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A convenient assay of glycoserum by nitroblue tetrazolium with iodoacetamide

Ya-Jie Xu[a,1], Xue-Qin Wu[b,1], Wei Liu[a], Xiao-Hong Lin[b], Jian-Wen Chen[b], Rong-qiao He[a,*]

[a] *Laboratory of Visual Information Processing, Centre for Brain and Cognitive Sciences, Institute of Biophysics, Chinese Academy of Sciences, 15 Da Tun Rd., Chaoyang District, Beijing 100101, China*

[b] *Zhongguancun Hospital, Haidian District, Beijing 100080, China*

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Abstract

Background: To determine glycoserum, the nitroblue tetrazolium (NBT) assay is quick, economical, convenient and easily automated. This method, however, is vulnerable to interference by thiol group, which should not be ignored during the analysis. *Methods*: Thiol group in glycoserum was blocked with iodoacetamide (IAM) before NBT was added. The reaction was carried out in a thermal bath and terminated on ice. The absorbance was measured at either 570 or 530 nm. *Results*: IAM (3–10 mmol/l) did not give any detectable interference in the NBT assay. The absorbance at both 570 and 530 nm was linearly related to the concentration of either glycoserum or 1-deoxy-1-morpholino-*D*-fructose (DMF) that had been treated with IAM. The assay showed a good discrimination between diabetic and normal subjects (*t*-test, $P<0.001$). Uric acid and lipemia under physiological conditions did not interfere with NBT reaction. The assay was affected by hyperlipemia and hematolysis. *Conclusions*: IAM-modified method prevented NBT assay from the interference by thiol group and uric acid.

Keywords: Glycoserum; Assay; Nitroblue tetrazolium; Iodoacetamide; Thiol; Uricase; Detergent

1. Introduction

It is an essential criterion to monitor the blood sugar for a diabetic. The amount of fructosamine in serum is increased in diabetes mellitus owing to the abnormally high concentration of sugar in blood. Fructosamine reflects the average blood sugar concentration over the past 2 to 3 weeks. Nitroblue tetrazolium (NBT) method is one of the analytic approaches to measure fructosamine. As it is fast, cheap and easily automated [1], the method has been used in many laboratories [2–7]. However, as reported by Siedel et al. [8], the fructosamine assay suffers from interferences due to uric acid and lipemia. They and Kruse-Jarres et al. [9] modified the colorimetric method by adding uricase and detergent, to remove uric acid and lipemia. In their reports, the modified test agreed well with fructosamine determination, confirmed by a furosine/HPLC method. Nevertheless, in the current NBT method, the fructosamine concentration falsely increases due to some

Abbreviations: NBT, nitroblue tetrazolium; IAM, iodoacetamide; DMF, 1-deoxy-1-morpholino-*D*-fructose.

* Corresponding author. Tel.: +86-10-6488-8531; fax: +86-10-6487-5055.

E-mail address: herq@sun5.ibp.ac.cn (R. He).

[1] These two authors equally contributed to this work.

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reducing components contained in serum, e.g., thiol group in the peptides and proteins [10–12]. These reducing components mainly reacted with NBT in the initial 10 min, and thus, Johnson and Baker [10] introduced a 10-min pre-incubation of the serum sample to avoid this interference. According to Liu and He [13], the interference that resulted from thiol group could not be undetectable throughout the assay. It detectably interfered with the fructosamine assay. Iodoacetamide (IAM) is commonly used to block thiol group, producing a stable carboxamidemethylated bound protein [14]. Fortunately, carboxamidemethylation does not disturb the reaction of NBT with fructosamine.

2. Materials and methods

2.1. Sera and materials

Fasting blood samples (0.2 ml for each, mixed with 1 mg/ml EDTA) from 109 normal and 60 abnormal (diabetic) subjects were taken to test their glycoserum. Both oral glucose tolerance test (OGTT) [15] and glycohemoglobin test (0.05-ml fresh blood) with *m*-aminophenylboronic-acid affinity column, as described by Mallia et al. [16], were used to distinguish normal and diabetic subjects. Hemolyzed blood was not used.

The reagent, 1-deoxy-1-morpholino-*D*-fructose (DMF), IAM, Triton X-100 and uricase (71.2 ku/g) were from Sigma. NBT was from Roche Molecular Biochemicals. All other reagents were analytic grade without further purification. The micro-column of *m*-aminophenylboronic acid for analysis of glycohemoglobin was from Seiagaku, Tokyo, Japan. Blood glucose of the subjects was determined with a glucose-dehydrogenase kit made by a local biotechnological company of Chinese Academy of Sciences in Beijing. Absorbance was measured on either a Perkin-Elmer λ-7 UV/VIS photospectrometer or a Beijing PUXI TU-1900 spectrophotometer.

