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Accumulation of Indoxyl Sulfate, an Inhibitor of Drug-Binding, in Uremic Serum as Demonstrated by Internal-Surface Reversed-Phase Liquid Chromatography

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We quantified indoxyl sulfate in uremic serum by using internal-surface reversed-phase high-performance liquid chromatography. Its concentrations were markedly increased in chronic hemodialysis patients, and were significantly but weakly correlated with the concentrations of creatinine and β_2-microglobulin in these patients' serum, and with the duration of their hemodialysis treatment. Indoxyl sulfate could not be removed effectively by conventional hemodialysis because of its strong binding to serum albumin. Equilibrium dialysis demonstrated that indoxyl sulfate inhibited the binding of salicylate to albumin, and that 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid inhibited the binding of indoxyl sulfate to albumin. In conclusion, indoxyl sulfate was markedly accumulated in uremic serum, and inhibited drug binding.

Additional Keyphrases: *hemodialysis · albumin binding of drugs · salicylate*

Albumin binding of many acidic drugs is well known to be decreased in serum from uremic patients (*1–3*). At present the reduction of drug-binding is considered to be due to the accumulation of several endogenous albumin-bound metabolites that competitively inhibit the albumin binding of drugs. So far, several endogenous metabolites have been proposed as inhibitors of drug-binding, including hippuric acid (*4–6*), indoxyl sulfate (*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*).

Indoxyl sulfate in human blood and urine results exclusively from metabolism by intestinal bacteria. Intestinal bacteria metabolize L-tryptophan to indole, which is absorbed into blood and metabolized in liver to indoxyl sulfate. Healthy human subjects excrete ~170 μmol of indoxyl sulfate daily into urine (*14*). In uremic patients the urinary excretion of indoxyl sulfate is decreased or virtually absent, causing accumulation of indoxyl sulfate in uremic blood.

We developed a rapid method for the analysis of protein-bound indoxyl sulfate in uremic serum by internal-surface reversed-phase (ISRP) HPLC. With our method we can determine the concentration of indoxyl sulfate directly in serum samples. We measured this concentration in uremic patients, and studied the correlation between these results and various other laboratory data for these patients. We also performed equilibrium dialysis to determine whether indoxyl sulfate inhibits the binding of salicylate to albumin, and whether 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid inhibits the binding of indoxyl sulfate to albumin.

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Materials and Methods

Chemicals. Indoxyl sulfate potassium salt, acetic acid, triethylamine, and HPLC-grade isopropanol, water, and tetrahydrofuran were obtained from Nakarai Chemical Co., Kyoto, Japan. Sodium salicylate was obtained from Yoneyama Yakuhin Kogyo Ltd., Osaka, Japan. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid was kindly supplied by Professor G. Spiteller, Bayreuth University, Bayreuth, F.R.G.

Samples and sample preparation. Serum samples were obtained from 10 healthy subjects and 80 uremic patients maintained on chronic hemodialysis. Serum samples were obtained from the uremic patients immediately before and after hemodialysis. Serum was separated from blood cells by centrifugation at 1000 × *g* for 10 min. Serum samples were stored at −20 °C until analysis. Authentic indoxyl sulfate potassium salt in water was quite stable, even at 4 °C for one month.

Serum samples were filtered through 0.20 μm (pore size) membrane filter (DISMIC-25cs, cellulose acetate, Toyo Roshi) to remove large insoluble particles prior to HPLC analysis. A 10-μL sample of the serum processed with the filter was injected into the chromatograph.

To analyze the compounds that were not bound to serum protein, we passed uremic serum and normal serum through a CF-25 ultrafiltration membrane filter (Amicon Co., Lexington, MA), then chromatographed a 10-μL sample of the ultrafiltrate. The ultrafiltration with the Amicon filter was facilitated by centrifuging at 1000 × *g* for 30 min.

