Laboratory Monitoring of Gestational Diabetes*

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ABSTRACT

The consequences of uncontrolled gestational diabetes is severe to both maternal and fetal well-being. An ideal laboratory test to monitor gestational diabetes should accurately reflect short-term glucose changes. Glycated albumin, by virtue of its short half-life of 14 to 19 days, lends itself as a test to monitor and control gestational diabetes. Analytical approaches for the measurement of glycated albumin are reviewed, and the non-specificity of the simple colorimetric fructosamine assay is stressed. The performance characteristics of fructosamine assay, and the merits of the one hour oral glucose screening test and three-hour oral glucose tolerance test are discussed. An objective strategy for laboratory monitoring of gestational diabetes would include other assays such as fasting plasma glucose to correlate with glycated albumin (fructosamine) and even to complement the less sensitive glycated hemoglobin assay.

Introduction

The development of gestational-onset diabetes in the second half of pregnancy is more likely since placenta during that period produces progressively increasing amounts of estrogen, progesterone, and human chorionic somatomammotropin also called human placental lactogen. Since these hormones are antagonistic to insulin, the pregnant mother develops insulin resistance leading to the onset of gestational diabetes. It is important that gestational-onset diabetes be brought under control, since otherwise the condition can have grave consequences to both mother and the fetus. Since maternal hyperglycemia exposes the fetus to high levels of glucose, the fetal pancreas has to produce increasing amounts of insulin to metabolize the increased glucose load. Since insulin promotes both fat and protein synthesis, the fetus may become so large as to cause complications (dystocia) during vaginal delivery, requiring delivery by caesarean section in many instances.

Diagnosis of Gestational-onset Diabetes

Gestational-onset diabetes is usually diagnosed in the second half of preg-
nancy. Those subjects who have not already been identified as having glucose intolerance before the 24th week of pregnancy should be screened. This is usually done between the 24th and 28th week of pregnancy. A oral glucose load of 50 grams is administered any time during the day without regard to the time of last meal. One hour later a blood sample is drawn, and if the plasma glucose level is greater than 140 milligrams per deciliter (mg per dl) or (7.8 mM per L–S.I. Unit), the subject should be given an oral glucose tolerance test. This is done by administering an oral glucose load of 100 grams after a fasting blood specimen is drawn. Three separate blood specimens, each after one hour interval, should be drawn after the oral administration of glucose is complete.

The diagnosis of gestational-onset diabetes is made if two or more of plasma glucose values given below are met or exceeded. Fasting: 105 mg per dl (5.9 mM per L), one hour after glucose load: 190 mg per dl (10.6 mM per L); two hours after glucose load: 165 mg per dl (9.2 mM per L); and three hours after glucose load: 145 mg per dl (8.1 mM per L).\(^9\) (Values in parenthesis are in S.I., units). The glucose values reported here were obtained with the classical Smogyi Nelson procedure. These values have been revised to reflect results that would be obtained with an oxygen uptake measurement-based glucose oxidase procedure.\(^2\) Using the corrected values subjects with a one hour plasma glucose value of 135 mg per dl (7.5 mM per L) or greater after a 50 gram oral glucose load will receive the three-hour oral glucose tolerance test. Two of the three following values should be met or exceeded to meet diagnostic criteria for gestational diabetes: Fasting: 95 mg per dl (5.3 mM per L); one hour after glucose load: 180 mg per dl (10 mM per L); two hour: 155 mg per dl (8.6 mM per L); three hour: 140 mg per dl (7.8 mM per L). Recent studies have utilized a one-hour glucose value after screening load as low as 130 mg per dl (7.2 mM per L) to institute a three-hour oral glucose tolerance test.\(^24\)

Laboratory Monitoring of Gestational Diabetes

An ideal test for monitoring of gestational diabetes should not be affected by the subject not fasting or the time of day of blood collection. It should also mirror short term changes in glucose levels. In this regard, glycated hemoglobin (HbA1C) is too insensitive, since the life time of erythrocyte is as long as 120 days. As such, the glycated hemoglobin value will include in its measurement window a period of normoglycemia prior to the onset of gestational diabetes. Because of this limitation, methodology and performance characteristics for HbA1C are not discussed in this paper. Suffice it to say, that two of the major methods for quantitating glycated hemoglobin, (ion exchange chromatography and affinity chromatography), each have their own limitations.

