Journal of Chromatography, 419 (1987) 75–83
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3713

MEASUREMENT OF GLYCATED ALBUMIN IN DIABETIC PATIENTS BY BIOSPECIFIC AFFINITY CHROMATOGRAPHY

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(First received December 19th, 1986; revised manuscript received March 19th, 1987)

SUMMARY

The percentage of glycated plasma albumin was measured by a procedure involving ammonium sulphate precipitation and Affi-Gel-Blue and phenylboronate chromatographies. The value correlates well with the amount of ketoamine-bound sugars determined by colorimetric assay ($r=0.98$, $n=39$). The normal mean value is $3.9 \pm 0.3\%$ (mean $\pm$ S.D., coefficient of variation $= 7.7\%$, $n=32$) and varies from 3.9 to 21% in diabetics ($n=54$). A good correlation is found with the mean blood glucose value of the preceding twenty days ($r=0.92$, $n=57$). Because of its relative ease of determination, glycated albumin constitutes a good short-term glycemic index and an alternative to glycated haemoglobin in some specific cases.

INTRODUCTION

The level of glycated ("glycosylated") haemoglobin is now routinely used to monitor the quality of blood glucose control in diabetic patients [1,2]. Because it is related to the haemoglobin life-span, it constitutes a long-term index (two months) that precludes the monitoring of short-term effects of a new therapy. Circumstances in which the lifespan of red blood cells is shortened (blood loss or haemolytic anaemia) are obvious interfering factors. Furthermore, haemoglobinopathies may create technological pitfalls in its determination [3].

Measurement of the glycated fraction of total plasma proteins or albumin might be an alternative, particularly when a short-term index of blood glucose control is sought. A colorimetric assay, measuring the hydroxymethylfurfural sugar derivatives liberated by acid hydrolysis of total plasma proteins [4] or albumin

0378-4347/87/$03.50

[5,6], is used most often. However, it is technically difficult and not very suitable for routine measurement. Plasma fructosamine, the rearranged form of protein-bound glucose, can also be determined [7,8]. Finally, phenylboronate affinity chromatography has been used to measure the percentage of glycated plasma proteins [9,10], and determination of albumin in the chromatographic fractions with a bromocresol green reagent has been reported [11]. Because plasma proteins are highly heterogeneous, with different half-lives and variable respective concentrations, it is obviously desirable to monitor the glycated fraction of a specific protein, such as albumin, rather than that of total plasma proteins.

Because of the controversial values reported in the literature [6,11] for glycated albumin and because the bromocresol green reaction is insensitive, we have devised a procedure to determine the percentage of glycated albumin by combining two biospecific affinity chromatographic steps, albumin purification on Affi-Gel-Blue and direct measurement of its glycated fraction on phenylboronate–agarose, respectively. The technical and clinical reliability of this approach to develop a short-term index of blood glucose control in diabetic patients has been ascertained.

EXPERIMENTAL

Materials

Affi-Gel-Blue (50–100 mesh), acrylamide and bisacrylamide were purchased from Bio-Rad (Richmond, CA, U.S.A.). Glycogel B kit was obtained from Pierce (Rockford, IL, U.S.A.). Thiobarbituric acid and ammonium sulphate were obtained from Merck (Darmstadt, F.R.G.). The Haemo-glukotest 20.800 R and the GOD/PAP glucose oxidase kit were supplied by Boehringer (Mannheim, F.R.G.). Cellulose acetate electrophoresis plates were purchased from Helena Labs. (Beaumont, TX, U.S.A.). All other reagents were of analytical grade. Absorbance was determined with a Model JY 201 spectrophotometer (Jobin et Yvon, Longjumeau, France) or a Model UV 111 LC detector (Gilson, Villiers le Bel, France).

Plasma samples

Samples were obtained from 32 healthy non-diabetic volunteers with normal fasting plasma glucose and normal glycated haemoglobin levels (Hb Alc, measured as in ref. 12) and from 57 patients with clinically established diabetes mellitus. Venous blood was collected on EDTA, and plasma was stored at −20°C until analysis. Samples from patients with various plasma protein electrophoretic pattern abnormalities were also included in the study.