HPLC apparatus and chromatographic conditions. We used a chromatographic assembly consisting of a Model BIP-1 pump, a Model VL-614 injector, and a Model 875-UV detector (all from Jasco, Tokyo, Japan). Results were analyzed by using a Model Chromatocorder 12 (Jasco). We used a 15 cm × 4.6 mm Pinkerton ISRP column (particle size 5 μm) equipped with a 1 cm × 3 mm ISRP guard cartridge (both from Regis Chemical Co., Morton Grove, IL). All solvents used in the mobile phase were filtered through 0.20-μm membrane (Advantec Tokyo, Tokyo, Japan) before use. The mobile phase, dilute acetic acid (0.2 mol/L in water)/isopropanol/tetrahydrofuran (84/10/6, by vol), adjusted to pH 6.5 with triethylamine, was delivered at a flow rate of 1.0 mL/min at ambient temperature. The eluate was monitored by detection of absorbance at 270 nm.

Assay procedure. To quantify indoxyl sulfate, we generated a calibration line by ISRP-HPLC, with seven serum standards prepared by adding 4.0 to 400 nmol of indoxyl sulfate to 1-mL aliquots of serum from apparently healthy subjects. The peak height for endogenous indoxyl sulfate in the nonsupplemented serum was subtracted from the peak heights for the standards. The corrected peak heights varied linearly with the sample concentrations and yielded a linear regression line (r = 0.9994). The intra-assay coefficient of variation (CV) for the assay of indoxyl sulfate at 120 μmol/L was 3.2% (n = 5), the inter-assay CV at that concentration was 5.1% (n = 5). Analytical recovery of indoxyl sulfate, added to give a concentration of 120 μmol/L, was 102.3 (SD

8.5)% (n = 5), with similar recovery of indoxyl sulfate at 12 μmol/L.

Figure 1 shows the HPLC chromatograms of authentic indoxyl sulfate potassium salt (*a*), protein-bound metabolites in uremic serum (*b*), protein-unbound metabolites in uremic serum ultrafiltrate (*c*), and protein-bound metabolites in normal serum (*d*) with an ISRP column. Indoxyl sulfate potassium salt showed the identical retention time as the unknown peak in Figure 1. We confirmed the identification of indoxyl sulfate by recording its ultraviolet spectrum on-line and by secondary-ion mass spectrometry.

Equilibrium dialysis. We used equilibrium dialysis to determine the degree of albumin binding. Into an equilibrium dialysis apparatus (Sanplatec Co., Osaka, Japan), we put 2-mL aliquots of 600 μmol/L albumin solution in 67 mmol/L phosphate-buffered saline, pH 7.4, and dialyzed against 3 mL of the phosphate-buffered saline with added salicylate (720 μmol/L). The dialysis was performed on a shaker at 4 °C for about 40 h. Drug-binding values obtained by this method were used to estimate the degree of binding defects induced by adding to the albumin solution various amounts of indoxyl sulfate: 0, 12.5, 50, 200, and 400 μmol/L (final concentrations). Concentration of salicylate was determined according to the method of Trinder (*15*). The albumin binding of salicylate was calculated as follows:

$$\text{albumin binding, \%} = [(a - b)/a] \times 100$$

where a = concentration of salicylate in the albumin solution, and b = concentration of salicylate in the dialysate.

To study the effect of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, a major protein-bound metabolite that accumulates in uremic serum, on the albumin binding of indoxyl sulfate, we added increasing amounts of the furancarboxylic acid to the albumin solution, and estimated the albumin binding of an indoxyl sulfate solution (120 μmol/L). To 2-mL aliquots of 600 μmol/L albumin in 67 mmol/L phosphate-buffered saline, pH 7.4, we added various amounts of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (0, 26, 52, 104, 208, and 416 μmol/L, final concentrations), and dialyzed these at 4 °C for about 40 h against phosphate-buffered saline containing 120 μmol of indoxyl sulfate per liter. Concentration of indoxyl sulfate was determined by ISRP-HPLC.

