Influence of Timing in the Fructosamine Assay

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The fructosamine assay, based on the measurement of the reducing activity in serum at alkaline pH, provides an index of protein glycation. The reducing activity is expressed in equivalents of 1-deoxy-1-morpholinofructose (DMF) by direct comparison with the activity either of this synthetic compound or with a secondary protein standard calibrated against DMF. This study reports the influence of assay timing on the apparent serum fructosamine concentration. The kinetics of alkaline reducing activity in serum differed from that in both DMF and a secondary protein standard. When compared with DMF, activity in serum increased but decreased relative to the protein standard as the pre-incubation interval of the assay was shortened. The use of secondary protein standards results in underestimation of serum fructosamine concentrations when the pre-incubation phase of the assay is shorter than that used for the calibration of the secondary standard. Ascorbate exerted an inhibitory effect in fructosamine assays with pre-incubation times exceeding 5 min. The inhibition increased with both the concentration of ascorbate and the duration of the pre-incubation.

The measurement of glycated hemoglobin, or HbA1c specifically, is well established as a means of monitoring the long-term control of diabetes mellitus. More recently, methods have been developed for assay of glycated proteins in serum and plasma, termed "fructosamines" by Johnson et al. (1). Fructosamine concentrations reflect the mean glucose concentration in blood over two to three weeks and thus provide a measure of the intermediate-term glycemic control. Different assays have been described for measuring fructosamines, but those based on nitroblue tetrazolium reduction, originally devised by Johnson et al. (1), appear to be the most widely used. Assays based on this procedure have gained popularity because of procedural simplicity and low operational cost.

Albumin is the main source of glycated protein in serum (2-4), but all serum proteins contribute in varying degrees to the total fructosamine concentration (5). The relative contribution of each protein is determined by its concentration, its content of free amino groups, and its turnover rate. The fructosamine reactivity of this heterogeneous group of compounds is expressed in equivalents of a synthetic fructosamine, 1-deoxy-1-morpholinofructose (DMF). The alkaline reducing activity of glycated proteins and that of DMF are, however, influenced to a different degree by changes in assay conditions including the pH, temperature, timing, and concentration of reactants. Thus fluctuations in assay conditions would affect the apparent serum fructosamine concentration. This source of variation was improved with the introduction of protein-based secondary standards (6, 7) but was not fully resolved, because the value of the secondary standard was assigned through calibration with DMF.

Recently a commercial fructosamine kit based on the use of secondary protein standards was introduced by Roche Diagnostics. The manufacturer's recommended assay timing involves a 10-min pre-incubation followed by measurement of the absorbance change during the next 5 min. This, however, is not feasible with all analyzers; e.g., the Technicon RA-1000 allows a pre-incubation of no longer than 7 min. In this study we undertook a detailed investigation of the effects of timing in the fructosamine assay. The primary aim was to evaluate the timing effect in assays based on standardization with secondary protein standards.

Methods and Materials

Reagents. The fructosamine test kit was from Roche Diagnostics, Baale, Switzerland. The kit comprised a protein-based standard and nitroblue tetrazolium (NBT) reagent (0.25 mmol/L NBT in carbonate buffer, 0.1 mol/L, pH 10.35). The protein-based standards used in this study (hereinafter called Roche standard) were assigned the values of 2.87 and 2.80 mmol/L DMF equivalents, respectively, by the manufacturer. Quality-control serum was from Gilford, Irvine, CA. DMF solutions were made in saline containing 40 g of human serum albumin per liter, from Biostest Pharma, Dreieich, F.R.G. Human sera were pooled from 35 normal blood donors.

Fructosamine assay. The fructosamine test kit is based on two-point spectrophotometric readings of the absorbance at 10 and 15 min with use of 20 µL of sample/standard, 50 µL of diluent (H2O), and 200 µL of NBT reagent. The reaction is carried out at 37 °C, and the absorbance is measured at 530 nm. The reaction is started by adding the NBT reagent; the time interval from the addition of the reagent to the first absorbance measurement is termed the "pre-incubation." Serum fructosamine concentrations are calculated as the ratio of absorbance increments of sample and standard, respectively, multiplied by the assigned concentration of the standard.

In this study we measured the incremental absorbance change per minute during an incubation period of 20 min, with a Cobas Bio centrifugal analyzer (Roche Diagnostic Systems). The reaction mixture was composed either as recommended by the manufacturer or with half the recommended volume of sample and standard with volume adjustment of the diluent to a total reaction volume of 270 µL.

Results

Glycated Protein vs DMF

The time course of alkaline reducing activity (expressed as ΔA/min) in DMF differed from that in glycated proteins (Figure 1). In the latter case, the time course was essentially monophasic for the Roche standard (glycated albumin), pooled human sera, and quality-control serum (Figure 1A). In contrast, the time course for the primary fructosamine standard, DMF, was biphasic, with a concentration-dependent optimal activity between 7 and 10 min (Figure 1B). The ratio of the activity in the Roche standard to that in DMF is depicted in Figure 2 with 20-µL samples (assay dilution factor 13.5) and 10-µL samples (assay dilution factor 27),
respectively. The absorbance changes per minute obtained with the Roche standard and DMF were calculated as a ratio. It is apparent from Figure 2 that the validity of the assigned DMF equivalence for the Roche standard does not extend beyond assay conditions under which the standard was calibrated against DMF.

