Glycosylated haemoglobin and plasma glycoprotein assays by affinity chromatography

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Summary. A rapid chromatography method for separating glycosylated protein from non-glycosylated protein, using a boronate-agarose affinity medium which selectively binds the cis-diol groups of glycoproteins, was used to quantitate plasma glycoprotein as well as glycohaemoglobin. The results were found to be independent of: (1) temperature from 16.5 to 29.8 °C; (2) haemoglobin variants, and (3) aldimine glycoprotein adducts. Thus several of the common problems occurring in existing haemoglobin A₁ assays are eliminated. There was a close correlation between glycohaemoglobin measured by af-

finity chromatography and haemoglobin A₁ by cation-exchange ($r=0.959$, $n=215$). Specimens from 103 diabetic patients and 112 healthy volunteers were assayed. The following reference ranges were established: glycohaemoglobin 5.5–8.4%; glycosylated total protein 11.5–16.2%; glycoalbumin 11.6–19.5%.

Key words: Glycohaemoglobin, plasma glycoprotein, affinity chromatography, diabetes, boronate-agarose resin.

Glycosylated haemoglobin, usually measured as HbA₁ or a subfraction of HbA₁ (e.g. HbA_{1c}), has wide acceptance as a long-term index of diabetic control. Limitations of the commonly used mini-column cation-exchange method include temperature dependence [1–4], influence of the results by haemoglobin variants [1, 3, 5], and interference by the 'labile' aldimine fraction [6] unless appropriate sample preparation steps are taken. Other methods, such as agar gel electrophoresis, suffer from a variety of limitations [7].

We have evaluated an affinity chromatographic method using boronic acid immobilized on an inert agarose medium to measure glycohaemoglobin. This method is specific for glycohaemoglobin [8] and avoids most of the difficulties seen in other procedures.

We also used the affinity method to measure plasma glycoprotein. Some investigators have suggested that plasma glycoprotein values reflect time-averaged blood glucose levels over 3–4 weeks (the protein turnover rate) and therefore indicate intermediate-term diabetic status [9–12].

Method correlations were made with an established commercial HbA₁ procedure (Isolab Fast Hb). Normal ranges were determined for glycohaemoglobin (GHb), plasma glycosylated total protein, and plasma glycoalbumin determined by affinity chromatography. GHb and HbA₁ are presented as different parameters since

affinity GHb also detects haemoglobin glycosylated at sites other than the N-terminus of the β chain.

Materials and methods

At Akron General Medical Center specimens from 103 diabetic patients and 112 healthy volunteers (71 females, 41 males) were assayed for HbA₁, GHb, glycosylated total protein, and glycoalbumin. The healthy volunteers ranged in age from 15 to 82 years. Blood specimens were collected in 5 ml evacuated tubes (Terumo Medical Corporation, Elkton, Maryland, USA) containing EDTA, mixed and delivered to the laboratory on ice, then promptly centrifuged to separate plasma from erythrocytes. Plasma was used for the affinity glycosylated total protein and glycoalbumin procedures. Haemolysates for affinity GHb were prepared from erythrocytes washed in saline (0.154 mol/l).

The affinity columns and associated reagents (Glyc-Affin) were obtained from Isolab, Akron, Ohio, USA, as were cation-exchange mini-columns and reagents used to measure HbA₁.

Elution buffer requirements for the affinity method were 2 ml for each fraction. Absorbance measurements on the separated fractions were used to calculate glycoprotein values as a percentage of total protein present. The dye-reagents provided to quantitate affinity glycosylated total protein and glycoalbumin were Coomassie Brilliant Blue G-250 and Bromocresol Green, respectively. The affinity columns were regenerated and used 10 times each.

To assess the effect of temperature on the affinity GHb measurements, we assayed three different samples representing different GHb levels at 16.5°, 23.0°, 26.8°, and 29.8 °C.

To assess the influence of labile HbA₁ in the affinity GHb assay, glucose was added to several whole blood samples to increase the labile HbA₁ levels artificially [13]. Affinity GHb measurements were

Table 1. Mean values and 95% confidence limits of assays

Method	Number of normal specimens	Mean value (%)	Normal range 95% confidence limits (%)
Isolab HbA ₁	112	6.4	5.3– 7.5
Glyc-Affin haemoglobin	112	6.9	5.5– 8.4
Glyc-Affin total protein	108	13.8	11.5–16.2
Glyc-Affin albumin	107	15.5	11.6–19.5

Fig. 1. Correlation between results by the affinity method (GHb) and the cation-exchange method (HbA₁). [Affinity glycohaemoglobin (%) = 1.557 x haemoglobin A₁ (%) - 2.955, $r=0.959$, $n=215$]

then compared to Isolab HbA₁ measurements before and after incubation with saline to remove the labile fraction.

