Radioimmunoassay for the determination of glycated haemoglobin

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Summary. A competitive radioimmunoassay for the quantitative determination of glycated haemoglobin was developed. The antiserum, obtained by immunizing guinea pigs with reduced glycated human albumin, was capable of identifying and quantitating the glucitolysine residues of glycated Hb after reduction with sodium borohydride. To simplify the sample preparation we introduced trichloroacetic acid precipitation to remove unreacted sodium borohydride instead of using dialysis or gel filtration. Using this procedure, our radioimmunoassay became relatively simple and provided satisfactory within- and between-run (1.3–2.8% and 1.9–5.4% coefficient of variation, respectively). The radioimmunoassay method was compared to the measurement of HbA_{1c} by high performance liquid chromatography which is the most widely used method for quantitating glycated Hb. For this purpose glycated Hb was measured in normal glucose tolerance, impaired glucose tolerance, and diabetes mellitus groups based on WHO criteria. Both assays were able to discriminate between the normal and diabetic

groups. In addition, while the determination of glycated Hb by the radioimmunoassay method was able to clearly discriminate between the normal and impaired glucose tolerance groups, the determination of HbA_{1c} by the high performance liquid chromatography method failed to discriminate between these two groups. Moreover, 15 of the 20 impaired glucose tolerance patients exceeded the upper normal range (mean normal values + 2 SD) in radioimmunoassay. But all 20 patients with impaired glucose tolerance were within the upper normal range in HbA_{1c} values.

These results demonstrate that the measurement of glycated Hb by radioimmunoassay is more sensitive than the measurement of HbA_{1c} by high performance liquid chromatography since it can discriminate between the normal and impaired glucose tolerance groups.

Key words: Radioimmunoassay, glycation, HbA_{1c}, glucose, glucitolysine

Glycated Hb is being used with increasing frequency to monitor long-term blood glucose control in diabetes mellitus [1–5]. The test of glycated Hb provides an accurate index of the mean concentration of blood glucose during the preceding two or three months, complementing more traditional measures of glucose control, such as glucose testing in urine and blood. A variety of methods have been described for the determination of glycated Hb, but many of them have serious pitfalls [6, 7]. The clinical utility of glycated Hb measurement will not achieve its full potential until appropriate references, standards, and degrees of accuracy and precision of these measurements are agreed upon.

HbA_{1c} is identical to HbA₀ except that in HbA_{1c} glucose is linked to the amino-terminal valine residue of the β chain [8]. Modification of Hb with glucose confers a change in charge of the Hb molecule. Most methods for quantitating glycated Hb utilize this charge difference to

separate the HbA_{1c} from HbA₀ by ion-exchange chromatography or high performance liquid chromatography (HPLC) [7]. The amount of HbA_{1c} is then expressed as a percentage of total Hb. Glucose can also condense with the ϵ -amino groups of lysine residues, and the amount of Hb modified at these sites, which is increased in erythrocytes from diabetic patients [9], is not always quantifiable by ion-exchange chromatography or HPLC.

We recently reported an enzyme-linked immunosorbent assay (ELISA) for detecting glycated serum proteins and a radioimmunoassay (RIA) for glycated serum albumin using an antiserum which was obtained by immunizing guinea pigs with reduced glycated human albumin [10, 11]. This antiserum contained antibodies which recognize glucitolysine epitopes in not only reduced glycated albumin and glycated plasma protein but also reduced glycated Hb. In this paper, we developed an RIA for glycated Hb using the antiserum

Fig. 1. Competition of glucitolysine with reduced glycosylated Hb and non-reduced glycosylated Hb which was prepared from a diabetic patient. Antiserum, at final dilution of 1:500, was added in the presence of glucitolysine, reduced glycosylated Hb, or non-reduced glycosylated Hb. Data were expressed as B/B_0 , in which B represents the count of ^{125}I -labelled reduced glycosylated HSA bound in the presence of competitors, and B_0 that bound in the absence of competitor. Glucitolysine (●), reduced glycosylated Hb (○), non-reduced glycosylated Hb (△). nmol glucitolysine added refers to glucitolysine (●). µg glycosylated Hb added refers to reduced (○) and non-reduced (△) glycosylated Hb

and present a comparison of this assay with HbA_{1c} measured by the HPLC method with respect to their discriminative power.