Bearing in mind that nearly half the glycation in hemoglobin is N-terminal, protein glycated solely at e-lysine groups with a pKa of 10 to 11 will be fractionated with the unglycated protein, and, as such, the full extent of hemoglobin glycation will be underestimated. As is well known, Schiff base, carbamylated hemoglobin, and abnormal hemoglobins such as hemoglobin F will comigrate with HbA1C on ion exchange chromatography.

In contrast, affinity chromatography of glycated hemoglobin on phenylboronate resins is much less sensitive to pH and temperature variations than ion-exchange chromatography and Schiff base and abnormal hemoglobins do not interfere, yet proteins glycated with phosphorylated sugar derivatives are retained weakly, and, are thus underestimated.\(^6\)
Glycated albumin, however, permits monitoring of short term changes in glucose levels since albumin has a half-life of 14 to 19 days and has greater relevance to the discussion of gestational-onset diabetes.

Laboratory methods for the measurement of glycated albumin are based on the measurement of the fructosamine moiety which results when glucose undergoes a non-enzymatic reaction with protein, which usually is albumin. An intermediate labile compound, an aldimine, or Schiff base is formed which undergoes rearrangement (amadori rearrangement) to give a stable ketoamine or fructosamine.\textsuperscript{1,6,21}

This non enzymatic glycation is dependant both on the time and the concentration of glucose, with the sugars glycating N-terminal amino acids or side chain lysine groups. The ketoamine that is formed is structurally a beta ketosylamine, whose ring structure is primarily in the pyranosyl form, although it does exist also in the furanosyl form.\textsuperscript{6}

**Approaches to Measurement of Fructosamine**

**Nitroblue Tetrazolium Dye Reduction**

The most popular approach that has been adapted to automation on several clinical chemistry analyzers is based on the reduction of nitrobluetetrazolium dye (NBT) by fructosamine under alkaline conditions. The basis of the assay is the ability of fructosamine to enolize rapidly under alkaline conditions to an eneaminol. The latter probably dehydrates to form a strongly reducing enediol, splitting off in the process the sugar-free protein moiety. It is this enediol that reduces NBT, and the resulting blue compound (formazan) is quantitated spectrophotometrically (figure 1).

The intensity of color formed in the reaction is dependant upon pH, temperature, NBT concentration and quantity of serum used in the assay. Standardization of variables is the key to the optimization of the assay which clearly has no endpoint, since the alkaline conditions promote the reaction to proceed indefinitely, by converting the enediol oxidation product resulting from NBT reduction to a deep blue-black chromogen.\textsuperscript{6} Although the exact mechanism of the reaction is still not quite well understood, it is likely that superoxide radicals are involved.\textsuperscript{21}

In a typical assay, serum is added to NBT reagent in buffer (carbonate buffer), at an optimized pH of 10.35 at 37^\textdegree C.\textsuperscript{1} Absorbance at 530 nm is measured generally 10 and 15 minutes after initiation of assay. The rationale for making a measurement after 10 minutes is to minimize

\[
\begin{array}{ccc}
\text{CH}_2\text{NH-PROTEIN} & \text{CH-NH-PROTEIN} & \text{CHOH} \\
| & | & | \\
C = 0 & C = 0 & \text{CHOH} \\
\text{ALKALI} & \text{ALKALI} & \text{ALKALI} \\
\text{HOC} & \text{HOC} & \text{HOC} \\
\text{HCOH} & \text{HCOH} & \text{HCOH} \\
\text{HCOH} & \text{HCOH} & \text{HCOH} \\
\text{CH}_2\text{OH} & \text{CH}_2\text{OH} & \text{CH}_2\text{OH} \\
\end{array}
\]

