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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES ON NON-MERCAPT ⇌ MERCAPT CONVERSION OF HUMAN SERUM ALBUMIN. II*

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SUMMARY

High-performance liquid chromatographic (HPLC) analysis of human serum albumin (HSA) on a GS-520 column with 0.03 M sodium phosphate buffer–0.15 M sodium sulphate (pH 6.87) showed three peaks, the principal component corresponding to human mercaptalbumin (HMA) and the secondary and tertiary components to nonmercaptalbumin (HNA). Using HPLC analysis, the nonmercapt → mercapt conversion of HSA during haemodialysis and the mercapt → nonmercapt conversion after haemodialysis in chronic renal failure were re-confirmed, indicating that HMA is a covalent carrier protein for sulphur-containing amino acids. Fractions of HMA in various liver diseases were significantly lower than those of healthy male adults.

INTRODUCTION

Plasma albumin is a mixture of mercaptalbumin and non-mercaptalbumin[1–6]. Mercaptalbumin is prepared by using mercuric dimer of mercaptalbumin[1,2,4,7,8],

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* For Part I, see ref. 6.

DEAE-cellulose or DEAE-Sephadex[9-11], SE-Sephadex[12-15] or activated thiol-Sepharose[16]. In these methods, it is necessary to fractionate mercaptalbumin starting at least from Fraction V (*ca.* 95% plasma albumin) and it is therefore difficult to determine the fraction of mercaptalbumin from a small volume of serum or plasma (5–20 μl) by chromatography. While conducting high-performance liquid chromatographic (HPLC) studies on human serum albumin (HSA), we found a convenient HPLC column for the analysis of fractions of human mercaptalbumin (HMA), non-mercaptalbumin (HNA) and iodoacetamide-blocked mercaptalbumin at neutral pH, without using any pH or salt concentration gradients[5,6]. HNA is composed of at least two kinds of mixed disulphide compounds[5,6,17-19], one with cysteine [HNA(Cys)] and the other with glutathione [HNA(Glut)]. During HPLC of HSA with various buffer solutions, we found a convenient buffer for the resolution of HNA into HNA(Cys) and HNA(Glut). Using a convenient HPLC column[5,6] and buffer solution, the non-mercapt $\rightleftharpoons$ mercapt (HNA $\rightleftharpoons$ HMA) conversion of HSA[6] was re-examined in haemodialysis patients with chronic renal failure and analysed HSA in various liver diseases.

EXPERIMENTAL

Materials

HSA preparations were obtained from Calbiochem Behring (La Jolla, CA, U.S.A.) (Lot 101150), Nippon Seiyaku (Tokyo, Japan) (Lot N471) and Hyland (Glendale, CA, U.S.A.) (Lot 0625M, 127AA). HNA(Cys) and HNA(Glut) were prepared by the intermolecular sulphydryl–disulphide exchange reaction, as described previously[5,6]. Synthesized HNA(Cys) and HNA(Glut) were extensively dialysed against distilled water. Circular dichroism-resolved secondary structures[5,20], that is, values of f_α (fraction of α-helix) and f_β (fraction of β-structure) of HSA and HNA were approximately 0.72 and 0.12, respectively. The major fraction of synthesized HNA(Cys) contained a mixed disulphide with cysteine. Synthesized HNA(Glut) was composed of two kinds of mixed disulphide compounds, one with cysteine and the other with glutathione, HNA(Cys) content being high (*ca.* 40%) in a starting HSA preparation.

Plasma or serum of haemodialysis patients with chronic renal failure before and after haemodialysis were kindly supplied by Hayatoku Hospital (Gifu, Japan) and Sawada Hospital (Gifu, Japan). The haemodialysis of patients was carried out for 5 h with a hollow-fibre haemodialyser as previously described[6] under the following operating conditions: circulation speed of blood, 120–200 ml/min; flow-rate of dialysis fluid, 500 ml/min; dialysis fluid, 132 mM Na$^+$, 2 mM K$^+$, 1.25 mM Ca^{2+}, 0.75 mM Mg^{2+}, 105 mM Cl$^-$, 33 mM CH$_3$COO$^-$, 11.1 mM glucose. Plasma of healthy male adults and patients with various liver diseases was obtained after overnight fasting.

