Use of Protein-Based Standards in Automated Colorimetric Determinations of Fructosamine in Serum

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We have developed an automated colorimetric assay for glycated serum proteins (fructosamines), measuring the reducing activity of serum in alkaline solution (pH 10.35) at 37 °C. The calibrants were prepared from a synthetic fructosamine (1-deoxy-1-morpholinofructose), although secondary standards of glycated bovine albumin were more robust in routine application. Interference was appreciable only with icteric specimens (bilirubin >60 μmol/L), and between-batch imprecision (CV) was less than 2%. The range of fructosamine concentrations measured in 502 healthy (nondiabetic) blood donors was 1.87–2.87 mmol/L. There were no significant (p >0.05) age- or sex-related differences in this population sample. Fructosamine accurately reflected blood glucose control as evidenced by the significant correlation with glucose concentrations in fasting plasma (r = 0.82, p <0.001) and with glycated hemoglobin (HbA1c) (r = 0.87, p <0.01) in 115 patients with type 2 (non-insulin-dependent) diabetes mellitus. The test is simple and rapid to perform (75 samples per hour) and provides an alternative to HbA1c determinations for monitoring blood glucose control and assessing the effects of changes in diabetes management.

Additional Keyphrases: diabetes • reference range • glycated bovine albumin as secondary standards

Glycated hemoglobin (HbA1c) is widely accepted as the single most reliable indicator of metabolic control in diabetes mellitus (1). Since the early 1970s, investigators have tried to develop routine assays that complement the technically difficult and time-consuming reference procedures of ion-exchange chromatography (2). At present, these routine assays are of three types: (a) electrophoresis or isoelectric focusing, (b) ion-exchange and affinity chromatography on short columns, and (c) colorimetry (2, 3). For the routine laboratory these methods are labor-intensive and difficult to standardize and control (4, 5). Moreover, they may be subject to interference from free glucose and other metabolites, and sensitive to changes in reaction temperature or pH (2).

We recently reported a manual colorimetric assay designed to measure glycated protein (fructosamine) concentrations in human serum (6). Fructosamine values provided similar clinical information to HbA1c measurements in patients with established diabetes mellitus (7, 8), and may present an alternative to estimates of blood glucose concentration in screening for diabetes (9, 10).

In the current study we describe a modified automated fructosamine assay, in which analytical performance is improved over that previously described by Lloyd and Marples (11). We examine specificity and control of the method, and report an amended reference range for nondiabetic individuals.

Materials and Methods

Patients

We based the reference range for fructosamine on results from 502 healthy, volunteer blood donors (242 men, 260 women) ages 16 to 73 (median 25) years. We compared fructosamine concentrations with the glucose concentrations in fasting plasma and with HbA1c concentrations in 115 type 2 (non-insulin-dependent) diabetic patients (68 men, 47 women) ages 31 to 84 (median 62) years, referred to the diabetes clinic for assessment.

Analytical Procedures

Fructosamine assay. Unless otherwise stated, all serum concentrations of fructosamine were determined with a Cobas Bio centrifugal analyzer (F. Hoffmann-La Roche, Basle, Switzerland) with the settings presented in Table 1. Specifications for a manual procedure and four alternative automated discrete analyzers are also described (Table 1). The reagent was carbonate buffer (0.1 mol/L, pH 10.35) containing 250 μmol of nitro-blue tetrazolium chloride (NBT) per liter.

Preparation of standards. 1-Deoxy-1-morpholinofructose (DMF) was synthesized as described by Hodge (12), and its purity confirmed by melting point determination (147 °C) and elemental analysis (calcd. for C10H19O6N: C, 48.2; H, 7.68; N, 5.62; found: C, 48.09; H, 8.14; N, 5.51). DMF is stable in the crystalline state at room temperature for more than six months. Absorbance change at 530 nm varied linearly with concentrations of DMF standards (0, 2, and 4 mmol/L) in a matrix of human serum albumin (40 g/L in isotonic saline, NaCl 150 mmol/L). We assayed the DMF standards with each batch of specimens and calculated a calibration factor after subtracting the contribution of the human albumin matrix. This alteration of the previous procedure (6) effectively increases individual patients’ results by about 0.8 mmol/L, compared with previously published values (6-11). Alternatively, we prepared secondary

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3 Nonstandard abbreviations: HbA1c, glycated hemoglobin; NBT, nitro-blue tetrazolium chloride; DMF, 1-deoxy-1-morpholinofructose.