Materials and methods

Reagents

Human serum albumin (HSA), human haemoglobin, CBZ-L-lysine, sodium borohydride (NaBH_4) and bovine gamma globulin (G-5009) were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Bovine serum albumin (BSA, A-6003) was from Calbiochem Behring Co. (La Jolla, Calif., USA). Bio-Rex 70 and Affi-Gel 15 were from Bio-Rad Laboratories (Richmond, Calif., USA). Glyco-Gel B was from Pierce Chemical Co. (Rockford, Ill., USA). The ^{125}I -radioiodination system was from New England Nuclear (Boston, Mass., USA). All other reagents were of the highest grade commercially available.

Preparation of glycosylated HSA, glycosylated Hb and glucitolysine

Reduced glycosylated HSA, reduced glycosylated Hb, non-reduced non-glycosylated HSA and non-reduced non-glycosylated Hb were prepared as described previously [10]. The extent of glycation of each sample was assessed by amino acid analysis as described by Trüeb et al. [12]. The percentage of lysine residue glycation was 62% in reduced glycosylated HSA and 35% in the reduced glycosylated Hb. Glucitolysine was synthesized by the method of Trüeb et al. [12]. Glucitolvaline was synthesized by the method of Schwarz and Gray [13].

Immunization and preparation of antiserum to glucitolysine

Male guinea pigs were immunized with reduced glycosylated HSA as described previously [10]. To remove cross-reactivity with native HSA, 1 ml of the immunized guinea pig serum was purified on an Affi-Gel 15 column coupled to native HSA, eluted with 0.1 mol/l sodium phosphate buffer, pH 8.0, and stored at -70°C .

Radiolabelling of HSA

Reduced glycosylated HSA and native HSA were iodinated by solid-phase lactoperoxidase using radioiodination system [11]. The free iodide was separated from the labelled protein by chromatography on Sephadex G-25, PD 10. The specific activities of the labelled proteins were about 5000 cpm/ng.

Chromatography on Bio-Rex 70

Erythrocyte haemolysates were prepared after overnight 0.9% NaCl incubation as described by Nathan et al. [14]. The haemolysates from a diabetic patient and a normal subject were then analysed on a Bio-Rex 70 column (1×30 cm) according to the method of Abraham et al. [15].

Subjects and sample preparation

Following a 75 g oral glucose tolerance test, subjects were classified as having normal glucose tolerance (N) ($n = 30$, age range 18–76 years), diabetes mellitus ($n = 30$, 36–75 years), and impaired glucose tolerance (IGT) ($n = 20$, 40–76 years) according to WHO criteria [16]. Whole blood was collected in EDTA and centrifuged at 4°C , 1500g, for 10 min. Erythrocytes were washed three times with isotonic saline (0.9%), then diluted 1:20, and incubated overnight at 22°C to remove the labile aldimine fraction. After centrifugation at 4°C , 1500g, for 10 min, the saline supernatant was removed and the erythrocytes were haemolyzed in 3 volume of distilled-deionized water and glycolipids were extracted with 3 volume of toluene. Separation of the haemolysate from toluene, glycolipids, and cell ghosts was accomplished by centrifugation at 1500g for 30 min. An aliquot of each sample was reduced at 37°C for 30 min with 50 mmol/l NaBH_4 . To remove unreacted NaBH_4 and to stop the reduction, glycosylated Hb was precipitated by addition of ice-cold trichloroacetic acid (TCA, 6% final concentration), followed by centrifugation at 4°C , 3000g, for 30 min. The supernatant was discarded by aspiration and the sedimented protein dissolved in 0.05 N NaOH (TCA-NaOH treatment). The pH of this solution was adjusted to pH 7.4 using 0.2 mol/l potassium phosphate-NaOH buffer. Reduced glycosylated Hb, appropriately diluted was also prepared by exhaustive dialysis against phosphate-buffered saline, pH 7.2 after NaBH_4 reduction instead of TCA-NaOH treatment as described above.

