Benzethonium Chloride Method for Proteins Adapted to Centrifugal Analysis

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A benzethonium chloride method for measuring protein concentrations in several types of body fluids has been adapted to the Cobas Bio centrifugal analyzer. The ability of this analyzer to add two reagents stepwise, measuring absorbances after each addition, is essential for proper blanking of urine specimens. The method has excellent curve stability and precision and yields results that correlate well with those of four other protein methods.

Additional Keyphrases: urine · cerebrospinal fluid · kinetic analysis

Over the years, requests for determination of total protein in urine, cerebrospinal fluid (CSF), and dialysate fluid have led to the development of many analytical methods. In my laboratory, proteins in urine were previously measured with a manual method involving trichloroacetic acid (TCA) or with the Du Pont acc two-pack benzethonium chloride method. Proteins in CSFs were measured by the manual TCA method, the rate biuret method in the Beckman Astra 8, or the acc method for cerebrospinal fluid, which is a bichromatic method involving TCA. Protein concentrations in dialysate fluids were usually measured by the manual TCA method. Calibrating these four methods so that results for each type of specimen were interchangeable between appropriate methods was difficult because of the differences in methodology and the different sensitivities to the albumin/globulin ratio.

I have developed an automated procedure, based on the benzethonium chloride method of Iwata and Nishikaze (1), that has replaced all the other methods in my laboratory. My adaptation of this method to the Cobas Bio centrifugal analyzer can be calibrated with the Urinary Protein Reference Material from the College of American Pathologists, and the standard curve can be stored at least 30 days without drifting. The reaction time has been shortened from the 40–60 min used by Iwata and Nishikaze (1) to a more convenient 10 min.

Benzethonium chloride belongs to a large class of chemicals known as benzalkonium compounds. Jacob (2), and later Seifler and Zymaris (3), reported the turbidimetry of serum alpha-globulins with octadecylmethylbenzylammonium chloride (Octab) at alkaline pH.

Materials and Methods

I compared the concentration of protein in the various fluids as measured with one or more of the following instruments: the acc (Du Pont Co., Wilmington, DE 19898) for CSF specimens (with acc "CFP" packs) and for urine and dialysate fluids (with acc "UP" packs); Astra 8, with use of the protein module (Beckman Instruments, Brea, CA 92621) for CSF specimens; Cobas Bio centrifugal analyzer (Roche Analytical Instruments, Nutley, NJ 07110) for CSF, urine, and dialysate fluids; and the manual TCA method, with a Stasar III spectrophotometer with flow-through cuvette (Gilford Instruments, Oberlin, OH 44074), for urine and dialysate fluids.

The manual TCA procedure is a modification of the method of Meulemans (4), involving 30 g/L TCA; I used a 1:10 (by vol) ratio of sample to TCA solution instead of a 1:4 ratio.

The procedure as used with the Cobas Bio is an adaptation of the method of Iwata and Nishikaze (1). The sample is mixed with the buffer solution (per liter, 0.5 mol of NaOH and 33 mmol of EDTA; pH 12.5) and the absorbance is measured at 660 nm for each sample (this is the blank). Benzethonium chloride (5) (2.0 g/L Sigma Chemical Co., St. Louis, MO 63178) is added and the absorbance measured again after 10 min. I used various dilutions of the Urine Protein Reference Material (College of American Pathologists, Skokie, IL 60077) to generate a six-point calibration curve. The complete Cobas Bio program is as follows: 1. Units, mg/L; 2. Calculation factor, 1000; 3. Standard concn 1, 50; 3. Standard concn 2, 275; 3. Standard concn 3, 550; 3. Standard concn 4, 1100; 3. Standard concn 5, 1650; 3. Standard concn 6, 2200; 6. Limit, 2200; 7. Temp, °C, 25.0; 8. Type of analysis, 7.6; 9. Wavelength, nm, 660; 10. Sample vol, μL, 5; 11. Diluent vol, μL, 25; 12. Reagent vol, μL, 200; 13. Incubation time, s, 10; 14. Start reagent vol, μL, 50; 15. Time of 1st read, s, 0.5; 16. Time interval, s, 600; 17. No. of readings, 2; 18. Blanking mode, 1; 19. Printout mode, 0 (2). Printout mode 2 is for storing a new standard curve; in printout mode 0 a previously stored curve is used for the calculations.

Results and Discussion

Effect of reaction time and temperature on curve shape. I assayed the standards for the six-point calibration curves at three temperatures and five reaction times, to determine the optimum conditions for the reaction in the Cobas Bio centrifugal analyzer. As Figure 1 shows, the reaction time affects the shape of the curves at all three temperatures. At the longest reaction time (10 min) the upper half of the curve is a nearly straight line, with increasing curvature in the lower half of the curve. As the reaction time is shortened, the curvature becomes more pronounced and includes the entire curve. The shape of the curve will affect the ability of the Cobas Bio analyzer to fit the curve with its dens option (Data Evaluation of Nonlinear Standard curves.
is a standard option with later models of the Cobas Bio). The DENS option can fit the data from any of the curves by using curve model 2, a five parameter log-logit model; fewer iterations are usually required for the data from the curves for 5- and 10-min reaction times.

