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A Micro-Method for Measuring Total Protein in Cerebrospinal Fluid by Using Benzethonium Chloride in Microtiter Plate Wells

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By using a benzethonium chloride concentration 12-fold that described originally (Clin Chem 1979;25:1317–9), we developed a reliable method suitable for routine measurement of total protein in cerebrospinal fluid. Only 10 μL of sample is required. Reactivity to immunoglobulin G (IgG) and to albumin (Alb) is similar, as is necessary for specimens that can have very varied IgG/Alb ratios. The assay, performed in a microtiter plate for ease of use, has a between-batch coefficient of variation of 3.4% for a protein concentration of 450 mg/L. This contrasts with a dye-binding technique with Ponceau S, for which the CV was 9% at the same concentration.

Additional Keyphrases: *albumin, globulin, ferritin tested · dye-binding method compared*

The fact that many different methods for measuring total protein in cerebrospinal fluid (CSF) have been described indicates that none overcomes all the associated problems. We required a method that was quick, simple, sensitive, inexpensive, and gave a similar response to each of the various proteins in CSF. This last point is especially important, because the relative concentrations of different proteins in CSF can vary greatly among samples. For example, IgG can constitute from 3% to 30% of the total protein in different samples.

Methods described for total-protein assays can be broadly divided into two main groups: dye-binding techniques and turbidimetric assays.

The former assays have the disadvantage that different proteins yield colored products with different molar absorbancies. Some of these methods also involve tedious precipitation and redissolving, especially inconvenient if many samples are to be processed. Examples of these are techniques in which Ponceau S (*1–3*) or Coomassie Brilliant Blue G-250 is used (*4–6*).

In the other main category of total-protein assays, precipitating agents are used, singly or in combination—for example, trichloroacetic acid and sulfosalicylic acid (*7*). With these techniques not all proteins are precipitated to the same extent. However, Iwata and Nishikaze (*8*) and Flachaire et al. (*9*) found no difference in the response of albumin and globulin when precipitated with benzethonium chloride (BTC), but McElderry et al. (*10*) disputed these findings.

McElderry et al. (*10*) found that the use of biuret reagent on a digest of protein after acid precipitation gave equal color and intensity for equal concentrations of albumin and globulin, but the method is tedious if many samples are to be processed. A further problem that makes many methods unsuitable for our laboratory is sample size. We perform a range of investigations on CSF samples, often of low volume, and so we need a method requiring little sample. Using a 12-fold increase in benzethonium chloride concentration over that used by Iwata and Nishikaze (*8*), we find that only 10 μL of CSF can be used for measuring total protein simply, quickly, reliably, and robustly, with good reproducibility.

Methods and Materials

Protein standards. Calibration standards were prepared by dissolving 150 mg of human albumin (Sigma, Poole, U.K.; cat. no. A-8763) and 50 mg of human gamma globulin (Sigma; cat. no. G-4386) in 100 mL of isotonic saline (NaCl, 0.85 g/L) containing 10 g of sodium azide per liter to give a stock 2000 mg/L standard for total protein. From this solution the following set of working standards was produced: 50, 100, 250, 500, 750, 1000, 1250, and 1500 mg/L.

For use in evaluating the response to different proteins, we prepared 1500 mg/L solutions of human albumin, human gamma globulin, and human transferrin (Sigma; cat. no. T-2252) in isotonic saline, then diluted to make working standards for each protein in the same concentrations as above.

Benzethonium chloride methods. To optimize sensitivity, we tested various concentrations of the reagents in the precipitating reagent. Final BTC (Sigma) and EDTA (BDH, Dagenham, U.K.; Analar) concentrations in the precipitating reagent were varied between 0.5 and 50 g/L and between 1 and 20 g/L, respectively. The concentration of sodium hydroxide (BDH, Analar) was kept constant at 10 g/L. Using a Boehringer "Dilutrend," we added 5 μL of a 1500 mg/L total-protein standard and 195 μL of precipitating reagent to a microtiter plate well (Dynatech, Billingshurst, U.K.). After all additions, the plate was left at room temperature for 15 min before the absorbance of each well was measured at 410 nm (Dynatech MR700 microtiter plate reader) after blanking the instrument with precipitating reagent.

We similarly determined the optimum sodium hydroxide concentration, varying its concentration between 1 and 50 g/L and keeping the final concentrations of BTC and EDTA constant at 5.0 g/L and 20 g/L, respectively, measuring absorbance as described above.

Using a precipitating reagent that contained 5.0 g of BTC, 20 g of EDTA, and 10 g of NaOH per liter, we evaluated the effect of sample volume: 2, 5, 7, 10, and 15 μL of standard was diluted to 200 μL final volume with precipitating reagent, and the absorbance was measured after 15 min.