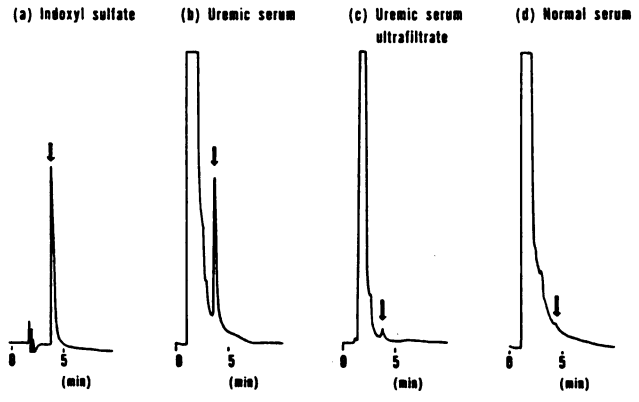

Fig. 1. HPLC chromatograms of authentic indoxyl sulfate potassium salt (*a*), protein-bound metabolites in uremic serum (*b*), non-protein-bound metabolites in uremic serum (*c*), and protein-bound metabolites in normal serum (*d*)

Table 1. Concentrations (Mean ± SD) of Indoxyl Sulfate in Serum from Healthy Subjects and Uremic Patients

	Healthy subjects (n = 10)	Uremic patients (n = 80)	
		Before HD	After HD
Total indoxyl sulfate, μmol/L	2.0 ± 1.1	129.9 ± 59.0	102.4 ± 48.2
Protein-unbound indoxyl sulfate, μmol/L	ND	15.1 ± 17.9	16.7 ± 13.1
Protein binding of indoxyl sulfate, %	≈100	89.4 ± 9.3	83.6 ± 9.1

ND, not detected. HD, hemodialysis.

Statistical analysis. We used Student's *t*-test to compare the difference between the concentrations of indoxyl sulfate in serum from healthy subjects and from uremic patients. The significance of a decrease in protein binding before and after hemodialysis was analyzed by Student's paired *t*-test.

Results

Table 1 shows the concentrations of total and protein-unbound indoxyl sulfate in serum from chronic hemodialysis patients, before and after hemodialysis. In serum from uremic patients the concentration of indoxyl sulfate was markedly more than that in serum from normal subjects ($P < 0.01$). The concentration of indoxyl sulfate in the serum did not decrease much after hemodialysis because of its strong protein binding. Interestingly, the protein binding of indoxyl sulfate after hemodialysis was slightly but significantly ($P < 0.01$) less than that before hemodialysis.

The concentration of indoxyl sulfate in serum from uremic patients before hemodialysis was weakly but significantly correlated with the concentrations of creatinine ($r = 0.304$, $P < 0.01$) and β_2-microglobulin ($r = 0.238$, $P < 0.05$) in these patients' serum, and with the duration of their hemodialysis treatment ($r = 0.275$, $P < 0.05$) (Figure 2). However, the concentration of indoxyl sulfate in the patients' serum did not show any significant correlation with the concentrations of urea, uric acid, potassium, phosphate, calcium, or parathyrin in the serum, or with the hematocrit.

To study the effect of indoxyl sulfate on albumin binding of salicylate, we added increasing amounts of indoxyl sulfate to albumin solution and determined how much salicylate was bound by albumin by using equilibrium dialysis. Figure 3 (top) shows the effect of indoxyl sulfate on the albumin binding of salicylate: increasing the concentration of indoxyl sulfate decreased the amount of salicylate bound.

