Assay of Cerebrospinal Fluid Protein: A Rate Biuret Method Evaluated

Paul R. Finley and R. Jane Williams

We evaluated a rate colorimetric method (Beckman) for measuring total protein in cerebrospinal fluid. The automated instrument we used was Beckman's ASTRA™. A 100-µL sample of spinal fluid is introduced into the biuret reagent in the reaction cell and the increase in absorbance at 545 nm is monitored for 20.5 s. Solid-state circuits determine the rate of alkaline biuret-protein chelate formation, which is directly proportional to the total protein concentration in the sample. The linear range of measurement is 120 to 7500 mg/L. Day-to-day precision (CV) over the range of 150 to 1200 mg/L ranged from 15.2 to 2.3%. The method was unaffected by radical alteration of the albumin/globulin ratio, but there is a positive interference in the presence of hemoglobin, a suppression in the presence of bilirubin, and no effect by xanthochromia. The method is precise, accurate, rapid, and convenient. The method was compared with the trichloroacetic acid method as performed on the Du Pont acu III, giving a correlation coefficient ($r^2$) of 0.9693. The method is precise, accurate, rapid, and convenient.

We report an evaluation of the first rate biuret assay of protein in human cerebrospinal fluid. The assay is performed in the total-protein module of the "ASTRA 8" (Automated Stat/Routine Analyzer; Beckman Instruments Inc., Brea, CA 92621). Previous techniques suffered a lack of sensitivity of the biuret chromogen in the visible range (1) or required complexing the protein with a dye (Ponceau S) and subsequent precipitation and analysis of the complex (2). Mauck (3) reported a method in which the spinal fluid is passed through an ion-exchange resin containing the biuret reagent; the resulting copper–protein complex is separated from low-$M_r$ (relative molecular mass) substances by gel filtration, and the copper in the eluted complex is determined colorimetrically with diethylthiobarbituric acid. The method has a limit of sensitivity of 20 mg of protein per liter. Artias et al. (4) have shown that the biuret reaction very efficiently measures protein in spinal fluid and that the contribution of low-$M_r$ proteoses and peptones to the color reaction is of minor significance.

Materials and Methods

**Instruments.** We used the ASTRA 8 total-protein chemistry module to measure the cerebrospinal fluid protein. The instrument was fitted with floppy discs.

The total-protein module (which is used for both serum and spinal-fluid protein) contains the total-protein board assembly as the electronic front end of the channel. The assembly depends on an external photodetector to supply its primary input signal, and provides a regulated voltage to use with an external incandescent source lamp. The primary output signal is a voltage that is proportional to the total protein concentration in a fluid sample. The rate of change, with respect to time, of the light absorbance of the sample (at 545 nm) is proportional to the protein concentration. The $da/dt$ signal is coarsely scaled to provide the output signal, which is analyzed by digital techniques in a master computer section that is not part of the assembly board.

The analog circuits consist of a current-to-voltage converter, pre-amplifier, log converter, buffer amplifier, multiplexer, differentiating output amplifier, and voltage regulator. The input signal to the pre-amplifier is a very low-level current generated by a vacuum photodiode located in the sample cell. A small beam of white light from the source lamp passes first through the sample cell and then through an interference filter (545 nm) before striking the detector. The output current from the detector is an analog of the light-transmitting property of the sample in the cell. The current, about 100 nA, is first converted to a percent transmittance ($T$) voltage signal in the pre-amplifier. The logarithm of the signal is converted into a low-level analog for absorbance.

Each decade change in percent $T$ generates about 0.065 V, which needs to be amplified. The buffer stage increases the signal to about 6.5 V. The absorbance signal is differentiated in the final output stage where a capacitor converts changes in input voltage ($da/dt$) into a low-level current, which is amplified by the final stage. The multiplexer input can be programmed by the external computer to select a high gain, to detect the low concentration of protein found in cerebrospinal fluid.

The instrument used in the comparison study was also a discrete analyzer, the acu III (Du Pont, Wilmington, DE 19888), in which cerebrospinal fluid protein is measured by trichloroacetic acid precipitation and endpoint turbidimetry.

**Reagents.** The biuret reagent and the liquid calibrators were supplied by Beckman Instruments, Inc. (Total Protein Reagent: per liter, 8.8 mmol of copper sulfate and 145 mmol of NaOH). The calibrators are prepared from human serum and stabilized with ethylene glycol. The assigned values are usually 200 and 900 mg/L. The ethylene glycol minimizes bacterial growth and stabilizes oxygen-labile constituents so that the calibrators are stable for 12 months at $-20\,^\circ C$ and for 20 days at $4\,^\circ C$.

