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Glycation of fibrinogen in diabetic patients: a practical colorimetric assay

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We have developed a practical method for the measurement of glycated fibrinogen which combines purification by glycine precipitation with a nitroblue tetrazolium assay at 56°C in the presence of Zwittergent 3–14. This detergent, at a concentration of 10 g/l, was chosen because it combines solubilization of fibrinogen and low coloration of blanks while increasing the sensitivity of the assay. A positive correlation ($r = 0.85$, $P < 0.02$) was found between this procedure and a previously reported one based on the thiobarbituric acid assay. To validate the method we then measured fibrinogen, glycated fibrinogen, serum fructosamine and erythrocyte glycated haemoglobin in a population of healthy euglycaemic subjects ($n = 30$) and a population of diabetic patients ($n = 40$). Glycated fibrinogen was significantly higher in diabetic patients than in control subjects (15.42 ± 0.70 *vs* 11.52 ± 0.27 nmol of deoxymorpholinofructose equiv. per mg, $P < 0.005$). Given the short half-life of fibrinogen, assay of its glycation may be useful as a short-term marker of glycaemic control.

Keywords: detergents, diabetes mellitus, fructosamine, HbA1c, non-enzymatic glycosylation

Introduction

Assay of glycated blood proteins has emerged as an indispensable aid in the management of glycaemic control in diabetic patients and has been the object of excellent and extensive reviews [1–6]. Glycated fibrinogen levels are higher in diabetic patients [7, 7bis] and change rapidly under either deteriorating or improving metabolic conditions [8]. *In vivo* and *in vitro* studies provide evidence for functional changes in the fibrinogen (Fb) molecule as a consequence of glycation that could favour hypercoagulability [9–11]. We describe in this paper an alternative method for the measurement of glycated Fb in plasma. It combines purification by glycine precipitation with a nitroblue tetrazolium (NBT) assay in the presence of an ionic detergent. To validate the procedure, we present evidence that the results obtained correlate with those obtained using a previously reported method [7], and we also compare glycated Fb with glycaemia, fructosamine and HbA1c in populations of healthy euglycaemic subjects and a population of diabetic patients.

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Materials and methods

Apparatus

Spectrophotometric measurements were made in a Shimadzu UV-150-02 double-beam spectrophotometer (Roucaire F-78143, Véllizy, France). Centrifugations were performed in a E 96 refrigerated centrifuge from Jouan (F-44805 Saint-Herblain, France). Electrophoresis equipment was from Pharmacia Fine Chemicals (F-78051 St.-Quentin-en-Ivelines, France). HbA1c was measured in a Diamat (Bio-Rad, F-94200 Ivry). Fructosamine

was analysed in a Hitachi 704 (Boehringer, F-38240 Meylan).

Reagents

To perform immunoelectrophoresis we used anti-total plasma proteins and anti-fibrinogen antisera from Hyland. Zwittergent 3–14 was purchased from Calbiochem (F-92190 Maudon, France). Polyacrylamide gels Excel SDS Gradient 8–18 were from Pharmacia. Other chemical products were analytical grade purchased from Sigma Chimie (F-38297 La Verpillière, France).

Specimens

Samples were obtained from the Laboratoire de Biochimie, Clinique Médicale B, CHU Strasbourg, France. Blood from fasted, euglycaemic healthy subjects and from type I and type II diabetic patients, whose informed consent had been previously obtained, was withdrawn into EDTA-containing tubes and centrifuged at 800 ***g*** and at 4°C for 15 min. Diabetic patients from the Clinique Médicale B attended for either primary diagnosis of diabetes or its subsequent management. Diabetes was diagnosed according to the criteria of the National Diabetes Data Group [12]: fasting plasma glucose before treatment at two different days was above 7.8 mmol/l. Plasma was analysed on the same day or stored at −20°C for no longer than 2 weeks. Glycated Fb was measured in 64 diabetic patients (36 men, 28 women) and 30 control non-diabetic subjects (12 men, 18 women). Glycaemia, erythrocyte glycated haemoglobin (HbA1c) serum fructosamine and plasma Fb concentration were also measured in all the subjects.