2.2. Fructosamine assay

Serum (50 μl) was suspended in 0.1 mol/l phosphate buffer (100 μl, pH 8.0) containing 3.2 mmol/l IAM, and allowed to stand in a thermal bath for 30 min, and then 1100 μl of NBT was added. NBT agent was freshly prepared with carbonate buffer (0.2 mol/l, pH 10.3). The reaction mixture was kept for another 30 min until the test tube was put into ice to terminate the reaction. After that, the absorbance was measured at either 570 or 530 nm. A sample without incubation with IAM was used as control. DMF was employed as a fructosamine standard compound as described by Johnson et al. [2]. The effect of IAM on the reaction was investigated under desirable concentrations. Under the same conditions, changes in the absorbance at either 530 or 570 nm were measured, following the addition of NBT. Serum, untreated with IAM, was used as control.

2.3. Measurements with uricase and detergent

To determine whether or not uric acid could interfere with this improved fructosamine assay, uricase was added to serum, as described previously by Siedel et al. [8] and Kruse-Jarres et al. [9]. Triton X-100 was commonly used as a detergent to release protein off liposome into aqua phase, as described by Hu et al. [17]. Consequently, Triton X-100 (1.7 and 30 mmol/l as final concentrations) was added to sera and incubated at room temperature for 1 h, and then centrifuged (12 000 rpm, 4 °C, 15 min) before taken for the assay.

3. Results and discussion

3.1. Effect of IAM on fructosamine assay

The effect of IAM modification on the measurement of glycoserum is shown in Fig. 1. The absorbance decreased by 19% at 530 nm and by 23% at 570 nm when IAM was used, indicating that thiol groups interfered distinctly with the assay and it could be removed by the treatment of IAM. It was also shown that IAM, other than blocking the thiol group, did not disturb the reaction of NBT with the glycated group of glycoserum. At the same time, IAM did not affect the reaction of NBT with the standard compound DMF (data not shown).

3.2. Determination of the fructosamine

The absorption spectra of IAM, NBT, DMF and serum were measured, respectively (Fig. 2). Serum had a light absorption around 530 nm, but the absorption

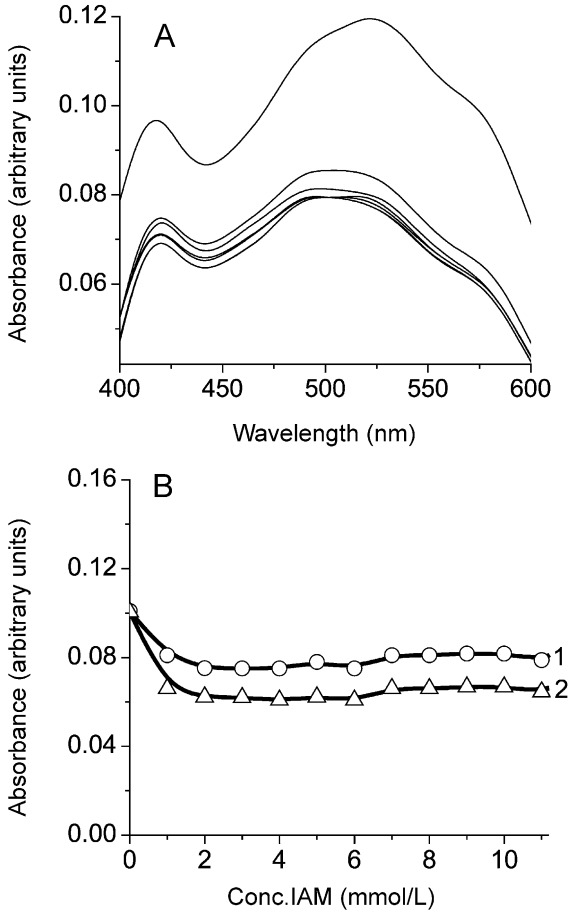

Fig. 1. The absorbance of human serum during reaction with NBT in the presence of IAM at different concentrations. Serum (50 μl) was incubated with IAM (100 μl) at different concentrations at 25 °C for 30 min before 1100 μl of NBT (10 mmol/l, as final concentration) was added. The reaction was carried out for another 30 min and then terminated in ice. (A) The absorption spectra. Curves from top to bottom at 530 nm represented the final IAM concentrations: 0.0, 1.0, 5.0, 2.0, 3.0, 4.0 and 6.0 mmol/l, respectively. (B) Absorbance at both 530 nm (curve 1) and 570 nm (curve 2) at different IAM concentrations.

