8iso PGF2α and α-Tocopheronolactone as Biomarkers of Oxidative Stress in Sepsis

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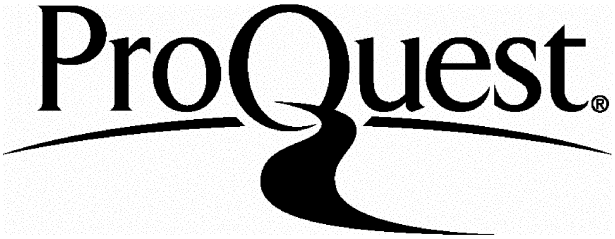
Mres Biomedicine



ABSTRACT

Sepsis is a state of severe illness caused by overwhelming infection of the blood stream by toxin producing bacteria. Oxidative stress has been indicated as an effect of the illness and not in its aetiology. 8isoPGF$_{2\alpha}$, is the most highly reliable and commonly used biomarker of oxidative stress in normal and diseased state individuals. It has also been suggested that metabolites of vitamin E such as α-Tocopheronolactone, may serve as an indicator of invivo oxidative stress. The aim of the current study was to re-establish and validate the methods using GC/MS for the analysis of 8isoPGF$_{2\alpha}$ and vitamin E metabolites and to utilize these assays in patients with sepsis (n=5) and control (n=5), to obtain evidence of oxidative stress from 8isoPGF$_{2\alpha}$ concentrations and to investigate whether α-Tocopheronolactone could be used as an invivo biomarker of oxidative stress. A marked increase with a mean ± ISD of 917.1 ± 238.6 and 2521 ± 442.1 pg/mg and 274 ± 148.1 and 29.29 ± 8.323 pmol/mmol was observed for the sepsis patients and controls subjects' urinary concentrations of 8isoPGF$_{2\alpha}$ and α-Tocopheronolactone respectively. No correlation was observed between the concentrations of the metabolites ($P > 0.05$), but a positive r value indicated the probability of a correlation with an increase in sample size. A high concentration of α-CEHC in the patients' urine (1940 ± 798.7 pmol/mmol) indicated a status of vitamin E status the reason for the increase is unknown and could be attributed to the diet (information unavailable) and also the sepsis state itself, but the formation of artefactual oxidation was ruled out; due to a poor correlation between α-CEHC and α-Tocopheronolactone. Thus the study validated the use of 8isoPGF$_{2\alpha}$ as an independent biomarker of oxidative stress and highlighted the possible role of α-Tocopheronolactone as a biomarker of oxidative stress. The study also suggests further investigation into the role of α-CEHC in oxidative stress and sepsis state.

ACKNOWLEDGEMENTS

I take this opportunity to thank my supervisor Proff. David Muller, for his support and encouragement, not only for my two projects; which he kindly took me under his wing, but also for my entire academic year at UCL. No amount of gratitude can be expressed in paper for the amount of time and patience he has put on my case.

The completion of this project would have been impossible without the help of Ms. Heather Green a Phd student who took time out despite her busy schedule to help me at every stage of this project, right from supervising my laboratory techniques to answering my innumerable phone calls for trouble shooting the problems faced during the course of this study.

Thanks are also due to Dr. Kevin Mills who helped me with my mini project and introduced me to the fascinating world of Mass Spectrometry, which has paved way for a career focus in my life.

I would like to thank every one in the Biochemisty department for their kindness and friendship without which it would have been impossible to love my stay in London.

I would like to express my gratitude to my parents, family and friends for their support throughout my stay in London.

Finally, I would like to thank the power above to help me achieve the best.

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ABBREVIATIONS

O_2	Oxygen
ATP	Adenosine triphosphate
NADH	Nicotinamide adenine dinucleotide (reduced)
$FADH_2$	Flavin adenine dinucleotide (reduced)
FMN	Flavin mono nucleotide
NADH-Q	NADH coenzyme Q
QH_2	Reduced coenzyme Q
(Q)	Coenzyme Q
Fe-S	Iron Sulphate
H_2O	Water
CO_2	Carbon dioxide
ADP	Adenosine di Phosphate
DNP	Dinitrophenol
ROS	Reactive Oxygen Species
·O-O·	Triplet Oxygen
O-O:	Singlet Oxygen
·O-O:	Superoxide ion
·O-O:H	Perhydroxy ion
H:O-O:H	Hydroperoxide
H:O·	Ozone
H:O:	Hydroxyl ion
HOCL	Hypochlorous acid
O_3	Ozone
OH	Hydroxyl Radical
NO·	Nitric oxide radical
NO2·	Nitric oxide
RNS	Reactive Nitrogen Species
SOD	Super Oxide Dismutase
Cu	Copper
Zn	Zinc
Fe	Iron
Mn	Manganese
DNA	Deoxyribo Nucleic Acid
NADPH	Nicotinamide di nucleotide Phosphate
TOH	α-Tocopherol
LOO.	Peroxyl Radical
TO.	Tocopheroxyl Radical
H	Hydrogen
R·	Carbon Radical Product
ROOH	Lipid Peroxide
OH·	Hydroxyl ion

TNF	**Tumour Necrosis Factor**
CO_2	**Carbon di oxide**
MODS	**Multiple Organ Dysfunction Syndrome**
TBARS	**Thiobarbituric acid reactive substances**
CAT	**Catalase**
GSH	**Glutathione**
NAC	**N-Acetylcysteine**
HCL	**Hydrocloric Acid**
8-OHdG	**8-hydroxydeoxy guanosine**
HPLC	**High Perforamance Liquid Chromatography**
ECD	**Electrochemical Detection**
GC-MS	**Gas chromatography- Mass Spectrometry**
MDA	**Melanaldehyde**
TBA	**Thiobarbituric Acid**
TBARS	**Thiobarbituric Acid Reactive Substances**
PUFA	**Polyunsaturated Fatty Acid**
PG	**Prostaglandins**
α	**Alpha**
β	**Beta**
γ	**Gamma**
δ	**Delta**
α- TL	**Tocopheronolactone**
α- CEHC	**α- carboxyethyl-6-hydroxychromans**
VLDL	**Very low density lipoproteins**
α- TA	**Tocopheronic Acid**
α-TQ	**Tocopheryl hydroquinone**
BSTFA	**N,O-bis(trimethylsilyl) Trifluoroacetamide**
MeOH	**Methanol**
SPE	**Solid Phase Extraction**
TLC	**Thin Layer Chromatography**
TIC	**Total Ion Chromatogram**
MS	**Mass Spectrometry**
TMS	**Trimethylsilyl**
QC	**Quality Control**
SIM	**Select Ion Mode**

INTRODUCTION

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1. Introduction

1.1. Oxygen

The element oxygen exists in the air as a diatomic molecule, O_2 which strictly should be called dioxygen. Over 99% of the O_2 in the atmosphere is the isotope oxygen-16 but there are traces of oxygen-17 (approximately 0.04%) and oxygen-18 (approximately 0.2%). O_2 appeared in significant amounts in the Earth's atmosphere over 2.5 X 10^9 years ago, and geological evidence suggests that this was due to the evolution of photosynthesis by blue green algae (cyanobacteria). Except for certain anaerobic and aero tolerant unicellular organisms, all animals, plants and bacteria require O_2 for efficient production of energy by the use of O_2 dependent electron transport chains, such as those in the mitochondria of eukaryotic cells. This need for O_2 obscures the fact that O_2 is also a toxic mutagenic gas as well as a serious fire risk; aerobes survive because they have antioxidant defences to protect against it (Gilbert 1981).

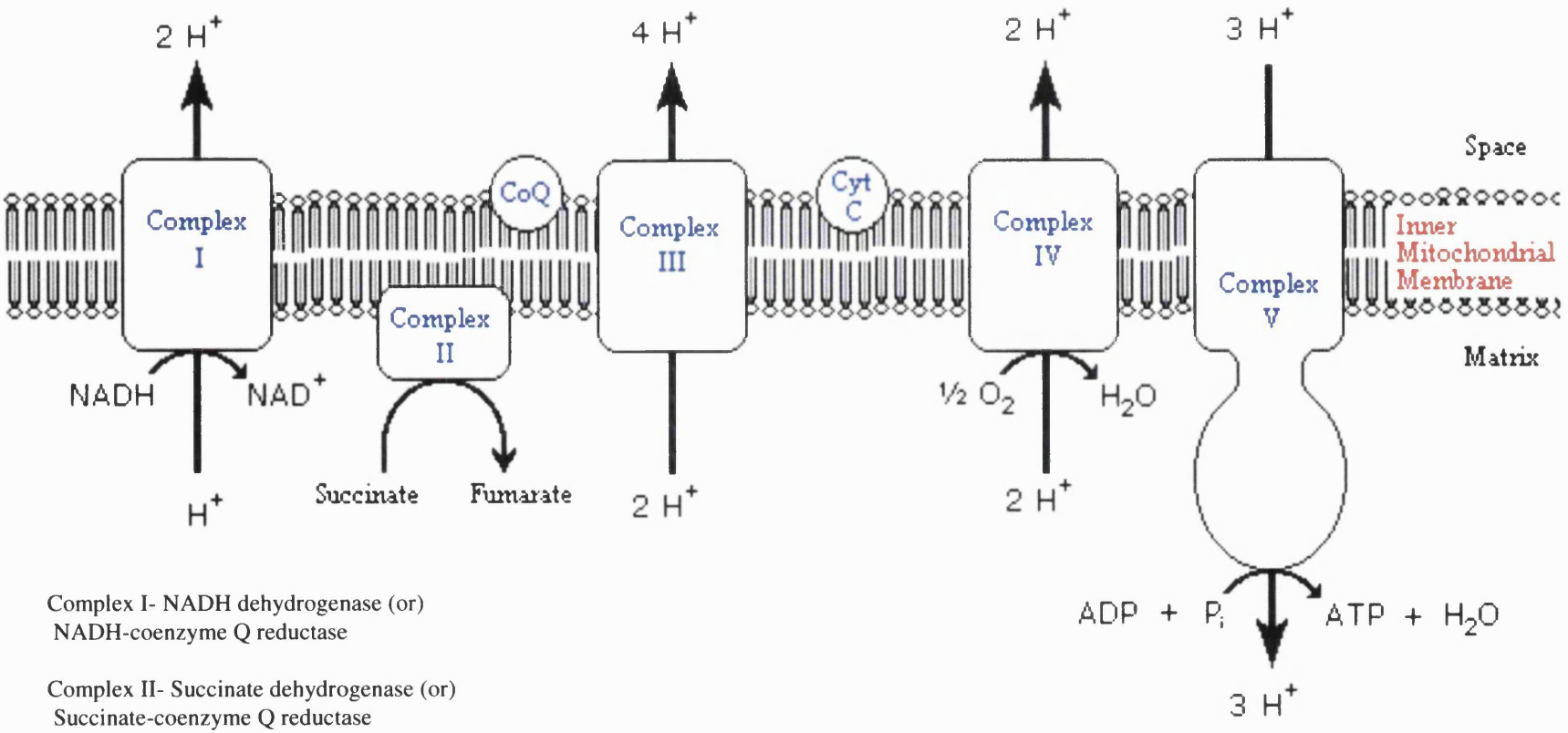
1.2. Energy Production

Complex multicellular organisms such as the mammals have evolved mechanisms to ensure that O_2 is delivered to all the cells that need it. Some O_2 travels dissolved in blood plasma, but most O_2 carried in the blood is transported by haemoglobin. About 85-90% of the O_2 taken up by the body is utilized by the mitochondria; these organelles are the major source of ATP in animals (Fig.1.1.) In oxidative phosphorylation, the synthesis of ATP is coupled to the flow of electrons from NADH

or $FADH_2$ to O_2 by a proton gradient across the inner mitochondrial membrane. Electron flow through three asymmetrically oriented transmembrane complexes results in the pumping of protons out of the mitochondrial matrix and the generation of a membrane potential. ATP is synthesised when protons flow back to the matrix through a channel in an ATP- synthesizing complex, called ATP synthase (also known as F_0F_1-ATPase or H+-ATPase). Oxidative phosphorylation exemplifies a fundamental theme of bioenergetics: the transmission of free energy by proton gradients (Halliwell 1984;Schapira & Afanas'ev 2004).

The electron carriers in the respiratory assembly of the inner mitochondrial membrane are flavins, iron-sulfur complexes, quinones, heme groups of cytochromes and copper ions. Electrons from NADH are transferred to the FMN prosthetic group of NADH-Q reductase, the first of three complexes. This reductase also contains Fe-S centres. The electrons emerge in QH_2, the reduced form of ubiquinone (Q). This highly mobile hydrophobic carrier transfers its electrons to cytochrome reductase, a complex that contains cytochrome b and c1 and Fe-S centre. This second complex reduces cytochrome c, a water-soluble peripheral membrane protein. Cytochrome c, like Q, is a mobile carrier of electrons, which it then transfers to cytochrome oxidase. This third complex contains cytochromes a and a_3 and two copper ions. A heme iron and a copper ion in this oxidase transfer electron to O_2, the ultimate acceptor, in form of H_2O. The flow of electrons through each of these complexes leads to the pumping of protons from the matrix side to the cytosolic side of the inner mitochondrial membrane. About 30 ATP are generated when a molecule of glucose is completely oxidised to CO_2 and H_2O. Electron transport is normally tightly coupled

tophosphorylation. NADH and $FADH_2$ are oxidised only if ADP is simultaneously phosphorylated to ATP. This coupling, called respiratory control, can be disrupted by uncouplers such as DNP, which dissipate the proton gradient by carrying protons across the inner mitochondrial membrane (Halliwell 1984;Schapira & Afanas'ev 2004).



Complex I- NADH dehydrogenase (or)
NADH-coenzyme Q reductase

Complex II- Succinate dehydrogenase (or)
Succinate-coenzyme Q reductase

Complex III- Cytochrome C - coenzyme Q oxidoreductase

Complex IV- Cytochrome oxidase

Complex V- ATP synthase

Fig.1.1. Oxidative Phosphorylation

1.3. Activation of Oxygen

One of the paradoxes of life on this planet is that the molecule that sustains aerobic life, oxygen, is not only fundamentally essential for energy metabolism and respiration, but it has been implicated in many diseases and degenerative conditions (Marx 1985). A common element in such diverse human disorders as ageing, arthritis, cancer, Lou Gehrig's disease and many others is the involvement of partially reduced forms of oxygen (Gutteridge 1996). The realisation of the significance of oxygen in disorders and stress is recent due in no small part to the difficulty in detecting and tracing oxygen molecules, to the multitude of forms and intermediates that oxygen can assume, and to the extreme reactivity and rate of the chemical reactions involved. As a consequence, experiments can only look for the "footprints" of oxygen reactions in attempts to determine cause-effect relationships in stress responses. (Gutteridge 1996) The following section describes the current understanding of the general principles of activated oxygen.

Atmospheric oxygen in its ground-state is distinctive among the gaseous elements because it is a biradical, or in other words it has two unpaired electrons. This feature makes oxygen paramagnetic; it also makes oxygen extremely unlikely to participate in reactions with organic molecules unless it is "activated". The requirement for activation occurs, as the unpaired electrons in oxygen have parallel spins. According to Pauli's exclusion principle, this precludes reactions with a divalent reductant, unless this reductant also has two unpaired electrons with parallel spin opposite to that of the oxygen, which is a very rare occurrence. Hence, oxygen is usually non-reactive to

organic molecules, which have paired electrons with opposite spins. This spin restriction means that the most common mechanisms of oxygen reduction in biochemical reactions are those involving transfer of only a single electron (monovalent reduction) (Afanas'ev 1985).

Activation of oxygen may occur by two different mechanisms: absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or monovalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state (O-O), in which the two electrons have opposite spins (Fig.1.2.). This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction). Since paired electrons are common in organic molecules, singlet oxygen is much more reactive towards organic molecules than its triplet counterpart (Afanas'ev, 1985). If a single electron is added to the ground state O_2 molecule, it must enter one of the antibonding orbitals. The product is the superoxide radical ($^{\cdot}$O-O) (Fig.1.2.). With only one unpaired electron, superoxide is less of a radical than is oxygen. Addition of another electron would give rise to peroxide ion, which is not a radical and its formation and properties will be dealt with in greater detail in the latter half of this section. A point to be noted is that, not all of the activated oxygen can be referred to as free radicals, as some are referred to as reactive oxygen species (ROS). Reactive oxygen species (ROS) on the other hand is a collective term often used by scientists to include not only the oxygen radicals but also some non radicals derivatives of oxygen. Eamples are shown in Fig 1.2. and include H_2O_2, hypochlorous acid (HOCL, an oxidizing and chlorinating

agent produced by activated phagocytes) and ozone (O_3) (Halliwell & Gutteridge 1999).

Triplet Oxygen (ground state)	·O-O·
Singlet Oxygen	O-O :
Superoxide	·O-O :
Perhydroxyl Radical	·O-O : H
Hydrogen Peroxide	H : O-O : H
Hydroxyl Radical	H : O ·
Hydroxyl Ion	H : O :
Hypochlorous acid	HOCL
Ozone	O_3

Fig.1.2. Nomenclature of various forms of reactive oxygen species

As shown in Fig.1.3. the stepwise monovalent reduction of oxygen to form superoxide (O^-_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH) forms water. The first step in the reduction of oxygen forming superoxide is endothermic but subsequent reductions are exothermic (Elstner 1982).

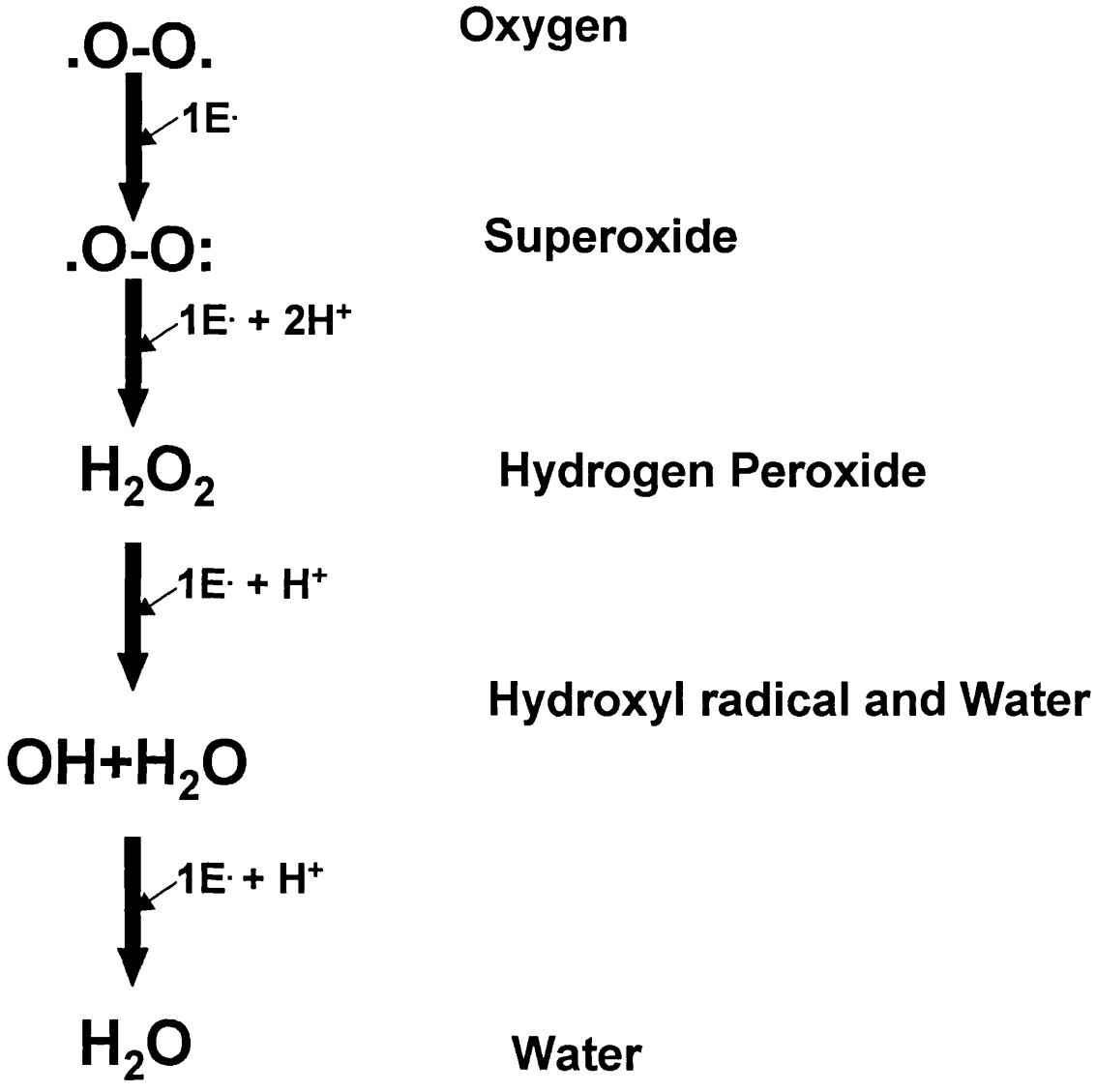


Fig 1.3. Reduction of Oxygen to water

Non-activated oxygen is a biradical. From this triplet state it can be activated by either reversing the spin on one of the unpaired electrons to form the singlet state or by reduction. The first reduction reaction is endothermic forming superoxide. Subsequent reductions form hydrogen peroxide, hydroxyl radical and water. The electronic state for each activation step is shown with the energy of the reaction in Kcal/mole (Fig.1.3) (Balentine 1982).

The univalent reduction of superoxide produces hydrogen peroxide, which is not a free radical because all of its electrons are paired (Fig. 1.3.) (Balentine 1982). (The formation of hydrogen peroxide has been dealt with in great detail in the latter half of the section)

Hydrogen peroxide is noteworthy because it readily permeates membranes and it is therefore not compartmentalised in the cell. Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecules. The well-known reactivity of hydrogen peroxide is not due to its reactivity per se, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical which is the strongest oxidizing agent known and reacts with organic molecules at diffusion-limited rates (Balentine 1982).

Fenton described in the late nineteenth century (Fenton 1894;Fenton 1899) the oxidising potential of hydrogen peroxide mixed with ferrous salts. Forty years later, Haber and Weiss (1934) identified the hydroxyl radical as the oxidising species in these reactions:

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$

In biological systems the availability of ferrous ions limits the rate of reaction, but the recycling of iron from the ferric to the ferrous form by a reducing agent can maintain an ongoing Fenton reaction leading to the generation of hydroxyl radicals. One suitable reducing agent is superoxide which participates in the overall reaction 2 as two half reactions shown in reactions 3 and 4:

(2) $\bullet O_2^- + H_2O_2 \rightarrow O_2 + \bullet OH + OH^-$

(3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$

(4) $\bullet O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

Therefore, in the presence of trace amounts of iron, the reaction of superoxide and hydrogen peroxide will form the destructive hydroxyl radical and initiate the oxidation of organic substrates. Metals other than iron may also participate in these electron transfer reactions by cycling between oxidised and reduced states.

The oxidation of organic substances (by OH^-) may proceed by two possible reactions: an addition of OH to the organic molecule, or abstraction of a hydrogen atom from it. In the addition reaction (reaction 5), the hydroxyl radical reacts with an organic substrate forming a hydroxylated product that is further oxidised by ferrous ions, oxygen or other agents to a stable, oxidised product (reactions 6 and 7). The hydroxylated products can also dismutate to form cross-linked products (reaction 8).

(5) $\bullet OH + R \rightarrow \bullet ROH$

(6) $\bullet ROH + Fe^{3+} \rightarrow ROH + Fe^{2+} + H^+$

(7) $\bullet ROH + O_2 \rightarrow ROH + \bullet O_2^- + H^+$

(8) $\bullet ROH + \bullet ROH \rightarrow R - R + 2H_2O$

In the abstraction reaction, the hydroxyl radical oxidises the organic substrate forming water and an organic radical (reaction 9). The latter product has a single unpaired electron and thus can react with oxygen in the triplet ground-state (reaction 10). The addition of triplet oxygen to the carbon radical can lead to the formation of a peroxyl radical which can readily abstract hydrogen from another organic molecule leading to the formation of a second carbon radical (reaction 11). This chain reaction is why oxygen free radicals cause damage far in excess of their initial concentration (Haber & Wiess 1934).

(9) $\bullet OH + RH \rightarrow R\bullet + H_2O$

(10) $\bullet R + O_2 \rightarrow ROO\bullet$

(11) $ROO\bullet + RH \rightarrow R\bullet + ROOH$

Fig.1.4. Chain reaction of Hydrogen Peroxide

1.4. Reactive Nitrogen species

Some oxides of nitrogen ($NO^{\cdot}$, $NO2^{\cdot}$) are also free radicals and just as the term ROS has been introduced in the earlier section, there exists a field in biology wherein products (radicals) of nitrogen react with reactive oxygen species resulting in a whole range of products which have been termed as reactive nitrogen species (RNS). RNS also includes a range of radicals and non-radical derivatives of nitrogen as illustrated in Table1.1. The most biologically relevant example is the fast reaction of nitric oxide radical ($NO^{\cdot}$) and ($^{\cdot}O\text{-}O$) to form peroxinitrite (reaction 12) (Beckman & Koppenol 1996).

(12) $NO^{\cdot} + {}^{\cdot}O\text{-}O \rightarrow ONOO^{-}$

Fig.1.5. Reaction of nitric oxide and activated oxygen.

REACTIVE NOTROGEN SPECIES	
RADICALS	Nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$)
NON- RADICALS	Nitrous acid (HNO_2) dinitrogen trioxide (N_2O_3) dinitrogen tetroxide (N_2O_4) nitronium (nitryl) ion (NO_2^+) peroxinitrite ($ONOO^-$) peroxynitrous acid (ONOOH) alkyl peroxinitrite ($ONOO^-$) nitroxyl anion (NO^-) nitrosyl cation (NO^+) nitryl chloride (NO_2CL)

Table 1.1. Reactive nitrogen species (RNS)

1.5. Defence Mechanisms

In order to prevent damage caused by oxygen in vivo the body has an array of antioxidant compounds and enzyme, which in healthy subjects are able to scavenge or prevent production of these highly reactive oxygen species. These antioxidants function in a variety of different ways and are localized within specific areas of the cell. These antioxidants include enzymes such as catalase, glutathione peroxidase, and superoxide dismutase (SOD) as well as small molecules such as the lipid-soluble vitamin E and water-soluble vitamin C (ascorbate), glutathione and urate (Burton & Ingold 1984).

1.5.1. Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) was isolated by Mann and Kleilin (1938) and thought to be a copper storage protein. SOD is now known to catalyse the dismutation of superoxide to hydrogen peroxide and oxygen:

(13) $\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{\text{Superoxide Dismutase}} H_2O_2 + O_2$

Fig.1.6. Reaction catalysed by Superoxide Dismutase.

Therefore, the activity of this enzyme determines the relative proportions of two constituents ($\cdot O_2^-$ and H_2O_2) of the Haber-Wiess reaction that generates hydroxyl radicals. Since SOD is present in all aerobic organisms and most (if not all) subcellular compartments that generate activated oxygen, it has been assumed that

SOD has a central role in the defence against oxidative stress. There are three distinct types of SOD classified on the basis of the metal cofactor: the copper/zinc (Cu/Zn – SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) forms. All forms of the SOD are nuclear encoded and are targeted to their respective subcellular compartments by an amino terminal targeting sequence (Beyer, Imlay & Fridovich 1991;Bowler & Inze 1992;Scandalias 1993).