Albumin purification

Solid ammonium sulphate (624 mg) was added to 2 ml of plasma (50% final saturation). The mixture was agitated (30 min at 4°C) and centrifuged (40 min at 10 000 *g*). A 2-ml volume of supernatant was dialysed overnight against 0.02 *M* sodium phosphate (pH 7.1) then applied to an Affi-Gel-Blue minicolumn (2 ml) equilibrated in the same buffer. The unbound material was washed off with

15 ml of this buffer. The bound albumin fraction was then eluted with 8 ml of buffer brought to 1.4 *M* sodium chloride. Before subsequent use, the columns were washed with 5 ml of 8 *M* urea, re-equilibrated with 15 ml of the starting phosphate buffer and stored at 4°C.

Glycated albumin assessment

Biospecific affinity chromatography. Albumin preparations were dialysed against a 0.25 *M* ammonium acetate, 0.05 *M* magnesium chloride buffer (pH 8.5). Aliquots of 5 ml (i.e. 20 mg of proteins), previously centrifuged to remove any particulate material, were applied on Glycogel B minicolumns (1 ml) at 4°C. The unbound fraction was eluted with 10 ml of the ammonium acetate buffer. The bound fraction (glycated albumin) was then eluted with 3 ml of a 0.25 *M* sodium citrate buffer (pH 4.6). Its percentage was determined by measuring the absorbance of each fraction at 280 nm. Before subsequent use, the columns were washed with 5 ml of 0.1 *M* hydrochloric acid, then 5 ml of 0.001 *M* hydrochloric acid and finally re-equilibrated with 15 ml of the ammonium acetate–magnesium chloride starting buffer. Unused columns were stored at 4°C.

Colorimetric assay. Albumin-bound sugars were measured according to the modified technique of Keeney and Bassette [13]. After dialysis against 0.9% sodium chloride, 3 ml of the albumin preparation (12–18 mg of protein) were treated with 0.5 ml of 15 *M* phosphoric acid at 100°C for 4 h. Digests were cooled in ice and proteins precipitated by adding 1 ml of trichloroacetic acid (400 g/l). Then 1 ml of the supernatant containing the 5-hydroxymethylfurfural (HMF) derivatives was incubated with 1 ml of 0.03 *M* thiobarbituric acid at 40°C for 30 min. Absorbance was measured at 443 nm. Results were expressed as nmol HMF per mg of albumin. Albumin concentration was calculated using an $A_{280}=5.5\%$ as the molar absorptivity.

Protein electrophoresis

Electrophoresis was performed both on cellulose acetate plates using a Tris–barbital–sodium barbital buffer (pH 8.6) and on polyacrylamide gels in the presence of sodium dodecylsulphate (SDS), using a Tris–glycine buffer, (pH 8.6). Plates were stained with Ponceau S and gels with Coomassie Blue R. Quantitation of the protein fractions was obtained by densitometric scanning on a Sebia Cellosystem (Issy les Moulineaux, France).

Blood glucose

For each diabetic patient ($n=47$), a mean arteriolocapillary blood glucose value was calculated from 46–120 fasting and post-prandial determinations (Haemoglukotest 20.800 R) for the preceding twenty days. For the extremely unstable patients ($n=7$), a mean value was calculated from six to fifteen venous blood glucose concentrations (GOD/PAP glucose oxidase kit).

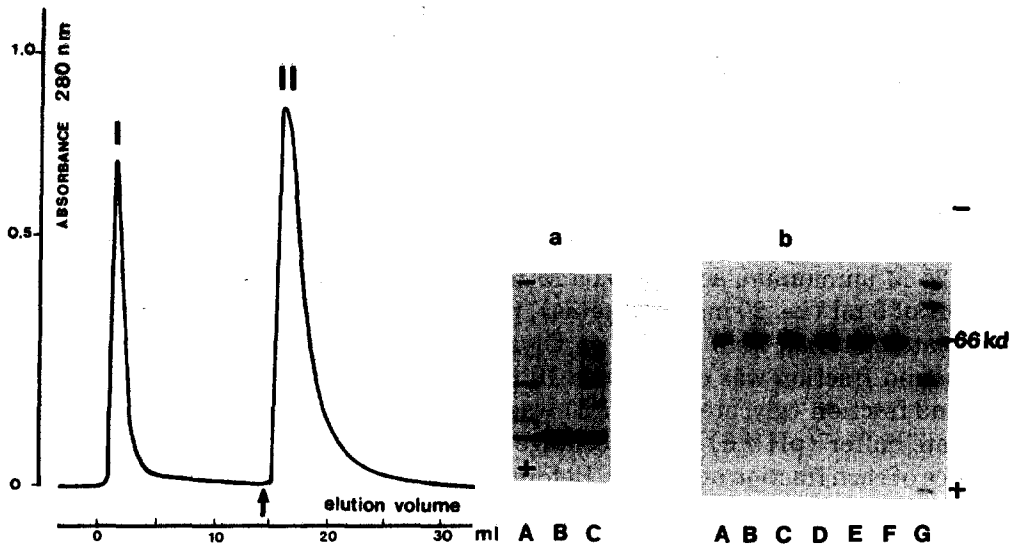

Fig. 1. Chromatography of human plasma on Affi-Gel-Blue. Absorbance was measured at 280 nm. The arrow indicates the change of initial buffer from 0.02 *M* sodium phosphate (pH 7.1) to 1.4 *M* sodium chloride.

