Is Serum Fructosamine Assay Specific for Determination of Glycated Serum Protein?
Erwin D. Schleicher, Richard Mayer, Eva M. Wagner, and Klaus-D. Gerbitz

We compared the fructosamine activity in sera from healthy and diabetic subjects with the degree of protein glycation detected by a liquid-chromatographic method. The latter technique measures frusose as a specific product after hydrolysis of ε-amino-fructose-lysine. Our results indicate that the fructosamine assay measures the extent of glycation of purified human serum albumin correctly. On the other hand, we found no correlation between the two methods for sera from healthy subjects, although for diabetics’ sera the values obtained with both methods were related. However, only about half of the reducing activity (fructosamine) was due to specific nonenzymatic glycation of proteins in healthy subjects and well-controlled diabetics. The remaining unspecific activity varied from serum to serum. It was not reducible with NaBH₄ and was independent of the glycation of albumin, which normally accounts for about 80% of glycated serum proteins. The fructosamine assay is therefore of limited specificity for the exact measurement of glycated proteins in serum.

Additional Keyphrases: diabetes • frusose • chromatography, liquid

Determination of glycated hemoglobin (Hb A₁) as an index to diabetic control is well established and widely used. Because the assays for measurement of glycated hemoglobin are expensive, laborious, and difficult to standardize (1), Johnson et al. (2) developed a rapid colorimetric assay for fructosamine in serum that is becoming widely used. The assay is based on the observation that nonenzymatic glycation of protein leads to protein-bound fructosamine residues that show reducing activity at alkaline pH. The reducing activity can be measured spectrophotometrically by using “redox” indicators such as nitroblue tetrazolium salts.

Unfortunately, this assay presents several methodological difficulties. Standardization involves the use of 1-deoxy-1-morpholinofructose (DMF) in aqueous albumin solution, although the authors have shown that the reducing activity of DMF is five- to sixfold higher than that of glycated proteins (3). Furthermore, several reports show that the apparent fructosamine activity is markedly influenced by the concentration of the indicator used, by the buffer pH, by the reaction temperature, and by the reading time, owing to nonlinearity of the color production (4-7).

We have compared the fructosamine assay with the furosine method recently described by our group (8). To obtain comparable results, we corrected values by both methods for protein concentration. We used a synthetic ε-amino-fructose-lysine standard for calibration of the furosine method, because it shares the structural features found in glycated proteins.

Materials and Methods

Patients. Blood specimens were obtained from all subjects after an overnight fast. All 25 normal controls had Hb A₁ values within the normal range (5–8%) and normal results for an oral glucose tolerance test (9). Sera from 60 unselected patients with type II diabetes were analyzed by both methods. Sera were stored for one or two days at 4 °C before analysis.

Analytical procedures. We measured Hb A₁ with the Boehringer (Mannheim, F.R.G.) assay kit and serum fructosamine with the kit of Hoffmann-La Roche (Basel, Switzerland) in nondialyzed serum specimens. Glycated serum protein was determined in parallel by the furosine method, with fructose-lysine used for calibration (8).

In vitro glycations were performed with pooled serum from nondiabetics and with human serum albumin (Behring, Marburg, F.R.G.). Protein solutions were incubated at 37 °C in phosphate-buffered saline (PBS) containing 0, 20, 45, 57, 82, or 103 mmol of n-glucose per liter. After 84 h, the samples were dialyzed extensively against PBS. Half of each sample was reduced with 0.5 mg of NaBH₄ per milligram of protein and dialyzed for at least 50 h in PBS (10).

Serum samples from patients were reduced with NaBH₄ and NaCN(BH₃) and dialyzed as described above. Na(CN)BH₃ specifically reduces the aldime bond, whereas NaBH₄ also reduces the keto group of glycated proteins.

Albumin was eliminated by passing the serum samples through a column of Cibacron Blue 3GA according to the manufacturer’s instructions (Bio-Rad, Munich, F.R.G.) Electrophoresis of serum protein, performed for all serum samples and all column eluates, showed that more than 95% of the serum albumin content was retained by the column. Protein was determined by the biuret method.

Statistical methods. Linear regression analysis was computed as the principal standardized component by the method proposed by Feldmann et al. (11).