**FRUCTOSAMINE** \quad **ENEAMINOL** \quad **ENEDIOL**

**Figure 1.** Nitroblue tetrazolium dye (NBT) reduction by fructosamine.
LABORATORY MONITORING OF GESTATIONAL DIABETES

the effect of interfering substances that are reported to reduce NBT during the first 10 minutes of the reaction time.\textsuperscript{8} Even so, the assay has been adapted to use on analyzers where the incubation time is only seven to eight minutes. A correction for albumin concentration is reported to increase the sensitivity of the measurement.\textsuperscript{26} There is considerable controversy with regard to the matrix effects of the standards that are used.\textsuperscript{1}

Originally 1-deoxy-1-morpholinofructose (DMF) which is a ketoamine was used as a standard in a 40 g per liter solution of albumin.\textsuperscript{1} Subsequently, matrix effects, depending on whether one uses DMF standards made up in serum or standards made up in albumin, have been addressed as well as variations owing to batch to batch differences of albumin.\textsuperscript{1} One approach to internal standardization is to assay the same serum sample twice, one aliquot with added DMF and the other without DMF.\textsuperscript{7} However, even this approach has been questioned.\textsuperscript{4} The fructosamine assay, when compared to the specific measurement of glycated amino acid residues of hydrolyzed serum proteins by HPLC, over estimates 10-fold the fructosamine concentration in serum.\textsuperscript{25}

Recently it was demonstrated by use of a secondary serum protein standard, calibrated with primary standards such as polylysine and glycated human serum albumin, that values obtained using DMF standards were indeed 10 times too high.\textsuperscript{20} The discrepancy was related to the DMF standardization being affected by variables such as pH, NBT concentration, and the instability of DMF standards.\textsuperscript{11} The reference interval for fructosamine of 0.205 to 0.285 mM per L obtained by use of serum protein standard calibrated with polylysine or glycated human serum albumin is said to agree with the mean glycation of three \(\mu\)mol per gram of protein determined by the furosine HPLC method with \(\epsilon\)-amino-fructosellysine as standard.\textsuperscript{20}

\textbf{Interferences in Fructosamine Assay.} Although neither the Schiff base intermediate nor glucose below pH 11 interfere with the fructosamine assay, carbonyl groups associated with collagen cross links could be a source of interference.\textsuperscript{6} Among reducing substances glutathione and uric acid have been known to interfere.\textsuperscript{6} When adapted to automation, numerous interferences have been reported, some varying from one analyzer to another. Thus, while on one discrete analyzer interferences were reported with bilirubin, heparin, cysteine, ethylenediamine tetraacetic acid (EDTA) and uric acid, on another discrete analyzer in addition to interferences by bilirubin, heparin, and cysteine, hemoglobin and glutathione were also reported to interfere.\textsuperscript{1}

The poor solubility of the diformazan formed during the NBT reduction reaction is also a variable in the fructosamine assay.\textsuperscript{11} Upon solubilization of diformazan in an optimized automated assay, it was noted that uric acid contributed significantly to the color, which was compensated for by a mathematical correction.\textsuperscript{25}

In addition to the interferences enumerated previously, some NBT reducing activity in serum has been attributed to non-specific substances other than fructosamine. Such non-specific NBT reducing activity varied from serum to serum and was not reducible with sodium borohydride.\textsuperscript{19} It has been reported that some of this non-specific NBT reducing activity is due to the presence of a vitamin: pyrroloquinoline quinone (PQQ).\textsuperscript{17} Since PQQ is not reduced by sodium borohydride, whereas fructosamine is reduced, a correction for non-specific fructosamine activity can be performed. The correction entails using two aliquots of serum to one of which sodium borohydride is added which should reduce only fructosamine, while to the other aliquot sodium borate is added which will leave both fructosamine and non-specific PQQ.
activity intact. After this preincubation step, acid is added; after neutralization, NBT assay is performed. The apparent fructosamine activity measured in the presence of sodium borohydride is subtracted from total fructosamine activity measured in the serum aliquot incubated with sodium borate to yield the true fructosamine activity.17

