National guidelines for analysis of cerebrospinal fluid for bilirubin in suspected subarachnoid haemorrhage

UK National External Quality Assessment Scheme for Immunochemistry Working Group

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Abstract

It is crucially important to detect subarachnoid haemorrhage (SAH) in all patients in whom it has occurred in order to select patients for angiography and preventative surgery. A computed tomography (CT) scan is positive in up to 98% of patients with SAH presenting within 12 h but is positive in only 50% of patients presenting within 1 week.

Cerebrospinal fluid (CSF) bilirubin spectrophotometry can be used to determine the need for angiography in those few CT-negative patients in whom clinical suspicion of a SAH remains high; it may remain positive for up to 2 weeks after the event. The lumbar puncture (LP) should only be performed >12 h after the onset of presenting symptoms. Whenever possible, collect four sequential CSF specimens. Always ensure that the last CSF sample taken is sent for bilirubin analysis. Protect the CSF from light and avoid vacuum tube transport systems if possible.

Always use spectrophotometry in preference to visual inspection. All CSF specimens are precious and should be analysed no matter how they were transported, where necessary with appropriate notice of the caveats regarding oxyhaemoglobin. Centrifuge the specimen at >2000 rpm for 5 min as soon as possible after receipt in the laboratory and in any case within 1 h of collection. Store the supernatant at 4°C in the dark until analysis.

An increase in CSF bilirubin is the key finding which supports the occurrence of SAH, but it is not specific for this. In most positive cases bilirubin will occur with oxyhaemoglobin. Oxyhaemoglobin occurring on its own is difficult to interpret and may be increased as a result of *in vitro* haemolysis of red cells introduced into the CSF during lumbar puncture. This process is exacerbated by vacuum tube transport systems. Results should be interpreted in the light of other investigations (e.g. if scan shows bilirubin then measure serum bilirubin and CSF oxyhaemoglobin and protein) and other confounding variables such as the time elapsed from presentation to LP.

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Introduction

Subarachnoid haemorrhage (SAH) is spontaneous arterial bleeding into the subarachnoid space, usually from a cerebral aneurysm.[1] Patients who have bled, and in whom the diagnosis is initially missed, often present with a further bleed, in a poorer condition and with a worse outcome than those in whom the correct diagnosis is made promptly.[2,3] It is thus crucially important to detect SAH in all patients in whom it has occurred.

The initial investigation, the demonstration of blood on a computed tomography (CT) scan, will, in experienced hands, be positive in 98% of patients with SAH presenting within the first 12 h after an event,[4] but positivity falls with time to about 50% in patients presenting after 1 week.[5] Patients with a positive CT usually proceed to catheter angiography to confirm the presence of an aneurysm and locate its site so that it can be treated to prevent a re-bleed. Catheter angiography, a resource-intensive and invasive procedure, carries a small but definite risk of morbidity and mortality.[6] There is thus a need for a procedure to

detect those CT-negative patients presenting with a history suggestive of SAH who actually have sustained a SAH[4] and to eliminate the diagnosis in the remainder without the need for catheter angiography. Best estimates are that a UK hospital may see up to 150 patients per annum with symptoms of SAH who are CT-negative; some 2–3% of these will be proven to have a ruptured aneurysm.[4]

