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Note

Determination of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid, a major endogenous ligand substance in uremic serum, by high-performance liquid chromatography with ultraviolet detection

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Serum protein binding of acidic drugs and some endogenous substances is decreased in patients with renal failure [1–3]. In recent years, many authors have reported that the accumulation of endogenous ligand substances that bind to serum protein compete with drug binding in uremic serum [4–9]. Several substances have been isolated and characterized as the endogenous drug-binding inhibitors present in uremic body fluids [7–9]. The drug-binding inhibitors present in uremic serum are essentially endogenous ligand substances that bind to serum protein.

We have recently used high-performance liquid chromatography (HPLC) to analyse and isolate a major endogenous ligand substance that inhibits the binding of diphenylhydantoin and tryptophan to plasma protein from uremic serum [10, 11]. Gas chromatographic–mass spectrometric (GC–MS) analysis revealed that this substance is 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), which is known to be a constituent of urine obtained from healthy subjects [12, 13].

In this paper, we present a rapid procedure for the estimation of the serum concentrations of CMPF by HPLC. The method is simple and does not require extensive sample treatment, such as extraction or derivatization. In addition, indole-3-acetic acid (IAA), which is another endogenous ligand substance in uremic serum [10], is simultaneously determined. Using the method described, the effect of haemodialysis (HD) on the serum concentrations of CMPF and IAA is evaluated.

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EXPERIMENTAL

Chemicals

The chemical synthesis of CMPF was performed using a method for other furanoid acids [13, 14]. The purity of CMPF was checked by thin-layer chromatography, HPLC and gas chromatography after derivatization with diazomethane.

All other chemicals including IAA were obtained from Nakarai (Kyoto, Japan) and were of HPLC or analytical grade.

Chromatography

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used, which included a Model SIL-1A injector with a 200-μl sample loop, a Model SPD-2A variable-wavelength UV detector equipped with an 8-μl flow-cell, and a Model SPD-M1A photodiode array UV–VIS spectrophotometric detector.

The HPLC analysis was performed according to the method previously reported, with some modifications [10, 11]. The HPLC column used was a Nucleosil 5-C_{18} (5 μm, 25×0.46 cm I.D.; Chemco, Osaka, Japan) in tandem with a guard column (3×0.4 cm I.D.). The elution solvent was acetonitrile–water–heptafluorobutyric acid (40:60:0.1, v/v/v). The elution was isocratic at a flow-rate of 1.0 ml/min at room temperature. The column effluent was monitored by UV absorbance at 215 nm. The sensitivity of the detector was set at 0.16 absorbance units full scale (a.u.f.s.). The UV spectra of chromatogram peaks were recorded on-line with an SPD-M1A photodiode array UV–VIS spectrophotometric detector. Identification of the IAA and CMPF peaks was based on the retention times, UV spectra and co-chromatography with the reference substances. Data were processed by a Chromatopac C-R1A recording integrator (Shimadzu). Quantitative calculations were based on peak-height measurements.

Sample preparation

Serum samples were obtained from thirteen haemodialysed patients (seven males and six females, aged 37–69 years), who underwent maintenance HD for 43–159 months, on the intake of few or no drugs, and from seven healthy subjects (three males and four females, aged 18–39 years). Fasting blood was collected by venipuncture. Blood was collected from patients either just prior to HD or just after HD. Since patients received heparin during HD, plasma was obtained after HD. The plasma was kept at 4°C for 48–72 h, and fibrin was rigorously removed. Serum was treated by the method previously reported [10, 11]. Briefly, serum was boiled at 100°C for 5 min to obtain total CMPF (free and bound fractions) and after centrifugation at 40 000 *g* for 60 min, the supernatant was subjected to HPLC analysis in a volume of 10 μl.

Because of the limited solubility of CMPF in distilled water, CMPF was dissolved in acetonitrile and diluted with distilled water, and used as a reference solution. This solution was stable at 4°C for at least two weeks. The reference solution of IAA was made in distilled water. This solution was unstable at 4°C. All samples, including the reference solutions, were stored at −70°C until use.