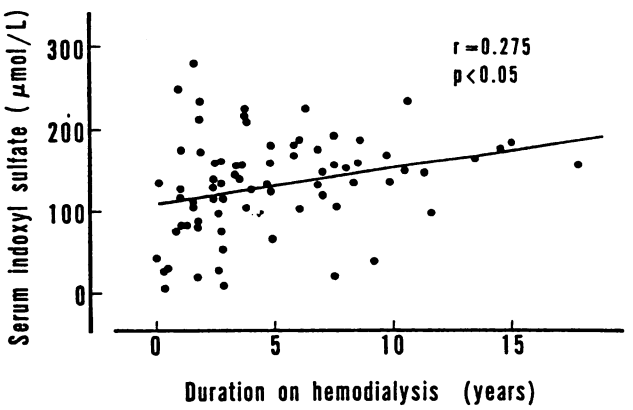

Fig. 2. Correlation between concentration of indoxyl sulfate in serum from chronic hemodialysis patients and their duration on hemodialysis

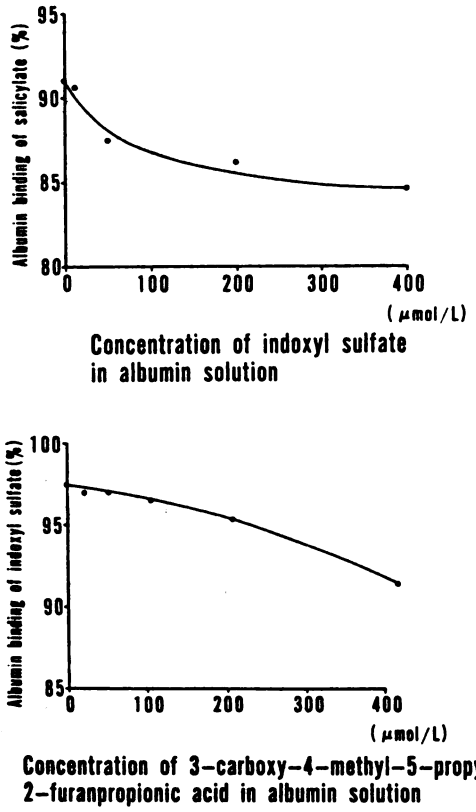

Fig. 3. (*top*) Effect of increasing concentrations of indoxyl sulfate on albumin binding of salicylate in vitro; (*bottom*) effect of increasing concentrations of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid on albumin binding of indoxyl sulfate in vitro

Figure 3 (bottom) shows the effect of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid on the albumin binding of indoxyl sulfate. With no furancarboxylic acid added, the albumin bound 97.4% of the indoxyl sulfate. Increasing the concentration of the furancarboxylic acid caused a decrease in the indoxyl sulfate bound by albumin.

Discussion

Indoxyl sulfate has so far been detected by spectrophotometry, spectrophotofluorometry (*16*), and reversed-phase HPLC (*17, 18*). However, sample preparation for spectrophotometry and spectrophotofluorometry is time-consuming and requires large sample volumes. Conventional reversed-phase HPLC analyses for protein-bound metabolites such as indoxyl sulfate require deproteinization of serum samples by heat and centrifugation. Our method requires only 10 μL of serum and does not require deproteinization before analysis.

We have demonstrated that the concentration of indoxyl sulfate in serum from uremic patients is significantly but weakly correlated with the concentrations of creatinine and β_2-microglobulin in these sera, and with the duration of the hemodialysis treatment. Because indoxyl sulfate is strongly albumin-bound, it is not removed effectively by conventional hemodialysis, and therefore accumulates in uremic serum as the hemodialysis treatment is continued. A method that can remove these protein-bound metabolites effectively from blood should be developed to prevent possible complications in uremic patients undergoing long-term hemodialysis because of the accumulation of protein-bound metabolites.

Indoxyl sulfate reportedly inhibits 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 furosemide (*19*). We showed that indoxyl sulfate also inhibits albumin binding of salicylate. Recently, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid has also been detected as a major endogenous inhibitor of albumin binding of drugs such as methyl red (*13*), salicylic acid (*12, 13*), L-tryptophan (*10, 13*), and phenytoin (*10, 12*). We demonstrated that the furancarboxylic acid also inhibits the albumin binding of indoxyl sulfate in a dose-dependent way. The protein binding of indoxyl sulfate in albumin solution in vitro was 97%, whereas that in uremic serum before hemodialysis was 89.4%. This suggests that the albumin binding of indoxyl sulfate in uremic serum is inhibited by the other endogenous albumin-bound metabolites such as 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid. Because the concentrations of indoxyl sulfate and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, which are strongly albumin-bound, are markedly increased in sera from uremic patients, we consider these the major inhibitors of drug-binding in uremic patients.

