcin-Normalwerte im Verhältnis zu Alter und Geschlecht

#### Kinder und Jugendliche

<table>
<thead>
<tr>
<th>Altersgruppe (Jahre)</th>
<th>1-10</th>
<th>11-15</th>
<th>16-20</th>
<th>Normbereich (nmol/l)</th>
<th>1,7-6,7</th>
<th>1,7-13,4*</th>
<th>1,7-8,4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ng/ml)</td>
<td>10-40</td>
<td>10-80*</td>
<td>10-50*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anzahl (n)</td>
<td>21</td>
<td>36</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* hohe Osteocalcin-Spiegel sind v. a. bei Wachstums schüben zu beobachten.

### Frauen

<table>
<thead>
<tr>
<th>Altersgruppe (Jahre)</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
<th>51-60</th>
<th>61-70</th>
<th>71-80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normbereich (nmol/l)</td>
<td>0,7-3,4</td>
<td>0,6-2,5</td>
<td>0,7-2,0</td>
<td>0,7-2,0</td>
<td>0,7-2,0</td>
<td>(0,7-2,0)</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>4-20</td>
<td>3,5-15</td>
<td>4-12</td>
<td>4-12</td>
<td>4-12</td>
<td>(4-12)</td>
</tr>
<tr>
<td>Anzahl (n)</td>
<td>82</td>
<td>61</td>
<td>113</td>
<td>96</td>
<td>40</td>
<td>3</td>
</tr>
</tbody>
</table>

### Männer

<table>
<thead>
<tr>
<th>Altersgruppe (Jahre)</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
<th>51-60</th>
<th>61-70</th>
<th>71-80</th>
<th>81-90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normbereich (nmol/l)</td>
<td>1,0-3,4</td>
<td>0,7-2,5</td>
<td>0,7-2,0</td>
<td>0,7-2,0</td>
<td>0,7-2,0</td>
<td>0,8-2,0</td>
<td>(0,7-2,0)</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>6-20</td>
<td>4-15</td>
<td>4-12</td>
<td>4-12</td>
<td>4-12</td>
<td>5-12</td>
<td>(4-12)</td>
</tr>
<tr>
<td>Anzahl (n)</td>
<td>79</td>
<td>76</td>
<td>81</td>
<td>32</td>
<td>18</td>
<td>18</td>
<td>7</td>
</tr>
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</table>
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