Identification and Quantification of a Protein-Bound Ligand in Uremic Serum
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A major protein-bound ligand in the serum of chronic hemodialysis patients was isolated from heat-deproteinized uremic serum by “high-performance” liquid chromatography (HPLC). The isolated compound was identified as 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid by use of liquid secondary-ion mass spectrometry and gas chromatography–mass spectrometry. On HPLC, an authentic sample of this compound showed a retention time identical to that of the protein-bound ligand peak. The concentration of the furan-carboxylic acid in serum, as estimated by HPLC, is markedly greater in chronic hemodialysis patients than in normal subjects.

Additional Keyphrases: chromatography, liquid · mass spectrometry · gas chromatography · hemodialysis patients · uremia

Binding to albumin of many acidic drugs and of some endogenous metabolites in serum is decreased in uremia (1–3). Because the intensity of a drug’s action is related to its concentration of plasma water, such a decrease can affect the therapeutic efficacy (or the toxicity) of acidic drugs.

In uremic patients the concentration of albumin in serum tends to be low, but the decrease in the binding of drugs cannot be explained by hypoalbuminemia alone (1). Two hypotheses have been proposed to explain the binding defect: albumin may undergo structural changes (4, 5), or some endogenous metabolites may bind the albumin, thereby inhibiting its binding of drugs (6–11). The latter possibility is supported by the correction of the defect in plasma binding by treating the acidified serum with charcoal (6–8), n-butyl chloride (9), hydrophobic resin (10), or anion-exchange resin (11).

The endogenous metabolites identified so far as inhibitors of albumin binding are indoxylsulfate (12, 13), hippuric acid (12, 14, 15), 2-hydroxybenzoylglycine (16), 3-(3-hydroxyphenyl)-3-hydroxypropanoic acid (14, 15), 4-hydroxyphenylacetic acid (14, 15), and indole-3-acetic acid (13). Recently, Mabuchi and Nakahashi (17) profiled the endogenous ligands in uremic serum by “high performance” liquid chromatography (HPLC). They proposed that an unidentified compound corresponding to "peak P" in the chromatogram was a major inhibitor of drug-binding in uremic patients. Here we report the isolation, chemical identification, and quantification of this substance.

Materials and Methods

Chemicals

Trifluoroacetic acid was supplied by E. Merck, Darmstadt, F.R.G. The water and acetonitrile used in the HPLC mobile phase were distilled, then filtered through a 0.45-μm (pore-size) membrane filter (Toyo Roshi Co. Ltd., Tokyo, Japan) before use. Authentic 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid was a gift (see Acknowledgment).

Samples and Sample Preparation

Serum samples were obtained from eight healthy subjects and from 27 uremic patients maintained on chronic hemodialysis. Venous blood was collected from the uremic patients immediately before hemodialysis.

We deproteinized the serum samples according to the method of Mabuchi and Nakahashi (17) by boiling 2 mL of sample at 100 °C for 5 min. On cooling to room temperature, the supernate was centrifuged at 30 min at 4280 x g to remove protein. After filtering the supernate through another 0.45-μm (pore-size) filter (Biofield Co. Ltd., Tokyo, Japan), we injected 100 μL of it onto the HPLC column.

To determine the ligands that were not bound to serum protein, we passed uremic serum through a CF-25 ultrafiltration membrane filter (Amicon Co., Lexington, MA), then chromatographed 100 μL of the ultrafiltrate.

HPLC Apparatus and Chromatographic Conditions

We used a chromatographic assembly from Jasco Co. Ltd., Tokyo, Japan. This consisted of a Model Trirotar-V pump equipped with a Rhedyne 7125 injection valve and a variable-wavelength detector (Uvidec-100V; Jasco Co. Ltd.). The column packing was Chemcosorb 5-ODS-H (particle size 5-μm, 300 x 7.5 mm i.d.), from Chemco Co. Ltd., Osaka, Japan. We eluted the column isocratically with acetonitrile/water/trifluoroacetic acid (40/60/0.08, by vol) at a flow rate of 2.2 mL/min at room temperature, and monitored the column effluent at 270 nm.

Quantification Assay

To quantify the furancarboxylic acid, we used the same analytical procedure as that used to profile the compounds in the uremic sera. We prepared a standard curve by assaying solutions of various amounts of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid in acetonitrile/water (4/6 by vol). Determination of the peak areas of the samples, relative to the peak area of the standard, yielded the linear regression equation, y = 0.465x + 0.015 (r = 0.99986).

