Measurement of Glycosylated Hemoglobin on Cellulose Acetate Membranes by Mobile Affinity Electrophoresis

Jeffrey Ambler,¹ Borek Janík,² and Geoffrey Walker¹

In this method for separating glycosylated from nonglycosylated hemoglobin in blood by electrophoresis on cellulose acetate membranes, we exploit the affinity of low-molecular-mass dextran sulfate for the nonglycosylated fraction, which increases the mobility of the latter relative to that of glycosylated hemoglobin. After the membrane strips are cleared and stained, the two fractions are quantified densitometrically. As evaluated by use with blood from diabetics, results compare well with those by chromatography on short columns and by electrophoresis in commercial agar gel films.

Glycosylation of hemoglobin, a nonenzyme-mediated, post-translational modification, occurs slowly in erythrocytes (1). Glucose is bound by a stable ketoamine linkage to the N-terminal valine residue of the hemoglobin beta-chain (2). The extent of glycosylation depends on the blood glucose concentration and measurements of it are made to monitor long-term control of blood glucose in patients with diabetes (3).

There have been several approaches to the measurement of glycosylated hemoglobin concentration. Long ion-exchange columns (1) and isoelectric focusing (4) are too complex and tedious for routine use. "High-performance" liquid chromatography (5) can be automated to carry out 50 analyses per day with good precision, provided the equipment is available in the laboratory. Estimations involving short ion-exchange columns (6) and an automated thiobarbituric acid (7) method have been used in clinical laboratories, but both have disadvantages. The short ion-exchange column method is capricious, being affected by small variations in temperature (8), pH (9), and buffer (10). The thiobarbituric acid method, although it has been optimized (11), has not performed well in our hands. More recently, an agar gel electrophoretic method with direct densitometry of unstained gels has been introduced (12). This method is not affected by temperature, buffer, or pH variations (13) and is extremely simple to use. However, films are tedious to prepare and costly when bought ready made.

We present a method based on a new concept, mobile affinity electrophoresis. It requires no special apparatus and exploits the convenience and economy of cellulose acetate membranes. Large batch analyses may be carried out and results correlate well with those obtained by the short-column method and by agar gel electrophoresis.

Materials and Methods

Mobile Affinity Electrophoresis

Reagents and equipment. The following equipment and methods were kindly supplied by Gelman Sciences Inc., Ann Arbor, MI 48106: Sepratek Electrophoresis Chamber, Sepratek Applicator (eight-sample size), Power Supply, ACD-18 Computing Densitometer, protein stain (Ponceau S, 5 g/L in trichloroacetic acid solution, 75 g/L), "Super Separclear" clearing solution, and "Super Sepaphore" membranes. In addition we also used the following solutions: sodium chloride solution, 8.5 g/L; hemolyzing solution (per liter, 1 g each of saponin and EDTA); affinity electrophoresis buffer, pH 6.4, 33 mmol/L citrate containing 2 µmol of dextran sulfate and 8 µmol of disodium EDTA per liter (obtainable as "Glyco-Phore Buffer" from Gelman Sciences, prod. no. 51261).

Sample preparation. Blood was collected into tubes containing EDTA as anticoagulant. We added 100 µL of whole blood to 5 mL of sodium chloride solution in a centrifuge tube, mixed gently, and centrifuged. The supernatant fluid was aspirated as completely as possible and 350 µL of hemolyzing solution was added.

Electrophoresis. Membranes were equilibrated with affinity electrophoresis buffer for at least 10 min and the chambers were filled with buffer. The applicator wells were loaded with hemolyzed samples (7 µL per sample well) by use of a Gilson "Pipetman" or similar device. The membranes were blotted and placed in the chamber and the samples were applied. To ensure an even application, the applicator was not removed from the membrane until the sample was totally absorbed. After electrophoresis at 150 V for 40 min the strips were promptly placed in stain for 10 min. Excess stain was washed out by constantly agitating in changes of 50 mL of acetic acid for a maximum of 5 min. The membranes were cleared in "Super Separclear" for 2 min, transferred to a glass plate, and placed in an oven at 80–90 °C for about 10 min until transparent. The separations were scanned from the anode with an ACD-18 Densitometer at a wavelength of 520 nm.