Special studies, including electrophoresis for HbS and alkali denaturation for HbF, were performed on any haemoglobinopathy specimens encountered to evaluate haemoglobinopathy effects on affinity GHb measurements.

Results

Flow rates of at least 0.2 ml/min gave rapid separation using the affinity columns. Total assay time for affinity GHb was usually <1 h. The extra colour development step needed for the plasma glycoprotein fractions added 30 min to these assay times.

Insignificant variations in affinity GHb values were seen for normal, elevated, and highly elevated levels measured at temperatures of 16.5°–29.8°C. The range of results for each of the three levels were 5.2–5.6%, 18.2–19.1%, and 47.5–48.0%.

No interference in the affinity GHb assay due to aldimine adducts was observed. In blood specimens with artificially elevated HbA₁, four tubes averaged GHb at 8.7% and HbA₁ at 29.5%. After these tubes were incubated at 37°C for 4 h to remove the labile fraction, the averages were (GHb) 8.7% and (HbA₁) 6.9%.

The manufacturers report within-run and between-run coefficients of variation of <7% for affinity determinations. In clinical usage over a period of 3 months, we saw coefficients of variation for glycoprotein (erythrocytes and plasma) of <9% in a normal volunteer from whom samples were drawn weekly. These higher coefficients of variation include fluctuations of in vivo glycosylation as well as analytical variables. Nevertheless the precision of the affinity procedure is comparable to that of cation-exchange mini-column methods, or better [14].

Normal ranges, calculated as mean $\pm$ 2 SD, are given in Table 1. Histograms showed Gaussian or near-Gaussian frequency distributions.

The normal range for affinity GHb was found to be 5.5–8.4%, showing close agreement with the manufacturer's normal range (5.5–8.5%). The plasma glycosylated total protein and glycoalbumin ranges are reasonably close to those reported for glycosylated human albumin by Day et al. (6%–15%) [15].

A good correlation was seen between GHb and HbA₁ for the combined patient and normal population ($r=0.959$, Fig. 1). A poorer correlation was seen between glycosylated total protein and HbA₁ for the combined population ($r=0.808$). No significant correlation was observed between glycosylated total protein and HbA₁ in normal subjects.

A definite advantage in using the affinity method was seen in two specimens from diabetic patients containing HbS (GHb 18.5%, HbA₁ 8.4% and GHb 19.6%, HbA₁ 7.0%). The HbA₁ values greatly underestimated the glycosylated haemoglobin status of these patients.

One other specimen contained HbF and gave the following results: GHb 6.4%, HbA₁ 10.3%. The HbF of this specimen was determined by alkali denaturation to be 4.0%. The falsely elevated result obtained by the cation-exchange method was apparent [1, 3].

Discussion

The good correlation between affinity GHb and cation-exchange HbA₁ supports the use of the affinity method in monitoring long-term glycaemic status, similar to the way in which HbA₁ is now used. The poor correlation between glycosylated total protein and HbA₁ may be due in part to significant individual differences in plasma protein turnover rate. In any case, they reflect glycosylation over different periods of time.

Dolhofer et al. [9, 16] have produced data supporting the clinical usefulness of plasma glycoprotein measurements in monitoring the effectiveness of diabetic

treatment over a relatively short period of time. Plasma glycoprotein levels may also be useful in monitoring gestational diabetes soon after its onset when erythrocytes have not had sufficient time to glycosylate fully. The normal ranges we report should provide useful guidelines.

A major advantage of the affinity GHb procedure was seen in patients with haemoglobinopathies. Since the affinity method measures the glycosylated form of any variant present, false indications of a patient's glycosylation status are avoided. It would be of interest to examine this effect in patients with other haemoglobin variants.

Analytically we have demonstrated that the affinity technique is relatively rapid, uncomplicated and reliable. The labile component is not measured and the assay can be run at ambient temperature in any laboratory.

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