Results

The lysine-bound glucose in pooled serum and in a solution of human serum albumin was determined by the fruose method. Both samples were glycated in vitro to various extents. When these samples were also measured with the fructosamine assay, the extent of glycation, expressed as nanomoles of lys-glù per milligram of protein, correlated well with the fructosamine activity (Figure 1). Both methods performed well in our laboratory, with CVs of 6.5% and 5.9% for the fructosamine and furosine assays, respectively. After we reduced the samples with NaBH₄, the fructosamine values were no longer dependent on the degree of glycation. Treatment of human serum albumin with NaBH₄ yielded a fructosamine activity that was near the detection limit of the assay, but a residual fructosamine activity corresponding to about 0.8 mmol/L was found in the pooled serum samples. After the NaBH₄ reduction, this residual reducing activity was different for different sera,


1 Nonstandard abbreviations: Hb A₁, glycated hemoglobin; DMF, 1-deoxy-1-morpholinofructose; PBS, phosphate-buffered saline.

Received October 7, 1987; accepted November 18, 1987.

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Fig. 1. Fructosamine activity in glycated human serum albumin (●) and dialyzed pooled serum (▲), both glycated in vitro
Glycation was performed as described in Methods and the extent of glycation was measured by the furosine method. After dialysis, half of each sample was reduced with NaBH₄ (open symbols), dialyzed again, and re-assayed by the fructosamine assay.

ranging from 25% to 60% of the activity before reduction in all serum samples (Table 1). In contrast, for all serum samples, furosine values were below the detection limit after reduction with NaBH₄. Treatment of the serum samples with Na(CN)BH₃ did not change the values measured by the two methods (data not shown).

Figure 2 demonstrates a correlation between the reducing activity determined by the fructosamine assay and the furosine values in untreated sera from 25 healthy and 60 diabetic subjects. Results obtained by the two methods were related to protein content of the sample and were expressed as nanomoles of reducing activity per milligram of protein, and nanomoles of lys-glup per milligram of protein, respectively. Regression analysis yielded \( y = 5.6x + 14.8 \) (\( r = 0.805 \)). Thus the correlation between the two methods was fairly good; however, the intercept of the regression line again demonstrates that they do not measure the same "activities." "Fructosamine" values, although expressed as nanomoles of reducing activity per milligram of protein, were about fivefold those obtained with our chromatographic procedure, a result of the use of DMF as a calibration standard for the secondary standard in the fructosamine assay (6). When the intercept of the regression line in Figure

**Table 1. Values for Glycation of Protein Samples after Reduction with NaBH₄**

<table>
<thead>
<tr>
<th>Fructosamine, nmol/mg protein</th>
<th>Residual activity, %</th>
<th>Furosine, nmol/mg protein</th>
<th>Residual activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before NaBH₄, After</td>
<td>Before NaBH₄, After</td>
<td>Before NaBH₄, After</td>
<td>Before NaBH₄, After</td>
</tr>
<tr>
<td>1</td>
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<td>59</td>
</tr>
<tr>
<td>2</td>
<td>23.9</td>
<td>8.9</td>
<td>37</td>
</tr>
<tr>
<td>3*</td>
<td>41.7</td>
<td>10.4</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>28.2</td>
<td>11.5</td>
<td>41</td>
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<tr>
<td>5*</td>
<td>40.8</td>
<td>13.5</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>27.6</td>
<td>11.7</td>
<td>42</td>
</tr>
<tr>
<td>Pooled serum</td>
<td>30.6</td>
<td>10.2</td>
<td>33</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>14.7</td>
<td>1.3</td>
<td>9</td>
</tr>
</tbody>
</table>

*Fructosamine was determined according to the manufacturer's instructions and the result was divided by the protein content of the serum sample.
*Patients with diabetes mellitus.

2, which corresponded to about 18 nmol of reducing activity per milligram of protein, was corrected for this calibration factor, the residual activity was still about 3.6 nmol per milligram of protein. This residual activity was not ascribable to the ketoamine linkage, as demonstrated in Figure 1.

Glycation of albumin purified from three normal human sera was also measured by both methods. The fructosamine assay gave values of 14–18 nmol of reducing activity per milligram of albumin, corresponding well to the 15 nmol per milligram of albumin reported by Johnson and Baker (3), while the furosine method resulted in 3.1–3.25 nmol of lys-glu per milligram of albumin.3 Corrected for the DMF-calibration factor (see above), the two methods gave similar results. Thus, the unspecific activity—i.e., activity not related to the ketoamine linkage—seemed not to be associated with serum albumin. This became also evident when fructosamine activity was measured in albumin-depleted serum: in terms of nanomoles of reducing activity per milligram of nonalbumin proteins, the values increased (Table 2). The reducing activity of the nonalbumin fraction ranged from 43% to 71% of the total reducing activity, whereas corresponding values for the furosine assay were 9%–22% (Table 2). Electrophoretic separation and determination of the isolated glycate protein fractions showed that about 80% of total serum glycation is due to albumin (12). All samples had been dialyzed extensively; therefore, the residual unspecific reducing activity in the assay system probably was related to substances of greater molecular mass.