Agar Electrophoresis

Prepared agar films were obtained as a kit (Glytrac; Corning Ltd., Halstead, Essex, U.K.) and the method performed according to the manufacturer's instructions, with the Corning cassette electrophoresis cell, variable power supply, applicator syringe, and drying oven. Whole-blood hemolysates (1 µL) were applied directly to the preformed wells in the agar. After electrophoresis at 60 V for 40 min, the gel was dried and scanned, unstained, with a Beckman Densitometer (R-112) fitted with a special 420-nm filter donated by Corning Ltd.

Short Ion-Exchange Columns

These were obtained from BioRad Ltd., Watford, Herts, U.K., and analyses were performed according to the manufacturer's instructions.

Results

A typical separation of specimens containing various amounts of glycosylated hemoglobin is shown in Figure 1; densitometric scans of tracks 1 and 2 are shown in Figure 2.

Sample Preparation

The results were not affected by the anticoagulant used for blood collection. Blood from five unselected individuals

¹ Department of Clinical Chemistry, University Hospital, Nottingham NG7 2UH, U.K.
² Gelman Sciences Inc., 600 South Wagner Rd., Ann Arbor, MI 48106.

Received May 25, 1982; accepted Oct. 26, 1982.
Effect of Variations in Temperature and Buffers

The performance of the method was little affected by changes in temperature or buffer pH and ionic strength, as shown by the following tests. Eight samples from diabetic patients were chosen without conscious bias, applied to membranes, and then subjected to electrophoresis under the conditions outlined in Table 1. It being impractical to examine all possible variations, an alteration in one parameter was examined while retaining the others at optimum conditions. The assay was very little affected by these changes, indicating a wide margin for error in dissolving the buffer powder. Change in the buffer concentration altered the distance of the separation rather than the distribution of protein between the two bands, as shown in Figure 3. High ionic strength, i.e., >75 mmol/L, produced no separation, just a single, very tight band. Progressive dilution of the buffer produced greater separation of the bands and an increasingly diffuse band for nonglycosylated hemoglobin. We found the optimum concentration of buffer was 30–40 mmol/L on the Super Sephaphore membrane. Other membranes may require slightly different conditions; for example, a buffer molarity of 25 mmol/L gave a good separation of the two bands with little diffusion on a Sephaphore III membrane.

Hemoglobin Variants

Only hemoglobin F (HbF), if present, interfered with the quantification by giving a spuriously high glycosylated fraction. When high concentrations of HbF were present, however, the glycosylated hemoglobin (HbA1) values could be corrected simply by subtracting the HbF percent from the apparent HbA1 value. When hemoglobin C was present, double bands were obtained, and the variant was easily recognizable by its slightly anodic mobility in comparison with nonglycosylated hemoglobin. By comparing results from the column and agar-electrophoretic methods, we

![Fig. 3. Effect of varying the buffer strength (mol/L) on the appearance of the separation](image)

Table 1. Effect of Varying the Electrophoresis Conditions on Glycohemoglobin Results for Eight Samples

<table>
<thead>
<tr>
<th>Buffer concn, mmol/L</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strength</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025 M</td>
<td>11.4</td>
<td>11.4</td>
</tr>
<tr>
<td>0.033 M</td>
<td>21.0</td>
<td>21.2</td>
</tr>
<tr>
<td>0.05 M</td>
<td>7.7</td>
<td>7.1</td>
</tr>
<tr>
<td>0.066 M</td>
<td>13.0</td>
<td>13.4</td>
</tr>
<tr>
<td>0.075 M</td>
<td>12.4</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of varying the buffer strength (mol/L) on the appearance of the separation

![Table 1](image)

