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Application of high-performance liquid chromatography in establishing an accurate index of blood glucose control

TADAO HOSHINO*, YUKO TAKAHASHI and MIKIKO SUZUKI
Pharmaceutical Institute, School of Medicine, Keio University, 35-Shinanomachi, Shinjuku-ku, Tokyo 160 (Japan)

ABSTRACT

A chromatographic method utilizing a carboxymethylated poly(vinyl alcohol) resin for a more accurate determination of stable haemoglobin A_{1c} (St-A_{1c}) has been developed. The complete separation between St-A_{1c}, labile HbA_{1c} (L-A_{1c}) and HbF was achieved by gradient elution with sodium chloride in phosphate buffer. This high resolution permits accurate quantitation of St-A_{1c}, even in the presence of high levels of HbF or L-A_{1c}. In 142 subjects with normal fasting plasma glucose and normal response to a 75-g oral glucose tolelance test, the reference interval of St-A_{1c} was 2.80–3.98%.

INTRODUCTION

Haemoglobin A_{1c} (HbA_{1c}) is considered to be a reliable indicator of long-term blood glucose regulation in diabetic subjects. However, the measured value of HbA_{1c} often changes, paralleling short-term fluctuations of blood glucose levels, and shows unexpectedly high levels[1,2]. Goldstein *et al.*[1] have shown that these acute changes depend on the concentration of labile A_{1c} (L-A_{1c}), which is not separated from stable A_{1c} (St-A_{1c}) by chromatography. Therefore, it was necessary to remove L-A_{1c} by incubation of erythrocytes in saline or by other methods prior to HbA_{1c} assay[3] Recently, many attempts have been made to improve the high-performance liquid chromatographic (HPLC) techniques used, and the resolution and accuracy of commercially available analysers for HbA_{1c} have been improved. However, the measured value does not yet reflect accurately the integrated level of blood glucose.

We have previously developed a liquid chromatographic method, based on an IEX-530 column, which enabled us to establish that HbA_{1c} consisted of six sub-fraction[4,5]. Although this method could measure L-A_{1c} and St-A_{1c} simultaneously, the resolution between the two peaks had a value of 0.49, and was still too poor for performing accurate measurements. More recently, we have employed a carboxymethylated poly(vinyl alcohol) resin for the analysis of HbA_{1c}, and it provided better resolution than that of the earlier chromatographic method. In this paper we report the improved method and some applications.

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EXPERIMENTAL

Apparatus

The chromatographic apparatus was obtained from Japan Spectroscopic Co. (Tokyo, Japan). The HPLC system was composed of a Trirotar VI pump, a system controller, a DG-3510 in-line degasser, a TU-300 column oven, a VL-614 sample injector and a Uvidec-100-VI variable-wavelength detector, equipped with a Model 7000A intelligent integrator (System Instruments, Tokyo, Japan). All flow lines were made of PTFE tubing (0.5 mm I.D.), except in the pump and injector. The column temperature was thermostatically controlled by passing water through the column jacket.

Reagents

All chemicals used for elution buffer and sample preparation were of special grade, purchased from Wako (Osaka, Japan). The weak and strong eluting buffers used were (A) 30 m*M* sodium phosphate contaning 0.01% sodium azide and (B) 30 m*M* sodium phosphate containing 0.6 *M* sodium chloride and 0.01% sodium azide. The pH was adjusted to 5.0–6.5 by adding the appropriate volume of 0.1 *M* sodium hydroxide solution. The water used was tap water purified with a system consisting of a Milli-Q/R and a Milli-Q filter (Millipore, Bedford, MA, U.S.A.).

Sample preparation

Blood samples from fasting subjects were obtained by venipuncture and placed in tubes containing EDTA or heparin. Haemolysates were prepared by a modification of the method of Trivelli *et al.*[26]. Blood was centrifuged at 800 *g* for 10 min and the packed erythrocytes were washed three times with four volumes of saline. The washed cells were lysed with four volumes of purified water and an equal volume of carbon tetrachloride was added. After vigorous mixing, cellular debris and carbon tetrachloride were settled by centrifugation under the same conditions. The haemolysate was analysed immediately or stored at −80°C until analysed. All of these procedures were carried out at 4°C.

