Determination of Indoxyl Sulfate in Plasma of Patients with Renal Failure by Use of Ion-Pairing Liquid Chromatography

Linda A. Stanfel, Paul F. Gulyassy,1 and Elizabeth A. Jarrard

Using our newly developed ion-pairing reversed-phase liquid-chromatographic method for assay of indoxyl sulfate, we measured its concentration in plasma of normal subjects and patients in various degrees of renal failure. Response was linear over the range of 50 to 25 000 pmol of indoxyl sulfate injected into the chromatograph. We demonstrated the specificity of the assay for azotemic plasma by using enzymatic conversion with a sulfatase. For a moderately above-normal indoxyl sulfate concentration in azotemic plasma of 134 μmol/L (29 mg/L), the within-day CV was 1.6%, the day-to-day CV 2.8%. Mean analytical recovery was 101.0% (CV = 2.8%). Over a range of 29 to 192 mg of creatinine per liter of plasma (X), indoxyl sulfate (Y) concentration (in μmol/L) was positively correlated (Y = 1.30x + 0.43). This method should prove valuable for further study of uremic toxins.

Additional Keyphrases: renal failure · uremia · reference interval · creatinine/indoxyl sulfate relationship

Indoxyl sulfate is mainly produced by bacterial decomposition of dietary tryptophan in the intestine. The initial product, indole, is absorbed, hydroxylated, conjugated with sulfate in the liver, and excreted by the kidneys (1, 2). Our interest in indoxyl sulfate concerns its possible role as a retained solute with toxic effects in patients with severe renal failure. We are studying impaired small-ligand binding by plasma proteins as a model of the many systemic disorders resulting from renal failure. From uremic body fluids we have prepared a potent extract, containing aromatic acids, which generates this binding defect when added to normal plasma (3, 4). Our next goal is to identify and measure the retained solutes in these extracts and in plasma of patients. In vitro studies by Bowmer and Lindup (5) suggest that indoxyl sulfate may be one of the uremic binding inhibitors in uremia. In reviewing the literature we could find no method for assay of indoxyl sulfate that appeared adequate to apply to such a complex solution as uremic plasma. We therefore developed and describe here an ion-pairing "high-performance" liquid-chromatographic (HPLC)1 method for use with normal and azotemic human plasma; it may be applicable to other fluids as well.

Materials and Methods

All chemicals and reagents were of the highest quality available commercially. Indoxyl sulfate (potassium salt) and sulfatase (EC 3.1.6.1; type H-2 from Helix pomatia) were obtained from Sigma Chemical Co., St. Louis, MO 63178.

"HPLC grade" acetonitrile was from E. Merck Science, Gibbstown, NJ 08027. "HPLC grade" water was freshly prepared by passing distilled, de-ionized water through a Norganic cartridge and 0.45-μm HA filter (Millipore Corp., Bedford, MA 01730). The ion-pairing agent, tetrabutylammonium phosphate (TBAP), was obtained as an aqueous 0.5 mol/L solution from Regis Chemical Co., Morton Grove, IL 60053.

Plasma or serum was obtained in the fasting state from normal volunteers and from patients with renal failure, with informed consent according to standards of the Human Subjects Committee of the University of California, Davis. Subjects had not ingested drugs, coffee, or tea for at least 12 h. The samples were stored at −60 °C until used. Plasma was deproteinized and bound indoxyl sulfate displaced by adding 1.5 mL of acetonitrile to 1.0 mL of plasma. After vigorous vortex-mixing, the sample was centrifuged at 10 000 × g for 20 min. An aliquot of the supernate was combined with an equal volume of 10 mmol/L aqueous TBAP. The samples were stored at −60 °C in glass vials with Teflon-lined caps. The vials were wrapped with aluminum foil to prevent light-induced degradation of indoxyl sulfate.

The mobile phase was prepared by mixing 300 mL of acetonitrile, 690 mL of "HPLC grade" water, and 10 mL of 0.5 mol/L aqueous TBAP. Indoxyl sulfate was quantified by ion-pairing HPLC in a Model 332 liquid chromatograph (Beckman Instruments, Inc., Berkeley, CA 94710). A 4.6 × 250 mm Beckman Ultrasphere® octyl silica column was used for early pilot studies and optimization of the TBAP concentration, but a 4.6 × 250 mm Ultrasphere C18 column coupled to a 4.6 × 30 mm guard column (type OD-GU, Brownlee Labs, Santa Clara, CA 95050) was used for the remaining part of method development and validation. Our HPLC system includes a Model 7125 injector (Rhodeyne Inc., Cotati, CA 94928) and a Model HM variable-wavelength detector (Gilion Medical Electronics, Inc., Middleton, WI 53562) set at 260 nm and 0.1 A full scale. A Model 4270 computing integrator (Spectra-Physics, San Jose, CA 95134) was used to quantify the indoxyl sulfate concentration.

