

## **A study on the use of two tetrazolium salts for determination of serum fructosamine**

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### **Abstract**

The determination of glycated serum proteins (fructosamine) has become an important tool for assessing the glycaemic status in diabetic patients. Methods involved in the laboratory assays are based on the ability of fructosamine to act as a reducing agent in alkaline solution. A colorimetry assay method was performed to compare the reduction ability of two tetrazolium salts namely p-Iodonitrotetrazolium violet (INT) and Nitroblue tetrazolium chloride (NBT) for estimation of serum fructosamine. Results revealed that the reduction ability of the ketoamine group of glycated protein (fructosamine) was more towards NBT than INT.

**Key Words :** Fructosamine, p-Iodonitrotetrazolium violet, nitrobluetetrazolium chloride.

### **1. Introduction**

The general principle for the non-enzymatic glycosylation of protein is the condensation of free amino acid groups in protein with the acyclic form of glucose or sugar aldehyde to yield a schiff-base intermediate which then undergo the Amadori rearrangement to form a stable ketoamine derivative (Hodge, 1955), generically termed as Fructosamine.

A number of proteins in the mammalian system eg. haemoglobin, serum proteins, membrane proteins and proteins in the eye lens etc. are glycated by non-enzymatic reaction to the extent which depends on the severity and duration of hyperglycaemia (Willms & Lehmann, 1990 & Benjamin & Sacks, 1994). As the largest fraction of serum proteins (about 60-70%) consist of albumin with a half-life of 2 -3 weeks, the estimation of fructosamine in serum provides a valuable index for assessing the metabolic control of diabetes over moderately shorted period (Johnson et al, 1982 Benjamin & Sacks, 1994).

The principle underlining the estimation of serum fructosamine is based on the property of ketoamine products to act as reducing agents in alkaline solution (Hodge & Rist, 1953). At present estimation of serum fructosamine is done using commercially available colorimetry assay method based on the reduction of tetrazolium salts mainly Iodonitrotetrazolium violet (INT) or Nitroblue tetrazolium Chloride (NBT) in alkaline medium. However, the reactive performance of these two tetrazolium salts in estimation of serum fructosamine has not been studied. Therefore, a colorimetry study was performed to compare the reactive performance of NBT & INT in estimation of serum fructosamine under local laboratory conditions.

## 2. Methods and Materials

Two Tetrazolium salts namely, p-Iodotetrazolium violet (Sigma USA cat No. I 18377) and Nitrobluetetrazolium chloride (Sigma USA cat. No. N 6846) were used in the present study and the Formazan product derived on reduction of INT and NBT was assayed by colorimetric method.

### *Preparation of Tetrazolium salts*

INT and NBT were prepared in different concentrations in carbonate buffer (pH 10.35) as follows.

INT concentrations of 0.2, 0.4, 0.6, 0.8, 1.2 and 1.6 mmol/L

NBT concentrations of 0.2, 0.3, 0.4, 0.6 and 0.8 mmol/L

The pH of the medium was decided after carrying out of few trial runs as suggested by Johnson et al 1982.

### *Preparation of 1-deoxy, 1-morpholino D-fructose (DMF) standards*

DMF (Sigma) USA cat no. D6 149), the synthetic Amadori rearrangement product was used as a standard (a calibrator) to measure the fructosamine concentrations of unknown serum specimens. The DMF standards of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mmol/L were made up according to the procedure described by Johnson et al 1982 by adding DMF to 0.14 mole of sodium chloride containing 40g albumin per litre in order to provide a suitable plasma environment for assay.

### *Collection of blood specimens for assay*

Based on the fasting blood glucose level recommended by the WHO 1980 (less than 110 mg/dl) thirty non diabetic subjects were selected using standard glucose oxidase assay method (DMA, USA) and their venous blood

was collected into collection vials without any anti-coagulant. Blood was allowed to clot and serum was separated and stored at -20°C approximately for 3-4 weeks pending fructosamine assay as the fructosamine is stable at this temperature indefinitely (Johnson et al 1982).

#### *Pre-treatment of serum samples for fructosamine assay*

A volume of 100µl of serum was added to 20µl of carbonate buffer (pH 13) and left approximately for 5 min. in order to prevent the interference from endogenous ascorbic acid and to minimize the matrix effect.

#### *Fructosamine assay method*

A volume of 100µl pre-treated serum sample was added to 1ml of carbonate buffer (pH 10.35) containing 1 ml of INT or NBT reagent and incubated at 37°C in a water bath and also at 30-32°C (room temperature) for 10, 15, 20 and 30 min. and the reaction was allowed to proceed to completion. The colour or Formazan product developed by the reduced NBT (purple colour) was measured colorimetrically at 530 nm and 500 nm respectively. The concentration of the fructosamine in the sample is proportional to the absorbance of formazan. These results were compared with the Sigma USA commercial assay method (cat. No. 465-A).

The thirty serum samples were subjected to each of the above experimental conditions. Each sample was divided into 3 and the serum fructosamine value was determined from each and the mean was calculated.

The DMF standards were also incubated in a similar manner using INT (1.2 mmol/L) and NBT (0.3 mmol/L) and the colour of the formazan developed was read at 500 and 530 nm respectively. The DMF standard curve was plotted according to Beer Lambert's law using the absorbance (Y) against the DMF concentration (X), which was subsequently used to determine the fructosamine concentrations in serum samples. These values were compared with the values obtained by using commercial assay method.