1.5.2. Catalase

Catalase is a heme-containing enzyme that catalyses the conversion of hydrogen peroxide into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes by oxidases involved in ß-oxidation of fatty acids, and purine catabolism. Catalase was one of the first enzymes to be isolated in a highly purified state. Careful examination of the structure of beef liver catalase has shown four NADPH binding sites per catalase tetramer(Fita & Rossmann 1985), but these sites were not in close association with the hydrogen peroxide reaction centre. Instead, NADPH functions in animal catalase to protect against inactivation by hydrogen peroxide (Kirkman, Galiano, & Gaetani 1987).

1.5.3 Ascorbate

L-ascorbic acid (vitamin C) is an important vitamin in the human diet. The most striking chemical property of ascorbate is its ability to act as a reducing agent. Ascorbate can directly scavenge oxygen free radicals with and without enzyme catalysts and can indirectly scavenge them by recycling tocopherol to the reduced form. By reacting with activated oxygen, ascorbate protects critical macromolecules

from oxidative damage. The reaction with the hydroxyl radical is limited only by diffusion. Fig. 1.7. illustrates the antioxidant nature of ascorbate. As an antioxidant, ascorbate will react with superoxide, hydrogen peroxide or the tocopheroxyl radical to form monodehydroascorbic acid and / dehydroascorbic acid. The reduced forms are recycled back to ascorbic acid by monodehydroascorbate reductase using reducing equivalents from NAD(P)H or glutathione, respectively. Dehydroascorbate may decompose to tartrate and oxalate (Halliwell & Gutteridge 1999).

The effects of dietary ascorbate depletion can be studied in experimental animals such as the guinea pigs. An earlier study on guinea pigs showed that a vitamin C deficient diet led to an exhalation of pentane and ethane, suggestive of an increased lipid peroxidation in vivo (Tanaka & Fukumara 1997). Levels of 8-hydroxydeoxyguanosine (an oxidative product of DNA and an index of DNA damage) were elevated in sperm from adult men seriously deficient in vitamin C and were normalized by adding ascorbate to the diet. Further evidence for an antioxidant role of ascorbate in vivo is provided by studies of its depletion of oxidative stress (Schorah, Amano, & Ames 1996;Verhagen, Beckman, & Beal 1999).

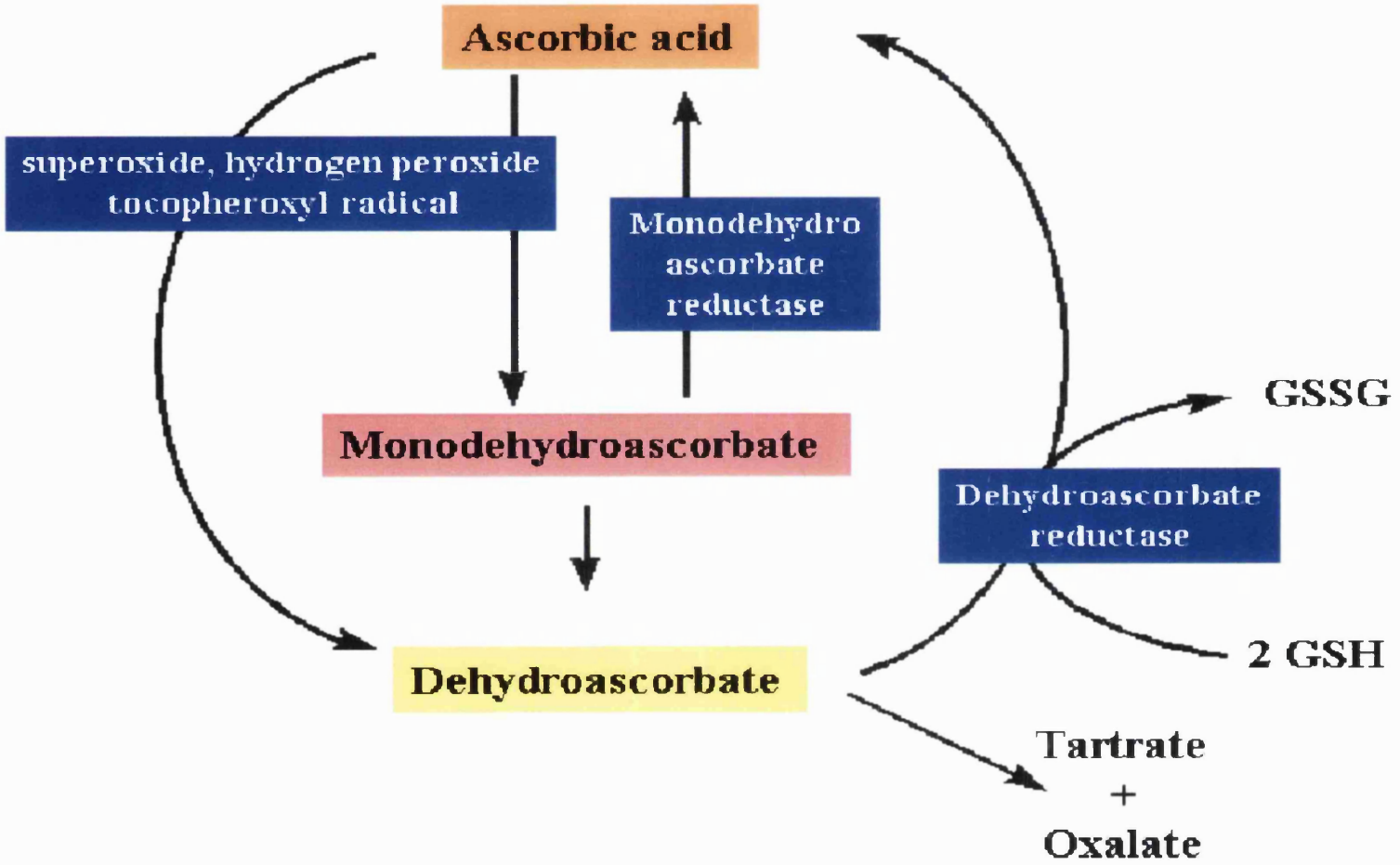


Fig. 1.7. Metabolism of Vitamin C(GSH-Glutathione and GSSG- oxidised Glutathione)

1.5.4. Vitamin E (α-tocopherol)

Vitamin E is a generic term for the tocopherols and tocotrienols, which have saturated and unsaturated side chains, respectively. Each group has α, β, γ and δ forms that differ according to the position and the number of the methyl groups on the hydroxychroman ring with α-tocopherol being the most abundant form of vitamin E invivo.

The biological activity of α-tocopherol has been attributed principally to its ability to act as an antioxidant, which prevents the peroxidation of lipids (reaction [14] and [15]). It is a chain breaking antioxidant, which interferes with one or more of the propagation steps after the initial peroxidative process. α-tocopherol is considered to be the principal lipid soluble chain breaking antioxidant in vivo(Burton & Ingold 1984).

(14) TOH + LOO$^{\cdot}$ ⟶ TO$^{\cdot}$ + LOOH

(15) TO$^{\cdot}$ + LOO$^{\cdot}$ ⟶ NRP + LOOH

Fig 1.8. Reaction of α-tocopherol (TOH) with lipid peroxyl radicals (LOO$^{\cdot}$).

TOH- α-tocopherol, **LOO$^{\cdot}$** – Peroxyl Radical, **TO$^{\cdot}$-** Tocopheroxyl Radical, **NRP-** Non- radical adduct.

Peroxyl radicals are probably the principal oxidants scavenged by α-tocopherol in biological systems. Phenols, such as α-tocopherol, typically trap peroxyl radicals by a two-step mechanism as indicated in general terms in Fig. 1.8. First a peroxyl radical

(obtained by oxidation by an oxygen species) abstracts a hydrogen atom from tocopherol to produce a hydroperoxide and tocopheroxyl radical. The tocopheroxyl The tocopheroxyl radical is unusually stable, owing to resonance stabilisation by the chroman ring, and therefore less likely to propagate the radical chain. Overall, each α-tocopherol molecule in capable of scavenging two radicals (Burton & Ingold 1984).

1.6. Oxidative stress

Oxidative stress refers to the situation where there is an imbalance between production of ROS/ RNS and antioxidant defences (Halliwell & Gutteridge 1999). Sies, who introduced the term from the title of the book he edited in 1985, Oxidative Stress, defined it in 1991 in the Introduction to the second edition as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage". Such damage is often, again loosely called oxidative damage (Sies H 1991). In principle, oxidative stress can result from:

1. Diminished antioxidants, e.g. mutations affecting antioxidant defence enzymes such as superoxide dismutase. Depletion of dietary antioxidants such as vitamin C and E and other antipxidants such as glutathione constituents can also lead to oxidative stress (Halliwell & Gutteridge 1999).

2. Increased production of ROS/RNS, e.g. by exposure to elevated O_2, the presence of toxins that are metabolised to produce ROS/RNS, or excessive activation of 'natural' ROS/RNS systems (e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases, such as rheumatoid arthritis ulcerative colitis and sepsis (Halliwell and Gutteridge, 1999).

1.7. Consequences of Oxidative stress

1.7.1. Classical Lipid Peroxidation

The peroxidation of lipids involves three distinct steps: initiation, propagation and termination. The initiation reaction between an unsaturated fatty acid (e.g. linoleate) and the hydroxyl radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid (reaction 9); in the case of linoleate this occurs at carbon-11. The remaining carbon centred radical, forms a resonance structure sharing this unpaired electron among carbons 9 to 13. In the propagation reactions, this resonance structure reacts with triplet oxygen, which is a biradical having two unpaired electrons and therefore reacts readily with other radicals. This reaction forms a peroxy radical (reaction 10). In the case of linoleate, addition occurs at either carbon-9 or -13. The peroxy radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another carbon centred free radical (reaction 11) that can participate in a second H abstraction (reaction 10). The peroxidation reactions in membrane lipids are terminated when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals, such as those shown in reactions 17 to 19:

(17) $R\bullet + R\bullet \rightarrow R - R$

(18) $R\bullet + ROO\bullet \rightarrow ROOR$

(19) $ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$

Typically high molecular weight, cross-linked fatty acids and phospholipids accumulate in peroxidised membrane lipid samples (Bradley & Min 1992).

Therefore, once one hydroxyl radical initiates the peroxidation reaction by abstracting a single H atom, it creates a carbon radical product (R·) that is capable of reacting with ground state oxygen in a chain reaction. The role of the hydroxyl radical is analogous to a "spark" that starts a fire. The basis for the hydroxyl radical's extreme reactivity in lipid systems is that at very low concentrations it initiates a chain reaction involving triplet oxygen, the most abundant form of oxygen in the cell (Burton & Ingold 1984).

The lipid hydroperoxide (ROOH) is unstable in the presence of Fe or other metal catalysts because ROOH will participate in a Fenton reaction leading to the formation of reactive alkoxy radicals:

(20) $ROOH + Fe^{2+} \rightarrow OH^{-} + RO\bullet + Fe^{3+}$

Fig.1.9. Reactions 17 to 20 depicting a classical lipid peroxidation reaction.

Therefore, in the presence of Fe, the chain reactions are not only propagated but amplified. Two radicals are produced by the summation of reactions 9 to 11 and 16. Among the degradation products of ROOH are aldehydes, such as malondialdehyde, and hydrocarbons, such as ethane and ethylene, which are commonly measured end products of lipid peroxidation.

Singlet oxygen can react readily with unsaturated fatty acids producing a complex mixture of hydroperoxides. Oxidation of unsaturated fatty acids by singlet oxygen produces distinctly different products than the hydroxyl radical (Bradley & Min 1992). Once formed the lipid hydroperoxides will decompose into a variety of

products, some of which can produce oxygen free radicals in the presence of metal catalysts (reaction 20)(Burton & Ingold 1984).

1.7.2. Oxidative Damage to Proteins

Oxidative attack on protein can result in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a peptide differ in their susceptibility to attack, and the various forms of activated oxygen differ in their potential reactivity. Primary, secondary, and tertiary protein structures alter the relative susceptibility of certain amino acids. In spite of this complexity, generalisations can be made. Sulphur containing amino acids, and thiol groups, are highly susceptible sites. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges. Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Reduction of both of these may be accomplished in microbial systems by thioredoxin and thioredoxin reductase (Farr & Kogoma 1991). A protein-methionine-S-oxide reductase has been measured in pea chloroplasts (Ferguson & Burke 1992).

Other forms of free radical attack on proteins are not reversible. For example, the oxidation of iron-sulphur centres by superoxide destroys enzymatic function (Gardner & Fridovich 1991). Many amino acids undergo specific irreversible modifications when a protein is oxidised. For example, tryptophan is readily cross-linked to form bityrosine products (Davies 1987). Histidine, lysine, proline, arginine, and serine form carbonyl groups on oxidation (Stadtman 1986). The oxidative degradation of protein

is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. In these cases, the metal binds to a divalent cation-binding site on the protein. The metal then reacts with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical that rapidly oxidises an amino acid residue at or near the cation-binding site of the protein (Stadtman 1986). This site-specific alteration of an amino acid can inactivate the enzyme by destruction of the cation-binding site.

Oxidative modification of specific amino acids is one mechanism of marking a protein for proteolysis (Stadtman 1986). In *E. coli* there are specific proteases that degrade oxidised proteins (Farr & Kogoma 1991) and similar specificity is expected in humans.

1.7.3. Oxidative Damage to DNA

Activated oxygen and agents that generate oxygen free radicals, such as ionising radiation, induce numerous lesions in DNA that can cause deletions, mutations and other lethal genetic effects. Characterisation of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986). Degradation of the base will produce numerous products, including 8-hydroxydeoxyguanisine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and -saturated products (Imlay & Linn 1986). Studies dealing with the oxidative damage to the DNA have been detailed in the Biomarkers of Oxidative stress section in the latter part of this Chapter.

The principal cause of single strand breaks is oxidation of the sugar moiety by the hydroxyl radical. *In vitro* neither hydrogen peroxide alone nor superoxide cause strand breaks under physiological conditions, and therefore, their toxicity *in vivo* is most likely the result of Fenton reactions with a metal catalyst. At least in *E. coli* these Fenton reactions can be driven by NADH. For example, the ndh mutant in *E. coli* accumulates NADH as a result of the mutant's inability to donate electrons from NADH to respiratory pathways; as a result, the mutant is hypersensitive to hydrogen peroxide. Studies of other *E. coli* mutants have led to the conclusion that a Fenton active metal is bound to DNA, probably chelated to a phosphodiester linkage. If the bound metal is reduced by a small diffusible molecule, such as NAD(P)H or superoxide, it will react with hydrogen peroxide to form the hydroxyl radical (Imlay & Linn 1986). The short-lived hydroxyl radical then oxidises an adjacent sugar or base causing breakage of the DNA chain (Imlay & Linn 1986).

Cross-linking of DNA to protein is another consequence of hydroxyl radical attack on either DNA or its associated proteins(Oleinick et al. 1986). Treatment with ionising radiation or other hydroxyl radical generating agents causes covalent linkages such as thymine-cysteine adduncts, between DNA and protein. When these cross-linkages exist, separation of protein from DNA by various extraction methods is ineffective. Although DNA-protein cross-links are about an order of magnitude less abundant than single strand breaks, they are not as readily repaired, and may be lethal if replication or transcription precedes repair (Oleinick et al 1986).

DNA is an obvious weak link in a cell's ability to tolerate oxygen free radical attack. First, it seems that DNA is effective in binding metals that are involved in Fenton reactions, and secondly less damage can be tolerated in DNA than other

macromolecules. As a consequence, the cell has a number of DNA repair enzymes (Beyer, Imlay, & Fridovich 1991). One reason why eukaryotic organisms have compartmentalised DNA in the nucleus, away from sites of redox cycling that are high in NAD(P)H and other reductants, may be to avoid oxidative damage (Oleinick et al 1986).

Superoxide, nitric oxide or H_2O_2, at physiologically relevant levels do not react with any of the DNA or RNA bases or sugars at significant rates, but by contrast exposure of DNA results to high levels of hydroxyl ($OH^{\cdot}$) results in multitude of products, since it attacks sugars, purines and pyrimidines (Breen & Murphy 1995). For example, $OH^{\cdot}$ can add on to guanine at positions 4, 5 and 8 in the purine ring. Addition to C-8 produces a C-8 OH- adduct radical that can be reduced to 8-hydroxy-7, 8-dihydroguanine, oxidised to 8-hydroxyguanine or undergo the opening of the imidazole ring, followed by one-electron reduction and protonation, to give 2,6-diamino-4-hydroxy-5-formanidopyrimidine (Breen & Murphy 1995;Von Sontang 1987). Similarly, $OH^{\cdot}$ can add on to the C-4, C-5, or C-8 of adenine. Pyrimidines are also attacked by $OH^{\cdot}$ to give a multiple of products. Thus thymine can suffer hydrogen atom abstraction from the ring or from the ring or from the methyl group. The resulting radicals are converted into various thymine peroxides, which can break down to cis- and trans- thymine glycols (5,6- dihydroxy-6-hydrothymines), and 5-hydroxy 5-methylhydantoin, 5-hydroxy-6-hydrothymine, 6-hydroxy-5-hydrothymine and 5-(hydroxymethyl) uracil. Cystosine can from several products, including cystosine glycol and 5,6- dihydroxycys.

1.7.4. Significance of oxidative stress in disease

As stated earlier, disease-associated oxidative stress could result from either (or both) diminished antioxidants (Golden 1987) or a state where there is an increased production of ROS/RNS (Halliwell & Gutteridge 1999). The latter mechanism is usually thought to be more relevant to disease and is frequently the target of attempted therapeutic intervention (Halliwell & Gutteridge, 1999).

Oxidative stress can result in either adaptation, where the target completely, partially or overtly protects itself against damage, or tissue damage where molecular targets like the DNA and protein are targeted or cell death where necrosis and apoptosis is observed. A point to be noted is that these three events follow each other chronologically, wherein the first line of action against oxidative stress is defence, when this fails tissue damage can occur which is then followed by necrosis and apoptosis of the cell (Halliwell & Gutteridge, 1999).

The large increase in the production of oxidants, leading to oxidative stress has been implicated in the aetiology of numerous diseases like atherosclerosis, diabeties and rheumatoid arthritis to name a few (Burton & Ingold 1984). Some human disease may be caused by oxidative stress. For example, ionising radiation generates $OH^{\cdot}$, by splitting water molecules and many of the biological consequences of exposure to excess radiation are probably due to oxidative damage to proteins, DNA and lipids. However, in most human diseases, oxidative stress is a consequence and not the primary cause of the disease process. Tissue damage by infection, trauma, toxins, abnormally high or low temperatures, and other causes usually leads to the formation of increased amount of putative 'injury mediators', such as prostaglandins, leukotrienes, interleukins and other cytokines such as tumour necrosis factor (TNFs).

All of these have, at various times, been suggested to play an important role in tissue damage. The presence of free radicals in these situations would exaggerate or aid in

1.8. Sepsis

Sepsis is defined as a severe illness caused by overwhelming infection of the blood stream by toxin producing bacteria. The systemic response to sepsis is manifested by two or more of the following conditions: temperature >38 or <36°C; heart rate >90 beats per min; respiratory rate >20 breaths per min, or pCO_2 <32 mm Hg; leukocyte count >12,000 cells per ml^3 or >10% immature (band) forms (Wang, Cristopher J.C., & Tracy 2004). If untreated, the patient may develop respiratory or renal failure, abnormalities of coagulation, and profound and unresponsive hypotension. A recent epidemiological study from North America found that the incidence was approximately 3.0 cases per 1,000 population, which translates into an annual burden of approximately 750,000 cases. The overall mortality is approximately 30%, rising to 40% in the elderly and is 50% or greater in patients with the more severe syndrome, septic shock (Agnus 2002). It is worth emphasizing that these figures represent mortality rates in patients admitted to hospital intensive care units and given antibiotics and the best available supportive care. The commonest sites of infection are the lungs, abdominal cavity, the urinary tract and primary infections of the blood stream. A microbiological diagnosis is made in about half the cases; Gram-negative bacteria account for about 60% of cases, Gram-positive for the remainder (Agnus 2002;Vincent, de Carvalho, & de Backer 2002)

The immunological response to sepsis is as follows and has been clearly illustrated in the Fig 1.6. The immunopathology of sepsis highlights the role of neutrophils, as compared to either macrophages or monocytes (Cohen J. 2002). Inefficient pathogen clearance (by neutrophils) can lead to systemic failure of cytokine regulation, resulting in overexpression of proinflammatory mediators of at least two distinct

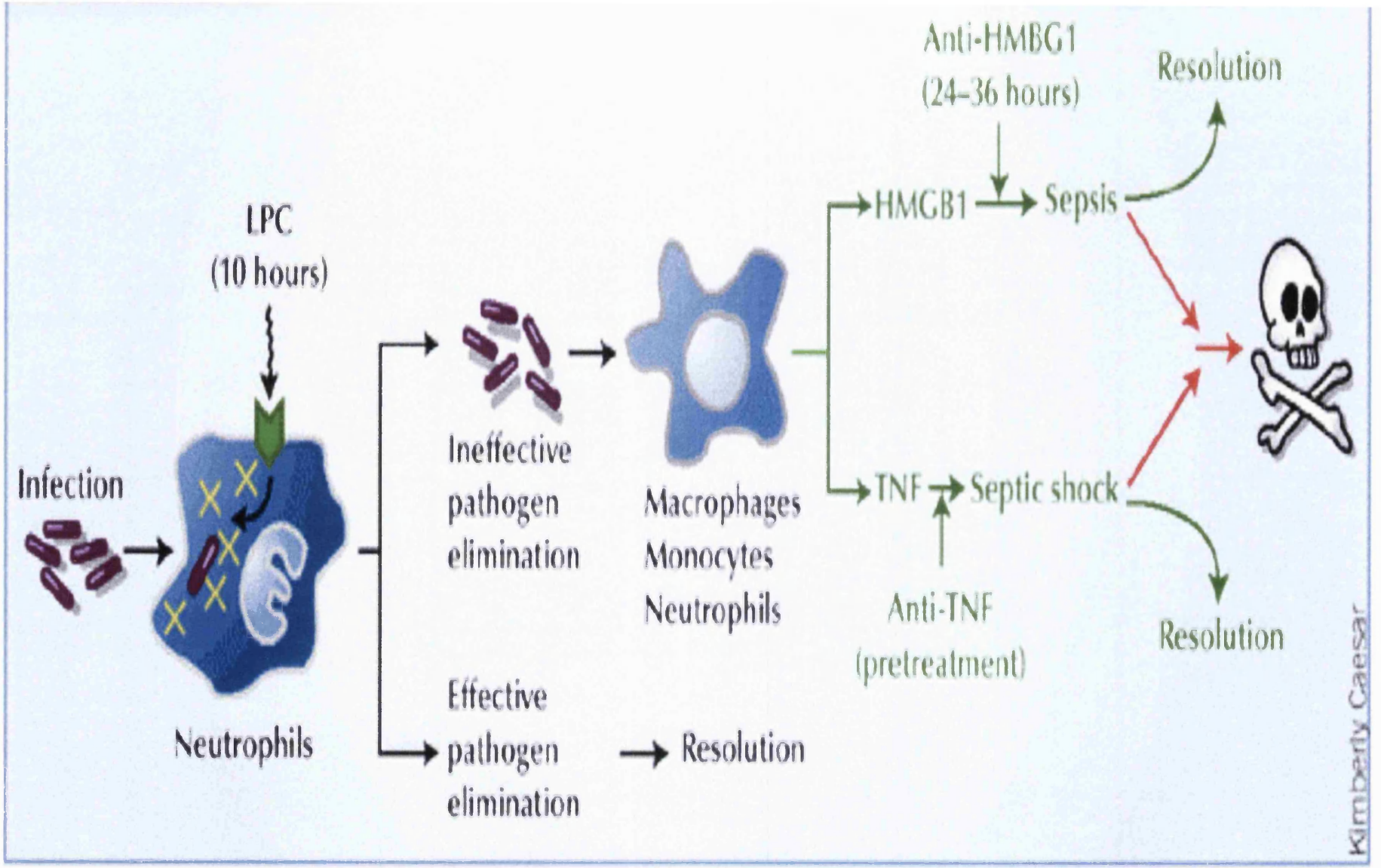


Fig.1.10. Chain of events from infection to sepsis and septic shock.

syndromes. If excess TNF is produced, the patient may develop acute septic shock syndrome. If excess HMGB1 is produced, the patient may develop severe sepsis syndrome. LPC enhances the bactericidal activity of neutrophils, enhancing bacterial killing and attenuating the inflammatory response(Cohen, 2002)

1.8.1. Oxidative Stress and Antioxidants in Sepsis

Although they have a potentially beneficial role in inflammatory cell function, the production of ROS, especially $NO^{\cdot}$, during sepsis may have deleterious effects on vascular control and enzymatic function. Albeit essential for infection containment, an overwhelming production of ROS is believed to contribute directly to endothelial and tissue injury via membrane lipid peroxidation, and cellular and DNA damage often results in multiple organ dysfunction syndrome (MODS), and finally death. In fact, vascular hyporeactivity to catecholamines, a key feature in the pathophysiology of septic shock leading to a high fatality rate, has been related to $ONOO^{-}$-mediated impairment of adrenoceptors (Takakura et al. 2003) as well as a deactivated oxidation of norepinephrine (Benkusky, Lewis, & Kooy 1999).

In sepsis as in other shock states, generation of ROS by inflammatory cells seems to contribute to cellular injury. Using various experimental methods, several investigators have shown enhanced production of markers of oxidative injury. An increase in lipid peroxidation was observed in dogs where septic shock was produced by E coli.(Morgan 1992). Takeda (1999) also reported a similar observation in experimentally induced sepsis in rats. Other investigators have used electron spin

resonance and spin trapping techniques to document ROS mediated changes in liver extracts of septic primates (Lloyd 1993). Formation of ROS during septic shock and organ failure has been attributed primarily to extracellular oxidant production by activated leukocytes, complement activation, or initiation of ischemia-perfusion mechanisms such as conversion of xanthine dehydrogenase to xanthine oxidase (Nanni et al. 1995). As corroboration, several investigators have shown increased survival of septic rats after intravenous administration of superoxide dismutase (SOD), a benefit attributed to scavenging of O_2^- by the antioxidant enzyme in the extracellular space (Warner, Hasselgren, & James 1996;Yoshikawa 2000).

A theory has been postulated that because ROS are produced even during normal metabolic conditions, it is likely that the demands of sepsis on cellular metabolism induce mitrochondria to generate increased quantities of oxygen-derived radicals. Thus mitochondria with good respiratory control generate greater amounts of $OH^·$ and H_2O_2 in succinate-stimulated organelles isolated from the livers of septic rats as compared with sham-operated animals (Taylor 1995). In addition to ROS production in response to increased energy demand by the cell, several other potential mechanisms could accelerate formation of ROS by mitochondria during sepsis. For instance, cytokine-associated changes in gene expression may alter the oxidant/ antioxidant balances within the cells or may even modify the functional activity of mitochondrial electron transport complexes themselves. As an example, several investigators have shown diminished activity of intracellular antioxidant enzymes like glutathione peroxidase and SOD during sepsis (Llsuy & Morgan 1994;Takeda & Avila 1986). In addition, serum MnSOD levels were found to increase in sepsis (Leff et al. 1993). When viewed in the context of a decline in markers of neutrophil

activation, this observation suggests that MnSOD may be released by damaged mitochondria during MODS (Leff et al.1993).

