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RAPID CHROMATOGRAPHIC QUANTITATION OF GLYCOSYLATED HAEMOGLOBINS

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SUMMARY

We have developed a rapid chromatographic method for determination of glycosylated haemoglobins by high-performance liquid chromatography with a new cation-exhange column. The haemoglobins are eluted with a three-step gradient in 7 min, and total assay time including re-equilibration of the column is 15 min. The method permits separation and quantitation of HbAlc, even in the presence of elevated levels of HbF. HbA1a, A1b and A2 can also be determined. The results correlate well ($r = 0.94$) with those obtained by the macro-column method of Trivelli *et al.* [*New Engl. J. Med.*, 284 (1971) 353] for determination of HbA1c. The method has been fully automated by the use of an automatic injector. The within-assay and between-assay coefficient of variation of the method is 2–3%.

INTRODUCTION

Several chromatographic methods for determination of glycosylated haemoglobins are in common use. The method described by Trivelli *et al.*[1] may still be considered a reference method. This method uses the cation-exchange resin Bio-Rex 70. More rapid variants of this method have been developed using the same ion exchanger in combination with high-performance liquid chromatography (HPLC). This shortens the time for one determination from 6 h to *ca.* 30–40 min[2,3]. Another method of shortening the assay time has been to use disposable micro-columns[4]. In micro-column methods the different glycosylated haemoglobins are not separated from each other. Another drawback of these and other chromatographic methods is the interference caused by fetal haemoglobin, which is especially important in pregnant women[5]. Other methods of assaying glycosylated haemoglobins include isoelectric focusing[6], affinity chromatography[7], and chemical determination of glucose conjugated with haemoglobin[8].

We have employed a new cation exchanger, Pharmacia Mono-S®, for the separation and quantitation of glycosylated haemoglobins and compared our results with those obtained by the macro-column method of Trivelli *et al.*[1]. Similar results are obtained, but the new method is more rapid and gives better separation than earlier chromatographic methods. It also lends itself to complete automation.

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MATERIALS AND METHODS

Patients

Blood samples were obtained from 99 apparently healthy pregnant women during routine antenatal care and from 50 pregnant diabetics.

Equipment

Mono-S cation-exchange columns were obtained from Pharmacia, Uppsala, Sweden. The chromatographic system consisted of a Spectra-Physics SP 8700 liquid chromatograph equipped with an SP-8300 dynamic mixer and an SP 770 variable-wavelength detector, a Hewlett-Packard 3390A integrator and a Waters WISP automatic injector. Alternatively, an LKB 2150 pump and an LKB 2152 controller, equipped with a low-pressure mixing valve and a manual Rheodyne injector with a 20-μl loop, were used. The column was thermostated by immersion in a 25°C waterbath.

Buffers

Buffer A contained 12.2 mmol/l of sodium dihydrogen phosphate, 2.8 mmol/l of disodium hydrogen phosphate (pH 6.8) and 4 mmol/l of potassium cyanide. This buffer was prepared as a 40-fold concentrated stock solution. Buffer B contained 0.26 mol/l of sodium dihydrogen phosphate and 0.12 mol/l of disodium hydrogen phosphate (pH 6.3). The buffers were filtered through a 0.5-μm filter and degassed at reduced pressure in an ultrasonic bath for 5 min. The buffers can be used for at least one week after dilution without further degassing. Cyanide solution contained 50 mmol/l of potassium cyanide. Saline buffer contained 150 mmol/l of sodium chloride. Hydrolysis buffer contained 25 mmol/l of sodium dihydrogen phosphate; its pH should be 4.8–4.9. Phthalate buffers (pH 5) contained either 20 or 50 mmol/l of potassium biphthalate.

Preparation of samples

Blood samples were drawn in EDTA tubes. Heparinized blood could also be used. The blood cells were sedimented by centrifugation at 1000 *g* for 10 min at room temperature. The packed cells were washed three times with saline buffer. One volume of packed red cells was lysed by addition of one volume of distilled water. Haemolysates were stored at −80°C unless assayed the same day. Delipidation was performed by addition of one volume of carbon tetrachloride to three volumes of haemolysate. After agitation in a Vortex mixer, the sample was centrifuged for 2 min at 6000 *g* in an Eppendorff 5414 microcentrifuge. Cyanide solution (15 μl) was added to 250 μl of haemolysate. Haemolysates were treated by two different methods before chromatography. In Method I an aliquot of the supernatant was diluted 40-fold with water before injection. To remove labile glycosylated haemoglobin, we used Method II. In this method the sample was diluted 40-fold with hydrolysis buffer and incubated for 15 min at 37°C or for 40 min at room temperature before injection. A 20-μl volume of haemolysate was injected either manually or by the automatic injector.

