Spectrophotometry for cerebrospinal fluid pigment analysis: a review

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Abstract

The use of spectrophotometry for the analysis of the cerebrospinal fluid (CSF) is reviewed. The clinically relevant CSF pigments — oxyhemoglobin and bilirubin — are introduced and discussed with regard to clinical differential diagnosis and potentially confounding variables (the four "T"s or: traumatic tap, timing, total protein and total bilirubin). The practical laboratory aspects of spectrophotometry and automated techniques are presented in the context of analytical and clinical specificity and sensitivity. The perceptual limitations of human color vision are highlighted and the use of visual assessment of the CSF is discouraged in light of recent evidence from a national audit in the United Kingdom. Finally, future perspectives including the need for longitudinal CSF profiling and routine spectrophotometric calibration are outlined.

Keywords spectrophotometry, xanthochromia, cerebrospinal fluid, subarachnoid hemorrhage

Contents

1 Introduction

Patient management decisions in medicine depend to an ever increasing degree on objective — evidence based — diagnostic guidelines. This is particularly true for neurocritical care where many patients are unconscious or unable to communicate adequately. Therefore, the diagnostic procedures underlying the guidelines must not only be correctly applied, but must also be legally and scientifically testable, to justify the management decisions.

Recently, concern has been raised that about 99% of over 3,000 laboratories participating in two independent US surveys[1,2] relied on visual assessment of the cerebrospinal fluid (CSF) for xanthochromia (Greek for "of yellow color"). Xanthochromia assessment is used to help decide whether or not an intracranial bleed has occurred, which is important for diagnosing a subarachnoid hemorrhage (SAH). Because a SAH is associated with a high mortality (50–60%), which increases significantly (to about 80%) if the initial diagnosis is missed and a rebleed occurs, a meticulous diagnostic approach is essential for rapid and appropriate treatment.

In this review, we argue for abandoning the visual assessment of the CSF for xanthochromia, because new evidence suggests that it is perceptually impossible or unreliable in about 80% of critical cases.[3,4] Instead, we argue for relying on the more sensitive and more specific spectrophotometry,[5] which is also documentable and therefore legally testable. Firstly, we review the clinical relevance of CSF pigment analysis. Secondly, we

summarize why the human visual system is unreliable for detecting xanthochromia. Thirdly, we review the laboratory techniques available for CSF analysis. Fourthly, we review the UK experience with spectrophotometric–based national guidelines and the current US recommendations for CSF pigment analysis. We conclude with an evidence based diagnostic flow–chart.

2 The clinical relevance of CSF pigment analysis

The normal CSF is clear and colorless, as shown by the roughly flat spectrophotometric trace depicted in Figure 1A. It consists of 99% water and has a much lower protein concentration ($\approx$ 350 mg/L) than the serum (70,000 mg/L). The color changes when additional substances such as bilirubin (yellow) or hemoglobin (red) enter the CSF. The correct identification of CSF pigments allows one to draw pertinent conclusions about pathology in the patient under examination. Although the major concern for the neurocritical care physician will be to correctly identify SAH, there are confounding factors and other conditions that need to be considered when making the diagnosis (for review see reference[6] and references therein).

The color changes associated with blood pigments released into the CSF, as the result of potentially life threatening conditions such as an aneurysmal SAH, are very rapid. Erythrocytes are haemolysed and release Hb (red colored) which dissociates into heme and globin. The heme

groups are, in turn, converted by heme oxygenase to biliverdin (bile green) and further by biliverdin reductase to bilirubin (canary yellow).[7] Bilirubin arises only *in vivo* which makes it the most specific metabolite for distinguishing a true bleed from a traumatic tap.[7–14] In fact, the presence of bilirubin in the CSF taken 6–12 hours after ictus in a patient with suspected SAH is virtually a fail–proof diagnostic.[9,12,15,16]

2.1 Subarachnoid hemorrhage (SAH)

Cerebral aneurysms are estimated to be present in about 5,000,000 North Americans of whom approximately 30,000 are likely to experience an aneurysmal SAH.[17,18] For these the mortality from a SAH rises to about 80% if a rebleed occurs.[19] The risk of a rebleed can be minimized by protection of the aneurysm either surgically ("clipping") or radiologically ("coiling").[19,20] Clearly, sensitive diagnostic tools are crucial for determining if an aneurysmal hemorrhage has occurred and to enable rapid and adequate patient management. At present the diagnosis of a SAH is predominantly made on the basis of non–enhancing computerized tomography (CT) of the brain.[6,19] There are, however, two situations under which the CT can be unreliable. Firstly, it may occur when the CT is performed some time after the bleed; Van Gjin *et al.* reported that the sensitivity of the CT falls from 97% in the first day after the bleed to less than 10% after three weeks.[21] Others have reported a negative CT in 14.6–20% of patients with a proven SAH,[22,23] which presumably must also be due in part to lack of prompt

scanning. Secondly, a caudally–located bleed or small bleed adjacent to bony structures (i.e. above the orbits) can be difficult to detect because of imaging artifacts. Thirdly, the scan may be suboptimal technically; beeing difficult due to severe anaemia or movement artefacts.[6] Even when optimally timed, using modern scanners and in the best of hands the CT still has a false negative rate of approximately 2-3% and confidence intervals are wide.[24,25] Not surprisingly, therefore, a body of literature provides evidence for the presence of a SAH by CSF analysis for xanthochromia in cases where the CT scan was negative.[15,16,23,24,26–29] In the future, MRI may be helpful in selected cases. There is preliminary evidence that its sensitivity is increased for detecting a bleed two to three weeks following a SAH.[30,31] Different sequences have been used with varying success, but more data are needed to recognize their potential pittfalls and their false positive and negative rates.[30–36] It would be important to compare the accuracy of the various MRI techniques with that of rigorous CSF analysis as outlined in this review.

2.2 The traumatic tap

The first traumatic tap was documented by Quincke on the 23rd of July 1891 whilst performing the fourth consecutive lumbar puncture (LP) in a 7 year old girl with hydrocephalus.[37] Overall a traumatic tap with a significant amount of blood can be expected in about 14–20% of standard LPs.[8,24,38,39] Importantly, this figure increases to 72% if a traumatic tap is

defined by an erythrocyte count of at least 100 cells (per μL, or $\times$ 10^6/L) in the sample.[38] There is no consensus about the precise definition of a traumatic tap. A pragmatic cut–off appears to be 400 $\times$ 10^6 erythrocytes/L.[8,24]

A traumatic tap causes contamination of the CSF sample with erythrocytes, possibly by puncture of the venous plexuses within the spinal sac or vessels adjacent to the cauda equina.[8] The recommendations for a correct lumbar tap are to collect four consecutive samples and use the last one for spectrophotometry. The samples should be transported to the laboratory as quickly as possible in order to minimize the risk of *in vitro* haemolysis of any erythrocytes. Only the last one should be used for assessment of CSF pigments and it should be centrifuged at $\geq$2000 rpm for 5 minutes. The supernatant should then be taken for spectrophotometric analysis as described below.