To prepare a control haemolysate, which was used to study the elution behaviour of haemoglobin components, packed erythrocytes were incubated with 5% glucose in isotonic phophate buffer (pH 7.0) for 1 h at 37°C prior to the saline washing.

For *in vitro* studies on the fluctuations of HbA_{1c}, packed erythrocytes and haemoglobin A_0 fraction, which was obtained by HPLC fractionation of the haemolysate, were incubated with 10% glucose at 37°C for 2 h or with saline at room temperature for 1 day.

Column preparation

Packing material based on carboxymethylated poly(vinyl alcohol) (DVT-119; exclusion limit = 30 000 daltons, particle size = 9 μm; ion-exchange capacity = 0.8 mequiv./g) was provided by Asahi Kasei Industries (Kawasaki, Japan). Dry resin (1 g) was dispersed in an adequate volume of buffer B and degassed by sonication under reduced pressure. The degassed slurry was pumped into a Pyrex glass column (150 × 8 mm I.D.) with a removable water-jacket and adjustable plunger. The packed resin reached a height of 50 mm.

RESULTS AND DISCUSSION

The elution behaviour of the haemoglobin components on the DVT-119 column was observed by using isoelectric elution at various pH and ionic strength values. The control haemolysate was used for this study. At pH > 6.0, St-A_{1c} was eluted together with other haemoglobin components but is was not eluted at pH < 5.0. The ionic strength of the mobile phase was also critical. At 0.12 *M* NaCl St-A_{1c} was eluted together with L-A_{1c}. As the ionic strength decreased from 0.12 to 0.06 *M* NaCl, the resolution of St-A_{1c} and L-A_{1c} was increased, but the k' value increased to more than 12.0. Moreover, HbA_0 is more readily retained than HbA_{1c} by cation-exchange. Therefore, we used an elution gradient consisting of buffers A and B (both at pH 5.5).

A chromatogram of a control haemolysate is shown in Fig. 1. The column was equilibrated with buffer A–buffer B (90:10). A 4-μl volume of haemolysate was injected into the column and eluted with the followed gradient: 10% B for 2 min; 10 to 12% B in 8 min; 12 to 15% B in 8 min; 15 to 20% B in 6 min; 20 to 40% B in 18 min; 40 to 80% B in 12 min and 80 to 100% B in 2 min. The flow-rate was 1.0 ml/min and the eluate was monitored at 415 nm. The column temperature was kept at 24°C. Before the next injection, the column had to be equilibrated with 10% buffer B for at least 20 min. Eight haemoglobin components were obtained from 4 μl of haemolysate and were arbitrarily numbered 1–8.

The identification of the peaks was carried out as described previously[4]. Fraction 3 was alkali-resistant and was eluted at the same position as foetal haemoglobin. Fractions 4 and 5 were identified as L-A_{1c} and St-A_{1c}, respectively. St-A_{1c} was well separated from HbF, and the resolution value of St-A_{1c} and L-A_{1c} was 0.98.

The influence of blood glucose fluctuations on the levels of haemoglobin components, as measured with our HPLC method, was investigated. The results are

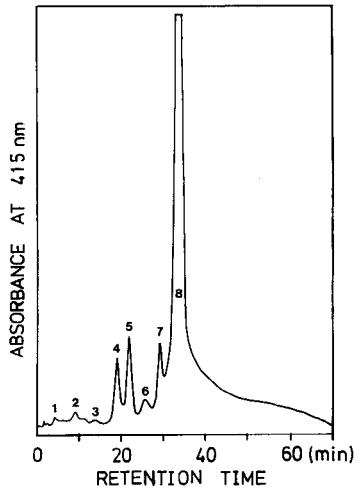

Fig. 1. Chromatogram of a control haemolysate on carboxymethylated poly(vinyl alcohol) resin. A 4-μl sample was applied to the column and eluted with a sodium chloride gradient (see text.) Four peaks were identified: 3 = HbF; 4 = L-A_{1c}; 5 = St-A_{1c}; 8 = HbA_0.