To determine the optimum length of incubation, we measured the absorbance of the turbidity produced with BTC for 50 and 1500 mg/L solutions of human albumin and human gamma globulin during 40 min.

For precision, reactivity, and sensitivity studies, we mixed 10 μL of sample with 190 μL of precipitating reagent (BTC 5.0 g/L, EDTA 20 g/L, NaOH 10 g/L). The absorbance of microtiter plate wells was measured at 410 nm after 10 min, as before.

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Ponceau S method [Pesce and Strande (*1*)]. Dilute 2 mL of Ponceau S (Raymond A. Lamb, 6 Sunbeam Road, London, U.K.) solution (4 g in 100 mL of distilled water) to 100 mL with 30 g/L trichloroacetic acid (BDH, Analar). Just before use dilute this solution 10-fold with distilled water. Mix 20 μL of sample with 500 μL of working Ponceau S reagent, centrifuge for 5 min, then remove the supernate carefully with a pipette and discard. Dissolve the remaining precipitate in 1 mL of 8 g/L sodium hydroxide, mix, and measure the color of the resulting solution at 560 nm in a spectrophotometer (we used a Beckman DU6; Beckman Instruments, Fullerton, CA).

Evaluation of methods. The BTC and Ponceau S methods were both investigated for precision, reactivity with the three major protein components of CSF, and sensitivity. We analyzed 100 CSF samples by both methods to check for method-to-method variability. All total-protein results for CSF were calculated by a computer, with use of a least squares, second-order best-fit program.

Results

Optimization of the BTC method

The optimum reagent concentrations were 5 g/L for BTC, 20 g/L for EDTA, and 10 g/L for NaOH, with 10 μL of sample. Turbidity was maximum after 8 min. In standards with a protein concentration >1000 mg/L the absorbance then gradually declined to 80% of maximum in 40 min, owing to flocculation of the precipitated protein.

Analytical Variables

Precision. The precision profile for both methods (Figure 1) was derived from 12 replicate readings for each standard. Table 1 shows the within-batch precision for the BTC method as evaluated by assaying two different lots of pooled samples, with 24 replicate readings in one batch. The same two pools were used to calculate the between-batch precision for 34 consecutive assays done during three months.

Reactivity. Figures 2 and 3 show the response to albumin, globulin, and transferrin in the two methods. Using a series of dilutions of a serum sample, we found that the response curve became flat at a 15 g/L concentration of total protein.

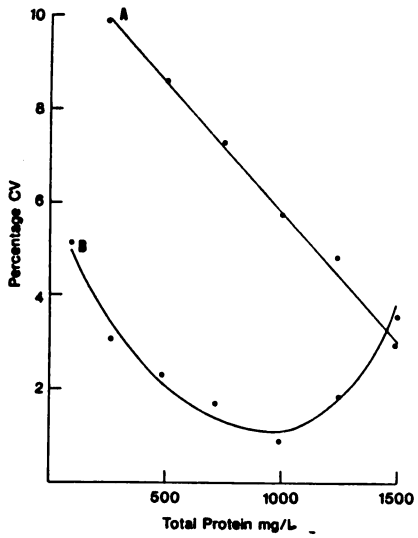

Fig. 1. Precision profile for the Ponceau S (*A*) and BTC (*B*) methods

Table 1. Between-Batch and Within-Batch Precision of the Present Method

	Within-batch		Between-batch	
n	24	24	34	34
Mean, mg/L	461.3	1070	450.2	1074
SD, mg/L	12.77	17.06	15.27	30.75
CV, %	2.77	1.59	3.40	2.86

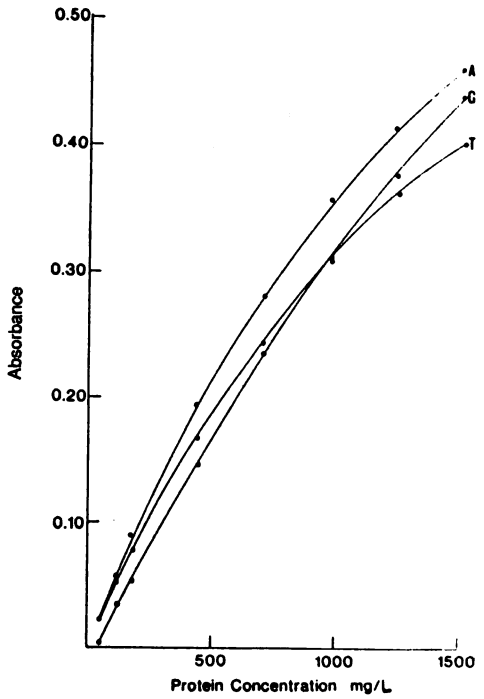

Fig. 2. Reactivity of human albumin (*A*), globulin (*G*), and transferrin (*T*) in the BTC method

Sensitivity. The sensitivity, calculated from calibration curves, is shown in Table 2. The absorbances obtained for each standard are related to the sample volume used, then are finally corrected for concentration of the standard. Results are expressed as the absorbance produced per microliter per milligram of total protein per liter.