Chromatographic procedures

In the HPLC method the column was regenerated for 10 min with buffer B

and then equilibrated with buffer A for at least 30 min before injection of the first samples. A low and a high control were run before and after the samples. A flow-rate of 2 ml/min was used. Elution was achieved by a step gradient, which was dependent on the equipment used (Table I). A new sample was injected every 15 min. The integrator was programmed to set the baseline at the start of the chromatogram, before the HbA1c peak and after the last peak at 6 min.

The Trivelli method[1] was performed as described earlier[5].

TABLE I

GRADIENT PROGRAM FOR SEPARATION OF HAEMOGLOBINS

Slightly different programs were used depending on the equipment used.

Spectra-Physics			*LKB*		
Time (min)	*% A*	*% B*	*Time (min)*	*% A*	*% B*
0	100	0	0	100	0
0.1	94	6	3	97	3
3.1	94	6	4	97	3
6.1	60	40	7	80	20
6.2	0	100	7.5	50	50
7.5	100	0	8.0	100	0

RESULTS

The separation of a sample with an elevated level of HbA1c is shown in Fig. 1. Three peaks are eluted within 1 min. These apparently correspond to HbA1a1, HbA1a2 and HbA1b, respectively[9]. HbA1c is eluted at *ca.* 2.2 min as a well-separated peak. The main component (HbA0) is eluted at 5.3 min, and HbA2 is partially separated at 6.3 min. In most samples one or two additional small peaks are eluted before HbA0. These apparently correspond to HbA1d and HbA1e[9], and their con-

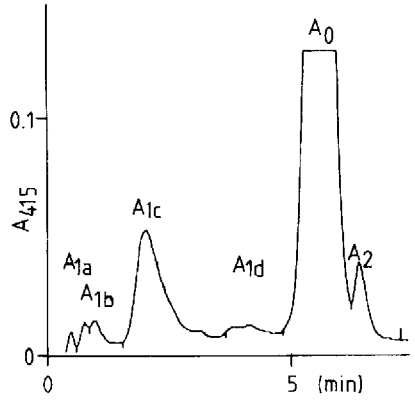
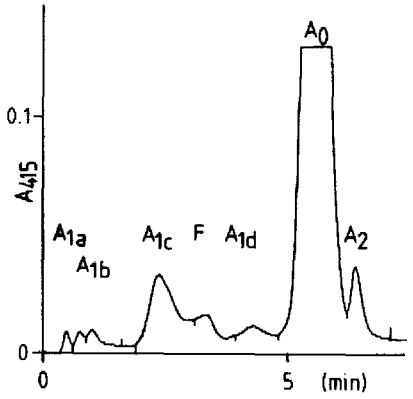


Fig. 1. Chromatography of haemoglobins in a sample with elevated level (10%) of HbA1c.

centration increased gradually when the sample was stored at −20°C. Although these peaks did not interfere with the quantitation of HbA1c, we routinely stored all samples at −80°C.

In samples from some pregnant women an extra peak is often eluted after HbA1c (Fig. 2). This peak was identified as HbF by addition of various amounts of cord blood haemolysate. HbF is eluted as a fairly broad peak when added in higher concentrations. This also causes a slight increase in retention time (Fig. 3). Because of this anomalous behaviour, HbF in low concentrations is not well separated from HbA1c.



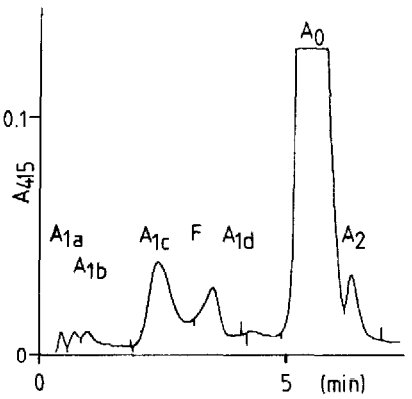


Fig. 2. Separation of haemoglobins in a sample with a normal level (4.7%) of HbA1c and an elevated level (1.2%) of HbF.

Fig. 3. Chromatography of a normal haemolysate supplemented with 0.1 volume of cord blood haemolysate.

Rechromatography of the peaks indicated that the first peak (tentatively identified as HbA1a1) was considerably (50–70%) contaminated by the other components and mainly by HbA0. The other peaks contained only small amounts of adjacent peaks and HbA0.

Incubation of the sample at pH 5 (Method II) caused a decrease in the HbA1c fraction compared with samples diluted with water (Method I). In 20 samples studied, the mean concentration without incubation (Method I) was 6.3% and in incubated samples 5.5%. The decrease in different samples varied from 0.1 to 1.2%. Incubation with phthalate buffer as originally described[3] (0.05 mol/l, pH 5.0) was not compatible with the present HPLC method, because the high ionic strength in the incubation buffer caused too early elution of the first peaks. With a phthalate concentration of 0.02 mol/l the same results for HbA1c was obtained as with acid phosphate, but the phthalate produced a buffer peak that obscured the three first peaks in the chromatogram.