Chromatography

HPLC analyses of 10 μl of serum or plasma, HSA and HNA were carried out with a Plasmagraph (Asahi Medical, Tokyo, Japan), assembled as follows: a Degasser Model ERC-3110 (Ermer Optical Works, Tokyo, Japan); a Jasco Twincle pump (Japan Spectroscopic, Tokyo, Japan); eight columns of Asahipak GS-520H (Asahi Chemical Industry, Kawasaki, Japan) (25 × 0.75 cm I.D.; 28 ± 1 °C); a Jasco Uvidec 100 IV UV monitor; and an autosampler (Model AS-48, Toyo Soda, Tokyo, Japan;

Model 638-08, Hitachi, Tokyo, Japan; or Model 710B, Japan Waters Assoc., Osaka, Japan). Eluent buffer solutions were 0.03 M sodium phosphate buffer–0.15 M sodium sulphate (pH 6.87) (sulphate buffer) and 0.10 M sodium phosphate buffer–0.30 M sodium chloride (pH 6.86) (chloride buffer). They were filtered through a Millipore Sterivex-GS filter unit (0.22 μm) or a Triton X-100-free Millipore membrane (0.45 μm) (Millipore, Bedford, MA, U.S.A.). The flow-rates of the sulphate and chloride buffer solutions were 0.80 and 0.50 ml/min, respectively, and the sample size was 10 μl. Fractions of HMA (f_{HMA}) and HNA (f_{HNA}) were obtained by HPLC on eight GS-520H columns (25 × 0.75 cm I.D.)[5,6]. The value of f_{HMA} was calculated by dividing the area under the peak corresponding to HMA by the total HSA area. To obtain these respective areas, a graphical method of symmetrical resolution was employed[6].

RESULTS AND DISCUSSION

Effect of buffer salts on the resolution of HSA into HMA, HNA(Cys) and HNA(Glut)

HPLC profiles of HSA in sulphate buffer showed two major peaks, one corresponding to HMA and the other to HNA (the lower profile in Fig. 1, f_{HMA} = 0.63)[5,6]. Mild reduction of a mixed disulphide bond by glutathione (GSH) [HSA:GSH = 1:5; 0.03 M sodium phosphate buffer–0.15 M sodium chloride (pH 7.09)] for 4.5 h at 25°C increased the principal peak in the HPLC profile, indicating the principal peak to be HMA (the upper profile in Fig. 1), as previously reported[5]*.

HNA is composed of at least two kinds of mixed disulphide compounds[5,6,15,17–19], one with cysteine [HNA(Cys)] and the other with glutathione [HNA(Glut)]. During HPLC experiments with Asahipak GS-520H, we found that retention time of HNA(Glut) is longer than that of HNA(Cys) in sulphate buffer, resulting in splitting of the HNA into two components, one corresponding to HNA-(Cys) and the other to HNA(Glut), as shown in Fig. 2. The major fraction of synthesized HNA(Cys) contained a mixed disulphide with cysteine. Synthesized HNA(Glut) was composed of two kinds of mixed disulphide compounds, one with cysteine and the other with glutathione, the HNA(Cys) content being high (*ca.* 40%) in the starting HSA preparation. Thus, HPLC profiles of synthesized HNA(Glut) showed two peaks in sulphate buffer, one corresponding to HNA(Cys) and the other to HNA(Glut), as shown in Figs. 2 and 4. However, in chloride buffer, synthesized HNA(Glut), showed a broad peak, as shown in the left upper part of Fig. 2.

The lower three examples of HPLC profiles in Fig. 2 also clearly indicate that the resolution of HSA into HMA, HNA(Cys) and HNA(Glut) is far better in sulphate buffer than in chloride buffer. We analysed approximately 150 ,era and/or plasma (f_{HMA} = 0.2–0.8) using both buffer solutions and found a good linear correlation between f_{HMA} values with sulphate buffer and those with chloride buffer (data