It is technically impossible to exclude that some erythrocytes may have haemolysed. Collecting multiple fractions helps reduce this problem. In the worst case scenario, if a traumatic tap has occurred and all the CSF is collected in one tube and the sample reaches the laboratory after a significant delay, then making a visual assessment for xanthochromia will be impossible as explained below. If this occurs, a spectrophotometric scan as shown in Figure 1 (B) may be observed. This scan provides evidence for the presence of oxyhemoglobin in the CSF, but not for bilirubin as no peak is seen at 455 nm *.

*The absorption maxima for bilirubin in the visible light is around 455 nm. The UK guidelines however recommend measuring the absorbance at 476 nm where the absorp-

Fluid from a LP which is, in fact a traumatic tap, when performed at least 12 hours after a SAH can still be used, because the presence of a peak at 476 nm (bilirubin) will still be indicative of SAH. For an example of this situation see Figure 1 (C). A similar scan may be expected if a second LP is performed subsequent to a traumatic tap but over 12 hours after ictus.

It is important to wait at least 12 hours after ictus before performing the LP to allow time for a sufficient conversion of heme to bilirubin to occur. A traumatic tap taken too early after ictus however precludes the option of a follow–up LP because any erythrocytes released into the lumbar sac as a consequence of the traumatic tap will subsequently lyse and may give rise to a spectrophotometric scan indistinguishable from that of a SAH (Figure 1 C).

It is perceptually impossible to see by eye a small amount of bilirubin in the presence of a large amount of haemolysed erythrocytes (red).[40] Hence, visual assessment of xanthochromia in the context of a traumatic tap is unreliable, as reported previously.[41,42]

2.3 The size of the problem

Currently about 30% of SAH patients are misdiagnosed at their initial visit.[6] Based on the above mentioned estimates[17,18] this would affect about 9,000 of those 30,000 patients who suffer from a SAH per year in North Amer-

tion curves for oxyhemoglobin and bilirubin are better separated.

ica alone. Optimal use of diagnostic tools may improve this percentage. However, given a false negative CT rate of approximately 2-3% with modern scanners,[24] up to 900 critical patients may be missed even in the best equipped specialist units. Thus, the real number is likely to be higher. These cases may profit from a LP, of which about 126 (12-14%) may be expected to turn into a traumatic tap; making the visual assessment extremely difficult or impossible.[8,24,38,39] In view of the perceptional limitations of human color vision, with an estimated 80% of critical samples not being seen as yellow,[3] CSF spectrophotometry would be helpful in diagnosing those 9,000 patients who are expected to bleed per year and essential for those 900 who are expected to have a “false negative” normal CT scan.

3 Human Color Vision and the Limitations of Visual Assessment

Under good viewing conditions, the human eye can discriminate about 150 wavelengths in the visible spectrum,[43,44] and as many as 20,000 non-spectral hues.[45] This ability depends upon comparisons of the differential photon absorptions by the three types of retinal photoreceptor cells, each containing a different photopigment: the short (S)-, middle (M)- and long (L)- wavelength sensitive cones. The absorbance spectra of the S-, M- and L-cones overlap considerably, but have their wavelengths of maximum absorbance in different parts of the visible spectrum; ca. 440, 545

and 556 nm, respectively, when estimated in vivo.[46] Although the probability that a photon will be absorbed by a given cone photoreceptor varies by many orders of magnitude with wavelength, once it is absorbed its effect is independent of wavelength. Thus, the individual cones are blind to the wavelength of capture; they only signal the rate at which photons are caught.

These physiological facts impose severe constraints on the visual assessment of CSF for xanthochromia. In fact, CSF samples of different spectral distributions, will appear visually identical, if they produce the same absorptions in the three different types of cone photoreceptors, and only different, if they do not.

3.1 Sensitivity of human colour vision

The concentration of bilirubin in an otherwise colorless CSF solution must exceed a certain threshold in terms of excitation purity[†] in order for it to be detected by xanthochromia. From earlier studies, we estimate that the human visual threshold lies at approximately 2.4% excitation purity; whereas spectrophotometry is reliably capable of detecting concentrations of bilirubin that produce excitation purities as low as 0.62%.

Our results are consistent with the raw data provided by Linn *et al.* who compared visual inspection with spectrophotometry for detecting low con-

[†]The excitation purity can be calculated from the spectrophotometric trace with respect to the viewing illuminant (usually daylight or a tungsten source) by using standard colorimetric procedures, which have been established by the Commission Internationale de l'Éclairage (CIE, or the International Commission on Illumination).[3,40]

centrations of bilirubin in the CSF.[47] Linn *et al.* spiked clear human CSF with different concentrations of bilirubin. The extinction was determined at 450-460 nm. Their subjects were required to assign the CSF samples visually to one of three categories: “yellow”, “colorless” or “doubtful”. Spectrophotometry, used as the gold standard, was capable of detecting a trace of bilirubin in samples with an extinction as low as 0.01; whereas human observers could not perform reliably below an extinction of 0.06 (see Table 2 [‡]).[47] It would be interesting to analyse the original absorption spectra of Linn *et al.*[47] to determine whether their visual threshold (extinction of 0.06) corresponds to ours (excitation purity of about 2.4%).[40]

3.2 Specificity of human colour vision

Any CSF sample will be perceived as having only a single hue or dominant wavelength regardless of how many wavelengths[§] actually comprise its spectral distribution. In other words, although the human visual system is easily capable of discriminating between the hues of different CSF samples, provided that the samples produce significantly different photon catches in the three cone classes, it is incapable of differentiating between the composite wavelengths within any given sample.

[‡]For comparison with our results, we only included the observed frequencies of their samples seen as “yellow” or as “colorless” in Table 2. Samples for which no decision could be made because the visual inspection was “doubtful” are not included into our post–hoc analysis.

[§]The dominant wavelength can be calculated from the spectrophotometric trace with respect to the viewing illuminant (usually daylight or a tungsten source) by using standard colorimetric procedures, which have been established by the Commission Internationale de l’Éclairage (CIE, or the International Commission on Illumination).[3,40]

Thus, a CSF sample containing bilirubin, which absorbs in the blue part of the spectrum and transmits in the yellow, may not appear xanthochromic or yellow, if the bilirubin concentration is low or if its concentration is exceeded by that of another pigment, such as oxyhemoglobin, which has characteristic absorbance signatures in other parts of the spectrum (e.g. red) to which the eye is sensitive. We have shown elsewhere[3,40] that as many as 80% of CSF samples containing substantial amounts of bilirubin may not be perceived as being xanthochromic. The presence of bilirubin therefore cannot reliably be ruled out in cases where CSF is visibly red.

3.3 Color vision is unreliable for detecting CSF pigments

In short, human vision is an unreliable or inappropriate means of assessing a CSF sample for the presence of bilirubin; not because the eye is insensitive to wavelength, but rather because it cannot differentiate quantitatively between the constituent absorbances determining the overall color appearance of the CSF sample. In contrast, spectrophotometry is specifically designed to do this job.