**Procedure.** The ASTRA operation has been described in
We followed the supplier’s instrument instructions: 1000 μL of biuret reagent is delivered to the measuring cuvet by the pumping system, after which the automatic probe samples and delivers 100 μL of cerebrospinal fluid. A typical reaction is shown in Figure 1. The injection of sample into the cuvet takes 3 s, and there is a 6-s period (“blind rate”) when no readings are taken, to avoid any aberrant signals owing to perturbation. Therefore the rate signal is monitored 9 s after injection and again 20.5 s after injection. The rate signal is verified at this time by a check of the absorbance to be certain that exhaustion of reagents has not occurred. The absorbance check also detects any exceeding of the specified spectral limitations.

**Results**

**Analytical Variables**

*Precision.* We tested within-day and between-day precision (CV) by using four pools prepared by diluting “Ortho Normal Unassayed Serum” (Ortho Diagnostics, Raritan, NJ 08869) with distilled water. The batch we used in this experiment is our usual serum control pool; we have established its total-protein content to be 62 g/L. The serum was diluted to give nominal concentrations of 1200, 600, 300, and 150 mg/L. Within-day precision was tested by assaying 20 replicates per run on four or five separate days. We assessed between-run precision by using single samples of the 150, 300, 600, and 1200 mg/L controls in 20 separate runs over 10 days. Typical within-day data were: mean 123.5 (SD 13.8) mg/L, CV 11.23%; mean 276.0 (SD 16.7), CV 6.04%; mean 561.5 (SD 15.3) mg/L, CV 2.73%; and mean 1167 (SD 11.3) mg/L, CV 0.967%. Between-run precision data were: mean 127.0 (SD 19.0) mg/L, CV 15.2%; mean 262.5 (SD 17.5) mg/L, CV 6.67%; mean 562.5 (SD 19.4) mg/L, CV 3.45%; and mean 1160.8 (SD 27.0) mg/L, CV 2.33%.

*Linearity.* For linearity studies, we measured eight albumin solutions with concentrations ranging from 125 to 7500 mg/L. Four separate experiments were carried out in triplicate on four separate days. Deviation from linearity ranged from −1.5 to +1.7%. We determined the lowest and highest limits of detectability of both systems (aca and ASTRA). The test solution was the “Ortho Normal Control” pool, suitably diluted with water. The lower and upper limits of the analytical response of the aca were: 100 (SD 15) mg/L, CV 15%, and 2500 (SD 48) mg/L, CV 1.9%. The ASTRA lower and upper limits were: 120 (SD 16) mg/L, CV 13.3%, and 7500 (SD 78) mg/L, CV 1.04%.

**Carryover studies.** We performed carryover studies according to the method of Passey et al. (5). On one carousel, a sample with a high concentration was assayed in triplicate followed by a low-concentration sample in triplicate, followed by a high-concentration sample in triplicate, and so on. In a typical carousel run a sample of 4000 mg/L (triplicate) was followed by a sample of 300 mg/L (triplicate), followed by 4000 mg/L in triplicate, followed by water in triplicate, and the 4000 mg/L sample in triplicate. Using such a scheme, we tested a full carousel (38 positions) and found no carryover. Expanding the study to include higher concentrations (10 000 mg/L) still showed no carryover to the 300 mg/L samples or in water. When we included a normal human serum [protein concentration of 62 g/L, followed by a sample of 300 mg/L, followed by a normal serum, followed by water, and so on (all in triplicate)] the calculated carryover was 0.18%.

**Calibrator cross analysis.** A cross analysis of controls and calibrators was carried out with both the ASTRA module and the aca III. The Ortho Unassayed Control Serum, which we use for the conventional serum protein assay, was diluted as previously mentioned to provide nominal concentrations of 1200, 600, 300, and 150 mg/L. The means of triplicate series analyzed in the aca were, respectively, 1230, 590, 280, and 130 mg/L. As already stated, analysis of this identical pool in the ASTRA (the between-day study) gave the following means: 1160.8, 562.5, 262.5, and 127.4 mg/L, respectively.

The Beckman calibrator, lot no. C009174, with an assigned value of 228.0 mg/L was determined with the aca to be 258 mg/L (mean of five replicates). The Beckman calibrator, lot no. C009175, with an assigned value of 998.0 mg/L gave a result of 1076 mg/L (mean of five replicates) in the aca.

The Du Pont Enzyme Verifier (the serum used for calibrating the cerebrospinal fluid protein assay in the aca, with an assigned value for total protein of 74 g/L), was diluted to provide nominal concentrations of 1480, 740, and 370 mg/L. The respective means of triplicate determinations were: 1436, 730, and 370 mg/L with the aca, and 1336, 630, and 290 mg/L with ASTRA.

**Interferences.** We examined the influence of hemoglobin and bilirubin on the rate biuret assay for spinal fluid protein.

