Quantitative Determination of Cerebrospinal Fluid Bilirubin on a High Throughput Chemistry Analyzer

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SUMMARY

Background: Subarachnoid hemorrhage is a condition with high rates of mortality and morbidity. The diagnosis requires an urgent cerebral computed tomography scan and also a lumbar puncture if the scan fails to demonstrate intracranial blood. In Sweden the cerebrospinal fluid (CSF) is analyzed by spectrophotometric scanning for the presence of hemoglobin and bilirubin. The aim of the study was to develop a quantitative diazo reagent based analysis of cerebrospinal fluid bilirubin as a replacement for spectrophotometric scanning.

Methods: The CSF bilirubin assay on an Architect C8000 chemistry analyzer was compared with spectrophotometry using patient samples.

Results: The method correlates with spectrophotometry, has a good linearity and precision.

Conclusions: Quantitative bilirubin measurement offers shorter turnaround times, simplifies the interpretation of the results and reduces work load in comparison with spectrophotometry.


KEY WORDS

Bilirubin, Cerebrospinal Fluid, Diagnosis, Humans, Subarachnoid Hemorrhage

INTRODUCTION

Subarachnoid haemorrhage (SAH) has an annual incidence of approximately 6-20/100,000 and accounts for 3-5% of all strokes [1,2]. SAH is associated with mortality rates up to 50% and many SAH patients will have sequelae such as neurological and cognitive deficits and psychosocial dysfunction [2]. Early diagnosis is of vital importance to minimize mortality and morbidity [3,4]. The investigation of choice for the diagnosis of SAH is computerised tomography (CT). However, CT scans are negative in 2% of patients [5], and cerebrospinal fluid (CSF) spectrophotometry for haem pigments (xanthochromia) is considered an essential investigation in these patients [6]. Some laboratories only perform visual inspection of CSF but it has previously been demonstrated that spectrophotometry has increased sensitivity over visual inspection [6]. The detection of CSF bilirubin is the basis for the laboratory identification of cerebral hemorrhage. Guidelines for the detection of bilirubin have recently been published [7]. In the guidelines, the preferred method for detection of bilirubin is spectrophotometric scanning. Spectrophotometric scanning for diagnosis of SAH is relatively expensive and requires expert interpretation which in our country often is difficult outside office hours.

Recently, quantitative measurement of CSF bilirubin on an automated instrument has been described [8,9]. At a cut-off of 359 nmol/L the method had a high sensitivity and specificity with a negative predictive value of 100% for excluding subarachnoid hemorrhage [8].

Considering the drawbacks of spectrophotometric scanning we wanted to develop a quantitative bilirubin assay that could be available at all hours. We thus modified our routine, diazo reagent based, plasma bilirubin assay on an Architect C8000 (Abbott Laboratories, Abbott Park, IL, USA) to increase the sensitivity of the assay enabling measurement of CSF bilirubin below 350
nmol/L. We also sought to acquire data on the stability of samples stored at different temperatures and exposure to electric or sun light.

**MATERIALS AND METHODS**

**Samples**
Materials from 200 CSF samples were used in the study. These were collected from routine requests that had been decoded prior to the study. The sample collection had been approved by the ethical board at Uppsala University (01-367). The samples were analyzed on an automated chemistry analyzer (Abbott C8000, Abbott Laboratories, Abbott Park, IL, USA) and a spectrophotometer (UV-1601 C, Shimadzu, Kyoto, Japan).

**Billirubin assay**
CSF bilirubin (2, 4-dichloroaniline reagent: REF 6L45-40, Abbott Laboratories) measurements were performed on an Architect C8000 analyzer (Abbott Laboratories) and reported using S.I. units. The total analytical imprecision of the plasma bilirubin assay was 1.4% at 22 μmol/L and 1.1% at 90 μmol/L and the measuring range was 1.7 - 427.5 μmol/L.

The plasma bilirubin method was modified by increasing the sample volume for CSF bilirubin measurements. CSF bilirubin measurements on an Architect C8000 were performed using the following instrument settings: Primary wavelength 548 nm, secondary wavelength 604 nm, 160μL reagent 1 and 40 μL reagent 2 were mixed with 35 μL sample, sample blank position 14-16, reading position 20-22 and linear calibration curve. The standard curve was prepared by diluting the plasma bilirubin calibrator so a standard curve ranging from 302.3 to 9479 nmol/L was obtained. Distilled water was used as blank.

**Linearity test of the CSF bilirubin assay**
A CSF pool was spiked with a plasma sample with high bilirubin content (approx 1000 μmol/L). The pool was then serially diluted 1:1.5, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 with 0.15 M NaCl. The results of the CSF bilirubin assays were compared with the calculated bilirubin concentrations for each dilution.