3.4 Other situations in which the CSF appears yellow (xanthochromic) to the eyes

There are a number of conditions and drugs which can give the CSF a yellowish appearance (Table 1). For clinical practice, a high total protein concentration is possibly the most relevant factor because it occurs in a broad spectrum of acute neurological disorders. Some of these, such as

acute encephalitis and meningitis, share clinical features (i.e. headache, meningism, etc.) with SAH.[6]

4 The laboratory analysis of CSF pigments

A number of laboratory techniques are available for measuring CSF pigments. Spectrophotometry is preferred because it allows the simultaneous assessment of a number of substances and we provide guidelines for its use.

4.1 Spectrophotometry

4.1.1 Principles

Each material has its specific color when viewed in white light because certain wavelengths are absorbed. Thus, a fluid containing a substance that absorbs blue light ($<$ 466 nm[4]) will appear yellow, when transluminated by white light, because only red and green light are being transmitted.

Spectrophotometry is a technique that measures the amount of light being transmitted at each wavelength. In a laboratory context, however, one refers to the absorbance rather than the transmittance at each wavelength; with absorbance being the inverse of transmittance. Confusion can be avoided by remembering that bilirubin which absorbs in the blue part of the spectrum (455 nm) will typically be perceived as yellow.

All haem proteins exhibit their maximal absorbance in the Sorét band region of 400 to 440 nm (thus they tend to appear yellow). Additional,

smaller peaks are seen between 500 and 600 nm.

4.1.2 Bilirubin

The key biochemical mechanism that makes CSF analysis such a useful diagnostic tool is the conversion of hemoglobin (Hb) to bilirubin, which can be easily detected by spectrophotometry. Essentially the erythrocytes released by the bleed into the CSF haemolyse. *In vitro* experiments show that haemolysis starts about 30 minutes after transfer of autologous red cells into CSF. Given the long half–life of about 120 days of erythrocytes *in vivo*, it is likely that the process starts by the early haemolysis of older and more fragile erythrocytes. Haemolysis leads to the release of Hb and oxyhemoglobin into the CSF. Figure 1 (B) shows the typical spectrophotometric curve of Hb and oxyhemoglobin. Clinical experience indicates that it takes about 12 hours to produce the critical amount of bilirubin *in vivo* required to become detectable by spectrophotometry.

The typical spectrophotometric scan of a CSF sample from a patient with proven SAH is shown in Figure 1 (C). The peak at 415 nm is the main peak indicating the presence of oxyhemoglobin or Hb in the CSF sample, with two secondary peaks between 500 to 600 nm¶. The broad absorbance shoulder band at the right handside of the Hb peak is bilirubin.

¶If these secondary peaks are absent then the first peak is likely to be caused by Hb alone.

4.1.3 Efficacy of spectrophotometry

The value of using spectrophotometry in the diagnosis of SAH as opposed to the widely practiced visual assessment for xanthochromia is probably best illustrated by a retrospective analysis of a scientific exchange that took place between 1988 and 1989. MacDonald and Mendlow reported that 46% of 68 SAH patients had a bloodstained, but not a xanthochromic CSF, on the basis of visual assessment. Accordingly, they concluded that a bloodstained but not xanthochromic CSF is the most sensitive sign of SAH.[23] This conclusion was immediately contested by Vermeulen *et al.* who pointed out that xanthochromia was present in 100% of samples taken between 12 hours to 2 weeks from the ictus.[15] What MacDonald and Mendlow, in fact experienced was that visual assessment of the CSF did not allow them to perceive the presence of bilirubin (yellow) in those 46% of samples *that were bloodstained.* It needs to be borne in mind that this was a retrospective study of patients with an angiographically confirmed aneurysmal SAH. Our own retrospective study of 632 CSF samples revealed that in about 80% of critical cases the color of the sample fell outside the hue category that would be perceived as yellow.[3,4] This is consistent with previous studies[14,41,42,48] and reinforces the point made by Vermeulen *et al.* that spectrophotometric assessment of the CSF should be the analytical method of choice.[15]

4.1.4 How to do it

The UK National Guidelines for analysis of CSF for bilirubin in suspected SAH give detailed instructions about sample acquisition, handling, analysis and interpretation of the results.[9] In brief, these are:

- Ideally take 4 sequential CSF samples with a time delay of at least 12 hours from ictus. Only the last one is to be used for spectrophotometry. Measurement of the plasma bilirubin level at the time of LP is advised.

- Transport sample as soon as possible to the laboratory, avoiding undue mechanical haemolysis (i.e. shaking, or delivery through a pneumatic tube system) and excessive light exposure.

- Centrifuge sample at $>$2000 rpm for 5 minutes after receipt in the laboratory and store supernatant at 4° C.

- Water (H_2O) is used as a blank and the undiluted supernatant is used for a zero–order spectrophotometric scan (350–750 nm). A cuvette with a 1-cm path length is recommended[‖]. Examples of such scans are shown in Figures 1 (A–B).

- Draw a baseline (see Figure 2) and measure the distance from the baseline to the scan at 476 nm, which is defined as the net biliru-

[‖]Small volumes (minimum of 50μL) can be measured using disposable cuvettes. For safety reasons we do not recommend the use of 10μL cuvettes, because they are difficult to clean after the handling of infectious material. About 1000μL of sample is required for a standard cuvette.

bin absorbance (NBA)**. Concurrently, the net oxyhemoglobin absorbance (NOA) should be measured at its highest peak between 410–418 nm.

- The interpretation is based on the simple combination of the AU for NBA and NOA, if the serum bilirubin is $<$20 μmol/L and the CSF total protein $<$1.0 g/L. Accordingly, there are three possible outcomes:

    1. No evidence to support SAH. NBA $\leq$0.023 AU and NOA =0 AU;
    2. SAH cannot be excluded, but the predictive value of the test is low. NBA $\leq$0.023 AU and 0$<$ NOA $\leq$0.1 AU (if NOA $\geq$0.1 AU a small increase of bilirubin may be masked. This may occur if the LP is performed too early.);
    3. The results are consistent with SAH. (A) NBA $>$0.023 AU and NOA =0 (i.e., seen one week after SAH), (B) NBA $>$0.023 AU and 0$<$ NOA $\leq$0.1 AU or NOA $\geq$0.1 AU (i.e., seen early after the SAH, or if a traumatic tap was performed with an appropriate time delay from the SAH, or a rebleed has occurred and the LP was performed directly after this complication).

- In the presence of a serum bilirubin $\geq$20 μmol/L or a CSF total protein $>$1.0 g/L additional calculations as outlined in the UK guidelines are

**For a straight forward absorbance a value of $>$0.023 absorbance units (AU) is taken as recommended by Vermeulen *and colleagues*.[15] The *corrected value* takes the plasma bilirubin levels alongside the total protein levels of the CSF and serum into account and refers to values of $>$0.007 AU, as explained elsewhere.[9,49,50] For simplicity we refer to the *uncorrected value* in this review.

recommended.[9]

A simplified diagnostic flow–chart is shown in Figure 3.