We first assayed pure hemoglobin solutions to assess the apparent concentrations as if they were samples of spinal fluid protein, and obtained the following results (hemoglobin concentration and assay result): a hemoglobin concentration of 940 mg/L read 230 mg/L (24% positive error); 1800 mg/L read 980 mg/L (52% positive error); and 3770 mg/L read 2470 mg/L (65% positive error). The same three solutions tested on the aca gave the following apparent concentrations: 1810 mg/L (192% positive error); 3390 mg/L (180% positive error); and 5400 mg/L (143% positive error). We also tested lower concentrations of the pure hemoglobin solution with the aca because of the more substantial hemoglobin interferences for this instrument. A hemoglobin concentration of 230 mg/L gave an assay result of 240 mg/L (104% positive error); and 470 mg/L gave an assay result of 700 mg/L (149% positive error). The interference of hemoglobin therefore was three- to eightfold greater for the aca than for the ASTRA.

We also mixed a crystal-clear pool of fresh spinal fluid (protein concentration 880 mg/L) with equal volumes of the pure hemoglobin solutions. The degree of interference with

---

**Fig. 1.** Typical rate and absorbance curves for the rate biuret protein reaction
each instrument confirmed the results of the previous experiment.

To test the influence of bilirubin, we added pure bilirubin solution to the previously mentioned spinal fluid pool (protein concentration 880 mg/L). The pure bilirubin solution used in the experiments was free of protein and gave no detectable signal when assayed in the ASTRA. The various mixtures of bilirubin solutions and the spinal fluid pool had final bilirubin concentrations of 300, 150, 70, and 35 mg/L and each mixture had a final concentration of 440 mg of protein per liter. At bilirubin concentrations of 300 and 150 mg/L there was suppression of the signal and no apparent protein was detected. At 70 mg of bilirubin per liter there was 73% suppression of the apparent concentration of protein, and at 35 mg/L there was a suppression in apparent protein concentration of 32%. No lesser bilirubin concentration was tested.

We tested the influence of xanthochromia in both systems. Four xanthochromic specimens were diluted with both water and clear spinal fluid of known protein concentration. All of these dilutions gave results close to those expected, when measured with either the aca or the ASTRA. Xanthochromic fluid therefore apparently has no discernible effect in either assay, because dilutions of the color down to a clear solution gave a linear response.

Finally, we tested the ability of the system to distinguish extreme variations in albumin/globulin ratios. Aqueous solutions of pure human albumin and globulin fractions were prepared to a target value of 1000 mg of total protein per liter. Unlike the aca spinal fluid assay, the ASTRA rate biuret reaction showed no deviation from the expected concentration, in spite of radical changes of the albumin/globulin ratio (Figure 2).

Clinical Samples

We used 131 fresh samples in a patient-correlation study, carried out over a period of three weeks. All of the samples were assayed in duplicate, and the variation between duplicates ranged from 1 to 4%. For the statistical analysis we used the first member of each pair. Every sample in the study had been recently (no more than 1 h earlier) assayed on the aca. The concentrations of the samples ranged from 100 to 5970 mg/L (aca-measured values) (Figure 3). Regression statistics

\[y = 1.034x + 26 \text{ mg/L; } r^2 = 0.9693; \] standard error of the estimate = 58 mg/L; mean of \(x\) (aca) = 565 mg/L; mean of \(y\) (ASTRA) = 619 mg/L; standard error of the slope = 0.017; 95% confidence limits of the slope = 1.00 to 1.068; standard error of the intercept = 9.6 mg/L; 95% confidence limits of the intercept = 6.8 to 45.2 mg/L.

Discussion

This report is the first evaluation of a rate biuret reaction for assay of total protein in human cerebrospinal fluid. The theoretical and experimental validity of measurement of total protein in spinal fluid by means of the biuret reaction is firmly established, although the sensitivity of the techniques has not always been satisfactory. Here, the precision, accuracy, analytical recovery, linearity, and comparison with an established method were quite satisfactory. The linearity range, in fact, is almost threefold that of the aca: the aca, however, has a slightly lower limit of detection than the ASTRA (100 vs 120 mg/L), although this does not alter the clinical utility of the ASTRA assay.

The present method has certain advantages not previously available. It is simple, rapid, specific, and adequately sensitive for clinical use. The use of substantial amplification in the electronic system affords a rapid assay over a wide concentration range (120 to 7500 mg/L). Further, the assay is unaffected by any radical change of the albumin/globulin ratio, unlike the assays involving sulfosalicylic acid or trichloroacetic acid. The influence of hemoglobin, although significant, is far less than that encountered in trichloroacetic acid turbidimetry (aca), a technique widely used to measure spinal fluid protein. Apparent protein concentration is decreased in the presence of bilirubin, but the bilirubin concentrations we tested would not usually be present in spinal fluid.

