Electroimmunoassay of Albumin in Human Serum: Accuracy and Long-Term Precision

Mark W. Pascucci, Daniel W. Grisley, Jr., and Roy N. Rand

An improved procedure for the Laurell "rocket" technique is described. Samples were electrophoresed in an agarose gel containing anti-human albumin. The gel plates were processed, the peaks stained, and peak heights used to calculate albumin concentrations. Factors affecting precision were (a) adequate heating of agarose gel before antibody is added, (b) accurate leveling of the gel surface during plate formation, (c) applied voltage during sample application, and (d) avoidance of the "edge" effect on sample placement in the gel. Multi-plate long-term precision (CV) for the method was 6.2% at a mean albumin concentration of 13 g/L and 3.0% at 37 g/L. Analytical recovery of 8 and 11 g of albumin per liter was 99 to 100%. There was negligible interference from hemoglobin and dextran as well as several common substances that bind to albumin—bilirubin and salicylate. Because of its high accuracy and good long-term precision, the method is a possible candidate reference method for serum albumin.

Additional Keyphrases: proteins • reference methods • analytical error • rocket immunoelectrophoresis

Immunochromatographic techniques for the measurement of albumin reportedly are more nearly accurate and specific than other methods (1). Electroimmunoassay (EIA; the Laurell "rocket" technique) was selected by the International Federation of Clinical Chemists as a candidate reference method, presumably because of practical advantages over immunoprecipitation and radial immunodiffusion (2). Although there are several seemingly excellent descriptions of the albumin electroimmunoassay method (3) and of the electroimmunoassay technique in general (4), our attempts to set up an electroimmunoassay according to published reports (5–7) produced a method having poor precision. Accordingly, we investigated various steps of the technique for their effects on variability, with the aim of improving the procedure. After a successful protocol had been defined that had acceptable within-run precision, we studied recovery, interferences, and long-term precision.

Materials and Methods

Materials

We obtained barbital buffer (preserved solid, packaged, B2 Buffer; Helena Laboratories, Beaumont, TX 77704); agarose, electrophoresis grade (Gibco, Grand Island, NY 14072); Coomassie Brilliant Blue R-250 and glacial acetic acid (both from Eastman Laboratory Chemicals, Rochester, NY 14650); and anti-human albumin (Atlantic Antibodies, Scarborough, ME 04074). Sodium salicylate, acetylsalicylic acid, bilirubin (Sigma grade), and dextran (clinical grade) were from Sigma Chemical Co., St. Louis, MO 63178.

Solutions. Reagent-grade water ("Milli-Q System"; Millipore Corp., Bedford, MA 01730) was used to prepare all aqueous solutions. Stock barbital buffer (75 mmol/L, pH 8.6) was prepared from preweighed packages; working barbital buffer (20 mmol/L, pH 8.6) was prepared by diluting this stock buffer and filtering it through a 0.45-µm pore-size filter. A 10 g/L agarose solution was divided into 20 mL aliquots, which were stored at 4°C. The destaining solution was 96% ethanol (960 mL/L)/glacial acetic acid/H2O (45/10/45 by vol). For staining we used a 5 g/L solution of Coomassie Brilliant Blue in destaining solution.

Solutions used in checking for interference were prepared in a pooled specimen of normal human serum as follows. For salicylate, acetylsalicylate, and dextran, an appropriate amount of solid was dissolved in the serum pool. For bilirubin we added 0.1 mL of a 20 g/L bilirubin solution in 0.1 mol/L NaOH to the serum pool to yield a final concentration of 200 mg/L. For hemoglobin, we added to the serum pool a clear hemolsate containing 137 g of hemoglobin per liter to yield a final hemoglobin concentration of 4 g/L.

Calibrators. Sources of human serum albumin were obtained from the following suppliers: lot no. 36, Pentex®, crystallized (Miles Laboratories, Elkhart, IN 46515); lot no. PRS-423, crystallized (Dade, Miami, FL 33152); lot no. 76C-7460, crystallized (Sigma Chemical Co.); lot no. 0945, crystallized (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, OH 44128); and Hyland (Hyland Diagnostics, Division of Travenol Laboratories, Inc., Deerfield, IL 60015).

Volatiles and ash content of albumin were determined thermogravimetrically at 120°C and 750°C, respectively, with the Du Pont TGA Model 951 (Du Pont Instruments, Wilmington, DE 19888). The monomeric purity was determined by electrophoresis in polyacrylamide gel, 75 g/L, after incubating the sample at 100°C for 5 min in sodium dodecyl sulfate, 20 g/L, to denature the proteins and render all molecules negatively charged. The globulin content was determined by electrophoresis on cellulose acetate.

