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Note

Rapid assay for indoxyl sulphate in uremic serum by internal-surface reversed-phase high-performance liquid chromatography

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Serum albumin binding of many acidic drugs is well known to be reduced in uremic patients [1–3]. The defect of drug-binding is now considered to be due to the accumulation of several endogenous albumin-bound metabolites that competitively inhibit the albumin binding of drugs. These metabolites include hippuric acid [4–6], indoxyl sulphate [4,7], 2-hydroxyhippuric acid [8], 3-(3-hydroxyphenyl)-3-hydroxypropanoic acid [5,6], 4-hydroxyphenylacetic acid [5,6], indole-3-acetic acid [7], 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid [9–13], 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid [9,12], 3-carboxy-4-methyl-5-ethyl-2-furanpropionic acid [12] and 3-carboxy-5-propyl-2-furanpropionic acid [12].

During a study of the endogenous protein-bound metabolites accumulated in uremic serum, we developed a new rapid method for the analysis of total indoxyl sulphate in uremic serum by high-performance liquid chromatography (HPLC) with an internal-surface reversed-phase (ISRP) column. Conventional reversed-phase HPLC analyses of protein-bound metabolites, such as indoxyl sulphate, require deproteinization by heating and centrifugation [10]. By using our method the serum level of indoxyl sulphate can be directly analysed from serum samples. The serum level of indoxyl sulphate was found to be highly elevated in uremic

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patients. Since a large portion of indoxyl sulphate was bound to serum protein, the serum level did not markedly decrease after hemodialysis.

EXPERIMENTAL

Chemicals

Indoxyl sulphate potassium salt, hippuric acid, 2-hydroxyhippuric acid, 4-hydroxyphenylacetic acid, indole-3-acetic acid, acetic acid and potassium dihydrogenphosphate were obtained from Nakarai (Kyoto, Japan). HPLC-grade 2-propanol, water and tetrahydrofuran were also obtained from Nakarai. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid was kindly supplied from Professor G. Spiteller, Bayreuth University (Bayreuth, F.R.G.).

Samples and sample preparation

Serum samples were obtained from ten healthy subjects and ten uremic patients maintained on chronic hemodialysis. Serum samples were obtained from the uremic patients immediately before and after hemodialysis, and samples filtered through a 0.20-μm membrane filter (DISMIC-25cs, cellulose acetate, Toyo Roshi) prior to HPLC analysis. A 10-μl sample of the serum was analysed by HPLC.

To analyse the compounds that were not bound to serum protein, uremic serum was ultrafiltrated using a CF-25 ultrafiltration membrane filter (Amicon, Lexington, MA, U.S.A.), and 10 μl of the ultrafiltrate were chromatographed.

High-performance liquid chromatography

The separation was carried out by HPLC using a Model CCPD pump from Toyo Soda (Tokyo, Japan), a Rheodyne Model 7125 injector (Berkeley, CA, U.S.A.) and a Model UV-8000, variable-wavelength detector (Toyo Soda). Results were analysed using a Model 7000A reporting integrator from SIC Instruments (Tokyo, Japan). The column used was a Pinkerton ISRP column (15 cm$\times$4.6 mm I.D., particle size 5 μm) equipped with an ISRP guard cartridge (1 cm$\times$3 mm I.D.) both from Regis (Morton Grove, IL, U.S.A.). All solvents used as mobile phases were filtered through a 0.20-μm membrane (Advantec Tokyo, Tokyo, Japan) prior to use. The HPLC mobile phase was 0.1 *M* potassium dihydrogenphosphate (pH 6.0)–2-propanol–tetrahydrofuran (84:10:6, v/v), delivered at a flow-rate of 1.0 ml/min at ambient temperature. The eluate was monitored by UV detection at 270 nm.

For collection and liquid secondary-ion mass spectrometry (SIMS), the mobile phase used for HPLC was 0.2 *M* acetic acid/triethylamine (pH 7.0)–2-propanol–tetrahdyrofuran (84:10:6, v/v).

Quantification

To quantify indoxyl sulphate, we used the same analytical procedure as that used for the compounds in the uremic sera. We prepared a standard curve by assaying solutions containing various amounts of indoxyl sulphate potassium salt in standard serum. Since the normal serum contains a small amount of indoxyl

sulphate, the peak height of indoxyl sulphate in the normal serum was subtracted from those in the standard serum solutions. The determination of the sample concentrations (x) using peak heights (y) yielded a calibration line ($y = -0.0165 + 1.542x$) with a correlation coefficient of 0.99936. The intra-assay coefficient of variation (C.V.) for the assay was 3.2% ($n = 5$), and the inter-assay C.V. was 5.1% ($n = 5$). The recovery was 102.3% (S.D. 8.5%); it was determined by assaying five aliquots of control serum solutions to which a known amount of indoxyl sulphate was added at a concentration of 120 μmol/l, and by comparing the peak heights for the serum samples with those obtained by a direct injection of the same amount of indoxyl sulphate in water.