Glycated fibrinogen analysis

Working solutions. The following working solutions were used: 2.68 mol/l glycine and 0.2 mol/l Na_2CO_3–$NaHCO_3$ buffer containing 10 g/l Zwittergent 3–14, pH 10.35, at 37°C (carbonate buffer).

The reagent for the NBT assay was prepared as follows. Stock solution A was 1 mmol/l NBT, 0.2 mol/l Na_2CO_3–$NaHCO_3$, pH 10.35, at 37°C. This solution must be kept in the dark and can be stored for 2 weeks at 4°C. Stock solution B was 0.2 mol/l Na_2CO_3–$NaHCO_3$ buffer containing 20 g/l Zwittergent 3–14, pH 10.35, at 37°C. This solution is stable and can be stored for at least 4 weeks at 4°C. The reagent consists of equal parts of solution A and B and should be mixed immediately before use to avoid the slight coloration that occurs in complete reagent upon storage at 4°C. Final concentrations are 0.5 mmol/l NBT, 0.2 mol/l Na_2CO_3–$NaHCO_3$, 10 g/l Zwittergent 3–14.

Fibrinogen purification. Fb was purified according to the procedure previously described [8]. Briefly, 250 µl of plasma was added to 900 µl of 2.68 mol/l glycine, then the mixture was vortexed and allowed to stand for 5 min at room temperature. After 10 min centrifugation at 1500 ***g***, the supernatant was carefully removed and the surface of the pellet and walls of the tube were washed gently with 2 ml of distilled water. Next, the precipitate was resuspended in 900 µl of 2.68 mol/l glycine and the precipitation and centrifugation steps were repeated. The final precipitate was redissolved in 1 ml of the carbonate buffer. Purified Fb was quantified by measuring absorbance at 280 nm [9]; 200 µl of Fb preparations was diluted to 1 ml with the carbonate buffer and read against a buffer blank. The absorbance coefficient (A, 1%/1 cm) employed was 15.5 [9].

NBT assay. Fb from control and diabetic patients samples was assayed by a modification of the method of Baker *et al.* [13]. Briefly, to 500 µl of complete NBT reagent, 400 µl of standards or samples containing 0.50 mg/ml Fb was added and the mixture was incubated at 56°C. Absorbance at 530 nm against a reagent blank was measured after 20 min incubation. Standard was freshly prepared deoxymorpholinofructose (DOMF) diluted in the carbonate buffer. The degree of glycation was correlated for the protein concentration and glycated Fb was then expressed as nmol of DOMF equivalents per mg of protein.

Comparison with an existing method

Glycation of purified Fb as measured by the proposed method was compared with a previously published procedure based on specific liberation of 5-hydroxymethylfurfural (HMF) by glycated proteins and its reaction with thiobarbituric acid (TBA) [7]. Briefly, after glycine precipitation, Fb was resuspended in 100 mmol/l sodium phosphate buffer pH 6.4. Fb (1–2 mg) was then hydrolysed in 0.6 mol/l oxalic acid (final concentration) for 1 h at 120°C and 202 kPa. Next, samples (1 ml) were cooled on ice and protein was precipitated by addition of 0.24 ml of 3.67 mol/l trichloroacetic acid. After 20 min centrifugation at 2000 ***g*** and 4°C, 0.9 ml of supernatant was transferred to another tube and reacted with 0.3 ml of 0.05 mol/l TBA for 15 min at 37°C and 20 min at room

temperature. A standard plot was run in parallel using fructose (which also yields HMF upon mild oxalic acid hydrolysis) in concentrations ranging from 0 to 100 μmol/l. Absorbance was read at 443 nm against a reagent blank. Purified Fb from 10 individual serum samples was analysed in triplicate.