We analyzed the material for albumin and globulins and established the albumin content by subtracting the weight of volatiles, ash, and globulins from the total weight of the commercial material.

We prepared stock calibrator solutions of albumin by dissolving a verified amount of albumin in reagent water, correcting appropriately for partial specific volume and purity (8). The solutions were preserved with sodium azide.

*This analysis was done by Dr. John Travis, Department of Biochemistry, University of Georgia, Athens, GA.
We prepared patients' samples and controls by diluting 15 μL of each with 5.0 mL of working barbital buffer. Typical working calibrator solutions were prepared by diluting 5, 10, 15, 20, and 24 μL of a 36 g/L stock solution with 5.0 mL of working barbital buffer to yield solutions having nominal albumin concentrations of 12.0, 24.0, 36.0, 48.0, and 57.6 g/L.

Control materials. As control materials, we used a human serum pool (MP 9044; Medical Products Division, Eastman Kodak Co.) with an albumin content of 13.1 g/L, and "Hyland II" (lot no. 3475U001A, lyophilized; Hyland Diagnostics) with an albumin content of 36.7 g/L.

Apparatus

For electroimmunoassay we used the Multiphor® System powered by a Model 2103 power supply (both from LKB Instruments Inc., Rockville, MD 20852). The electrophoresis chamber was cooled by a circulating cooling water bath. A precision level (Starrett® no. 97; L. S. Starrett Co., Athol, MA 01331) was used in adjusting the leveling table.

For electrophoresis of albumin on cellulose acetate we used a Helena electrophoresis system (Helena Laboratories, Beaumont, TX 77704) and scanned the resulting electropherograms with a Corning 750 densitometer (Corning Medical and Scientific, Corning, NY 14830). For dilutions we used a Model 25000 pipettor (Micromedic Systems, Horsham, PA 19044). A "Micro/Pettor" (Scientific Manufacturing Industries, Inc., Emeryville, CA 94608) was used to deliver 10 μL of each sample dilution to the immunoelectrophoresis gel.

Procedures

Plate preparation. Liquify two 20-mL aliquots of the 10 g/L agarose gel in a bath of boiling water for 45 min. Place the cooling plate of the Multiphor System on the leveling table, and level the table with the aid of the precision level. Warm the plate to 55 °C by connecting it to a circulating cooling water bath. Place a 125 x 260 mm glass plate on the warmed plate and allow it to come to 55 °C.

Immerse the tubes of hot agarose solution in the 55 °C water bath for 5 min. Transfer the agarose to a mixing vessel, add about 0.16 mL of the anti-human albumin, and mix thoroughly. The exact volume of anti-human albumin used depends on the titer of antibody, which is determined by adjusting the amount of antibody so that the resulting peaks are approximately 1 and 4 cm high for 10 and 50 g/L calibrators, respectively.

Pour the warm agarose–antibody mixture onto the warm, leveled plate. Spread the liquid quickly and evenly to the edges of the plate with a glass rod. Solidify the gel by immediately circulating cold water through the cooling plate. At 6-mm intervals (measured center to center) punch 36 wells, 4 mm in diameter, into the gel, in a line parallel to and 30 mm from the long edge of the plate. Place the end wells at least 15 mm from the short edges of the plate.

Electrophoresis. Dilute 15 μL of each unknown and control with 5.0 mL of working barbital buffer. Set up the Multiphor system according to the manufacturer's instructions, with special attention to leveling the buffer tank and placement of the wicks. Using a volt/ohm meter attached to the LKB gel probe, adjust the power supply so that 3–4 V/cm is applied across the gel.

Pipet 10 μL of each dilution per well. Place calibrators, controls, and patients' samples in random order in positions 3 through 18. Place duplicates in the same pattern in positions 19 through 34. To avoid increased imprecision from an edge effect, place "dummy" samples in positions 1, 2, 35, 36 (see below). Electrophorese for 16–18 h at 8 °C, press, dry, stain, and destain the gel (5).

Calculations. Measure the peak heights to the nearest 0.5 mm, determine the linear regression equation for calibrator concentrations vs calibrator peak height, and use the equation to calculate the concentrations of the unknowns. Figure 1 shows a typical calibration curve.

