Methodological problems with the assay of fructosamine in serum—standardization, matrix effects, and dependence on buffer pH—have been minimized with a method involving colorimetric assay of each specimen and subsequent re-assay after standard addition of 1-deoxy-1-morpholinofructose. Absorbance at optimum wavelength of 540 nm varies linearly with fructosamine concentration to at least 5.5 mmol/L, and between-run precision is about 6% for both patients' specimens and quality control materials. Correction of fructosamine to serum albumin of 40 g/L minimizes the effect of albumin while maintaining transferability of data and reference values. From data on biological variation, the analytical goal for precision (CV) is <2.6%. The square root of the ratio of intra- to interindividual variance is low, indicating that fructosamine concentrations have a high index of individuality; thus conventional population-based reference values are of limited use. Although this assay may be useful in monitoring disease, we doubt that it provides a valid screening test for diabetes.

Johnson et al. (1) described a colorimetric assay of fructosamine in serum, designed to quantify the glycated proteins in serum as an index of glucose homeostasis over the preceding one to three weeks. Advocated as a method of screening for diabetes in pregnant (2) and nonpregnant adults (3), the assay has been widely applied to monitoring diabetes; these uses would be facilitated by the development of automated techniques (4–10).

Unfortunately, the assay presents several basic methodological difficulties. Standardization usually involves use of synthetic 1-deoxy-1-morpholinofructose (DMF) in aqueous albumin solution; however, Hindle et al. reported (8) a nonlinear relationship between color production and the concentration of DMF, and others have found that the change in absorbance with time is influenced by the type and source of the albumin in which the DMF standards are prepared (1, 6, 8, 9, 11). If standards of DMF in solution of albumin in saline solution are used, the apparent concentration of fructosamine in serum is markedly affected by the concentration of the indicator used in the assay, nitroblue tetrazolium (NBT), by the buffer pH, and by the reaction temperature (9).

Although these difficulties are alleged to be alleviated by adoption of secondary standards of glycated albumin (9), or lyophilized or liquid serum (6, 7, 9–11), the fructosamine concentrations of these standards are derived from prior analyses performed with DMF as standard, thus leaving these problems not truly resolved. In addition, the inherent reactivity of the albumin solution used in preparation of the standard ought to be taken into account (6, 9–14).

Fructosamine originates from the non-enzymic glycation of albumin. Its concentration therefore depends on the concentrations of glucose and albumin. Because the assay of fructosamine is used as an index of prior concentrations of glucose, it seems logical to correct the measured fructosamine for expression in terms of constant albumin concentration. Recently, Van Dieijen-Visser et al. (15) that the fructosamine concentration depends on the concentration of albumin in serum, and suggested that fructosamine values be corrected by subtracting 0.023 mmol of fructosamine for every gram of albumin per liter.

In view of these difficulties, we investigated the basic methodological variables, and here propose a simple, rapid method which includes use of DMF as standard, minimizes matrix effects, is independent of buffer pH, and is potentially adaptable to a variety of automated analyzers. Moreover, because we firmly believe that the components of biological variation found in healthy individuals should be defined early in the development of any new assay so as to delineate objective analytical goals for precision (16) and to assess the usefulness of conventional population-based reference values (17), we investigated biological variation of fructosamine in a cohort of 16 ostensibly healthy individuals. We adjusted these data for serum albumin content by making (a) no correction, (b) a minor modification of the correction described by Van Dieijen-Visser et al. (15), or (c) a correction according to the formula:

\[
\text{Corrected fructosamine, mmol/L} = \left[ \text{measured fructosamine} + 0.03 (40 - \text{serum albumin concn, g/L}) \right], \text{mmol/L}
\]

Materials and Methods

Analytical Methods

NBT and DMF were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Except where indicated, carbonate buffer (0.1 mol/L, pH 10.8) was used throughout this study.

In preliminary experiments, the manual method used was exactly as previously described (1). We measured absorbance with a Gilford 300-N spectrometer (Corning Medical and Scientific, Halstead, Essex, U.K.). The change in absorbance with wavelength was monitored with a Model SP 8000 scanning spectrophotometer (Pye Unicam, York St., Cambridge, U.K.); wavelength accuracy was validated with "Spectrosol" potassium dichromate standard solutions (BDH Chemicals Ltd., Poole, U.K.).