In vitro *glycation and recovery of glycated fibrinogen in precipitates*

To rule out a direct effect of glycation on Fb recovery in glycine precipitates, we ran the following experiment: pooled EDTA plasma from control subjects was made up to 1 mmol/l phenyl methyl sulphonyl fluoride and 1 mmol/l NaN_3 and incubated in the absence (control plasma) or in the presence (glycated plasma) of 100 mmol/l glucose for 4 days at 37°C. Immediately prior to Fb purification 100 mmol/l glucose was added to the control samples to compensate for any interference of glucose in the subsequent steps. Three 250-μl aliquots of incubated control plasma as well as three 250-μl aliquots of incubated glycated plasma were used for the purification procedure. Recovery of protein was measured in this way for control and *in vitro* glycated Fb. Samples were also analysed for glycation by both our method and the TBA assay [7].

HbA1c was measured by ion-exchange high-performance liquid chromatography (HPLC), with a Diamat [14].

Plasma fructosamine was measured according to the method of Baker *et al.* [13].

Glycaemia was measured by the glucose oxidase–peroxidase method [15].

Statistics

Results are presented as mean ± SEM. Data were compared using the Mann–Whitney test. Correlations were performed using either Spearman's correlation or linear regression as appropriate. Statistics were calculated using StatWorks from Cricket Software (Philadelphia, PA, USA).

Results

Choice of reaction conditions

Detergent. Addition of a detergent to the NBT reagent increases the sensitivity of the classical NBT reaction, thus allowing its use with samples containing low protein concentrations. Detergents have, on the other hand, the disadvantage of producing high coloration of blanks. In this regard, we first studied the reaction conditions with several detergents using DOMF, a synthetic fructosamine. Figure 1a shows absorbance data for a blank and a standard (10 μmol/l DOMF) when the reagent contained either no detergent or 10 g/l of different detergents, and Figure 1b shows absorbance of blank and standard as a function of Zwittergent 3–14 concentration. Triton X-100, Tween 20 and Brij were discarded because of the high coloration of blanks they produce. Nonidet P-40 and Zwittergent 3–14 at a concentration of 10 g/l show similar behaviour to the DOMF standard as shown in Figure 1a, but fibrinogen solutions in Nonidet P-40 cause problems upon incubation at 56°C. Hence, Zwittergent 3–14 was chosen as the most appropriate for our purposes; it solubilizes fibrinogen, accelerates the reaction and produces low coloration of blanks and no turbidity.

Time and temperature. Because only low amounts of pure Fb can be isolated from 250 μl of plasma, good absorbance values require incubation for 2 h at 37°C, when the reaction reaches a plateau (data not shown). To reduce the assay time we investigate the course of the reaction at 56°C (Figure 1c). The incubation time at 56°C can be shortened to 20 min. To rule out artifacts that could appear at 56°C in a reaction originally characterized at 37°C, we investigated the absorbances of 20 purified Fb samples incubated at either 37°C or 56°C. Figure 1d shows that this correlation is excellent ($r = 0.990$, $P < 0.001$).

Glycated Fb assay

Purity of the isolated Fb was confirmed by denaturing sodium dodecyl sulphate (SDS)–polyacrylamide gradient electrophoresis, in which only the three bands of Fb chains could be detected, and by immunoelectrophoresis, in which Fb showed only one precipitation band with either anti-total human plasma proteins antibodies or anti-Fb antibodies. The modification of the NBT assay we describe is useful for the microassay of the glycation of purified protein. It has a detection limit (determined as three times the standard deviation of the non-glycated Fb control, obtained by sodium borohydride reduction) of 2 nmol DOMF equivalents per mg of protein. Linearity was between 2 and 30 nmol DOMF equivalents per mg of protein. The intra-assay CV was 5.1% and 4.7%