Liquid secondary-ion mass spectrometry

The HPLC fraction obtained from 1 ml of uremic serum was evaporated and the residue was redissolved in methanol. The solution was filtered through a 0.45-μm membrane filter (Chromatodisc 4A, Kurabou K.K.) and evaporated under a nitrogen stream. The residue was dissolved in 20 μl of water. A 1-μl sample of this solution was mixed with ca. 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-8089 high-field magnet, SIMS source and Model M-0101 data system (all from Hitachi, Tokyo, Japan). The operating conditions were: primary ion, Xe^+; accelerating voltage, 8 kV (primary) and −3 kV (secondary); and source at ambient temperature.

RESULTS

Fig. 1 shows HPLC profiles of protein-bound compounds in uremic serum (a) and normal serum (b), and of protein-unbound compounds in uremic serum ultrafiltrate (c) with an ISRP column. Two peaks with retention times of 4.8 and 10.9 min were found to be increased in the uremic serum compared with the normal serum. The compounds were almost all bound to serum albumin, since the peaks were very small in the chromatogram of the uremic serum ultrafiltrate.

The peak at 4.8 min was tentatively identified as 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, since the authentic compound showed an identical retention time.

The other protein-bound compounds that have been reported to accumulate in uremic serum were analysed by ISRP-HPLC. The compounds analysed include 4-hydroxyphenylacetic acid, hippuric acid, 2-hydroxyhippuric acid, indole-3-acetic acid and indoxyl sulphate potassium salt. Among these compounds only the last had an identical retention time with the unknown peak in Fig. 1. Fig. 2 shows the UV spectra of the unknown peak (a) and indoxyl sulphate potassium salt (b) recorded on-line: the two spectra appear identical.

To confirm the presence of indoxyl sulphate, the unknown peak was collected, evaporated and then analysed by liquid SIMS. Fig. 3 shows the negative-ion mass spectrum of the samples isolated from the uremic serum (a), and indoxyl sulphate potassium salt standard (b). The negative secondary-ion mass spectrum

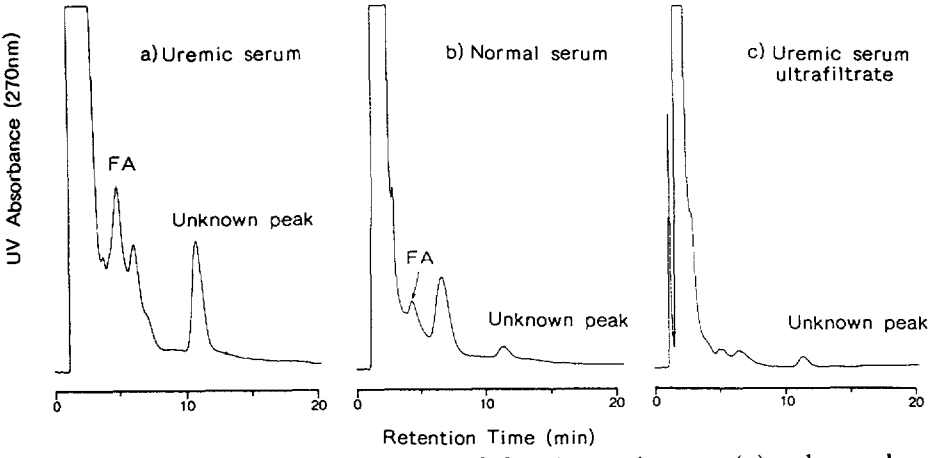

Fig. 1. Chromatograms of protein-bound metabolites in uremic serum (a) and normal serum (b), and of protein-unbound metabolites in uremic serum ultrafiltrate (c). Peak FA = 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid.

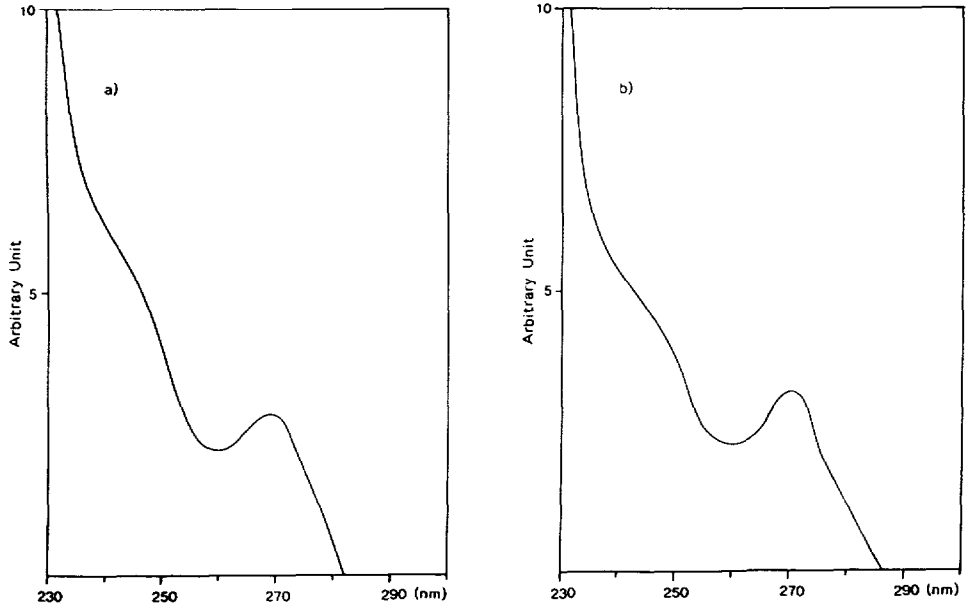

Fig. 2. On-line UV spectra of the unknown peak in Fig. 1 (a), and indoxyl sulphate potassium salt (b).

of the unknown peak showed an intense ion at m/z 212, which was the $[M-K]^-$ ion of indoxyl sulphate potassium salt.

