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Multi-wavelength spectrophotometric analysis for detection of xanthochromia in cerebrospinal fluid and accuracy for the diagnosis of subarachnoid hemorrhage

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ABSTRACT

Background: Cerebrospinal fluid (CSF) was examined for bilirubin, an important indicator for diagnosis of subarachnoid hemorrhage (SAH).
Methods: A multi-wavelength (340, 415, and 460 nm) spectrophotometric assay was developed for the quantitative measurement of bilirubin in CSF, enabling the mathematical correction for absorbance of hemoglobin and proteins. Bilirubin and hemoglobin results were correlated to HPLC and a standard colorimetric assay, respectively. A subset of samples was sent for an absorbance reading at 450 nm following baseline correction. The multi-wavelength bilirubin assay was validated on 70 patients with confirmed SAH and 70 patients with neurologic symptoms who ruled out for SAH.
Results: The multi-wavelength spectrophometric assay demonstrated no interferences due to proteins (albumin) up to 30 g/l or oxyhemoglobin up to 260 mg/l. The assay limit of detection was 0.2 mg/l, linear to 20 mg/l, and CVs ranged from 1 to 6% at bilirubin concentrations of 0.84 and 2.1 mg/l. The spectrophotometric assay correlated to HPLC and the colorimetric assay for bilirubin and hemoglobin, respectively. Results also correlated to the absorbance method (with removal of samples with high hemoglobin and proteins). The area under the ROC curve for diagnosis of SAH was 0.971 and 0.954 for the HPLC and spectrophotometric assay, respectively. At a cutoff of 0.2 mg/l, the clinical specificity was 100% for both assays, and the clinical sensitivity was 94.3% and 88.6% for SAH for the HPLC and spectrophotometric asays, respectively.
Conclusions: The multi-wavelength spectrophotometric assay is an objective alternative to visual inspection, HPLC, and absorbance for CSF bilirubin.

1. Introduction

The visual and spectral analysis of cerebrospinal fluid (CSF) for the presence of xanthochromia is useful for detection of subarachnoid hemorrhage (SAH) [1–4]. Xanthochromia following SAH is due to bilirubin, but other pigments such as hemoglobin, and protein, may be present [1,2]. A traumatic tap can cause peripheral blood to be collected along with CSF, and a false positive indicator of a SAH. CSF bilirubin is an indication of in vivo breakdown of hemoglobin, and is used to differentiate cerebral hemorrhage from a traumatic tap. CSF bilirubin indicates bleeding that occurred >12 h prior to lumbar puncture [3–5]. Computer-aided topography (CT) is the gold standard, but after 24 h, it is less sensitive and diagnostically useful. CSF bilirubin measurement is most useful in patients who present later [5,6].

Since the hemoglobin component of xanthochromia produces a false positive diagnosis of SAH, a specific assay for CSF bilirubin will improve the diagnosis of SAH. It is difficult for the human eye to differentiate the colors of hemoglobin and bilirubin, particularly at low concentrations. The use of a spectrophotometer can improve the sensitivity through detection of wavelengths that bilirubin absorbs (440–460 nm) [3,4]. In the spectral analysis of CSF, oxyhemoglobin interferes with the absorption peak of bilirubin. Many procedures have been developed to remove this interference. Current methods employ a spectrophotometric scan of CSF from 350 to 650 nm to identify the peaks of interest. In the Chalmers et al.'s method hemoglobin interference is corrected by subtracting background absorbance by drawing a tangent line from 360 to 530 nm, and the corrected bilirubin

Abbreviations: CSF, cerebrospinal fluid; SAH, subarachnoid hemorrhage; HPLC, high performance liquid chromatography; CV, coefficient of variance; UK NEQAS, United Kingdom National External Quality Assessment Service; CT, computer-aided tomography; AU, absorbance unit; ARUP, Association of Regional University Pathologists; AUC-ROC, area under the receiver operating characteristic curve; STARD, Studies on Diagnostic Accuracy; UCSF, University of California, San Francisco; SFGH, San Francisco General Hospital; SE, standard error; $S_{y|x}$, standard deviation of residuals; CNS, central nervous system.