All samples were analyzed with a Rotochrom II parallel centrifugal analyzer (Aminco, Silver Spring, MA 20910), with the settings listed in Table 1. In summary: 50 μL of specimen was diluted with 250 μL of the carbonate buffer (diluent), mixed with 250 μL of reagent NBT (0.5 mmol/L, in diluent), and the absorbance was measured at 540 nm after 5 min for a further 2 min at 15-s intervals. We then
Table 1. Rotochem Instrument Settings for Fructosamine Assay

<table>
<thead>
<tr>
<th>Program type</th>
<th>Kinetic Rate I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Determination</td>
<td>Fructosamine</td>
</tr>
<tr>
<td>Sample size, μL</td>
<td>50</td>
</tr>
<tr>
<td>Run temp, °C</td>
<td>37</td>
</tr>
<tr>
<td>Conversion factor</td>
<td>1000</td>
</tr>
<tr>
<td>Abs. change</td>
<td>increase</td>
</tr>
<tr>
<td>No. decimal places</td>
<td>2</td>
</tr>
<tr>
<td>Units</td>
<td>mmol/L</td>
</tr>
<tr>
<td>Lag time, s</td>
<td>300</td>
</tr>
<tr>
<td>Wavelength, nm</td>
<td>540</td>
</tr>
<tr>
<td>Sampling interval, s</td>
<td>15</td>
</tr>
</tbody>
</table>

assayed replicates of the samples again at identical instrument settings but with 50 nmol of standard in the 250 μL of diluent (DMF concentration, 0.2 mmol/L).

The fructosamine concentration in each specimen was calculated as follows:

\[ \text{Fructosamine, mmol/L} = \Delta A_1 / (\Delta A_2 - \Delta A_1), \]

where \( \Delta A_1 \) = absorbance change per minute in first run (without DMF), and \( \Delta A_2 \) = absorbance change per minute in second run (with DMF).

The albumin concentration in serum was determined with either continuous-flow (smac) or RA-1000 discrete analyzers (Technicon Instruments Corp., Tarrytown, NY 10591), by the bromcresol green method, with Technicon reagents; the two methods were calibrated to give identical results.

Biological Variation

We recruited apparently healthy laboratory staff members (seven men and nine women, ages 23–46 years) for the study; all were nondiabetic according to the criteria of the World Health Organization (18). Ten 10-mL specimens of venous blood were collected from each subject at regular intervals during five weeks. To minimize preanalytical variance, the same phlebotomist collected the blood at the same time each day (between 08:30 and 09:30 hours) from seated subjects. All serum specimens were frozen at \(-40\) °C until the day of analysis; all those from one subject were thawed at room temperature, and each one was mixed thoroughly before assay with the centrifugal analyzer. To minimize analytical variation, we assayed each of the sets of 10 specimens in random order, in duplicate, within the same analytical batch; reagents, standard, and quality-control materials from the same sources were used throughout. We estimated analytical variance (SD_\text{A}^2) from the duplicate results for each specimen. The variance of the set of first results for each subject was used to calculate the average intra-individual biological variance (SD_\text{B}^2) by simple subtraction of SD_\text{A}^2 from the observed dispersion (equal to SD_\text{D}^2 + SD_\text{B}^2). After calculating the overall variance of the set of first results for all 16 subjects, we subtracted SD_\text{B}^2 and SD_\text{A}^2 to determine the interindividual biological variance (SD_\text{D}^2).

Results and Discussion

Assay Optimization

*Choice of wavelength.* In previously published methods, wavelengths for the absorbance measurements ranged from 530 to 550 nm. To determine the optimum wavelength for the assay, we assessed absorbance vs wavelength from 400 to 650 nm for manual assays of duplicate samples from diabetic and nondiabetic patients, and a 40 g/L solution of human serum albumin in isotonic saline (NaCl, 9 g/L), with one of each pair of samples containing added DMF (2.0 mmol/L) and one not. Without exception, each sample displayed a sharp absorbance peak at 538 to 540 nm. We therefore concluded that the optimum wavelength for this assay was 540 nm, in contrast to the findings of Johnson et al. (1).

