CHAPTER VII

SERUM AND SALIVARY FRUCTOSAMINE
INTRODUCTION

Body proteins undergo increased non-enzymatic glycosylation also known as glycation under protracted hyperglycemic conditions (116). The reaction predominantly involves the interaction of glucose with the free amino groups of proteins to form Schiff’s base and subsequent irreversible Amadori rearrangement resulting in ketamine derivative. Measurement of glycated hemoglobin has been widely used as an index of long-term diabetic control (259, 260, 261). Because of the relative long half-life of hemoglobin, the concentration of glycated hemoglobin reflects the average level of control over a period of the preceding 2-3 months (262). An assessment of diabetic control over a shorter period would also be of value in certain situations. This would be the case for example, during adjustment of therapy or during pregnancy, where good control reduces the fetal problems associated with diabetic pregnancy (218). Glycated albumin level is suggested to be a more sensitive index than glycated hemoglobin during short-term control of hyperglycemia (202, 263). Serum fructosamine concentration is a measure of total glycated serum proteins, and reflects the integrated glycemia over 2-3 weeks prior to its estimation (264, 265, 266).

Fructosamine is the generic name for plasma protein ketamines. The name refers to the structure of the ketamine rearrangement product formed by the interaction of glucose with the ε-amino group of lysine residues of albumin and other serum proteins. Analogous to glycated hemoglobin, measurement of fructosamine may be used to monitor the average concentration of blood glucose for an extended period of time, and several clinical studies have validated serum fructosamine level as an index of glycemic control (185).

In 1982 Johnson and Baker (185) developed a simple colorimetric method for measuring glycated serum proteins, which were summarized under the name ‘fructosamines’. The method is based on reduction of nitrobluetetrazolium
chloride (NBT) to its colored diformazan by glycated proteins in alkaline condition. The method relies on the ability of ketamines to act as reducing agents in alkaline condition (pH 10.35). Deoxymorpholino fructose a synthetic ketamine, is used as standard. Johnson et al (185) did not find an impressive correlation between serum fructosamine values and glycated proteins when determined by thiobarbituric acid colorimetric method. Ross et al (267) showed fructosamine values were able to distinguish between the best and worst controlled diabetics by NBT method, but thiobarbituric acid method was not able to do so. Johnson et al (186) reported that albumin accounts for 80% of the serum fructosamine activity, whereas Walker et al (268) indicated that only 50% of the activity is accounted for by albumin. Thus, a serious criticism of the method is that it is applied to a complex mixture of proteins with different half-lives and concentrations which can contribute unequally to the final value. Another major problem with reference to quantitation of glycated proteins in this method is the lack of an appropriate standard. Deoxymorpholino fructose used as a standard is claimed to show five times less reducing activity compared to protein ketamine (205). Glutathione, ascorbate and glucose can interfere depending on the pH used for the reduction reaction (185, 268). Serum fructosamine levels in normal subjects is reported to be in the range of 2.0-2.7 mmol/L (205) after modifications of the method compared to 1.3-1.9 mmol/L by the original method (185). Assuming a total protein concentration of 70 g/L in serum and that albumin contributes to 80% of the fructosamine activity, it can be computed that 3.0- 4.3 mol glucose is attached non-enzymatically to one mol albumin. Caines et al (191) have developed a modified fructosamine assay for glycated albumin. These workers used 3-(-4, 5-dimethyl thiazolyl) 2,4-diphenyltetrazolium bromide instead of nitrobluetetrazolium which under similar conditions, showed 3 fold increase in sensitivity compared to the latter. Go et al (269) have developed an
enzyme linked immunosorbent assay for the measurement of non-enzymatically bound glycated proteins in serum and tissues.

It is well known that the total proteins of human saliva averaged around 200 mg/dl (229), only about 3% of the protein concentration in plasma (229). Proteins originate from oral microorganisms, crevicular fluid, epithelial cells, polymorphonuclear leukocytes and dietary constituents. The salivary proteins include enzymes, immunoglobulins, antibacterial factors, mucous glycoproteins (mucin), traces of albumin and certain polypeptides and oligopeptides of importance in oral health (229). The serum proteins present in saliva amounted to 20% including IgG, IgM, IgA in addition to albumin and some alpha and beta globulins. The albumin content of the saliva is around 8 ± 5 mg/100ml (270).

No study is available on fructosamine values in saliva in normal and diabetic patients. Since measurement of salivary fructosamine will be relatively simple than in serum, a systematic study has been carried out.

MATERIALS

Nitrobluetetrazolium (NBT) stock solution (1.0 mmol/L)

81.76 mg of nitrobluetetrazolium chloride (NBT) was dissolved in 100 ml of 0.1 M carbonate buffer, pH 10.35. (75 mmol of sodium carbonate plus 25 mmol of sodium bicarbonate per liter).