In addition ROS that elude mitochondrial defences are capable of significant intracellular damage, including direct impairment of mitochondrial function like electron transport (Zhang et al. 1994). Therefore, mounting evidence from basic studies supports the hypothesis that both mitochondrial injury and enhanced generation of ROS by mitochondria contribute to cell injury and organ system failure in sepsis (Taylor 1995).

A number of reports have documented reduced level of antioxidants in sepsis which is further evidence for its involvement in oxidative stress. Thus in patients with septic shock, high plasma concentrations of thiobarbituric acid-reactive substances (TBARS)—an indicator for lipid peroxidation—and low levels of circulating antioxidants such as vitamin E, C and carotenoids have been reported (Goode et al. 1995). In a further study, the plasma "antioxidant potential" of patients with sepsis and secondary organ dysfunction who survived rapidly attained normal or supranormal values over time although initial values were reduced (Cowley et al. 1996). In contrast, non-survivors never reached the normal range. Pascual et al. (1998) found the plasma antioxidative capacity—including α-tocopherol, ascorbate, urate, bilirubin, and SH-containing proteins—to be lower in patients with sepsis whereas in septic shock this antioxidative capacity was significantly increased (Pascual et al. 1998).

In an issue of *Intensive Care Medicine*, Ritter et al. (2003) reported alterations of both the antioxidant capacity and the indicates free radical damage in rats with sepsis. In addition to an increase in TBARS and protein carbonyl groups (an indicator of oxidative damage to proteins), an increased SOD activity was found in plasma and various organ sites, i.e., heart, lung, liver, and kidney along with a concomitant $O_2^{\cdot -}$ production. Furthermore, plasma SOD activity, lipid peroxidation, and protein carbonyls predicted fatal outcome as early as 3 h after sepsis induction. Although plasma catalase (CAT) activities were significantly increased in rats with sepsis, no difference was present between survivors and non-survivors. In contrast to plasma, the CAT activities in organ tissues were diminished in animals with sepsis, indicating a different modulation of this enzyme in the course of sepsis as compared to SOD(Ritter et al. 2003).

In animals with sepsis decreased cellular glutathione (GSH) levels correlated with increased organ damage and fatality (Carbonell 2000). Consistent with this, the administration of exogenous N-acetylcysteine (NAC), which acts as a GSH precursor and also a direct scavenger of free radicals, was demonstrated to improve several "physiological" parameters, such as cardiac output (Spapen et al. 1999) and oxygen delivery (Spapen et al. 1998). However, in clinical studies NAC showed rather equivocal results: although a significant improvement in organ function and a reduction of biochemical markers of the oxidative stress was reported in patients, in none of these trials was survival improved (Molnar et al. 2004).

Some times conflicting results have been stated with respect to the effect of antioxidants and stress where, circulating concentrations of vitamins C and E are

markedly depleted in patients with sepsis (Quinlan et al. 1998). Whereas high doses of ascorbic acid and α-tocopherol were shown to be protective in a number of animal studies, these vitamins failed to ameliorate free radical-mediated damage and outcome in patients (Quinlan et al. 1998). Although interestingly, Nathens and co-workers (Nathens et al. 2002) demonstrated a reduced incidence of organ failure and shortened ICU stays with prophylactic and combined administration of α-tocopherol and ascorbic acid in a large randomized prospective in critically ill patients. The role of other natural and synthetic antioxidants such as dimethylsulphoxide, lazaroids, pyrrolidine dithiocarbamate, tempol, etc., in the management of sepsis has been investigated in animal experiments (reviewed by Macdonald 2003) but appropriate in controlled clinical studies are still missing (Ritter et al 2003).

1.9. Biomarkers of oxidative stress

Oxidative stress is propagated by free radicals (**Section 1.6.**). Free radicals are extremely reactive and short-lived and it is difficult to measure these species, and therefore, oxidative stress directly. Electron spin resonance (ESR) can theoretically be used to detect free radicals(Gilbert 1981). However, in practice it has been found to be necessary to use indirect spin trap methods to detect radical adducts.This method is not feasible for application to sample batches in clinical trials. Most methodologies therefore, measure putative products of oxidative stress. Products of oxidative stress are various because as discussed previously free radicals attack all cell components resulting in peroxidation of lipids, oxidation of protein and carbohydrates, and oxidative damage to DNA. Guanine is the DNA base m prone to oxidative damage resulting in the most predominant lesion, 8-hydroxydeoxy guanosine (8-OHdG). 8-OHdG has been studied widely in both cellular DNA analyses and in non-invasive urinary analysis (Helbock et al. 1998). The most commonly used analytical procedures are high performance liquid chromatography with electrochemical detection (HPLC-ECD) (Von Sontang 1987)and gas chromatography-mass spectrometry (GC-MS) (Jenner et al., 1998)

Other frequently used markers of oxidative stress are products of lipid peroxidation. A direct approach would involve the measurement of primary products of peroxidation, the hydroperoxides. However, hydroperoxides are unstable, so indirect measures are frequently used which employ determination of secondary or end products derived from further oxidation of the hydroperoxides. The most frequently quoted index of lipid peroxidation is the aldehyde, malondialdehyde (MDA). MDA is a three carbon,

low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Documented methods to measure MDA either involve the measurement of free MDA or an MDA derivative. The most common and simple method employs measurement of an MDA derivative, where MDA reacts with thiobarbituric acid (TBA) at low pH and elevated temperature to produce fluorescent and pigmented adducts, referred to as thiobarbituric acid reactive substances (TBARS). This method is however non-specific, when analyzing extracts from biological fluids as other low molecular weight aldehydes are also able to react with TBA (Asakawa Matsushita 1980).

A hydrocarbon breath test is a method to measure exhalation of a group of volatile hydrocarbons, the alkanes, formed by in vivo lipid peroxidation of polyunsaturated fatty acids (PUFAs). The alkanes, most frequently measured, include ethane and pentane. In 1981, Kivits et al demonstrated that an increased recovery of exhaled ethane resulted from increased oxidation of ω-3-PUFA, and oxidation of ω-6-PUFAs was responsible for increased pentane recovery. Practical limitations of this non-invasive approach include the lack of standardized methods of collecting, processing and analyzing expired air. Ambient air ethane and pentane, which are of higher concentration than in expired air alone and , therefore, can easily contaminate the breath samples.

Many studies have measured a group of compounds called the F_2-isoprostanes, a family of eicosanoid like structures, as a sensitive marker of in vivo oxidative stress. The F_2-isoprostanes are lipids and are non-enzymatically derived isomers of the prostaglandins. In this study I have measured urinary isoprostanes (as discussed later)

now recognised as a good biomarker of lipid peroxidation and therefore of oxidative stress.

1.10. Isoprostanes

Isoprostanes (IsoPs) are prostaglandin (PG)-like compounds that are produced independent of the cyclooxygenase enzyme by free radical catalysed peroxidation of arachidonic acid. The notion that PG-like compounds could be generated in vitro non-enzymatically as products of autoxidation of fatty acids was actually first demonstrated over 40 years ago (Nugteren 1976).

1.10.1. Formation of Isoprostanes

The mechanism for the formation of these compounds is shown in Fig.1.10. Abstraction of bis-allylic hydrogens of arachidonic acid by free radicals leads to the formation of the three-arachidonoyl radicals as shown. Subsequent attack by O_2 results in the formation of four peroxyl radical derivatives of arachidonic acid. These peroxyl radical derivatives then undergo endocyclization followed by further addition of O_2 to form PGG_2-like bycyloendoperoxides. Reduction of these PGG_2-like compounds results in the formation of PGF_2-like compounds. Depending on the location of the peroxyl radical derivatives of arachidonic acid, four regioisomers are formed as noted (I–IV). There are also eight possible racemic disastereoisomers of each regioisomers. Thus, a multiplicity of compounds can be generated by this process, although the formation of some is more favoured over the others (Nugteren 1976).

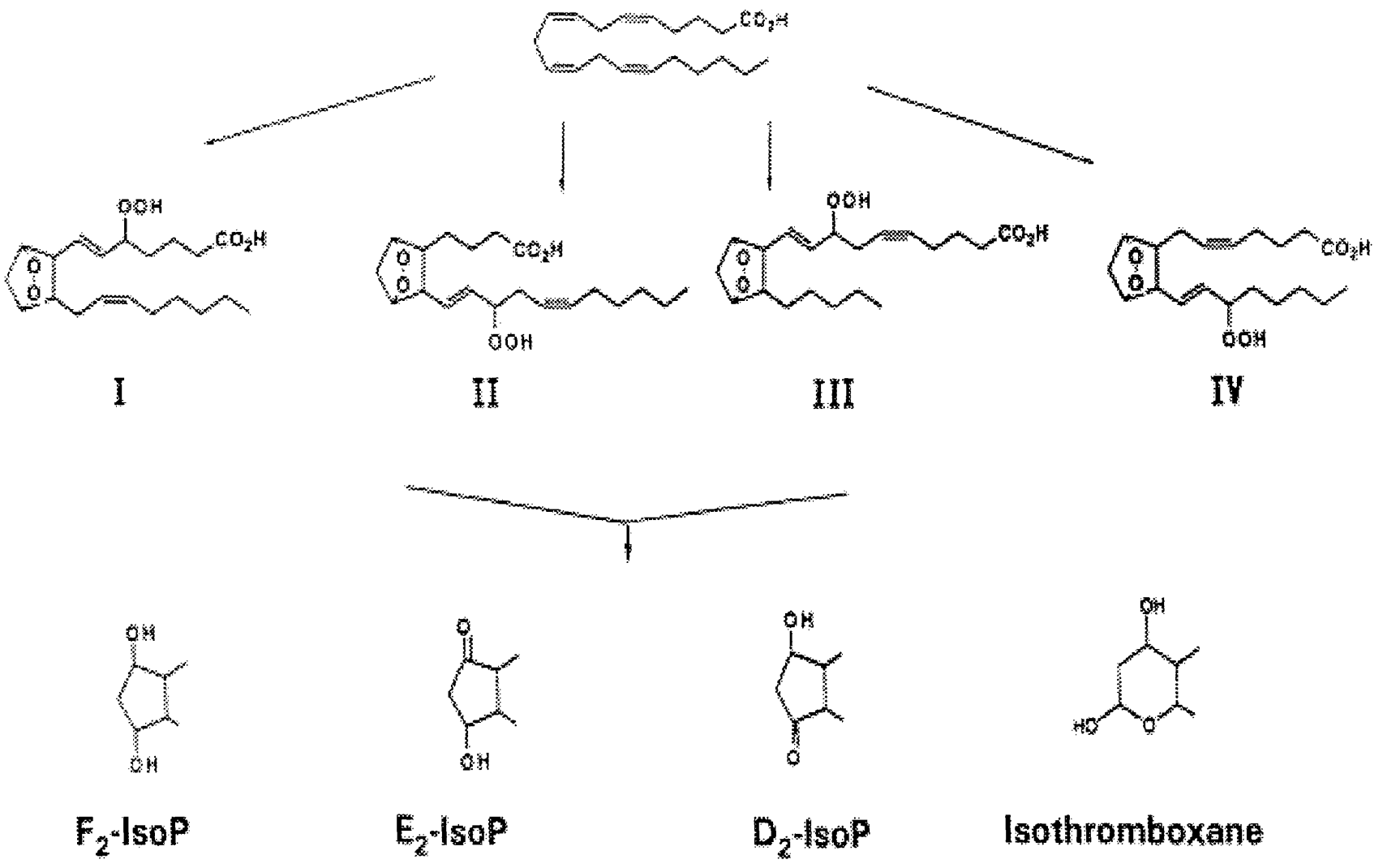


Fig.1.11 . Potential fates of the bicycloendoperoxide intermediates of the isoprostanes (I–IV) derived from the peroxidation of arachidonic acid. The endoperoxides can either undergo reduction to F_2-IsoPs or rearrangement to E_2/D_2-IsoPs and isothromboxanes .

1.10.2. Metabolism of Isoprostanes

The knowledge of the metabolic fate and kinetics of metabolic disposition of IsoPs is somewhat limited. Morrow (1992) from studies in rats found that the $t_{1/2}$ of the clearance of 8-iso-$PGF_{2\alpha}$ from the circulation was ≈16 min. Although not examined directly, it is likely, analogous to the metabolism of other prostanoids, that the lung is the major site of metabolic clearance of F_2-IsoPs from the circulation. This postulation, is supported by the finding that the creation of a portal caval shunt and ligation of the hepatic artery in rats, completely eliminating clearance of 8-iso$PGF_{2\alpha}$ by the liver, only prolonged the $t_{1/2}$ of the clearance of 8-iso$PGF_{2\alpha}$ from the circulation from 16 to 21 min (Morrow et al. 1992).

The metabolic fate of 8-iso$PGF_{2\alpha}$ in humans using radiolabelled 8-iso$PGF_{2\alpha}$ was explored (Robert 1997). Interestingly, ≈43% of excreted radioactivity was unextractable into ethyl acetate, suggesting the presence of very polar material, perhaps polar conjugates (Roberts & Morrow 1994). The major urinary metabolite of 8-iso$PGF_{2\alpha}$ was identified as 2,3-dinor-5,6-dihydro-8-iso$PGF_{2\alpha}$ The importance of the identification of the major urinary metabolite of 8-iso$PGF_{2\alpha}$ is that it provides the basis for development of methods of assay for 2,3-dinor-5,6-dihydro-8-iso$PGF_{2\alpha}$ as a means to obtain an integrated assessment of total endogenous F_2-IsoP production in humans (Roberts 1997).

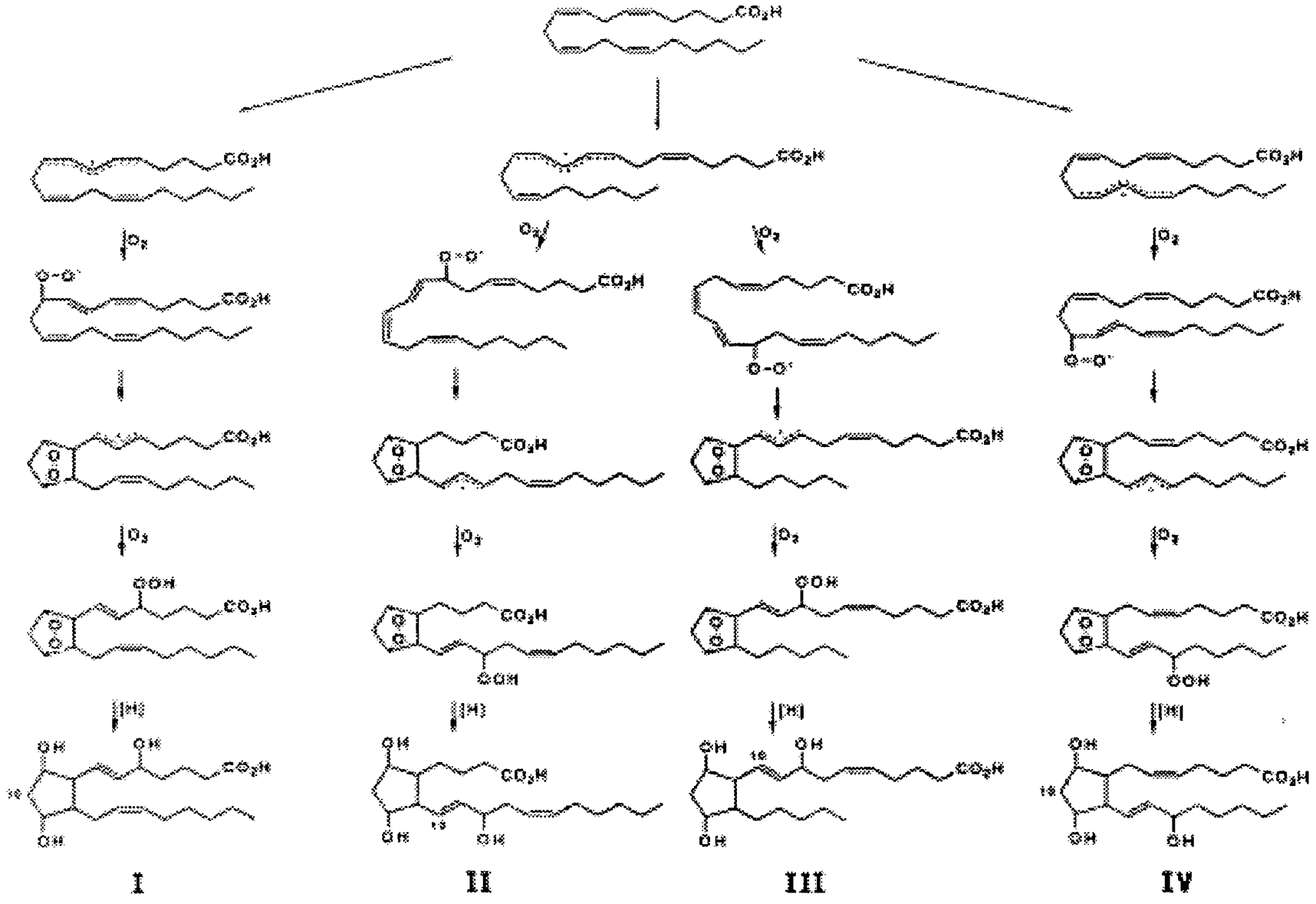


Fig.1. 12. Mechanism of the F_2- Iso Ps

1.10.3. Biological actions of Isoprostanes

As IsoPs are isomeric to cyclooxygenase-derived PGs, which exert potent biological activity, it is of considerable interest to explore whether IsoPs may also possess biological activity, in which case they may participate as mediators of oxidant injury. A unique structural feature of IsoPs is that, in contrast to cyclooxygenase-derived PGs, the side chains are predominantly oriented *cis* in relation to the prostane ring (Roberts & Morrow 1994). Because the levels of individual unmetabolized F_2-IsoPs in urine are very high, between 1–2 ng/ml (Robert & Morrow 1994), which may in part derive from local production in the kidney, it was initially examined whether 8-isoPGF$_{2\alpha}$ exerted any biological effects on renal function in the rat. Interestingly, it was found that 8-isoPGF$_{2\alpha}$ was an extremely potent renal vasoconstrictor, reducing glomerular filtration rate and renal blood flow by 40–45% in the low nanomolar range (Morrow et al 1992;Takahashi et al. 1992). 8-isoPGF$_{2\alpha}$ has also been found to be a potent pulmonary artery vasoconstrictor in rabbits and rats and causes bronchoconstriction in the rat lung (Banerjee & Lund 2004;Kang, Schini-Kerth, & kim 1995). Interestingly, 8-isoPGF$_{2\alpha}$ has also been shown to induce mitogenesis in vascular smooth muscle cells and induce endothelin-1 release from bovine aortic endothelial cells (Fukunaga, Yura, & Badr 1995;Fukunaga, Takahashi, & Badr 1993). Of considerable interest was the finding that 8-iso-PGE$_2$ was also a potent renal vasoconstrictor, approximately equipotent with that of 8-isoPGF$_{2\alpha}$ (Morrow 1992). This was an unexpected finding because in most systems, cyclooxygenase-derived PGE$_2$ and PGF$_{2\alpha}$ have opposing biological effects, which has been attributed to the differences in ring structure (Campbell C.J., Takahuka, & Fukunaka 1996). The finding that 8-iso-PGE$_2$ and 8-isoPGF$_{2\alpha}$ are both potent vasoconstrictors in the renal vascular bed suggests that the stereochemistry of the side chains, rather than ring

structure, may be an important determinant of this biological actions of IsoPs (Robert 1997).

A series of investigations aimed at elucidating the mechanism by which 8-isoPGF$_{2\alpha}$ and 8-iso-PGE$_2$ exert their biological actions on vascular smooth muscle have led to provocative findings, namely that these IsoPs may exert their effects by interacting with a unique receptor. Initially, reportings were cited that, the renal vasoconstricting actions of these compounds could be abrogated by SQ29548, a thromboxane receptor antagonist, suggesting that these compounds interacted with thromboxane receptors (Morrow et al. 1990;Takahashi et al. 1992). Interestingly, however, when incubated with platelets, 8-isoPGF$_{2\alpha}$ (10^{-6} and 10^{-5} M) caused only a shape change and at very high concentrations (10^{-4} M) only induced reversible but not irreversible aggregation (Morrow 1992). 8-iso-PGE$_2$ caused a modest degree of irreversible aggregation of platelets from some individuals at concentrations of 10^{-5} and 10^{-4} M but in most, it only caused reversible aggregation at these concentrations (Longmire, Roberts, & Morrow 1994). In contrast, both IsoP's were more potent as antagonists of thromboxane receptor agonist-induced platelet aggregation. These findings, namely that these compounds acted primarily as antagonists of the thromboxane receptor in platelets, would not be consistent with these compounds interacting with thromboxane receptors unless the platelet and vascular smooth muscle thromboxane receptors were different. Although this would be one possible explanation for these findings, it is not very attractive owing to the fact that only a single thromboxane receptor gene has been identified (Hirta 1991) Splicing variants of the thromboxane receptor have been identified which appear to have similar ligand binding characteristics and phospholipase C activation but oppositely regulate adenylyl cyclase activity

(Raychowdhury et al. 1995). However, evidence suggests that 8-isoPGF$_{2\alpha}$ does interact with either of these isoforms of the thromboxane receptor (Hirta 1991).

Another explanation for the above findings was the possibility that the IsoPs interacted with a unique receptor on vascular smooth muscle distinct from the thromboxane receptor. In order to explain the fact that the vascular effects of these compounds is abrogated by SQ29548, one would envision that this novel receptor must be structurally similar to the thromboxane receptor. Initial clues consistent with the presence of a receptor for IsoPs distinct from the thromboxane receptor was the finding that, whereas the IsoPs were more potent in inducing a functional response on vascular smooth muscle cells, e.g. phosphoinosotide turnover, than thromboxane receptor agonists, they were much weaker than thromboxane receptor agonists in IsoPs devoid cells(Takahashi et al. 1992).

1.10.4. Value of Measuring Isoprostanes to assess oxidative stress in vivo

Most non-invasive methods developed to detect free radical injury in vivo have been found to be unreliable. However, a considerable body of evidence has been obtained that suggests strongly that measurement of IsoPs represents an important advance in our ability to assess oxidative stress status in vivo (Robert 1997).

First, it is important to point out that IsoPs are almost entirely products of lipid peroxidation. In this regard, however, it is known that minute quantities of the F_2-IsoP, 8-isoPGF$_{2\alpha}$ can be produced as a minor by-product of the cyclooxygenase enzyme (Robert 1997). More recently, it was demonstrated that very small quantities of 8-isoPGF$_{2\alpha}$ are formed by Prostaglandin H synthase-1 (PGH synthase-1) during aggregation of human platelets in vitro and by PGH synthase-2 in human monocytes

((Pratico et al. 1998). Importantly, it was demonstrated that the administration of high doses of cyclooxygenase inhibitors to normal humans does not significantly reduce 8-isoPGF$_{2\alpha}$ concentrations (Morrow et al. 1990). This indicates that the relative contribution of enzymatic generation of 8-isoPGF$_{2\alpha}$ in vivo is inconsequential compared to the amounts formed non-enzymatically (Robert 1997). Further evidence that 8-isoPGF$_{2\alpha}$ is formed non enzymatically comes from where in pathologic situations associated with a profound increase in cyclooxygenase activity, enzymatic generation of 8-isoPGF$_{2\alpha}$ remains insignificant in relation to the amounts formed non-enzymatically in vivo (Robert 1997).

Initial clues that measurement of IsoPs may provide a valuable approach to assess oxidative stress status in vivo emerged from some of the early studies carried out related by Morrow (1990). Importantly, as mentioned previously, measurable levels of IsoPs can be detected in virtually every animal and human biological fluid and tissues that have been analyzed. This allows the definition of a normal range such that even small increases in the formation of IsoPs can be detected. Further, overproduction of IsoPs has been well documented to occur in settings of oxidant injury. For example, in earlier studies it was found that the formation of IsoPs increased as much as 200-fold above normal in two well established models of oxidant injury, namely the administration of CCl_4 to normal rats and the administration of the herbicide, diquat, to Selinium-deficient rats (Morrow et al. 1990). These data indicate that measuring 8-isoPGF$_{2\alpha}$ can be used as a biomarker (quantification) of oxidative stress (lipid peroxidation) in normal and diseased state individuals. This study will thus look into the quantification of 8-isoPGF$_{2\alpha}$ in sepsis patients.

1.11. Vitamin E metabolites

As mentioned previously Vitamin E is a group pf compounds-tocopherols and tocotrienols, each having four families (α, β, γ and δ) depending on number and position of methyl groups.

Vitamin E has a general role as a lipid soluble antioxidant that helps prevent lipid peroxidation. Vitamin E has also been proposed to have an important protective role against disease where increased oxidative damage has been observed (Burton et al. 1995). There is currently interest in measuring urinary metabolites of vitamin E, as it has been suggested that α- tocopheronolactone (α- TL) with an oxidised chroman ring may be an indicator of oxidative stress and that the α- carboxyethyl-6-hydroxychromans (α- CEHC) with a shortened phytyl side chain may provide a measure of adequate vitamin E status (Schultz et al. 1995).

1.11.1. Vitamin E metabolism

Although vitamin E was discovered in the 1920's relatively little is known about its metabolites. Vitamin E metabolites are likely to form two distinct groups. The first of these contains metabolites that are produced after reaction of vitamin E with oxidants while the second contains metabolites that are produced in the liver from excess vitamin E that is not loaded onto nascent VLDL, for transportation into the circulation (Schultz et al 1995)

Vitamin E metabolites are excreted as conjugates but virtually all of the research into the metabolism of vitamin E has concentrated on urinary metabolites, after their deconjugation. Urinary metabolites of vitamin E, were first reported by Simon et al (a,b). They described two metabolites of α-tocopherol, tocopheronic acid, (α- TA) and

its lactone (tocopheronolactone α- TL) which were produced by both rabbits and humans (Fig 1.12.). Enzymatic deconjugation of these metabolites with β-glucuronidase suggested that α- TA and α- TL were excreted as glucuronide conjugates. Owing to their quinone structures, it was suggested that α-TA and α- TL were derived form the known α- tocopherol oxidation product, α- tocopherylquinone (α-TQ) (Simon et al. 1956b;Simon et al. 1956a). It was proposed that these metabolites were produced after reduction of α- TQ to α-tocopheryl hydroquinone (α-THQ), conjugation and then subsequent β-oxidation of the phytyl side chain. Indeed, an unknown conjugate of α- TA, released after acid hydrolysis, was the main metabolite observed after the injection of rats with C14- α- TQ (Gloor & Wiss 1966). Oral administration of α- TQ to humans also resulted in the excretion of α- TA in the urine but the level of excretion only accounted for 0.3% of the original dose (Gloor & Wiss 1966). This low conversion to α- TA was probably because of poor intestinal absorption of α- TQ.