**Curve stability.** The effect of temperature on the reaction is noticed primarily in the day-to-day stability of the standard curve. The standard curve for the 10-min reaction at 25 °C is very stable, and it can be used for at least 30 days without recalibration. The time and expense saved by omitting standard curves from routine runs is considerable in view of the multipoint curve necessitated by the non-linearity of the reaction. The total analyzer time required is 15–20 min, depending on the number of specimens and number of iterations required for the calculations. Standard curves for reactions at 30 and at 37 °C are not as stable as for those at 25 °C.

**Sensitivity.** The DENS option allows for calculating only values that fall between the lowest and highest standards: thus the sensitivity of the method is best for a 10-min reaction time. With the 10-min reaction time, the lowest usable standard (the sensitivity limit) is 50 mg/L; at shorter reaction times, the lowest reproducible standard is about 100 mg/L.

**Precision.** The precision of the procedure is excellent throughout the range of standards: the within-run CV was 3.7% at 160 mg/L and 1.0% at 1080 mg/L: the between-day CV was 1.7% at 660 mg/L and 1.5% at 1110 mg/L.

**Method comparison.** Table 1 summarizes the results of method-comparison studies between the Cobas Bio benzethonium chloride method and the manual TCA method (for urines and dialysate solutions), the Astra 8 biuret method (for CSF specimens), the aca with "CFP" packs (for CSF specimens), and the aca with "UP" packs (for urines and dialysate solutions). Results for the Cobas Bio method correlate well with those by the other methods except for the aca "CFP" packs for CSF specimens when calibrated with the Urine Protein Reference Material. Results with the aca "CFP" packs agree with those by the Cobas Bio when the aca is calibrated with a diluted serum that has a normal ratio of albumin to globulin. This is not surprising: the Urine Protein Reference Material is a solution of human albumin (Cohn Fraction V), whereas Du Pont recommends calibrating the "CFP" channel with a protein solution containing albumin and globulins in the normal ratio (1.8:1.0).

A few urines gave widely divergent results by Cobas Bio as compared with the aca and manual TCA results. Repeating the analysis of these samples failed to resolve the differences. Because in the aca method the first pack is used for a blank, with benzethonium chloride in the second pack, I tried matching the short reaction time of the aca (about 45 s) on the Cobas Bio, but this also failed to resolve the differences between the two methods. When results by these methods differed substantially, the Cobas Bio values were always higher.

**Additional comments.** The Cobas Bio method is very versatile because two reagents can be added sequentially, with the absorbance being measured after each addition. This blanking procedure is essential for accuracy in measuring proteins in urine.

The stability of the standard curve makes the procedure attractive by obviating the time-consuming preparation of accurately diluted standards. However, the DENS option does have to recalculate the five parameters after each run, even when using a stored curve. Apparently the analyzer stores only the data points from the stored curve and not the five parameters.

This benzethonium chloride-based method can probably be successfully adapted to other automated chemistry analyzers that have the required blanking and calculating capabilities. The data for absorbance vs concentration obtained with the Cobas Bio can be manually entered in the Ames-Gilford "Optimate" by using the immunoassey 33 test code. Fitting data by a four-parameter log-logit model gives results identical to those derived with the Cobas Bio analyzer. However, the Optimate does not have the two-reactant capability needed for the proper blanking of the urine specimens. Plotting the Cobas Bio data on graph paper and drawing a smooth curve manually will give acceptable precision for reading unknown concentrations.

I have not done reference-range studies with the present Cobas Bio procedure. With appropriate calibration, results by the three CSF protein methods studied differed by less than 30 mg/L in the range of 250 to 1000 mg/L. However, the positive bias of the Cobas Bio method could cause an

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**Table 1. Summary of Linear Regression Analysis**