4.2 Automated measurements

Ungerer *and colleagues* reported an automated method for measuring CSF bilirubin, which is based on the classical Jendrsik-Gróf principle which utilizes the reaction of bilirubin with diazotised sulfanilic acid to yield blue–green coloured azobilirubin.[51] The authors modified their routine serum bilirubin assay[51,52] and the results were compared with those obtained by spectrophotometry as outlined in the UK guidelines.[9] The intra–assay CV was 20% for bilirubin concentrations at 100 nmol/L and the detection limit was 35 nmol/L. The two methods seem to yield a comparable diagnostic performance for samples with a significant increase of CSF bilirubin levels. However, for small amounts of bilirubin, this may not necessarily be the case, because the reported upper reference limit of the automated method is over 2-fold higher (359 nmol/L) than the one defined by the UK NEQUAS (166 nmol/L, corresponding to a corrected absorbance of 0.007 at 476 nm)[††].

If validated this method may be of use to those laboratories that do not have access to a spectrophotometer. It should be borne in mind that the sample volume required will depend on the dead space of the particular system and may be large.

[††]The values were taken from the study by Unger *et al.*,[51] but the interpretation on sensitivity is our own.

5 Guidelines for spectrophotometric analysis of CSF pigments

5.1 The UK experience

The UK guidelines were published in 2003 by the UK National External Quality Assessment Scheme (NEQAS) for the Immunochemistry Working Group, of which one of the authors is a member (GK).[9] The effect that these guidelines had on laboratory practice in the UK was evaluated through UK–NEQAS over the following year.[53] The results revealed that the implementation of the guidelines had a major impact on clinical practice in the UK. Prior to the publication of the guidelines, 77/101 (76%) of laboratories used spectrophotometry as opposed to visual inspection. But thereafter, the proportion using spectrophotometry increased significantly to 108/115 (94%). Moreover, the approximated error rate decreased from 40% to 9%.

5.2 The US recommendations

At the time of this review, neither the American Academy of Neurology (AAN) nor the American Neurology Association (ANA) provide advice on the CSF analysis for xanthochromia. At present, there are no national US guidelines.

A recent US survey by Edlow and colleagues revealed that a large number of laboratories evaluate CSF for xanthochromia (1944/2551, 76.2%).[1] Of these, 91.8% report routinely on xanthochromia. An impressive 87.4%

of laboratories achieved a turnaround time of $\leq$ 60 minutes and 97.3% offered the test 24 hours a day, 7 days a week. However, the analytical method chosen by 99.7% of the participating laboratories was *visual inspection*. This figure is comparable to that reported in an independent survey.[2] An algorithm for evaluation of possible SAH was proposed [8, Figure 2] and advice for the CSF analysis was given by these authors on the "real life" basis that most US laboratories do not presently perform spectrophotometry.[8]

5.3 Future perspectives

CSF bilirubin A longitudinal profile of the bilirubin levels in patients with SAH is needed. This would allow one to establish the time–frame during which CSF analysis for bilirubin is reliable. Because bilirubin is metabolized, it is expected that after a certain time has elapsed no bilirubin will be detectable in the CSF. The study by Vermeulen *et al.* suggests that, after 2 weeks, 32/32 (100%) of patients still have traces of bilirubin in their CSF. After 3 weeks, this falls to 20/22 (91%) and after 4 weeks to 10/14 (71%).[15] The decrease of CSF bilirubin is likely to depend on the initial blood load, which is not known in the Vermeulen *et al.* study. A conservative study should include patients with only a small amount of blood visible on CT (i.e., a Fischer grade II).[54]

CSF oxyhemoglobin A longitudinal profile for oxyhemoglobin after SAH may also be relevant. Our own quantitative data indicate a secondary

increase of CSF oxyhemoglobin between days 5 and 6 in 10 patients with aneurysmal SAH.[55] This result was consistent with an animal study by Pluta *et al.*[56] However, because we did not perform re–imaging, it remains unknown whether the mild secondary increase of CSF oxyhemoglobin was due to micro–rebleeds.

Spectrophotometer calibration It is important to ensure that the spectrophotometers performing the CSF sample analysis are regularly calibrated. Ideally this should be managed through an external quality control body such as UK–NEQAS. Typically, holmium oxide is used to calibrate the wavelength response of spectrophotometers. For practical reasons holmium oxide is dissolved in perchloric acid. Holmium (Ho–165) is a stable rare earth, discovered by the same J.L. Sorét (1878) who gave his name to the Sorét–band (see above). A 4% solution of Holmium Oxide in 10% perchloric acid has 14 peaks at the following wavelengths: 241.08, 249.98, 278.03, 287.47, 333.40, 345.49, 361.16, 358.86, 416.62, 451.24, 467.94, 485.33, 536.97 and 640.84 nm.

Quantitative techniques CSF spectrophotometry is semi–quantitative.[57] A range of more quantitative techniques based on derivate spectrophotometry have been described.[58–60] These techniques may be relevant for determining the longitudinal profile of CSF bilirubin as proposed above.

Timing of lumbar puncture The conversion of heme to bilirubin starts immediately after the haemoylsis of the erythrocytes. The current UK guidelines advice waiting about 12 hours after ictus to allow for a sufficient quantity of heme be converted into bilirubin.[9] Across the world, however, there are considerable differences in speed of patient management. In certain situations, it may be difficult or contrary to accepted medical emergency practice to wait for 12 hours. From a clinical point of view there are two important scenarios. Firstly, a patient may present with antecedent symptoms prior to ictus that may already have been owing to a small degree of aneurysmal hemorrhage, a so–called "warning" bleed.[6,61] An earlier LP in such patients may be considered, based on the assumption that a sufficient amount of bilirubin (or ferritin as discussed below) is already present. There are no published studies directly examining this point, but, if verified, its application would potentially have important management implications because such patients may be more vulnerable to vasospasm. Typically, vasospasm appears not earlier than 3-4 days after ictus.[62,63] However, very occasionally vasospasm is observed before that. It should be investigated whether or not the presence of bilirubin in the CSF of patients with sentinel symptoms, possibly due to an earlier warning bleed may help to identify those at higher risk for vasospasm during or following angiography, coiling and clipping. The results of such studies could potentially have management implications.

Secondly, a patient may be admitted with a clinical presentation that

is suspiciously indicative of a SAH, but with no sentinel symptoms, and a negative CT brain scan. It seems probable that the use of highly sensitive, quantitative techniques[58–60] may permit one to detect small amounts bilirubin at an earlier time; allowing for a laboratory diagnosis. However, no data are yet available on early LPs; unfortunately, preventing any statement about the expected levels of bilirubin within the first 12 hours after a bleed. Thus, given the lack of data and the risk that a traumatic tap may hinder a later, more adequately timed LP, such practice cannot be recommended at present. In such cases, if there is a high clinical suspicion of a SAH, it may be more appropriate to perform magnetic resonance angiography or conventional angiography.

CSF ferritin Keir *et al.* noted that high CSF ferritin levels were detected in 14 patients who had no evidence for xanthochromia (Tables 2 & 3 in reference[64]); five of whom suffered from an intracranial bleed. In fact, we routinely use CSF ferritin as a biomarker for an occult bleed. Because the need for ferritin arises downstream from the metabolism of bilirubin, CSF ferritin may be detectable for a longer period following a SAH than bilirubin. It should be noted that high CSF levels of ferritin are unlikely to be specific for SAH because they have also been associated with intraparenchymal cerebrovascular events, miscellaneous central nervous system infections and vasculitis.[65–68]

6 Summary

The relevance of CSF pigment analysis was reviewed and its clinical and diagnostic pitfalls were discussed (The 4 “T”s in Figure 3). The physiological basis for the limitations of human color vision for CSF analysis was presented. The different practical aspects of spectrophotometry as the analytical method of choice were reviewed in detail. Special attention was given to published guidelines and their impact on clinical practise and pertinent questions to be addressed in future studies were presented.