Results and Discussion

Pentex (lot 36) was selected as the calibrator material because it had the lowest globulin content of all the albumins tested (Table 1). Its ash content was acceptably low, < 1.0%. Volatiles content averaged 6.5%. We evaluated the purity of the albumin rather than depending on information from the vendor, because of the large bottle-to-bottle variation (± 1.5%) in the volatiles content and discrepancies with the vendor-assigned value of 2.2%.

Aggregate analysis of the material showed it to contain 90% monomer, 5% dimer, and 5% polymer. We estimated that this proportion of nonmonomeric albumin in the calibrator material caused an average positive error of 2.5% in the apparent albumin concentration of serum samples (9).

The first factor improving within-run precision was adequate heating of the agarose gel. The agarose gel had to be
liquified in a boiling water bath for about 45 min before the antibody–agarose mixture was prepared. Heating the agarose for only 15 min resulted in a visually homogeneous mixture but unacceptably high within-run variability (Table 2). The improvement in variation was significant ($\alpha = 0.05$).

The second factor was accurate leveling. We found that a plate prepared with a deviation of only 0.07" off-level produced significantly ($\alpha = 0.05$) less precise analyses (Table 3). The ability to level the plate to this tolerance with the level provided in the Multiphor kit was difficult; use of a precision level was necessary to produce plates with the desired quality.

The third factor was applying voltage to the gel plate during sample pipetting. For example, at a concentration of 22 g/L, the within-run CV ($n = 14$) was 1.3% with applied voltage as opposed to 2.4% without applied voltage during sample pipetting. Pipetting time was less than 10 min. Weeke (6) recommended that the current be on during sample application to avoid diffusion. Laurell (10), however, suggested that if the interval between the first and last sample pipetting did not exceed 10 min, diffusion would not affect the results.

### Table 2. Effect of Duration of Heating of Agarose Gel on the Within-Run Precision of Controls*

<table>
<thead>
<tr>
<th>Mean albumin, g/L</th>
<th>CV, % after heating for</th>
<th>45 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td></td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>2.0</td>
<td>9.3</td>
</tr>
</tbody>
</table>

*The positions of the two controls were alternated on the plate. 
$n = 10$ each.

### Table 3. Effect of Accurate Leveling on Within-run Precision

<table>
<thead>
<tr>
<th>Mean albumin, g/L</th>
<th>CV, % for wells prepared</th>
<th>Accurately leveled</th>
<th>0.07&quot; off level</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td></td>
<td>2.2</td>
<td>12.1</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>1.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

$n = 11$ each.

The last factor studied was the “edge” effect (see Procedure). This phenomenon is illustrated in Figure 2 in which the same sample (39.8 g/L) was placed in wells 1–34, with the end wells 15 mm from the edge of the plate. Toward the edge the peak heights have a rising trend with a maximum bias of 1 mm (2.5%) from the mean peak height. Laurell and MacKay reported a similar effect (4). Contrary to our results, Weeke (5) reported that the edge effect could be avoided by placing the end wells at least 10 mm from the gel edge.

Although brief published statements have been made concerning the precision and accuracy of this method (1, 7, 10), no systematic study has been reported. Within-run, day-to-day, and total precision were determined by running controls in triplicate, one run per day for 35 days along with patients’ samples (Table 4).

Long-term CV at 13 g/L was less impressive than that obtained at 37 g/L. Both within-run and day-to-day components of variation contributed about equally to the total. Recoveries of crystallized albumin added to human serum pools at 8 and 11 g/L were essentially quantitative (Table 5). Although immunoelectrophoresis is considered a highly specific method for the determination of proteins, we expected that substances that bind strongly to albumin, and also high-Mr substances, might alter the immunoprecipitation mechanism during electrophoresis. Therefore, we tested several commonly occurring compounds in these categories for possible interference with the assay by adding them to normal human serum pools at realistically high therapeutic and physiological concentrations (Table 6). Of the compounds studied, only bilirubin showed a small, but statistically significant, effect.

We believe that the electroimmunoassay for albumin, performed as described here, is accurate, reliable, and reasonably precise over long periods of time; therefore, it is a reasonable candidate for a reference method. Further studies are planned: (a) lot-to-lot variations of reagents, (b) further means of reducing variance, (c) the influence of

### Table 4. Long-Term Precision

<table>
<thead>
<tr>
<th>Mean albumin, g/L</th>
<th>CV, %</th>
<th>Within run</th>
<th>Day to day</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>3.4</td>
<td>4.6</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>1.8</td>
<td>2.5</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

$n = 105$, over a 35-day period.