References

1. Reidenberg MM, Odar-Cederlöf I, von Bahr C, Borga O, Sjoquist F. Protein-binding of diphenylhydantoin and desmethylimipramine in plasma from patients with poor renal function. N Engl J Med 1971;285:264–7.

2. Reidenberg MM. The binding of plasma proteins and the interpretation of measurements of plasma concentrations of drugs in patients with poor renal function. Am J Med 1977;62:466–70.

3. Øie S, Lowenthal DT, Levy G. Protein binding of bilirubin in plasma of anephric patients. J Dialysis 1980;4:91–100.

4. McNamara PJ, Lalka PJ, Gibaldi M. Endogenous accumulation products and serum protein binding in uremia. J Lab Clin Med 1981;98:730–40.

5. Gulyassy PF, Bottini AT, Jarrard EA, Stanfel LA. Isolation of inhibitors of ligand:albumin binding from uremic body fluids and normal urine. Kidney Int 1983;24:s238–42.

6. Gulyassy PF, Bottini AT, Stanfel LA, Jarrard EA, Depner TA. Isolation and chemical identification of inhibitors of plasma ligand binding. Kidney Int 1986;30:391–8.

7. Bowmer CJ, Lindup WE. Decreased drug binding in uremia: effect of indoxyl sulfate and other endogenous substances on the binding of drugs and dyes to human albumin. Biochem Pharmacol 1982;31:319–23.

8. Lichtenwalner DM, Suh B. Isolation and chemical characterization of 2-hydroxybenzoylglycine as a drug binding inhibitor in uremia. J Clin Invest 1983;71:1289–96.

9. Liebich HM, Pickert A, Tetschner B. Gas chromatographic and gas chromatographic–mass spectrometric analysis of organic acids in plasma of patients with chronic renal failure. J Chromatogr 1984;289:259–66.

10. Mabuchi H, Nakahashi H. Isolation and characterization of an endogenous drug-binding inhibitor present in uremic serum. Nephron 1986;44:277–81.

11. Takeda N, Niwa T, Tatematsu A, Suzuki M. Identification and quantitation of a protein-bound ligand in uremic serum. Clin Chem 1987;33:682–5.

12. Niwa T, Takeda N, Maeda K, Shibata M, Tatematsu A. Accumulation of furancarboxylic acids in uremic serum as inhibitors of drug-binding. Clin Chim Acta 1988;173:127–38.

13. Collier R, Lindup WE, Liebich HM, Spiteller G. Inhibitory effect of the uraemic metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid on plasma protein binding. J Clin Pharmacol 1986;21:610p–1p.

14. Kirkland JL, Vargas E, Lye M. Indican excretion in the elderly. Postgrad Med J 1983;59:717–9.

15. Trinder P. Rapid determination of salicylate in biological fluids. Biochem J 1954;57:301–3.

16. Ludwig GD, Senesky D, Bluemle LW, Elkinton JR. Indoles in uremia: identification by countercurrent distribution and paper chromatography. Am J Clin Nutr 1968;21:436–50.

17. Schoots AC, Homan HR, Gladdines MM, Cramers CAMG, de Smet R, Ringoir SMG. Screening of UV-absorbing solutes in uremic serum by reversed phase HPLC—change of blood levels in different therapies. Clin Chim Acta 1985;146:37–51.

18. Mabuchi H, Nakahashi H. Profiling of endogenous ligand solutes that bind to serum proteins in sera of patients with uremia. Nephron 1986;43:110–6.

19. Ikeda K, Yoshitomi H, Nakayama T, Goto S, Kimura T. Plasma protein binding of frusemide in renal failure rabbits: investigation of endogenous protein binding inhibitors. J Pharm Pharmacol 1984;36:663–7.