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Short Communication

Rapid analysis of human serum albumin by high-performance liquid chromatography

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ABSTRACT

High-performance liquid chromatographic analysis of human serum albumin, using a column containing quaternized dimethylaminomethylstyrene–ethylene glycol dimethacrylate, was performed by isocratic elution. This column afforded resolution of albumin components, such as human mercaptalbumin and human nonmercaptalbumin. The method is an alternative to gradient chromatography, and allows rapid determination of the albumin components.

INTRODUCTION

Human serum albumin is a mixture of mercaptalbumin (HMA) and nonmercaptalbumin (HNA) [1–6]. HMA in serum is equilibrated with HNA, which is a mixed disulphide with cysteine or glutathione [7,8]. The equilibrium is of interest in connection with physiology and diagnosis, since the HMA content changes in the elderly and in some diseases [9–11]. The resolution of both components by high-performance liquid chromatography (HPLC) was first reported by Sogami and co-workers [10,12], using an Asahipak GS-520H column. However, these methods were rather time-consuming for clinical applications.

Recently, we reported that an N-methylpyridinium polymer (4VP-EG-Me) column could resolve the albumin components [13,14], but owing to the use of gradient elution one sample required at least 1 h for analysis. Therefore, this analytical method was not practical for clinical use. The quaternized dimethylaminomethylstyrene–ethylene glycol dimethacrylate (PDAMS-Q) column, developed by us, could also resolve [15] albumin components by the same method. However, we found that both components could be completely resolved by isocratic elution.

This paper describes a rapid HPLC analysis by isocratic elution using a PDAMS-Q column for the determination of HSA components.

EXPERIMENTAL

Materials

HSA was kindly provided by the Chemo-Sero Therapeutic Research Institute (Kumamoto, Ja-

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pan). All other chemicals were of reagent grade. Human sera, obtained from healthy volunteers and patients, were diluted (1:9, v/v) with distilled water, and a 5-μl volume was injected directly onto the column.

Preparation of the column packing

Polymerization was performed under a nitrogen atmosphere. Toluene containing dimethylaminomethylstyrene, ethylene glycol dimethacrylate and azo-bis-isobutyronitrile (0.2% monomers) was suspended in 1% aqueous hydroxyethylcellulose containing 20% sodium chloride. After stirring for 7 h at 70°C, the resulting resin (PDAMS) was washed with hot water until free from adhering stabilizer. Finally, it was fractionated in methanol, and the fraction containing particles with a diameter of 10–15 μm was allowed to react with methyl bromide in a pressurized bottle for 20 h at 60°C. The product (PDAMS-Q) was filtered, then washed successively with methanol, acetone and water.

Chromatography

Chromatography was performed with a Hitachi L-6200 intelligent pump equipped with a Hitachi L-4000 UV detector and a Hitachi D-2000 chromato-integrator.

PDAMS-Q was packed into stainless-steel guard column (10 mm × 4 mm I.D.) and analytical column (150 mm × 4 mm I.D.), then conditioned with 0.05 *M* tris(hydroxymethyl)aminomethane–acetic acid (Tris–HAc) buffer (pH 7.0) containing 0.5 *M* sodium acetate. Unless stated otherwise, serum samples were eluted with 0.05 *M* Tris–HAc (pH 7.0) buffer containing 0.1 *M* sodium acetate at a flow-rate of 0.5 ml/min. The chromatographic procedure was performed at room temperature, with detection at 280 nm.

RESULTS AND DISCUSSION

Analysis time

The PDAMS-Q column separated albumin components with a 30-min linear gradient of sodium acetate from 0 to 0.5 *M* in 0.05 *M* Tris–HAc at various pH values (5.0–7.0) [15]. Sharp peaks were obtained, but the resolution of each component was incomplete.

In order to achieve a rapid separation of HSA suitable for clinical analysis, several buffer systems for isocratic elution were investigated. Fig. 1 shows typical HPLC profiles of HSA at pH 5.0 and 7.0. The best separation of HMA and HNA was achieved using isocratic elution with 0.05 *M* Tris–HAc buffer (pH 7.0) containing 0.1 *M* sodium acetate. The three main peaks observed in the chromatograms were characterized according to the original method [10,11].