### Table 5. Analytical Recovery of Albumin

<table>
<thead>
<tr>
<th>Albumin concn, g/L</th>
<th>% recovered</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>In pool</td>
<td>Added</td>
<td>Mean</td>
</tr>
<tr>
<td>23</td>
<td>8</td>
<td>99.6</td>
</tr>
<tr>
<td>37</td>
<td>11</td>
<td>100.4</td>
</tr>
</tbody>
</table>

$n = 8$ for each pool.

### Table 6. Interference Study*

<table>
<thead>
<tr>
<th>Substance</th>
<th>Conc. g/L</th>
<th>g/L</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>0.35</td>
<td>+0.4</td>
<td>+1.1</td>
</tr>
<tr>
<td>Acetylsalicylate</td>
<td>5.00</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.20</td>
<td>−0.8</td>
<td>−2.1</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>4.00</td>
<td>−0.3</td>
<td>−1.1</td>
</tr>
<tr>
<td>Dextran</td>
<td>10.00</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Results are the mean of eight applications of supplemented and base pools on a single plate.

*Significant ($\alpha = 0.05$) by Student’s t-Test.
aggregates in lyophilized albumin preparations, and (d) sample-to-sample interactions during electrophoresis.

We thank the Industrial Laboratory of Eastman Kodak Co. for performing volatile and ash assays on albumin. We also thank Dr. Richard B. Coolen for critical and helpful comments in the writing of the manuscript.

References

Semi-Automated Continuous-Flow Enzyme Immunoassay for Antiepileptic Drugs in Serum
Sadao Hayashi, Shigeru Kurooka, Kazutaka Arisue, Kadzuma Kohda, and Chozo Hayashi

We have developed a semi-automated method for measuring five kinds of antiepileptic drugs in serum by successfully adapting commercial competitive-binding enzyme immunoassay kits (MARKIT®; Dainippon) for use with a continuous-flow analyzer (Technicon AutoAnalyzer II equipped with a dialyzer). The free enzyme-labeled drug is automatically separated by a microfilter from the competitive immunoreaction mixture between labeled and unlabeled drug for anti-drug immunoglobulin coupled to bacterial cell walls. The concentrations of the antiepileptic drugs in serum samples can be determined by automated measurement of enzyme activity of the enzyme-labeled drugs. Results of the semi-automated method correlated well with those obtained by manual enzyme immunoassay, gas–liquid chromatography, and "high-pressure" liquid chromatography. The correlation coefficients were all >0.95, showing the practicality of this method for therapeutic monitoring of antiepileptic drugs.

Additional Keyphrases: phenytoin • phenobarbital • primidone • carbamazepine • valproic acid • monitoring therapy

For routine therapeutic monitoring of antiepileptic drugs (AED) in the serum of patients with epilepsy (1), automated methods are desired. Concentrations of AED in serum, as measured with commercial enzyme immunoassay (EIA) kits (MARKIT®, Dainippon Pharmaceutical Co., Ltd., Osaka, 541 Japan) based on a competitive-binding EIA (2, 3), agree well with results by radioimmunoassay, fluoroimmunoassay, gas–liquid chromatography, and "high-pressure" liquid chromatographic methods and are used for therapeutic drug monitoring in Japan (4, 5).

Recently, we have succeeded in adapting these EIA kits for use with a Technicon AutoAnalyzer II with a microfilter FM 65 (Fuji Photo Film Co., Tokyo 106), and now can measure the serum AED semi-automatically. In the manual (kit) method, the competitive immuno-reaction mixture between AED in samples and E-AED against anti-AED immunoglobulin coupled to carrier bacterial cell walls in test tubes with tight rubber caps is centrifuged upside down. To the free E-AED recovered in the supernate after the tubes are set upright on the test tube rack, one adds substrate for measurement of the enzyme activity, which has a stoichiometric relationship with AED concentrations in the samples (Figure 1).

In semi-automation of the EIA, the mixture containing the competitive immuno-reaction between AED in samples and E-AED against the anti-AED is aspirated into the pumping tube (Figure 2), and the free E-AED, separated from the bound E-AED by a membrane (B/F separation),

![Fig 1. Assay principle of manual EIA with MARKIT reagents](#)

1. The Central Laboratory for Clinical Investigation, Osaka University Hospital, 1-1-50, Fukuishinakku, Osaka, 555 Japan.
3. Nonstandard abbreviations: AED, antiepileptic drugs; E-AED, enzyme-labeled AED; and EIA, enzyme immunoassay.