Automated Measurement of Cerebrospinal Fluid Bilirubin in Suspected Subarachnoid Hemorrhage. 

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Early detection of subarachnoid hemorrhage (SAH) can improve clinical outcome (1). After hemorrhage into the cerebrospinal fluid (CSF), erythrocytes lyse, releasing oxyhemoglobin, which is metabolized to bilirubin. The detection of increased CSF bilirubin is the basis for the laboratory identification of in vivo hemorrhage (2). Guidelines for the detection of CSF bilirubin in suspected SAH have recently been published (2). In the guidelines, the preferred method for detection of bilirubin is spectrophotometric scanning, with the net bilirubin absorbance (NBA) calculated according to Chalmers’ modification (3, 4). The procedure is not necessarily ideally suited for a clinical laboratory environment, and most hospital laboratories in the United States still use subjective visual inspection to identify CSF xanthochromia (5). The visual interpretation of spectrophotometric spectra is also subjective (which complicates clinical interpretation), although an iterative model has been proposed to circumvent this problem (6). We evaluated the measurement of CSF bilirubin on an automated instrument, using the Jendrassik–Gröf method, calibrated to measure lower concentrations. We report our experience with this method and compare the results with those of spectrophotometric scanning.

Spectrophotometric scanning was performed according to the guidelines (2), and a cutoff value of >0.007 for NBA was defined as positive. In all positive cases, a corrected value was calculated using the CSF and serum protein concentrations and the serum bilirubin concentration (2). Bilirubin was measured by a modification of our routine serum bilirubin assay, which consists of an in-house method on an Aeroset analyzer (Abbott Laboratories).

The method is based on the Jendrassik–Gröf principle as described by Doumas et al. (7), with a modification of the diazo reagent according to Chan et al. (8). To measure bilirubin at the concentrations typically found in CSF, we increased the ratio of sample to reagent volume from 0.043 to 0.150. The calibrator was diluted to give a 7-point calibration curve ranging from 0 to 10 550 nmol/L. We tested assay linearity by use of polynomial regression according to NCCLS document EP6-A. Oxyhemoglobin interference on CSF bilirubin and NBA determinations were investigated in samples prepared from purified bilirubin to concentrations of 340 and 6000 nmol/L. Hemoglobin was prepared from patient erythrocytes, and the concentration was verified by measurement on a Coulter Stack S analyzer. The hemoglobin was added to the two pure bilirubin solutions to give concentrations of 500, 1000, 2500, and 5000 mg/L. A 1:10 dilution of our serum bilirubin quality-control sample, Multiqual Level 1 (Bio-Rad) was used for continuous internal quality control.

The CSF bilirubin method had an intraassay CV of 20% (n = 20) at 100 nmol/L, based on repeat analysis of dilutions of Multiqual Level 1 (Bio-Rad). At 200 nmol/L, the CV was 11% (n = 20), and at 400 nmol/L it was 7% (n = 20). The detection limit was 35 nmol/L, based on replicate analysis (n = 25) of saline within batch and calculation of the mean plus 2 SD. The diluted serum quality control had mean value of 800 nmol/L and a CV of 6% (n = 140 days). The response was linear from 100 to 2400 nmol/L, encompassing the clinically relevant interval. We found no significant interference at a hemoglobin concentration ≤100 mg/L (10 mg/dL). Higher hemoglobin was associated with increasing negative interference.

To estimate a reference interval, we measured CSF bilirubin on samples from patients in which SAH was not suspected and which were visually clear and had normal CSF protein (≤400 mg/L). For 172 samples, the mean (SD) CSF bilirubin was 234 (62) nmol/L.

We studied 144 CSF samples for which xanthochromia testing had been requested (Fig. 1A). Results were positive in 23 by spectrophotometric analysis. The ability of CSF bilirubin measurements to correctly identify the presence of xanthochromia assigned in this way was investigated by ROC curve analysis.

ROC curve analysis gave an area under the curve of 0.99 (95% confidence interval, 0.95–1.00). At our upper reference limit for CSF bilirubin (359 nmol/L), the sensitivity was 100% and specificity was 92%. The sensitivity and specificity were calculated at various cutoffs (Table 1).