From the chromatographic changes of HSA with dithiothreitol (DTT) reduction and treatment with cystine, it was established that the first peak was HMA and the second was HNA coupled with cysteine. The peak of HNA coupled with glutathione, HNA(Glu), reported by King [7] and Anderson [8], appeared as the fourth peak after treatment with oxidized glutathione. However, commercial HSA preparations contain only very small amounts of HNA(Glu), therefore the peak was not observed in the case of HSA alone. Since the third peak was unaltered by DTT reduction and treatment with cystine or oxidized glutathione, it is likely to be an oxidized form of HMA containing a sulphinic acid or sulphonic acid group [16]. Isocratic elution with 0.05 *M* Tris–HAc without sodium acetate at pH 5.1–5.5 separated the third peak (oxidized form), but the

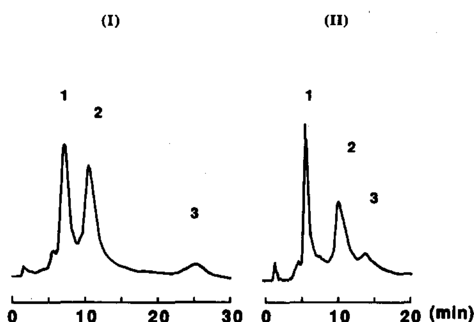

Fig. 1. Typical chromatograms of HSA at pH 5.1 (I) and pH 7.0 (II). Peaks: 1 = mercaptalbumin; 2 = nonmercaptalbumin; 3 = oxidized form. Column, PDAMS-Q (150 mm × 4 mm I.D.); eluent, (I) 0.05 *M* Tris–HAc at pH 5.1, (II) 0.05 *M* Tris–HAc containing 0.1 *M* sodium acetate (pH 7.0); flow-rate, 0.5 ml/min; UV detection wavelength, 280 nm; column temperature, ambient.

separation of HMA and HNA peaks was incomplete.

Regulation of retention

Fig. 2A shows the effect of the pH of the mobile phase without sodium acetate on the retention of the albumin components. The pH markedly affected the retention behaviour in the range 5.1–5.5. In this buffer system, the contribution of other factors, such as partition and hydrophobic interaction, other than ion exchange on the retention of HSA, is considered from the isoelectric point of HSA, in the range of 4.7–5.2. Fig. 2B shows the effect of the sodium acetate concentration added to Tris–HAc buffer (pH 7.0) on the retention of HSA components. The concentration of sodium acetate also significantly influenced the retention of albumin components. It seems that ion exchange is retentive at this pH. Thus the retention times of HSA components can be affected by a combination of pH values or by changing the concentration of salt in the buffer system.

Fig. 3A shows a chromatogram of a normal human serum sample on a PDAMS-Q column at a flow-rate of 0.5 ml/min. A large peak that eluted before HMA was mainly globulin and transferrin fractions, but it did not interfere with the quantification of the albumin components.

The effect of the flow-rate on the resolution of

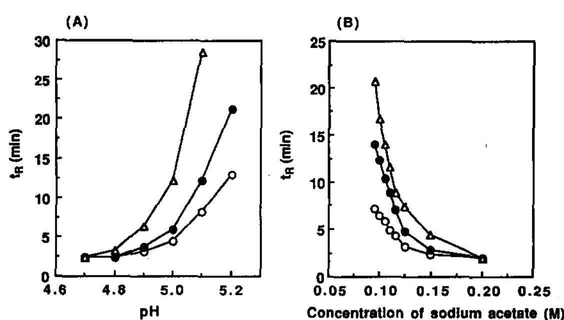

Fig. 2. Effect of (A) pH and (B) concentration of sodium acetate in the mobile phase on resolution of HSA components. HSA components: (○) mercaptalbumin; (●) nonmercaptalbumin; (△) oxidized form. Eluent, (A) 0.05 *M* Tris–HAc at various pH values, (B) 0.05 *M* Tris–HAc (pH 7.0) containing various concentrations of sodium acetate. Other chromatographic conditions as in Fig. 1.

