Simple Colorimetry of Glycated Serum Protein in a Centrifugal Analyzer

David Lloyd and John Marples

A recently described (Clin Chim Acta 127: 87-95, 1982) colorimetric assay for glycated proteins in serum exploits their ketoamine activity to reduce 3,3',4,4'-biphenylidene)biss[2-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) (nitroblue tetrazolium) in alkaline solution; the authors termed this the "fructosamine assay." The method is simple, requiring only the addition of one reagent and measurement of the absorbance change during 5 min; results are expressed relative to a synthetic standard. We have adapted this for use in a centrifugal analyzer and report its performance, both analytically and as an index of hyperglycemia. Precision is good (between-batch CV 2.1%), the reagent is stable and inexpensive, and the procedure is rapid (75 samples per hour). Albumin influences the measurement, but for concentrations \( >35 \) g/L this was not a serious problem. Normal and diabetic populations can be clearly discriminated \((p < 0.001)\). The test detected 25 (84%) of the 30 untreated diabetics studied and gave four false positives (8%). The results correlate well with those for glucose in plasma of fasting subjects \((r = 0.87)\) and for hemoglobin \(A_1\) \((r = 0.80)\).

Additional Keyphrases: diabetes • fructosamine • albumin • glycated hemoglobin

With increasing recognition of the possible importance of strict glycemic control for the prevention or delay of diabetic complications, there is much interest in methods for monitoring the therapeutic control of blood glucose concentrations. Of these methods, those for glycated hemoglobin and glycated serum proteins have attracted considerable attention. Glycated hemoglobin can be assayed chromatographically, electrophoretically, or colorimetrically (1); glycated protein, colorimetrically or chromatographically (2, 3). The colorimetric methods, which are based on the thioribarbituric acid reaction (4) and thus require an acid-hydrolysis step, reportedly are subject to interference from free glucose in the serum. Thus a dialysis step has been recommended (5). Affinity chromatography has been used to separate glycated proteins in plasma from their nonglycated counterparts, with subsequent colorimetry (6).

All of the methods require good technical skill and are not amenable to automation. The recently described "fructosamine assay" (7), however, requires only simple colorimetry. We have adapted this method for use in a centrifugal analyzer and describe its performance both analytically and as an index of hyperglycemia.

Materials and Methods

Apparatus. We used a centrifugal analyzer (Cobas Bio; Roche Products Ltd., Welwyn Garden City, U.K.), a spectrophotometer (Model SP1800; Pye Unicam Ltd., Cambridge, U.K.), a continuous-flow analyzer (SMA II; Technicon Instruments Corp., Tarrytown, NY 10591), and a glucose analyzer (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA 92621).

Reagents and standards. Nitroblue tetrazolium (Sigma Chemical Co., Poole, Dorset, U.K.), \(0.25 \text{ mmol/L} \), was prepared in carbonate buffer \((0.1 \text{ mol/L} , \text{pH 10.80 at } 20^\circ\text{C})\). This reagent is stable for four months in the dark at \(4^\circ\text{C}\).

A 40 mmol/L stock solution of 1-deoxy-1-morpholine-2-fructose (DMF; Sigma) was prepared in human albumin solution \((40 \text{ g/L in } 155 \text{ mmol/L saline}; \text{ Blood Products Laboratory, Elstree, Herts., U.K.) \). Appropriate aliquots of this stock standard were diluted with human albumin solution to prepare a series of DMF standards ranging from 0.5 to 4.0 mmol/L.

Lyophilized human quality-control sera (External Quality Assessment Scheme, Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Birmingham, U.K.) was diluted with appropriate amounts of 155 mmol/L saline to give three pools containing high, medium, and low concentrations of fructosamine. These pools were stable for up to four months if stored frozen at \(-20^\circ\text{C}\).

Subjects. Untreated diabetics were a group of 30 ambulant patients \((18 \text{ women and } 12 \text{ men}, \text{ ages } 32-72, \text{ mean } 54 \text{ years})\) in whom a glucose tolerance test \((75\text{-g glucose load})\) revealed diabetes mellitus, as classified by WHO criteria (8).