Received February 11, 1985; accepted May 20, 1985.
standard solutions of glycated bovine albumin (40 g/L in isotonic saline) (13) and calibrated them with DMF as above. The concentrations of the solutions (assigned values of 0.98, 2.74, and 4.52 mmol/L) were linearly related to absorbance change and were stable at -20 °C for six months.

Other tests. We determined glucose concentrations by a hexokinase/glucose-6-phosphate dehydrogenase method (14) (Glucose Rapid Test; Roche Diagnostics) with the Cobas Bio. Proportions of HbA1c were measured by isoelectric focusing on commercial polyacrylamide gels and quantification with a laser densitometer (LKB, Bromma, Sweden). The between-batch imprecision (CV) was 1.0% for glucose estimation and 3.9% for HbA1c. Statistical significance was estimated by Student's t-test for paired or unpaired data, as appropriate.

Results

Method Evaluation

Calibration standards. To examine the effect of error in reagent preparation and specimen analysis, we determined serum fructosamine values for 25 patients' specimens by using NBT at either 100 or 500 μmol/L in the reagent. Results differed markedly (Figure 1A) from values obtained

Fig. 1. Effect of NBT dye concentration (A), buffer pH (B), and reaction temperature (C) on fructosamine concentration of 25 diabetic and nondiabetic sera (O) assayed with DMF standards.

Fructosamine estimates expressed as a proportion of values obtained with standard assay conditions: i.e., NBT 250 μmol/L, buffer pH 10.35, 37 °C. O, median values for the 25 patients' specimens.
with standard reagent, which contains 250 μmol of NBT per liter. Alterations in pH (Figure 1B) and reaction temperature (Figure 1C) also affected results. By contrast, use of glycated bovine albumin (40 g/L in isotonic saline) as a standard material minimized the effects of these manipulations (Figure 2).

Species source of albumin for secondary standards. We incubated separately human serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) and bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 40 g/L in a solution of 100 mmol of NaCl and 50 mmol of Na₂HPO₄/NaH₂PO₄ per liter (pH 7.40), at 37 °C with glucose (50 mmol/L) and (U-¹⁴C)-glucose (1–2 μCi/L). We removed aliquots daily and dialyzed them overnight against isotonic saline (NaCl, 150 mmol/L) before precipitating the protein with trichloroacetic acid (0.5 mol/L) and washing. Radioactivity was quantified in a Beckman LS 7500 spectrometer (Beckman Instruments, Irvine, CA). We related radiolabel and glucose concentrations by determining the radioactivity in the incubation medium. Fructosamine concentration (expressed as change in absorbance between 10 and 15 min, Table 1, manual method) in the dried material correlated significantly both with ¹⁴C-uptake in bovine albumin (fructosamine = (21.9 × ¹⁴C) + 4.41, r = 0.98, p < 0.001, n = 16) and with ¹⁴C-uptake in human albumin (fructosamine = (24.6 × ¹⁴C) + 2.27, r = 0.96, p < 0.001, n = 16). There was no significant difference (p > 0.05) in the slopes of these lines, indicating equivalent reactivity of the two glycated albumin solutions under our conditions of assay. The intercepts were significantly different (p < 0.001), reflecting different fructosamine concentrations in the starting materials.

Control material. We compared the homogeneity and stability of commercial quality-control sera (Table 2). Fructosamine concentrations were increased even in normal sera and ranged from diabetic (3.37 mmol/L) to suprapathological (6.77 mmol/L) values. Vial-to-vial variation exceeded stated criteria (CV < 0.25%) (15) for six of the eight sera, although only the three materials with CV >1% proved unsatisfactory as precision controls. Most specimens were stable at 4 °C in the dry state and did not deteriorate after reconstitution during a standard 12-h working day. We did not consider pools of human sera as control materials for routine use because of the risk of contamination with hepatitis and other biological hazards.

