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Note

Separation of albumin-binding ligands present in uremic serum by high-performance affinity chromatography

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The decreased binding of anionic drugs and several endogenous substances to serum protein in patients with renal failure is now well documented [1,2]. The free fraction of the drugs is considered to interact with receptor sites to produce pharmacological responses. It is, therefore, clinically important to elucidate the mechanism of the inhibition of drug-binding to serum protein that is observed in renal failure. Considerable evidence has suggested that the accumulation of endogenous ligands that bind to serum albumin compete with the drugs for specific sites on albumin molecule [3–8]. Several such substances have been reported to be retained in uremic serum [9–12].

We and others have recently demonstrated that 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) is a major endogenous ligand retained in uremic serum [13–15]. CMPF inhibits phenytoin and dyes binding to serum protein or to human serum albumin (HSA) at concentrations usually observed in uremic serum [16,17]. Taking into consideration hypoalbuminemia usually found in uremia, however, CMPF does not entirely account for the impaired phenytoin binding observed in uremic serum [17]. Gulyassy et al. [18] also reported that the addition of hippuric acid and indoxyl sulphate separately or together to normal plasma showed that these ligands could account for only 15% of the impaired binding of salicylate by azotemic plasma. These observations suggest the presence of other potent drug-binding inhibitors, not yet

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identified, in uremic serum. Drug-binding inhibitors present in uremic serum are essentially endogenous ligands that bind to serum albumin.

This paper describes the separation of albumin-binding ligands present in both normal and uremic serum by high-performance affinity chromatography, and their analysis by reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials and samples

TSK-GEL Tresyl-5PW columns (7.5 cm×0.75 cm I.D.) for high-performance affinity chromatography were obtained from Tosoh (Tokyo, Japan). A prepacked Nucleosil $5C_{18}$ column (particle size 5 μm, 25 cm×0.46 cm I.D.), for reversed-phase HPLC, was from Chemco (Osaka, Japan). HSA, essentially fatty acid-free, was from Sigma (St. Louis, MO, U.S.A.). CMPF was synthesized in our laboratory [19]. Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and were of HPLC grade or of analytical grade.

Serum samples were obtained from five patients with chronic renal failure, who had been on maintenance hemodialysis three times a week for at least six months, and from three healthy persons. None of the subjects was receiving medication. The serum samples were stored at −70°C until use.

HPLC apparatus

We used a Shimadzu LC-6A system (Shimadzu, Kyoto, Japan), consisting of two Model LC-6A pumps, a Model SPD-6AV detector equipped with an 8-μl flow-cell, a Model SPD-M1A photodiode array UV–VIS spectrophotometric detector and a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.). Data were processed with a Shimadzu Model C-R3A recording integrator.

Preparation of high-performance affinity chromatography column

The coupling of HSA to the TSK-GEL Tresyl-5PW column support was carried out in situ according to the manufacturer's instructions at room temperature (20±2°C). The column was washed first with ten column volumes of distilled water, then with ten column volumes of 0.1 *M* phosphate buffer containing 0.5 *M* sodium chloride (pH 7.4) (coupling buffer). The HSA solution, 1.5 mg/ml in the coupling buffer, was then recycled overnight at a flow-rate of 0.5 ml/min. The remaining tresyl groups were deactivated with ca. 50 column volumes of 0.1 *M* Tris–HCl buffer (pH 8.0). The amount of coupled HSA was determined by the UV absorbance at 280 nm of the HSA solutions before and after coupling.

Separation of HSA-binding ligands

Serum was deproteinized by adding a two-fold volume of ethanol. After vortex-mixing, the sample was centrifuged at 35 000 *g* for 60 min and the supernatant was lyophilized. The lyophilized sample, obtained from 1 ml of serum, was reconstituted in 500 μl of 1/15 *M* phosphate buffer (pH 7.4) and subjected to high-performance affinity chromatographic separation in a sample volume of 100 μl.

We used 1/15 *M* phosphate buffer (pH 7.4) as the binding buffer. Several solvents, including 0.1 *M* acetate [20], were tested as the eluting solvent, with CMPF as a representative ligand. CMPF was applied to the affinity chromatography column and its detection in the eluate fractions was performed by the method previously reported [19]. We added the chaotrophic ion, CF_3COO^-, and an organic modifier, 2-propanol, to the eluting solvent to elute ligands tightly bound to HSA. We finally adopted an "aggressive" mobile phase, water–2-propanol–acetic acid–trifluoroacetic acid (1000:100:20:2, v/v) as the eluting solvent. In this eluting solvent, a loss in column-binding capacity was observed within twenty separations. The elution was performed at a flow-rate of 0.8 ml/min and the column effluent was monitored by UV absorbance at 280 nm at a sensitivity of 0.16 a.u.f.s.