The major urinary metabolite observed after injection of rats with radiolabelled δ-tocopherol, was δ- carboxy- ethyl- hydrochroman (δ-CEHC) (Chiku, Hamamura, & Nakamura 1984) and was proposed to be formed by β- oxidation of the phytyl side chain of δ- tocopherol (Fig.1.12.).

The α-tocopherol homologue of δ-CEHC was characterised in human urine by Schonfeld et al (1993) and detailed study, was conducted by Schultz et al (1995). Enzymatic studies indicate α- CEHC was excreted as a sulphate conjugate and since this metabolite was only detected after a daily intake of 50-150mg of α- tocopherol, it was proposed to be an indicator of excess vitamin E. Care was taken to use non-oxidative conditions and neither α- TL nor α- TA were observed (Schinfeld et al.

1993). Therefore, they proposed that α- TL nor α- TA described previously were oxidation artefacts of the experimental procedure. This was shown to be plausible by the oxidative conversion of α- CEHC to α- tocopheronolactone in the presence of air and acid (Schinfeld et al. 1993).

In 1996, Wechter et al isolated a new endogenous natriuretic factor (a factor that controls the body's pool of extracellular fluid), which was characterised as unconjugated trimethyl-carboxyethyl-hydroxychroman (γ-CEHC) and therefore presumed to be a metabolite of γ- tocopherol. Unconjugated γ- CEHC acts as a natriuretic factor by inhibiting the 70 pS ATP- sensitive K+ channel of the thick ascending limb cells of the kidney ((Wechter et al. 1996).

The excretion of conjugated γ- CEHC in the urine, was reported by Traber et al (1998). They proposed that CEHC metabolites were synthesised from excess vitamin E in the liver. Contrary to previous evidence by Schultz et al (1995), α- CEHC was shown to be excreted in the urine at all levels of vitamin E intake and not just when supplementation reached a certain threshold ((Lodge et al. 2000;Schinfeld et al.1993)

Vitamin E metabolism as mentioned above, are expected to be excreted in the urine as sulphate or glucuronide conjugates. However, no definitive characterisation of the intact conjugates has been reported owing mainly to a lack of standards and suitable techniques to analyse these polar metabolites directly.

In this study we investigate whether urinary α- TL the oxidised metabolite of α- tocopherol would be a biomarker of oxidative stress.

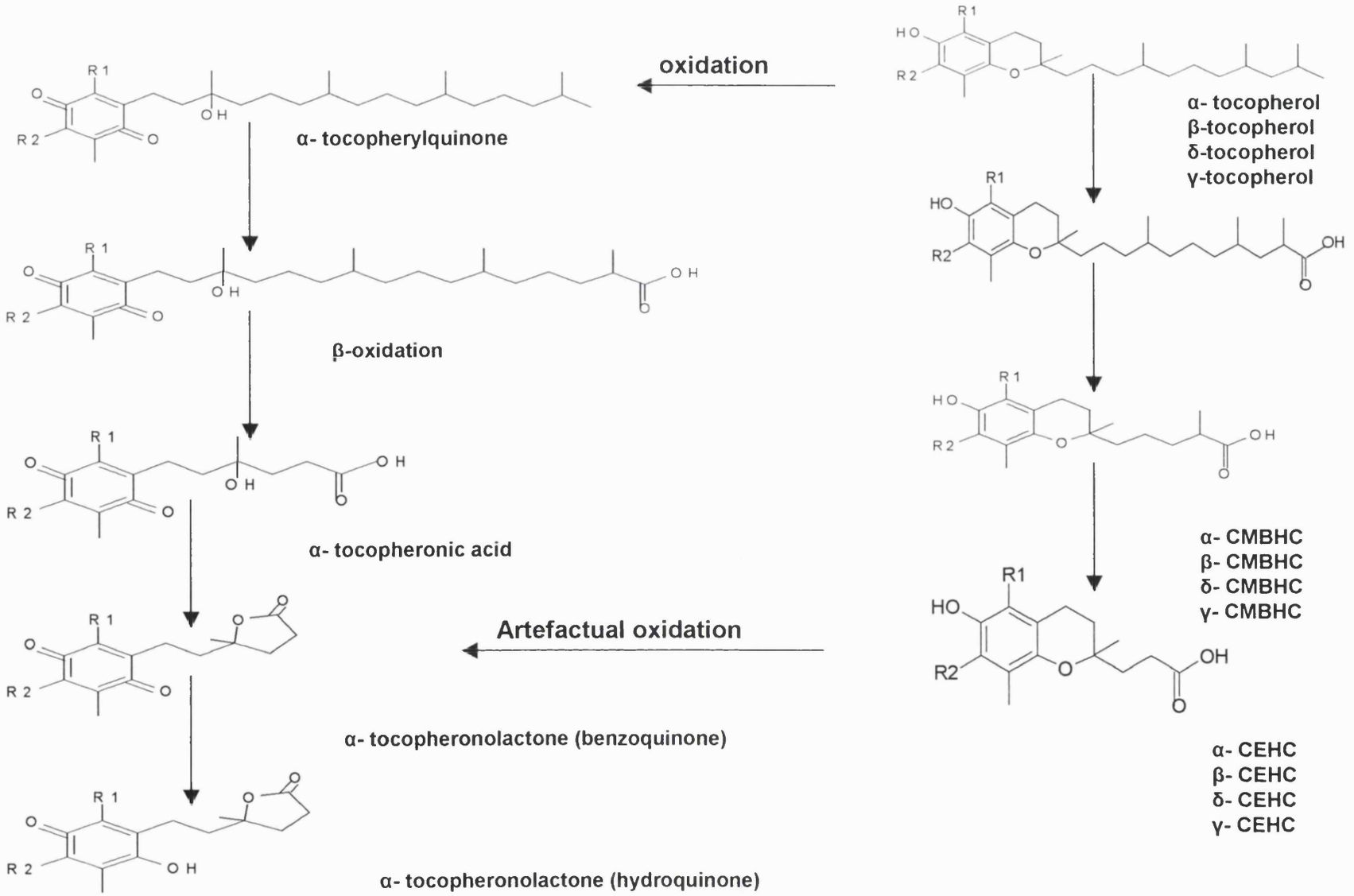


Fig 1. 13. Overview of vitamin E metabolism

1.2. Aims of the study

1. To re-establish and validate the urinary analysis of 8-isoPGF$_{2\alpha}$ and vitamin E metabolites using gas chromatography/ mass spectrometry.
2. To utilise these assays in patients with sepsis and controls to obtain evidence of oxidative stress.
3. To correlate urinary 8-isoPGF$_{2\alpha}$ concentrations (an established biomarker of oxidative stress) with urinary concentrations of α-tocopheronolactone to see whether the latter could also be used as an invivo biomarker of oxidative stress.

METHODOLOGY

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2. Methodology

The effect of oxidative stress in the pathology of sepsis has been discussed in the previous sections. α-Tocopheronolactone has been proposed to be a potential biomarker of in vivo oxidative stress. This hypothesis was investigated by analysing the urinary samples of sepsis patients for the lactone using a recently standardised methodology. The lactone values were evaluated and correlated to that of 8-isoPGF2α, a well-established biomarker of oxidative stress. This chapter describes the methodology used in the analysis of these two biomarkers.

The subjects used in this study were compartmentalised as Controls and Sepsis patients and their details have been tabulated in Table 2.1 A and B respectively. The sepsis patients and control subject's urine were kindly supplied by Simon Eaton of the Surgery Institute of Child Health, Unit of Great Ormond Street Hospital, London U.K.

Patients	Age (mths)	Nutrition	Diagnosis
P1	39.233	SI-1	Belchet syndrome, Respiratory failure
P2	21.933	SI-2	Meningococcol septicaemia
P3	29.133	D-1	Meningococcol septicaemia
P4	38.7	NA	Streptococcus A sepsis
P5	23.1	NA	pneumonia

SI-1 Saline Infusion 28 ml/hr + 10 ml SMA gold 1hr before study.
SI-2 Saline Infusion 29 ml/hr.
D-1 Dextrose 4 % 20 ml/hr + 10 ml SMA gold 1 hr before the study.
NA Data Not Available

SMA gold contained 0.74mg/100ml of vitamin E (tocopherol-rich extract)

Table 2.1. A Sepsis patient Subjects Information

Control	Age (mths)	Nutrition	Diagnosis
C1	32	NA	NA
C2	122.2	NA	NA
C3	75.2	NA	NA
C4	105.7	NA	NA
C5	14	NA	NA

NA Data Not Available

Table 2.1. B Control Subjects Information.

2.1.Urinary Vitamin E Metabolites- Extraction and Analysis

2.1.1.Materials

Trolox was purchased from Sigma-Aldrich Company Ltd and d9-α-CEHC was a kind gift from Dr Burton (Steacie Institute of Molecular Sciences, National Research Council, Ottawa, Canada). Isolate C4 solid phase extraction cartridges (500 mg sorbent mass, 6 ml reservoir volume) were supplied by Jones Chromatography. The TMS derivitizing agent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Chemicals Ltd. All other chemicals were purchased from Sigma-Aldrich unless otherwise mentioned.

2.1.2. Method overview

The overview of the method has been shown in Fig 2.1 and will be briefly described. The basis of the method will then be discussed.

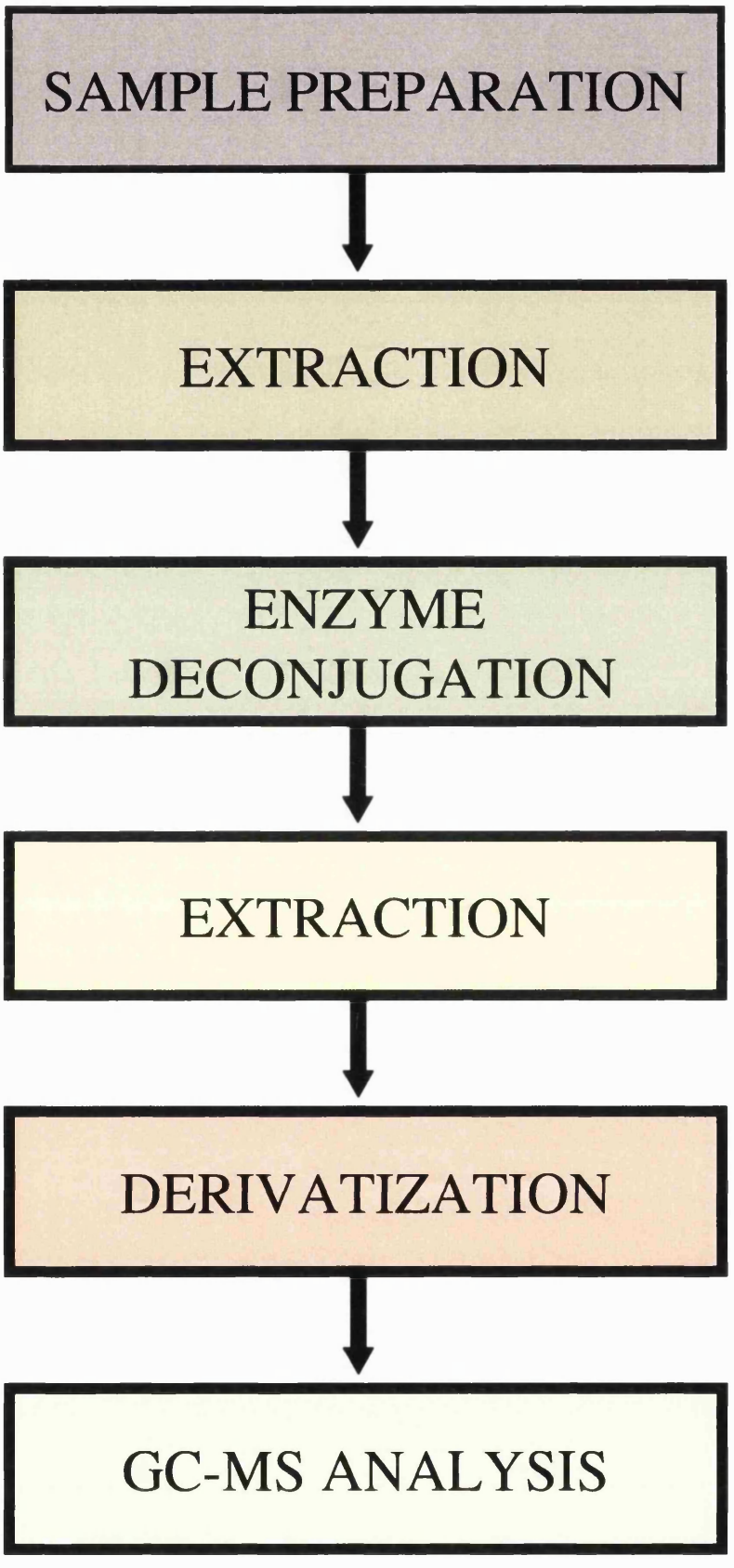


Fig.2.1. Overview of the vitamin E methodology

2.1.2.1. Preparation of urine samples

1ml of urine (centrifuged) sample was spiked with 10nmoles d9- α- CEHC and 5 nmoles trolox in ethanol, as internal standards. The use of these internal standards enabled the quantification of endogenous urinary metabolites and estimation of the percentage artefactual oxidation, which is obtained in the course of the experimental procedure (has been explained in the latter part of the section). The pH was acidified to 2.5 to protonate the metabolites and increase their affinity for the C4 sorbent.

2.1.2.2. Sample 'clean-up' by solid phase extraction

C4 solid phase cartridges were primed with 5ml of MeOH, followed by 5ml dH2O (pH2.5). The sample was then loaded and allowed to run through the sorbent under gravity. This enabled the metabolites to bind to the sorbent. The cartridges were then washed with 5ml of dH2O (pH2.5), to remove any unbound compounds. The vitamin E metabolites were eluted using 5ml of MeOH and evaporated.

2.1.2.3. Enzymatic deconjugation of vitamin E metabolites

As vitamin E metabolites are excreted in urine in a conjugated form, either as glucuronide or sulphated conjugates, the extracted sample was deconjugated to liberate the unconjugated forms of the metabolites. The eluate was dissolved in deconjugation solution, 500μl 0.5M sodium acetate (pH 4.5) and 25 μl suspension of β-glucuronidase/ sulphatase (activity of 143,400 units per ml). It was then incubated

at 37°C for three hours, after which the samples were acidified to pH 2.5 to stop enzymatic hydrolysis and to protonate the metabolites.

2.1.2.4. Sample Extraction

The deconjugated samples were extracted as before using new C4 solid phase extraction cartridges and the eluate was again evaporated to dryness under nitrogen stream.

2.1.2.5. Derivatization

The extracted metabolites were derivatized to silyl derivatives by redissolving in derivitizing solution, 200µl acetonitrile/BSFTA (1:1), and incubated at 60°C, for an hour.

2.1.2.6. Analysis

The analysis was made, by using gas chromatography –mass spectrometry. The derivatized sample (1 µl) was injected by splitless mode onto a HP-1 methyl siloxane column (30m, 0.25 µm film thickness), in a Hewlett-Packard 6890 GC. The GC was linked to a Hewlitt-Packard mass selective detector and run by a Chem station data system. The oven was maintained at 120°C for 2mins at the start of the run and the injection. It was then ramped to 200°C, at a rate of 20°C/min, then to 240°C, at 2°C/min and finally to 300°C at 50°C/min the temperature was held at 300°C for 5

mins. The total time per injection was 32.8min. The flow rate of the column was maintained at 54.3 ml/ min and the pressure was maintained at 11.80 psi.

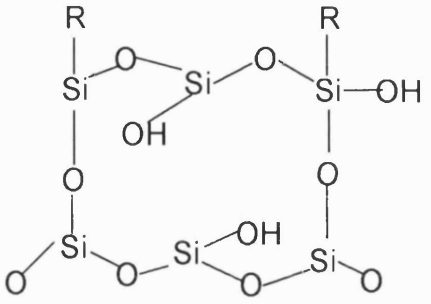
2.1.3. Basis of the method

2.1.3.1. Extraction

Extraction of the vitamin E metabolites was required both before and after deconjugation. Solid phase extraction was used in this procedure. Solid phase extraction (SPE) is described as " the separation or removal of an analyte or analytes from a mixture of compounds by selective partitioning of the compounds between solid phase (sorbent) and a liquid phase (solvent) " (Jones Chomatography 1998). A classical liquid-liquid extraction relies on a marked difference in the solubility of the analyte(s) between the two solvents. Problems with liquid-liquid extractions include the relatively large solvent volumes required, the possible formation of emulsions or mixtures resulting from partial miscibility of the solvents, the possible oxygenation and subsequent oxidation of the sample resulting from partial miscibility of the solvents, the possible oxygenation and subsequent oxidation of the sample resulting from vigorous shaking and a lack of selectivity. In contrast, solid phase extraction requires small solvent volumes, has inherent immiscibility, can show selectivity for specific functional groups and is applicable to batch processing.

Most solid phase extraction cartridges are based on silica chemistry. Cross-bonded silanols form the bases of the solid phase with changes in the R group affecting specificity (Fig.2.2 A). The most commonly used SPE cartridges are probably C18 SPEs. These consist of the silanol backbone with octadecyl side-chains allowing hydrophobic interactions to occur with the analytes of interest. Sorbents containing other functional groups, which form polar or ionic interactions, are sometimes used to allow greater selectivity. Priming the cartridges with a n organic solvent such as

A



C_{18}, R=$C_{18}H_{37}$

C_4, R=C_4H_9

B

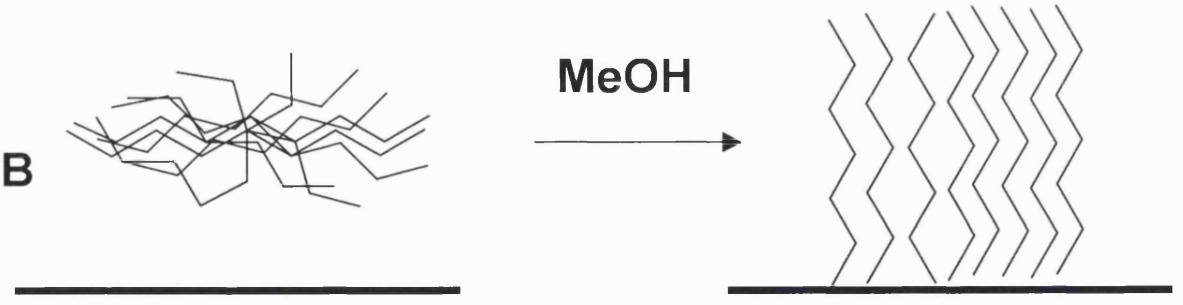
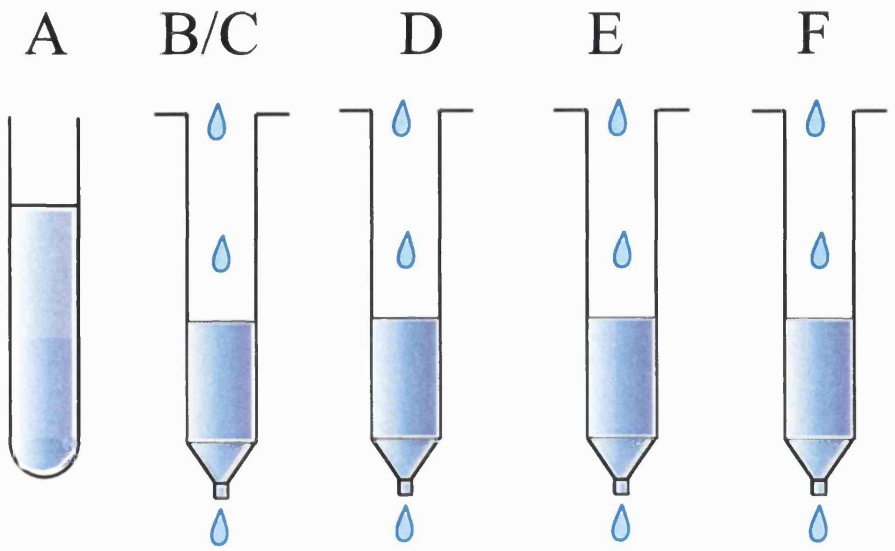


Fig. 2.2 A The structure of silanol based SPE cartridges. B Priming of the cartriges to increase the retention capacity.

methanol stops aggregation of the hydrophobic side chains and thereby increases retention of molecules with hydrophobic areas (Fig 2.2. A)

The six steps involved in a typical solid phase extraction are shown are in figure 2.3. The first step is sample pre-treatment (Fig 2.3 A). This may involve removal of solid material from the sample, ionisation/ deionisation of the sample to increase/ decrease interactions with the solid phase or derivatization. The next step is the solvation or priming (conditioning) of the sorbent bed of the SPE cartridge (Fig 2.3 B). This involves adding an organic solvent, such as methanol, to the cartridge so that interactions between the branches of the solid phase are minimised allowing greater interaction with analytes. The hydrophobicity of solid phase such as C18 tends to make the chains aggregate together if they are not first primed. The cartridge is then washed with the solvent that will be used to apply the sample (Fig 2.3 C). The sample is then loaded onto the column (Fig 2.3 D). Interfering compounds are washed off, often using the same solvent that was used to apply the sample (Fig 2.3 E) and then the analytes are eluted using a suitable solvent- often the initial priming solvent (Fig 2.3 F). Variations can include sequential elution using greater amounts of organic modifier in order to achieve better separation.



A . Sample pre- treament e.g.acidification

B. Column conditioning e.g. MeOH

C. Column equlibration e.g. water

D. Sample application

E. Elution of interfering compounds e.g. water

F. Analyte elution e.g. MeOH

Fig. 2.3. The six steps involved in a typical solid phase extraction

2.1.3.2.Deconjugation

The deconjugation step in the analysis of vitamin E metabolites is required for the removal of sulphate and glucuronide residues, which are attached in vivo to the metabolites to facilitate their excretion in urine. Previously this step has been carried out using rather harsh conditions like acid hydrolysis (Gloor & Wiss 1966;Simon et al. 1956b;Simon et al. 1956a). The acid (HCL) hydrolysis reaction under ordinary conditions (i.e. under oxygen) has led invariably to the oxidation of α- CEHC to α-tocopheronolactone and α-tocopheronic acid, and has frequently resulted in a failure to identify α-CEHC as the major metabolite of α-tocopherol (Simon et al., 1956a; Schultz et al., 1995).

Recently the problem of artefactual oxidation has been minimised, either by using short periods of enzymatic deconjugation under an inert gas, such argon, or by using methanolic HCL that theoretically stops oxidation to α-tocopheronic acid/ α-tocopheronolactone by esterifying the carboxyl group of α- CEHC (Chiku et al. 1984). The most frequent method of enzymatic deconjugation has been to use a mixture of β-glucuronidase and sulphatase enzymes. The use of 'pure' enzymes by some groups has been employed to study the types of conjugates present, especially in animal studies where radiolabelled vitamin E has been administered (Chiku et al. 1984). Owing to the difference in polarity between the conjugated and deconjugated metabolites, Thin layer chromatography (TLC) of the radiolabelled metabolites before and after specific enzymatic deconjugation is a simple way to ascertain the type of conjugates present. However, these studies have not produced unequivocal results. Simon et al in 1956 have illustrated that 70-90 % of α-tocopherol metabolites in the

rabbit were released by β-glucuronidase treatment (Simon et al a and b), where as Chiku et al (1984) demonstrated δ-CEHC to be almost exclusively sulphated in the rat. Other work on enzymatic digestion of non-radiolabelled metabolites has also produced unclear results, although the lack of radiolabel makes it difficult to confirm the proportion of conjugates that have been deconjugated (Stahl et al. 1999;Swanson et al. 1999). The difference between the types of conjugates observed may result from the "impure" nature of the supposedly pure enzyme preparations or the lability of some conjugates under certain conditions. Another reason for anomalous findings may result from metabolite and species differences.

2.1.3.3. Separation and detection

Following a further extraction step, the deconjugated metabolites can be analysed using either HPLC or GC-MS. The choice of GC-MS or HPLC analysis is largely a matter of the equipment available in the laboratory. Nevertheless GC-MS is generally the more sensitive technique, although HPLC can approach this sensitivity if the detection method is suitable, as in the case of electrochemical detection (Lodge et al. 2000) or fluorescence detection of derivatives. In this chapter only GC-MS technique is discussed.

GC-MS is an established technique for the analysis of complex mixtures and is particularly popular because of its selectivity, sensitivity and versatility. The basic principles underlying GC-MS are relatively simple. Essentially, the sample mixture is separated on a gas chromatography column and each peak eluting from the column is ionised and analysed by the mass spectrometer. This technique allows the separation

and quantification of a large number of different compounds in one sample. The separation of a mixture of compounds is achieved by choosing a suitable column and temperature gradient. It is often necessary to derivatise samples using trimethysilyl derivatising agents to produce trimethylsilyl (TMS) derivatives so that polar interactions are minimised, hence reducing the temperature needed to elute the metabolites from the column.

GC-MS, unlike gas chromatography alone, also provides important mass spectral information about unknown peaks, which is extremely helpful in the identification of novel compounds. At each instant in time, a mass spectrum is produced from the compound(s) eluting from the column at that time. If the abundances of all the masses in the spectrum at each time point are added, the total ion abundance is obtained and a total ion chromatogram (TIC) can then be produced, which shows the total ion abundance over the entire course of the analysis. If the TIC is complicated because of co-eluting peaks or large contaminating peaks, the data analysis software can be used to produce an extracted ion chromatogram. This allows a specific compound or group of compounds to be visualised by plotting the abundance of ions which are present only in their spectra. An example of an extracted ion chromatogram is shown in Fig 2.4. which only shows peaks containing ions of masses specific to urinary vitamin E metabolites.

2.1.3.4. Quantification

The quantitative estimation of compounds by mass spectrometry (MS) is not straightforward because measurements are not exactly reproducible. The response of a

sample at the detector is dependent on a number of variables that are difficult to control. The conditions, such as the temperature and pressure in the ion source affect the observed response. For this reason the mass spectrometer must be calibrated with known amounts of the compound under investigation either just before the assay or in a manner which makes the measurement independent of instrument variability.

The two most common methods for quantitative analysis employ the use of either an external or an internal standard (Fig 2.4). Sucessively different, known amounts of an external standard can be assayed and a plot of signal intensity verses quantity can provide data for a calibration curve. It is an external standard since it is not added to the sample to be analysed. Quantities of the compound in a sample can be determined by relating the signal intensity obtained to the calibration curve. With GC-MS, external standard methods are prone to error resulting from the poor reproducibility of sample volume injections and changes in the condition of the instrument affecting the magnitude of signals.