#### *Recovery Studies*

Known amounts of fructosamine (0.55, 1.11 and 2.23 of Sigma commercial calibrator) were added to 10 serum samples (in triplicates) with known fructosamine concentration and incubated with NBT and INT separately in a similar manner and the recovery rate was calculated using the DMF standard curve.

#### *Statistical Analysis*

The statistical significance of each variable (concentrations, incubation time and temperature) was assessed by Student's t-test for paired or unpaired data as appropriate.

### 3. Results

#### *Effect of incubation time and the NBT and INT concentration on Fructosamine Value;*

Table I. Shows the results of the mean fructosamine concentration of 30 non diabetic serum samples assayed using different concentration of NBT and INT incubated at different temperatures and for varying time intervals. Results indicated that the intensity of formazan dye product derived upon the reduction of both tetrazolium salts increased with the prolonged incubation time and by increasing the tetrazolium salts concentration. Furthermore, the results revealed that the fructosamine values of samples when treated with either 0.3 mmol/L NBT or 1.2 mmol/L INT concentration in carbonate buffer and incubated for 15 to 20 min at 30-32°C (room temperature) for 10 to 15 min at 37°C were statistically (2 DS) comparable with the corresponding values obtained with the commercial assay method.

**Table I.** The effect of temperature, incubation time and concentration of Iodonitro tetrazolium violet (INT) and Nitroblue tetrazolium chloride (NBT) of serum Fructosamine value (u.mol/l)

Incubation Temperature 30-32°C (Room Temperature)						37°C				
Incubation Time (minutes)	5	10	15	20	30	5	10	15	20	30
<b>Mean INT (u.mol/L)</b>										
0.2	56	58	68	76	78	68	76	78	80	81
0.4	66	70	78	86	88	76	82	88	93	98
0.6	120	138	146	150	158	124	138	148	160	172
0.8	160	172	196	208	230	190	218	236	258	262
1.2	180	198	208	220	236	190	218	236	258	262
1.6	186	202	210	230	238	198	228	240	258	272
<b>Mean NBT (U.mol/L)</b>										
0.2	168	180	188	199	203	176	198	208	238	253
0.3	192	198	216	228	237	208	220	232	248	264
0.4	210	226	238	250	270	228	240	268	280	296
0.6	238	257	278	293	317	246	268	289	310	329
0.8	258	274	292	318	337	269	291	320	343	372
<b>Commercial Assay Method (SD).</b>										
	178	190	218	223	256	208	221	224	258	282
	±6	±5	±5	±4	+6	±4	±5	±3	±6	±6

**Note :** Sample size is 30 per each experimental treatment.

*Performance Characteristics on NBT and INT*

The recovery studies indicated that there was a higher recovery rate of 89% with 90% reproducibility for NBT, compared to a low recovery rate (68%) and reproducibility (77%) for INT (Table II)

**Table II** The mean (n=10) recovery rate and reproducibility performance characteristics of nitroblue tetrazolium chloride (NBT) and Iodonitro Tetrazolium violet (INT)

Tetrazolium salt	% Recovery Rate (SD)	% Reproducibility (SD)
NBT (0.3 mmol/L)	88.7 ± 3.1	90.5 ± 1.6
INT (1.2 mmol/L)	67.5 ± 4.3	77.5 ± 2.1

*The accuracy of predicting results using DMF standards*

The two assay methods used for estimation of serum fructosamine (the DMF and commercial assay method) shown very similar results giving a high correlation coefficient of 0.91.

**4. Discussion**

In general, the laboratory estimation of Amadori rearrangement products have been based upon their ability to reduce certain tetrazolium salts, methylene blue, dichlorophenol indophenol and o-dinitrobenzene in alkaline medium (Hodge and Rist 1953). The present study revealed that by increasing the incubation time, temperature and the concentration of NBT and INT increased the intensity of formation of formazan product, which shows that the reduction was a function of incubation time and temperature and the concentration of tetrazolium salts.

The reduction performance by the amadori products suggest that the reactive efficiency towards NBT at 0.3 mmol/L and INT at 1.2 mmol/L when incubated at 37°C for 15-20 min. or 30-32°C (under room temperature) was within the 2 SD range of the standard commercial assay method. However, by considering the overall productive performance of INT and NBT shows that the ability of the amadori product (fructosamine/DMF) to reduce NBT (0.3 mmol/L) was higher in NBT than INT (1.2 mmol/L). Few comparison studies confirmed that the ability of DMF to reduce NBT was greater than that of methylene blue, dichlorophenol indophenol o-

dinitrobenzene (Hodge and Rist 1953 and Johnson et al, 1982). However, the information available on the reduction ability upon the property of fructosamine on NBT and INT are lacking. Based on the cost analysis of Sigma USA product use in the Present study, it was revealed that the use of NBT for estimation of serum fructosamine appeared to be more cost effective (Rs. 959 for 100 tests) compared to INT (Rs. 1324 for 100 tests).

The present study suggests that the reduction ability of glycated serum protein (fructosamine) was more efficient towards NBT than INT. Further more, the colorimetric method described in the present study using NBT (0.3 mmol/l) could be used as costeffective assay method for estimation of serum fructosamine under local laboratory conditions.

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