Finally, the use of the term “xanthochromia” is to be discouraged. Rather, it is recommended that the clinician should ask for a “CSF pigment analysis”. The laboratory should use spectrophotometry to evaluate the presence of CSF pigments such as bilirubin and oxyhemoglobin.

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References

[1] J.A. Edlow, K.S. Bruner, and G.L. Horowitz. Xanthochromia. *Arch Pathol Lab Med*, 126:413–405, 2002.

[2] B Judge. Laboratory Analysis of xanthochromia in patients with suspected subarachnoid hemorrhage: a national survey. *Pa: Scientific*

Assembly, American College of Physicians, 2000.

[3] A Petzold, G Keir, and LT Sharpe. Spectrophotometry for xanthochromia. *N Engl J Med*, 351:1695–1696, 2004.

[4] A Petzold and LT Sharpe. Hue memory and discrimination in young children. *Vision Research*, 38:3759–3772, 1998.

[5] R. Beetham, M.N. Fahie-Wilson, and D. Park. What is the role of CSF spectrophotometry in the diagnosis of subarachnoid haemorrhage? *Ann Clin Biochem*, 35:1–4, 1998.

[6] J.A. Edlow. Diagnosis of subarachnoid hemorrhage. *Neurocrit Care*, 2:99–109, 2005.

[7] LJ Barrows, FT Hunter, and BQ Banker. The nature and clinical significance of pigments in the cerebrospinal fluid. *Brain*, 78:59–80, 1955.

[8] K.H. Shah and J.A. Edlow. Distinguishing traumatic lumbar puncture from true subarachnoid hemorrhage. *J Emerg Med*, 23:67–74, 2002.

[9] UK NEQAS. National guidelines for analysis of cerebrospinal fluid for bilirubin in suspected subarachnoid haemorrhage. *Ann Clin Biochem*, 40:481–408, 2003.

[10] J.A. Edlow and L.R. Caplan. Avoiding pitfalls in the diagnosis of subarachnoid hemorrhage. *N Engl J Med*, 342:29–36, 2000.

[11] M. Vermeulen and J. van Gijn. The diagnosis of subarachnoid haemorrhage. *J Neurol Neurosurg Psychiatry*, 53:365–372, 1990.

[12] J. van Gijn. Slip-ups in diagnosis of subarachnoid haemorrhage. *Lancet*, 349:1492–1400, 1997.

[13] NG Wahlgren and C Lindquist. Haem derivates in the cerebrospinal fluid after intracranial haemorrhage. *Eur Neurol*, 26:216–221, 1987.

[14] OJS Buruma, H Janson, F Den Bergh, and G Bots. Blood-stained crebrospinal fluid: traumatic puncture or haemorrhage? *J Neurol Neurosurg Psychiatry*, (44):144–147, 1981.

[15] M. Vermeulen, D. Hasan, B.G. Blijenberg, A. Hijdra, and J. van Gijn. Xanthochromia after subarachnoid haemorrhage needs no revisitation. *J Neurol Neurosurg Psychiatry*, 52:826–808, 1989.

[16] N. van der Wee, G.J. Rinkel, D. Hasan, and J. van Gijn. Detection of subarachnoid haemorrhage on early CT: is lumbar puncture still needed after a negative scan? *J Neurol Neurosurg Psychiatry*, 58:357–309, 1995.

[17] F.H. Linn, G.J. Rinkel, A. Algra, and J. van Gijn. Incidence of subarachnoid hemorrhage: role of region, year, and rate of computed tomography: a meta-analysis. *Stroke*, 27:625–609, 1996.

[18] M.R. Mayberg, H.H. Batjer, R. Dacey, M. Diringer, E.C. Haley, R.C. Heros, L.L. Sternau, J. Torner, J.r. Adams HP, W. Feinberg, and al. et.

Guidelines for the management of aneurysmal subarachnoid hemorrhage. A statement for healthcare professionals from a special writing group of the Stroke Council, American Heart Association. *Stroke*, 25:2315–2328, 1994.

[19] J van Gijn and GJ Rinkel. Subarachnoid haemorrhage: diagnosis, causes and management. *Brain*, 124:249–278, 2001.

[20] A. Molyneux, R. Kerr, I. Stratton, P. Sandercock, M. Clarke, J. Shrimpton, and R. Holman. International Subarachnoid Aneurysm Trial (ISAT) of neurosurgical clipping versus endovascular coiling in 2143 patients with ruptured intracranial aneurysms: a randomised trial. *Lancet*, 360:1267–1274, 2002.

[21] J. van Gijn and K.J. van Dongen. The time course of aneurysmal haemorrhage on computed tomograms. *Neuroradiology*, 23:153–106, 1982.

[22] HP Adams, NF Kassel, JC Torner, et al. CT and clinical correlations in recent aneurysmal subarachnoid: a preliminary report or the Cooperative Aneurysm Study. *Neurology*, 33:981–988, 1983.

[23] A. MacDonald and A.D. Mendelow. Xanthochromia revisited: a re-evaluation of lumbar puncture and CT scanning in the diagnosis of subarachnoid haemorrhage. *J Neurol Neurosurg Psychiatry*, 51:342–304, 1988.

[24] M.J. Wood, G. Dimeski, and A.M. Nowitzke. CSF spectrophotometry in the diagnosis and exclusion of spontaneous subarachnoid haemorrhage. *J Clin Neurosci*, 12:142–106, 2005.

[25] J.A. Edlow and P.C. Wyer. Evidence-based emergency medicine/clinical question. How good is a negative cranial computed tomographic scan result in excluding subarachnoid hemorrhage? *Ann Emerg Med*, 36:507–516, 2000.

[26] A. Cruickshank, R. Beetham, I. Holbrook, I. Watson, P. Wenham, G. Keir, P. White, and W. Egner. Spectrophotometry of cerebrospinal fluid in suspected subarachnoid haemorrhage. *BMJ*, 330:138–100, 2005.

[27] A.E. Berstad, S.H. Bo, and O. Sortland. [Cerebral computer tomography in subarachnoid hemorrhage]. *Tidsskr Nor Laegeforen*, 122:267–271, 2002.

[28] R. Sidman, E. Connolly, and T. Lemke. Subarachnoid hemorrhage diagnosis: lumbar puncture is still needed when the computed tomography scan is normal. *Acad Emerg Med*, 3:827–831, 1996.

[29] H. Gunawardena, R. Beetham, N. Scolding, and S.D. Lhatoo. Is cerebrospinal fluid spectrophotometry useful in CT scan-negative suspected subarachnoid haemorrage? *Eur Neurol*, 52:226–209, 2004.