We assessed analytical recovery by assaying five 2-mL aliquots of control serum to which known amounts of the furan carboxylic acid had been added and comparing the peak areas from the heat-deproteinized samples with those obtained from a direct injection of the same amount of authentic standard in acetonitrile/water (4/6 by vol). Recoveries exceeded 85% in most instances.

Gas Chromatography–Mass Spectrometry (GC–MS)

Authentic 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid and the HPLC effluent fraction obtained from the supernate of the heat-deproteinized serum were used in

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3 Nonstandard abbreviations: HPLC, "high-performance" liquid chromatography; GC–MS, gas chromatography–mass spectrometry; sisma, secondary-ion mass spectrometry.
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identifying a protein-bound ligand.

We injected 0.3 mL of the supernate of the heat-deproteinized serum into the HPLC system and so obtained a sufficient amount of sample for GC-MS analysis. The HPLC eluate was evaporated under reduced pressure and a solution of diazomethane in diethyl ether was added to the residue dropwise until the reaction mixture became yellow. The reaction mixture was left at room temperature for 12 h, then evaporated under nitrogen. After reconstituting the residue in 15 μL of ethyl acetate, we injected 5-μL aliquots into the GC–MS. Authentic 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, methylated by the same procedure, was redissolved in ethyl acetate to give a concentration of 5 g/L and 1 μL was injected into the GC–MS.

We used a Model 9A gas chromatograph coupled to a GSMS-QP1000 quadrupole mass spectrometer (both from Shimadzu Co. Ltd., Kyoto, Japan). The mass spectra from automatic repetitive scanning at 3-s intervals were recorded over the mass range m/z 50–350 and stored on a floppy disk. The GC conditions were as follows. A 1-m glass column packed with Gas Chrom Q coated with 2% OV-101, 100–120 mesh (Shimadzu Co.); carrier gas, helium; flow rate 50 mL/min; column temperature, 80 °C for 1 min, then programmed to 270 °C at 5 °C/min; injection block temperature, 300 °C. The MS conditions were as follows: ionization energy for the mass spectra, 70 eV; ion source temperature, 250 °C.

Liquid Secondary-Ion Mass Spectrometry (SIMS)

The HPLC fraction obtained from 1.1 mL of heat-deproteinized serum supernate was evaporated and the residue was redissolved in 20 μL of water. We mixed 1 μL of this solution with about 1 μL of glycerol on a silver-plated SIMS probe.

For liquid SIMS we used a double-focusing Model M-80B mass spectrometer fitted with a Model M-8099 high-field magnet, SIMS source, and Model M-0101 data system (all from Hitachi Co. Ltd., Tokyo, Japan). Operating conditions were: primary ion, Xe⁺; accelerating voltage, 8 kV (primary) and −3 kV (secondary); and source at ambient temperature.

Results

Figure 1 shows typical liquid chromatograms for equal volumes of supernate from heat-deproteinized uremic serum, ultrafiltrate of uremic serum, and supernatate from heat-deproteinized nonuremic serum. The characteristic peak being investigated, designated as "peak P" by Mabuchi and Nakahashi (17), is eluted 17.6 min after sample injection. The heat-deproteinized samples from chronic hemodialysis patients (Figure 1b) show high concentrations of peak P for sera, but a just-detectable peak P in the ultrafiltrate of this uremic serum (Figure 1c). Evidently the substance corresponding to peak P is tightly bound to protein.

To clarify the structure of this protein-bound ligand accumulated in uremic serum, we collected and combined eluates of serum samples containing peak P, concentrated this by evaporation, and then subjected the residue to mass spectral analysis. Figure 2 shows the positive and negative ion SIMS spectra for this residue. Determination of molecular
mass is less straightforward from the spectrum of positive ion mode than of the negative ion mode. The peak at m/z 239 observed in the negative ionization mode corresponds to the quasi-molecular ion, [M − H]−, of the intact protein-bound ligand, whereas the peaks at m/z 245 and 223 in the positive ionization mode could be assigned to the dehydrated ions [(M + Na) − H2O]+ and [(M − H − H2O)]+, respectively.

Methylation with diazomethane followed by GC-MS was a powerful technique for making structural identifications. The electron-ionization mass spectrum of the GC peak eluted at 17.0 min exhibited [M]+ at m/z 268 and fragments at m/z 239, 237, 208, 195, 179, and 147 (Figure 3b), consistent with its assignment as 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid dimethyl ester (18). The total and selected ion monitoring chromatograms of methylated residue from the uremic serum showed responses identical with those for the authentic dimethyl ester (Figure 3). In fact, the electron-ionization mass spectrum of the authentic furanarboxylic acid dimethyl ester (Figure 4a) was identical with that shown in Figure 4b. Furthermore, the HPLC retention time for the authentic furancarboxylic acid was identical to that of peak P (Figure 1a).