Using the internal standard approach, a known constant amount of the internal standard is added to increasing amounts of the compound of interest. The different mass spectrometric responses to the varying quantities of the compound under investigation are measured in relation to the internal standard. The ratio of the two responses is plotted against the amount of compound to give a calibration curve. For the actual analysis of samples of interest, the same amount of internal standard is

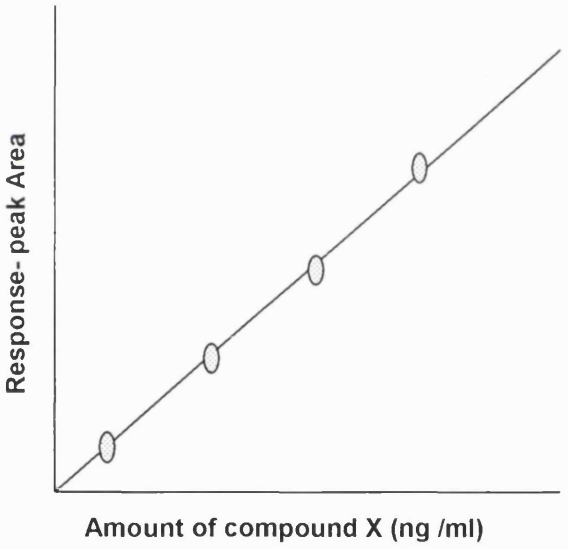
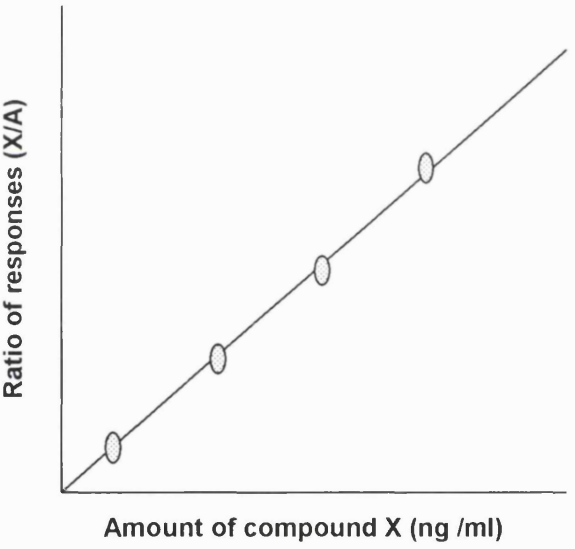
External standard method	Internal standard method
Standard (X) is not added to The sample	Known amount of standard (X) is added to sample prior to Extraction/ derivatisation steps
Calibration curve of quantity Of standard (X) verses Response.	Constant known amount of standard (A) added to various known amount of the compound to be measured (X) Calibration curve of ratio of response (X/A) verses quantity of compound X.
	
Response to compound X in sample related to quantity From calibration curve	Ratio of responses (X/A) in sample Related to quantity from calibrated curve

Fig 2.4 A comparison of quantitation methods using external and Internal standardisation.

added to each sample and the ratio rather than an absolute value is used to quantitate the compound of interest from the calibration curve.

The internal standard (whether a homologue or an isotopically labelled analogue) is added to the sample at the beginning of the procedure. The adoption of this method is also advantageous, since it can account for loss of material in the original sample during the whole procedure from extraction to analysis. In order to achieve the most reliable information, the internal standard should be as similar in structure as possible to the compound of interest.

Trolox is a water-soluble α- tocopherol analogue without the phytyl side-chain, which is structurally similar to α- CEHC. It is routinely used as a standard in both HPLC and GC-MS analysis of vitamin E metabolites. Lodge et al (2000) showed that the recoveries of trolox and α- CEHC standards added to unsupplemented urine were virtually identical. In some procedures deuterated internal standards have been used for quantitative purposes (Parker, Sontag, & Swanson 2000;Swanson J.E., Ben, Burton, & Parker 1999). Deuterated internal standards are ideal since they account precisely for losses in the work up procedure and should behave identically in the mass spectrometer.

Typically, various metabolites of vitamin E have been measured in urine samples ranging in volume from 1 to 10 ml. These previously accounted data have been related to daily levels of vitamin E excretion either by taking 24hr urine samples and/or relating the concentration of metabolites to that of creatinine. Daily creatinine excretion is fairly constant and there is little variation either between days or between subjects. Therefore, it is frequently employed as an index of the urine concentration to

enable approximate daily excretion levels of urinary metabolites to be calculated. 24 hr urine samples are theoretically ideal since they allow the measurement of exact daily excretion values. However, the inconvenience and difficulty in obtaining 24hr urine often outweigh the advantages and thus it was considered to use spot urinary method.

2.1.3.5. Artefactual oxidation

One of the major considerations in the development of a method to measure vitamin E metabolites is the possibility of errors arising from artefactual oxidation. Schultz et al. (1995) demonstrated that α- CEHC can be oxidised to α- tocopheronolactone in the presence of acid and/ or oxygen and proposed that any α- tocopheronolactone observed was likely to be the product of artefactual oxidation of α- CEHC during the extraction procedure. They therefore took extra care to exclude oxygen especially during the deconjugation step, which was performed under argon. This modification eliminated the presence of any detectable amounts of α- tocopheronolactone.

Other intervention to reduce artefactual oxidation utilise derivatization procedures of α- CEHC that prevent its conversion to α- tocopheronolactone. An example of such a derivatization is the use of methanolic HCL to esterify the carboxyl group of α- CEHC while simultaneously deconjugating (Chiku et al. 1984; Schulz et al.1995). The use of d9 CEHC in this study has enabled us to calculate the correction for the artefactual formation.

2.1.4. Identification

The chromatographic peaks for both α-CEHC and α-Tocopheronolactone (α-TL) were successfully identified (in a quality control sample) by the fragment ions, which are specific to each peak in their respective mass spectra. Each peak identified in the chromatogram (Fig 2.5) is discussed below.

Fig 2.5 illustrates that, deuterated and undeuterated α-CEHC were identified at 21.89 and 22.106 min respectively, indicated as A and B in Fig 2.5. The deuterated undeuterated α- TL were recognised at 25.182 and 25.383 min respectively in Fig 2.5 and has been labelled as C and D. The undeuterated and deuterated metabolites were further confirmed according to their characteristic ions found in the mass spectra.

TMS-esters/ ether of α-TL and α-CEHC are isobaric compounds, both showing a large molecule ion of m/z of 422 for the undeuterated metabolite (Fig 2.7) and m/z 431 for the d9-deuterated internal standard (Fig 2.6). All TMS ethers/esters show characteristic fragments of 73 m/z representing a TMS group that breaks between the oxygen and silicon atom. The deuterated α-CEHC as illustrated in Fig 2.6 A was confirmed in the mass spectra by its characteristic fragment ions of 431 m/z and 246 m/z and the deuterated α-TL was differentiated from the acid by its distinguishing fragment of 318 m/z. The undeuterated α-CEHC as illustrated in Fig 2.7 A was confirmed in the mass spectra by its characteristic fragments of 422 m/z and 237 m/z and the undeuterated α-TL was differentiated from α-CEHC by its distinguishing fragment ion of 309 m/z.

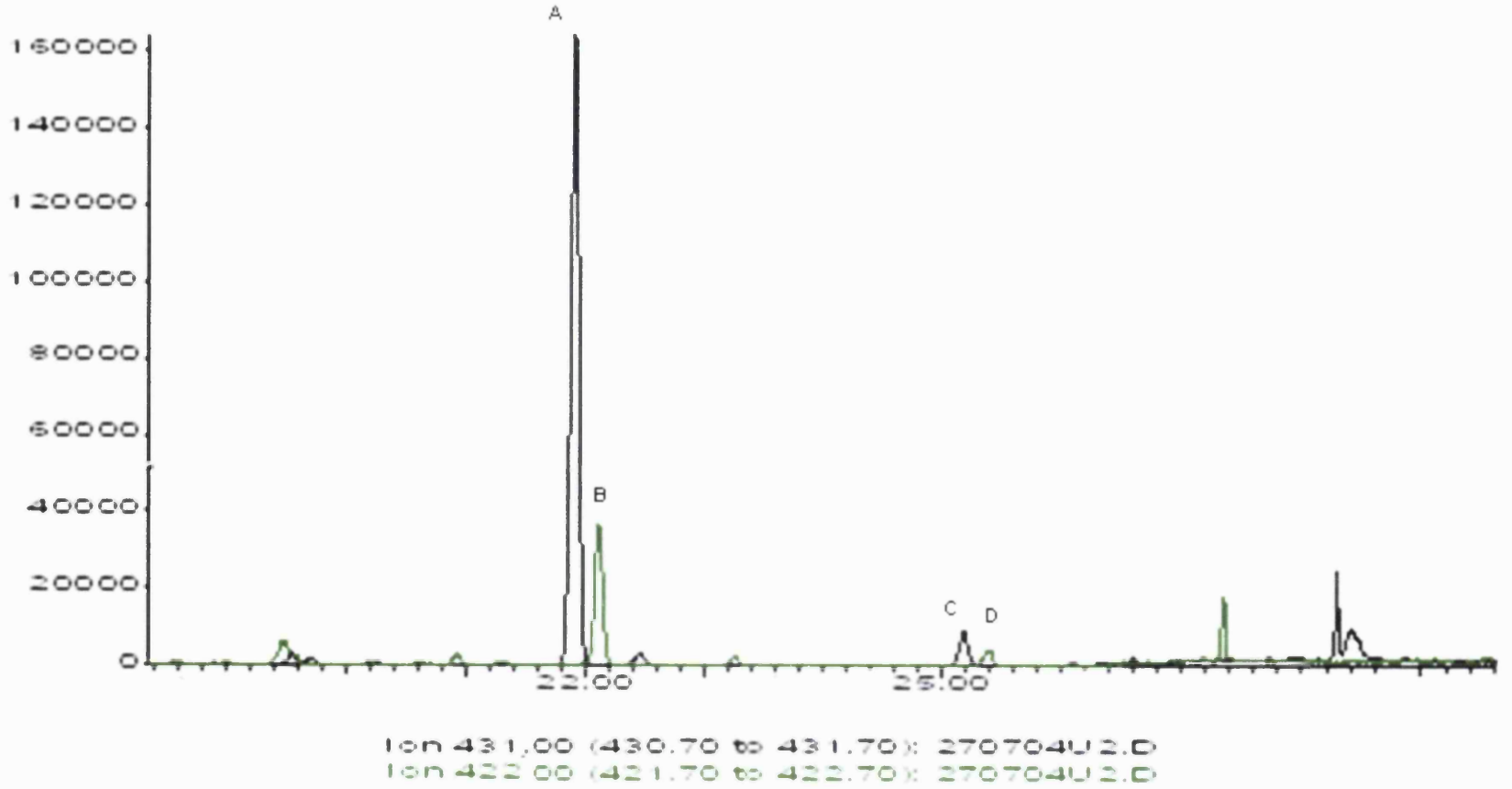


Fig 2.5 The GC-MS of α- CEHC and α- Tocopheronolactone identified as deuterated and undeuterated metabolites with an eluting time of 22 min and 25 min respectively. Peaks A and C represent the deuterated and B and D the undeuterated metabolites of which α- CEHC and α- Tocopheronolactone are represented as A,B and C,D respectively.

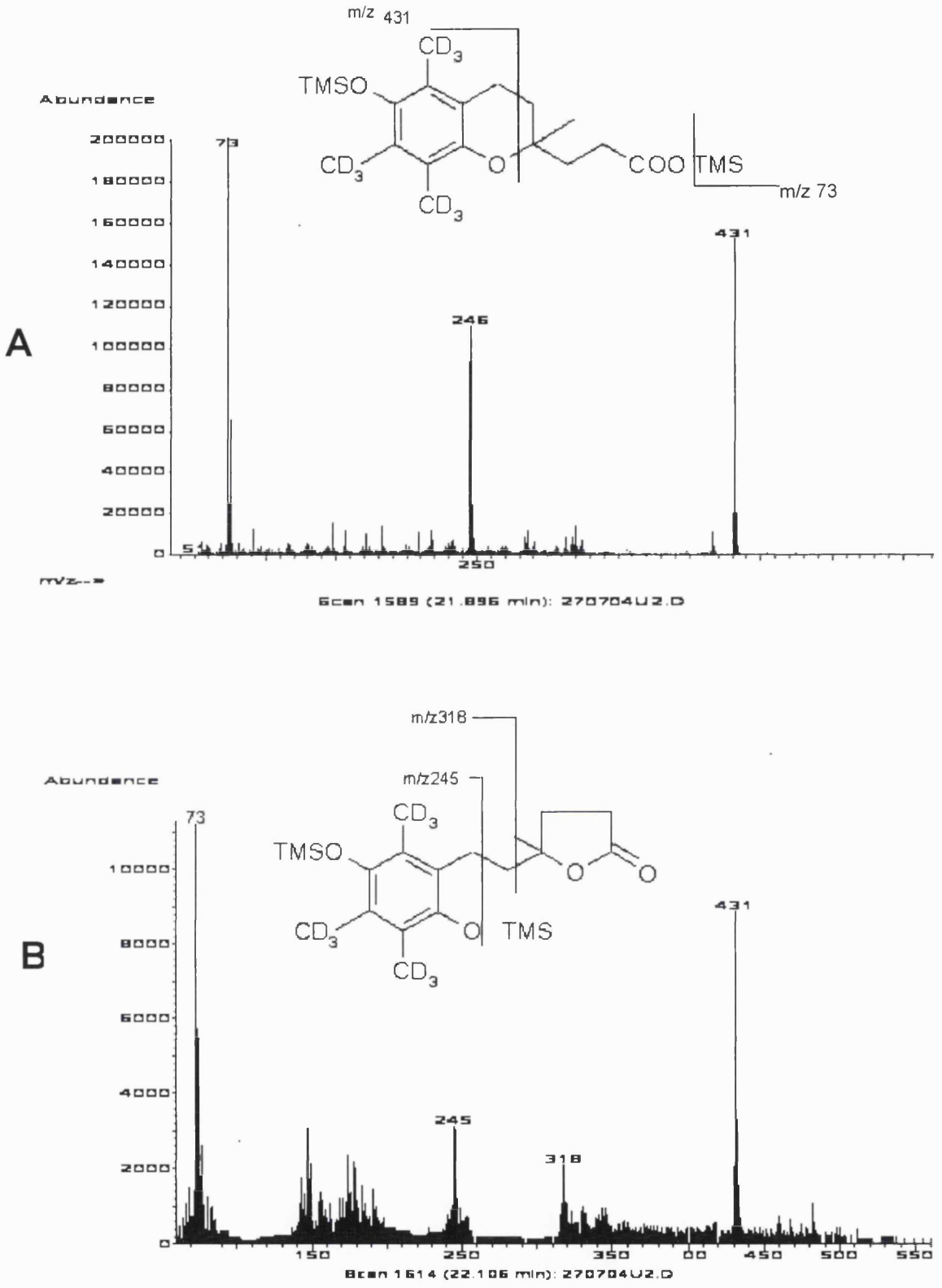


Fig.2.6. A and B represent the mass spectra for the deuterated α-CEHC and α-TL respectively. The α-TL is differentiated from α-CEHC by the characteristic 318m/z ion

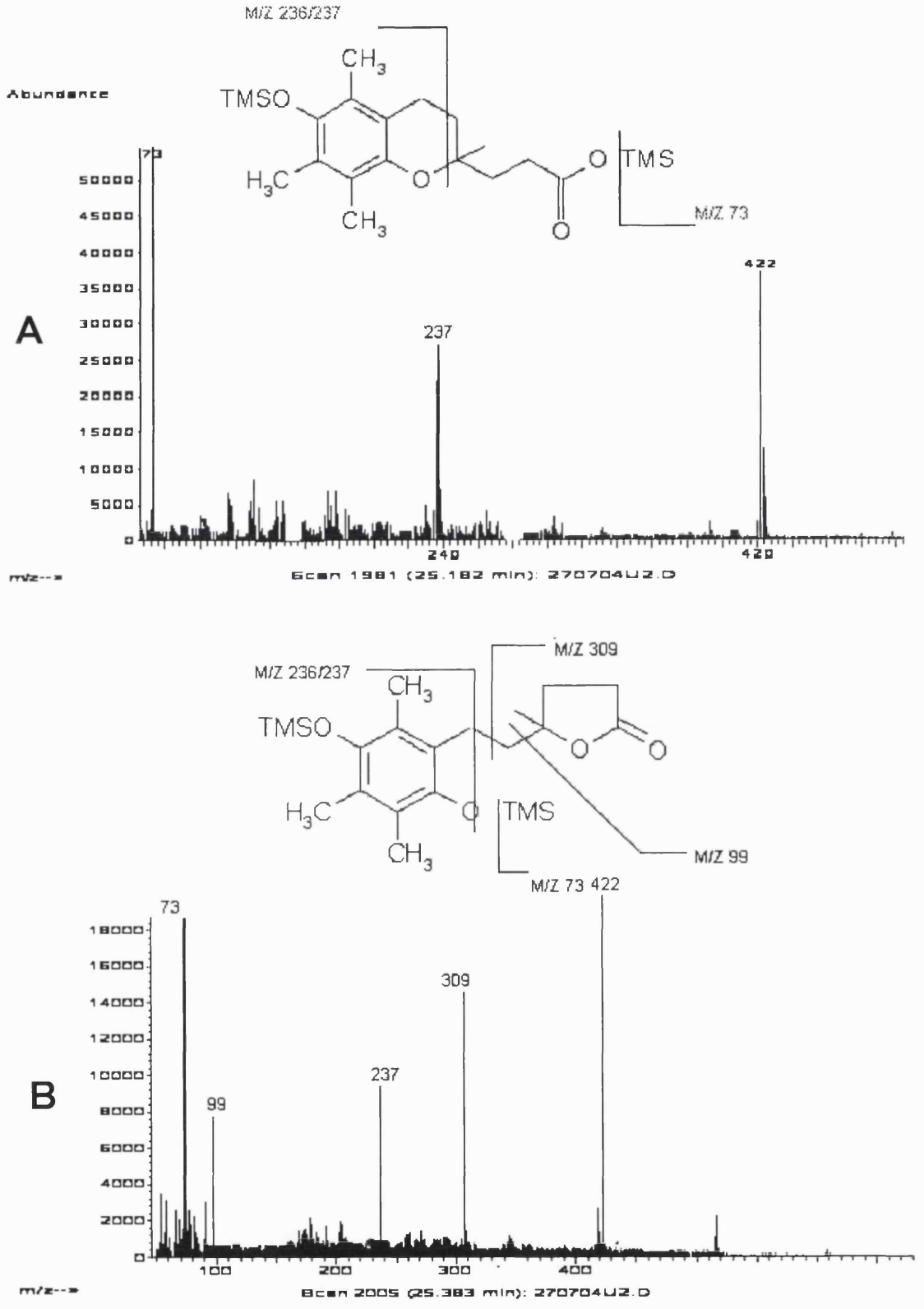


Fig.2.7. A and B represent the mass spectra for the undeuterated α-CEHC and α-TL respectively. The α-TL is differentiated by the characteristic 309 m/z ion.

After identification of these peaks, which corresponded to the peaks and fragments identified previously by Pope et al (2000), it was possible to proceed to the next stage of the validification of the method(Pope, Clayton, & Muller 2000).

2.1.5. Replication studies

Standards (d9-CEHC) of a fixed volume (10 µl) but different concentrations ranging from 0.1 to 20 µM (where 0.1 µM was the first and 20 µM was the eighth injections) were dervitised injected onto the GC-MS. The area obtained for each concentration form the chromatogram was plotted against their respective concentration in the graph Fig 2.8. As can be observed there was a direct increase in the area obtained with an increase in the standard concentration, but the method was not sufficiently sensitive to quantitate concentrations less than 5 µM. The gradual increase of the area in relation to the increase in the standard concentration gave confirmation to the reproducibility and validity of the method.

A pilot study consisting of five quality control (QC) samples (made from the same urine pool) was conducted. As observed in Fig 2.9 the concentrations of both the α-TL and α-CEHC in their deuterated and undeuterated forms were similar in all five samples, with a mean ± ISD of 9.23 ± 0.19, 0.76 ± 0.19, 2.69 ± 0.27 and 0.65 ± .69 for the deuterated and undeuterated α-CEHC and α-TL metabolites respectively. It was also noted that the 5th QC sample had a higher concentration of the lactone (α-TL) in both the d_9 standard and the metabolite which could have been caused by artefactual oxidation. It was then concluded after this test that the methodology used was consistent and confirmed its reproducibility.

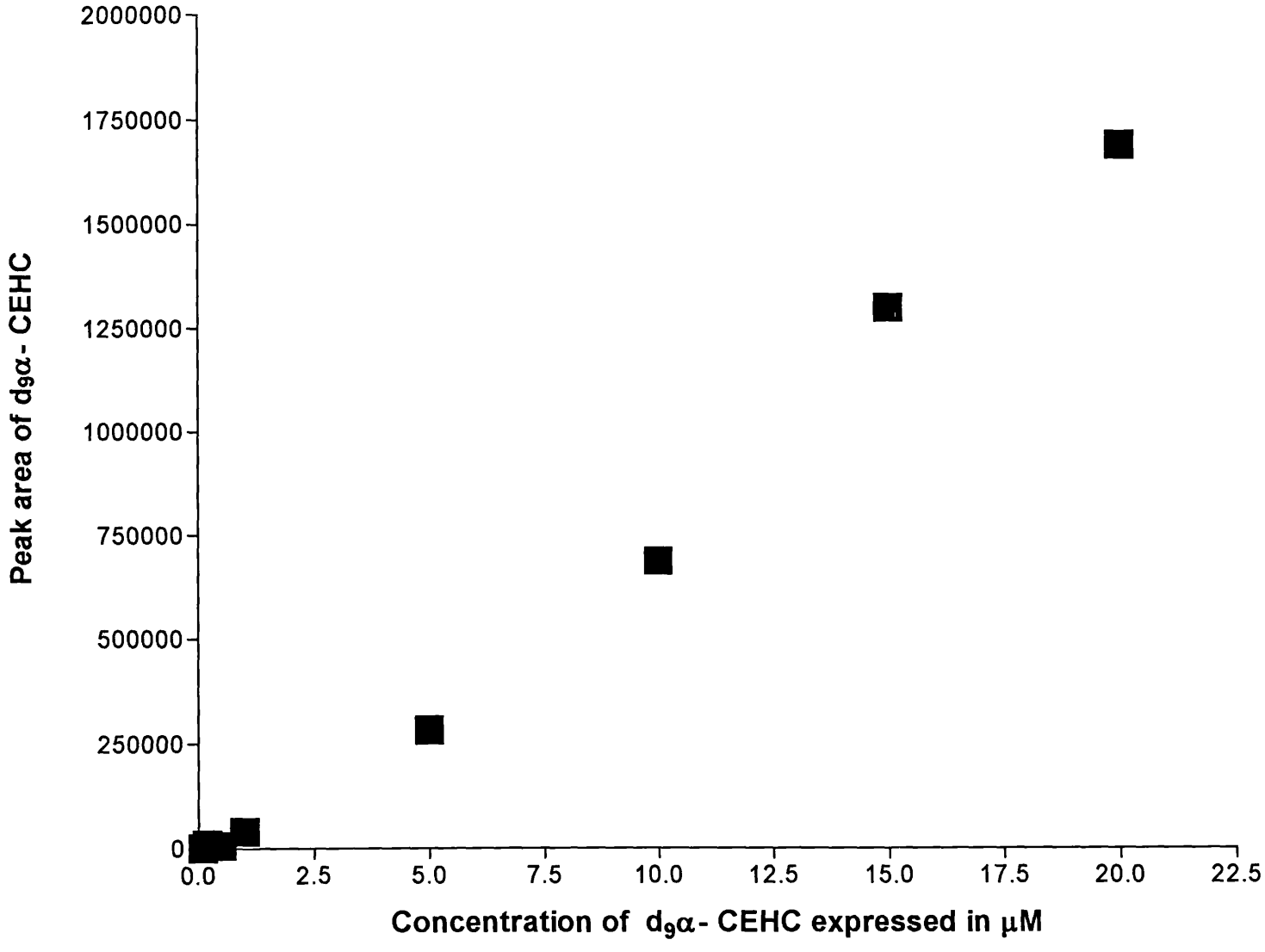


Fig 2.8 Standard curve for d_9 α-CEHC

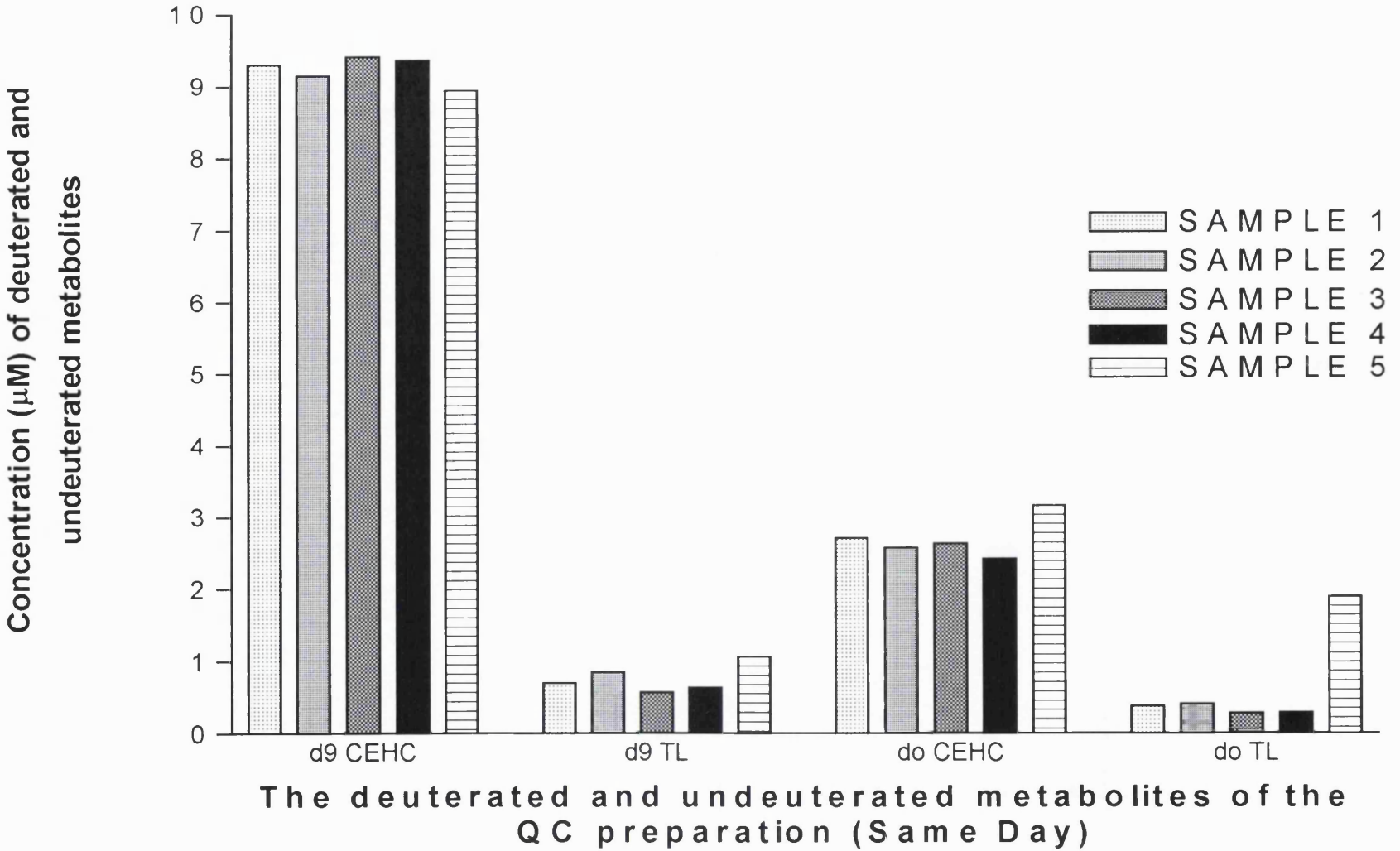


Fig. 2.9 Replication studies of Vitamin E metabolites

2.1.6. Trouble Shooting

Following the validification studies described above very poor chromatograms were obtained after the first run of the samples. In order to trouble shoot the problem, multiple injections of a single standard of $d_9\alpha$-CEHC (derivatisation only) were conducted to rule out a fault on the machine and the standards. With the exception of the first injections reproducible results were obtained as shown in Table 2.2 A. The first injection of a run tends to give problems and is always repeated. The problem with the patients' sample was proposed to have arisen due to faulty extraction cartridges, as the extraction time taken was observed to be extremely long. A fresh batch of cartridges was then used and four samples were analysed. The four samples analysed were a control and a QC which were subjected to the entire extraction procedure (i.e. two SPE cartridges) and a QC and a water sample spiked with $d_9\alpha$-CEHC which were analysed after extraction (with only one SPE cartridge). All the samples were spiked with 10 μM of $d_9\alpha$-CEHC and derivatized. Apart from the control urine sample where the peak area obtained for $d_9\alpha$-CEHC was considerably lower, the results obtained were found to be consistent with the 10 μM value of the standard curve (Fig 2.8) and it was then decided to run the rest of the samples.