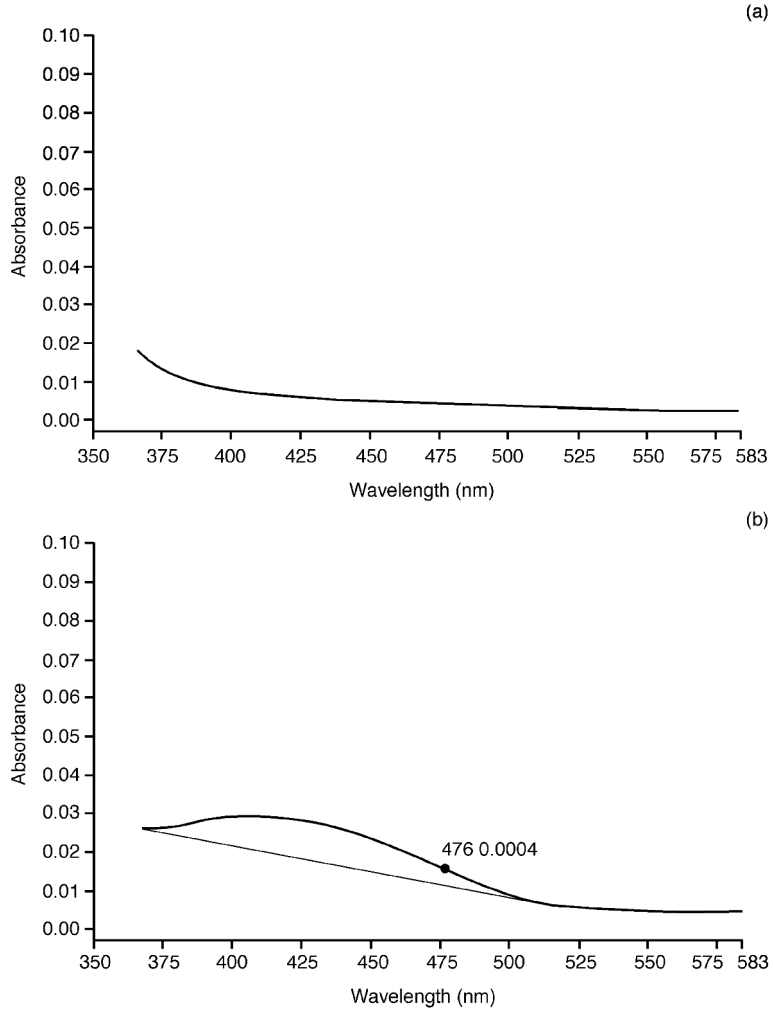
Following haemorrhage into the CSF, red blood cells undergo lysis and phagocytosis; the liberated oxyhaemoglobin is converted *in vivo* in a time-dependent manner into bilirubin[7] and sometimes methaemoglobin.[8] Of these three pigments, only bilirubin arises solely from *in vivo* conversion. Oxyhaemoglobin and methaemoglobin may both be produced *in vitro* as well as *in vivo*.[9]

Bilirubin may be detected in CSF by spectrophotometry or by visual inspection for the yellow discoloration (xanthochromia) it imparts to CSF. Evidence clearly indicates that visual inspection is not a reliable method.[10,11] Spectrophotometry to detect bilirubin is of particular value in the investigation of a CSF sample with an increased erythrocyte count as there is no other reliable way for distinguishing between SAH and a traumatic lumbar puncture. It is also of value in the investigation of CSF with a normal red cell count from a patient presenting several days after an event, by which time the cells may no longer be present.

We now propose guidelines for the specimen requirements, transport, handling, analysis of CSF and interpretation in patients with a suspected SAH but a negative CT scan. Notes to these guidelines, printed as Appendix 1, provide the reasoning behind our recommendations.

Specimen requirements and transport

A protocol for specimen requirements and transport is provided in Appendix 2, although modification may be required to meet local needs. Readers should also refer to Appendix 1, Notes to the Guidelines. Essentially, the requirements are:



- Whenever possible, collect four sequential specimens.
- The specimen for spectrophotometry should always be the last fraction of CSF to be taken, and ideally at least the fourth.
- The volume requested must be that which enables the analysis to be undertaken without dilution, and will be determined by local requirements.
- The specimen should be protected from light.
- Use of pneumatic tube systems to transport the specimen to the laboratory should be avoided.[12]