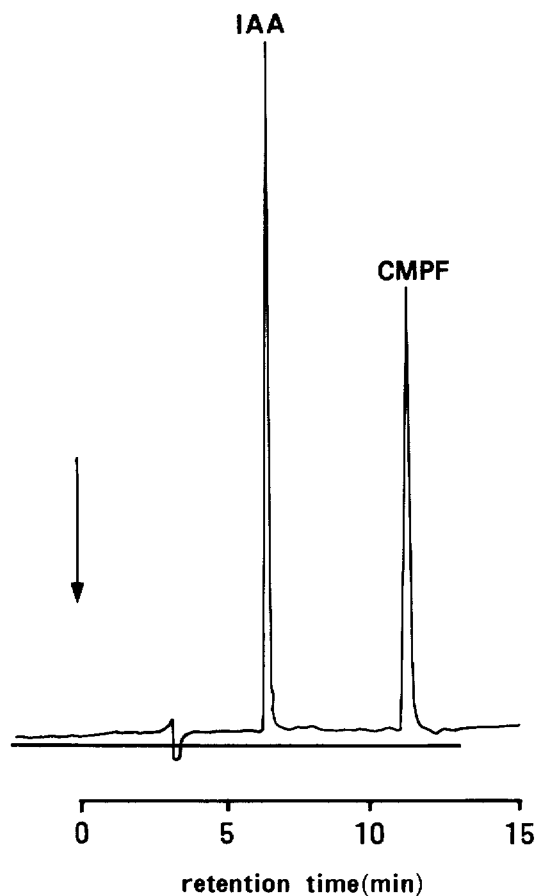

Fig. 1. Chromatogram of a standard mixture containing 122 ng of IAA and 330 ng of CMPF.

Determination of serum albumin

Serum albumin concentration was determined by laser nephelometry using a Behring Nephelometer analyzer (Hoechst Japan, Tokyo, Japan). The molar ratios of albumin to IAA and albumin to CMPF were used as a "serum IAA index" and a "serum CMPF index", respectively.

Statistics

The results are expressed as means ± S.D., and the probability was determined using the Student's t-test.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of IAA and CMPF standards. The analysis time was ca. 13 min. Peaks were identified by matching the retention times and the UV spectra of peaks from serum samples with those of the reference substances, and co-chromatography with the reference substances. The calibration curves of

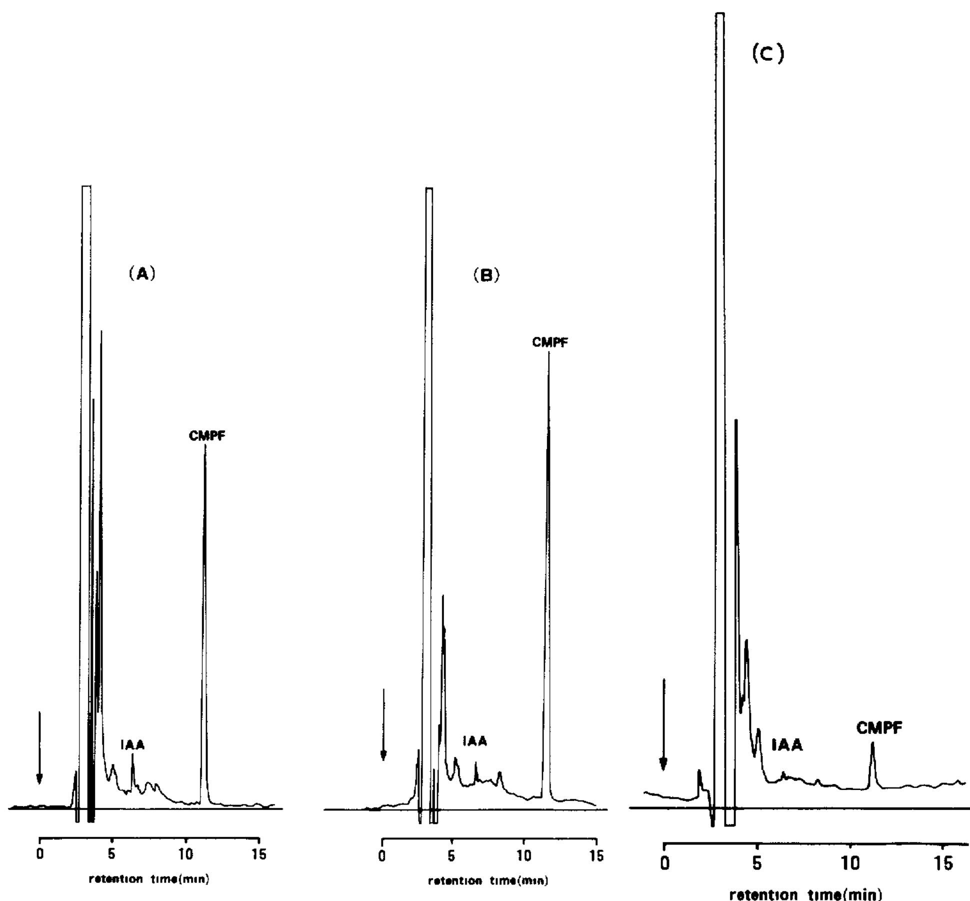

Fig. 2. Chromatograms of serum samples: (A) pre-HD sample; (B) post-HD sample; (C) a healthy subject. Serum concentrations of IAA of pre- and post-HD samples are 8.7 and 5.7 μmol/l, respectively. Serum concentrations of CMPF of pre-HD, post-HD and a healthy subject are 0.227, 0.291 and 0.023 mmol/l, respectively.