A

	Retention Time	Peak Area d_9CEHC
Standard 1	21.87	156494
Standard 2	21.87	339635
Standard 3	21.87	298947
Standard 4	21.87	298420
Standard 5	21.87	298716

B

Urine/water	Retention Time	Peak Area d_9CEHC
Control (entire extraction)	21.96	3448824
QC (entire extraction)	21.91	7014446
QC (half extraction)	21.91	7020711
Spiked water (half extraction)	21.86	7902071

Table 2.2 Table A and B indicate the reproducibility of the Standards and the extraction method respectively

2.2.Urinary 8-isoPGF$_{2\alpha}$ – Extraction and Analysis

2.2.1.Materials

Cayman Chemicals supplied 8-isoprostane standards and deuterated 8-isoProstane $F_{2\alpha}$ (CAY-16350) and 8-isoProstane $F_{2\alpha}$-d4 (CAY-316350). These standards were made up into stock solutions of 20ng/ml in methanol and stored at –20°C. In this state they are reported by the manufacturer to be stable for up to six months. The TMS derivitizing agent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Chemicals Ltd. Affinity sorbents supplied by Cayman Chemicals. All other chemicals were purchased from Sigma-Aldrich unless otherwise mentioned.

2.2.2. Method Preparation

The overview of the method is shown in Fig 2.10 and will be briefly described. The basis of the method will then be discussed.

2.2.2.1. Preparation of urine samples

- Pool fresh urine, divide in to 10ml glass vials
- Centrifuge at 1000g for 5 minutes to remove 4ml of particulate matter
- Spike urine aliquots (4ml) with d4-8-isoPGF2α standard
- Vortex all samples for 30 seconds.

2.2.2.2. Sorbent Preparation

- Suspend sorbent in column buffer, by gently pipetting and expelling to make the sample homogenous

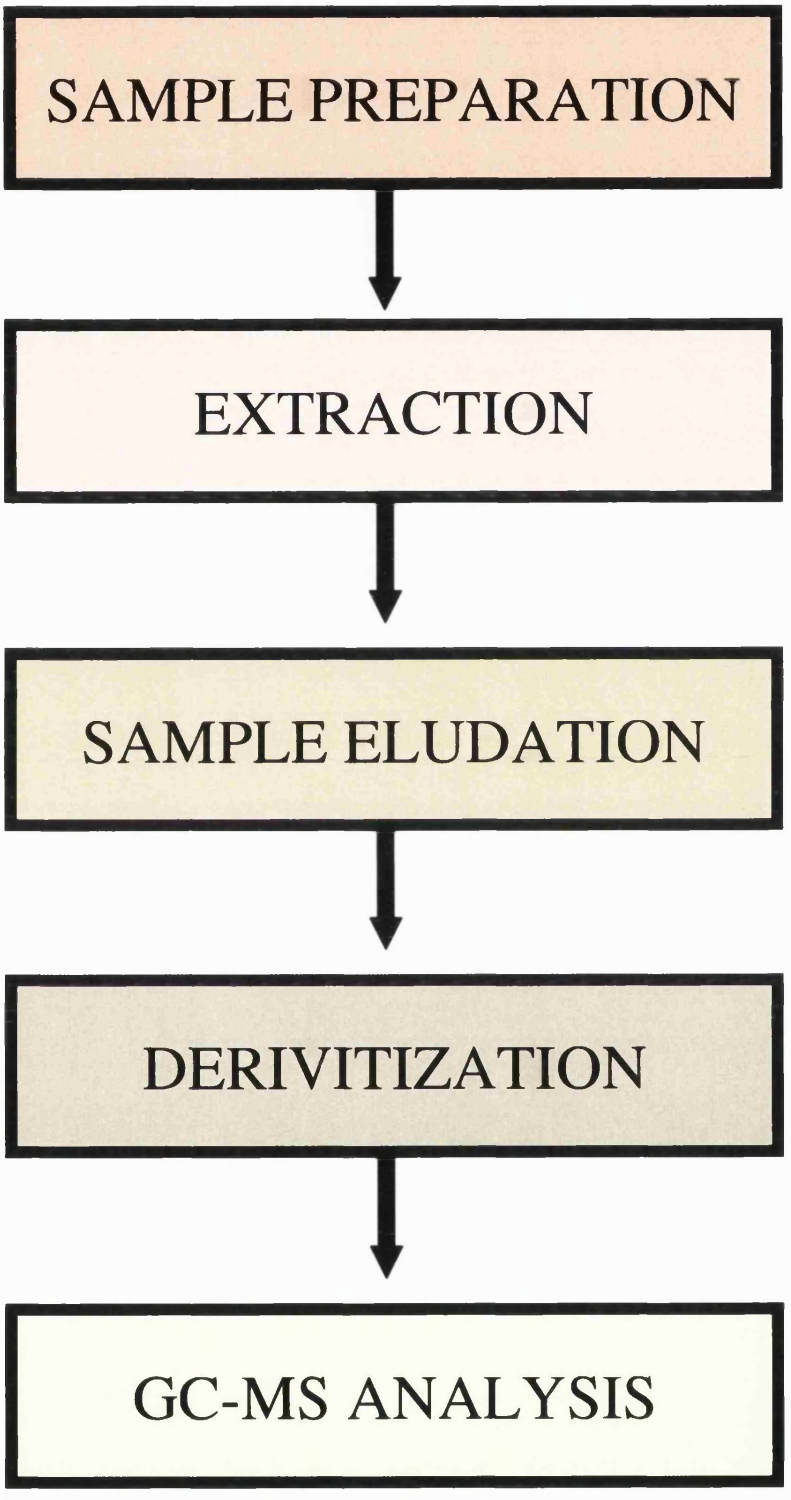


Fig. 2.10. Overview of the Isoprostane Methodology

- Aliquot all 2ml sorbent suspension to urine sample
- Mix well by inverting, rotating for 90 minutes.

2.2.2.3. Extraction

- Centrifuge at 2,000g for 5 minutes, to sediment the sorbent
- Remove supernatant (devoid of 8-isoPGF2α)
- Wash sorbent with 5ml column buffer (0.1M phosphate buffer)
- Vortex for 30 seconds
- Centrifuge at 2,000g for 5minutes, to sediment the sorbent
- Remove supernatant (devoid of 8-isoPGF2α) and repeat
- Repeat wash procedure with 0.1M phosphate buffer.

- Wash sorbent with 5ml of ultrapure distilled water
- Vortex for 30 seconds
- Centrifuge at 2,000g for 5mins, to sediment sorbent
- Remove supernatant, repeat twice (supernatant devoid of 8-isoPGF2α)

- Resuspend sorbent pellet in 2.5ml of elution solution (95% EtOH)
- Vortex for 30 seconds
- Centrifuge at 2,000g for 5mins, to sediment sorbent
- Remove EtOH phase, carefully (so as not to disturb sorbent)
- Collect in 10ml glass tube
- Repeat twice

2.2.2.4. Derivatization of eluate

- Evaporate EtOH phase to dryness (NOTE; when approximately 1 ml remained this was transferred to a derivitizing vial before completing evaporation)
- Add derivitizing agent, 60µl Acetonitrile/ BSTFA TCMS(1:1)
- Incubate at 45^0C for 90 minutes.

2.2.2.5. Run sequence on GC-MS in both scan and SIM acquisition modes

Analysis was performed in electron impact mode at 70 eV, using a Hewlett Packard GC-MS system (HP-5973 mass selective detector, HP 6890 series GC instrument with an automatic liquid sampler, and HP-Chem station for data acquisition).). The initial oven temperature of 130°C was maintained for 1 minute, then increased to 300°C at 18°C/min, which was then held for 2minutes. The oven temperature was then increased to 310°C at 4°C/min and maintained at this temperature for 5 minutes to purge the column. The injector system was run in splitless mode. The carrier gas, helium, was kept at a constant flow rate of 1ml/min. GC-MS temperatures were set as follows; injector at 250°C, interface at 300°C, source at 220°C and the quadrupole at 100 °C. The run length was set to 19.94 min, the injection temperature being 250°C with a flow rate of 54.5 ml/min and the pressure maintained at 15.4 psi.

2.2.3. Basis of the Method

2.2.3.1. Extraction

An extraction method was required to enable an efficient measurement of both deuterated and undeuterated forms of 8-iso-PGF2α from the urine. The extraction was carried out using affinity sorbents supplied by Cayman Chemicals for the purification of 8-isoPGF2α from urine, which consists of mouse anti-8-isoprostanes covalently bound to Sepharose 4B. The manufacturer reported the specificity of the sorbents to be 100% for 8-isoPGF2α and the binding capacity per millilitre of sorbent as 10ng. In order to extract a sufficient level of endogenous 8-isoPGF2α along with a deuterated internal standard to enable detection by GC-MS, while not exceeding the sorbent binding capacity, 4ml of urine was used. The Isoprostanes were extracted using the sorbent in a batch procedure. This approach was used to make maximum utilization of the sorbent's specificity. During the extraction procedure it was necessary to wash the sorbent with water before elution as the phosphates in the buffer have shown to hinder the derivatization step.

2.2.3.2. Analysis

The analysis of the isoprostanes by GC-MS, was carried out by a modification of the method of Bessard et al (2001). The standards were derivatized to their trimethyl-silyl derivatives using 60μl BSTFA (+1%TMCS). Bessard's method involved no incubation for the derivatization process suggesting immediate derivatization.

Derivatisation conditions were adopted in this study and conducted at 45°C for 90 minutes to ensure derivatization reached completion.

The mass spectrometer (MS) was programmed to run in both complete scan mode and in selected ion-monitoring (SIM) mode. For the selected ion-monitoring mode the principal fragment ion were selected for identification. The selected fragments of d0-8-isoPGF$_{2\alpha}$ were 571 m/z and 485 m/z. Quantification was achieved by the peak height of the highest abundance mass ions for both undeuterated and deuterated 8-isoPGF$_{2\alpha}$, which were the ions 481m/z and 485m/z respectively in the selected ion mode (SIM). Peak heights were used to quantify 8-isoPGF$_{2\alpha}$ to reduce interference of other species with similar retention times, for example prostaglandin F$_{2\alpha}$ described by Bessard et al (2001), and other species that may also be extracted. The chromatographic peaks of both undeuterated and deuterated 8-isoPGF$_{2\alpha}$ standards were successfully identified according to their charecteristic fragmentation patterns.

2.2.4. Identification

The chromatographic peaks for both the undeuterated and deuterated 8-isoPGF2α standards were identified as illustrated in Fig 2.11 at 10.5 min. The deuterated (485 m/z) and undeuterated 8-isoPGF2α (481 m/z) were differentiated according to their characteristic fragmentation patterns as shown in Fig. 2.12. The undeuterated 8-isoPGF2α (B) had characteristic peaks at 391, 462, 537, 552 and 571 m/z with the deuterated compound (A) having ions with masses + 4.

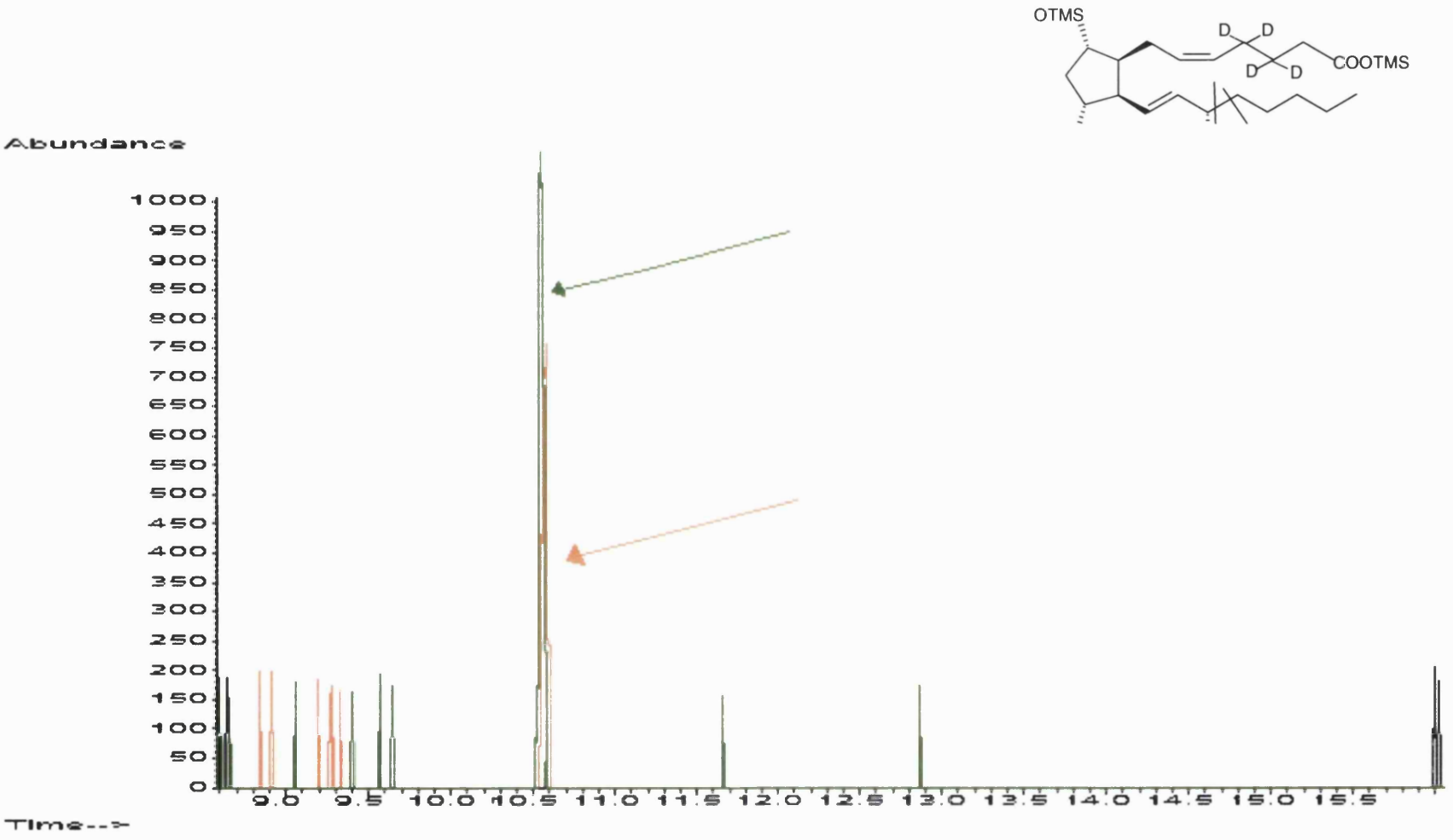
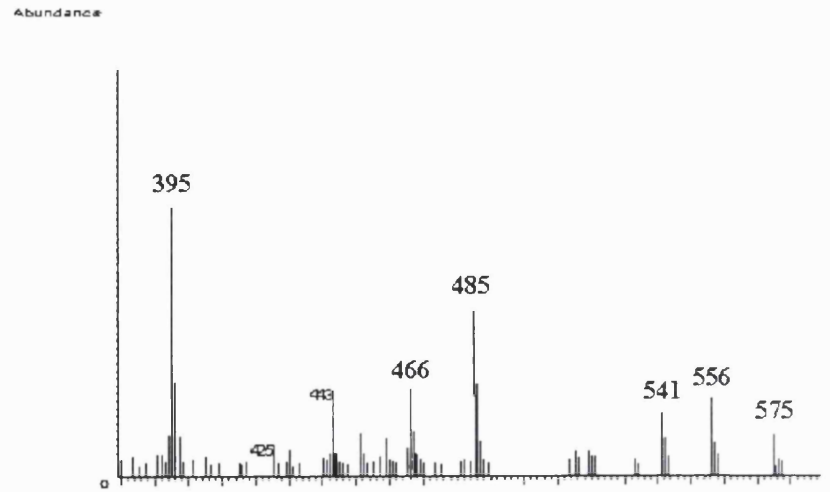
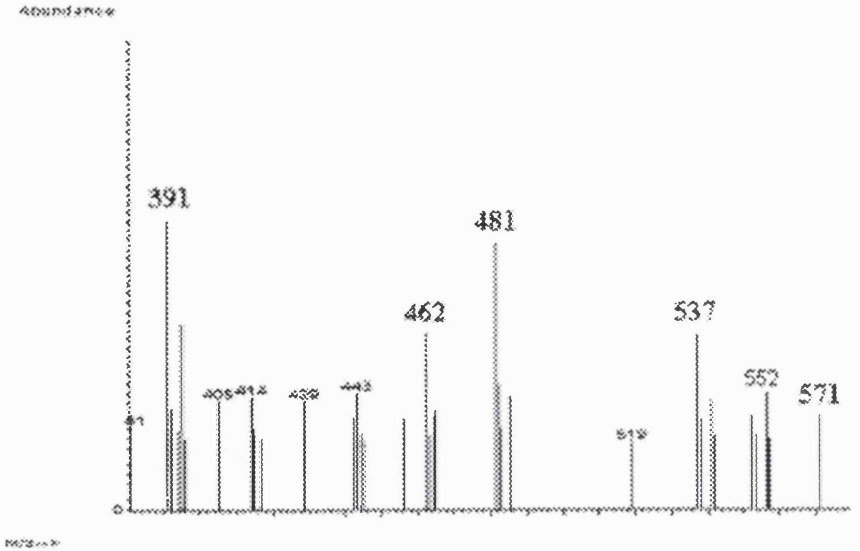


Fig 2.11 Extracted ion chromatogram of d0- and d4- 8-isoPGF2a



d4-8-isoPGF Fragment ions:	m/z
[M-C5H11-2TMSOH]+	:395
[M-2TMSOH]+	:466
[M-C5H11-TMSOH]+	:485
[M-TMSOH-CH3]+	:541
[M-TMSOH]+	:556
[M-C5H11]+	:575
[M-CH3]+	:631
M+	:646



d0-8-isoPGF Fragment ions:	m/z
[M-C5H11-2TMSOH]+	:391
[M-2TMSOH]+	:462
[M-C5H11-TMSOH]+	:481
[M-TMSOH-CH3]+	:537
[M-TMSOH]+	:552
[M-C5H11]+	:571
[M-CH3]+	:627
M+	:642

Fig 2.12 A and B represent the mass spectra for deuterated and undeuted 8-isoPGF2a

2.2.5. Replication Studies

Standards (8-isoPGF$_{2\alpha}$) of a fixed volume (100 µl) but of different concentrations ranging from 50 to 50000 pg/ml were derivitized and injected onto the GC-MS. The peak height obtained for each concentration was plotted against their respective concentration as shown in the Fig 2.13. As can be observed there was an increase in the area obtained with an increase in the standard concentration, but the method was not sufficiently sensitive to quantitate concentrations less than 500 pg/ml. The gradual increase of the area in relation to the increase in the standard concentration gave confirmation to the reproducibility and validity of the method.

A pilot study, consisting of four quality control (QC) samples (made from the same urine pool) was conducted after the first test. As observed in Fig 2.14 the concentrations of 8-isoPGF2α in its deuterated and undeuterated forms were shown to be similar with a mean ± ISD of 24.75 ± 8.62 and 28.75 ± 8.12 of the deuterated (485) and undeuterated (481) metabolites respectively. The reproducibility in this experiment was not as good as hoped for but was considered adequate for the proposed studies.

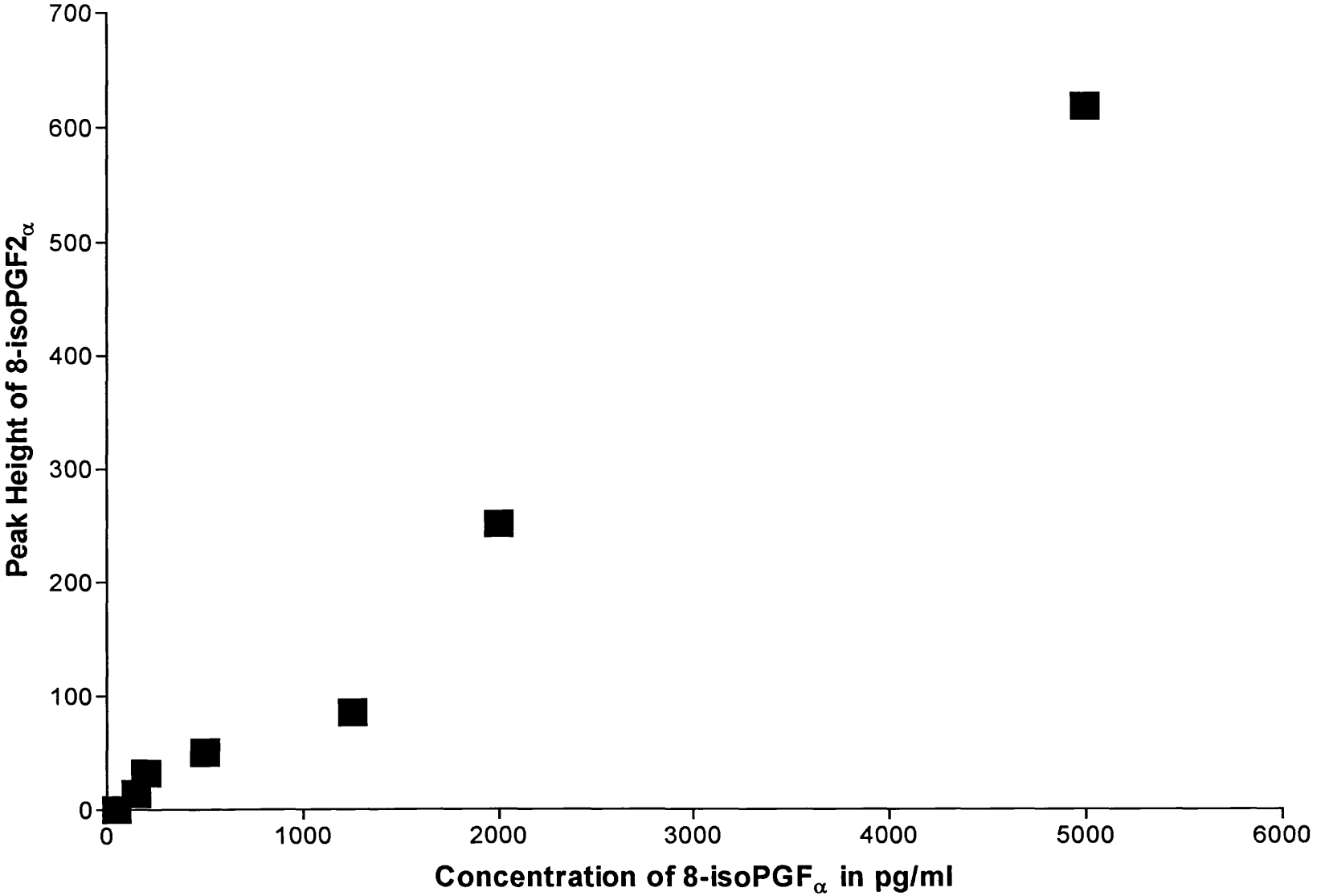


Fig. 2.13 Standard curve of 8-isoPGF2α standard

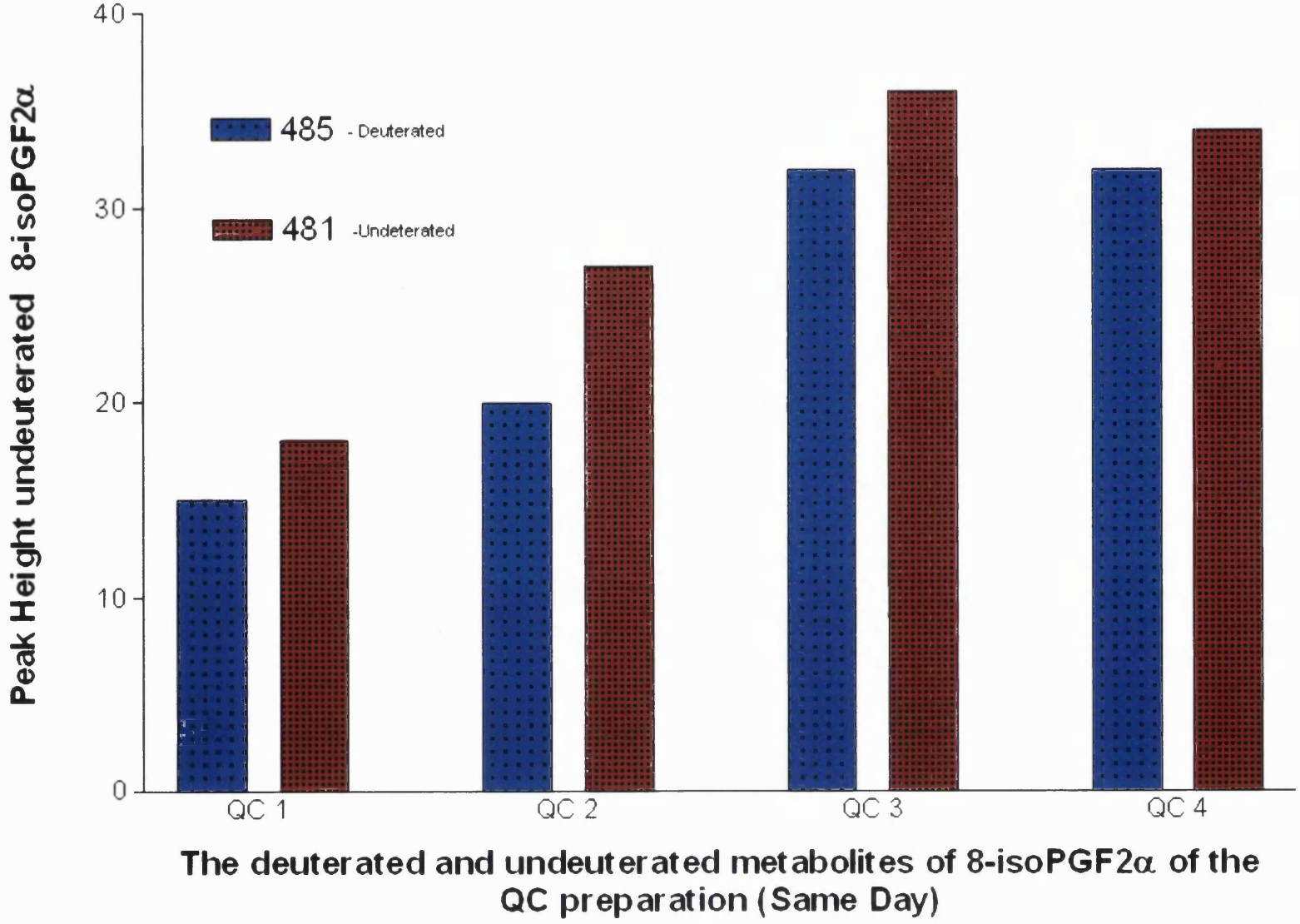


Fig. 2. 14 Replication studies of 8-isoPGF2α

2.2.6. Trouble Shooting

As for the vitamin E metabolites there were problems with the first run of patient samples. In order to rule out problems with the GC-MS, duplicate injections of a single standard were injected onto the GC-MS. The results obtained, are shown in Table 2.3 A and proved to be almost identical and consistent with that obtained in the standard curve of the same concentration. The next step taken was to perform a QC analysis. Here again duplicate injections of the same QC sample were made onto the GC-MS. The result obtained proved to be very similar to each other as shown in Table 2.3 B and was also similar to the results obtained in the replication study. The reason why the earlier run did not work is not known but may be due to the sorbents used.