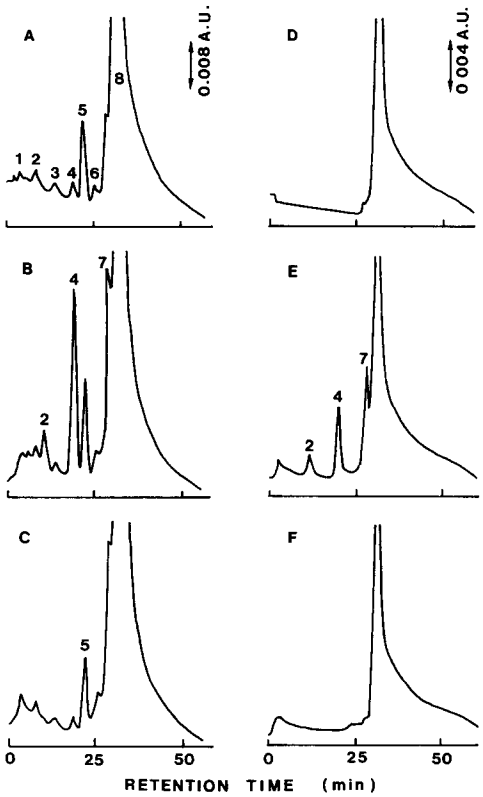

Fig. 2. Acute changes of L-A_{1c} *in vitro*. (A) Normal adult blood; (B) normal adult blood after incubation with 10% glucose at 37°C for 2 h; (C) normal adult blood after washing with saline for 1 day at room temperature; (D) HbA_0 fraction; (E) HbA_0 fraction after incubation with glucose; (F) HbA_0 fraction after following wash with saline.

shown in Fig. 2. Haemolysate from a healthy person and the HbA_0 fraction obtained by HPLC in advance were incubated in 10% glucose at 37°C for 2 h, then further dialysed against saline for 1 day at room temperature. The elution pattern of fraction 5 was hardly affected by incubation in glucose or dialysis against saline. In contrast, fractions 2, 4 and 7 increased after incubation in glucose and decreased after dialysis against saline (Fig. 2A, B, C). In the case of a HbA_0 fraction containing no fraction 2, 4 and 7 components (Fig. 2D), after incubation with glucose Fractions 2, 4 and 7 newly appeared, as shown in Fig. 2E. These peaks disappeared when the HbA_0 fraction was further dialysed against saline for 1 day. Fraction 4 of the haemolysate sample decreased after dialysis against saline, but did not disappear, as in the HbA_0 fraction sample (Fig. 2E). It may represent various other forms of haemoglobin which are eluted together with L-A_{1c} in fraction 4. These results suggest that in patients who have wide fluctuations of blood glucose, the levels of fractions 2, 4 and 7 can be highly variable. In contrast, the level of fraction 5 is very stable. Therefore, to obtain an accurate reflection of long-term blood glucose control, it may be necessary to measure only St-A_{1c}. This method enabled us to measure St-A_{1c}, distinguished from L-A_{1c} and

HbF, without pretreatment to remove L-A_{1c}. In 142 subjects with normal fasting plasma glucose and normal response to a 75-g oral glucose tolerance test, the range of St-A_{1c} levels (mean ± 2S.D.) was 2.80–3.98%.

One of the advantages of the method presented here is an improvement in the purity of St-A_{1c}. This is clearly illustrated in the chromatographic patterns of haemoglobin samples from normal subjects and patients, as shown in Fig. 3. In diabetic patients the level of fraction 5 was high and uninfluenced by incubation with glucose or dialysis against saline (Fig. 3B).

