Measurement of Cerebrospinal Fluid Bilirubin in Suspected Subarachnoid Hemorrhage, *Jacobus Petrus Johannes Ungerer,* Sandra Jayne Southby, Christopher Michael Florkowski, and Peter Myles George* (Canterbury Health Laboratories, PO Box 151, Christchurch, Canterbury, New Zealand; * author for correspondence: fax 64-3-364-0750, e-mail ungerer@mweb.co.za)

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–Gróf 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 Jendrasik–Gróf 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 was 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 = ??) 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 = ??), and at 400 nmol/L it was 7% (n = ??). 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 (x = NBA-derived CSF bilirubin; y = CSF bilirubin) was: $y = 1.05x + 249$ 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 anal-

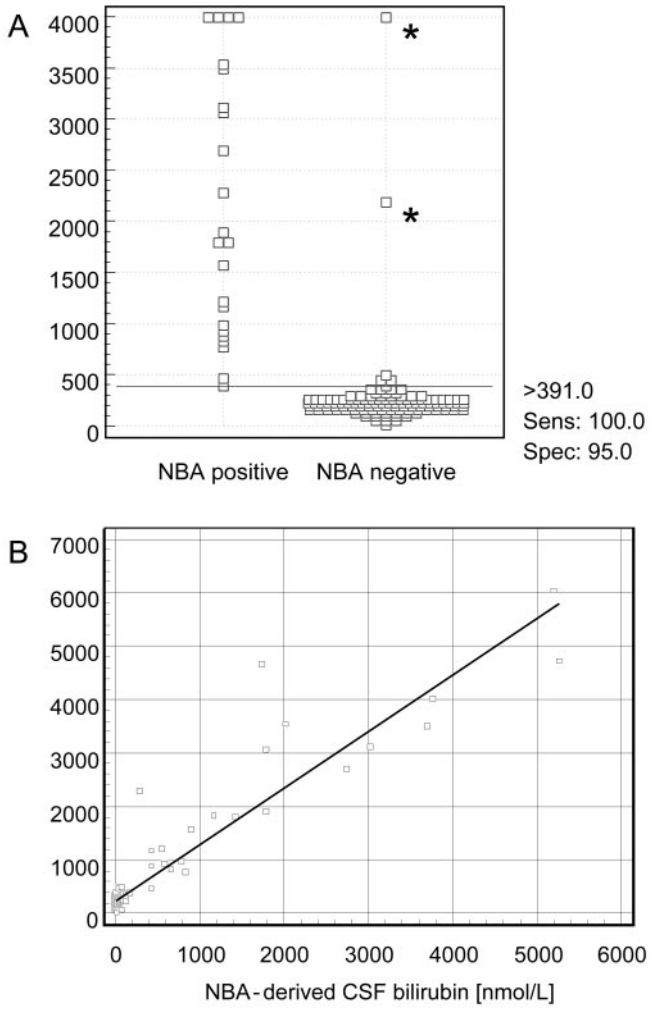

Fig. 1. Representative results from 144 specimens tested for xanthochromia.

(*A*), CSF bilirubin values in xanthochromia-positive and -negative groups; ∗, two specimens unsuitable for spectrophotometry. (*B*), scattergram showing the regression line for comparison of the two methods.

ysis (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

Table 1. Statistical parameters at different CSF bilirubin cutoffs.[a]

Cutoff,[b] nmol/L	Sensitivity, %	Specificity, %	Predictive value,[c] %	
			Positive	Negative
300	100	85	56	100
359	100	92	70	100
400	96	96	82	99
500	91	98	88	98
800	87	98	91	98
1000	70	98	89	94

[a] NBA >0.007 is defined as positive.

[b] Cutoff at 359 nmol/L refers to the upper limit of the reference interval.

[c] Positive, predictive value of a positive result; Negative, predictive value of a negative result.

of 24 nmol/L, which is lower than expected. The expected normal bilirubin in CSF is ~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 assay 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

1. Edlow JA, Caplan L. Avoiding pitfalls in the diagnosis of subarachnoid hemorrhage. N Engl J Med 2000;342:9–36.
2. UK National External Quality Assessment Scheme for Immunochemistry Working Group. National guidelines for analysis of cerebrospinal fluid for bilirubin in suspected subarachnoid haemorrhage. Ann Clin Biochem 2003; 40(Pt 5):481–8.
3. Chalmers AH, Kiley M. Detection of xanthochromia in cerebrospinal fluid. Clin Chem 1998;44:1740–2.
4. Chalmers AH. Cerebrospinal fluid xanthochromia testing simplified. Clin Chem 2001;47:147–8.
5. Edlow JA, Bruner KS, Horowitz GL. Xanthochromia. Arch Pathol Lab Med 2002;126:413–5.
6. Duiser HJ, Roelandse FWC, Lentjes EGWM, van Loon J, Souverijn JHM, Sturk A. Iterative model for the calculation of oxyhemoglobin, methemoglobin, and bilirubin in absorbance spectra of cerebrospinal fluid. Clin Chem 2001;47: 338–41.
7. Doumas BT, Kwok-Cheung PP, Perrry BW, Jendrzejczak B, McComb RB, Schaffer R, et al. Candidate reference method for determination of total bilirubin in serum: development and validation. Clin Chem 1985;31:1779–89.
8. Chan K-M, Scott MG, Wu T-W, Clouse RE, Calvin DR, Koenig J, et al. Inaccurate values for direct bilirubin with some commonly used direct bilirubin procedures. Clin Chem 1985;31:1560–3.
9. Gimpel JA, van Rijn HJ, Putters J. Reference interval for the bilirubin excess in cerebrospinal fluid by derivative spectrophotometry. J Clin Chem Clin Biochem 1989;27:217–9.

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