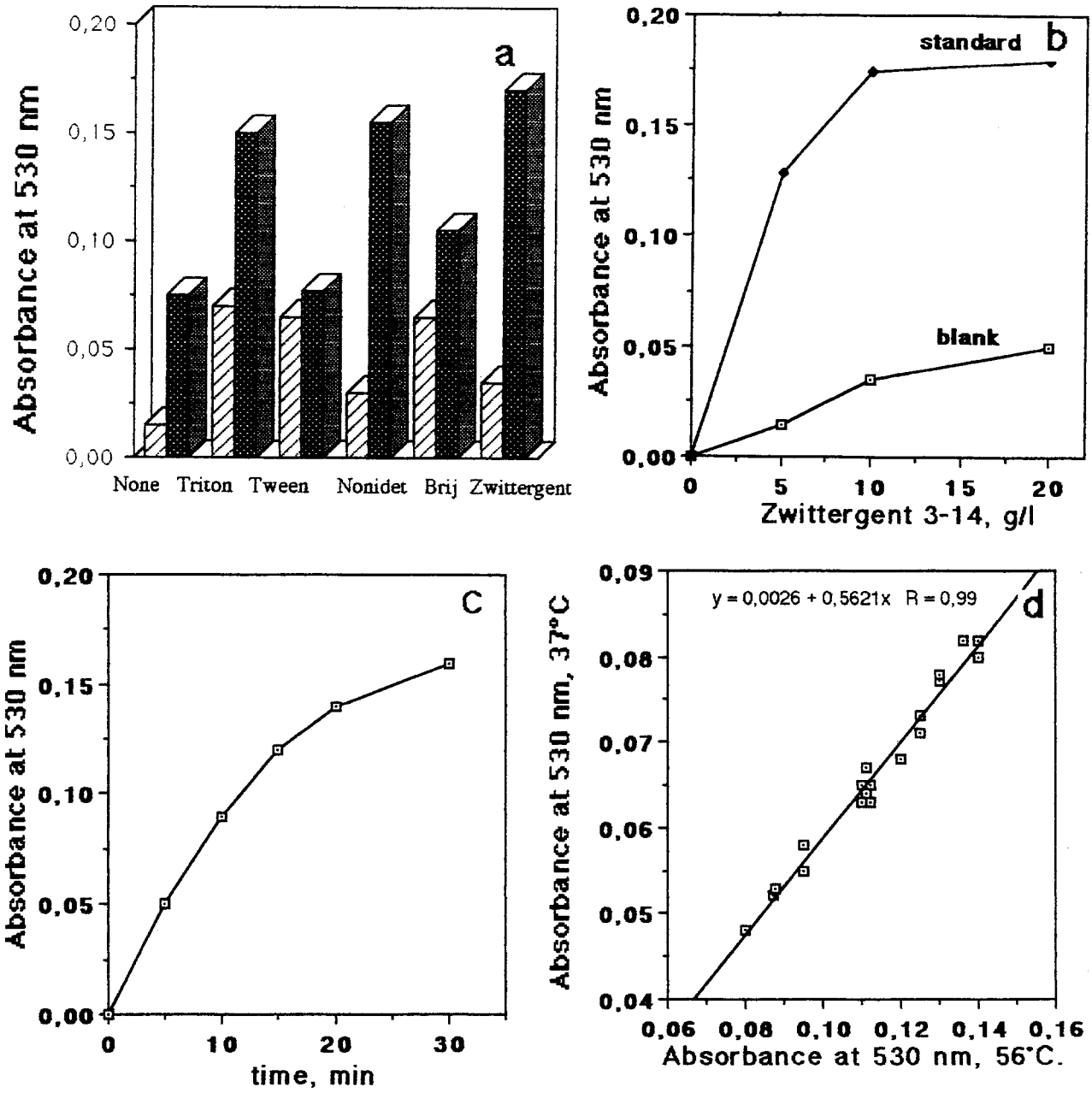

Figure 1. Choice of detergent and reaction conditions for the measurement of glycation on purified fibrinogen by the nitroblue tetrazolium assay. (a) Absorbance of blank and standard after 20 min incubation at 56°C with NBT reagent in the absence (none) or in the presence of 10 g/l of different detergents (Triton X-100, Tween 20, Nonidet P-40, Brij, Zwittergent 3–14; standard 10 μmol/l DOMF). ▨, blank; ▩, standard. (b) Absorbance of blank and standard after 20 min incubation at 56°C with NBT reagent containing increasing concentrations of Zwittergent 3–14. (c) Time course of the reaction at 56°C of purified fibrinogen (200 μg) with NBT reagent containing 10 g/l Zwittergent 3–14. Glycated fibrinogen contained 20 nmol DOMF equivalents per mg of protein. (d) Correlation of absorbances of 20 purified fibrinogen samples incubated for either 2 h at 37°C or 20 min at 56°C with NBT reagent containing 10 g/l Zwittergent 3–14.

for levels of 10.0 and 20.0 nmol DOMF equivalents per mg of protein respectively ($n = 30$). Inter-assay CV was 7.6% for levels of 15 nmol DOMF equivalents per mg of protein ($n = 30$).

Correlation with the thiobarbituric acid assay

A positive correlation ($r = 0.85$, $P < 0.02$, $n = 15$) was found between the values of glycated Fb obtained by our method and a previously reported

TBA assay [7]. The range of glycated Fb values explored was between 10.2 and 16.1 nmol DOMF equivalents per mg of protein.

Recovery of in vitro *glycated Fb*

Plasma from control subjects was incubated in the presence (glycated) or in the absence (control) of 100 mmol/l glucose for 4 days at 37°C. Recovery of protein in the final precipitate was the same for either control (1.82 ± 0.09 mg/ml, $n = 6$) or glycated (1.83 ± 0.16 mg/ml, $n = 6$) samples. Control Fb showed glycation rates of 11.9 ± 0.07 nmol DOMF equiv./mg against 23.7 ± 0.11 nmol DOMF equiv./mg for *in vitro* glycated Fb ($P < 0.001$, $n = 6$).

After *in vitro* incubation in the above-mentioned conditions, the glycation of Fb was nearly 100% higher than that of the control, and even this strong modification did not change Fb recovery.