**Evaluation of suitable controls for the CSF bilirubin measurements**
Two control materials were evaluated: Liquichek Unassayed Chemistry Control (18.4 μmol/L bilirubin, Level I, Bio-Rad Laboratories, Hercules, CA, USA) and Autonorm Human Liquid L-1 (19.1 μmol/L bilirubin, Sero, Billingstad, Norway). The controls were diluted 1:50 each day or diluted and then stored at +8°C. The dilutions were made in distilled water and the controls were tested for up to ten days. Intra-assay CV was calculated by diluting Autonorm Human Liquid L-1 controls and analyzing them ten times.

**Method comparison between bilirubin measurements and spectrophotometry**
80 patient samples were analyzed using an Architect C8000 and spectrophotometry. Absorbance at 360, 415, 455 and 530 nm were measured with the spectrophotometer. The quantitative results from the Architect were compared with the delta absorbance for A455 after subtraction of the baseline between 360 and 530 nm on the Shimadzu UV-1601 C.

**Sample stability**
Sample stability was tested by storing samples for 48 h at 8°C, 6 h at room temperature in the dark, 6 h at room temperature in electric light and 6 h at room temperature in a window exposed to sunlight.

**Hemoglobin effects on bilirubin results**
Red blood cells were first washed in 0.9% NaCl to remove plasma proteins and then diluted with distilled water to lyse the erythrocytes. The hemoglobin concentration of the haemolsate was analyzed on a Celdyn 3200 (Abbott Laboratories). The concentration of the hemoglobin lysate was 40 g/L. A 1:2 serial dilution in 0.15 M NaCl was prepared and the dilutions were mixed with two CSF samples with bilirubin concentrations of approximately 1000 and 8000 nmol/L. The samples were analyzed on the Architect C8000.

**RESULTS**

**Linearity test of the CSF bilirubin assay**
There was a good correlation between calculated and measured CSF bilirubin in serially diluted samples with a slope close to 1.0 (Figure 1).

**Evaluation of suitable controls for the CSF bilirubin measurements**
Autonorm Human Liquid L-1 showed a lower day to day variation than the Liquichek Unassayed Chemistry Control. The Autonorm control showed good stability for more than a week both when prediluted and diluted each day (Figure 2). Intraassay CV calculated from ten repeated tests from the same sample cup with diluted controls was 0.45% at 1720 nmol/L and 1.01% at 303 nmol/L.

**Method comparison between bilirubin measurements and spectrophotometry**
There was a good correlation between bilirubin quantities on the Architect and with spectrophotometry ($R^2 = 0.9852$) (Figure 3).

**Sample stability**
Sample storage for 48 h at 8°C had no effect on the test results. Comparison with samples run after 48 hours storage at 8°C showed a slope of 1.010 and $R^2 = 0.9947$. Sample storage at room temperature in the dark...
Figure 1: Correlation between calculated bilirubin values and measured bilirubin values of a serially diluted sample from the Architect

Figure 2: Stability of control. Comparison between prediluted Autonorm controls (□) used for up to ten days or controls diluted each day (+)
Figure 3: Method comparison between bilirubin measurements on Architect C8000 (y-axis) and spectrophotometry (x-axis)

Figure 4: Comparison between samples analysed prior and after six hour storage at room temperature without exposure to light (nmol/L bilirubin)
for 6 h also showed good agreement with prestorage values (slope 1.0099, \( R^2 = 0.9947 \)) (Fig. 4). Storage at room temperature for six hours with the samples exposed to electric light caused a reduction of the test results by almost 30% (slope 0.733, \( R^2 = 0.9855 \)) while 6 hours exposure of the sample for daylight caused a reduction of the test results by almost 60% (slope 0.413, \( R^2 = 0.9018 \)).

### Hemoglobin effects on bilirubin results

The effect of hemoglobin (range 0.0024–20 g/L) on bilirubin results were tested at approximately 490 and 3800 nmol bilirubin/L. At the lower bilirubin concentration a hemoglobin concentration of 1.25 g/L resulted in an inhibition of 13.5% in comparison with the sample with the lowest hemoglobin concentration. Hemoglobin had less effect when the bilirubin concentration was 3800 nmol/L and the inhibitory effect of 2.5 g hemoglobin/L reduced the bilirubin value by 11%. Lower hemoglobin concentrations had limited effects on the bilirubin analysis.