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample</th>
<th>Range, mg/L</th>
<th>n</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
<th>SE, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>aca (UP)</td>
<td>Urine</td>
<td>140–6850</td>
<td>42</td>
<td>1.031</td>
<td>132.9</td>
<td>.9969</td>
<td>163.8</td>
</tr>
<tr>
<td>Manual TCA</td>
<td>Urine</td>
<td>20–8150</td>
<td>43</td>
<td>1.061</td>
<td>183.9</td>
<td>.9956</td>
<td>175.0</td>
</tr>
<tr>
<td>aca (UP)</td>
<td>Dialysate</td>
<td>270–1890</td>
<td>11</td>
<td>1.084</td>
<td>51.1</td>
<td>.9953</td>
<td>56.8</td>
</tr>
<tr>
<td>Manual TCA</td>
<td>Dialysate</td>
<td>280–2180</td>
<td>22</td>
<td>0.951</td>
<td>68.8</td>
<td>.9850</td>
<td>82.7</td>
</tr>
<tr>
<td>Astra 8</td>
<td>CSF</td>
<td>130–1390</td>
<td>46</td>
<td>0.942</td>
<td>45.8</td>
<td>.9927</td>
<td>33.1</td>
</tr>
<tr>
<td>aca (CFP)</td>
<td>CSF</td>
<td>110–1490</td>
<td>24</td>
<td>0.821</td>
<td>142.9</td>
<td>.9837</td>
<td>49.0</td>
</tr>
<tr>
<td>aca (CFP)</td>
<td>CSF</td>
<td>230–1280</td>
<td>20</td>
<td>0.935</td>
<td>43.6</td>
<td>.9868</td>
<td>44.8</td>
</tr>
</tbody>
</table>

*y = benzethonium chloride method used in the Cobas Bio analyzer.

*Calibrated with Urine Protein Reference Material.

*Calibrated with diluted human serum.
increase in the urine protein reference range. Each laboratory should establish its own reference range for urinary protein.

References

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Increased Serum Sulfate in Pregnancy: Relationship to Gestational Age
David E. C. Cole,1,3 Lesley S. Baldwin,1 and Linda J. Stirk2

Controlled-flow ion chromatography has significantly improved the precision with which inorganic sulfate (SO4) can be measured in serum. In this study, we have shown that serum SO4 is increased in pregnancy. The increase appears to follow gestational age, resulting in a 39% higher value by the middle of the third trimester. We suggest that this increase is a natural physiological process, which enhances SO4 availability to the growing fetus and placenta.

Until recently, the relative imprecision of standard assay methods has prevented a detailed analysis of factors affecting concentrations of sulfate (SO4) in the circulating blood (1). Renewed interest in this metabolite has been stimulated by the discovery of new classes of sulfate conjugated molecules, including the tyrosine residues of selected proteins (2), bile acids, and an expanding family of glycolipids and glycoproteins (3).

Furthermore, new physiological roles for the sulfonconjugation process have been described. The growing fetus is a net consumer of SO4 (4), obtaining the metabolite as free inorganic SO4 after transfer from maternal to fetal circulation via active transplacental transport (5, 6). Several earlier reports described an increase in SO4 in the serum of pregnant women (7, 8), but offered little information about its relation to gestational age. We assayed serum SO4 in 144 pregnant women and 44 non-pregnant controls. The aggregate data demonstrate a strong positive correlation between gestational age and SO4 concentration, irrespective of the group into which the subject was categorized.

Materials and Methods

Samples were obtained from five groups of women: (a) controls, (b) pregnancy terminations, (c) clinic pregnancies, (d) high-risk pregnancies, and (e) women in labor. The 44 non-pregnant controls were laboratory staff volunteers, 17 to 44 years old. Pregnancy terminations (n = 28) were women of ages 13 to 39 years, who were admitted to hospital in the mid-second trimester for therapeutic abortion. All of these women were ostensibly in good health. Clinic pregnancies (n = 39) were healthy, ambulatory women, ages 15 to 35 years, who were seen for routine antenatal care at the walk-in clinic of the Grace Maternity Hospital. The 31 high-risk pregnancies were women of ages 19 to 38 years, who were admitted to hospital during the second and third trimester with potential obstetric complications. Patients with hypertension or renal failure were excluded. None of the women was in any acute distress at the time of study; a complete clinical description of this group appears elsewhere (9). The last group consisted of 46 women, ages 19 to 39 years, who were sampled at the time of delivery. A summary of their clinical features also appears elsewhere (8).

Methods

Blood sampling was not timed, although most specimens were taken during the morning hours. Serum was stored at -20 °C until analysis.

Inorganic sulfate was separated and quantified by controlled-flow ion chromatography according to procedures described previously (10). Serum samples were diluted with a 1 mmol/L solution of NaOH and injected directly. The D-10 Ion Analyzer (Dionex Instruments, Sunnyvale, CA) was equipped with two guard pre-columns. Chromatographic profiles of sera from pregnant women were quantified by peak height against known standards.

Statistical analyses included Student's t-test, one-way analysis of variance, and multiple linear regression analysis. A MINTAS statistical package (University of Pennsylvania, 1981) was used for data reduction.

Results

Table 1 shows the statistics describing each group. Values for non-pregnant controls are not different from those we have described previously (10). Mean serum SO4 was higher in all the groups of pregnant women as compared with controls. In the case of the pregnancy-termination group the difference was small, but the other groups were significantly higher (p <.05), as judged by one-way analysis of variance. Because there are differences among the mean gestational intervals for each of the four pregnancy groups, we used