Method Comparison

We measured the total protein in 100 CSF samples by both techniques to evaluate the correlation between methods (Figure 4). The equation of the regression line of best fit was: BTC = 0.838 Ponceau S − 0.004 mg/L (standard error of the slope = 0.0493, standard error of the intercept = 2.8334, r = 0.8741, mean BTC = 44.68 mg/L, mean Ponceau = 53.36 mg/L).

Discussion

This optimized micro-method for total protein in CSF requires only a 10-μL sample. For routine use we keep a stock BTC reagent (100 g/L in distilled water), which we dilute 10-fold with water, then mix with an equal volume of 40 g/L EDTA solution in 20 g/L NaOH. This reagent is prepared freshly for each batch.

Use of a microtiter plate enables many samples to be processed quickly and easily. For fewer samples, or where no microtiter plate reader is available, the method can be

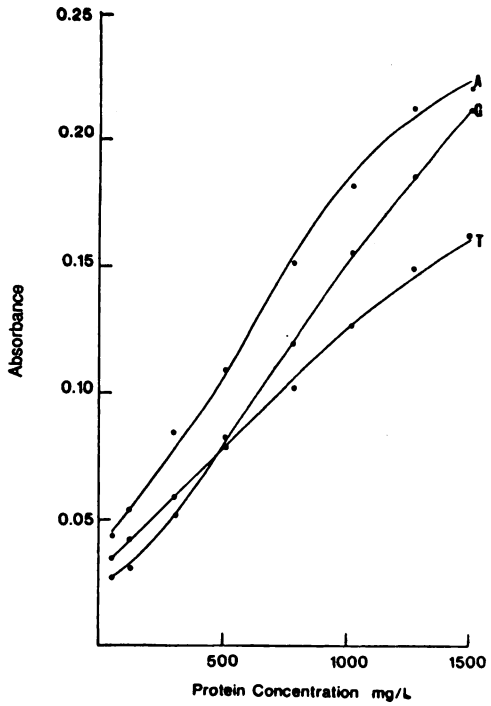

Fig. 3. Reactivity of human albumin (*A*), globulin (*G*), and transferrin (T) in the Ponceau S method

Note different scale on *y*-axis than used in Fig. 2

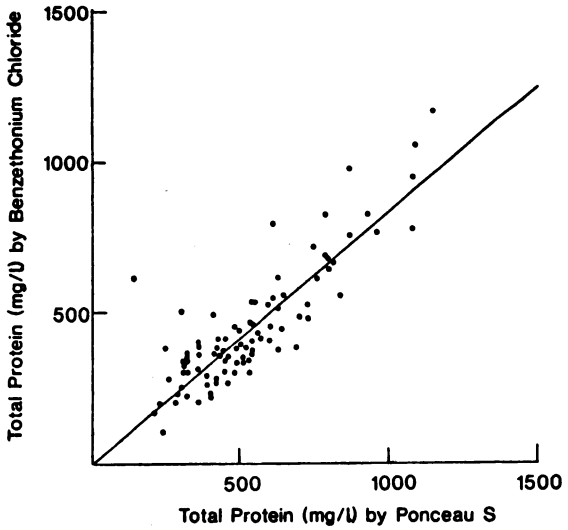

Fig. 4. Regression line for CSF total protein by the Ponceau S method and the BTC method

$r = 0.8741$ ($t = 16.97$). Mean of $x = 53.36$. Mean of $y = 44.68$

scaled up to measure 25-μL samples in 500 μL of reagent in microcuvettes, with absorbance being measured in a conventional spectrophotometer.