Fig. 3C is a chromatogram of a haemolysate from a normal subject, which shows high level of fraction 3. This peak was identified as HbF by its alkaline resistance and elution behaviour. Samples showing elevated HbF values, as in Fig. 3C, accounted for about 6% of all the samples analysed. Even in these instances the method made it possible to determine St-A_{1c} accurately.

An elevation of fractions 4 and 7 in the haemolysate from patients with renal failure was observed, as shown in Fig. 3D. Increases in these fractions were also observed when glucose incubation of erythrocytes was carried out as stated above. However, in this instance, the blood glucose levels of the patients were within the normal range. Hence the increase was not due to hyperglycaemia. Whereas L-A_{1c} disappeared after saline incubation of erythrocytes or dialysis of a haemolysate before measurement, the amounts of these fractions hardly decreased. Hence it seems that carbamylated haemoglobins which is formed by combination of haemoglobin and cyanic acid, originating from urea, was eluted in the same position as L-A_{1c}. Several other investigators have reported that in patients with renal failure HbA_1 or HbA_{1c} was often observed to be present in large amounts in site of normal blood glucose levels[7,8]. These unreasonable results are considered to be caused by a failure to separate fraction 4, whose amount increases in cases of renal failure, from HbA_1 or HbA_{1c} by their method.

A chromatogram of haemoglobin from a patient with β-thalassaemia is shown in Fig. 3E. It was different from that from a normal person. HbS was eluted after HbA_0

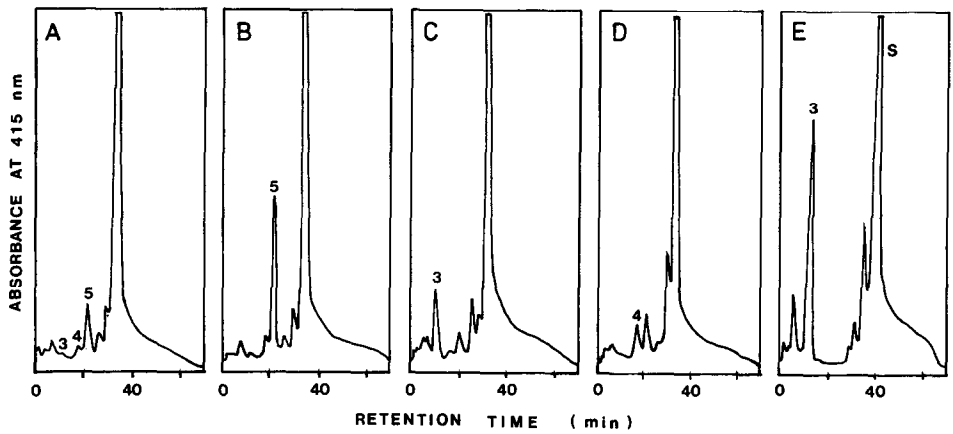

Fig. 3. Separations of haemoglobins from normal persons and patients. (A) Normal adult; (B) diabetic subject; (C) normal subject with high level of HbF; (D) patient with renal failure; (E) patient with β-thalassaemia.

and the amount of HbF was found to be high. Although this method was not designed for the detection of abnormal haemoglobins, it is considered that it could be a useful method for this purpose with further modification of the chromatographic conditions.

In summary, the chromatographic method developed here gave excellent resolution between L-A_{1c} and St-A_{1c}. This made it possible to determine St-A_{1c} levels accurately in normal persons and patients. This method enables characteristic chromatographic patterns of haemoglobin in patients to be obtained. However, there is a minor problem concerning custom-made columns and the difficulty that might arise with some clinical applications.

Prepacked CM columns, ES-502C (Asahi Kasei Industries) IEX-530CM (TOSOH, Tokyo, Japan), which are now commercially available, gave similar chromatographic patterns of haemoglobin in normal persons and patients to those obtained with the custom-made column packed with DVT-119. However, the resolution between St-A_{1c} and L-A_{1c} was very poor in the prepacked columns compared with that from the custom-made column.

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