<table>
<thead>
<tr>
<th>Optimum conditions*</th>
<th>Temp, °C</th>
<th>pH</th>
<th>Buffer concn, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>23.6</td>
<td>9.0</td>
<td>8.5</td>
</tr>
<tr>
<td>13.3</td>
<td>13.3</td>
<td>13.7</td>
<td>12.5</td>
</tr>
<tr>
<td>14.5</td>
<td>14.0</td>
<td>14.9</td>
<td>14.3</td>
</tr>
<tr>
<td>8.5</td>
<td>8.5</td>
<td>8.4</td>
<td>7.8</td>
</tr>
<tr>
<td>14.0</td>
<td>13.7</td>
<td>14.3</td>
<td>13.4</td>
</tr>
<tr>
<td>12.5</td>
<td>13.2</td>
<td>13.3</td>
<td>12.8</td>
</tr>
<tr>
<td>13.2</td>
<td>13.2</td>
<td>13.3</td>
<td>12.8</td>
</tr>
</tbody>
</table>

*Optimum conditions are buffer, 33 mmol/L, pH 6.4, and temperature 18 °C.

CLINICAL CHEMISTRY, Vol. 29, No. 2, 1983 341
found that glycosylated hemoglobin S (HbS) co-migrated with HbA1 and that the two nonglycosylated fractions, HbA and HbS, also co-migrated electrophoretically.

Precision

Coefficients of variation of 4.9 and 3.8% were obtained for 16 replicate analyses of samples with 7.5% and 15% glycosylated hemoglobin content, respectively. The precision of the method was therefore satisfactory for the routine estimation of glycosylated hemoglobin in the clinical chemistry laboratory.

Comparison with the Agar Gel Electrophoresis Method

The hemoglobin bands obtained by the new method were more compact and better defined than the agar gel electrophoresis pattern (Figure 4). Excellent correlation was obtained between paired results of the two methods \( r = 0.94 \), as shown in Figure 5, and results by the two methods can be used interchangeably.

Comparison with the Short Column Method

The correlation between 76 paired results by chromatography on short columns and by mobile affinity electrophoresis was also excellent \( r = 0.94 \) when the columns were temperature controlled at 22 °C. Operating the columns at room temperature and correcting for temperature did not produce such good results \( r = 0.89 \).

Discussion

Affinity electrophoresis is a relatively new concept and hitherto has usually been carried out in agarose (14) or polyacrylamide gels (15) with the active ligand either bound or entrapped in the matrix of the gel. In the method we describe, the active ligand is in the mobile phase and no special modification of the support medium is necessary. This allows use of a simple procedure that is ideally suitable for routine use.

The membrane we used, "Super Sepraphore," is a Mylar-supported cellulose strip. We obtained better separation of the bands with this particular membrane than with the more usual type of nonsupported membrane. Although the electrophoresis could be carried out in any electrophoresis chamber, we used the Gelman Sepratek System because it allowed the simple automatic application of eight samples at one time. Running four chambers from one power pack, we could analyze 32 specimens per batch. Alternatively, one might use the Gelman Semi Micro II Chamber, which takes three membranes.

We did not stain for protein in the initial development work. Instead, a whole-blood hemolysate was taken and applied to the membrane. After electrophoresis the proteins were fixed in trichloroacetic acid and the membrane was dried in an oven. The separation was scanned at 420 nm without protein staining, but the precision of densitometry was not good for a noncleared membrane and a normal glycosylated hemoglobin value. Because the membrane could not be cleared without protein staining, we used washed erythrocyte hemolysates to avoid interference by serum protein staining.

It is becoming increasingly recognized that most simple methods for measuring total glycosylated hemoglobin are subject to interference by an unstable intermediate, which may cause a spuriously high glycosylated percentage to be recorded, especially where grossly above-normal concentrations of blood glucose are present just before the blood is withdrawn. The intermediate may be removed by incubating the blood in a glucose-free medium before estimation (16), a step that has been applied to the agar gel electrophoresis method (17) in which whole-blood hemolysates are normally used. Our preliminary results confirm that the unstable intermediate may be removed by incubating the cells at 37 °C for 5 h in the saline wash before they are centrifuged and hemolyzed.