*Reaction kinetics.* To several serum specimens, encompassing a range of fructosamine concentrations, we added DMF in carbonate buffer to give final concentrations of 1, 2, and 4 mmol/L, and assayed these with the manual method. Typical absorbances for 45 min after the start of the reaction are shown in Figure 1. To determine a suitable incubation period, we followed a similar procedure for the centrifugal-analyzer method, but determined the absorbance change over different time periods, namely, between 30 s and 5 min, 5 and 25 min, and 2 and 10 min after mixing the sample and reagent. The results did not differ from those in Figure 1. We therefore concluded, in contrast to the proposal of Lim and Staley (19), that the assay could be run with use of a lag period of 5 min and monitoring the absorbance change over the subsequent 2 min.

*Standardization.* To minimize standardization and matrix effects, we used an internal standardization technique in which samples were analyzed twice, once without and once with added DMF in a final concentration of 1.0 mmol/L. This concentration of DMF was chosen so as to keep in the same range the changes in absorbance for samples with and without the added DMF. To assess whether our method did minimize matrix effects, and to a similar extent in specimens from diabetics and nondiabetics, we examined the difference in absorbance change per minute with and without added DMF (\( \Delta A_2 - \Delta A_1 \)). This quantity should be constant if matrix effects are negligible. In 76 diabetics, \( \Delta A_2 - \Delta A_1 \) was 23.4 (SD 1.84), and in 43 nondiabetics, \( \Delta A_2 - \Delta A_1 \) was 23.0 (SD 1.90). The means and standard deviations were not statistically significantly different, by Student's t-test (\( t = 0.92, 0.4 > p > 0.3 \)) and the F-test (\( F = 1.06, p > 0.5 \)) respectively.

*Influence of pH.* There has been controversy regarding the optimum pH of the reaction buffer; the authors of the original method (1) and some other workers used a pH of 10.8, but this was later shown and admitted (11, 20–23) to be incorrect, and a pH of 10.35 was advocated. To investigate the effect of buffer pH, we assayed samples from 22 nondiabetics, using the automated method and carbonate buffer.
Table 2. Precision* of Assay

<table>
<thead>
<tr>
<th>Fructosamine concn in control material</th>
<th>Within-batch</th>
<th>Between-batch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (and SD), mmol/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>Low</td>
<td>1.21 (0.03)</td>
<td>2.9</td>
</tr>
<tr>
<td>Medium</td>
<td>1.56 (0.03)</td>
<td>2.2</td>
</tr>
<tr>
<td>High I</td>
<td>2.16 (0.04)</td>
<td>2.1</td>
</tr>
<tr>
<td>High II</td>
<td>3.30 (0.11)</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* n = 20 each.

Buffers of pH 10.8 and 10.35. The respective mean values of 1.77 and 1.78 mmol/L were statistically identical by paired t-test (t = 5.5, p < 0.001). In our method, therefore, buffer pH had no discernible effect.

Precision studies. Within-run precision was assessed by 20 analyses of four specimens of pooled sera from patients. Between-run precision was assessed by analysis of thawed aliquots of the same four stored-frozen serum pools, 20 times each, in separate analytical runs, over a five-week period. The results are shown in Table 2. To confirm that this precision was obtainable with patients' specimens, we assayed sera from diabetic and nondiabetic patients in duplicate, placing the duplicates in different analytical batches. The CV as calculated from the differences between the duplicates for diabetics was 4.5% (n = 32; mean = 2.41 mmol/L) and for nondiabetics 5.4% (n = 14; mean = 1.89 mmol/L). These values compare favorably with those of some previous reports: 6.5% (1), 7.4% (7), and 5.5% (8).

Linearity. To assess the linear range, we prepared two series of six samples each by diluting aliquots of serum from a diabetic with a high concentration of fructosamine (a) with carbonate buffer and (b) with aliquots of serum from a nondiabetic with a low concentration of fructosamine. All the samples were analyzed in duplicate, in different analytical batches. By the criterion that the found value should lie within ± 2 SD (mean ± 2 SD) of the expected value, the plot of found vs expected values was linear up to at least 5.5 mmol/L.