Fig. 2. Electrophoresis of the Affi-Gel-Blue fractions. (a) Cellulose acetate electrophoresis of (A) fraction I, (B) fraction II, (C) normal plasma; (b) SDS polyacrylamide gel electrophoresis (fraction II) of albumin preparations from normal and pathological sera: (A) normal sample, (B) polyclonal gammopathy, (C) monoclonal gammopathy, (D) hepatic cirrhosis, (E) nephrotic syndrome, (F) acute phase reaction, (G) molecular weight markers.

Statistical analysis

Least-squares analysis of the correlation curves, mean values, standard deviation (S.D.) and coefficient of variation (C.V.) were calculated by classical methods.

RESULTS

Albumin purification

Direct purification of albumin from plasma by Affi-Gel-Blue chromatography gave a degree of purity of 80% only. Therefore, we have introduced a 50% ammonium sulphate saturation cut. After this first step, albumin represented 85% of the proteins in the plasma supernatant. Fig. 1 shows the chromatographic profile of this fraction on Affi-Gel-Blue. Absorbance was first monitored continuously at 280 nm, and the eluate was fractionated to determine the exact volumes needed to obtain a standardized and reproducible separation between the unbound (I) and bound (II) fractions. Analysed by cellulose acetate electrophoresis, fraction I contained globulins and less than 9% of albumin, whereas fraction II was composed of more than 97% albumin (Fig. 2a). The procedure was applied to sera presenting with an abnormal electrophoretic profile and/or albumin content (nephrotic syndrome, acute and chronic inflammation, mono- and polyclonal

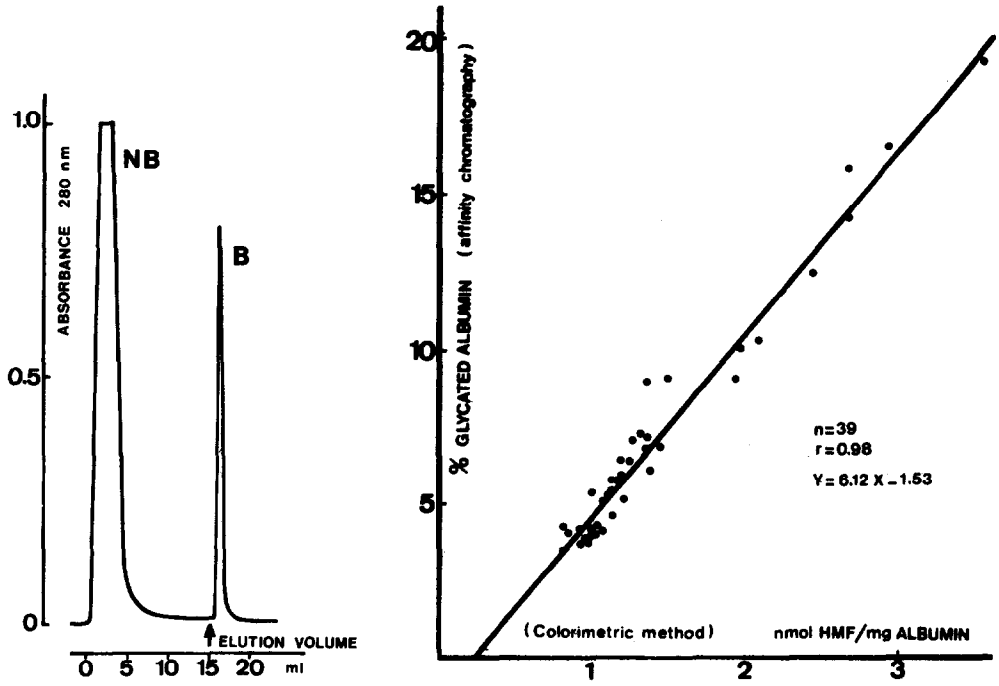

Fig. 3. Chromatography of purified albumin on phenylboronate-agarose. Absorbance was measured at 280 nm. Sample in ammonium acetate buffer (pH 8.5) was applied to the column equilibrated in the same buffer. The NB fraction was eluted with 15 ml of ammonium acetate buffer; the arrow indicates the change to a citrate buffer (pH 4.6). Glycated albumin (B) is eluted in 3 ml of this buffer.