The new method performed well in comparison with agar electrophoresis, as expected, because the principles of the two methods are similar. In the agar electrophoretic method, the nonglycosylated hemoglobin binds to the sulfate groups in the medium and its mobility is retarded, while the glycosylated fraction is carried towards the cathode by electroendosmosis. In the cellulose acetate method, the nonglycosylated fraction combines with the sulfate groups on the carrier molecule and is separated from the nonglycosylated hemoglobin by electrophoresis. A real advantage of our method is the economy of cellulose acetate membranes. The total cost per test is about 20–30 p, compared with about 80 p per test for the agar electrophoretic method. The new method, however, has the disadvantage of involving more technical steps, e.g., preparing washed cell hemolysates and protein staining. In comparison with the short ion-exchange columns, the cellulose acetate method is easier to use for large batches, has better precision, and shows no interference from changes in temperature, pH, or buffer concentration.

The principle may be applied to any electrophoretic medium such as agarose or polyacrylamide gel that does not bind the carrier molecule. Furthermore, other active groups such as antibodies, enzyme substrates, or lectins might also be bound by existing methods to dextran sulfate. The charged nature of the carrier molecule could then succeed in producing the desired specific separation.
Assessment of the Benzethonium Chloride Method for Routine Determination of Protein in Cerebrospinal Fluid and Urine

Elisabeth Flachaire,1 Odile Damour,2 Jacques Bienvenu,2 Tarak Aoulti,2 and Roger Later1

We have tested the characteristics of the method of Iwata and Nishikaze (Clin Chem 25: 1317, 1979). The linearity, sensitivity, and precision are satisfactory and the reactivity of benzethonium chloride with various proteins (albumin, immunoglobulins) is the same. The method has been compared with Meuleman's technique (Clin Chim Acta 5: 757, 1960), routinely used in our laboratories, by analysis of 82 samples of cerebrospinal fluid (CSF) and 119 samples of urine. Our results for cerebrospinal fluid agree well with those of Iwata and Nishikaze (r = 0.976; y = 0.992x - 0.013), but we find their method unsuitable for urinary protein determination, probably because of interfering compounds in urine.

Additional Keyphrase: turbidimetry

We have been very interested in the new turbidimetric method described by Iwata and Nishikaze (1) for determination of proteins in cerebrospinal fluid (CSF) and urine. Benzethonium chloride in alkali is used as a flocculation reagent. Iwata and Nishikaze say that this method has a higher sensitivity than the classic method of Lowry et al. (2) and shows satisfactory reproducibility and recovery, and that the turbidity produced is the same for albumin and gamma-globulins, and is more stable than in Meuleman's method (3) or in the method of Bossak et al. (4).

We have evaluated their method, and we have compared the results with those by the method we routinely use in our laboratories, the sulfosalicylic acid method (3).

Materials and Methods
Preparation and Storage of Specimens
CSF and urine specimens were promptly centrifuged after collection. Protein was determined concurrently by the two methods, either immediately or after storage at -20°C (the specimen being centrifuged just before analysis). Spectrophotometers used were the Model 25 Beckman (at Pierre Benite4) and Model 1800 Philips (at Lyon4).

Reagents
Iwata/Nishikaze (I/N) method: Benzethonium chloride (Sigma Chemical Co., St. Louis, MO 63178) 2 g/L, sodium hydroxide 0.5 mol/L in 33 mmol/L tetrasodium ethylenediaminetetraacetate (EDTA) (Prolabo, Paris, France).
Meuleman's method: (a) Dissolve 6 g of sulfosalicylic acid in 100 mL of distilled water. (b) Dissolve 14 g of anhydrous sodium sulfate in 100 mL of water. Mix equal volumes of a and b for use in the protein determination.
Standard and control. Human albumin (crystallized and lyophilized; Sigma A9511 and human globulin (Gamma 16; Institut Merieux, Marcy l'Etoile, France).