Glycated Fb rates in non-diabetic subjects and in diabetic patients

Table 1 shows the levels of glycaemia, glycated Fb, fructosamine and HbA1c in diabetic patients and in control subjects. Glycated Fb was significantly higher in diabetic patients than in control subjects. The mean increment was 34% while fructosamine rates showed an 18% increase. The reference range for glycated Fb was from 8.82 to 14.22 nmol DOMF equiv./mg (mean ± SD). The range of values found in diabetic patients was from 9.52 to 20.32 nmol DOMF equiv./mg. Total Fb levels showed a tendency to be higher in diabetic patients, but the difference found was without statistical significance. A weak positive Spearman's correlation between glycated Fb and HbA1c was demonstrated ($r_s = 0.33$, $P < 0.05$). For the same patient, the correlation was slightly better when a shorter marker of diabetic control was considered: fructosamine values versus glycated Fb showed an r_s of 0.47 ($P < 0.05$).

Discussion

In this work we set out to study fibrinogen glycation in diabetic patients by introducing a new and convenient assay. As we currently use the nitroblue tetrazolium (NBT) assay for the measurement of glycation of other purified proteins [16, 17], we decided to combine the purification procedure previously described for fibrinogen [8] with a colorimetric NBT assay in the presence of detergents to enhance the reaction. This method has several advantages over those previously reported. In one previous method, glycation was measured on urokinase digests of precipitated fibrin by a colorimetric assay [18]. In another approach Fb is precipitated by glycine, and either assayed with TBA [7] or the glycated Fb is separated by affinity chromatography on aminophenylboronate agarose (PBA), the protein in the eluates being measured by a Coomassie blue colorimetric assay [8]. The method is a long procedure, which has the problem of imprecision associated with PBA chromatography, and requires an appropriate column recycling method. Measurement of HMF liberated from glycated Fb by hydrolysis with oxalic acid also has the problem of long, cumbersome manipulations and the need to do triplicates in order to obtain adequate precision.