Various amounts of 10 and 500 μmol/L solutions of indoxyl sulfate dissolved in the mobile phase were injected and the results were used to construct a standard regression line covering the range of concentrations found in test samples. We injected 100-μL plasma extracts, equivalent to 20 μL of plasma. The flow rate of the mobile phase was 1.0 mL/min. To evaluate the specificity of the method for indoxyl sulfate, we subjected plasma extracts from azotemic patients to digestion with sulfatase. Plasma was de-proteinized as described above and the supernate was evaporated under a stream of nitrogen gas. The residue was dissolved in phosphate buffer (50 mmol/L, pH 5.3), then titrated to pH 5.0 with 2.0 mol/L HCl. To one sample we added 20 μL of sulfatase (102 U); 20 μL of water was added to its duplicate. Samples were incubated at 37 °C for 130 min. The acetonitrile was added at a volume ratio of 3:2 (acetonitrile:sample).
and, after mixing, the solution was centrifuged at 10,000 × g. The supernate was removed and diluted with an equal volume of 10 mmol/L aqueous TBAP. We injected 100 μL into the HPLC system for quantification of indoxyl sulfate.

**Results**

Figure 1 shows the retention time of indoxyl sulfate as a function of TBAP concentration on an Ultrasphere octyl silica column. We used a constant acetonitrile concentration and varied the concentration of TBAP from 0.5 to 10 mmol/L. At a TBAP concentration of 10 mmol/L, indoxyl sulfate was not eluted within a reasonable period of time. At the lowest concentrations of TBAP the reproducibility of retention time was poor. To maintain reproducible retention times and still keep the assay cost effective, we used a TBAP concentration of 5 mmol/L.

The Ultrasphere octyl column re-equilibrated very slowly when the TBAP concentration was changed, such that the previous retention time of indoxyl sulfate could not be reproduced if the chromatography was repeated at a lower TBAP concentration. We used an Ultrasphere C18 column for our final method development, owing to its widespread use and ready availability. Jandera et al. (6) have shown that the capacity factor for a C18 column is similar to that of an octyl column. Using the Ultrasphere C18 column, a mobile phase consisting of 5 mmol of TBAP per liter of acetonitrile/water (30/70 by vol), and a flow rate of 1.0 mL/min, we observed a sharp, symmetrical peak, which appeared at 15 min. After these column conditions were established they were always maintained and never changed for the life of the column, which in our hands was at least 550 injections during three months.

Variability of retention times was evaluated on separate days 15 times during 60 days. We recorded the retention time of the first daily injection of the indoxyl sulfate standard solution and compared it with the second and the last daily injection of the same standard solution. Because the column temperature was uncontrolled, it varied with room temperature, which was in the range of 26 to 30 °C. The mean retention time for the initial daily injection was 15.20 (SD 0.57) min and for the second daily injection 14.99 (SD 0.24) min. The ratio of the last daily injection to the second injection was 0.989 (SD 0.017). This small change may have been due to temperature effects alone.

A linear response of concentration to peak area was established over two ranges: 50–1000 pmol per injection (n = 8 points) and 500–25 000 pmol per injection (n = 9 points). The regression equations relating peak area (y) in area units to indoxyl sulfate injection (x), in nanomoles, were y = 42.3x – 34.4 and y = 43.1x – 1469, respectively. Regression coefficients were 0.99954 and 0.99998, respectively. The concentration in unknown samples was determined by reference to standard curves, which were constructed daily through at least three points. Daily standard curves showed correlation coefficients of 0.99805 or better.

Precision was evaluated by adding two different amounts of indoxyl sulfate to two normal plasma pools before proceeding with extraction and analytical HPLC. The concentrations simulated those in plasma from patients with early and advanced renal failure. Six replicate aliquots of each pool were all extracted on the same day, followed by analytical HPLC one to three days later. For the samples supplemented with a small additional amount of indoxyl sulfate, the mean within-day determined value per 100 μL injected was 490.2 (SD 15.0) pmol and the CV was 3.06%. For the plasma supplemented to give a high concentration of indoxyl sulfate, the mean within-day value was 4996.2 (SD 56.3) pmol and the resulting CV was 1.13%. We evaluated day-to-day precision over seven weeks, using pools of supplemented plasma. The mean values were 479.7 (SD 16.0) and 5069.3 (SD 122.5) pmol per 100 μL injected for the low- and high-concentration samples, respectively; the CVs were 3.34% and 2.42%, respectively. During the day-to-day precision study the retention times (n = 12) averaged 14.71 (SD 0.285) min with a CV of 1.94%.