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absorbance measured at 440 nm [7]. Later, these investigators changed the bilirubin measurement wavelength to 476 nm since the absorptivity of hemoglobin at this wavelength is negligible [8]. This technique has been adopted by the UK National External Quality Assessment Service (UK NEQAS) Specialist Advisory Group for External Quality Assessment of CSF Proteins and Biochemistry [4]. Bhardri et al. created an algorithm developed from spectroscopic absorption curves for bilirubin and hemoglobin [9]. Ahmed et al. modified a serum-based bilirubin assay to quantitatively measure CSF bilirubin on a chemistry analyzer [10]. However, this assay has interference with hemoglobin and does not correct for CSF proteins.

The UK NEQAS recommends "interpret with caution" when the CSF total protein concentration exceeds 1.0 g/l [4]. Although they have a correction formula for adjustment of net bilirubin absorbance in the presence of increased serum total proteins, they do not recommend use of this equation when there is an increased in CSF proteins alone, or when there is an increase in serum bilirubin and CSF proteins >1.0 g/l. High CSF proteins are observed in neurologic diseases such as meningitis, neurosyphilis, polyneuritis, cerebral hemorrhage and trauma [11]. Fishman suggested that CSF proteins >1500 mg/l produce significant xanthochromia that can be misinterpreted as CSF bilirubin [11].

We established molar absorptivity parameters in developing a multi-wavelength measurement of bilirubin in CSF fluid. This assay is similar in principle to the measurement of hemoglobin species in co-oximeters [12]. We compared results of the photometric assay against an HPLC assay and a graphically-corrected absorbance assay at 460 nm. The clinical sensitivity and specificity of both methods were determined from a cohort of CSF samples from confirmed SAH and non-SAH cases. We also correlated CSF hemoglobin results against a standard colorimetric assay (Drabkin reagent). We did not correlate the CSF protein as this is a widely available test within clinical laboratories.

2. Materials and methods

2.1. Reagents

To determine molar absorptivity, standards were prepared for each analyte (unconjugated bilirubin, oxyhemoglobin, and albumin used as a surrogate for total CSF proteins) [13]. Albumin-bound bilirubin was prepared according to Jacobsen et al. [14]. Five milligrams of crystalline unconjugated bilirubin (Calbiochem) was dissolved into 1 ml of 0.1 mol/l NaOH. The pH was adjusted to 8.5 using 0.5 mol/l HCl to avoid colloid formation of bilirubin. The albumin concentration was adjusted to 2.50 g/l so that bilirubin would be saturated with albumin to create protein-bound bilirubin. Previous studies showed that two bilirubin molecules can be reversibly bound to one albumin molecule [15]. The mixture was incubated for 10 min, then adjusted to pH 7.4 with 0.5 mol/l HCl. The concentration of bilirubin standards was determined from the absorptivity of albumin-bound bilirubin (48,500 mol/l at 460 nm) [16]. These values were verified using the Verichem Laboratories bilirubin calibrator reagent. A blank with equal albumin concentration was used to remove the protein interference.

Albumin standards of about 25 g/l were made from lyophilized human albumin (Sigma) dissolved in phosphate-buffered saline, pH 7.4. The albumin concentration was determined by the absorbance at 280 nm. Equil Plus Hemoglobin Control (RNA Medical) was used to determine the absorptivity of oxyhemoglobin (O_2Hb level >95%, and tHb of 169 g/l), diluted 1:20 in phosphate-buffered saline, pH 7.4.