The working NBT reagents, 0.25 mmol/L, 0.50 mmol/L and 0.75 mmol/L were prepared by diluting stock solution with carbonate buffer, pH 10.35.
1-Deoxy 1-morpholino D-fructose (DMF) standard (10 mmol/L)

250 mg of DMF is dissolved in 100 ml of bovine serum albumin (40g/L). The working standards (0.5 - 4 mmol/L) were prepared by appropriate dilution. The albumin solution served as control in constructing the standard graph.

For salivary fructosamine estimation DMF stock (1.0 mmol/L) and working standards (40-200μmol/L) were prepared in distilled water without containing albumin.

All other materials are listed in Chapter II.

METHODS

Unless stated otherwise, the following routine procedures were employed.

Measurement of fructosamine in serum

Fructosamine assay was performed with the method described by Johnson et al (185). To 0.3 ml of serum sample, 3.0 ml of 0.25 mmol/L (0.75 μ moles) nitrobuletetrazolium (NBT) reagent, pH 10.35 was added and the reaction mixture was incubated at 37°C in a water bath. The absorbance was measured at 530 nm at 10 and 15 minutes. The change in absorbance between 10 and 15 minutes is proportional to the amount of fructosamine in the serum sample. Normal saline (0.30 ml) with 3.0 ml of NBT reagent served as blank. The values of fructosamine were calculated from standard graph and are expressed in terms of mmoles of DMF equivalents.

Similarly, a series of 1-deoxy 1-morpholino D-fructose (DMF) standards ranging from 0.5 mmol/L to 4.0 mmol/L (0.15 - 1.2 μ moles) in a volume of 0.3 ml containing bovine serum albumin (40 g/L) were treated with 3.0 ml of NBT reagent (0.25 mmol/L). The reaction mixture was incubated at 37°C in a
water bath. The rate readings were measured at 530 nm at 10 and 15 minutes. The change in absorbance between 10 and 15 minutes was used for the construction of a standard graph (Figure 7.1).

**Measurement of fructosamine in saliva**

Fructosamine content of saliva was estimated by the method described by Johnson et al (185) with major modifications. Preliminary studies with salivary fructosamine showed that no measurable change in absorbance was noticed when the volume of saliva, concentration of NBT reagent (0.25 mmol/L) and measurement time of reaction rate readings were same as described for serum.

A systematic study was conducted to know the effects of NBT concentration on reaction rates of DMF standard and saliva, to understand how these factors affect the values obtained for saliva and also to determine the suitable concentration of NBT for the assay. Concentration of DMF used for constructing a standard graph was in the range, 40-200 μmol/L (0.04 – 0.2 μmoles). Three concentrations of NBT (0.25, 0.50 and 0.75 mmol/L) were used (Figure 7.2).

Salivary fructosamine was estimated by the following method. One ml of saliva was mixed with 3.0 ml of NBT reagent (0.75 mmol/liter) and incubated at 37°C. The absorbance was measured at 530 nm at 10 and 60 minutes. The change in absorbance at 530 nm is a measure of fructosamine values. Salivary fructosamine values were calculated by using standard graph (Figure 7.3).

Methods, for collection of serum and saliva samples have been described in Chapter VI. Method for estimation of salivary protein is also described in Chapter VI.
Figure 7.1. Standard graph for the estimation of serum fructosamine.
Figure 7.2 Effect of NBT concentration on fructosamine assay

- NBT (2.25 μ moles)
- NBT (1.50 μ moles)
- NBT (0.75 μ moles)
Figure 7.3 Standard graph for the estimation of salivary fructosamine.
RESULTS

The data obtained for serum fructosamine as absorbance units for 1.0ml sample are shown in Table 7.1. The mean absorbance values for control subjects and diabetics are 0.226 ± 0.016 (range 0.193-0.259) and 0.399 ± 0.053 (range 0.306-0.509) respectively. There is an increase of 1.79 fold in serum fructosamine value for diabetics compared to normal subjects and the increase is highly significant (P<0.001, t=15.72). The serum fructosamine values expressed in terms of DMF equivalents are shown in Table 7.3. The mean values for control subjects and diabetics are 1.69 ± 0.124 μ moles of DMF equivalents (range 1.45-1.95) and 2.99±0.399 μ moles of DMF equivalents (range 2.29 - 3.82) respectively.

Table 7.2 shows the values of salivary fructosamine expressed in terms of absorbance units for 1.0 ml of saliva in normal and diabetic subjects. The mean absorbance values are 0.17 ± 0.01 (range 0.16-0.20) and 0.25 ± 0.04 (range 0.19-0.37) respectively for normal and diabetic subjects. There is an increase of 1.47 fold in diabetics compared to normal subjects and the increase is statistically significant (P<0.001, t-9.39).