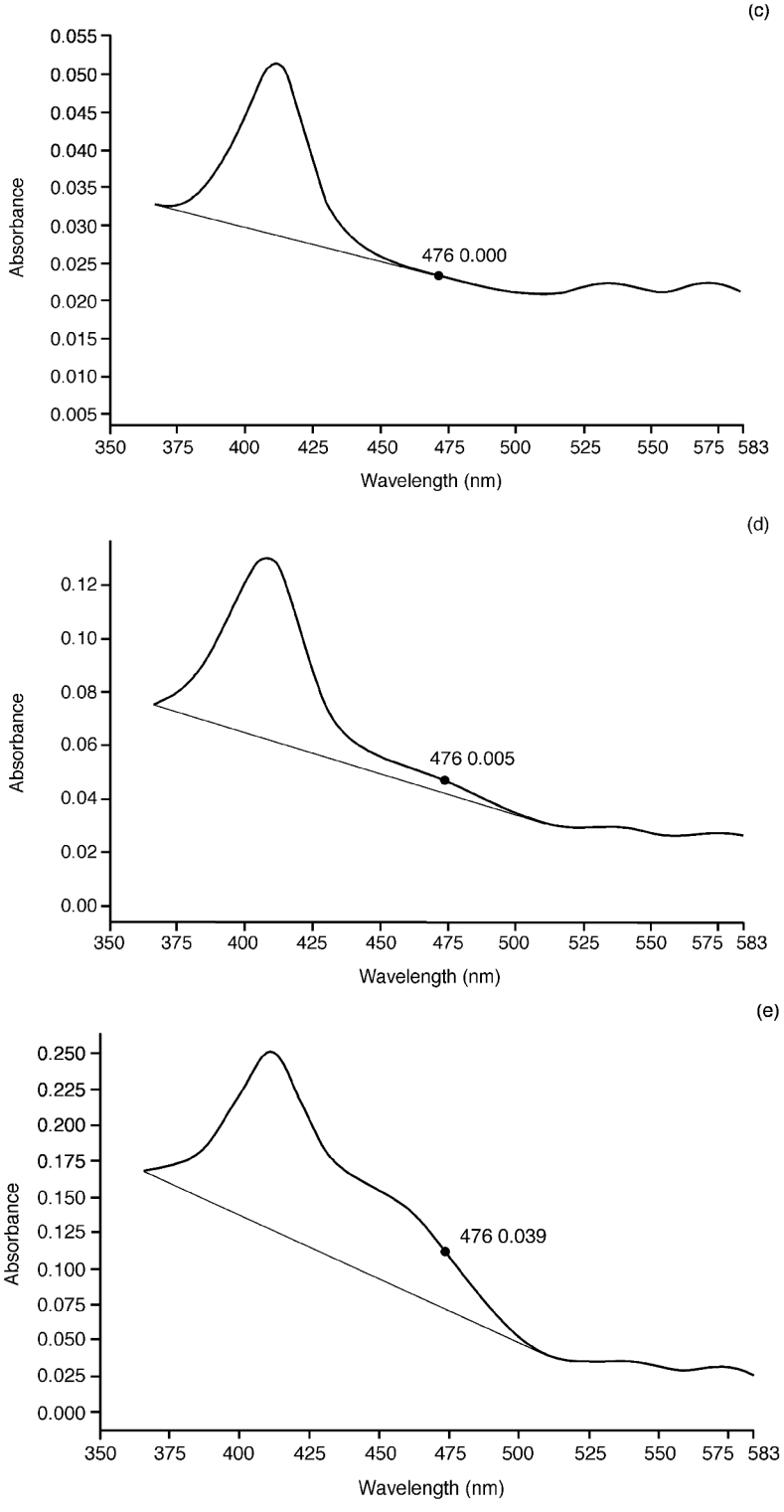


Figure 1(a) to (e). *Representative spectrophotometric scans showing net bilirubin absorbance (NBA) at 476 nm above a tangential baseline, as described in the text. (a) Normal cerebrospinal fluid with essentially no bilirubin; scan and baseline (not drawn) are superimposable. (b) NBA within the reference range. (c) Oxyhaemoglobin with zero NBA. (d) Oxyhaemoglobin with NBA within the reference range. (e) Oxyhaemoglobin with an increased NBA. (In practice, such scans are best visualized filling the whole of an A4 page in landscape mode.)*

- A simultaneous blood specimen should be taken for serum bilirubin and total protein measurement.
- Record the timing of sampling relative to that of possible haemorrhage. This should be no less than 12 h.