Treated diabetics consisted of 35 insulin-treated diabetics \((22 \text{ women and } 13 \text{ men}, \text{ ages } 28-71, \text{ mean } 51 \text{ years})\) attending a diabetic clinic.

The nondiabetic control group comprised 89 ambulant volunteers \((54 \text{ women and } 35 \text{ men}, \text{ ages } 18-71, \text{ mean } 42 \text{ years})\) who attended the Laboratory for blood testing but who had no history of diabetes and whose random venous plasma glucose was less than 6 mmol/L.

Procedures. Blood samples for fructosamine were collected in plain tubes. The concentrations of fructosamine in sera were stable for up to three months if stored at \(-20^\circ\text{C}\).

We assayed fructosamine by the method of Johnson et al. (7), adapted for the Cobas Bio centrifugal analyzer as in Table 1. The assay was calibrated with a 2.5 mmol/L

| Table 1. Cobas Bio Centrifugal Analyzer Settings for the Assay for Fructosamine |
|---------------------------------|--------|
| Calibration factor              | 0      |
| Standard 1 concn                | 2.5 mmol/L |
| Standard 2 concn                | 2.5 mmol/L |
| Temp                            | 37.0 °C |
| Type of analysis                | 5      |
| Wavelength                      | 530 nm |
| Sample vol                      | 30 µL  |
| Diluent vol                     | 10 µL  |
| Reagent vol                     | 300 µL |
| Incubation time                 | 0      |
| Start reagent vol               | 0      |
| Time of first reading           | 600 s  |
| Time interval                   | 300 s  |
| No. of readings                 | 2      |
| Blanking mode                   | 1      |
| Printout mode                   | 1      |

Department of Clinical Biochemistry, Royal Albert Edward Infirmary, Wigan WN1 2NN, U.K.

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solution of DMF in human albumin solution (40 g/L), with blank correction for the albumin solution.

Hemoglobin A1c was estimated with the chromatographic method of Schnek and Schroeder (9), with commercially obtainable columns (Boehringer Corp. Ltd., Bell Lane, Lewes, East Sussex, U.K.).

Statistical analyses consisted of an unpaired Student's t-test and regression analysis for bivariate data sets by the procedure of Bartlett (10).

Results

Calibration. We found that the absorbance change for a known concentration of DMF standard depends on the type of albumin used in its preparation. Use of a liquid albumin preparation gave results similar to those reported by Baker et al. (11), whereas a lyophilized albumin preparation gave results approximately 24% higher.

Linearity. Plotting absorbance change vs concentration for eight DMF standards (0.5–4.0 mmol/L) gave a straight line of slope 0.077 (95% confidence interval: 0.072–0.080) with an x-intercept of −0.018 mmol/L and a y-intercept of 0.014. Plotting absorbance change vs dilution for serum serially diluted with 155 mmol/L saline also gave a straight line (slope 0.079, 95% confidence interval: 0.065–0.092).

Analytical recovery. DMF (0.96 μmol) was added to 1-mL serum samples from 10 nondiabetic and 10 diabetic subjects. The mean percentage recovery (and ranges) for the nondiabetics was 99 (96–103)% and for the diabetics 103 (96–105)%.

Interference studies. The effect of hemolysis (hemoglobin up to 4.3 g/L), lipemia (to a degree producing an initial reaction absorbance of 1.0), and glucose (up to 50 mmol/L) was investigated by adding hemolysate, latex particles, and glucose, respectively, to serum samples from nondiabetics.

At these concentrations we detected no interference. To study any possible interference from uremic, jaundiced, or uricemic sera, we assayed groups of samples from nondiabetics (n = 20) in which the mean content (and range) of the respective constituent was as follows: uremic group, urea 45 mmol/L (38–50); jaundiced group, total bilirubin 70 μmol/L (55–100); uremic group, urate 0.72 mmol/L (0.5–0.8). The mean fructosamine values for these groups were 1.53, 1.58, and 1.57 mmol/L, respectively. There was no significant difference (p > 0.05) between any of these values and that for the reference population.