was in background at 570 nm, i.e., changes in the absorbance at 570 nm just resulted from the reaction of NBT with glycoserum. The absorbance was linearly related to the concentration of glycoserum incubated either with or without IAM (Fig. 3). Furthermore, the slope of the absorption curve in the absence of IAM was observed to be higher than that of the samples in the presence of IAM, suggesting again that thiol group definitely interfered with the reaction.

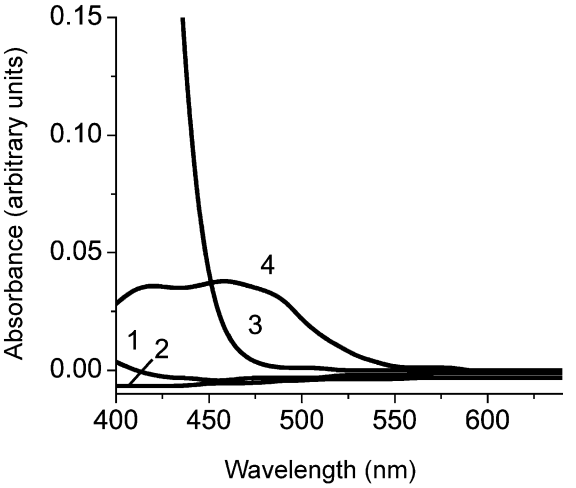

Fig. 2. The absorption spectra of IAM, DMF, NBT and human serum. Absorbance of IAM (3.2 mmol/l), DMF (1.6 mmol/l), NBT (10 mmol/l) and serum was measured. The concentrations of the reagents were the same as used in the assay of fructosamine. Curves 1 through 4 represented IAM, DMF, NBT and serum, respectively.

The slope k_1 represented changes in absorbance of DMF per mmol/l (data not shown). The slope k_2 represented changes in absorbance percentage of serum. k_2 should be divided by the maximum volume of serum (50 μl) to get the slope of absorbance resulted from 1 μl of serum. The reaction mixture

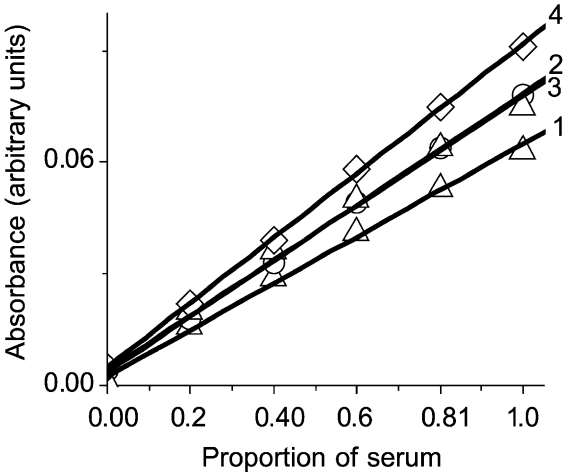

Fig. 3. Absorbance of NBT reacting with serum at different concentrations. Regression lines of the results were obtained at 570 nm with (curve 1) and without IAM (curve 2); those at 530 nm with (curve 3) and without IAM (curve 4).

Table 1
Contrast of the fructosamine concentrations between the normal subjects and diabetics

Subjects	Reaction temperature (°C)	Reaction time (min)	Aver(x) ± s (mmol/l)	Aver(x) ± 2s (mmol/l)	S_x
Normal	35 (109 cases)	15	2.50 ± 0.23	2.50 ± 0.46	0.021
Normal	25 (109 cases)	30	2.35 ± 0.21	2.35 ± 0.42	0.021
Diabetics	35 (60 cases)	15	3.38 ± 0.38	3.38 ± 0.76	0.048
Diabetics	25 (60 cases)	30	3.27 ± 0.35	3.27 ± 0.70	0.048

All data were measured at 570 nm. The reaction was terminated by putting the sample into ice. Distilled water was used as the background measurement. Data were showed in mean ± standard deviation.