Assay of glycosylated Hb

Test samples (100 µl) which were reduced with NaBH_4 and treated with TCA-NaOH as mentioned before were incubated with 200 µl of 0.2 mol/l potassium phosphate-NaOH buffer, pH 7.4 containing 0.25% BSA, 100 µl of antiserum [final dilution 1:500 at 50% inhibition (B/B_0)], and 100 µl of ^{125}I -labelled reduced glycosylated HSA, diluted to about 20,000 cpm, in polystyrene tubes for 24 h at 4°C . After the incubation, bound tracer was precipitated by adding 10 µl (1 mg) of bovine gamma globulin and 500 µl of ice-cold 25% polyethylene glycol 6000 containing 0.1% Tween 20 and centrifuging at 3000 rev/min for 45 min at 4°C . The radioactivity of the precipitate was counted in an LKB 1282 gamma counter. Glucitolysine at 0.005–5 nmol/100 µl was used as the standard. The Hb protein concentrations were determined according to Lowry et al. [18].

Fig. 2. The linear range of the RIA. Reduced glycosylated Hb with a high concentration of glucitolysine was obtained by using an affinity chromatography. A series of six solutions was prepared by diluting the assay buffer. Points and bars are mean $\pm$ SD for four determinations

HbA_{1c} determination by HPLC

Determination of HbA_{1c} was performed using HPLC, AUTO A1C (Kyoto Daiichi Kagaku Co., Kyoto, Japan). The samples for the HPLC were prepared by using 0.9% NaCl incubation to remove the labile aldime fraction as mentioned before. The HPLC column used was the same one during all sample measurements. In this HPLC method, within-run precision (coefficient of variation, CV) was 5.5%, and between-run precision was 7.2%.

Statistical analysis

Statistical significance was analysed by the Student's *t*-test.

Results

Specificity of antiserum

The characterization of the antiserum was studied in a competitive RIA in our previous paper [10, 11]. The antiserum recognized glucitolysine. However, lysine, valine, mannitol and sorbitol were not recognized even when added in a large excess. Glucitolvaline was less than 1% cross-reactive when compared with glucitolysine. Reduced glycosylated Hb which was prepared from a diabetic patient was also recognized, and the shape of the inhibition curve was parallel with glucitolysine (Fig. 1). Non-reduced glycosylated Hb was not recognized even when added in a large excess. The binding of ¹²⁵I-labelled native human albumin and ¹²⁵I-labelled non-reduced glycosylated HSA to the antiserum were less than 1% of the total amount (data not shown). These results indicated that cross-reactivity to native HSA was eliminated from the antiserum preparation and that the dominant epitope recognized by the antiserum was identified as glucitolysine; the reduced hexose alcohol form of glucose conjugated to the ϵ -amino group of lysine. The antiserum was capable of identifying and quantitating glucitolysine residues on Hb after reduction with NaBH₄.

For sample preparation we introduced TCA-NaOH treatment. Glucitolysine has been thought to be stable against acid like TCA [12, 19]. In order to make sure of the

stability of glucitolysine against 0.05 N NaOH we compared the effect of treatment with 0.05 N NaOH on a standard curve of glucitolysine, and we found no discernible difference between glucitolysine standards treated or not treated with NaOH (data not shown).

Discrepancies of estimated reduced glycosylated Hb values by RIA with or without TCA-NaOH treatment

To investigate the effect of TCA-NaOH treatment, we assayed samples from two normal subjects and two diabetic patients, using the TCA-NaOH treatment method and the dialysis method after reduction of the samples (Table 1). The values using our TCA-NaOH method were more than 20-fold higher than the values using the dialysis method. Therefore, it is possible that TCA-NaOH treatment denatured Hb, and exposed hidden glucitolysine epitopes.