Interference. Assessing 28 potential interfering substances by an approach similar to that of Passey et al. (14), we encountered significant interference from exogenous bilirubin (428 μmol/L), and lesser effects from added EDTA, heparin, cysteine, ascorbate, and urate (Table 3). A comparison of fructosamine and bilirubin concentrations in specimens from nondiabetic patients (not shown) confirmed interference with icteric specimens (bilirubin >60 μmol/L).

Precision. We analyzed pools of nondiabetic and diabetic human sera 20 times for within- and between-batch imprecision. The respective CVs were 1.3% and 1.4% for the diabetic pool (mean fructosamine, 5.23 mmol/L) and 0.5%
Linarity. Linearity was assessed by serially diluting in saline a diabetic serum with a fructosamine activity of 5.52 mmol/L. The equation for the regression line for the serum–saline dilutions was: observed fructosamine = 1.009 x expected fructosamine + 0.051 (r = 1.00). The standard deviations of the slope and intercept were 0.001 and 0.006, respectively, and the standard error of estimate was 0.010.

Method comparison. We assayed 100 diabetic and nondiabetic sera for fructosamine concentration by a manual procedure (6) with a DMF standard curve (2), and by our automated method with glycated albumin standards (4). The regression equation was y = 1.03x – 0.08 (r = 0.98). The standard deviations of the slope and intercept were 0.018 and 0.055, respectively; the standard error of estimate was 0.153.

Patients’ Data

Biological variation. The range of fructosamine values in 502 healthy nondiabetic volunteers was 1.87 to 2.87 (median 2.37) mmol/L (Figure 3). Results were distributed normally, confirmed by normal plot and Shapiro–Wilks statistic of 0.995 (16). There were no apparent age- or sex-dependent changes in this population sample (p > 0.05).

Diabetic values. Fructosamine concentrations from 115 patients with type 2 diabetes mellitus ranged from 2.26 to 5.22 (median 3.02) mmol/L. Corresponding glucose results in plasma from fasting subjects were 5.2 to 23.9 (median 9.3) mmol/L, and HbA1c proportions were 4.3 to 18.2% (median 7.3%). Fructosamine concentrations correlated significantly both with glucose in these plasma samples (fructosamine = 0.13 glucose + 1.90, r = 0.82, p < 0.001) and with HbA1c (fructosamine = 0.19 HbA1c + 1.64, r = 0.87, p < 0.001).

Discussion

Alkaline-reducing activity at room temperature is a characteristic property of Amadori rearrangement products, and has long been used to identify fructosamines in the presence of free glucose and N-glucosylamine (Schiff-base) derivatives (12, 17, 18). In a preliminary experiment, we confirmed the reducing activity of serum in cold alkaline solution, although direct quantification of fructosamines was impractical because of interference from ascorbic acid and glutathione (6). We found that kinetic analysis, after an initial preincubation of serum and reagent, eliminated the contributions of most nonspecific reductants and allowed us to estimate the glycated protein concentrations in serum. We chose DMF as a primary standard because it can be obtained in pure crystalline form (12), is stable at room temperature (12), and shows similar reducing activity to that of serum under our conditions of assay (6).

Direct automation of our manual method (6) has already been described by Lloyd and Marples (11), who retained our initial approach of standardization by DMF. Here, however, we have used glycated protein secondary standards in routine application. We made this change because the reaction of DMF with NBT is very sensitive to reaction conditions (Figure 1), leading to inaccuracy in the assay and to difficulty in intercorrelating results for the method from different automated analyzers, which require modifications of the original procedure (Table 1). By contrast, results based on use of standards of glycated albumin appeared far more robust (Figure 2). We prefer to use glycated bovine albumin as the secondary standard material; it is less expensive than human albumin and shows equivalent reactivity under our conditions of assay.