[30] T. Ogawa, A. Inugami, H. Fujita, J. Hatazawa, E. Shimosegawa, K. Noguchi, T. Okudera, I. Kanno, K. Uemura, A. Suzuki, and al. et. MR diagnosis of subacute and chronic subarachnoid hemorrhage: comparison with CT. *AJR Am J Roentgenol*, 165:1257–1262, 1995.

[31] K. Noguchi, T. Ogawa, H. Seto, A. Inugami, H. Hadeishi, H. Fujita, J. Hatazawa, E. Shimosegawa, T. Okudera, and K. Uemura. Subacute and chronic subarachnoid hemorrhage: diagnosis with fluid-attenuated inversion-recovery MR imaging. *Radiology*, 203:257–262, 1997.

[32] P.D. Griffiths, I.D. Wilkinson, P. Mitchell, M.C. Patel, M.N. Paley, C.A. Romanowski, T. Powell, T.J. Hodgson, N. Hoggard, and D. Jellinek. Multimodality MR imaging depiction of hemodynamic changes and cerebral ischemia in subarachnoid hemorrhage. *AJNR Am J Neuroradiol*, 22:1690–1607, 2001.

[33] P. Mitchell, I.D. Wilkinson, N. Hoggard, M.N. Paley, D.A. Jellinek, T. Powell, C. Romanowski, T. Hodgson, and P.D. Griffiths. Detection of subarachnoid haemorrhage with magnetic resonance imaging. *J Neurol Neurosurg Psychiatry*, 70:205–211, 2001.

[34] Z. Rumboldt, M. Kalousek, and M. Castillo. Hyperacute subarachnoid hemorrhage on T2-weighted MR images. *AJNR Am J Neuroradiol*, 24:472–405, 2003.

[35] RJ Woodcock Jr, J Short, HM Do, ME Jensen, and DF Kallmes. Imaging of acute subarachnoid hemorrhage with a fluid-attenuated inversion recovery sequence in an animal model: comparison with non-contrast-enhanced CT. *AJNR Am J Neuroradiol*, 22:1698–2303, 2001.

[36] M. Wiesmann, T.E. Mayer, I. Yousry, R. Medele, G.F. Hamann, and H. Bruckmann. Detection of hyperacute subarachnoid hemorrhage of the brain by using magnetic resonance imaging. *J Neurosurg*, 96:684–609, 2002.

[37] H Quincke. Die Lumbalpunktion des Hydrocephalus. *Berliner Klinische Wochenschrift*, 28:929–932, 1891.

[38] C.J. Eskey and C.S. Ogilvy. Fluoroscopy-guided lumbar puncture: decreased frequency of traumatic tap and implications for the assessment of CT-negative acute subarachnoid hemorrhage. *AJNR Am J Neuroradiol*, 22:571–506, 2001.

[39] KI Marton and AD Gean. The spinal tap: a new look at an old test. *Ann Intern Med*, 104:840–848, 1986.

[40] A. Petzold, G. Keir, and T.L. Sharpe. Why human color vision cannot reliably detect cerebrospinal fluid xanthochromia. *Stroke*, 36:1295–1297, 2005.

[41] WW Tourtelotte, LN Metz, ER Bryan, and DeJong RN. Spontaneous subarachnoid hemorrhage — Factors affecting the rate of clearing of the cerebrospinal fluid. *Neurology*, 14:302–306, 1964.

[42] KG Kjellin and CE Soderstrom. Diagnostic significance of CSF spectrophotometry in cerebrovascular diseases. *J Neurol Sci*, 23:359–369, 1974.

[43] WD Wright and FHG Pitt. Hue discrimination in normal colour-vision. *Proceedings of the Physical Society (London)*, 46:459–473, 1934.

[44] EG Boring. *Sensation and Perception in the history of Experimental Psychology*. Appleton-Century-Crofts, New York, USA., 1942.

[45] M.E. Chevreul. De la loi du contraste simultane des couleurs. *Pitois Levreault (Paris)*, 1839.

[46] A Stockman and L T Sharpe. Spectral sensitivities of the middle- and long-wavelength sensitive cones derived from measurements in observers of known genotype. *Vision Research*, 40:1711–1737, 2000.

[47] F.H. Linn, H.A. Voorbij, G.J. Rinkel, A. Algra, and J. van Gijn. Visual inspection versus spectrophotometry in detecting bilirubin in cerebrospinal fluid. *J Neurol Neurosurg Psychiatry*, 76:1452–1404, 2005.

[48] C.E. Soderstrom. Diagnostic significance of CSF spectrophotometry and computer tomography in cerebrovascular disease. A comparative study in 231 cases. *Stroke*, 8:606–612, 1977.

[49] A.H. Chalmers and M. Kiley. Detection of xanthochromia in cerebrospinal fluid. *Clin Chem*, 44:1740–1702, 1998.

[50] AH Chalmers. Cerebrospinal fluid xanthochromia testing simplified. *Clin Chem*, 47:147–148, 2001.

[51] JPJ Ungerer, SH Southby, M Florkowski, and PM George. Automated measurement of cerebrospinal fluid bilirubin in suspected subarachnoid hemorrhage. *Clin Chem*, pages 1854–1856, 2004.

[52] KM Chan, MG Scott, TW Wu, et al. Inaccurate values for direct bilirubin with some commonly used direct bilirubin procedures. *Clin Chem*, 31:1560–1563, 1985.

[53] D Patel, P White, W Egner, and R Beetham. National guidelines for analysis of CSF bilirubin and their impact. *Proceedings of the ACB National Meeting*, ISSN 0959-9029:116, 18-20 May 2005.

[54] CM Fisher, JP Kistler, and JM Davis. Relation of cerebral vasospasm to subarachnoid hemorrhage visualized by computerized tomographic scanning. *Neurosurgery*, 6:1–9, 1980.

[55] K Rejdak, A Petzold, MA Sharpe, et al. Cerebrospinal fluid nitrite/nitrate correlated with oxyhemoglobin and outcome in patients with subarachnoid hemorrhage. *J Neurol Sci*, 219:71–6, 2004.

[56] RM Pluta, JK Afshar, RJ Boock, and EH Oldfield. Temporal changes in perivascular concentrations of oxyhemoglobin, deoxyhemoglobin,

and methemoglobin after subarachnoidhemorrhage. *J Neurosurg*, 88:557–561, 1998.

[57] N.G. Wahlgren and K. Bergstrom. Determination of haem derivatives in the cerebrospinal fluid–a. *J Neurol Neurosurg Psychiatry*, 46:653–608, 1983.

[58] J.W. Stroes and H.J. van Rijn. Quantitative measurement of blood pigments in cerebrospinal fluid by derivative spectrophotometry. *Ann Clin Biochem*, 24 (Pt 2):189–97, 1987.

[59] H.J. Duiser, F.W. Roelandse, E.G. Lentjes, J. van Loon, J.H. Souverijn, and A. Sturk. Iterative model for the calculation of oxyhemoglobin, methemoglobin, and bilirubin in absorbance spectra of cerebrospinal fluid. *Clin Chem*, 47:338–341, 2001.

[60] O.F. Laterza, C.H. Smith, T.R. Wilhite, and M. Landt. Accurate direct spectrophotometric bilirubin measurement combined with blood gas analysis. *Clin Chim Acta*, 323:115–20, 2002.