To investigate the influence of albumin concentration on the assay, we measured the fructosamine concentration in a group of nondiabetic patients (n = 70) in whom the concentration of albumin varied from 20 to 55 g/L. Plotting fructosamine vs albumin gave a line of slope 0.019 mmol/g (confidence interval: 0.015–0.024) with a y-intercept of 0.61 mmol/L (r = 0.75, p < 0.001).

Reagent pH. The absorbance change of the nitroblue tetrazolium reaction is pH dependent, with sera and DMF standards giving proportionally different absorbance changes as the pH of the reaction changes. For good between-assay precision, therefore, it is important that buffer pH is accurately standardized.

Precision. To evaluate within-assay and between-assay precision, we assayed aliquots of frozen quality-control material at three concentrations, both in one batch and repeatedly over a four-month period (Table 2).

Reference interval. Plotting serum fructosamine concentration against frequency for the reference population resulted in an essentially normal distribution 1.56 ± 0.01 mmol/L (mean ± SEM). The reference interval (mean ± 1.96 SD) was 1.33–1.80 mmol/L.

Comparison of fructosamine concentrations with other indices of glycemia. The results of fructosamine measurements in the sera of 90 patients in whom diabetes mellitus was suspected clinically and who were referred for glucose tolerance testing are shown in Figure 1. There was a significant difference (p < 0.001) in fructosamine concentrations between those with proven diabetes mellitus (2.17 ± 0.09 mmol/L, mean ± SEM) and the nondiabetics (1.56 ± 0.01). Using a discrimination value of 1.8 mmol/L, we detected 25 of the 30 diabetics (84%). For the 50 nondiabetics there were four false-positive results (8%).

Fructosamine concentrations were significantly correlated with the concentrations of glycated hemoglobin (fructosamine = 0.12 glycated hemoglobin + 0.88, r = 0.80, p < 0.001, n = 53) and glucose in fasting plasma (fructosamine = 0.094 glucose + 1.14, r = 0.87, p < 0.001, n = 53). Of the 10 patients found to have impaired glucose tolerance, only three had fructosamine concentrations above the discrimination value. The mean fructosamine value for these 10 patients was 1.70 mmol/L. Fructosamine concentrations measured in insulin-treated diabetics (n = 30) were also significantly correlated with glycated hemoglobin (fructosamine = 0.127 glycated hemoglobin + 1.1, r = 0.61, p < 0.001).

![Fig. 1. Fructosamine measured in the sera of (a) nondiabetic controls, (b) patients with impaired glucose tolerance, (c) untreated diabetics, and (d) insulin-treated diabetics](image-url)
Discussion

The measurement of fructosamine in serum with a centrifugal analyzer is a simple procedure capable of good precision; it should be capable of being easily transferred to other types of discrete analyzers. With a Ciba-Corning centrifugal analyzer one can assay 75 samples per hour at a total reagent cost of approximately 5¢ per sample. The reagent is stable, and samples can be stored frozen for as long as four months. The method was found not to be subject to any serious interference other than albumin. Variations in albumin concentration within the reference interval for this serum protein (35–53 g/L) are unlikely to produce fructosamine values that are clinically misleading. If albumin concentrations are below or exceed the reference interval, the fructosamine assay may give significantly different values that do not reflect the true degree of glyceremia. Under these conditions some form of albumin correction may be appropriate.

Calibrating the assay required care because apparently the type of albumin used in preparing the DMF standards can affect the absorbance change of the nitroblue tetrazolium reaction. When we used a liquid preparation of human albumin, the reference interval for fructosamine we determined agreed with that reported by Baker et al. (11).

In a group of untreated diabetics and nondiabetics the fructosamine concentration in serum correlated well with the concentrations of glucose and glycated hemoglobin in plasma after fasting. The correlation between fructosamine and glycated hemoglobin was also significant in insulin-treated diabetics.

According to previous studies (11), the measurement of fructosamine gives information regarding the degree of glyceremia in the preceding two to three weeks. This period is similar to that found by Dolhofer and Wieland (12) for glycated albumin and by Yue et al. (2) for glycated protein in serum.

In conclusion, because of the simplicity, good precision, and low cost of the fructosamine assay in comparison with established methods for measuring glycated proteins and glycated hemoglobin, we recommend undertaking further studies to evaluate its clinical utility.

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References

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