It is advised that prospective protocols are discussed with users of the service.

Specimen handling

The specimen designated for spectrophotometry should be centrifuged at >2000 rpm for 5 min as soon as possible after receipt in the laboratory and in any case within 1 h of collection. The supernatant should be stored in the dark at 4°C until analysis.

Analysis

- Perform a zero-order spectrophotometric scan on the supernatant between 350 and 600 nm using a recording spectrophotometer and a cuvette with a 1-cm path length. Use an initial full-scale deflection (FSD) of 0.1 absorbance units (AU). If any peaks exceed 0.1 AU, scale as appropriate but never use a FSD less than 0.1 AU.
- The specimen should not be diluted.

Inspect the scan and identify and record the presence of the following haem pigments:

- *Oxyhaemoglobin*: absorbance maximum between 410 and 418 nm.
- *Bilirubin*: either a broad peak in the range 450 to 460 nm or a shoulder adjacent to an oxyhaemoglobin peak if present.
- *Methaemoglobin:* the rarest pigment, and if present usually manifest as a broader peak than oxyhaemoglobin, occurring between 403 and 410 nm.

Calculate the net bilirubin absorbance (NBA) according to Chalmers' modification[13] to the original method of Chalmers and Kiley[14] as follows:

- Draw a predicted baseline which forms a tangent to the scan between 350 and 400 nm and again between 430 and 530 nm. This baseline should never cut the scan.
- Measure the absorbance of the scan above this predicted baseline at 476 nm; this is the NBA. If the baseline forms a tangent to the scan before 476 nm, then the measured NBA is by definition zero.
- Also measure the absorbance of any oxyhaemoglobin peak above this predicted baseline; this is the net oxyhaemoglobin absorbance (NOA).

Illustrative zero-order spectra are shown in Figs 1(a) to (e).

Reporting and interpretation

The following is the most appropriate advice that we can provide regarding reporting and suggested interpretative comments. Readers should refer to Appendix 1, Notes to the Guidelines, for further details. For each case, the final interpretation should take into account the known dynamic production of haem pigments following a bleed, as outlined in the Introduction. Thus, most positive cases exhibit both oxyhaemoglobin and bilirubin. Oxyhaemoglobin occurring on its own may, unusually, be found early on after a bleed (particularly if the absorbance of the oxyhaemoglobin peak is sufficiently great to obscure a small but significant amount of bilirubin – see below). Bilirubin occurring on its own would not be expected within the first few days, but becomes an increasingly possible finding as the second week progresses.

NBA $\leqslant 0.007$ AU and no oxyhaemoglobin present

Report as: 'No significant bilirubin and no oxyhaemoglobin present. No evidence to support subarachnoid haemorrhage.'

NBA $\leqslant 0.007$ AU and oxyhaemoglobin present but NOA < 0.1 AU

Report as: 'Oxyhaemoglobin present but no significant bilirubin. Oxyhaemoglobin on its own has a low predictive value for subarachnoid haemorrhage but does not exclude.'

NBA $\leqslant 0.007$ AU and NOA $\geqslant 0.1$ AU

Report as: 'Oxyhaemoglobin present but no significant bilirubin. NB The concentration of oxyhaemoglobin may mask a small but significant increase in bilirubin. Subarachnoid haemorrhage not excluded.'

NBA > 0.007 AU and no oxyhaemoglobin present

(a) Serum bilirubin $\leqslant 20\,\mu$mol/L and CSF protein $\leqslant 1.0$ g/L.

Report as: 'Increased CSF bilirubin but no oxyhaemoglobin. Consistent with subarachnoid haemorrhage.' (NB This would be an unusual pattern within the first week after an event.)

(b) Serum bilirubin $> 20\,\mu$mol/L and CSF protein $\leqslant 1.0$ g/L.