The cross calibrator experiments showed minimum discrepancies between the two instruments. The modest bias (about 7%) demonstrated in the assay of patients’ samples can in part be explained by these discrepancies and would suggest that the calibrator assignments are at slight variance. The differences are, however, not clinically significant.

The novel detection system, the analysis time (one sample per minute), the automated aspects of the technique, and the satisfactory comparison with an established method all make
the rate biuret assay a useful addition to methods for spinal fluid protein.

References


CLIN. CHEM. 29/1, 129-132 (1983)

Multilayer Film Analysis: Evaluation of Ion-Selective Electrolyte Slides

Patricia Costello, Norman P. Kubasik, Bernard B. Brody, Harrison E. Sine, James A. Bertsch, and John P. D'Souza

We evaluated the Kodak Ektachem multilayer ion-selective electrolyte slides. For various types and concentrations of control material the precision (CV) within- and between-day ranged from 0.5 to 1.3% (1.7-2.1%) for sodium, 1.2 to 2.2% (2.7-2.9%) for potassium, 2.9 to 4.6% (5.9-6.7%) for carbon dioxide, and 0.7 to 1.6% (1.3-1.4%) for chloride. For all these analytes, analytical recovery was about 100%, except in the supra-physiological ranges, for which carbon dioxide recovery was about 110-120%. Either serum or heparin-treated plasma can be used, interchangeably, for analysis; use of serum treated with lithium iodoacetate is unacceptable. Comparisons with results by continuous-flow procedures demonstrated good correlation for sodium, potassium, and chloride; carbon dioxide comparisons indicate an Ektachem calibrator bias may be required. Abnormally low protein concentrations or lipemia had no observed effects on results for electrolytes. Abnormally high protein concentrations affect sodium results slightly (∼5 mmol/L).

We have previously described some basic laboratory performance criteria for the Ektachem GLU/BUN Analyzer (Eastman Kodak, Rochester, NY 14650), in which discrete, dry, multilayer films are used for glucose or urea measurements (1). Here we report our evaluation of the analytical performance for the Ektachem multilayer ion-selective electrolyte slides (2). The instrument we used was an experimental, manually operated Ektachem Electrolyte Analyzer (EEA), which cannot be obtained commercially. However, the same slide system and chemical principles are used in the commercially available Ektachem 400 Analyzer.

Materials and Methods

The Analyzer, calibrators, and ion-selective slides were obtained from Eastman Kodak. Briefly, the EEA is a manually operated instrument in which discrete, dry, thin-film, single-use, disposable, ion-selective electrodes are used for assay of sodium, potassium, chloride, or carbon dioxide. Each slide contains a reference electrode and sample electrode. About 10 μL of reference fluid is applied to the reference electrode at the same time that 10 μL of sample is applied to the sample electrode. After a liquid junction is formed, and the reaction has stabilized (3 min), an electrometer measures the potential difference between the two half-cells created by the reference and sample fluids. A microcomputer then calculates the results, based on calibration with known standards that relates measured millivolt response to analyte concentration.

Standard Reference Materials for sodium chloride (SRM 919) and potassium chloride (SRM 918) were obtained from the National Bureau of Standards (NBS), Washington, DC 20234, for use in calibration and analytical-recovery studies.

Venous blood specimens for the plasma/serum comparison study were collected from volunteers. Serum was collected into two evacuated blood-collection tubes (Venoject KT200 from Kimble-Terino, Inc., Elkton, MD 21921; Vacutainer Tube 6474, iodoacetic acid-lithium salt, from Becton Dickinson, Rutherford, NJ 07070). Plasma samples were collected in Vacutainer Tube 3200-XF183 (ammonium heparin, Becton Dickinson). All samples were either assayed promptly or stored frozen until analysis.

For method comparison, we analyzed samples with the EEA and by continuous-flow (AutoAnalyzer II; Technicon Instruments Corp., Tarrytown, NY 10591) on the same day. Our continuous-flow method for sodium and potassium is flame photometry; the chloride method is based on mercuric thiocyanate (3); and the carbon dioxide method is based on cresol red (3). For sodium, potassium, and chloride, we calibrated the AutoAnalyzer II with NBS material. Analytical-grade sodium carbonate, anhydrous (no. 5-2602; J.T. Baker Chemical Co., Phillipsburg, NJ 08865), was used for carbon dioxide standardization.

The EEA was operated according to the manufacturers' instructions and was calibrated weekly with Ektachem Calibrators.

Statistical analysis of the data included regression analysis and Student's t-test; t-test significance was determined from standard tables (4).