The salivary fructosamine values expressed in terms of DMF equivalents are shown in Table 7.3. The mean values for normal and diabetics are 0.034 ± 0.002 μ moles of DMF equivalents (range 0.032-0.040) and 0.050 ± 0.008 μ moles of DMF equivalents (range 0.038-0.074) respectively. The data obtained with salivary fructosamine expressed in terms of absorbance units for 50 minutes per mg of salivary protein are shown in Table 7.4. The mean absorbance values for normal and diabetics are 0.149 ± 0.010 (range 0.110-0.180) and 0.150±0.010 (range 0.120-0.190) respectively. The data show that when the values were expressed per mg protein of saliva, there is no difference between normals and diabetics. It has been shown earlier (Table 6.1) that salivary protein level is increased in diabetes mellitus.
**TABLE 7.1**  
ABSORBANCE VALUES OF SERUM FRUCTOSAMINE AT 530 nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Normals mean ± S.D (range) n=25</th>
<th>Diabetics mean ± S.D (range) n=25</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.226 ± 0.016 (0.193 - 0.259)</td>
<td>0.399 ± 0.053 (0.306 - 0.509)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t = 15.12</td>
</tr>
</tbody>
</table>

The values are in optical density units for one ml of serum, as differences in values measured at 10 and 15 minutes.
TABLE 7.2

ABSORBANCE VALUES OF SALIVARY FRUCTOSAMINE AT 530 nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Normals mean ± S.D (range) n=25</th>
<th>Diabetics mean ± S.D (range) n=25</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva</td>
<td>0.17 ± 0.01 (0.16 - 0.20)</td>
<td>0.25 ± 0.04 (0.19 - 0.37)</td>
<td>P &lt; 0.001 t = 9.39</td>
</tr>
</tbody>
</table>

The values are in optical density units for one-ml saliva as differences in values measured at 10 and 60 minutes.
TABLE 7.3
FRUCTOSAMINE VALUES FOR SERUM AND SALIVA EXPRESSED IN TERMS OF μ moles OF DMF EQUIVALENCES PER ml OF FLUID

<table>
<thead>
<tr>
<th>Sample</th>
<th>Normals mean ± S.D (range) n=25</th>
<th>Diabetics mean ± S.D (range) n=25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1.69 ± 0.124 (1.45 - 1.95)</td>
<td>2.99 ± 0.399 (2.29 - 3.82)</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.034 ± 0.002 (0.032 - 0.040)</td>
<td>0.050 ± 0.008 (0.038-0.074)</td>
</tr>
</tbody>
</table>

Salivary fructosamine values were calculated using the 50 minutes standard graph. A correction factor of 0.55 was applied to normalize the values to 0.75 mmol/L of NBT, based on the patterns of DMF at different NBT concentration (Figure 7.2).
### TABLE 7.4
**ABSORBANCE VALUES OF SALIVARY FRUCTOSAMINE**  
**(SPECIFIC ACTIVITY)**

| Sample | Normals mean ± S.D  
| (range)  
| n=25 | Diabetics mean ± S.D  
| (range)  
| n=25 |
|-------|-------------------------------|-------------------------------|
| Saliva | 0.149 ± 0.010  
| (0.110 - 0.180) | 0.150 ± 0.010  
| (0.120 -0.190) |

The values are in optical density units for saliva as differences in values measured at 10 and 60 minutes, expressed in terms of absorbance units per mg protein.
DISCUSSION

There have been several reports describing the value of fructosamine assay as an index of diabetic control (264, 266, 271). The method has several important advantages over alternate techniques for the measurement of glycated plasma proteins. These include its speed and precision, as well as significant cost advantages. It has been observed that fructosamine activity is a convenient measure of glycosyl protein concentration in serum/plasma and that the index responds mainly to glycated albumin. In our present study, the method developed by Johnson et al., (185) is used for the estimation of serum fructosamine and the same method is exploited for the measurement of salivary fructosamine with some major modifications.

The data obtained with serum fructosamine when expressed in terms of DMF equivalents (mmol/L) are in agreement with the values reported by several workers (266, 271). The method described by Johnson et al., (185) for the estimation of serum fructosamine, when applied for the measurement of salivary fructosamine, no measurable amount of chromogen was noticed. This is due to the low concentration of protein in saliva compared to serum, since, the measurement of fructosamine is dependent on the concentration of protein. Further, the contribution from plasma proteins to salivary proteins is as low as 10-15% of the total salivary protein (229). A modified method for fructosamine estimation in saliva has been developed using, higher reaction time, higher concentration of NBT and larger volume of test samples.

Salivary fructosamine values were also found significantly increased in diabetics. Since estimation of salivary fructosamine is easier than that of serum, this can be employed as a routine test for hyperglycemic detection. When salivary fructosamine values were expressed as a function of protein concentration, no difference between normal and diabetics was noticed. This is
because of a significant increase in salivary protein in diabetics unlike in normals. The time available for nonenzymatic glycation of proteins in saliva is limited. It can be concluded that serum proteins contribute bulk of salivary fructosamine value that reaches saliva due to permeability changes of the basal membrane of salivary glands.

When the serum and salivary fructosamine values are compared in terms of DMF equivalents, it was observed that serum values are nearly 50 fold higher than salivary values both in control subjects and diabetics. This is roughly in the same range as far as protein concentration in the two fluids is concerned. This further supports that salivary fructosamine has its origin mainly from serum.