The reference interval for serum for the furosine method is 3.0 ± 0.6 (2 SD) nmol of lys-glu per milligram of protein (12). For sera from 25 healthy subjects we found a range of 31 ± 8.5 (2 SD) for the fructosamine assay. Probably because of the varying degree with which the unspecific reducing components reacted in the fructosamine assay (Table 1), there was no clear correlation between values obtained with the two methods within the reference interval. In three of 60 sera from diabetic patients, the furosine method yielded normal values, whereas in 19 of 60 diabetic sera the values for fructosamine were within the reference interval.

*Corresponds to ca. 0.22 mol/mol albumin; see also ref. 12.
The higher sensitivity of the furosine method—i.e., discrimination between a normal and a diabetic glucose balance—became also evident with respect to Hb A1c. 18 of 60 diabetic patients showed normal values for Hb A1c.

Discussion

Although several workers (2–5, 14–16) proposed measuring serum fructosamine activity as a quick and reproducible indication of antecedent glycemic control in diabetic subjects, there are some reports on limitations of the procedure (17–20). Our results indicate that the fructosamine assay accurately measures the extent of glycation of purified human serum albumin. On the other hand, we have also shown that an unspecific reducing activity in serum, which is not due to protein glycation, is also measured by the fructosamine assay. The interference is not albumin-associated and differs from patient to patient. Evidence that a substantial amount of reducing activity is not albumin-associated is also presented by Howey et al. (17). Correlating fructosamine activity with albumin concentration, they found an intercept of 0.9 mmol/L for reducing activity. Comparing fructosamine activity with glucose concentrations measured preprandially and with determination of glycated serum protein by the hydroxymethylfurfural method, Johnson et al. (2) found intercepts of 1.1 and 2.1 mmol/L, respectively, indicating the presence of an unspecific reducing component that amounts to at least half the normal range.

Further indirect evidence that an unspecific reducing activity is measured by the fructosamine assay comes from comparison of the median of 2.37 mmol/L for healthy subjects with a median of 3.02 mmol/L found for diabetic patients whose median preprandial blood glucose value was 9.3 mmol/L (6). In spite of about twofold increased blood glucose values, their fructosamine activity was increased by only 27%. If fructosamine values were only a result of the specific reducing activity caused by protein glycation, a proportional increase with increasing glucose concentrations would be expected. Because in vitro experiments have shown that nonenzymatic glycation of human serum albumin and other serum protein is proportional to glucose concentration (12, 13), these considerations again support our evidence that measurement of serum fructosamine activity also includes the measurement of a variable unspecific reducing activity. A high index of individuality of fructosamine activity in serum from normal subjects was also found by Howey et al. (17). Although the nature of the variable unspecific reducing activity is not clear, it may be in part explained by results of Jones et al. (18), who found that serum ceruloplasmin concentration influences fructosamine activity. Different degrees of interference by various serum proteins in different assay procedures of the fructosamine assay were reported by Blair et al. (19).

In an earlier publication (12) we showed that glycated serum protein measured by the furosine method is a suitable screening test for diabetes and that it represents a more sensitive measure for long-term control than Hb A1c. Both advantages are probably due to the narrow reference range and the low individuality in glycated serum protein. On the other hand, the furosine method does not replace the glucose tolerance test for diagnosis of impaired glucose tolerance, probably because, in such situations, no significant manifest hyperglycemia is found under normal life conditions.

In conclusion: our results indicate that the reducing activity in serum of normal, borderline, or diabetic subjects may be misinterpreted, owing to unspecific component(s). If this influence could be eliminated, the fructosamine assay probably would be a good index to glycated serum proteins.

References


Table 2. Glycation Values for Serum Samples after Cibacron Blue Chromatography

<table>
<thead>
<tr>
<th>Fructosamine, nmol/mg protein*</th>
<th>Furosine, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Cibacron blue</td>
<td>After Cibacron blue</td>
</tr>
<tr>
<td>Before Cibacron blue</td>
<td>After Cibacron blue</td>
</tr>
<tr>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>8</td>
<td>51.9</td>
</tr>
</tbody>
</table>

*Same as in Table 1.

Values obtained by multiplying the glycation values for the nonalbumin fraction (B, E) by the percentage of total serum protein content (G). The values obtained were divided by the total protein glycation (A, D) and multiplied by 100.