2.2. Instruments

For the multiwavelength determinations, samples were analyzed with the Nanodrop 2000c Spectrophotometer (Thermo Scientific), using micro volume UV transparent disposable cuvettes. All samples were filtered with 0.22 μM Spin-X centrifuge tube filters prior to analysis (Corning). HPLC analysis was performed using Model 1200 with photodiode array detector (Agilent), and a Onyx Monolithic 100 × 4.6 mm C18 column (Phenomenex), direct sample injections. For the scanning absorbance assay conducted at ARUP Laboratories (Salt Lake City, UT), the absorbance of CSF was measured from 350 to 550 nm using a DU-800 Spectrophotometer (Beckman Coulter) using a 1-cm pathlength cuvette. The absorbance units (AU) that the curve deviated from a baseline at ~414 and 476 nm were recorded as the net bilirubin and oxyhemoglobin absorbance.

2.3. Multi-wavelength analysis (index test)

The molar absorptivity of bilirubin, oxyhemoglobin, and albumin was calculated at 330 and 340 nm for proteins, 415 and 576 nm for oxyhemoglobin, and 460 nm for bilirubin, in order to find the best three (one for each analyte measured) for the multi-wavelength assay. We prepared mixtures by spiking CSF with increasing concentrations of oxyhemoglobin and albumin. This was done individually and with the two interferrents combined. The bilirubin was kept at the same concentration, and the oxyhemoglobin and albumin doubling in concentration for a total of five dilutions. The best combination of wavelength was selected by the one showing the highest precision for the unchanged bilirubin measurement.

From Beer's law, the absorbance at one wavelength produced an equation which is the sum of the absorptions of each analyte:

$$A_{\lambda 1} = \varepsilon_{1,1} * c_1 * l + \varepsilon_{2,1} * c_2 * l + \varepsilon_{3,1} * c_3 * l$$

where $\varepsilon_{1,1}$ is the molar absorptivity of analyte 1 at wavelength 1, $\varepsilon_{2,1}$ is the molar absorptivity of analyte 2 etc., c is the concentration of each species and l is the spectrophotometer pathlength.

The measurement of absorbance at three wavelengths produced three equations. The concentration of each analyte is determined using linear algebra. Both disposable UV transparent cells with a 10 mm and a 1 mm pathlength were used. With the final selection of wavelengths and derivation of the multi-wavelength equation, the CSF bilirubin and hemoglobin assays were validated for within-run and total precision, linearity, correlation to predicate assays, and clinical outcomes from SAH and non-SAH patients.

2.4. Reference analysis

As a reference standard comparative method, a sensitive HPLC assay for CSF bilirubin was validated, selected because it has better analytical sensitivity and specificity than any clinical or research bilirubin assay. While the HPLC method may be more definitive, it is not as practical for routine clinical analysis for CSF bilirubin. We used a solvent gradient assay since methanol precipitates proteins. The aqueous buffer was started to allow proteins to flow ahead of the more hydrophobic bilirubin molecules. The mobile phase was switched to methanol to allow the bilirubin to flow through the column and detector. Mobile phase A was 100 mmol/l acetate buffer, pH 4.5. Mobile phase B was HPLC grade methanol. Mobile phase A was run for 1 min, then switched to the gradient. At 3 min the mixture was 50/50 and held for 1 min. The gradient was changed to 100% mobile phase B by 6 min and held for a total of 10 min. The detector was set to 453 nm. A 5-point standard curve ranged from 0 to 13 mg/l was created using the prepared bilirubin solution. Dilutions were made with a 500 mg/l albumin solution in phosphate-buffered saline. A representative standard curve produced a linear regression correlation of $r^2 = 0.999$. The bilirubin value of 13 mg/l was calculated by an absorbance at 460 nm to standardize with the spectrophotometric assay. This HPLC bilirubin assay had a total precision of 4.8% and 2.5% at 1.1 and 3.7 mg/l of conjugated bilirubin, respectively.

Following manufacturer's instructions CSF hemoglobin results were compared against a colorimetric assay using the Drabkin reagent