Effect of albumin on fructosamine. Sera from 60 patients contained albumin in concentrations ranging from 19 to 48 g/L, and fructosamine from 1.49 to 3.34 mmol/L. Simple linear regression showed that these concentrations were correlated, r = 0.57, in the following relationship:

Fructosamine (mmol/L) = 0.03 albumin (g/L) + 0.9 (mmol/L)

Van Dieijen-Visser et al. (15) advocate correcting the fructosamine by subtracting 0.023 of fructosamine per gram of albumin per liter. However, this corrects to a concentration of zero serum albumin, which would lead to low fructosamine values.

In contrast, correcting fructosamine concentrations with reference to a serum albumin concentration of 40 g/L by using the formula we listed in the introduction does result in values similar to those previously reported, an independence of fructosamine from serum albumin concentration (r = 0.004), and the relationship:

Fructosamine (mmol/L) = 0.0002 albumin (g/L) + 2.1 (mmol/L)

Biological Variation

Figure 2 shows the mean and range of uncorrected values of fructosamine for each of the 16 nondiabetic subjects. The overall mean concentration of fructosamine was 2.07 mmol/L. We used the data to calculate intra- and inter-individual variances, and the percentage that each component contributes to the total variance; the results are shown in Table 3.

The corresponding values for the variances and the mean concentration of fructosamine, corrected by the technique of Van Dieijen-Visser et al. (15), and the formula we developed, are also shown in Table 3.

As stated earlier, correction to zero albumin yields values lower than either uncorrected values or those corrected to an albumin concentration in serum of 40 g/L, and we do not support use of this technique. The components of variance are similar, however, whether corrected or not.

Analytical goals for precision are best defined by using data from studies of biological variation (16). Given the current view that, to facilitate optimal patient care, analytical variability should be equal to or less than half of the intra-individual biological variation (16), the analytical goal for the precision (CV) of serum fructosamine assays is thus

Table 3. Estimated Average Analytical Variance (V₀), Intra-Individual Variance (Vᵢ), and Interindividual Variance (V₀), and Mean Concentrations of Fructosamine

<table>
<thead>
<tr>
<th>Variance, (mmol/L)²</th>
<th>Mean concn of fructosamine, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>V₀</td>
<td>0.009 (15)* 0.012 (21) 0.037 (64)</td>
</tr>
<tr>
<td>Corrected to serum albumin of zero</td>
<td>0.007 (15) 0.013 (27) 0.027 (58)</td>
</tr>
<tr>
<td>Corrected to serum albumin of 40 g/L</td>
<td>0.007 (15) 0.013 (27) 0.027 (58)</td>
</tr>
</tbody>
</table>

* Percentages of total variance in parentheses.

Fig. 2. Parametric means and absolute ranges for uncorrected concentrations of fructosamine in sera from 16 healthy subjects.
<2.6%. Although the assay we have developed does not meet this theoretically desirable level of performance, we believe that the methodological principles detailed here are sound and that the goal is feasible with current technology.

The wide range of population-based reference intervals quoted for fructosamine (2-10) include both intra- and interindividual variation. The index of individuality—i.e., the square root of the ratio of intra- to interindividual variance—allows assessment of the usefulness of such conventional reference intervals (17), which are of real value only when intra-individual variation exceeds interindividual variation. The ratio of individuality is 0.56 for the fructosamine determinations reported here, or 0.69 when corrected with our formula. This high index of individuality means that population-based reference intervals are of little value and may be misleading. Consequently, we believe that measurement of fructosamine concentrations in serum is not suitable as a screening test for diabetes, as has been suggested (2, 3). However, because effective monitoring of diabetic control is done by consideration of an individual as his or her own reference, with subsequent interpretation of the difference seen in sequential results, the assay of fructosamine is undoubtedly of potential value in the long-term monitoring of diabetic patients. It is important for this use that laboratory results for an individual should be comparable over time and locale. The wide range in the values obtained by different published methods is undoubtedly attributable in large part to deficiencies in standardization techniques. We consider that wide adoption of our analytical approach—relating concentration to absorbance change from the reduction of NBT by pure DMF added to serum, and correcting to a serum albumin concentration of 40 g/L—would result in greater transferability of data.

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References