Fig. 4. Comparison of the percentage of glycated albumin determined by affinity chromatography on phenylboronate-agarose with the amount of albumin-bound sugars measured by the colorimetric thiobarbituric acid assay.

gammopathies or liver cirrhosis). In all cases, the albumin fraction was more than 97% pure on cellulose acetate plates and presented a single band on SDS polyacrylamide gels (Fig. 2b).

Assessment of glycated albumin

Purified albumin was submitted to phenylboronate affinity chromatography on Glycogel B (Fig. 3). The unbound fraction was entirely eluted by 15 ml of the starting ammonium acetate buffer (pH 8.5). Then 3 ml of citrate buffer (pH 4.6) were sufficient to elute all the bound albumin, even when it amounted to 21% of total albumin. For this last sample, the unbound fraction was collected, concentrated and re-chromatographed, so as to test for an eventual overload of the column. No detectable absorbance at 280 nm was observed in the fraction corresponding to the bound species.

Using this standardized procedure, a value (mean $\pm$ S.D.) of $3.9 \pm 0.3\%$ (C.V. $= 7.7\%$) was found for the percentage of bound albumin fraction in 32 controls with a normal level of Hb Alc and a normal fasting blood glucose. In samples

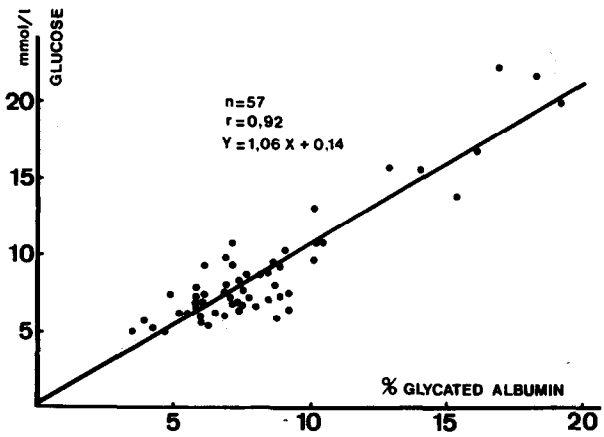

Fig. 5. Correlation between the levels of glycated albumin determined by affinity chromatography and the mean value of blood glucose during the preceding twenty days in diabetic patients.

from 57 diabetic patients, this percentage varied from 3.9 to 21%. The intra-assay C.V. values were 4.2% (mean 3.8%, S.D. = 0.16%, $n = 16$) and 2.1% (mean 10.2%, S.D. = 0.21%, $n = 16$) for the normal controls and diabetic patients, respectively. The inter-assay C.V. was 5.0% (mean 4.2%, S.D. = 0.21%, $n = 11$). The columns can be used up to twenty times without significant variation of the inter-assay C.V.

To appreciate if the percentage of the fraction bound to phenylboronate indeed reflects the degree of non-enzymic glycation of albumin, we compared these values with the amounts of protein-bound sugars measured on the same albumin preparations by using the thiobarbituric acid colorimetric assay. This assay, specific for sugars bound to proteins through ketoamine linkages, is the reference method for non-enzymic glycation. Comparison of the results obtained by the two procedures shows a good correlation ($r = 0.98$) between the two sets of values (Fig. 4).

To explore further the relation between the percentage of albumin bound to phenylboronate and the degree of blood glucose control, the former value was compared with the mean value of fasting and post-prandial glycemias during the preceding three weeks. A good correlation ($r = 0.92$) was indeed observed between these two parameters (Fig. 5).

DISCUSSION

Limits to the usefulness of the determination of glycated haemoglobin in following up diabetic patients have been well documented [3,14]. When the red blood cell lifespan is shortened, the level of glycated haemoglobin does not reflect the quality of blood glucose control. This is the case in haemolytic anaemias of diverse origins, blood loss [14] or when blood deprivation is used therapeutically in haemochromatosis patients. The value of Hb Alc also under-represents global glycation when young red blood cells are elevated after treatment of iron deficiency [15]. Furthermore, the level of glycated haemoglobin reflects the quality

of blood glucose control over a period of six to eight weeks, but imbalances or improvements of the carbohydrate metabolism for a few days only are not perceived. For all these cases, measurement of the extent of non-enzymic glycation of plasma protein has been proposed as a substitute.