[61] FJ Gillingham. The management of ruptured intracranial aneurysm. *Ann R Coll Surg (English)*, 23:89–117, 1958.

[62] B Weir, M Grace, J Hansen, and C Rothberg. Time course of vasospasm in man. *J Neurosurg*, 48:173–108, 1978.

[63] N Janjua and SA Mayer. Cerebral vasospasm after subarachnoid hemorrhage. *Curr Opin Crit Care*, 9:113–109, 2003.

[64] G Keir, N Tasdemir, and EJ Thompson. Cerebrospinal-fluid ferritin in brain necrosis - evidence for local synthesis. *Clin Chim Acta*, 216:153–166, 1993.

[65] DR Campbell, BS Skikne, and JD Cook. Cerebrospinal fluid ferritin levels in screening for meningism. *Arch Neurol*, 43:1257–1260, 1986.

[66] CJ Sindic, D Collet-Cassart, CL Cambiaso, PL Masson, and EC Laterre. The clinical relevance of ferritin concentration in the cerebrospinal fluid. *J Neurol Neurosurg Psychiatry*, 44:329–333, 1981.

[67] R Hallgren, A Terent, L Wide, K Bergstrom, and G Birgegard. Cerebrospinal fluid ferritin in patients with cerebral infarction or bleeding. *Acta Neurol Scand*, 61:384–392, 1980.

[68] N Gruener, O Gozlan, T Goldstein, J Davis, I Besner, and TC Iancu. Iron, transferrin, and ferritin in cerebrospinal fluid of children. *Clin Chem*, 37:263–205, 1991.

[69] A Petzold and LT Sharpe. Visual assessment for xanthochromia needs no revisitation. *J Neurol Neurosurg Psychiatry*, 76:http://jnnp.bmjjournals.com/cgi/eletters/76/10/1452, 2005.

Table 1: *Other conditions that may potentially give rise to a "xanthochromic" CSF.*

Carotinoids
Compounds containing Bromide
Contamination with betadine or iodine (i.e. during surgery)
High CSF total protein
High systemic bilirubin
Other sources of a bleed in the neuroxis (i.e. haemosiderosis)
Rifampicin
Tissue necrosis within the CSF

Table 2: *Human color vision is less sensitive than spectrophotometry for detecting small amounts of bilirubin from the CSF (data from references[40,47]). The table is reproduced with permission from reference.[69]*

Petzold *et al.*					Linn *et al.*				
Excitation purity (%)	Color vision (observed frequency)	Spectrophotometry (observed frequency)	χ^2	P	Extinction (at 450-460 nm)	Color vision (observed frequency)	Spectrophotometry (observed frequency)	χ^2	P
36.6	1.00	1.00	...	NS	—	—	—	—	
18.2	1.00	1.00	...	NS	—	—	—	—	
9.3	1.00	1.00	...	NS	0.09	1.00	1.00	...	NS[a]
4.8	0.90	1.00	...	NS	0.07	1.00	1.00	...	NS
2.4	0.45	1.00	8.25	$<$0.01	0.06	0.95	1.00	4.67	$<$0.05
1.66	0.00	1.00	22.0	$<$0.001	0.04	0.72	1.00	31.7	$<$0.0001
1.04	0.00	1.00	22.0	$<$0.001	0.02	0.09	1.00	157.9	$<$0.0001
0.62	0.00	1.00	22.0	$<$0.001	0.01	0.12	1.00	148.1	$<$0.0001

[a]The statistics are based on the numbers from Table 1 in Linn *et al.*: for an extinction of 0.09 none of the clinicians or medical students saw the sample as colorless (n=0), 50 clinicians and 49 students saw the sample as yellow (n=99), and 1 clinician and 2 students were undecided and therefore left out of the analysis. In contrast, spectrophotometry clearly revealed the presence of bilirubin and all clinicians and students would have interpreted the spectrophotometric trace in the same way (n=102). The numbers for the other extinctions were analyzed analogously.

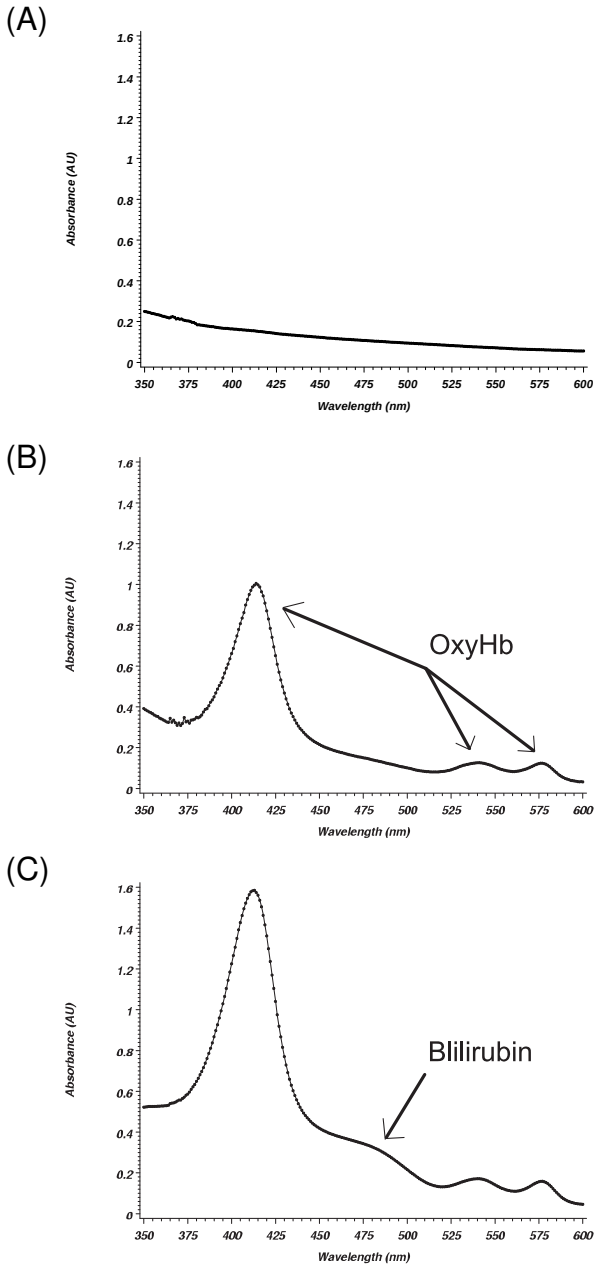

Figure 1: *Examples for (A) a spectrophotometric scan showing a normal CSF. (B) a spectrophotometric scan showing the presence of oxyhemoglobin in the CSF. The highest peak for oxyhemoglobin is observed at 415 nm and secondary, much smaller peaks are observed between 525–600 nm. (C) a spectrophotometric scan showing the presence of bilirubin and oxyhemoglobin in the CSF. This scan is consistent with a SAH. For practical reasons the absorbance for bilirubin will be read at 476 nm, which is outside the absorbance for oxyhemoglobin. It should be noted that the maximum of the broad bilirubin broad peak lies within 450–460 nm.*