Linearity and precision of RIA

To assess the linear range, we prepared a series of six solutions which were obtained from the glycosylated Hb fraction by using a Glyco-Gel B boronate affinity chromatography. All the samples were analysed four times by the RIA in different analytical batches (Fig. 2). The plot of values was linear from 0.1 to as high as 6 nmol glucitolysine. Therefore, all samples were measured in this region.

Within-run precision (CV) assessed by five analyses of three specimens and between-run precision assessed by five analyses of three specimens, in separate analytical runs, over a three-week period was as shown in Table 2.

Bio-Rex 70 chromatographic fractionation of Hb

In order to elucidate the localization of glucitolysine, we assayed all Bio-Rex 70 chromatographic fractions by our RIA. The chromatograms of Hbs from a normal subject and a diabetic subject are shown respectively in Figures 3 and 4. A large amount of glucitolysine was incorporated into the leading edge of the major Hb components (HbA₀) as reported by Bunn et al. [8]. Furthermore, it was clearly shown that the HbA_{1a+b} and HbA_{1c} peaks from the normal and diabetic subjects contained glucitolysine. It was therefore confirmed that HbA_{1c} is glycosylated not only at the β -amino terminus but also at the lysine ϵ -amino groups.

Table 1. Discrepancies in estimated reduced glycosylated Hb values by RIA with and without TCA-NaOH treatment

Sample	HbA _{1c} (%)	Glycosylated Hb (nmol glucitolysine/mg Hb)	
		Dialysis	TCA-NaOH ^a
1	5.0	0.11	3.0
2	5.2	0.12	3.2
3	9.8	0.69	13.8
4	11.0	0.75	16.7

^a TCA-NaOH was used instead of dialysis for the removal of excess NaBH₄. The reduced glycosylated Hb was TCA precipitated and the precipitated protein was dissolved in 0.05 N NaOH for analysis

Fig. 3. Bio-Rex 70 chromatographic separation of the Hbs in haemolysate from a normal adult. 300 μ l (25 mg) of haemolysate was applied to a column (1 $\times$ 30 cm). Fraction size was 1.5 ml and the flow rate was 15 ml/h. At the point of fraction number 40, stripping buffer was applied. (●—●) protein (415 nm); (○—○) glucitolysine (RIA)

Fig. 4. Bio-Rex 70 chromatographic separation of the Hbs in a haemolysate from a diabetic patient. The details are those described in the legend to Figure 3 and in Materials and methods

Table 2. Precision of assay

Sample	Within-run ($n=5$)		Between-run ($n=5$)	
	Mean $\pm$ SD ^a CV (%)		Mean $\pm$ SD ^a CV (%)	
1	3.6 $\pm$ 0.1	2.8	3.7 $\pm$ 0.2	5.4
2	7.7 $\pm$ 0.2	2.6	7.5 $\pm$ 0.4	5.3
3	15.3 $\pm$ 0.2	1.3	15.6 $\pm$ 0.3	1.9

^a nmol glucitolysine/mg Hb

Comparison of glyated Hb by RIA with HPLC

The results of assays for glyated Hb, carried out on the N, IGT and diabetic subjects, are presented in Figure 5. The mean glyated Hb levels by RIA were 3.3 ± 0.4 nmol glucitolysine/mgHb (mean $\pm$ SD) in N, 4.7 ± 0.5 in IGT and 14.4 ± 7.5 in the diabetic group. The differences between the three groups were highly significant ($p < 0.001$). On the other hand, the mean HbA_{1c} levels by HPLC were

N: $5.0 \pm 0.4\%$ (mean $\pm$ SD), IGT: $5.1 \pm 0.3\%$, and there was no significant difference between the N group and IGT group. Fifteen of the 20 IGT patients exceeded the upper normal range (mean normal values + 2 SD) for RIA values. But all IGT patients were within the upper normal range for HbA_{1c} values.

Discussion

It has been generally accepted that the level of glyated Hb is a clinically useful measure of the average long-term glycaemic control in a diabetic patient. While the development of a quantitative methodology for the measurement of glyated Hb has been extensively carried out, there is still a need for better methods of measurement with appropriate references and standards with which accuracy and precision can be maintained. Recently, the National Diabetes Data Group in the United States has concluded that a major problem with all methods is that there are no agreed upon references and standards [6].