Another interference that has been reported is due to immunoglobulin-A (IgA), which, since it is also highly glycated, gives spuriously high fructosamine values at pathological concentrations.22

THIOBARBITURIC ACID PROCEDURE

Several variations of this procedure have been utilized.1,21 Suffice it to say that they all involve prolonged heating for at least eight hours at 115°C to convert the keatoamine linkage in the fructosamine molecule to 5-hydroxymethyl furfural (5-HMF). The latter compound is then reacted with thiobarbituric acid for 30 to 50 minutes, and the resulting color is measured in a spectrophotometer at 443 nm. Results are reported as fructose equivalents using fructose as standard. Glucose in the patient’s sample is a major interferent and has to be removed either by dialysis or by precipitation of proteins including fructosamine with trichloroacetic acid prior to conversion to 5-HMF. A serum blank has to be included to compensate for non-specific fructosamine activity by reducing the true fructosamine with sodium borohydride. Even with the various modifications that this assay has undergone in an effort to optimize the assay and reduce the analysis time, the procedure is still involved, and, as such, is not amenable to routine usage.1

FUROSINE PROCEDURE

Furosine (epsilon N-(2-furoylmethyl)-L-lysine) is one of the products that results when the epsilon amino group of lysine in the protein molecule that is bound to glucose to form fructosamine is hydrolyzed with six molar hydrochloric acid at 95°C to 100°C for 18 hours. Furosine is then separated on reverse phase high performance liquid chromatography (HPLC) with phosphoric acid (7 mol per liter) as the mobile phase and quantified by dual U.V. wavelength measurements at 254 and 280 nm.21 Although this procedure offers a specific measurement of fructosamine since Schiff base, enzymatically bound sugar, or collagen cross link compounds do not interfere, the prolonged hydrolysis step coupled with the use of a HPLC instrument is the major stumbling block for its use in the routine clinical laboratory.

AFFINITY CHROMATOGRAPHY

This procedure is based on the ability of compounds containing Cis-hydroxy groups, such as glycated proteins, to form a complex with boronic acid which is used as an affinity support. However, it should be borne in mind that phosphorylated sugars are hindered from binding to boronate resins. As such, not all glycated species of a protein are estimated by phenyl boronate affinity chromatography. Essentially, the procedure involves application of plasma or serum diluted preferably with buffer to a column containing immobilized m-aminophenyl boronic acid.12,13 Typically an ammonium acetate buffer 0.25 molar containing 0.05 molar magnesium chloride adjusted to a pH varying from pH 8.013 to pH 8.812 is used to elute non-glycated albumin. Glycated albumin is subsequently eluted with either sodium citrate buffer at acid pH (0.2 molar, pH 4.5),13 or sorbitol buffer containing 0.2 molar sorbitol, 0.05 molar EDTA and 0.1 molar Tris adjusted to a pH of 8.8, the same pH as the buffer used to elute non-glycated albumin.12 Albumin in both glycated and
non-glycated fractions can be measured either by dye-binding techniques using bromocresol green at 630 nm\textsuperscript{13} or by immunoturbidimetry at 340 nm.\textsuperscript{12} Results are reported as percent glycated albumin. The affinity procedure can be performed manually with commercially available column kits or using an automated HPLC apparatus. The procedure, since it is free from major interferences including glucose, and the fact that Schiff base dissociates during chromatography and as such does not interfere, is the procedure of choice. However, the manual manipulations using kit-based procedures, or alternatively the unavailability of HPLC apparatus in many clinical laboratories, limits the application of the affinity procedure to routine use. This leaves the laboratorian with little choice but to rely on the popular and simple procedure utilizing NBT dye reduction for the measurement of fructosamine in spite of its limitations.