Figure 2 shows representative chromatograms of normal

![Fig. 1. Retention of indoxyl sulfate by an Ultrasphere octyl silica HPLC column as a function of TBAP concentration. The eluent was acetonitrile/water (30/70 by vol), with various concentrations of TBAP](image)

![Fig. 2. Separation of indoxyl sulfate (retention times of 14.56–14.60 min) in normal plasma extracts. A, an extract of normal plasma containing approximately 6.0 μmol of indoxyl sulfate per liter, yielding an area of 3460 units. B and C: the same sample supplemented with 19.2 and 225 μmol of indoxyl sulfate per liter, with areas of 19 670 and 198 700 units](image)

*One area unit = 0.5 μV·s.*
plasma before and after addition of indoxyl sulfate. We evaluated analytical recovery, using six individual samples of normal plasma. Indoxyl sulfate concentration was measured in each sample before and after being supplemented to two concentrations, yielding increments of 19.2 and 225 μmol/L (increments of 387 and 4864 pmol/100 μL injected). Recoveries were 100.6 (SD 2.3)% and 107.9 (SD 1.9)% with CVs of 2.29% and 1.76%. The recovery probably exceeded 100% because no correction was made for the water content of plasma (about 96%).

Concentrations of indoxyl sulfate in normal healthy volunteers were generally too low to quantify accurately with the above methods. A pool of plasma from normal fasting volunteers was extracted, as described above, in replicate aliquots (n = 6) and each extract was chromatographed in duplicate. A mean concentration of 5.4 (SD 1.2) μmol/L or 1.15 (SD 0.026) mg/L was derived from a mean area of 3303 (SD 917) peak-measurement units (a distinguishable peak containing 50 pmol has an area of approximately 1500 units). The mean area units of 2700–4300 were too low to be within the accurate range of the integrator; therefore, the CV (22.4%) was very large. If this procedure is to be used to quantitate indoxyl sulfate accurately in plasma of normal humans, it would be necessary to include a concentration step, inject a larger volume, and (or) increase the detector sensitivity to greater than 0.1 A full scale. Our studies, as well as those of other investigators, entail measurement of much higher concentrations in plasma or urine.

Representative chromatograms from two patients with severe renal failure (Figure 3) show sharp, symmetrical peaks for indoxyl sulfate. We further evaluated precision, using a pool of serum obtained from uremic patients. We used eight aliquots of this pool to evaluate the within-day precision and carried each serum aliquot through the protein precipitation, dilution, and analytical HPLC steps. The mean indoxyl sulfate concentration was 133.6 (SD 2.1) μmol/L or 28.5 (SD 0.45) mg/L (within-day CV, 1.6%). Daily-to-day precision was evaluated during five weeks. The mean value for eight determinations was 29.5 (SD 0.8) mg/L (CV, 2.65%).

Table 1 gives analytical recovery data for azotemic patients. Indoxyl sulfate was quantified in seven individual patients' samples over a range of 21 to 285 μmol/L, or 4.5 to 60.8 mg/L. Plasma was then supplemented with additional indoxyl sulfate so that the concentration was increased by 117 μmol/L (or 2.34 mmol/20 μL) of plasma. Mean recovery was 101.0 (SD 2.8)% and the CV was 2.8%.

The effect of long-term storage of the sample extract was evaluated during eight weeks. Two patients' samples were deproteinized and diluted as described above. The indoxyl sulfate concentration was determined immediately by analytical HPLC and the extracts were stored at −60 °C. Aliquots were subsequently thawed and analyzed an additional four times. The chromatographic peak for indoxyl sulfate remained sharp and clearly distinguishable. The initial and subsequent results were 184, 188, 188, 185, and 197 μmol/L for one sample and 190, 186, 204, 187, and 201 μmol/L for the other. Evidently indoxyl sulfate is stable for at least eight weeks when stored under these conditions.

Assay specificity was analyzed by using a sulfatase to convert indoxyl sulfate to 3-hydroxyindole, thereby removing it from our area of interest on the chromatogram. We could then observe any co-eluting substances that might be misinterpreted to be indoxyl sulfate. Representative chromatograms are displayed in Figure 4, showing complete disappearance of the indoxyl sulfate peak and the absence of any closely eluting interfering substance. Of eight different patients' samples similarly analyzed, none contained any substances that would interfere with the specificity of our method.