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* Although evidence is indirect, the HMA subfraction prepared by SP-Toyopearl chromatography (Toyo Soda) at pH 4.30 and 3°C corresponded exactly to the HMA peak in Figs. 1–5 (sulphate buffer). As previously reported by us[5], synthesized HNA(Cys) monomer, prepared by Toyopearl HW-55 chromatography (chloride buffer), also corresponded exactly to the HNA peak (chloride buffer) and synthesized glucosylated HSA mainly showed the principal peak, indicated as HMA in the figures, because of reduction of a mixed disulphide bond of HNA by glucose. However, carbamylated HSA and the γ-glutamylcysteine derivative of HNA were not analysed with a GS-520H column. HPLC profiles of HSA were not affected by bound impurities, such as fatty acids[5].
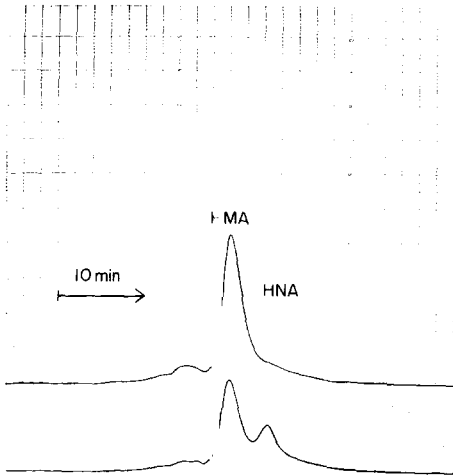
Fig. 1. The lower and upper HPLC profiles (280 nm) show HSA (f_{HMA} = 0.63) and glutathione-treated HSA (HSA:GSH = 1: i, mild reduction of a mixed disulphide bond for 4.5 h at 25°C in 0.03 M sodium phosphate buffer–0.15 M sodium chloride, pH 7.09), respectively. Flow-rate, 0.80 ml/min. Sample, 10 μl. Eluent, 0.03 M sodium phosphate buffer–0.15 M sodium sulphate, pH 6.87 (sulphate buffer). Column temperature, 28 ± 1°C. HMA and HNA indicate human mercaptalbumin and non-mercaptalbumin, respectively.

not shown). Unless stated otherwise, only results with sulphate buffer are given here. As shown in Table I, the mean value of f_{HMA} ± standard deviation (S.D.) for 28 healthy male adults of 0.75 ± 0.028 was similar to the 0.76 ± 0.025 (chloride buffer) previously reported by us[6].
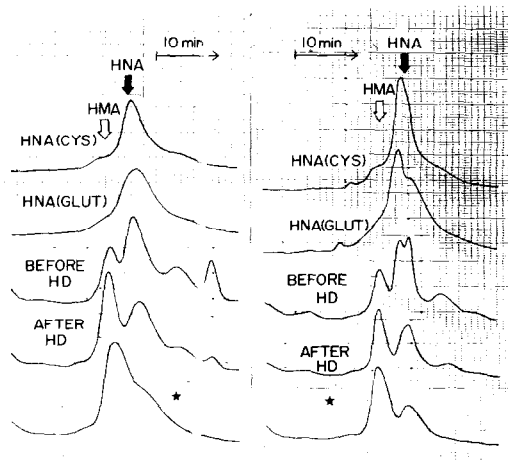

Fig. 2. HPLC profiles of synthesized HNA(Cys), synthesized HNA(Glut), plasma before and after haemodialysis (HD) and a rare example of HSA ($\star$), which did not show a clear resolution into HMA and HNA with chloride buffer. See text on synthesized HNA. The primary and secondary peaks in HNA (sulphate buffer) might correspond to HNA(Cys) and HNA(Glut), respectively. Left (0.10 M sodium phosphate buffer–0.30 M sodium chloride, pH 6.86): sample, 10 μl; flow-rate, 0.50 ml/min. Right (0.03 M sodium phosphate buffer–0.15 M sodium sulphate, pH 6.87): sample, 10 μl; flow-rate, 0.80 ml/min.

TABLE I

f_{HMA} VALUES IN PATIENTS WITH VARIOUS LIVER DISEASES*

Parameter	Control	CH	LC(c)	LC(d)	LC(c) and HCC	AH	FHF
f_{HMA}	0.75 ± 0.028	0.64 ± 0.053	0.64 ± 0.050	0.49 ± 0.076	0.52 ± 0.073	0.64 ± 0.046	0.33
n	28	23	9	9	10	5	2

(Significance brackets below the table, each marked ★★★ except one marked ★★, spanning various group comparisons.)

* CH = chronic hepatitis; LC(c) = liver cirrhosis (compensated); LC(d) = liver cirrhosis (decompensated); HCC = hepatocellular carcinoma; AH = acute hepatitis; FHF = fulminant hepatic failure.
** $P < 0.01$.
*** $P < 0.001$.

Non-mercapt ⇌ mercapt conversion of HSA in haemodialysis patients

Robins et al.[21] and Wilcken et al.[22] reported that cystine and homocysteine–cysteine mixed disulphide were significantly increased in patients with chronic renal failure and were decreased by haemodialysis for several hours. Isles and Jocelyn[23] and Sogami and co-workers[5,6] also reported that mercaptalbumin is easily converted into non-mercaptalbumin after incubation with cystine at neutral pH. We therefore analysed sera* of chronic renal failure patients by HPLC on Asahipak GS-520H. The mean value of f_{HMA} ± S.D. for 28 patients with chronic renal failure was 0.51 ± 0.096, indicating a significant increase in HNA content**. The present results support those of Isles and Jocelyn[23] as well as our own[6].