Non-enzymic glycation depends both on the lifespan of the considered protein and the mean value of blood glucose during this period. Plasma proteins are composed of highly heterogeneous species of different lifespans. The number of glycation sites varies from one protein to another, and Dolhofer and Wieland [5] have shown that the amounts of HMF derivatives released from albumin or the non-albumin fraction are different. Furthermore, their ratio varies in normal and diabetic subjects. We found a variation from 1.35 to 2.31 in the ratio of the sugars released from total proteins over those liberated from the albumin fraction in our panel of normal and abnormal samples. In addition, the relative concentration of the respective fractions varies in some physiological or pathological conditions. Therefore, in order to be used as a fine index of blood glucose control, the extent of glycation must be determined on a specific protein rather than on total plasma proteins. Albumin, the major plasma protein, is the obvious candidate because it is relatively easy to isolate and because its glycated form has been thoroughly characterized [16].

Ammonium sulphate precipitation and Affi-Gel-Blue chromatography easily and reproducibly generate albumin fractions with a purity higher than 97%, even after ten usages of the columns. Although albumin most often represents 60–70% of total proteins, its level decreases in circumstances such as nephrotic syndrome, liver failure, acute or chronic inflammation and mono- or polyclonal gammopathies. We checked that none of these circumstances affected the results of our procedure. The extent of glycation of this fraction can then be estimated by several approaches. Although the thiobarbituric assay is the reference method, it is demanding, difficult to adapt for routine use and extremely dependent on the experimental conditions [17]. Therefore, there is a great variation in the reported values. We found an excellent correlation between the percentage of the albumin fraction bound to phenylboronate–agarose and the amount of HMF liberated from albumin, indicating that, indeed, this fraction directly reflects the extent of non-enzymic glycation. Dialysis of the albumin fraction before the chromatography most probably eliminates small molecules such as catecholamines, epinephrine and norepinephrine, which bind to phenylboronate–agarose through hydrophobic interactions and shorten the column lifespan.

We found a normal mean value for glycated albumin ($3.9 \pm 0.3\%$) higher than that previously reported by Rendell et al. [11] ($1.64 \pm 0.06\%$). These authors measured glycated albumin by phenylboronate chromatography of total plasma using the bromocresol green reaction. Large protein loads (80 mg) had to be used, hence there was a risk of overloading the column [9].

Besides being reliable, precise, inexpensive and independent of variations in temperature and pH, this technique also has the advantage for its use as a routine clinical test that its results, expressed as percentages, are easily perceived by the clinicians. Expression of results of the colorimetric assay varies greatly, i.e. five authors [4,6,9,18,19] use four different modes of expression. It is, therefore, most

difficult to compare results, routinely, from one laboratory to another, and clinicians are likely to be confused. A similar situation is encountered for the measurement of plasma fructosamine [7,8]. Consequently, phenylboronate affinity chromatography seems an adequate and specific method for monitoring the extent of plasma albumin non-enzymic glycation.

We found an excellent correlation ($r=0.92$) between the percentage of glycated albumin and the mean value of fasting and postprandial glycemias during the twenty days preceding the assay, i.e. over a period corresponding to albumin half-life. These results are in agreement with those of Dolhofer and Wieland [5], who compared the amount of albumin-bound sugars with the mean blood glucose level of the preceding four to five days. They are in contrast, however, to the poor correlation found between glycated plasma proteins measured either by the colorimetric assay [4] or by biospecific affinity chromatography [9] and the level of blood glucose in the sample analysed for glycated fractions. The extent of albumin glycation, therefore, constitutes a good short-term index of the carbohydrate metabolism balance, and can be most precious in follow-up work with diabetic patients when the determination of glycated haemoglobin is impaired by interfering factors. Furthermore, when a new therapy is initiated, the level of glycated albumin could permit its effects to be monitored more accurately and more rapidly than the measurement of glycated haemoglobin. Therefore, if the determination of glycated albumin should not be considered as a systematic replacement of that of glycated haemoglobin, it constitutes a valuable additional tool in selected cases.

ACKNOWLEDGEMENTS

This work was supported by grants from the Faculté de Médecine Xavier Bichat, Université Paris 7, and from the Institut National de la Santé et de la Recherche Médicale (Grant No. 845008).

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