IAA and CMPF are linear over a wide range of concentrations and passed through the origin (data not shown). The detection limits of serum concentrations in a sample volume of 10 μl are 2 μmol/l for IAA and 3 μmol/l for CMPF at a signal-to-noise ratio of 3:1. The serum concentrations of IAA obtained from healthy subjects are below the detection limit with the method described. Increased sensitivity can be obtained by increasing the sample volume and/or the sensitivity setting of the detector.

Preliminary experiments revealed that the boiling at 100°C for 5 min had no effect on the reference solutions of IAA and CMPF. Also, denaturation of serum protein with the use of trifluoroacetic acid or heptafluorobutyric acid led to the same results as the boiling procedure. Therefore, we adopted the boiling method for serum treatment to avoid sample dilution.

Representative chromatograms of serum samples are shown in Fig. 2. IAA and CMPF are sufficiently resolved to allow accurate quantification in uremic serum

TABLE I

PRECISION OF THE ASSAY FOR IAA AND CMPF IN SERUM SAMPLES

Sample	Compound	Concentration (μmol/l)	n	Coefficient of variation (%)
HD patient	IAA	8.9±0.1	5	1.2
	CMPF	175.0±1.0	5	0.6
Normal	CMPF	24.8±0.4	5	1.8

samples as previously reported [10, 11]. Serum samples obtained from a uremic and a healthy subject were used for precision studies. The precision of the assay is shown in Table I. The analytical results of serum samples from hemodialysed patients and healthy subjects are given in Table II. The results reveal the marked elevation of the serum concentrations of CMPF in uremic compared with healthy subjects. The serum concentrations of IAA in uremia is compatible with the value previously reported [15].

The effect of HD on the serum concentrations of IAA and CMPF is studied. Fig. 2A and Fig. 2B show typical chromatograms of pre- and post-HD samples, respectively. Despite a decrease of the peak height of IAA, the peak height of CMPF is increased after HD. This phenomenon is probably due to haemoconcentration following dialytic therapy. To evaluate this hypothesis, the molar ratio of serum albumin to CMPF, which is designated as a serum CMPF index, is compared. Serum albumin concentrations before and after HD are 37.9±3.6 and 43±4.9 g/l, respectively ($P<0.0001$). The changes of serum CMPF indices following HD are not significant, despite apparent serum concentrations are significantly increased after HD (Fig. 3). These observations indicate that the increase of the apparent serum concentrations of CMPF is accompanied by an increase of serum albumin concentrations due to haemoconcentration. These results also suggest that CMPF is dialysed to a very limited extent, if at all, because of its high affinity for serum protein. Our results do not agree with those of Liebich et al. [16], who reported that the mean elimination rate of CMPF following HD is 21% by GC–MS analysis. The results reported here, however, indicate that the

TABLE II

DETERMINATION OF IAA AND CMPF IN SERUM FROM HAEMODIALYSED PATIENTS AND HEALTHY SUBJECTS

N.D. = not detectable

Subject	IAA (μmol/l)	IAA index ($\times 10^{-3}$)	CMPF (mmol/l)	CMPF index
Normal ($n=7$)	N.D.	N.D.	0.02±0.009	0.033±0.014
HD patients ($n=13$)				
Pre-HD	12.0±4.1	21.6±9.0	0.183±0.038	0.321±0 060
Post-HD	9.1±2.9	13.0±6.1	0.210±0.047	0.323±0.065