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* Kindly supplied by professor T. Koshiba (School of Medicine, Kitazato University, Sagamihara, Japan).
** We found that sera and/or plasma of chronic renal failure patients, stored at 3°C, should be analysed by HPLC within a few days or should be stored at −70 to −80°C, because the sera or plasma of the patients before haemodialysis were almost completely converted into HNA on storage at −20°C for 60 days. The major mechanism for HMA → HNA conversion on storage at −20°C might be due to the unfrozen water around the protein, as extensively studied by ^{1}H NMR spectroscopy, etc. (see ref. 24 and references cited therein).

In chronic haemodialysis patients, the HPLC profiles of HSA were completely different from those of healthy adults (Table I), as shown in Figs. 3–5. The mean values of f_{HMA} ± S.D. before haemodialysis for 293 patients (1983–84) and 122 patients (1984–85) were 0.41 ± 0.073 and 0.40 ± 0.093, respectively. These mean values were similar to the value of 0.45 ± 0.08 ($n = 112$) previously reported by Sogami et al.[6] (chloride buffer). After haemodialysis for 5 h, mean values of Δf_{HMA} ± S.D. [(f_{HMA} after haemodialysis) − (f_{HMA} before haemodialysis)] for 291 patients (1983–84) and 110 patients (1984–85) were 0.15 ± 0.086 and 0.17 ± 0.077, respectively. The Δf_{HMA} values were similar to the value of 0.16 ± 0.054 ($n = 100$) previously reported by Sogami et al.[6] (chloride buffer).

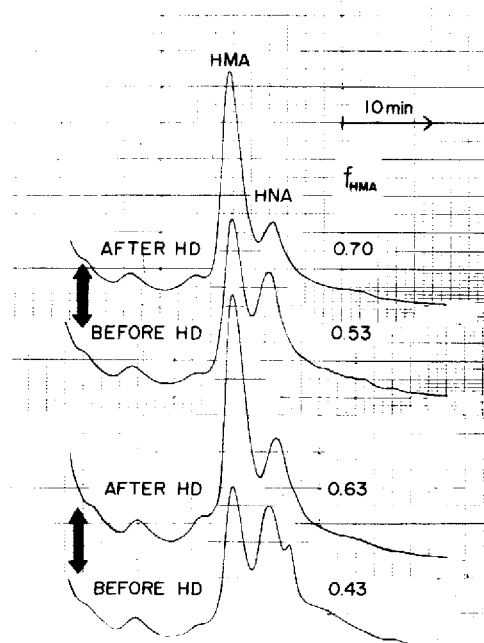Fig. 3. Two examples (HPLC profiles) of non-mercapt → mercapt conversion (HNA → HMA) of HSA by haemodialysis for 5 h in haemodialysis patients. The double arrow indicates data on the same patient before and after haemodialysis (HD) for 5 h. Eluent, sulphate buffer; sample, 10 μl; flow-rate, 0.80 ml/min.

Two examples of HPLC profiles of HSA in chronic renal failure before and after haemodialysis are shown in Fig. 3 and an example of the time course of HNA → HMA conversion of HSA during haemodialysis for 300 min is shown in Fig. 4.

As we have previously reported[5,6], the HPLC profiles of HSA, eluted from a GS-520H column, were not affected by tightly bound lipid impurities, such as fatty acids, and the retention time of HSA disulphide dimer was very different from those indicated as HMA and HNA. Mild reduction of HSA preparations [plasma before haemodialysis, such as the samples in Fig. 3 ($f_{HMA} = 0.43$) and Fig. 4 ($f_{HMA} = 0.32$)] by glutathione (HSA:GSH ≈ 1:5, 0.15 M sodium chloride, pH 7.09) for 100 min at 25°C increased the f_{HMA} values, indicating the principal peak of HSA to be HMA
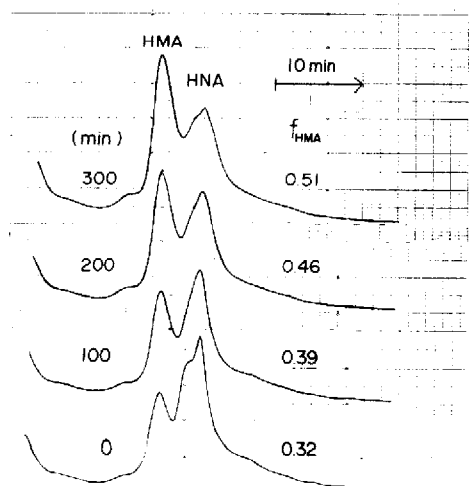
Fig. 4. Time course of non-mercapt → mercapt conversion of HSA in a haemodialysis patient during haemodialysis for 5 h. Eluent, sulphate buffer; sample, 10 μl; flow-rate, 0.80 ml/min.