Apply formula to calculate an adjusted NBA (see Appendix 3).

If adjusted NBA $\geqslant 0.007$ AU, report as: 'Increased CSF bilirubin but no oxyhaemoglobin. Consistent with subarachnoid haemorrhage.' (NB This would be an unusual pattern within the first week after an event).

If adjusted NBA < 0.007 AU, report as: 'Increased CSF bilirubin but probably totally accounted for by increase in serum bilirubin. No oxyhaemoglobin. Not supportive of subarachnoid haemorrhage.'

(c) CSF protein >1.0 g/L, whatever the serum bilirubin.

Report as: 'Increased CSF bilirubin, but no oxyhaemoglobin. This finding may be consistent with: subarachnoid haemorrhage; an increased bilirubin accompanying the increased CSF protein; or other source of CSF blood. Interpret result with caution in relation to subarachnoid haemorrhage, especially if within first week of event.'

NBA >0.007 AU and oxyhaemoglobin present but NOA $\leqslant 0.1$ AU

(a) Serum bilirubin $\leqslant 20$ μmol/L and CSF protein $\leqslant 1.0$ g/L.

Report as: 'Increased CSF bilirubin with oxyhaemoglobin present. Consistent with subarachnoid haemorrhage.'

(b) Serum bilirubin >20 μmol/L and CSF protein $\leqslant 1.0$ g/L.

Apply formula (Appendix 3) to calculate adjusted NBA.

If adjusted NBA $\geqslant 0.007$ AU, report as: 'Increased CSF bilirubin with oxyhaemoglobin present. Consistent with subarachnoid haemorrhage.'

If adjusted NBA <0.007 AU, report as: 'Increased CSF bilirubin but probably totally accounted for by an increased serum bilirubin. Oxyhaemoglobin present which on its own has a low predictive value for subarachnoid haemorrhage, although does not totally exclude.'

(c) CSF protein >1.0 g/L, whatever the serum bilirubin.

Report as: 'Increased CSF bilirubin with oxyhaemoglobin present. This finding may be consistent with: subarachnoid haemorrhage; an increased bilirubin accompanying the increased CSF protein; or other source of CSF blood.'

NBA >0.007 AU and NOA >0.1 AU

(a) CSF protein $\leqslant 1.0$ g/L, whatever the serum bilirubin.

Report as: 'Increased CSF bilirubin with oxyhaemoglobin present. Consistent with subarachnoid haemorrhage.'

(b) CSF protein >1.0 g/L, whatever the serum bilirubin.

Report as: 'Increased CSF bilirubin with oxyhaemoglobin present. This finding may be consistent with: subarachnoid haemorrhage; an increased

