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

Toshimitsu Niwa, Naohito Takeda, Akira Tatematsu, and Kenji Maeda

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 β2-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

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 Yonemaya 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 CP-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 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 
\mu\text{mol/L}.

Figure 1 shows the HPLC chromatograms of authentic 
indoxyl sulfate potassium salt (a), protein-bound metabo-
lites in uremic serum (b), protein-unbound metabolites in 
uremic serum ultrafiltrate (c), and protein-bound metabo-
lites 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 equilibri-
um dialysis apparatus (Sanplatec Co., Osaka, Japan), we 
put 2-mL aliquots of 600 \mu\text{mol/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 \mu\text{mol/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 \mu\text{mol/L} 
(final concentrations). Concentration of salicylate was deter-
mined according to the method of Trinder [15]. The albumin 
bindigng of salicylate was calculated as follows:

\[
\text{albumin binding, } \% = \left(\frac{a - b}{a}\right) \times 100
\]

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-furan-
propionic acid, a major protein-bound metabolite that 
accumulates in uremic serum, on the albumin binding of 
indoxyl sulfate, we added increasing amounts of the furan-
carboxylic acid to the albumin solution, and estimated the 
albumin binding of an indoxyl sulfate solution (120 \mu\text{mol/L}). 
To 2-mL aliquots of 600 \mu\text{mol/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 \mu\text{mol/L}, final concentra-
tions), and dialyzed these at 4 °C for about 40 h against 
phosphate-buffered saline containing 120 \mu\text{mol of indoxyl} 
sulfate per liter. Concentration of indoxyl sulfate was deter-
mined by ISRP-HPLC.

| Table 1. Concentrations (Mean ± SD) of Indoxyl Sulfate 
 in Serum from Healthy Subjects and Uremic Patients |
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<tr>
<td></td>
<td>Healthy subjects</td>
<td>Uremic patients</td>
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<td></td>
<td>( n = 10 )</td>
<td>( n = 80 )</td>
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<tr>
<td><strong>Total indoxyl sulfate, \mu\text{mol/L}</strong></td>
<td>2.0 ± 1.1</td>
<td>129.9 ± 59.0</td>
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<td><strong>Protein-unbound indoxyl sulfate, \mu\text{mol/L}</strong></td>
<td>ND</td>
<td>15.1 ± 17.9</td>
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<tr>
<td><strong>Protein binding of indoxyl sulfate, %</strong></td>
<td>=100</td>
<td>89.4 ± 9.3</td>
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<td>ND, not detected. HD, hemodialysis.</td>
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**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 hemodial-
ysis 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 signifi-
cantly \((P < 0.01)\) 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 \(\beta_2\)-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 para-
thyrin 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.

![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).](image)

![Correlation between concentration of indoxyl sulfate in serum from chronic hemodialysis patients and their duration on hemodialysis](image)
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 β2-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-furancarboxylic 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-furancarboxylic acid. Because the concentrations of indoxyl sulfate and 3-carboxy-4-methyl-5-propyl-2-furancarboxylic 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