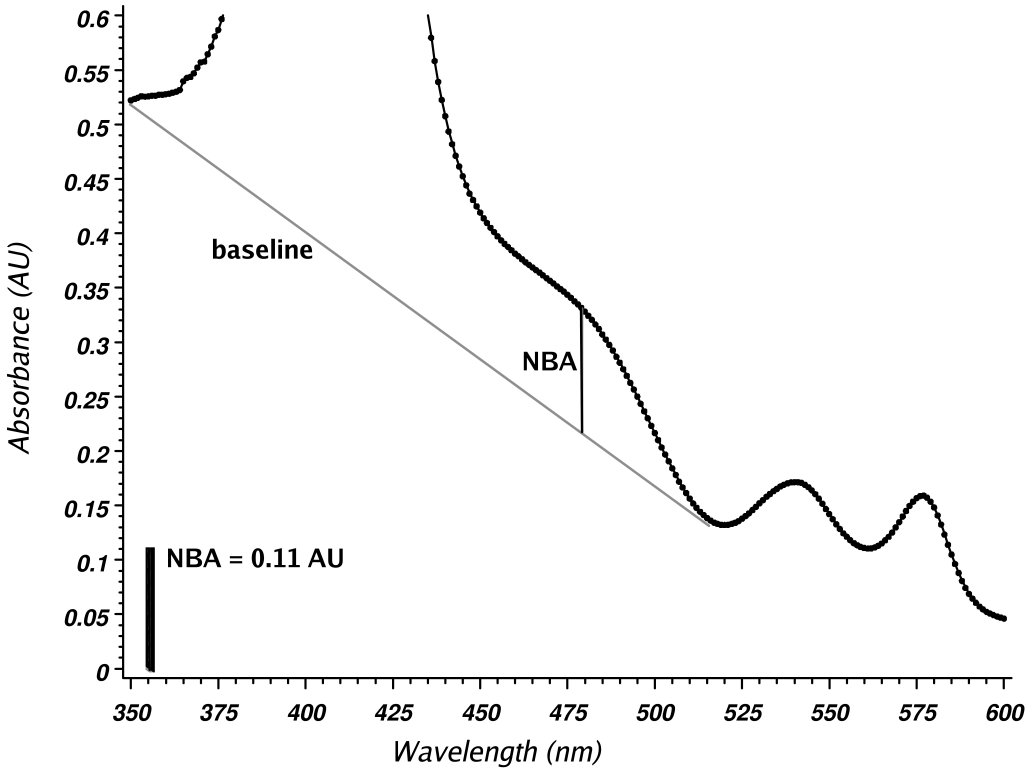

Figure 2: *An example of "how to" calculate the absorbance (AU) of bilirubin. A baseline is drawn from the beginning of the scan at 300 nm to the bottom deflection at about 510 nm. The distance from this baseline to the scan is measured at 476 nm to obtain the net bilirubin absorbance (NBA). In this example, which represents an enlarged view of Figure 1 (C), a NBA of 0.11 AU is calculated. For illustration, the line drawn to determine the NBA is also demonstrated in the bottom left corner of the graph.*

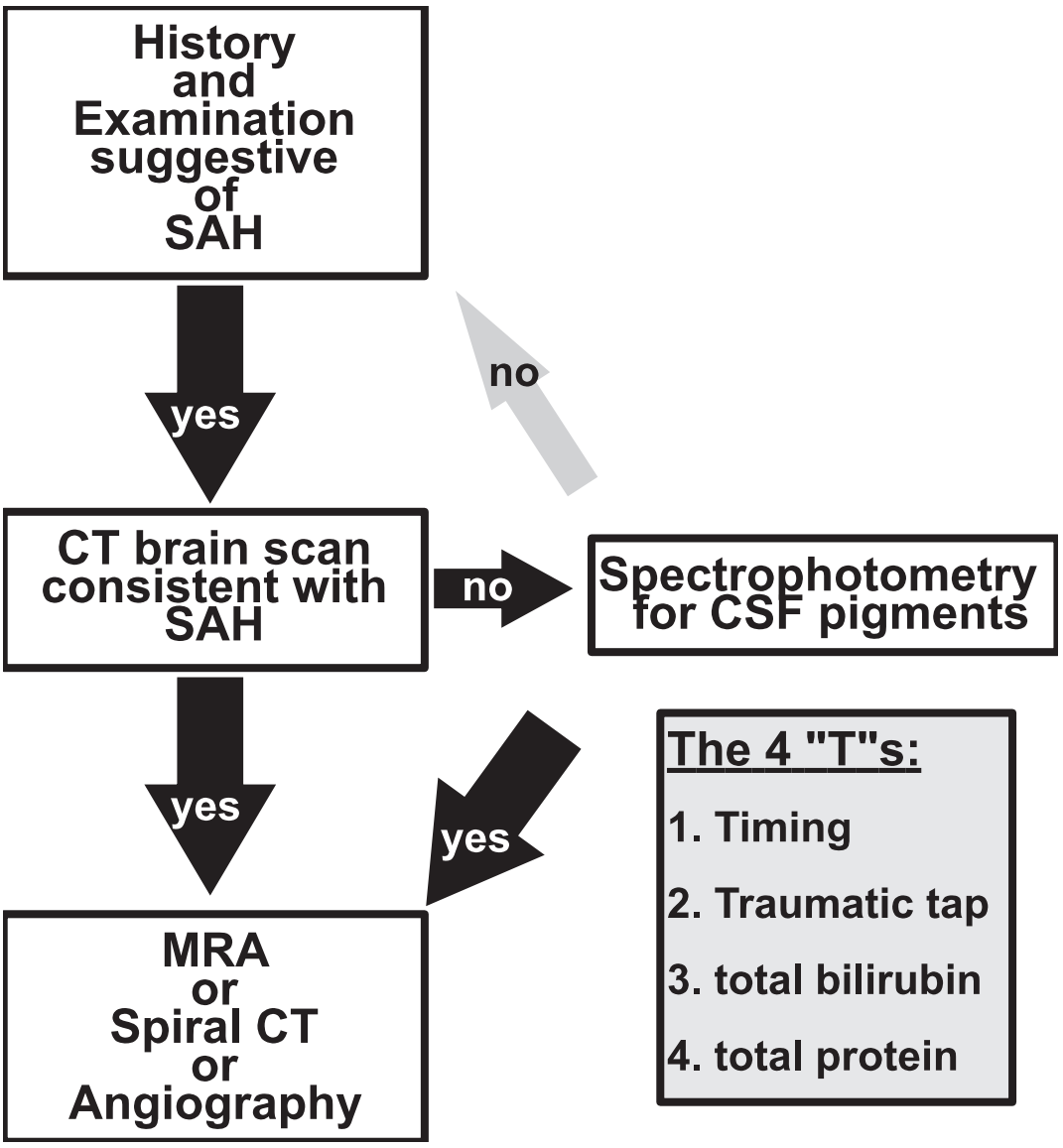

Figure 3: *A diagnostic flow–chart for assessment of a patient with suspected SAH. CT = non–enhancing computed tomography of the brain, CSF = cerebrospinal fluid, MRA = magnetic resonance angiography, SAH = subarachnoid hemorrhage.*