In 5 of the 23 positive cases, the NBA corrected to <0.007. The five samples had initial NBA results ranging from 0.007 to 0.218, and all had CSF bilirubin concentrations (391–6037 nmol/L) above the reference interval. When corrected, four of the five CSF bilirubin results returned to below the upper reference limit. The fifth result had a value of 1219 nmol/L and corrected to 431 nmol/L. These findings suggest that the principle of correcting for the NBA that would be expected if no...
bleeding into the CSF occurred would also apply in the case of CSF bilirubin.

Two xanthochromia-negative specimens with NBA values of 0 showed grossly increased CSF bilirubin (2195 and 8927 nmol/L; Fig. 1A). Both specimens showed gross macroscopic hemolysis, and retrospective inspection of the scanning spectra indicated that the determination of NBA was impossible. The oxyhemoglobin peak obscured the bilirubin peak at 476 nm. These two samples can be viewed as unsuitable for testing and were not included in further analysis.

To facilitate comparison of NBA and diazo bilirubin results (Fig. 1B), the NBA values were transformed to NBA-derived CSF bilirubin in nmol/L by use of the molar absorptivity of 0.000042 (2). The regression equation \( y = 1.05x + 249 \) nmol/L \( (r = 0.94; 95\% \) confidence intervals for the slope and intercept were 0.99–1.12 and 191–308 nmol/L, respectively). Weighted Deming regression analysis (assuming 3% error for both methods) gave a relationship of: \( y = 1.09x + 239 \), 95% confidence intervals for the slope of 0.89–1.28 and for the \( y \)-intercept of 204–274 nmol/L. The reason for the bias in the regression equations is uncertain. Spectrophotometric scanning gives a derivative that can be converted to NBA for the purposes of determining the presence or absence of xanthochromia, but has not itself been validated as an accurate measure of CSF bilirubin concentration. Approximately 50 of our NBA results were zero. A zero CSF bilirubin is unlikely, and the sensitivity of scanning at low concentrations may be problematic. An absorbance of 0.007 at 476nm corresponds to a bilirubin of 166 nmol/L. The mean CSF bilirubin for the samples with NBA <0.007 was 234 nmol/L. This may also explain the \( y \)-intercept of 249 nmol/L found with regression because 85% of the CSF samples tested gave negative NBA results. The mean NBA in this group was 0.001, corresponding to a bilirubin of 24 nmol/L, which is lower than expected. The expected normal bilirubin in CSF is \( \sim 30–150 \) nmol/L, with an expected mean of 90 nmol/L (9). The observed bias should not adversely affect the utility of the test for clinical decision-making, given the performance data that we have shown. It is imperative, however, that each laboratory validate its own method, reference interval, and decision limits. It would be advantageous if manufacturers could validate a modification of their bilirubin assays for use in CSF specimens.

The ROC analysis showed a 100% negative predictive value at a CSF bilirubin cutoff of 359 nmol/L (Table 1), suggesting that SAH can be reliably excluded at lower values and that spectrophotometric scanning is not necessary. The test is therefore ideally suited as a screening test, where samples with a bilirubin greater than the upper decision limit can be submitted to scanning. The two methods seem to be comparable and CSF bilirubin analysis can be considered an alternative marker of xanthochromia. This may be particularly relevant to laboratories where spectrophotometric analysis is unavailable and where there is still reliance on the less rigorous method of visual inspection (5). Although our findings suggest that the diagnostic performance would be similar,
larger studies in which both methods are evaluated against clinical outcome are needed.

Most authorities agree that the presence of xanthochromia is the primary biochemical criterion for a diagnosis of SAH, but it may be absent, especially if the lumbar puncture was done <12 h after the event (1). In these cases, an increase in CSF oxyhemoglobin may be the only biochemical abnormality. Unfortunately, CSF oxyhemoglobin may be increased by traumatic lumbar puncture (2).

In conclusion, automated bilirubin measurement is an easy and robust test and can be used in a stat environment to screen patients for increased NBA as defined by spectrophotometric scanning. The method can easily be performed on automated chemistry analyzers, using the on-board serum bilirubin reagent, although individual laboratories should validate their own decision limits.