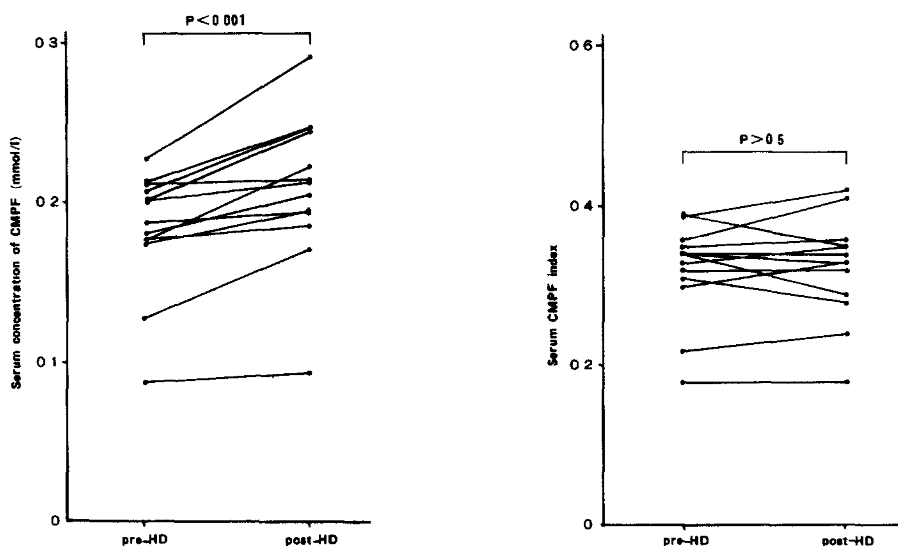

Fig. 3. Serum concentration of CMPF and serum CMPF index of pre- and post-HD samples.

elimination rate of ligand substances present in uremic serum cannot be calculated simply by comparing the changes of apparent serum concentrations. Fig. 4 shows the change of apparent serum concentrations of IAA and serum IAA indices following HD. These results indicate that IAA is a dialysable ligand substance as previously reported [17, 18]. Calculation of the percentage decrease of IAA following HD is based on the changes of apparent serum concentrations and serum IAA indices, which are 32.0 ± 8.9 and 39.8 ± 9.7, respectively. The value based on serum IAA indices is significantly larger than those of apparent serum concentrations ($P < 0.0001$). It might be expected that the elimination rate of IAA is larger than the value estimated by the changes of apparent serum concentrations.

In recent years, several attempts have been made to isolate and characterize the endogenous ligand substances present in uremic body fluids that inhibit the binding of drugs to serum protein [7–9]. Indoxylsulphate, 2-hydroxybenzoylglycine, *p*-hydroxyphenylacetic acid and 3-(3-hydroxyphenol)-3-hydroxypropanoic acid have been isolated and reported to be the endogenous drug-binding inhibitors in uremic serum [7–9]. Some of these substances cannot always be detected in uremic serum by HPLC [10], and the serum concentrations have not been adequately evaluated except for indoxylsulphate [19]. Our previously report demonstrated the presence of many endogenous ligand substances in uremic serum [10]. Despite the fact that no single substance is responsible for the drug-binding inhibition in uremic serum, it might be expected that CMPF is a major endogenous ligand substance and a drug-binding inhibitor because of its high affinity for serum protein and its abundance in uremic serum.

It is important to investigate CMPF as a major endogenous ligand substance and one of the so-called "uremic toxins" to understand a variety of biochemical and metabolic abnormalities that are observed in uremia despite adequate dialytic therapy. Until now, no specific quantitative correlation has been shown between

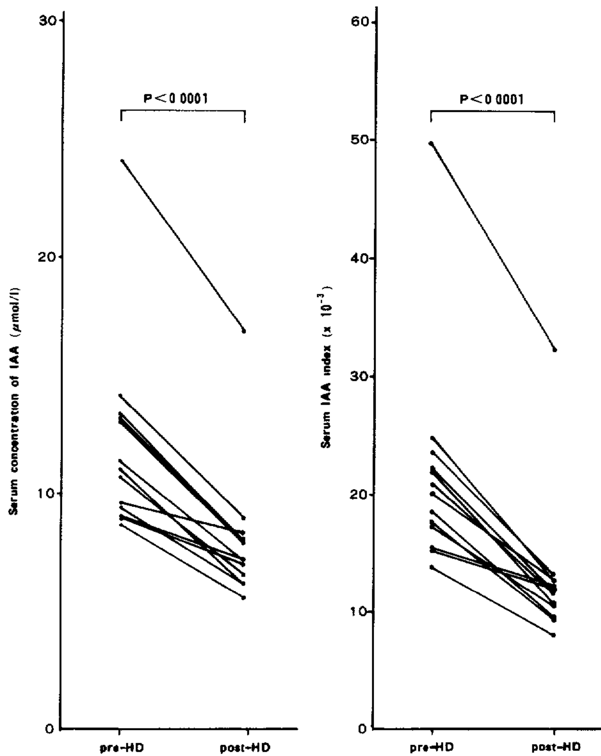

Fig. 4. Serum concentration of IAA and serum IAA index of pre- and post-HD samples.

the serum concentrations of the uremic toxins and observed uremic toxicities. Therefore, it is very important to elucidate the specific quantitative correlation between the serum concentrations of CMPF and the drug inhibition observed in uremic serum. This work is now in progress in our laboratory.

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