Urinary concentrations of the vitamin E metabolites and isoprostanes were expressed per mmol creatinine measured on a Cobas Fara using kits supplied by Cayman Chemicals.

A

Standard observed in Sim mode	Retention Time	Peak Height 8-isoPGF2α
Standard 1	10.58	50
Standard 2	10.59	51

B

QC observed in Sim mode	Retention Time	Peak Height 8-isoPGF2α
QC 1	10.58	29
QC 2	10.59	31

Table 2.3. Tables A and B indicate the reproducibility of the standards and the extraction method respectively.

RESULTS

3. Results

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3. Results

The methods described in chapter 2 for the measurement of the vitamin E metabolites and 8-isoPGF2α was used in the analysis of urine from patients with sepsis and controls.

3.1. Analysis of 8-isoPGF$_{2\alpha}$ in the urinary samples of control and sepsis patient subjects

Fig 3.1 shows the urinary α-CEHC concentration in the controls and patients with sepsis. The control subjects indicated had lower concentrations of 8isoPGF$_{2\alpha}$ (range varying from 156.9 to 1628.41 pg/mg creatinine) as compared to the patients (ranging from 204.54 to 4627 pg/mg creatinine) with mean concentrations of 917.1 ± 238.6 and 2521 ± 442.1 pg/mg of creatinine in the two groups respectively with a significant P value of 0.013.

3.2. Analysis of α-Tocopheronolactone in the urinary samples of control and sepsis patient

The figure 3.2 shows the urinary α- tocopheronolactone concentrations in the control and patients with sepsis. The figure clearly illustrates the difference in the α-tocopheronolactone range obtained for the control and sepsis patient subjects. The control subjects' range varied from 8.9 to 59.6 pmol/mmol creatinine, with a mean value of 29.29 ± 8.32 pmol/mmol creatinine. The sepsis patients' urinary α-tocopheronolactone concentration was noted to be highly variable with a range from 12 nmol/mmol of creatinine to 212.22 pmol/mmol of creatinine with a mean of 274 ±

148.1. The P values were not found to significant in an unpaired t test with P value of 0.1368 ($P > 0.05$).

3. 3. Analysis of α-CEHC in the urinary samples of control and sepsis patient subjects

Fig 3.3 shows the urinary α-CEHC concentrations in the controls and patients with sepsis. The concentrations in the controls were observed to be similar with a mean ± ISD of 230.8 ± 28.78 pmol/mmol. The concentration of α-CEHC in patients' urine was noted to be highly variable ranging from, 204.54 nmol/mmol to 2826 nmol/mmol with a mean of 1940 ± 798.7. The means were not found to be significant in an unpaired t test with $P > 0.05$ (P value 0.0649).

After conducting preliminary statistical analysis using the mean, standard deviation and unpaired t test in the data obtained, the next step taken was to conduct correlation tests between the variables wherein the concentrations of the different metabolites were plotted against each other and also against the age of the subjects. The results of the correlation studies are as follows.

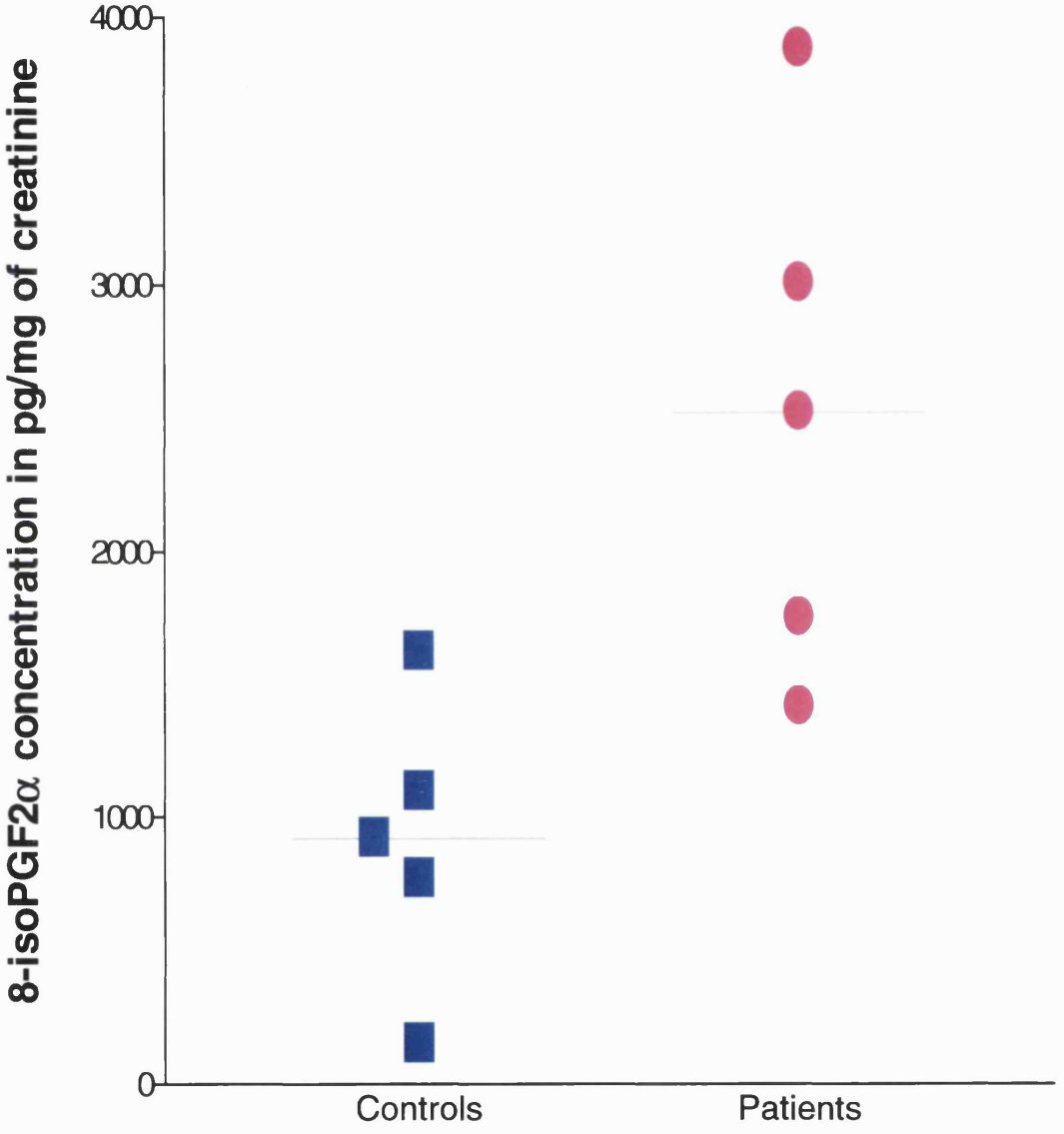

Fig. 3.1 Urinary 8-isoPGF2α concentration in control and sepsis patients.

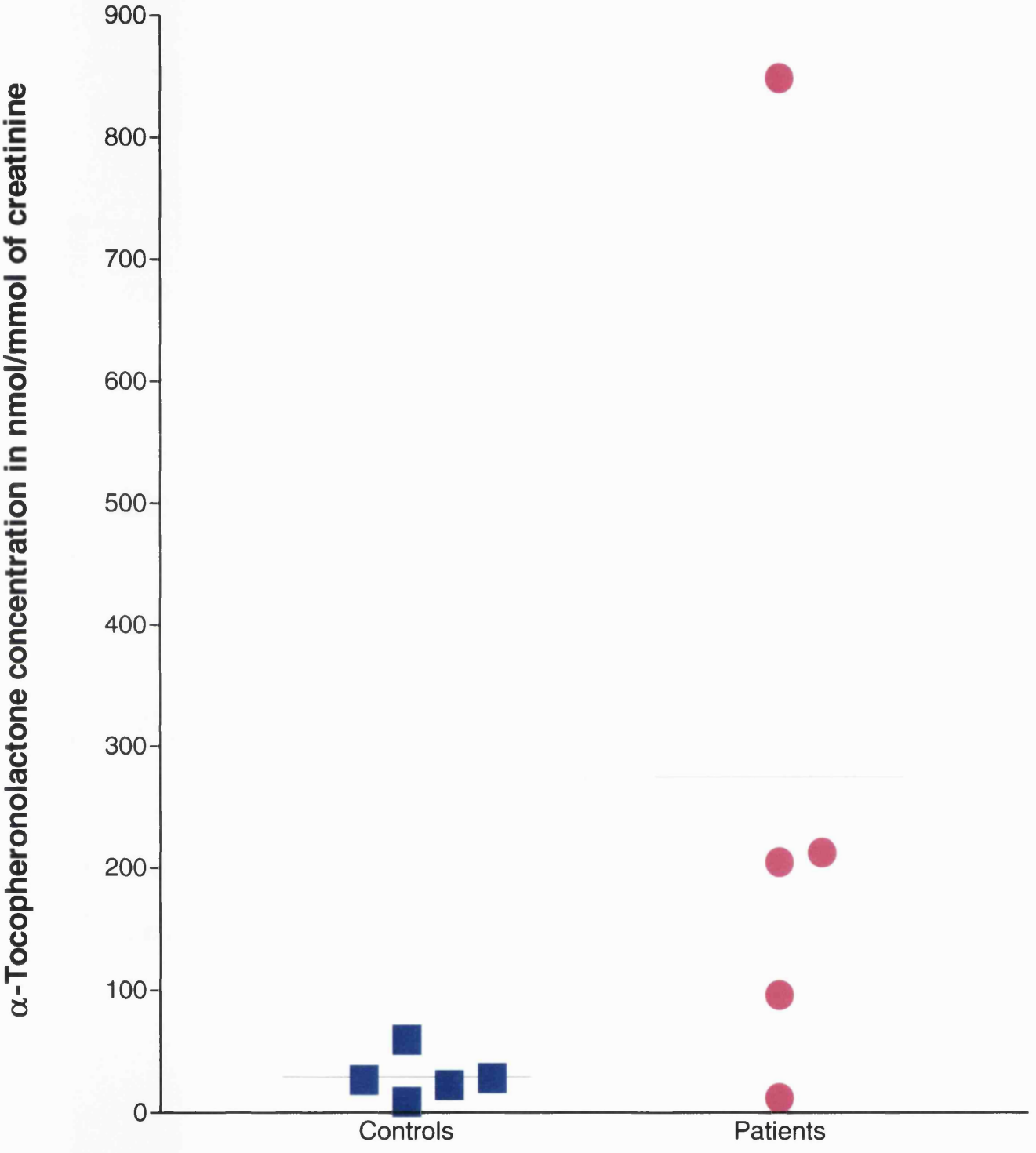

Fig. 3.2 Urinary α-Tocopheronolactone concentration in control and sepsis patients.

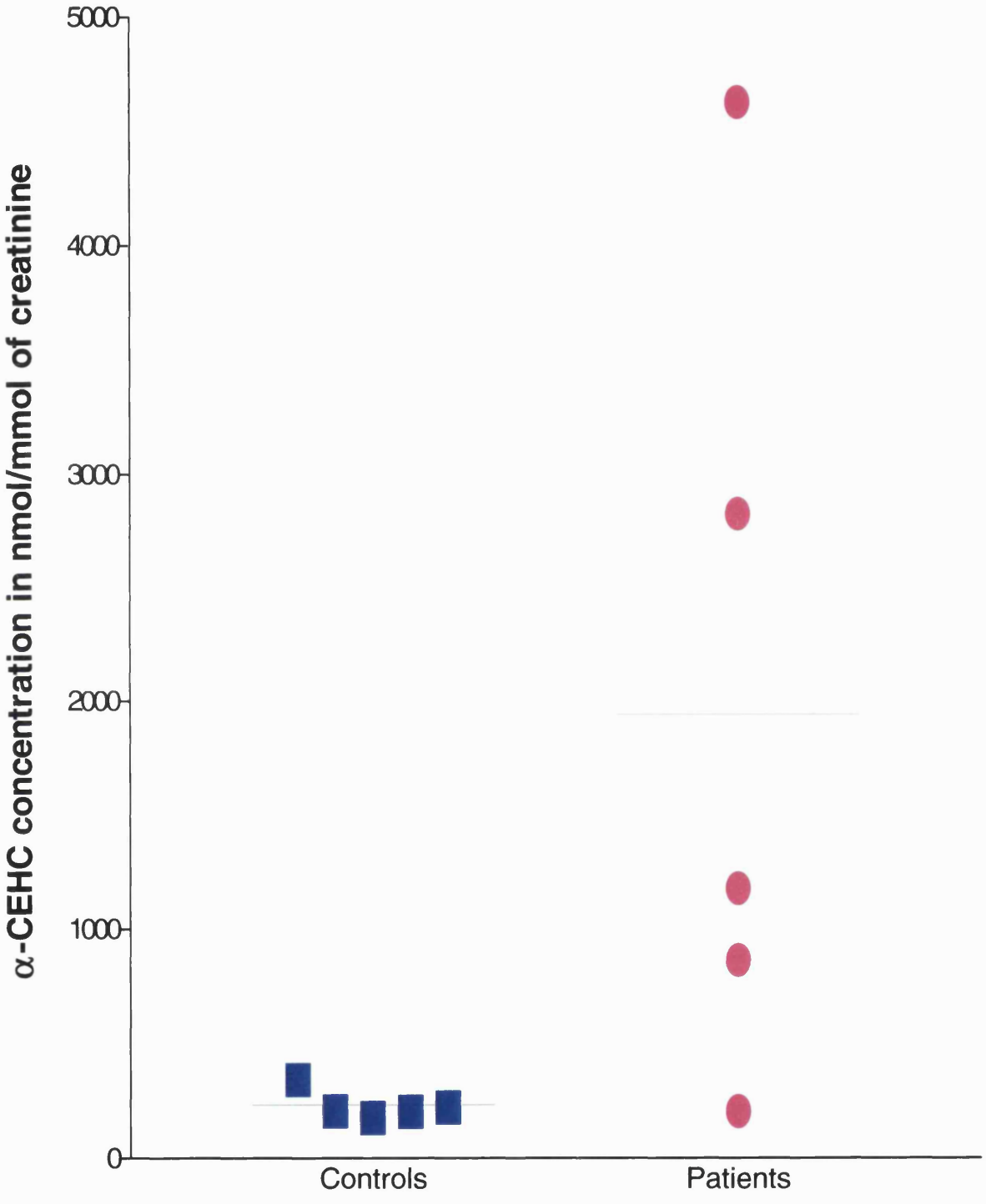

Fig. 3.3 Urinary α-CEHC concentration in control and sepsis patients

3.4. Correlation studies of the concentrations of the metabolites

3.4.1. Concentrations of 8-isoPGF$_{2\alpha}$ expressed in terms of α-Tocopheronolactone

Fig 3.4 shows the correlation of urinary concentrations of 8-isoPGF$_{2\alpha}$ and α-tocopheronolactone in controls (A) and sepsis patients (B), whereas Fig 3.5 illustrates the correlation of urinary concentrations of 8-isoPGF2α and α-tocopheronolactone in the entire population. Figures 3.4 A & B and Fig.3.5 clearly indicate that there was no correlation between the concentrations of 8-isoPGF$_{2\alpha}$ and α-tocopheronolactone in the controls', patients' or the entire sample urine sample where the correlation coefficient (r) was - 0.20, – 0.003 and 0.4 for the controls', sepsis patients' or the entire sample respectively and the p value obtained was found to be not significant in all the cases ($P > 0.05$).

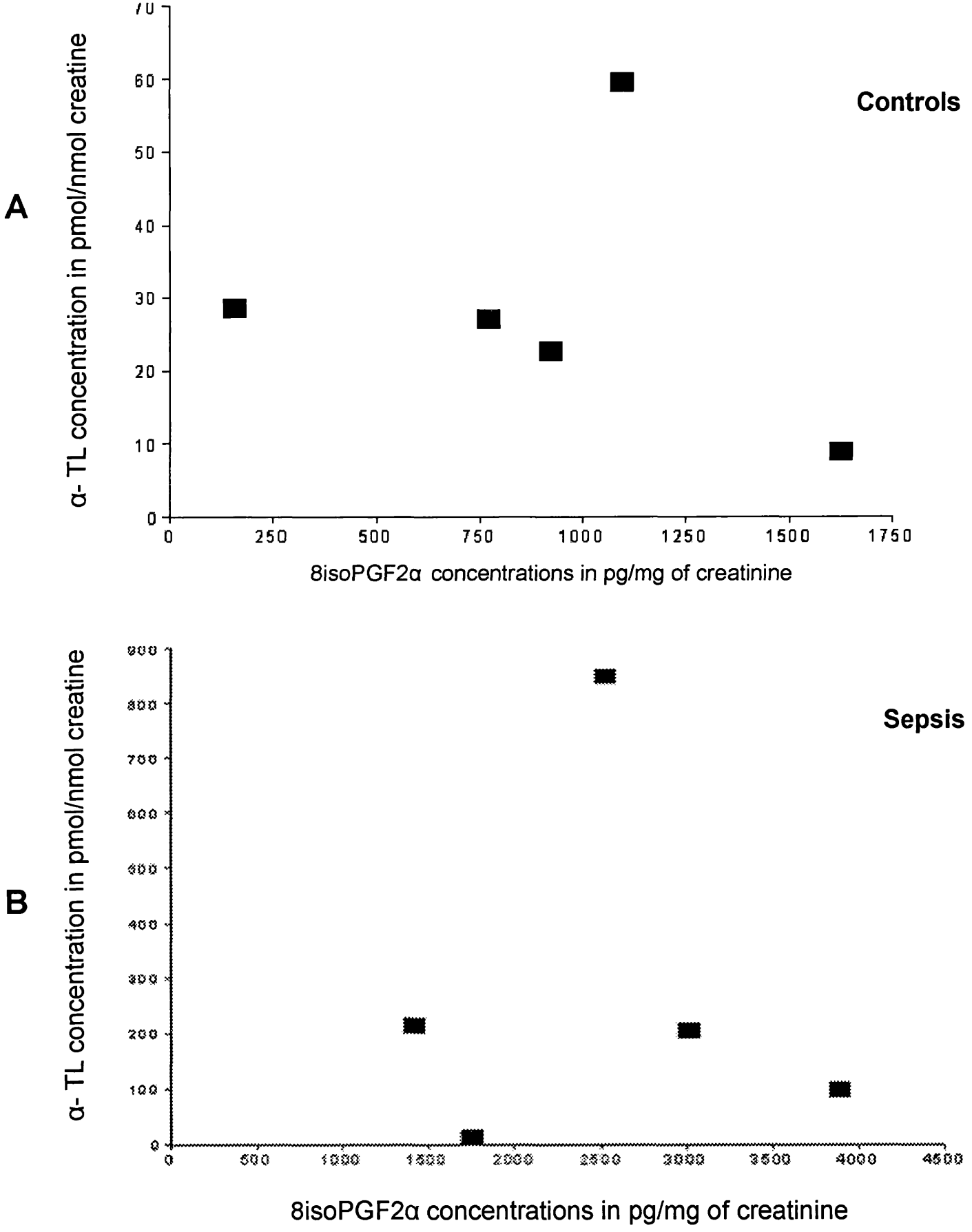

Fig.3.4 Correlation of urinary concentrations of 8isoPGF2α and α- TL in Controls (A) and Patients (B)

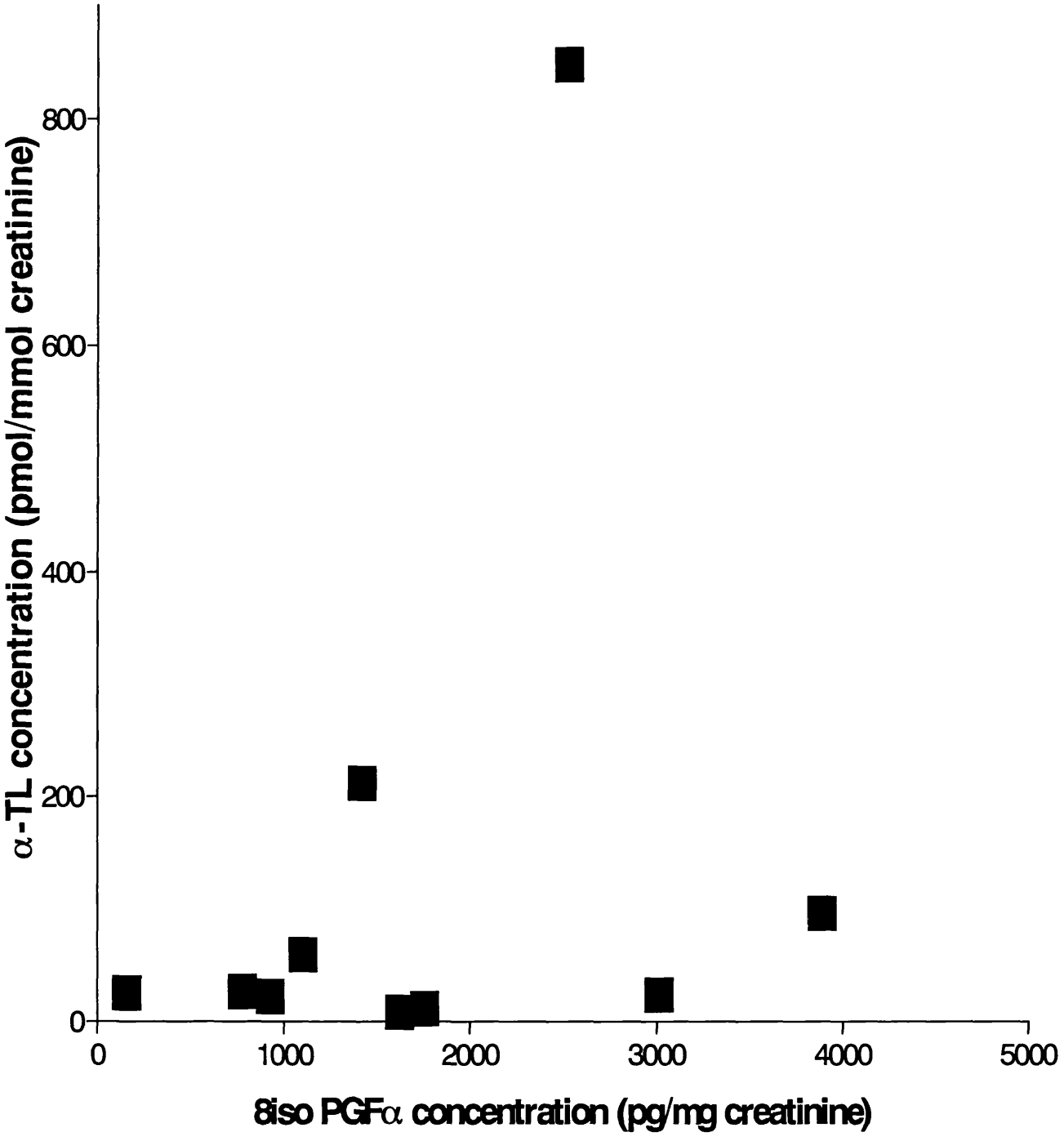

Fig 3.5. Correlation of urinary concentrations of 8isoPGF. and . -TL in control and sepsis patients

3.4.2. Concentrations of 8-isoPGF$_{2\alpha}$ expressed in terms of Age (months)

The Fig. 3.6 illustrates the correlation of urinary 8-isoPGF$_{2\alpha}$ and age in controls (A) and sepsis patients (B). The concentration of 8-isoPGF$_{2\alpha}$, when plotted against the age of the control and sepsis patients samples as illustrated in Fig 3.6 A and B; it was observed that there was no significant correlation ($P > 0.05$). The r value was expressed as 0.24 and -0.004

3.4.3. Concentrations of α-Tocopheronolactone expressed in terms of Age (months)

The Fig 3.7 illustrates the correlation of urinary α-tocopheronolactone and age in controls (A) and sepsis patients (B). Fig 3.7 A and B shows that when the concentrations of α-tocopheronolactone was plotted against the age of the control and sepsis patients samples, no significant correlation was observed ($P > 0.05$). The r value was 0.24 and 0.24 in control and sepsis patient's samples respectively.

3.4.4. Concentrations of α-CEHC expressed in terms of Age (months)

Fig 3.8 illustrates the correlation of urinary α-CEHC and age in controls (A) and sepsis patients (B). Fig.3.8 A and B illustrates that when the concentrations of α-CEHC was plotted against the age of the control and sepsis patients, there was no significant correlation ($P > 0.05$). The r value was -0.64 for the control and -0.29 for the sepsis patients.