Further departures from our previous procedure (6) include the correction for human albumin matrix in the DMF primary standard series. We made this change, also described by Lloyd and Marples (11), because we observed marked batch-to-batch variation in the reducing activity of different human albumin preparations. Moreover, we used milder conditions (pH 10.35) than Lloyd and Marples (pH 10.8), to minimize interference from glucose and other analytes (Table 3). As a result, our reference range of 1.87–2.87 mmol/L is higher than that reported previously (6–11).

The practical advantages of automated fructosamine assay over other glycated protein methods (2–5) include stable calibration materials, rapid analysis on a range of discrete analyzers (Table 1), and acceptable imprecision (2% between batches) compared with intr-individual biological variation of approximately 3% (10). Further advantages are the suitability of commercial quality-assurance materials as high-concentration controls, which can be diluted to obtain appropriately low control values (see section on Linarity).

The close correlation between fructosamine measurement and both the concentrations of glucose in plasma from fasting subjects and the proportions of HbA1c, together with the ease of fructosamine analysis, have encouraged us to examine fructosamine as a screening test for diabetes mellitus. Clinical studies in progress should give further information on the usefulness of this simple test in diabetic pregnancy and in other patients at risk of developing diabetes mellitus.

References
Determinations of Immunoglobulins G, A, and M in the Technicon RA-1000

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We evaluated the Technicon RA-1000 "Random Access" analyzer for the measurements of immunoglobulins G, A, and M in serum by turbidimetry. For various concentrations of serum pools the total CV ranged from 3.0 to 4.5% for IgG, from 3.1 to 3.2% for IgA, and from 4.8 to 5.4% for IgM. The linearity of the standard curves was good in the clinically useful ranges. Results for patients' samples correlated well with those determined with the Beckman Auto ICS (rate nephelometric method). Normal reference intervals (based on data for 200 healthy blood donors) are 7.67 to 16.87 g/L for IgG, 0.87 to 3.89 g/L for IgA, and 0.61 to 2.70 g/L for IgM.

The RA-1000 requires only 8 μL for all three tests and can analyze 25 specimens for all three proteins in 37 min. Compared with other methods for immunoglobulin assays, the RA-1000 has higher throughput and offers significant savings in labor and reagent costs.

Additional Keyphrases: random-access analysis · reference interval · turbidimetry · rate nephelometry compared

Radial immunodiffusion (RID) and rate nephelometry have been widely used for measuring IgG, IgA, and IgM. The former, relatively tedious to perform, lacks precision. The latter, although faster, usually requires a dedicated instrument, and its throughput is lower than that of most common chemistry analyzers. Turbidimetric methods for measuring IgG, IgA, and IgM have been recently introduced for the RA-1000® analyzer (Technicon Instruments Corp., Tarrytown, NY 10591), a bench-top, computer-controlled analyzer that can perform, without batching, single or multiple tests in any combination or sequence (1). Here we report our evaluation of the analytical and operational performance of the three immunoglobulin assays used in the RA-1000.

Materials and Methods

Instruments and principles of assays. In the turbidimetric method used in the RA-1000, polyethylene glycol is used to accelerate the antigen–antibody reaction (2). The insoluble complex formed by the specific antisera and the immunoglobulin in the specimen is quantified by an equilibrium method as the amount of turbidity at 340 nm after a 5-min incubation. The rate-nephelometric method used in the Auto ICS system (Beckman Instruments, Inc., Brea, CA 92621) measures the intensity of light scattered by the antigen–antibody complex.

Calibration. The RA-1000 was calibrated once a week, and each time new working reagent was prepared. The calibrator (Technicon "Reference Serum") and antisera were diluted according to the recommendations of the manufacturer. Each calibration involved six concentrations of calibrator. The Technicon calibrator (usually containing about 27 g of IgG, 5 g of IgA, and 4 g of IgM per liter) was routinely diluted to 0, 12.5, 25, 50, 75, and 100% of its original concentration, and all three assays were calibrated with use of the same calibrator preparations. We tested the stability of the calibration curves by assaying the calibrators as unknown samples seven days after a calibration.

Received May 7, 1985; accepted June 19, 1985.

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CLIN. CHEM. 31/9, 1554–1557 (1985)