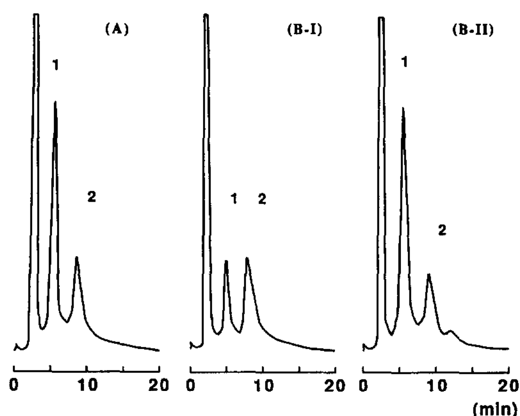

Fig. 3. Typical chromatograms of (A) healthy human serum and (B) haemodialysis patient serum (I) before and (II) after haemodialysis. Peaks: 1 = mercaptalbumin; 2 = nonmercaptalbumin. Eluent, 0.05 *M* Tris–HAc containing 0.1 *M* sodium acetate at pH 7.0. Other chromatographic conditions as in Fig. 1.

the albumin components is given in Table I, which shows that the degree of resolution of albumin components in serum is esssestially unchanged.

Regeneration of the column with 0.05 *M* Tris–HAc buffer containing 0.5 *M* sodium acetate was necessary for every 25 injections, which corresponded to the number of injections during one day. This simple treatment did not affect the resolution for at least 1000 injections.

Determination of the HMA fraction

The fraction of HMA in HSA (f_{HMA}) was determined by dividing the area under the peak corresponding to HMA by the total HSA area. The f_{HMA} value of the HSA preparation obtained by using Tris–HAc buffer (pH 7.0) at a flow-rate of 0.5 ml/min, was 0.49; this value was nearly equal to the sulphydryl group content determined by spectrophotometry with 2,2′-dithiodipyridine [17]. The mean ± S.D. value of f_{HMA} of eight healthy human sera was 0.76 ± 0.04, which was in reasonable agreement with published data (0.70 [8], 0.75 ± 0.025 [10]).

The precision of the method was assessed by repeated analysis of HSA and of a normal human serum. The mean ± S.D. values of f_{HMA} ± for HSA and the normal human serum were 0.49 ± 0.01 ($n = 10$) and 0.70 ± 0.03 ($n = 10$), respective-

TABLE I

PEAK RESOLUTION (R_s) OF HSA COMPONENTS IN SERUM AT VARIOUS FLOW-RATES

Chromatographic conditions as in Fig. 3A, except for the flow-rate.

Flow-rate (ml/min)	Retention time (min)			R_s	
	Globulin	HMA	HNA	Globulin-HMA	HMA-HNA
0.5	1.88	4.71	7.98	2.28	1.95
0.75	1.25	3.44	5.77	2.43	1.82
1.0	0.94	2.44	4.03	2.59	1.62

ly, and the coefficients of variation (C.V.) were 2.04 and 4.29%, respectively.

Application to chronic haemodialysis patient sera

We previously reported that the 4VP-EG-Me column could detect changes in the amounts of HMA in renal failure patient sera [13]. In order to further elucidate the validity of the proposed method, it was applied to the analysis of human sera obtained from haemodialysis patients with chronic renal failure. A typical HPLC profile of HSA in patient serum before and after haemodialysis is shown in Fig. 3B. Table II shows the values of f_{HMA} for fifteen haemodialysis patients. The f_{HMA} value increased after haemodialysis. The mean values were nearly equal to those reported previously [9,10].

TABLE II

VALUES OF f_{HMA} IN HAEMODIALYSIS PATIENT SERA

Chromatographic conditions as in Fig. 3A.

Sample No.	f_{HMA}	
	Before HD[a]	After HD
1	0.5812	0.7482
2	0.5830	0.7647
3	0.4934	0.7713
4	0.3737	0.6479
5	0.4896	0.7756
6	0.5558	0.7657
7	0.5270	0.7170
8	0.5016	0.7233
9	0.4421	0.6842
10	0.5264	0.7100
11	0.6204	0.7886
12	0.6365	0.7773
13	0.5356	0.7160
14	0.4824	0.6734
15	0.5883	0.7503

[a] HD = haemodialysis. Average ± S.D.: before HD, 0.53 ± 0.07; after HD, 0.73 ± 0.04.

CONCLUSION

The PDAMS-Q column used in an isocratic elution mode resolved HSA components within a short time. The proposed method is very simple and is suitable for clinical analysis. Application to the diagnosis of some diseases associated with a change of albumin components seems promising.

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