**Oral Glucose Screening Test and Glucose Tolerance Test.** How good is the one-hour 50 gram oral glucose test for screening gestational diabetes? If the study is applied to a population of gestational diabetes subjects 25 years and older, the one-hour 50 gram oral glucose screening test can achieve a sensitivity of 88 percent and a specificity of 82 percent.\textsuperscript{10} However, if screening is conducted based on the presence of one or more of the clinical history factors, such as a family history of diabetes, hypertension, weight gain, or proteinuria, although the specificity rises to 87 percent, the sensitivity drops to 62 percent, making the screening test inadequate.\textsuperscript{10} In a recent study, subjects with a high risk for gestational diabetes were chosen and were given an adequate carbohydrate diet (a minimum of 300 g per day) three days prior to administering a 50 g oral glucose screening load, with the subjects fasting from midnight up to the time of collection of the fasting blood speci-
Comparison of Results Obtained on Serum Fructosamine and Glycated Hemoglobin (HbA1C) at Base Line Venipuncture for the 100 Gram Glucose Tolerance Test

**Table I**

<table>
<thead>
<tr>
<th>Test:</th>
<th>13 Subjects with Gestational Diabetes</th>
<th>84 Non-diabetic Pregnant Subjects (Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subjects at 26th to 28th week of pregnancy</td>
<td></td>
</tr>
<tr>
<td>Fructosamine (mM)</td>
<td>2.02 ± 0.08</td>
<td>1.98 ± 0.02</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>4.42 ± 0.2</td>
<td>4.60 ± 0.3</td>
</tr>
</tbody>
</table>

3 hour glucose tolerance test.
Mean glucose values in mg per dl; values in (mM per liter) (S.I. Units)

| Fasting | 122 (6.8) | 90 (5.0) | P < 0.005 |
| 1 hour  | 234 (13.0) | 158 (8.8) | P < 0.001 |
| 2 hour  | 194 (10.8) | 135 (7.5) | P < 0.001 |
| 3 hour  | 140 (7.8) | 99 (5.5) | P < 0.005 |


ing test for gestational diabetes. However, during follow up studies of diabetic subjects at two-week intervals, there was a significant correlation between fasting blood glucose and serum fructosamine levels (r = 0.81; P < 0.001). As expected, there was no correlation between glycated hemoglobin (HbA1C) and fasting glucose levels (r = 0.11). These data would suggest that the fructosamine assay reflects short-time glycemic control, and, as such, may be useful in monitoring the glycemic state of the pregnant mother. In another study, serum fructosamine levels correlated significantly with mean plasma glucose levels over a one to three week interval, with the closest association found in the preceding week. Eighty-five percent of women with gestational diabetes in this study had peak serum fructosamine values that were over the normal reference interval. In these pregnant women, there was an 88 percent chance of an abnormal glucose tolerance test, post-partum, when fructosamine levels were greater than 3.2 mM per L. Patients who had their fructosamine levels reduced to less than 2.5 mM per L had fewer obese babies and lower cord insulin and C-peptide levels as compared to neonates of mothers with higher fructosamine concentrations.

In discussing performance characteristics of a method, the reference intervals, clinical sensitivity and specificity, analytical characteristics such as precision, and interferences need to be delineated. Attempts to summarize some of the performance characteristics of the fructosamine assay are shown in table II.

In terms of establishing reference intervals, it has been pointed out from a study on 40 non-diabetic pregnant women that there is a large inter-individual variation for plasma fructosamine that comes in the way of adapting an overall reference range for pregnant women. In this study, although the interrun precision at two levels (1.46 mM per L and 2.8 mM per L) of plasma fructosamine were similar (C.V. of 4.8 percent), the reference range for plasma fructosamine was between 0.3 to 0.9 mM per L, with a mean of 0.53 mM per L. Reference ranges established for normal pregnant women vary from study to study. As noted earlier, reference intervals would also depend on the stan-
TABLE II