(data not shown). The results in Figs. 3 and 4 suggested the conversion of HNA to HMA during haemodialysis, that is, non-mercapt → mercapt conversion (HNA → HMA) of HSA, as previously reported by Sogami et al.[6].

Sogami et al.[6] reported that no HNA → HMA conversion of HSA solution (f_{HMA} = 0.30) was observed on dialysis at 37°C when a haemodialyser was used under the same operating conditions. In order to study the HNA → HMA conversion mechanism, we carried out HPLC analyses of the inlet and outlet blood plasma of
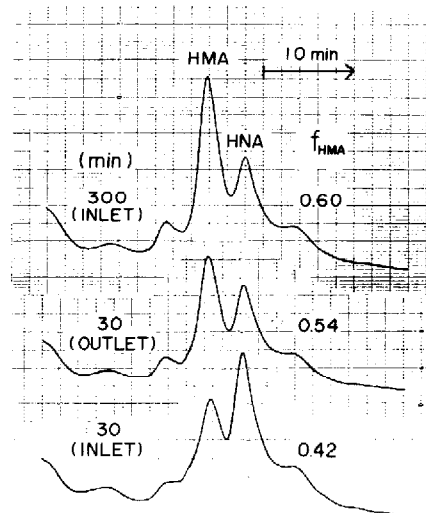

Fig. 5. Time course of non-mercapt → mercapt conversion of HSA in a haemodialysis patient during haemodialysis for 5 h. The lower and middle HPLC profiles are those for HSA of the inlet and outlet blood plasma of a hollow-fibre dialyser ca. 30 min after the start of haemodialysis, respectively. Eluent, sulphate buffer; sample, 10 μl; flow-rate, 0.80 ml/min.

the hollow-fibre dialyser *ca.* 30 min after the start of haemodialysis. The mean value of [(f_{HMA} of outlet plasma) − (f_{HMA} of inlet plasma)] ± S.D. for 122 haemodialysis patients was 0.092 ± 0.053, indicating the HNA → HMA conversion to be statistically significant ($P < 0.001$), as shown in Fig. 5. These results suggest some contribution of blood cells to HNA → HMA conversion of HSA during haemodialysis.

The concentration of cystine is extremely high in haemodialysis patients and is decreased by haemodialysis[21,22]. With decreasing cystine concentration on haemodialysis, HNA might be converted into HMA by certain cells, such as blood cells. With increasing cystine concentration in plasma after haemodialysis, the HMA → HNA conversion might be accelerated, as reported by Isles and Jocelyn[23] and Sogami et al.[6], according to

$$HMA + cystine \rightleftharpoons HNA + cysteine \tag{1}$$
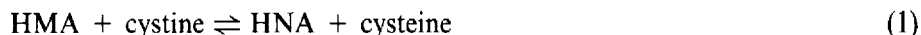
and the cysteine produced might be rapidly reoxidized to cystine for re-utilization[6,23]. The present results confirm our reported conclusion that HMA might be the important covalent carrier protein for sulphur-containing amino acids in haemodialysis patients, resulting in decreases of free concentrations of cystine, glutathione disulphide and mixed disulphide compounds, such as homocysteine–cysteine and cysteine–glutathione[6].

HPLC analyses of HSA in various liver diseases

Luetscher[25,26] studied HSA in chronic renal failure, nephrotic syndrome and liver cirrhosis by moving-boundary electrophoresis in acidic medium (pH range of the N–F transition[3]) and found significant differences in the N–F transition between HSA of normal healthy adults and those of the pathological states. We therefore re-examined HSA in various liver diseases, such as chronic hepatitis, liver cirrhosis, by HPLC analyses with a GS-520H column (sulphate buffer). The HPLC profiles of HSA in various liver diseases were completely different from those of normal healthy adults (HPLC profiles not shown). As shown in Table I, f_{HMA} values in various liver diseases were significantly lower than those of healthy male adults*.

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* Final manuscript now in preparation by Professor Y. Muto's group (1st Department of Internal Medicine, School of Medicine, Gifu University) and M. Sogami's group.

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