The correlation of results obtained by HPLC and by the method of Trivelli is good, $y = 1.12x - 0.69, r = 0.97$ (y = HPLC, x = Trivelli). The mean concentration of HbA1c in 99 samples from healthy pregnant women is 5.44 ± 0.57%. The reference range for the different Hb variants is shown in Table II. The precision of the

TABLE II

CONTENT OF HbA COMPONENTS IN HEALTHY PREGNANT WOMEN

The reference ranges are based on the central 95% reference interval in samples from 99 women.

Component	*Range (%)*	*Median (%)*	*Reference range (%)*
HbA1a	0.5 1.1	0.6	0.5–1.0
HbA1b	0.6–2.3	1.0	0.7–1.5
HbA1c	3.9–7.1	5.4	4.2–6.6
HbA2	2.2–4.5	3.4	3.0 4.2

method is acceptable (Table III). Fetal haemoglobin was detected at levels from 0.3 to 5.1% in 21 out of the 99 samples from healthy pregnant women.

The level of HbA2 is overestimated because the peak is only partially separated and the integrator was not programmed to take this into account. Although the method is not suitable for quantitation of HbA2, it allows identification of patients with clearly elevated HbA2 levels.

TABLE III

REPRODUCIBILITY OF HbA1c DETERMINATION BY HPLC ON MONO-S ION EXCHANGER

Concentration of HbA1c (%)	*n*	*Coefficient of variation (%)*	
		Intra-assay	*Inter-assay*
5.6	10	2.4	3.2
10.2	10	2.7	3.3

DISCUSSION

Most HPLC methods for determination of HbA1c use the cation exchanger Bio-Rex 70[2,3]. With this resin, separation requires 20–30 min and total assay time is 30–40 min. By the present method, separation is achieved in 7 min, and the whole assay takes 15 min. In addition, HbA1a and A1b are separated from each other, and HbA1c from HbF, when the latter occurs in measurable concentrations. Separation of HbF is important, because we use the method for measurement of HbA1c in pregnant patients, who occasionally may have increased concentrations of HbF[5]. In this study 21% of the pregnant non-diabetic women had measurable levels of HbF and 15% had levels higher than 1%. In most rapid methods based on cation-exchange chromatography, elevated levels of HbF cause a false elevation of the glycosylated haemoglobin.

The results for HbA1c obtained by the present method are similar to those obtained by other methods based on ion-exchange chromatography. The correlation with the macro-column method of Trivelli is good. The reference range for pregnant women, 4.2–6.6%, is slightly higher than the range, 4.0–6.0%, used with the macro-column method[5]. The false elevation of the HbA1c fraction caused by reversibly

bound glucose was eliminated by incubation at low pH. The phthalate buffer used earlier for this purpose[5] was not compatible with our chromatographic method. We therefore used a dilute phosphate buffer giving a final pH of 5, as used in the original method[3]. Because the samples will be kept at room temperature for several hours when an automatic sample injector is used, we also evaluated the effect of incubation at room temperature, *ca.* 23°C. After 40 min at this temperature, no further decrease in HbA1c level was observed. We preferred this method, because samples incubated at 37°C were less stable when standing in the injector for over 10 h. This was evident as an increase in the HbA1d and e fractions. Samples incubated at room temperature were stable for at least 20 h.

Application of the present method to various kinds of HPLC equipment will require adjustment of the gradient program to obtain the same separation as in this study. This is demonstrated by the fact that slightly different programs had to be used with the two chromatographs used in the present study (Table I). The most time-consuming phase of the method is the reequilibration of the column. About 8–10 min was a minimum with the buffers used. We attempted to shorten the separation time by increasing the flow-rate above 2 ml/min (the highest flow-rate recommended by the manufacturer). This was possible with retained separation, but the column was gradually compressed. It retained its original volume within some hours when the flow was stopped. With a flow-rate of 2 ml/min one column has been used for more than 1000 assays. Regeneration of the column with sodium dodecyl sulphate has been performed once a week according to instructions by the manufacturer. So far, we have used four different columns with little difference in performance.

The temperature control is important for separation of Hbs on Bio-Rex columns[10], and we observed a strong dependence on temperature in initial studies with linear gradients. With the step gradient used in the present method the temperature is less critical, but it is necessary to use thermostatic conditions to ensure reproducible results. The concentration and pH of the buffers are very critical. A small increase in the concentration of buffer A causes an increase in the fractions eluted first.

The column used in the present study has allowed a considerable improvement in the assay of HbA1c, and this method could easily be adapted for the separation and quantitation of HbA2 and abnormal Hbs simply by making the increase in concentration of buffer B after 3 min less steep.

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