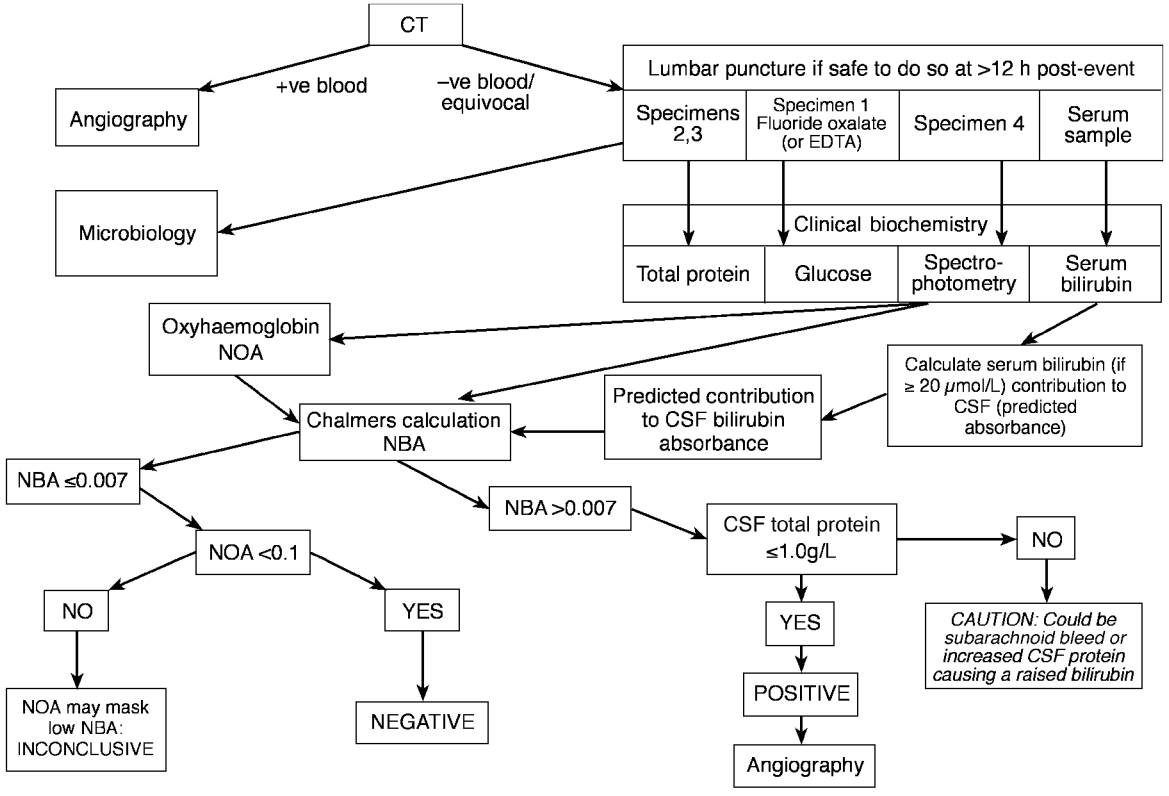


Figure 2. *Bilirubin absorbance in cerebrospinal fluid (CSF) for detection of subarachnoid bleeding. CT = computed tomography; +ve = positive; −ve = negative; NOA = net oxyhaemoglobin absorbance; NBA = net bilirubin absorbance. (NB. The disposition of specimens for microbiological examination and total protein will be subject to local arrangement.)*

bilirubin accompanying the increased CSF protein; or other source of CSF blood.'

Methaemoglobin detected

This is an unusual finding and probably related to artefactual conversion of oxyhaemoglobin. Therefore, when methaemoglobin is present the significance of the finding is the same as if oxyhaemoglobin had been detected. Report as under 2, 3, 5 and 6, substituting methaemoglobin for oxyhaemoglobin.

NB When reporting on spectrophotometry, bear in mind that: (a) a normal erythrocyte count in a CSF taken definitely between 12 and 72 h after an event is evidence against a SAH; and (b) spectrophotometric findings on a CSF taken at a second or subsequent lumbar puncture some hours or more after the previous puncture only reflect the probability that blood has been introduced traumatically into the subarachnoid space at an earlier puncture.

Decision tree

A decision tree (*see* Fig. 2) outlines the steps involved in producing the key laboratory information for the detection of a subarachnoid bleed. (A colour version of Fig. 2 is available from the corresponding author.)

Standards based on these guidelines

- The laboratory should provide instructions for users which provide details of requesting, specimen requirements, transport and interpretation (see example in Appendix 2).
- There should be in place Standard Operating Procedures (SOPs) for specimen handling, analysis, reporting and interpretation.
- The laboratory must participate in an appropriate external quality assurance scheme.
- It is unlikely that a laboratory will build up sufficient expertise unless a minimum of 25 specimens are analysed annually.
- The nature of the analytical service which a laboratory provides, e.g. whether it is available only within certain hours or at all times, will be dependent upon local needs. In particular, these will be determined by the tertiary centre's referral policy, access to its beds and availability of angiography. Both the analytical and interpretative aspects of the service should be provided together.
- To meet the requirements of clinical governance, all spectrophotometric scans should be kept in an appropriate form for recall for a minimum of 2 years.
- Spectrophotometers should be serviced regularly and undergo regular absorption and wavelength checks.