Fig. 5. Glycated Hb levels in normal (N) ($n = 30$), impaired glucose tolerance (IGT) ($n = 20$), and diabetes mellitus (Dm) ($n = 30$), determined by RIA and HPLC. The horizontal lines indicate the mean $\pm$ SD of each group. Mean values significantly different in RIA: N vs IGT ($p < 0.001$); IGT vs Dm ($p < 0.001$). Mean values significantly different in HPLC: N vs IGT (NS); IGT vs Dm ($p < 0.001$)

The RIA method presented in this report has the advantage of using glucitolysine which is chemically stable. Shapiro et al. found that sites of Hb glycation were different in vitro and in vivo [9], so if glycosylated Hb was synthesized in vitro as a standard, it is chemically different from Hb glycosylated in vivo. On the other hand, glucitolysine was easily synthesized and was resistant to acidic and alkaline conditions [12, 19]. Since our RIA method used glucitolysine as a standard substance, this method has a chemically defined and stable reference standard.

In the sample preparation, it was necessary to reduce glycosylated Hb. After reduction, dialysis or gel filtration was generally used to stop the reduction and to remove unreacted NaBH_4 [8, 20]. Since this is too time consuming for routine use we introduced the TCA-NaOH treatment after reduction as has been described previously [10]. By using the TCA-NaOH treatment, the assay was relatively simple. Moreover, the TCA-NaOH treatment seemed to have another advantage in that it offered greater sensitivity (Table 1). A possible explanation for this increased sensitivity has been reported in a study of murine monoclonal antibodies recognizing glucitolysine [20]. Utilizing this monoclonal antibody, the levels of glucitolysine quantitated were lower than previous estimates of the amount of glucose bound to total plasma proteins and it was thought that the presence of hidden glucitolysine epitopes not accessible to the antibodies could account for the difference. As shown in Table 1, the values determined by TCA-NaOH treatment were about 25 times higher than those by dialysis. The amount of glucose assayed RIA as bound to

Hb in TCA-NaOH treated samples was similar to previous estimates by the HPLC analysis of furosine, a specific quantitation of ϵ -amino group lysine-bound glucose in Hb [21, 22]. Moreover, the synthesized reduced glycosylated Hb contained 35% of the lysine residues. Thus, 15 moles of 44 moles of lysine residues were glycosylated as determined by amino acid analysis which is equivalent to about 200 nmol glucitolysine/mgHb. This value was similar to the 178 nmol glucitolysine/mgHb which was measured using the RIA with TCA-NaOH treatment. By contrast, the dialysis method revealed a far lower value of 7.0 nmol glucitolysine/mgHb. Thus, it is plausible that the increased values found following TCA-NaOH treatment in comparison with the dialysis method for sample preparation is a consequence of the unfolding of the Hb molecule, thereby exposing the hidden glucitolysine epitopes.

It has been shown previously that glucose is conjugated to the ϵ -amino group of lysine of Hb which exists in the HbA₀ [8, 21]. We are able to clearly demonstrate that glucose conjugated to the ϵ -amino group of Hb exists not only in the HbA₀ but also in the HbA_{1c} (Figs. 3 and 4).

The diagnostic value of the RIA method and the HPLC method was compared in the N, IGT, and diabetes groups. HbA_{1c} as determined by HPLC could clearly separate a group of N from a group of diabetic patients, but could not separate a group of N from a group of IGT. On the other hand, by the RIA method there was a significant difference between the groups of N and IGT. Moreover, all of the 20 IGT patients were within the upper normal range for HbA_{1c} by the HPLC method, but 15 of the 20 IGT patients exceeded the upper normal range for our RIA method. Thus the RIA method might be able to be used to detect IGT patients in a mass screening test.

A quantitative ELISA for glycosylated serum albumin by using the same antiserum [10] was recently developed in our laboratory and the RIA method seems to be applicable to quantitate any kind of glycosylated protein with the desired precision for clinical use [11] (Table 2).

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