### DISCUSSION

It is vitally important not to miss the diagnosis of SAH in order to avoid morbidity and mortality related to rebleeding and vasospasm. The main reasons for misdiagnosis are failure to recognize the symptoms, erroneous interpretation of CT data and inappropriate interpretation of CSF blood pigment analysis [3,10]. Once the SAH diagnosis is entertained, the first choice of investigation is a CT scan to detect extravasated blood. In a large, prospective study from the 1980s, CT was normal in 5% of patients with SAH who were investigated within one day [11]. Since then CT methods have improved and ten years later the number of normal CTs had decreased to 2% [5]. Even though the new generation scanners may return an even higher sensitivity [12] it is still widely accepted that a negative CT scan does not exclude an SAH [3]. In this situation lumbar puncture and CSF analysis of blood pigments is the investigation of choice.

Considering the problems of distinguishing between a true acute hemorrhage and a traumatic tap, it has been advocated that the lumbar puncture be delayed until at least 12 hours after the suspected bleed to allow the formation of bilirubin from hemoglobin by hemoglobinase in brain cells [7,13,14]. Such a change in diagnostic strategy takes advantage of CSF bilirubin as a more specific marker of SAH compared to oxyhemoglobin that may be released from lysing erythrocytes in the test tube after a traumatic tap.

Chao et al [8] proposed, based on their own prospective validation study of 193 suspected SAH patients, that quantitative analysis of CSF bilirubin may be used as a screening method to determine which samples need spectrophotometric scanning for confirmation of SAH (using the NBA method according to the UK National Guidelines) [7]. The authors found that the bilirubin assay had a 100% negative predictive value for prediction of elevated NBA at a cut-off of 359 nmol/L (corresponding to a NBA of 0.007). According to clinical records (n=162) there was no evidence of any missed SAH diagnosis in their material. In an editorial comment, Beetham [15] remarks that this approach challenges the accuracy and external quality control needs of the analytical method given the very low bilirubin levels in CSF, and runs the risk of spectrophotometric scanning eventually being performed only at a few centers of excellence leading to an unacceptable analytical delay.

In our region (catchment area of 1.9 million inhabitants) the referral of patients with suspected SAH and a negative CT scan to the surgical centre in Uppsala is based on spectrophotometric scanning of CSF samples. The subjective evaluation of spectrophotometry data according to our current routine complicates clinical interpretation and the dialog between the referring physician and the neurosurgeon on call. This leads to an over-referral of patients and many unnecessary angiographic investigations.

We are therefore aiming at replacing spectrophotometric scanning with a CSF bilirubin assay combined with a single absorbance measurement at 415 nm for estimation of oxyhemoglobin content. These analyses can be performed in all local hospitals in our region on a 24 hour basis and would provide a quantitative basis for a referral decision. The first step in this process was to validate a modified, widely used plasma bilirubin assay against spectrophotometry. The results suggest that the accuracy of the modified Architect bilirubin assay is sufficient for this purpose and may be introduced in our entire region.

Presently there is no Swedish external quality assurance program for CSF bilirubin and haemoglobin. Such a program should be developed regardless if the laboratories use quantitative measurements or scanning spectrophotometry. We have initiated a discussion with the Swedish external quality assurance organization on this issue.

The reasons for combining quantitative bilirubin measurements with an absorbance measurement at 415 nm in the initial phase are two-fold. Our colleagues in the region have a long tradition of using oxyhemoglobin rather than bilirubin as a basis for their referral decision and this may not be changed over night. Presently all CSF samples with suspected SAH are screened at 415 nm. Only if the A415 is above a certain threshold (0.025-0.040) scanning spectrophotometry is performed. Secondly, the presence of hemoglobin in the sample calls for caution regarding a possible interaction with the bilirubin assay. However, according to the present data we believe that a significant inhibition occurs at a hemoglobin level that is unrealistically high in patients with a negative CT scan. Also, the haemoglobin levels that cause interference are considerably higher than could be expected due to a traumatic tap.
Interfering haemoglobin should thus be due to a recent bleeding. Inhibition of haemoglobin from a recent bleeding will reduce the bilirubin value thus changing the ratio between haemoglobin and bilirubin thus causing the bleeding to appear to be more recent. This will be of minor importance as the clinical decision and treatment will not change.

By tradition, CSF samples have been protected against light to avoid breakdown of the bilirubin. The present data showed stable sample bilirubin levels after storage at room temperature in the dark for 6 h (Figure 4), whereas exposure to electric light or daylight caused a reduction of the test results by 30 and 60%, respectively. Shorter exposures to electric light (<1 h) should thus not cause major changes in bilirubin results.

In conclusion, diazo reagent based automated CSF bilirubin measurement is an accurate, convenient and cost-effective method that has the potential of replacing spectrophotometric scanning for the diagnosis of suspected SAH patients with a negative CT scan.

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References:

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