References


Previously published online at DOI: 10.1373/clinchem.2004.033282

Anti-Transglutaminase Antibodies and Age, Valentina Baldas,1 Tarcisio Not,1* Alberto Tommasini,1 Filippo Ansaldo,2 Sergio Demarinii; Daniele Sblattero,4 Roberto Marzari,4 Lucio Torelli,5 Alberto Burlina,6 Claudio Tiribelli,7 and Alessandro Ventura1 (1) Clinica Pediatrica, 2 Istituto d’Igiene, 3 Dipartimento di Biologia, 4 Dipartimento di Scienze Matematiche, and 7 Centro Studi Fegato and Dipartimento di Biochimica, Biofisica, Chimica delle Macromolecole, Università di Trieste, Trieste, Italy; 3 Divisione di Neonatologia IRCCS Burlo Garofolo Trieste, Trieste, Italy; 6 Dipartimento di Pediatria, Università di Padova, Padua, Italy; * address correspondence to this author at: Clinica Pediatrica, IRCCS “Burlo Garofolo”, Via dell’Istria 65/1, 34100 Trieste, Italy; fax 39-043785210, e-mail not@burlo.trieste.it

The tendency within the general population to produce both organ and non-organ-specific autoantibodies is well recognized (1). It is not clear whether these autoantibodies represent the subclinical spectrum of certain autoimmune diseases or whether they are one effect of aging on the autoimmune system (2, 3). Celiac disease (CD) is an autoimmune disorder triggered by ingestion of gluten; it occurs mainly in individuals carrying the celiac-related HLA DQ2/8 (4). The discovery that the enzyme tissue transglutaminase (tTG) was the CD autoantigen has led to the design of various human tTG-based immunoassays to measure the specific antibodies of CD for diagnostic purposes. The widespread adoption of these assays indicates the diagnostic value of anti-human (TG, with its high sensitivity (98%) and specificity (95%) (5–7). Epidemiologic studies using this test have revealed that CD is one of the most common lifelong disorders, with a childhood prevalence of 1 in 90 individuals (8, 9). We investigated the relationship between age and the serum concentrations of anti-human-tTG autoantibodies in apparently healthy individuals, focusing in particular on identifying the age-dependent cutoff limits for the general population.

The study was performed retrospectively on sera from 4575 individuals (2431 females and 2144 males; median age, 9 years; range, 3 days–82 years) participating in two large screening programs, one for CD in the general population and the other for metabolic diseases in newborns and children. Anti-human-tTG values of screened individuals diagnosed as having CD (46 of 4575) were excluded.

In addition, we compared antibody concentrations in the study group with the IgA and IgG antibody concentrations of 144 patients with biopsy-confirmed CD diagnosed at the Children’s Hospital “Burlo Garofolo” from January 2002 to September 2003.

The study population enrolled included both healthy individuals and untreated CD patients classified as follows:

Group 1 included 145 sera from unselected newborns at the third day of life (75 females and 50 males) and 49 sera from healthy children (29 females and 20 males; mean age, 1.8 years; range, 1–5 years) obtained as part of a nutritional study.

Group 2 included 2774 sera from schoolchildren (1393 females and 1381 males; median age, 9 years; range, 5–12 years) in Trieste as part of a screening for CD (9) and 257 sera from teachers collected during the school-based screening (127 females and 130 males; median age, 35 years; range, 22–55 years).

Group 3 included 1350 serum samples collected in 1991 (797 females and 553 males; median age, 48 years; range, 12–82 years) from the population of Cormons as part of the Dionysos Study (10). Individuals were without risk factors for liver disease, such as alcohol use or hepatotropic viruses. The sera were stored at −30°C and thawed only once before use.

Group 4 included 144 sera from patients with biopsy-confirmed CD (82 females and 62 males; median age, 12 years; range, 1–68 years). Eleven patients (8 females and 3 males; median age, 10 years; range, 2–20 years) had total IgA serum deficiency (IgA <50 mg/L).