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Appendix 1. Notes to the guidelines

(a) In addition to the oxyhaemoglobin which appears after a SAH, it also commonly arises either from the *in vitro* lysis of red cells in the CSF obtained following puncture, or from the trauma of the puncture itself. As explained in note (g) below, such oxyhaemoglobin may interfere with the detection of bilirubin and is a confounding element in interpretation. Therefore, every effort should be made to eliminate it. It is for this reason that CSF taken for spectrophotometry should be collected into a separate container to those in which the first few mL of fluid are placed, and also why transport by pneumatic tube is not recommended.

(b) As explained in note (j), even small increases above the reference range are sufficient to be consistent with a SAH and therefore indicate the need for angiography. Dilution of the specimen will decrease the certainty with which such increases may be detected.

(c) Stability studies have shown that CSF stored in a plastic tube and exposed to spring daylight through a north-facing window showed a bilirubin decay rate of at least 0.005 AU/h. CSF specimens must therefore be protected from light to avoid this phenomenon, which may lead to false negative results.

(d) Current consensus is that CSF should not be examined for bilirubin earlier than 12 h after an event. This is based on two strands of evidence:

(i) That bilirubin forms 9–15 h after a bleed.[7] We have been unable to review the evidence on which this statement has been made.
(ii) That in a series of 111 patients positive for blood on CT, all subject to lumbar puncture after 12 h, xanthochromia was present in all.[19] This evidence must be reviewed with caution due to the ambiguous definition of xanthochromia.

It is also commonly believed that xanthochromia will be evident in all patients up to 2 weeks following a bleed. Again, this is based on an inappropriate

group – those who were positive for blood on CT.[19] In patients who are negative for blood on CT who may be negative due to late presentation or small bleeds, we cannot be certain about this period of 2 weeks. In our experience, we have detected an increased CSF bilirubin in two patients subsequently shown to have ruptured aneurysms, in whom the CSF was taken at 11 and 14 days after the onset of symptoms.

(e) Derivative spectroscopy has been found to be of value by some analysts, but requires considerable experience in interpretation. It is therefore not recommended.

(f) We have confirmed that, on 58 CSF specimens with bilirubin NBA 0.003–0.251 AU (24 of which contained oxyhaemoglobin in addition to bilirubin), there was no significant difference between the NBA obtained by the original Chalmers and Kiley method[14] and that by the modification of Chalmers.[13]

(g) Out of 740 spectrophotometric scans reviewed from CT-negative patients in four participating centres, 425 had no oxyhaemoglobin and NBA $\leqslant 0.007$ AU. Angiograms were performed in 31 of these 425 patients and no aneurysms were found.

(h) From the same series, 204 CSF samples were reported as containing oxyhaemoglobin with NBA $\leqslant 0.007$ AU. Twenty-nine of these patients had angiography, and in only two instances was an aneurysm found. Oxyhaemoglobin thus has a low predictive value for SAH. However, we recognize that rarely, early on after a bleed, oxyhaemoglobin may be present without bilirubin.

(i) Experiments using a combination of increasing bilirubin and oxyhaemoglobin concentrations have indicated that oxyhaemoglobin causes an underestimation of NBA by approximately 0.001 AU for every 0.030 AU of NOA. To prevent undue complexity, we incorporate this into interpretation for NOA > 0.1 AU.

(j) Originally, Chalmers and Kiley[14] indicated a reference range for NBA of 0 to 0.007 AU; values of 0.010 to 0.015 AU were classed as equivocal and values > 0.015 AU as positive. In the series quoted above, CSFs from three patients with proven ruptured aneurysms yielded NBAs of 0.008, 0.015 and 0.016 AU. In addition, we are aware of three CT-positive patients with proven aneurysms whose CSFs yielded NBAs of 0.008, 0.012 and 0.019 AU. We therefore recommend that NBA values > 0.007 AU are a clear indication for angiography. In the series quoted above, 27 patients with NBA > 0.007 AU proceeded to angiography, of which 12 were found to have aneurysms.