Indoxyl sulfate was quantified in plasma of 15 uremic patients with various degrees of chronic renal failure and the results are shown in Figure 5. Concentrations ranged from 4.5 to 60.8 mg/L, or 21 to 285 μmol/L. The concentration of indoxyl sulfate (γ) in uremic patient plasma was positively correlated within the plasma creatinine (z) in the range of 29 to 192 mg of creatinine per liter (γ = 1.30z + 0.43). The indoxyl sulfate concentration in the two patients with extreme uremia was probably lower than predicted because they were eating little. This explanation is suggested by findings in three patients with severe renal failure who were receiving hemodialysis therapy, had not taken food by mouth for several weeks, and were receiving only parenteral nutrition. Their plasma indoxyl sulfate values were 0, 0, and 5 μmol/L.

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<th>Creatinine, mg/L</th>
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Mean (SD) 101.0 (2.8)
Discussion

Of the various naturally occurring aromatic acids, indoxyl sulfate presents an unusual analytical challenge. Because of the extreme acidity of the sulfate group (pKₐ about -5 for sulfuric acid) the most popular method for separation of aromatic acids—ion-suppressed reversed-phase HPLC—is not effective. We have found that indoxyl sulfate is only slightly retarded by a C₁₈ HPLC column, even when very low concentrations of the organic solvent are used in the mobile phase. Indoxyl sulfate is therefore a strong candidate for HPLC determination with use of an ion-pairing agent at neutral pH. When plasma is analyzed, this procedure would follow a deproteinization step. Both the aromatic structure and the highly ionized sulfate group account for its very high affinity for plasma proteins, to which more than 90% of it is bound (unpublished observation).

We have found previously published quantitative techniques impossible to utilize for accurate estimation of indoxyl sulfate in whole plasma, owing to the strong protein binding. The colorimetric method of Bryan (7), developed for urine, is preceded by only bulk group separation, is relatively nonspecific, and is very sensitive to acidity as was suggested by Bryan and reconfirmed in our laboratory. The standard acidification methods for deproteinizing plasma with trichloroacetic and (or) sulfosalicylic acid yield extremely high blanks. Plasma from uremic patients contains many retained solutes that ordinarily are excreted by normal kidneys. These retained solutes are likely also to interfere with the method of Bryan.

Deproteinization with acetonitrile is complete and technically simple. We found analytical recovery and reproducibility to be satisfactory. Resolution is excellent, even with the most complicated plasma samples from uremic patients.

The most important issue to resolve for uremic plasma samples was specificity. Currently, there is no method that reasonably can be designated the reference method. Older methods consist of nonspecific color reactions, with only crude or no pre-analysis separation steps applied to the samples (7, 8). We therefore used a modification of the method reported by Bryan et al. (9) of enzymatic conversion of indoxyl sulfate to 3-hydroxyindole, which is eluted considerably earlier than indoxyl sulfate in the present method. We modified conditions of incubation with the enzyme from those used by Swan et al., because in their procedure indoxyl sulfate was decreased but not completely eliminated, as is essential in studies of specificity. We found in preliminary studies of standard solutions that this enzyme’s action is very slow at pH 7.4 but proceeds rapidly at pH 5.0. Similar results, with complete abolition of the indoxyl sulfate peak, were obtained for extracts of uremic samples (Figure 4).

The mean indoxyl sulfate concentration in plasma of normal subjects is about 5 μmol/L; for our patients with renal failure the range of concentrations was 21 to 285 μmol/L, four- to 53-fold the normal mean. Plasma creatinine and indoxyl sulfate concentrations were positively correlated. Because indoxyl sulfate is substantially bound to plasma proteins, tubular secretion may be more important than glomerular filtration as the mechanism of its renal elimination. Using isolated perfused rat kidney, we currently are evaluating this question.

The indoxyl sulfate concentrations found in the patients with moderate to severe uremia may be sufficiently high to at least partly account for the impaired binding of small ligands to albumin in uremia. Bowmer and Lindup (5) found that 200 μmol of indoxyl sulfate per liter decreased tryptophan binding to human albumin (10 g/L solution) in vitro from 76.4% to 25.9%. Similarly, in our laboratory Tavares-Almeida et al. (10) found strong inhibition by indoxyl sulfate of tryptophan, salicylate, and warfarin binding to whole human plasma.

We intend to apply this method to measure indoxyl sulfate concentration in plasma samples from additional patients with advanced renal failure, before and after starting dialysis therapy. The concentrations of this ligand will be correlated with the extent of binding inhibition of model
probes. We will also correlate the fluorescence of uremic samples with the indoxyl sulfate concentration before and after its enzymatic destruction. We strongly suspect that the increased fluorescence of uremic plasma (11, 12) is due not to a single compound, but to a large number of retained ligands. Finally, this method could be applied to measurement of plasma and urinary indoxyl sulfate concentrations for studies of intestinal malabsorption (13).

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References