contained 1250 μl of the carbonate buffer, and then the serum fructosamine concentration (mmol/l) should be $25k_2/k_1$. At the same time, protein fructosamine was about fivefold more actively reducing than equimolar concentrations of DMF [10]. The concentration of glycoserum was expressed by the following equation:

$$\text{serum fructosamine (mmol/l)} = 5k_2/k_1 \quad (1)$$

All fructosamine-reacted data were obtained by measuring the absorbance at 570 nm (Table 1). When the fructosamine assay was performed at 25 °C for 30 min, the glycoserum concentration of normal subjects was 2.35 ± 0.21 mmol/l (n = 109), and one of the subjects was falsely positive. The fructosamine concentration among diabetic subjects was 3.27 ± 0.35 mmol/l (n = 60), and there were 3 were false negatives. We had also performed the NBT assays at different temperatures for different times (Table 2). Glycoserum concentration was 2.56 ± 0.61 mmol/l (1.68–3.07 mmol/l) in normal subjects and 3.71 ± 0.85 mmol/l (2.49–4.88 mmol/l) in diabetic subjects when the reaction was carried out at 35 °C for 15 min. Glycoserum concentration in 5 subjects of 109 normal subjects was >3.0 mmol/l and that in 4 subjects of 60 diabetics was <2.40 mmol/l. Glycoserum concentration between normal group and diabetic group exhibited significant discrimination by t-test (P < 0.001). It suggested that this improved fructosamine assay with IAM could be conveniently used as a fructosamine test.

Table 3
The mean concentration of fructosamine from the uricase- and Triton-X-treated and untreated sera

Group	Mean	Variance	P
With uricase	3.79	2.20	
Without uricase	3.87	1.71	NS
With Triton-X	3.24	1.35	
Without Triton-X	3.21	1.25	NS

Fasting blood samples were taken from 11 (uricase study) and 12 (Triton-X study) random subjects.
Uricase and detergent were used in the fructosamine assay as described by Siedel et al. [8] and Kruse-Jarres et al. [9].

3.3. Assay in the presence of uric acid and lipids

To determine whether urate influences the NBT reaction or not, the following procedures were performed: (1) uricase (4 U/ml) was added to the serum and kept at 25 °C for 1 h before the assay was carried out. Then, the sera (fasting blood from 11 subjects) were diluted with PBS buffer at different proportions (20%, 40%, 60%, 80% and 100%). The results were obtained on the basis of Eq. (1), as shown in Table 3. The serum untreated with uricase was used as a control. The results were analyzed by the t-test, indicating that there was no significant difference

Table 2
The fructosamine concentrations from the normal subjects and diabetics at different times and temperatures

Temperature of reaction (°C)	33		35		37	
Reaction time (min)	15	25	15	25	15	25
Normal subjects (mmol/l)	2.64 ± 0.44	2.91 ± 0.53	2.56 ± 0.61	2.85 ± 0.70	2.61 ± 0.40	2.84 ± 0.46
Diabetics (mmol/l)	3.28 ± 0.57	3.48 ± 0.57	3.71 ± 0.85	4.61 ± 0.98	4.97 ± 1.10	5.07 ± 1.67

All data were measured at 570 nm. Reaction was terminated by putting the sample into ice. Distilled water was used as the background measurement. Data were showed in mean ± standard deviation.

between the uricase-treated and uricase-untreated sera ($t < t_{(10)}$ 0.05). It was suggested that urate in the serum did not affect the NBT assay in the presence of IAM.

Previous reports have shown that lipemia is another interferent to the fructosamine assay [8,9]. Here, we incubated the serum with Triton X-100 (1.7 and 30 mmol/l, respectively) at room temperature for 1 h before assaying with NBT. Different specimens from fasting blood of 12 subjects were assayed. Although the results were significantly different ($t > t_{(11)}$ 0.01) when the detergent concentration was as high as 30 mmol/l, Triton X-100 slightly increased the fructosamine concentration (difference) by ~ 6% on average (data not shown). Comparison of the data in Tables 1 and 2, the average difference was less than the standard deviation of the fructosamine concentrations when the lipemia was normal. However, when the sera were treated by 1.70 mmol/l of the detergent, there were not any significant differences, since triglycerides are the main lipid component in blood with a normal range of 0.56–1.70 mmol/l (World Health Organization: Manual of Basic Techniques for a Health Laboratory, Geneva, 1980). Similar to those reported by Johnson et al. [2], 1.7 mmol/l of Triton X-100 did not disturb the reaction (Table 3), suggesting that the fructosamine assay was not interfered by a normal triglyceride concentration. On the other hand, similar to the results as describe by Johnson et al. [2], hemoglobinemic sera could interfere (data not shown) because of the glycated hemoglobin released from red blood cells.

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