(k) While there is documented evidence for the production of methaemoglobin following SAH, it was such an uncommon finding in the series quoted (in four patients, one of whom was angiography-positive) that no clear indication of its significance could be obtained. Very recent work, which needs to be confirmed, has implicated high levels of iodine (widely used as a skin disinfectant) as being involved in *in vitro* methaemoglobin formation.

Appendix 2. Exemplar protocol for the collection, handling and transport to the laboratory of cerebrospinal fluid requiring spectrophotometric scanning for the detection of bilirubin

Principle

This test is performed to try to identify those patients who have had a SAH but in whom the CT scan is negative. The spectrophotometric scan detects bilirubin in CSF, and this finding is consistent with a bleed into the CSF.

The formation of bilirubin after haemorrhage is a time-dependent process and bilirubin may not be detectable soon after the event (e.g. onset of severe headache). On current evidence, it is recommended that CSF is not sampled until at least 12 h after a suspected event. The opening pressure should always be recorded when performing a lumbar puncture. Lumbar puncture is contra-indicated in patients with papilloedema, focal neurological deficit or reduced consciousness.

Please indicate on the request form:

- Clinical indication for request
- Result of CT scan
- Time of onset of symptoms/event
- Time of lumbar puncture
- If the differential diagnosis includes meningitis

Specimens

- Cerebrospinal fluid may also be required for microbiological examination and for protein and glucose estimation. Sufficient CSF will therefore be needed for all of these required investigations.
- Label three 28-mL sterile universal containers and one *yellow-top fluoride EDTA tube* with the patient's name, hospital number, ward, date of birth, the time that the CSF was obtained and the sequence order of sampling.
- The first specimen should be a *minimum of 0.5 mL* of CSF placed in a *yellow-top fluoride EDTA tube for glucose* and *protein* estimations. This specimen should be sent to the *clinical biochemistry department*.
- *Microbiology* requires *at least* 5 *mL* of CSF divided into *two* sequentially numbered, sterile 28-mL universal containers labelled 'second' and 'third'.

These two specimens must be delivered to the *microbiology* department as soon as possible. *Use of the pneumatic tube delivery system should be avoided.*

- A further minimum of *1 mL* of CSF should be placed in the final (labelled 'fourth') sterile 28-mL universal container for the spectrophotometric scan. (NB 1 mL is about 20 drops from the Luer connector on a needle). Protect this sample from the light by placing it in a thick brown envelope outside the usual plastic specimen bag.
- A blood specimen should be taken at the same time for serum bilirubin, total protein and glucose estimation, which are needed to aid interpretation.

NB. These samples must also be delivered to the *clinical biochemistry* department as soon as possible. Use of the pneumatic tube delivery system should be avoided.

If this procedure is not followed, analysis is likely to be compromised.

Text in italics indicates those details subject to local requirements.

Appendix 3. Adjustment of net bilirubin absorbance for an increase in serum bilirubin

The predicted absorbance (PA) of a CSF at 476 nm due to bilirubin can be calculated according to the following equation:[15–17]

$$\mathrm{PA} = \frac{\text{CSF total protein (g/L)}}{\text{Serum total protein (g/L)}} \times \text{serum bilirubin } (\mu\text{mol/L}) \times 0.042 \text{ AU} \quad (1)$$

$$\text{Then adjusted NBA} = \text{measured NBA} - \text{PA} \quad (2)$$

We recommend use of this formula because it has been validated for use:

(a) in neonatal jaundice,[18] albeit often at higher bilirubin concentrations than are encountered in adults, and

(b) in a group of 12 patients with increased serum bilirubin and CSF protein up to 1.0 g/L, in whom predicted − actual NBA produced a mean value of −0.002 AU.

We do not recommend adjustment of NBA where the CSF bilirubin is increased due to an increased CSF protein concentration alone, or where there is an increased serum bilirubin concentration and the CSF protein is greater than 1.0 g/L, because of lack of validation.

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