Performance Characteristics of Fructosamine Assay

1. Reference Intervals for Pregnant Women (in mMol per Liter)

<table>
<thead>
<tr>
<th>Number of Subjects</th>
<th>Normal Non-diabetic</th>
<th>Diabetic</th>
<th>Literature Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>79</td>
<td>1.18 - 1.9</td>
<td>1.54 - 2.26</td>
<td>16, 27</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>16, 27</td>
</tr>
<tr>
<td>87</td>
<td>1.83 - 2.54</td>
<td>1.78 - 2.82</td>
<td>5, 27</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td>5, 27</td>
</tr>
<tr>
<td>40</td>
<td>Mean of 0.532</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>(Minimum 0.3; maximum 0.9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. N  Sensitivity  Specificity  Prevalence (Gestational Diabetes)  Literature Reference

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>86 %</td>
<td>95 %</td>
<td>11 %</td>
<td>14, 27</td>
</tr>
</tbody>
</table>

3. Interferences

Glutathione, uric acid, carbonyl groups associated with collagen cross links, bilirubin, heparin, cysteine, ethylene diamine tetraacetic acid (EDTA), hemoglobin, pyrroloquinoline quinone (PQQ), and immunoglobulin-A (IgA)

 standards used for calibration. Overlap between normal and diabetic pregnant subjects have been reported (table II), reflecting the insensitivity of serum fructosamine as a screening test to detect gestational diabetics.

As to sensitivity and specificity, a recent critical assessment of current literature on the fructosamine assay in the diagnosis and control of diabetes in general lamented on paucity of information on the subject. Of 65 articles from literature that the authors evaluated, only seven of them stated the sensitivity and specificity of the test or provided data to perform such calculations. Even these studies had limitations to the extent that standards such as selection of patients were not clearly stated, and relating fructosamine levels to patient categories were not mutually blind to the extent that the diagnosis was known even before the test was performed.

Even so, in spite of the numerous interferences and technical problems associated with the procedure, which were noted previously, the fructosamine assay, although not useful as a screening test for gestational diabetes, has merit in the follow-up of the maternal glycemic state.

The recent critical assessment of literature leaves much room for improvement, and through validation of the fructosamine assay, to remove doubts in the minds of the laboratorian on the validity of the fructosamine test.

Choice of Laboratory Procedures for Monitoring Gestational Diabetes. Given the fact that glycated albumin (fructosamine) measures short term glucose changes, it is superior to glycated hemoglobin, which because of the long life time of the erythrocyte (120 days) measures a period of normoglycemia that may have preceded the onset of gestational diabetes. So the averaged glycated hemoglobin value may be too insensitive to accurately reflect the state of glycemia in the pregnant individual.

Although the affinity chromatography procedure for glycated albumin or glycated protein offers specificity, the lack
of access to HPLC apparatus in many clinical laboratories limits its use on a routine basis. Although inter-assay C.V. of 6.4 percent and 5.8 percent have been reported for glycated albumin and glycationed protein, respectively, on a manual affinity chromatography procedure, doubts have been cast on the ability to distinguish between gestational diabetes patients and normal controls based on these measurements. As such, it would appear that the affinity chromatography procedure for glycated albumin and glycated protein requires further critical validation.

Because of the lack of specificity of the convenient NBT reduction assay for fructosamine, it has limited usefulness as a screening test for gestational diabetes. In this context, neither the fructosamine assay nor the glycated albumin and glycated protein affinity chromatography assays should replace the one-hour 50 gram oral glucose screening test for gestational diabetes. However, the fructosamine assay is useful to monitor the glycemic state in the mother, as judged by tests such as fasting blood glucose and mean outpatient blood glucose especially in the third trimester of pregnancy.

Conclusions

Glycated albumin assessed by a measurement of fructosamine lends itself to monitor and control gestational diabetes. For purposes of base line comparison and to assess improvement in the glycemic state of the pregnant diabetic, it could even serve as a complementary test to glycated hemoglobin, even though the latter is not quite sensitive to mirror short term changes in glycemia. The rationale is to see if there is a shift in both the glycated albumin and the glycated hemoglobin value compared to a base line value, and to determine if this shift correlates with the level of fasting blood glucose in the subject, thus providing a stricter laboratory monitoring of gestational diabetes.

References