Reversed-phase HPLC

The albumin-binding ligands separated by high-performance affinity chromatography were analysed by reversed-phase HPLC on a Nucleosil $5C_{18}$ column. The elution was achieved by the use of a linear gradient from acetonitrile–water–heptafluorobutyric acid (50:950:1, v/v; solvent A) to acetonitrile–water–heptafluorobutyric acid (500:500:1, v/v; solvent B) over 75 min, followed by isocratic elution with solvent B for 5 min at a flow-rate of 1.0 ml/min at room temperature. The column effluent was monitored by UV absorbance at 265 nm at a sensitivity of 0.04 a.u.f.s. We recorded the on-line UV spectra of the chromatographic peaks with a Model SPD-M1A photodiode array UV–VIS spectrophotometric detector. In addition, we analysed supernatants from ethanol-deproteinized sera by reversed-phase HPLC.

RESULTS AND DISCUSSION

HSA was coupled in situ to the high-performance affinity chromatography column by pumping the HSA solution through the column. The amount of coupled HSA calculated was 40 mg. The binding buffer was 1/15 *M* phosphate buffer (pH 7.4), and the eluting solvent was water–2-propanol–acetic acid–trifluoroacetic acid (1000:100:20:2, v/v). Despite the use of this "aggressive" solvent, CMPF still showed broad elution (Fig. 1). The elution profiles of normal and uremic serum samples are shown in Fig. 1. We chose UV absorbance at 280 nm to minimize the baseline elevation. The eluate fraction was collected

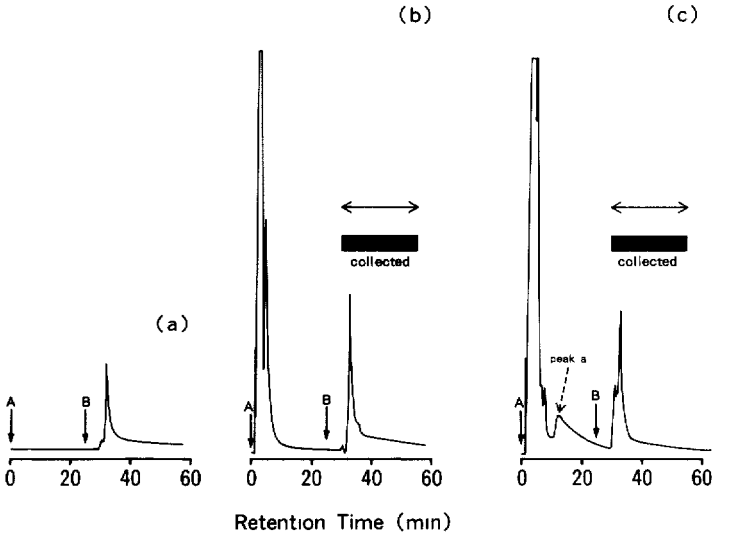

Fig. 1. Separation of HSA-binding ligands by high-performance affinity chromatography. (a) Blank run; (b) normal samples; (c) uremic samples. Double-headed arrows indicate the elution range of CMPF. Single-headed arrows A and B indicate the binding buffer and the eluting solvent, respectively.

as marked, lyophilized, reconstituted in 500 μl of solvent A and subjected to analysis by reversed-phase HPLC in a sample volume of 200 μl.

The elution profiles of normal and uremic samples obtained from affinity chromatographic separations are shown in Fig. 2. Peaks showing signal-to-noise ratios of over 5:1 were assumed to be significant. The numbered peaks were detected in all uremic samples, and some of these peaks were also detected in normal samples. However, we could not rigorously determine whether or not the peaks showing the same retention times in both normal and uremic samples were the same substances. We identified the peaks by comparison of the retention times and the UV spectra of the peaks with those of the authentic substances.

Peak 3 and peak 8 had the highest UV absorbance among uremic samples (Fig. 2). The retention time and the UV spectrum (Fig. 3) indicated that peak 8 was CMPF. The retention time and the UV spectrum (data not shown) of peak 3, however, indicated that this is a hitherto unknown ligand in uremic serum.

Previously known endogenous ligands, other than CMPF, present in uremic serum, such as indoxyl sulphate, hippuric acid, indole-3-acetic acid or tryptophan [9–12], were not detected in the samples separated by affinity chromatography (Fig. 2). The affinity of these ligands for HSA may be weak compared with that of CMPF, based on the degree of free fractions in uremic serum [21].