We quantified indoxyl sulphate by ISRP-HPLC without using an internal standard. In uremic serum the mean ($\pm$S.D.) concentration of indoxyl sulphate before hemodialysis was 153.0 ± 46.2 μmol/l ($n=10$), and after hemodialysis, 116.7 ± 33.1 μmol/l, compared with 2.0 ± 1.1 μmol/l ($n=10$) in normal serum.

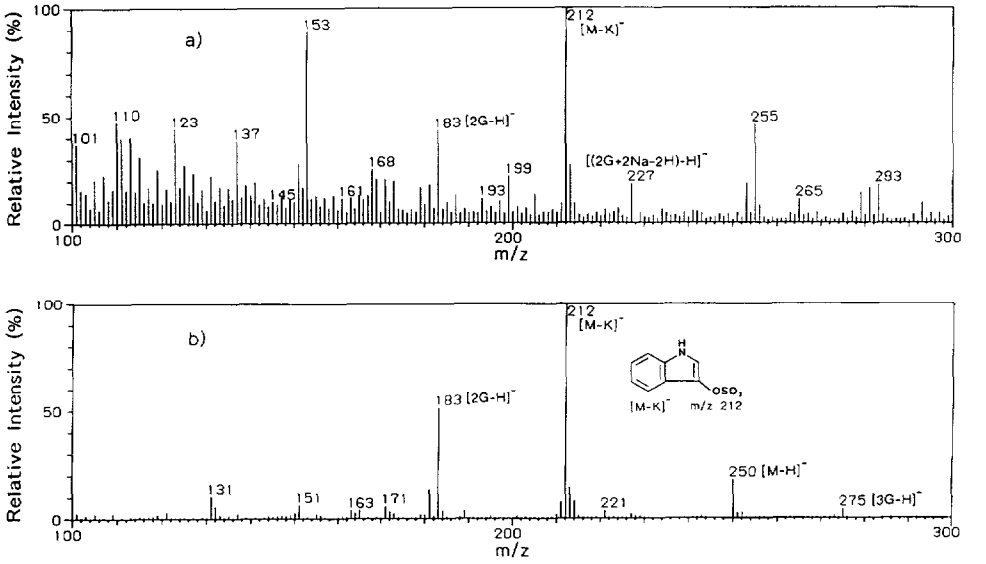

Fig. 3. Negative secondary-ion mass spectra of the unknown peak in Fig. 1 (a) and indoxyl sulphate potassium salt (b).

DISCUSSION

Indoxyl sulphate in uremic serum has so far been analysed by spectrophotometry, spectrophotofluorometry [14] and reversed-phase HPLC [15,16]. However, sample preparation for spectrophotometry and spectrophotofluorometry is laborious and time-consuming and needs large amounts of serum samples. Conventional reversed-phase HPLC needs only small volumes of serum samples, but requires deproteinization of serum prior to analysis. Only the serum level of unbound indoxyl sulphate in uremic patients has been reported by HPLC analysis of deproteinized serum ultrafiltrates [15]. Mabuchi and Nakahashi [16] described the profile of the serum protein-bound metabolites by reversed-phase HPLC using deproteinization by heat and centrifugation of the serum samples before analysis.

Our new rapid method for HPLC analysis of indoxyl sulphate requires only 10 μl of serum and does not require deproteinization before analysis. Peak identification was carried out by liquid SIMS and UV spectrometry. Our results revealed that the serum level of indoxyl sulphate was markedly increased in uremic patients and did not decrease sufficiently after hemodialysis owing to the protein binding of indoxyl sulphate. Since conventional hemodialysis cannot efficiently remove the protein-bound metabolite, a novel hemodialysis method that can remove the protein-bound metabolite should be developed to prevent possible complications due to accumulation of indoxyl sulphate in uremic patients on long-term hemodialysis.

Indoxyl sulphate was reported to inhibit albumin binding of many drugs, such as diazepam [4], warfarin [4], methyl red [7], methyl orange [7], 2-(4'-hydroxybenzeneazo)benzoic acid (HABA) [7], L-tryptophan [7], and furosem-

ide [17]. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid was also reported to inhibit albumin binding of methyl red [13], salicylic acid [12,13], L-tryptophan [10,13] and phenytoin [10,12]. Since the serum levels of indoxyl sulphate and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid are highly increased in uremic patients, and both are strongly albumin-bound, thereby inhibiting albumin binding of drugs, the defect of drug-binding in uremic patients is mainly due to the accumulation of indoxyl sulphate and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid.

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