We quantified the furancarboxylic acid by HPLC without using an internal standard. In nonuremic sera the mean concentration of furancarboxylic acid was 6.6 (SD 2.8) mg/L (n = 8), as compared with the 38.6 (SD 11.4) mg/L in heat-deproteinized uremic serum (n = 27).

Fig. 3. The total and selected ion monitoring chromatograms of authentic 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid dimethyl ester (a) and methylated residue from the supernate of a heat-deproteinized uremic serum (b)

Discussion

The decreased binding of various acidic drugs to protein in sera from uremic patients is generally attributed to the accumulation of endogenous metabolites that bind to serum albumin and competitively inhibit the binding of drugs.

Mabuchi and Nakahashi (17), using HPLC, considered peak P in their chromatograms to correspond to a major inhibitor of drug-binding in uremia.

Using the same HPLC method, we have now isolated peak P from uremic serum, and have identified it as 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid by liquid GC, and GC-MS. We also used the HPLC method to quantify the concentrations of furancarboxylic acid in sera from uremic patients and healthy subjects. Deproteinization of serum by heating at 100 °C for 5 min was appropriate for this procedure: not only is it easy and rapid, it also gives high analytical recoveries of added authentic compound. The concentrations of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid in sera were highly increased in all uremic patients as compared with healthy subjects, and the acid was bound to serum protein, being almost absent from ultrafiltrate of uremic serum.

The accumulated 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid in uremic serum cannot be removed efficiently by conventional hemodialysis, because the lipophilic acids bind tightly to serum protein (<5% is unbound in uremic serum).

3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid, first detected in urine from healthy subjects by Spiteller and Spiteller (18), was also detected and quantified in sera from uremic patients by Liebich et al. (19), who used preparative thin-layer chromatography and GC-MS. Their quantification method is laborious and time-consuming. Our HPLC method is rapid, and requires only 20 µL of supernate from heat-deproteinized serum.

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References

Enhanced-Latex-Agglutination Assay for C-Reactive Protein in Serum, with Use of a Centrifugal Analyzer

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This is an improved assay of C-reactive protein in serum, for use with the Baker "Encore" centrifugal analyzer. Features of this assay include: 250-specimen throughput per hour, within-batch CV 2.2%, between-batch CV 2.7%, no antigen-excess problems up to 1000 mg/L, negligible interference from rheumatoid factor, and good correlation (r = 0.99) with radial immunodiffusion. The method is inexpensive and automated, involving no predilution steps. It can be adapted for use in a wide range of systems and can be used for single urgent estimations.

Additional Keyphrases: acute-phase proteins • radial immunodiffusion compared

C-reactive protein (CRP; \(M_r 110,000-140,000\)), the classic acute-phase protein of human serum, is synthesized by hepatocytes. Normally, it is present only in trace amounts in serum, but it can increase by as much as 1000-fold in response to injury or infection (1,2). In vitro, complexes with CRP can activate the complement system, promote phagocytosis, inhibit platelet aggregation, and interact with and activate certain sub-populations of lymphocytes (3–5). Despite these findings, the actual biological function of CRP has not yet been defined, although it probably acts primarily as a protective agent during the onset of inflammation and tissue damage. The clinical measurement of CRP in serum therefore appears to be a valuable screening test for organic disease and a sensitive index of disease activity in inflammatory, infective, and ischemic conditions. In rheumatology, CRP seems to be the best single parameter for estimating disease activity and response to therapy (6). CRP is now increasingly quantified to differentiate bacterial from viral infections (7,8), and it can also be useful in diseases such as myocardial infarction, appendicitis, and postoperative complications (7). Given this wide role of CRP in clinical diagnosis and treatment, a reliable, inexpensive automated method is needed for its determination in biological fluids.

Here we report a method in which antibodies to human CRP are simply adsorbed to latex particles and uncoated latex sites are saturated with bovine serum albumin. The tendency of the coated latex particles to aggregate in the absence of CRP antigen, particularly during the process of preparing the stock reagent, is alleviated by sonication. The assay consists of monitoring the increase in turbidity that results from the interaction of coated latex beads with CRP at optimum conditions.

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