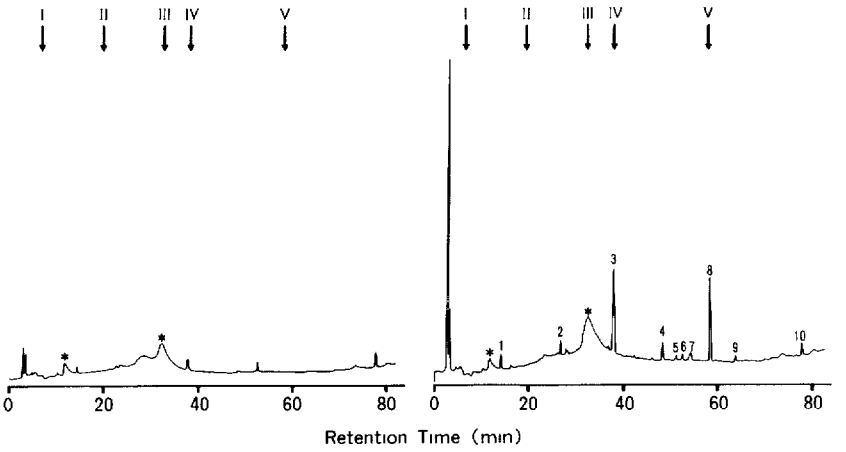

Fig. 2. Typical reversed-phase HPLC profiles of normal (left) and uremic (right) samples obtained from affinity chromatographic separation. Arrows I–V indicate the elution positions of indoxyl sulphate, hippuric acid, tryptophan, indole-3-acetic acid and CMPF, respectively. Asterisks indicate ghost peaks.

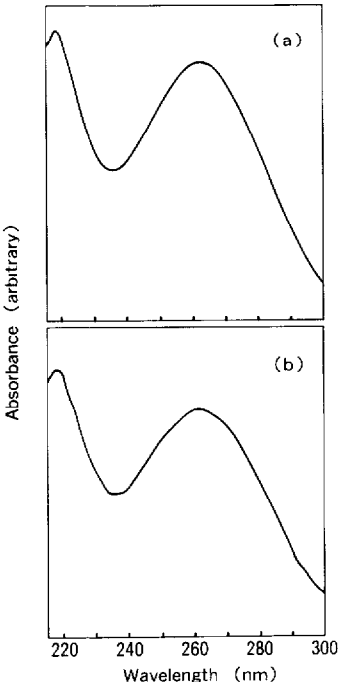

Fig. 3. UV spectra, recorded on-line, of (a) authentic CMPF and (b) peak 8 in Fig. 2.

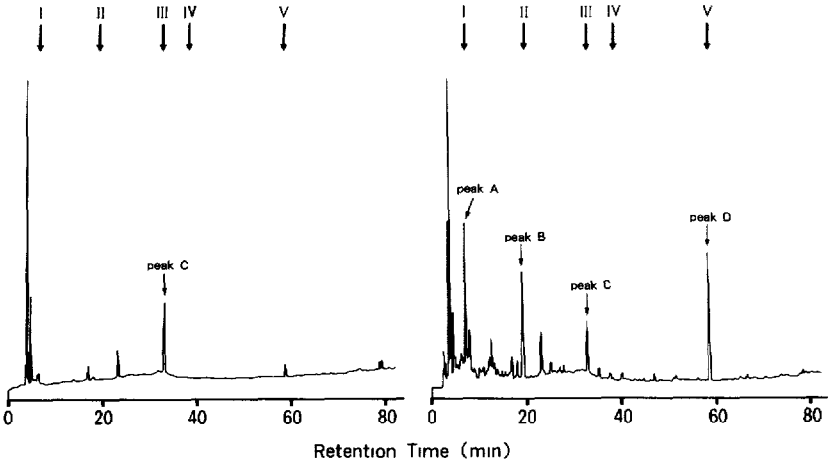

Fig. 4. Typical reversed-phase HPLC chromatograms of supernatants from ethanol-deproteinized normal (left) and uremic (right) sera. Arrows I–V are as in Fig. 2. For identification of peaks A–D, see text. Sample volume, 20 μl.

It can, therefore, be assumed that the endogenous ligands detected in this study may have strong affinity for HSA.

In recent years, immobilized bovine serum albumin (BSA) on silica has been used as a chiral stationary phase for the resolution of enantiomers [22–24]. The results of enantiomer analysis on BSA-silica columns indicate that weakly bound ligands are eluted with the binding buffer, and "peak a" in Fig. 1c contains these ligands.

The elution profiles of supernatants from ethanol-deproteinized sera are shown in Fig. 4. The retention times and the UV spectra (data not shown) indicated that peaks A–D were indoxyl sulphate, hippuric acid, tryptophan and CMPF, respectively. We could not find several ligands, detected in the uremic samples separated by affinity chromatography, by direct analysis of supernatants from ethanol-deproteinized sera. Therefore, it remains to be clarified whether or not these ligands are drug-binding inhibitors in uremic serum.

In conclusion, our results indicate that HSA coupled to a high-performance affinity chromatography column can be used for the detection and separation of HSA-binding ligands with strong affinity for HSA in uremic serum, and that these ligands detected are candidates for drug-binding inhibitors other than CMPF in uremic serum.

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