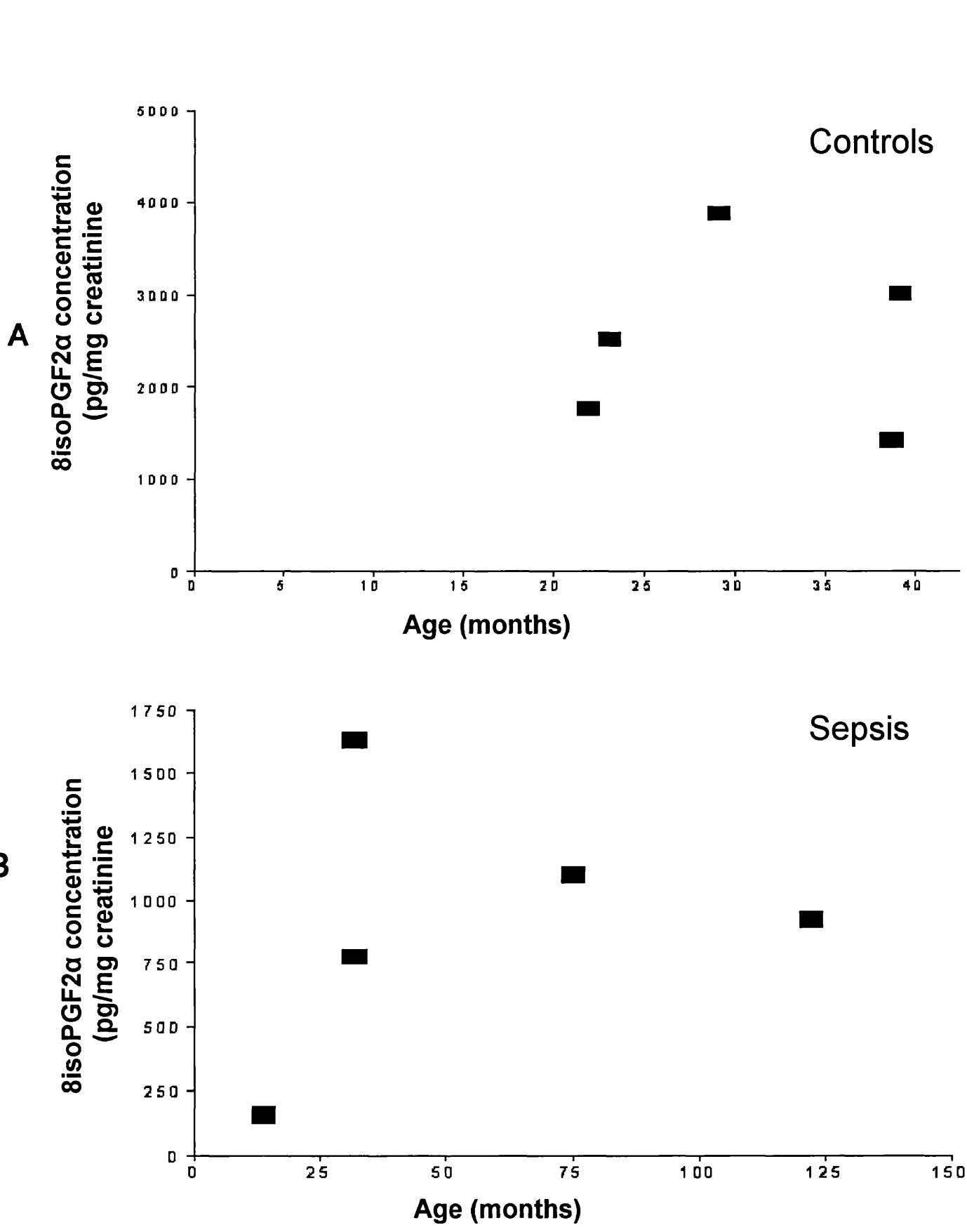

Fig.3.6. Urinary concentrations of 8-isoPGF2α expressed in terms Of age in controls (A) and patients (B) samples

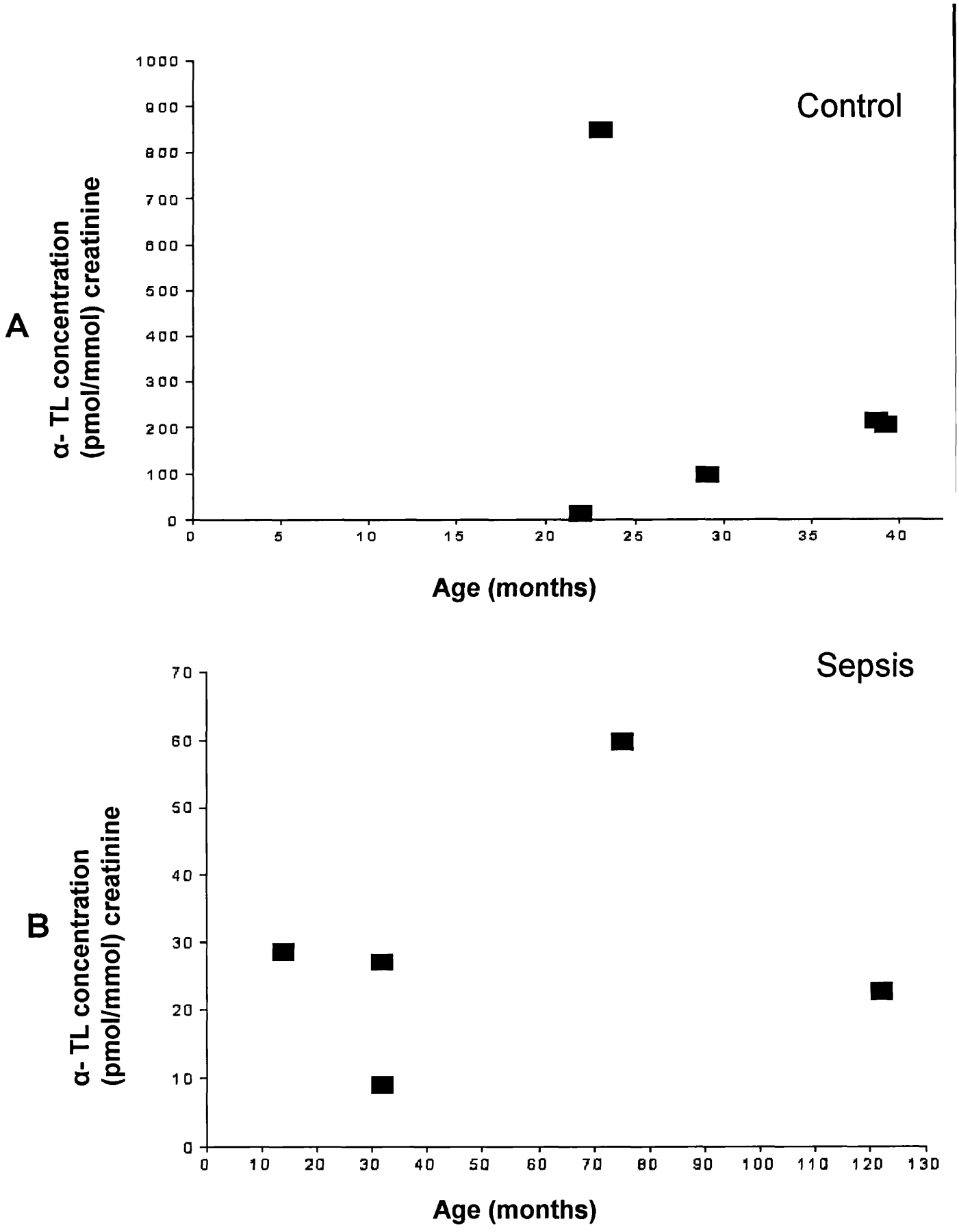

Fig.3.7 Urinary concentrations of α-Tocopheronolactone expressed in terms of age in controls (A) and patients (B) samples

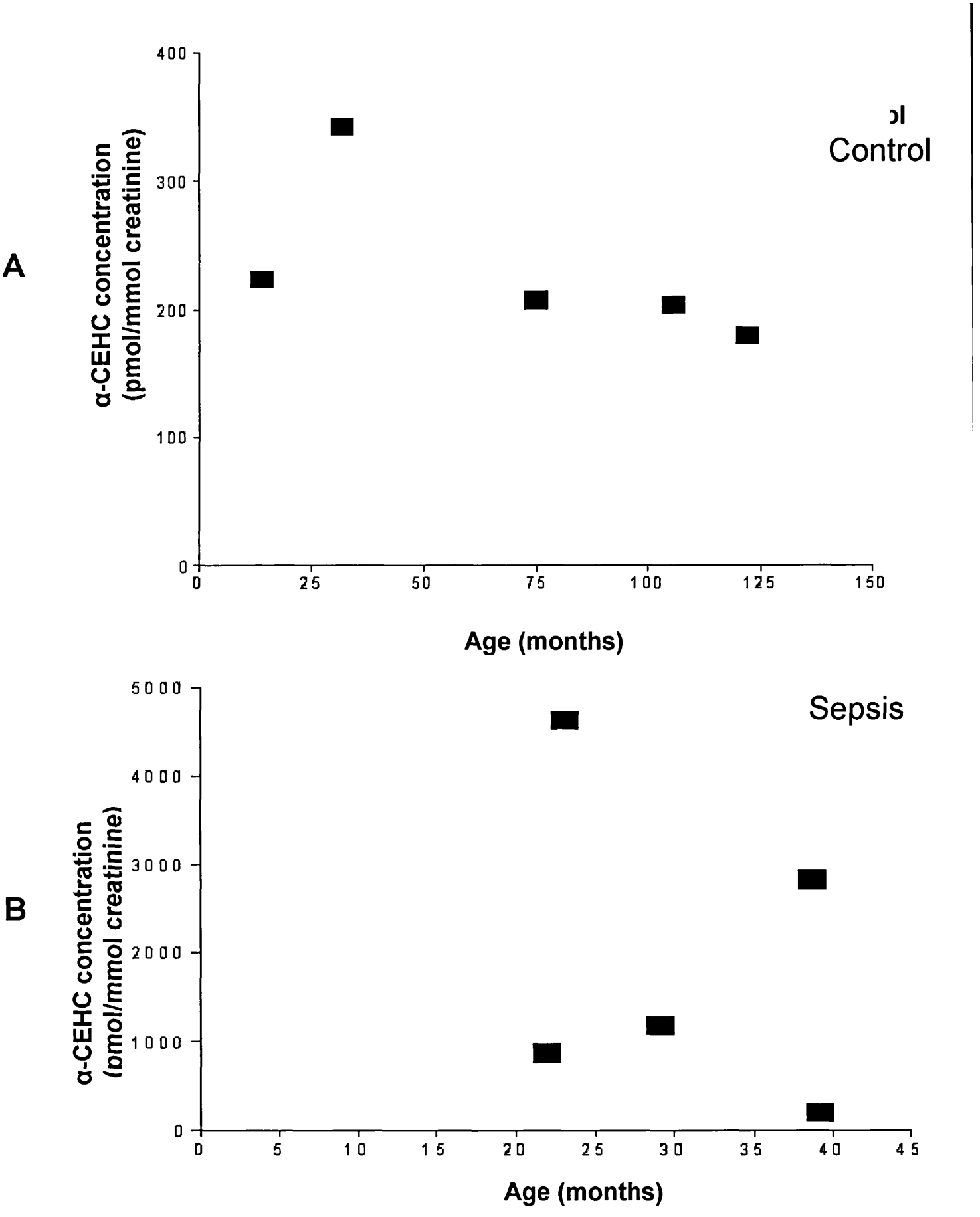

Fig.3.8. Urinary concentrations of α-CEHC expressed in terms Of age in controls (A) and patients (B) samples

3.4.5. Concentrations of α-CEHC expressed in terms of α-Tocopheronolactone

Fig 3.9 illustrates the correlation of urinary α-CEHC and α-tocopheronolactone in control (A) and sepsis patients (B), whereas Fig 3.10 illustrates the correlation of urinary concentrations of α-CEHC and α-tocopheronolactone in the entire population. The figures (Fig 3.9 A & B and Fig 3.10) clearly indicate that no correlation was observed between the urinary concentrations of α-CEHC and α-tocopherol in either the control, patients or the entire populations' urinary sample with the correlation coefficient (r) expressed as 0.1758, -0.5393, 0.3148 for controls and sepsis patients and the entire population respectively with $P > 0.05$ in all the cases.

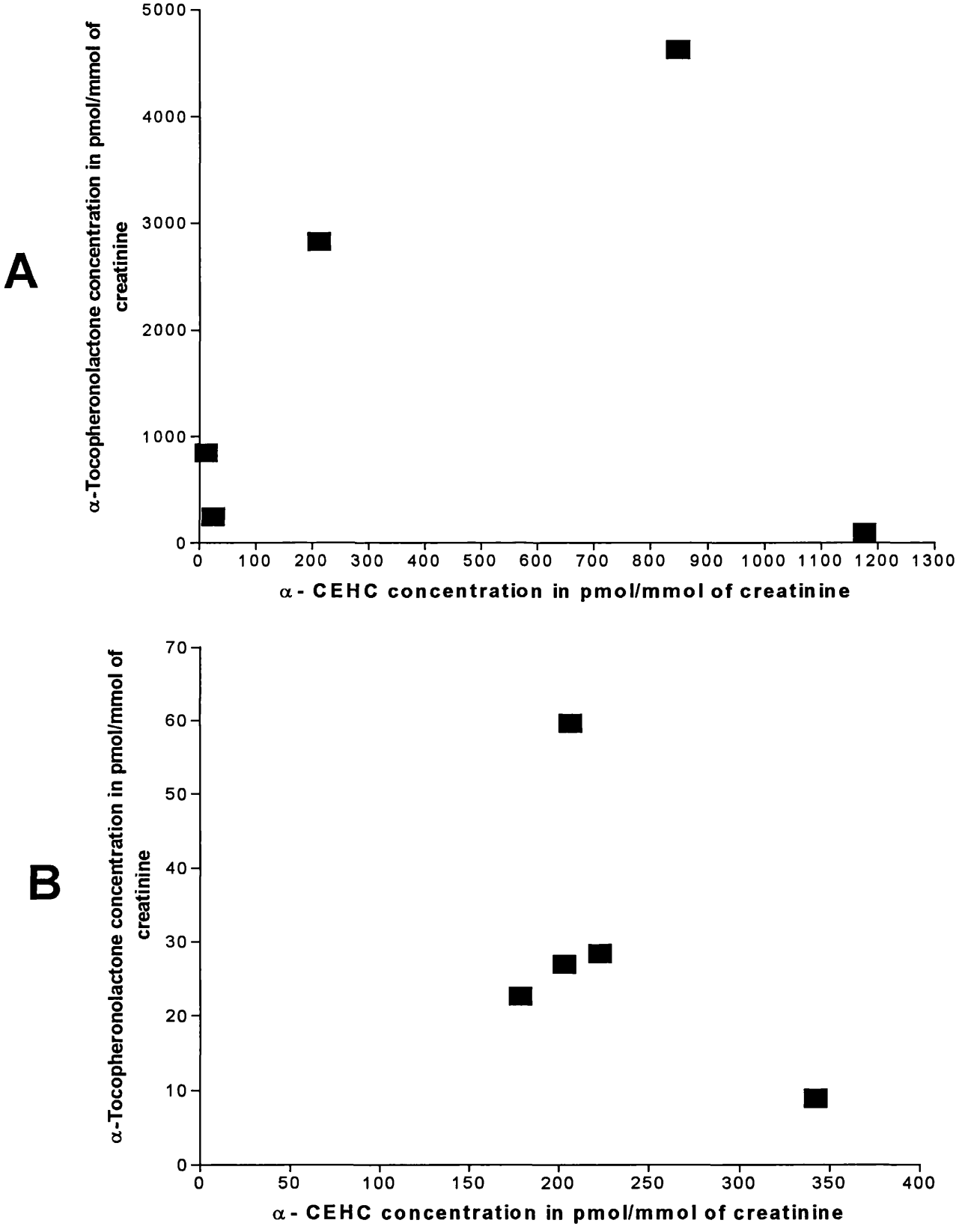

Fig.3.9. Correlation of urinary concentrations of α-TL and α-CEHC of Control (A) and Patients (B).

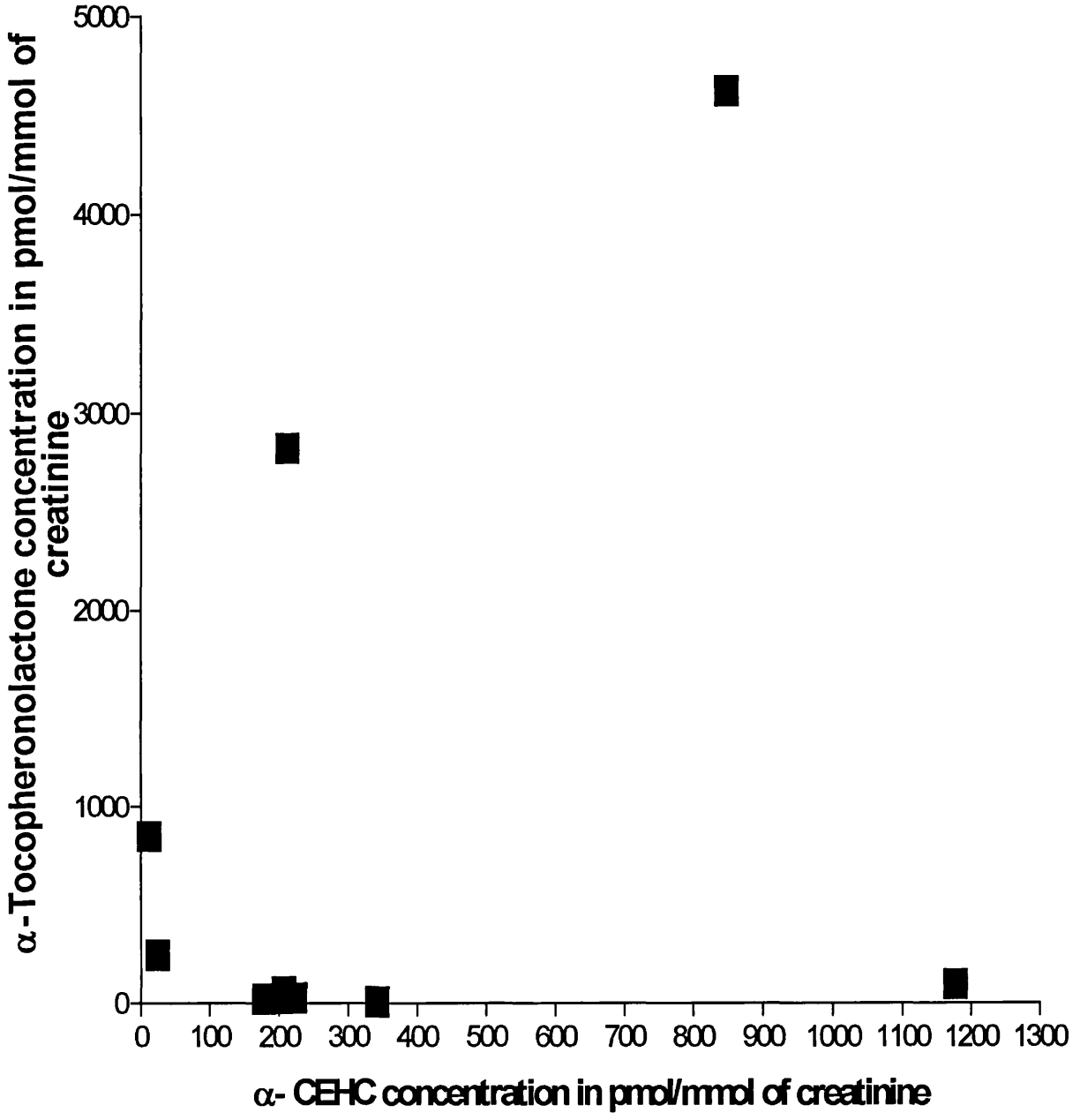

Fig.3.10. Correlation of urinary concentration of α-TL and α-CEHC in controls and patients.

DISCUSSION AND CONCLUSION

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4. Discussion and Conclusion

4.1. Introduction

The aims of the current study was

- To re-establish and validate the methods for the urinary analysis of 8-isoPGF$_{2\alpha}$ and vitamin E metabolites using gas chromatography/ mass spectrometry
- To utilize these assays in patients with sepsis and controls.
- To obtain evidence of oxidative stress from 8-isoPGF$_{2\alpha}$ concentrations and to investigate whether α-tocopheronolactone to see whether the latter could also be used as an invivo biomarker of oxidative stress.

4.2. Methods for the analysis of vitamin E metabolites

A method using solid phase extraction and enzymatic hydrolysis, coupled with GC-MS was re-established for the measurement of the vitamin E metabolites (α-CEHC and α-TL). The deuterated and undeuterated metabolites were successfully identified by their respective peaks in the chromatogram **(Section 2.1.4. Fig. 2.5)**. The metabolites were later confirmed and differentiated by their respective mass spectra **(Section 2.1.4. Fig. 2.6)**. The eluting time of the chromatogram and the ions of the mass spectra were confirmed by comparing it to the results obtained by previous researchers (Murphy 1987, Liebler, 1996; and Mottier 2001). The sensitivity of the method was tested using derivitized standards (d_9- CEHC) of a known volume (10 μl) and increasing concentration (0.1μM to 20 μM). The results obtained **(Section 2.1.5. Fig. 2.8)** showed a linear correlation to the concentration of the standards injected to the GC-MS with the sensitivity increasing only after 5 μl and this linear increase was

similar to that observed by Schultz (1997). Replication studies were also conducted to validate the method prior to testing the samples (**Section 2.1.5. Fig 2.9**). The results obtained were considered consistent and confirmed the methods reproducibility.

4.3. Method for analysis of 8-isoPGF$_{2\alpha}$

The use of sorbent extraction, enzymatic hydrolysis, coupled with a GC-MS temperature programme that allowed greater separation of the components in the urine made the method for the 8-isoPGF$_{2\alpha}$ metabolite. After extraction the metabolites were derivatized and then later analysed by the GC-MS.

Deuterated and undeuterated 8-isoPGF$_{2\alpha}$ was identified by their chromatogram and they were confirmed by their fragments ions in the mass spectra (**Section 2.2.4**. **Fig 2.11**). The metabolites were later differentiated and confirmed by their characteristic fragmentation ions (**Section 2.2.4. Fig 2.12**). The chromatograms and fragment ions observed proved to be comparable to the results obtained by Roberts et al., 1996 ; Lawson et al., 1998 ; Proudfoot et al., 1999 ; Bessard et al., 2001a The sensitivity test conducted, was similar to the test conducted for vitamin E metabolites where standards of a fixed volume (derivitized only) with increasing concentration (50 to 5000 pg/ml) were injected onto the GC-MS. The results obtained (**Section 2.2.5. Fig 2.13**) showed that, a linear increase was observed with an increase in the concentration of the standard with the sensitivity increasing only after 500 pg/ml and this linear increase was similar to that observed by Robert (1996). Replication studies were also conducted to validate the method prior to testing the samples (**Section 2.2.5.**

Fig 2.14). The results obtained were considered consistent and confirmed the methods reproducibility.

4.4. Urinary concentrations of 8-isoPGF$_{2\alpha}$ and vitamin E metabolites in patients with sepsis and control.

Initially the urinary, concentrations of 8-isoPGF$_{2\alpha}$ and of the vitamin E metabolites (α-CEHC and α- TLHQ) of the patients with sepsis and controls were expressed in terms of their age. None of the analytes showed any significant correlation with age (i.e. $P > 0.05$) and thus the samples could be analysed in groups of control and sepsis patients.

There was a significant difference (P value of 0.013) in the concentrations of 8-isoPGF$_{2\alpha}$ between the control and sepsis patients with means ± ISD of 917.1 ± 238.6 and 2521 ± 442.1 pg/mg for the controls and the patients' respectively(**Section 3.1. Fig. 3.1**). A consistency was also observed in the values within the groups (control and sepsis). This increase in the urinary 8-isoPGF$_{2\alpha}$ concentrations observed in this study agrees with the postulated theory of an increase in the oxidative stress levels in sepsis patients (Cowley et al. 1996) and also relates well to data observed earlier like an increase in OH and H2O2 (Taylor 1995), NO., ROS and vascular control and enzymatic functioning in sepsis patients (Takakura et al 2003). Increased levels of 8-isoPGF$_{2\alpha}$ were reported earlier in conditions like Alzeimer's (Montine et al. 1999;Pratico et al. 1998), Asthma (Dworski et al. 2001), Diabetes (Devaraj et al. 2001). In light of the previously obtained results, the findings of the current study also suggest a possibility for using isoprostanes for monitoring antioxidant therapies. The

data obtained also substantiates Morrow's (1990) suggestion of considering the use of 8-isoPGF$_{2\alpha}$ as an independent biomarker of stress.

A marked increase was also observed in the concentration of α-tocopheronolactone, in the sepsis patients as compared to that of the controls, with means ± ISD of 274 ± 148.1 and 29.29 ± 8.323 pmol/mmol for the sepsis patients and controls respectively. The control samples' α-tocopheronolactone concentration was observed to be extremely consistent being in a range of 8.9 to 59.6 pmol/mmol and apart from one sample the results obtained for the sepsis patients' was also similar (range being 12 to 212.22 pmol/mmol) (**Section 3.2. Fig 3.2**). The consistency in the overlapping data suggest an accountable increase in the α-tocopheronolactone production in the sepsis state, thus proposing the relevance of measuring α-tocopheronolactone as an indicator of oxidative stress. No correlation was observed between the concentrations of 8-isoPGF$_{2\alpha}$ and α-tocopheronolactone in either the controls', sepsis patients' or the entire sample (**Fig 3.4 A and B and Fig 3.5**). The reason why no correlation was observed in all the three cases could be due to the small sample size, but what is highlighted from the data is that when the two groups i.e. control and sepsis patients data were grouped together the r value obtained was positive indicating that there could have been correlation between the two values if the sample size was bigger. This suggested that α-tocopheronolactone may be an independent marker of oxidative stress.

It was also of interest that there was a marked increase in the urinary α-CEHC concentrations in the sepsis patients' where a range of 204 to 2826 pmol/mmol with a mean of 1940 ± 798.7 compared with the controls' where the α- CEHC concentrations

ranged from 178.63 to 342.6 pmol/mmol with a mean concentration of 230 ± 28.78 pmol/mmol (**Section 3.3 Fig.3.3**). α- CEHC is considered as an indicator of adequate or excess vitamin E status and was expected to be low in a state of oxidative stress (Pope et al, 2000). The reason for an increase in α-CEHC concentrations is not known but there was the theoretical consideration, where possibilities are that it reflected the dietary intake of vitamin E. But this assumption was unlikely as there was no evidence from available information that the patients with sepsis had received greater than normal levels of dietary α-tocopherol (vitamin E). Another reason postulated for the increase in the α-CEHC concentration could be due to the sepsis state itself. The investigation of this increase in sepsis is beyond the scope of this study and should be examined in future research. The concentration of α-CEHC were then plotted against that of α-tocopheronolactone of the sepsis patients', controls' and combined. No significant correlations were observed when the data was observed. A point to be noted is that, if there was a positive correlation between the two vitamin E metabolites, it could have indicated an underestimation of the artefactual oxidation in the methodology used.

4.5. Conclusion

In conclusion the study has re-established and validated the urinary analysis of 8-isoPGF$_{2\alpha}$ and vitamin E metabolites using gas chromatography/ mass spectrometry. The study has clearly obtained evidence of an increased 8-isoPGF$_{2\alpha}$ excretion in sepsis patients confirming, not only an oxidative stress status in sepsis but also the use of 8-isoPGF$_{2\alpha}$ as an appropriate method for measuring oxidative stress. It is not still not clear from this study whether α-tocopheronolactone can be used as an independent biomarker of oxidative stress even though an increase in the concentration of α-tocopheronolactone was observed in the sepsis patients' urine, there was no significance correlation between 8-isoPGF$_{2\alpha}$ and α-tocopheronolactone. A larger sample size might have provided an answer to this question. The study also indicates the need of looking into the role of α-CEHC in oxidative stress and sepsis as the increase in excretion of this metabolite in this study is not fully understood.

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