Figure 3A, derived in the same way as Figure 2, shows the time course of the activity ratio between serum (pooled sera) and DMF. Owing to the different time course of the activity in DMF and glycated protein, the protein can be assigned a range of DMF equivalence values, each representing a fixed set of assay conditions, e.g., a fixed pre-incubation time and a fixed timing for absorbance measurements. No particular assay timing is per se more appropriate than the other, and each DMF equivalence value is therefore equally valid. The Roche standard was calibrated and assigned a DMF equivalence by the manufacturer, using 20-μL samples, a 10-min pre-incubation time, and absorbance increments during the subsequent 5 min. The implications of using the Roche kit with a shorter pre-incubation time than that recommended merit consideration. Figure 2 reveals that the relative activity between the Roche standard and DMF increases as the pre-incubation time is shortened. Thus a hypothetical recalibration of the Roche standard at shortened pre-incubation time would result in a higher DMF equivalence. As a consequence, fructosamine concentrations measured in sera with the Roche kit are underestimated when pre-incubation times of <10 min are used.

Serum vs Roche Standard

Secondary protein-based standards were introduced in the fructosamine assay to improve the robustness of the assay and thereby minimize the effect of small fluctuations in the reaction conditions such as day-to-day variations in pH, temperature, and timing (6). It appears from Figure 1A that the alkaline reducing activity in serum is decreased relative to that in the Roche standard as the pre-incubation time is shortened. The same finding is illustrated more clearly in Figure 3B, which depicts the ratio of activities in sera and Roche standard as a function of pre-incubation time. The ratio \( \frac{\Delta A_{\text{serum}}}{\Delta A_{\text{Roche std}}} \) decreased as the pre-incubation time was shortened, irrespective of the sample volume used (10 or 20 μL). Serum fructosamine concentrations are determined from the simple expression \( \Delta A_{\text{serum}}/\Delta A_{\text{Roche std}} \times k \), where \( k \) is the DMF concentration assigned to the standard. Consequently the sliding activity ratio (serum/ std) increases the underestimation of serum fructosamine concentrations at shortened pre-incubation times.

Effect of Sample Volume

We measured the alkaline reducing activities in a quality-control serum, the Roche standard, DMF, and pooled sera, with 10- and 20-μL samples, respectively. The ratio of absorbance changes at various pre-incubation times is shown in Table 1. All the ratios fell significantly short of the theoretical value, 2.00, irrespective of the time interval and source of fructosamine used.

Effect of Ascorbate

Serum constituents other than glycated proteins can reduce nitroblue tetrazolium at alkaline pH and thus potentially interfere in the fructosamine assay. Ascorbate may be the most important potentially interfering substance. The time course of the reducing activity of ascorbate is brisker than that of glycated protein. Two-point spectrophotometric readings at 10 and 15 min were originally described (7), to minimize this potential interference. We observed in this study that addition of ascorbate (0.12–0.47 mmol/L) to serum resulted in a paradoxically reduced fructosamine activity when measured with the above-described assay timing (Table 2). In contrast, much less interference was seen with a shortened assay time (11), with use of a 4-min pre-incubation and measurement of the absorbance change during the following 3 min. The percentage inhibition of \( \Delta A \) in Table 2 is identical to the percentage inhibition of the apparent fructosamine concentration, because the concen-
tation is determined as $\Delta A_{\text{serum}}/\Delta A_{\text{Roche std}} \times \text{assigned concentration}$. Figure 4 illustrates the kinetics of the ascorbate effect. During the initial 4-min incubation with ascorbate the reaction velocity increased, as would be expected owing to the increased reducing potential. After 4 min all the ascorbate appeared to be oxidized, as shown by the intersection of the lines in Figure 4B depicting the slope ($\Delta A$/min) of the absorbance increase as a function of time shown in Figure 4A. For incubation times longer than 5 min, the slope (and therefore the apparent fructosamine activity) decreased with increasing ascorbate concentrations. The most likely explanation for the decreased activity in the presence of ascorbate may appear from the simple equations describing the kinetics of absorbance change and formazan production, respectively:

$$\frac{dA}{dt} = k \times \frac{d[\text{formazan}]}{dt}$$  \hspace{1cm} (1)

$$\frac{d[\text{formazan}]}{dt} = k' \times [\text{NBT}] \times [\text{fructosamine}]$$  \hspace{1cm} (2)

Owing to the initial ascorbate-induced reduction of NBT to formazan (Figure 4A), the NBT concentration may be sufficiently lowered to explain the subsequent reduction in reaction velocity by serum fructosamine (Figure 4B, equations 1 and 2). An inhibitory effect of ascorbate was likewise reported by Baker et al. (6) in a comprehensive interference study involving a 10-min pre-incubation.