The precision studies on the BTC method agree well with those reported by Iwata and Nishikaze (*8*) and by Flachaire et al. (*9*). The precision profile on the BTC method shows that the best precision (CV 1%) is obtained with a total protein of about 1000 mg/L. At lower concentrations of total protein the assay becomes less precise, but the CV is still between 3% and 4% at the top of the normal reference interval for CSF total protein (350 mg/L) and <5% for the middle of the reference interval. The results for within-batch precision fit well on the precision-profile curve. The profile produced for the Ponceau S method shows that it is not optimized over this concentration range of total protein. Using these same reagents, Pesce and Strande (*1*) achieved better precision, but our results agree well with those of McElderry et al. (*10*). Differences in the reactivity of albumin and globulin were also seen by McElderry et al. in their BTC method, but not by Iwata and Nishikaze (*8*), who used a similar method. Our micro-scale method shows little difference in reactivity between albumin, globulin, and transferrin. The standard curves for albumin and globulin are almost parallel, showing a constant difference in reactivity, whereas the relative reactivity with transferrin decreases as the concentration exceeds 1000 mg/L. However, this concentration of transferrin would mean that the CSF total protein would have to be about 10 g/L, necessitating routine dilution of the sample; thus, transferrin is not a problem. At a protein concentration of 1000 mg/L, there is an 11% difference between the absorbances produced by albumin and globulin, compared with an 18% difference by the Ponceau S method.

Samples with the same total protein concentration but various proportions of albumin, globulin, and transferrin will show a wider spread of results by the Ponceau S method than by the BTC method. These differences in reactivity of the different proteins cause one of the biggest problems in measuring total protein in CSF, namely, deciding which is the best calibrant to use. It is inappropriate to use a single protein as a standard for analyses for total protein. Also, the composition of a secondary standard, such as diluted serum, may vary between batches, making it difficult to determine exactly. For this reason, we use a mixture of purified albumin and globulin to make a primary standard for which we know, precisely, the protein composition.

The calibration line produced is slightly curved, requiring the use of a second-order curve-fit program to obtain the results. The samples with a very high total protein concentration, which will flocculate and give falsely low values, are easy to detect, because the precipitate forms in the

Table 2. Sensitivity of the Benzethonium Chloride (BTC) Method Compared with the Ponceau S (Pon S) Method for CSF Total Protein

Standard, mg/L	Absorbance		Absorbance/vol		(Absorbance/vol)/concn	
	Pon S	BTC	Pon S	BTC	Pon S	BTC
100	19.0	33.5	0.95	3.35	0.0095	0.0335
500	51.1	155.6	2.55	15.56	0.0051	0.0311
1000	114.8	270.4	5.74	27.04	0.0057	0.0270
1500	165.3	363.8	8.26	36.38	0.0055	0.0243
				Mean values	0.0065	0.0289

microtiter wells. Such samples must be diluted and reanalyzed.

As Table 2 shows, the BTC method is 4.4 times more sensitive than the Ponceau S method. It is seen from the regression analysis, however, that BTC shows a negative bias compared with Ponceau S. The statistical data show significant proportional bias between the two methods at the 95% confidence level. The r value of 0.8741 reflects the spread in the results, presumably attributable to the differing ratios of proteins in the samples. Again, this underlines the problem associated with measuring total proteins in CSF.

In conclusion, our modified BTC method is quick, simple, sensitive, and precise, with acceptable reactivity with albumin, globulin, and transferrin. The BTC method requires fewer manipulations than the dye-binding technique with Ponceau S, thereby reducing potential errors. Although Flachaire et al. (*9*) found BTC not suited to the measurement of urine protein, we found its use advantageous in measurement of total protein in CSF.

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Accuracy and Precision of Analyses for Total Cholesterol as Measured with the Reflotron Cholesterol Method

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We compared plasma cholesterol measurements made with the Boehringer Mannheim Reflotron reflectance photometric analyzer in 1298 capillary blood samples with measurements made in venous blood samples collected at the same time and analyzed in four standardized Lipid Research Clinics laboratories. The Reflotron measurements averaged 0.8% to 7.8% lower than the laboratory values. Correlations (r) between the two sets of measurements ranged from 0.92 to 0.96. In some samples, however, the Reflotron values differed from the laboratory values by ≥12%; the cholesterol concentrations in these samples tended to be higher than in those for which better agreement was observed. The smaller negative biases were observed when test strips were used that were calibrated with reference to the Centers for Disease Control Reference Method for cholesterol. The agreement between sequential Reflotron values averaged ≤4.3%. There was an average difference of ≤1.0% between Reflotron measurements made in each of two sequential capillary blood samples taken from a single finger puncture.

Additional Keyphrases: *cholesterol measurement in capillary blood, venous blood · reflectance photometry · test strips · screening · variation, sources of*

The National Institutes of Health Cholesterol Consensus Conference concluded that a causal relationship exists between increased concentrations of blood cholesterol and atherosclerotic cardiovascular disease, and that individuals whose concentration of cholesterol in plasma exceeds the 75th percentile have an unacceptably high risk for this disease (*1*). Subsequently, revised risk-linked concentrations for cholesterol were recommended by the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Desirable cholesterol concentrations are those <2000 mg/L, or about the 50th percentile; concentrations

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