As previously reported [8] Fb is extracted free of contaminants by two glycine precipitations. The sensitivity of this reaction can be greatly enhanced by addition of detergents. Zwittergent 3–14 at a concentration of 10 g/l was chosen because it solubilizes Fb and produces low coloration of blanks and sufficient enhancement of the reaction. In

Table 1. Glycated fibrinogen and other parameters of glycaemic control in diabetic patients and control subjects

	Fasting plasma glucose (mmol/l)	Plasma glycated fibrinogen (nmol DOMF equiv./mg)	Serum fructosamine (mmol/l)	HbAlc (%)	Plasma fibrinogen (g/l)
Control subjects ($n = 30$)	5.17 ± 0.21	11.52 ± 0.27	2.41 ± 0.22	5.15 ± 0.06	2.54 ± 0.17
Diabetic patients ($n = 64$)	8.28 ± 0.91[a]	15.42 ± 0.70[a]	2.86 ± 0.52[b]	9.83 ± 0.23[b]	2.71 ± 0.18

[a] $P < 0.005$ *vs* control subjects
[b] $P < 0.001$ *vs* control subjects
} Mann–Whitney test.

All values are shown as mean ± SEM.

order to shorten the reaction time and therefore the practicability of the test we introduced an incubation at 56°C instead of 37°C.

In the first report demonstrating increased levels of glycated Fb in diabetic patients, the authors measured HMF released by mild hydrolysis of purified protein [8]. We found a positive correlation between this method (which has an intra-assay CV higher than 10%) and our current procedure.

During the process of Fb purification, potential interferences with the colorimetric NBT reaction (*i.e.* uric acid, bilirubin, glucose, etc.) were eliminated, thus allowing longer incubation times and higher temperatures to be employed. This permitted a decrease in the detection limit of the method and its application to low protein concentrations. Our results showed that in diabetic patients glycated Fb levels are up to 34% higher than in control subjects. These data confirmed those previously reported in the literature by different approaches [7, 8, 18].

To confirm that highly glycated Fb did not remain in solution during the purification step, we performed *in vitro* experiments as follows: pooled plasma from healthy subjects was incubated in the presence (*in vitro* glycated) or in the absence (control) of 100 mmol/l glucose. Recovery of Fb after the purification steps was the same for either the control or the *in vitro* glycated samples. Glycation of Fb was nearly 100% higher in the latter samples. We calculated (data not shown) that 3.5 mol of HMF was liberated per mol of Fb, which means that a mean of 3.5 glucose molecules had been incorporated into each Fb molecule. This is in agreement with the data shown by Ney *et al.* [19], in which Fb was glycated *in vitro* in the presence of [^{3}H]glucose.

There was a low positive correlation between glycated Fb and glycated haemoglobin, which reflects the different information provided by both parameters and the difference in half-life. When fructosamine values, which reflect glycaemic control over a period of 2 or 3 weeks, were compared with glycated Fb, this correlation was better.

Although the first reports on glycated Fb failed to show any effect on the function of the protein [19, 20], more recent work suggests that platelet and clotting abnormalities in diabetics might be linked with modifications in the function of Fb produced by glycation [9–11].

As shown by Hammer *et al.* [8], glycated Fb levels respond rapidly to changes in metabolic control. Our data, however, show overlap between control and diabetic values, suggesting that our method may only be useful in longitudinal studies. The level of glycated Fb could also be affected by infection or inflammation because Fb is an acute-phase reactant. We chose to express Fb glycation as a ratio and we always used the same amount of Fb for reaction with NBT (200 mg). In this way results are less affected by variations in total Fb concentration. Nevertheless, if acute infection exists, results should be interpreted in the light of total Fb concentration.

The method we propose provides a simple procedure for the evaluation of the levels of glycated Fb in small plasma samples. Its clinical usefulness as a short-term marker of glycaemic control requires further longitudinal studies. On the other hand, it could be useful in studies attempting to correlate coagulation disorders in diabetic patients with the degree of Fb glycation.

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