No ascorbate interference was seen with a recent modification of the fructosamine assay (18) in which blank-corrected absorbance is measured after 15 min. With this procedure, samples and calibrators are left for 30 min at ambient temperature in alkaline medium before the redox indicator is added. This incubation step may explain the lack of ascorbate effect that otherwise would be expected, because ascorbate is highly unstable at alkaline pH.

Discussion

Recent reports on the fructosamine assay (7-9) advised against using pre-incubation times shorter than 10 min (I), on the grounds that this may diminish the specificity of the assay. Blair et al. (9) reported that glutathione caused a large positive bias in assays carried out with the Technicon RA-1000 analyzer and 7 min pre-incubation. No bias from this analyte was apparent in an earlier study by Baker et al. (6), who used a 10-min pre-incubation. However, the discrepancy between these two studies may be ascribable to factors other than the duration of pre-incubation. In a more
systematic study of this temporal relationship (10), the effect of each potentially interfering analyte was investigated, and no positive bias of glutathione was noted.

Higher apparent serum fructosamine concentrations were reported to result from decreasing the length of pre-incubation time (5, 8, 11, 12). This effect has been attributed to interference by non-ketoamine reducing constituents of serum (7–9). We propose another explanation based on the results of this investigation, which showed a different time-course of alkaline reducing activity in the primary fructosamine standard, DMF, and in glycated proteins. The activity ratio of reducing activity in protein over that in DMF increased with decreasing pre-incubation time. Thus, the apparently higher concentrations of serum fructosamines in assays with short pre-incubation times may in part reflect the different chemical nature of sample and DMF standard rather than assay interference alone. The predominant source of interference may stem from the presence of ascorbate in serum. In our study of the effects of ascorbate, pre-incubation times >4 min produced an inhibitory effect, which increased with the duration of the pre-incubation. A positive interference of ascorbate was only evident at pre-incubation times <4 min. The assay of San-Gil et al. (13) was carried out with only a 1-min pre-incubation in the presence of NBT, but our findings on ascorbate interference do not apply to this study in which the serum samples were exposed to alkaline pH for 5 min before NBT was added.

In the present study, the ratio of the reducing activity in pooled normal sera to that in the Roche standard decreased as the pre-incubation was shortened. We suggest that this effect may be due to the difference in the composition of the protein matrix. A sliding activity ratio would be expected if glycated proteins of different structure showed different alkaline reducing kinetics. It does not appear likely that the shift in the activity ratio can be ascribed to a hypothetical interference by reducing non-ketoamine serum constituents, because such interference would increase the activity ratio (serum/Roche standard) with decreasing duration of pre-incubation.

The proportional relationship between alkaline reducing activity and the concentration of DMF (13, 15, 16) and glycated proteins (14–16), respectively, is well established. We observed a disproportionate relationship between the reducing activity and the sample volume for all standards and sera alike in this study. This observation appears to be at variance with established findings, but the discrepancy is more apparent than real. In the studies demonstrating a linear relationship, the total albumin concentration was fixed. In our study the concentration of albumin in the assay mixture varied with the sample volume. We previously noted that albumin exerted a negative bias in the fructosamine assay (15), and such a bias was minimized at final assay concentrations of <1.5 g/L. A similar effect of the protein matrix was noted by van Dieijen-Visser et al. (5). In contrast, a linear dilution effect was reported by Baker et al. (17) and Johnson et al. (1).

It appears from the present study that shortening pre-incubation times will result in higher apparent values in assays in which DMF is the standard and lower values in assays based on secondary protein standards calibrated at a longer pre-incubation time than used in the assay. This agrees with previously reported findings (15). The proliferation of assay modifications has led to a disconcerting variation in reported fructosamine concentrations. The expression of serum fructosamine concentrations in DMF equiva-

lents is inherently erroneous when these concentrations are calculated from tests with secondary standards and assay conditions deviating from those at which the secondary standard was calibrated. However, in practical terms such error detracts little from the clinical usefulness of the assay if each laboratory establishes its own reference values and if diabetic control is assessed accordingly. Inter-laboratory variations in reference intervals are of little import considering the low ratio of intraindividual to interindividual variance in fructosamine concentrations shown by Howey et al. (14). The primary purpose of the fructosamine assay is to provide a tool for monitoring diabetic patients. In this respect each patient is his or her own reference. Fructosamine assays may help the clinician to assess the progression of disease and the effect of treatment modalities, and to evaluate patient compliance.

References
5. Johnson RN, Baker JR. The alkaline reducing activity of glycat-
70.
10. Frandsen EK, Baccus RA. Effect of duration of pre-incuba-
11. Hindle EJ, Rostron GM, Gatt JA. The estimation of serum fructosamine an alternative measurement to glycated hae-
13. San-Gil F, Schier GM, Moses RG, Gan IET. Improved estima-
17. Baker JR, O'Connor JP, Metcalf PA, Lawson MR, Johnson RN. Clinical usefulness of estimation of serum fructosamine concentra-