laboratory. This method may be particularly well suited to large diabetic screening studies and as an adjunct to routine diabetes care where patients are carrying out home monitoring of capillary blood glucose. In this situation, patients could collect whole-blood samples at home and send them directly to the central laboratory.

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References


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Liquid-Chromatographic Study of Fluorescent Materials in Uremic Fluids

James S. Swan,1 Edith Y. Kragten,2 and Hans Veening3

Using reversed-phase “high-performance” liquid chromatography with fluorescence detection, we separated and identified some naturally fluorescent compounds in uremic serum and hemodialysate from patients with chronic renal disease. Several of the naturally fluorescent compounds were identified as indole derivatives by co-chromatography with authentic standards. In one specific case, the identity was confirmed by an enzymatic peak-shift method. Compounds identified included indican, kynurenic acid, tryptophan, and 5-hydroxyindole-3-acetic acid. Comparison of normal and uremic serum showed that the fluorescent materials are present in significantly greater concentrations in samples from uremic patients.

Additional Keyphrases: chromatography, reversed-phase • hemodialysis patients • chronic renal disease • indole derivatives • enzymatic peak-shift method

Recent Letters to this journal have discussed the presence of unidentified naturally fluorescent compounds in the serum, urine, hemodialysate, and hemofiltrate of patients with chronic renal failure (1–4). Vladutiu et al. (1) reported finding one or more fluorescent substances (maximum emission near 340 nm) in the serum of patients with chronic renal failure but not in the serum of those with acute renal failure. They concluded that the substances were not vitamins or anabolic steroids and could be bound to a serum protein. Schwertner et al. (2) found a fluorescent compound (excitation maximum 322 nm, emission maximum 415 nm) not only in serum, but also in hemofiltrate, hemodialysate, and urine of patients with chronic renal disease. The substance was 80-fold more concentrated in uremic serum than in normal serum. They also determined (by gel filtration) that the relative molecular mass ($M_r$) of the substance was probably <1000, and that it was not a drug or drug metabolite. Digenis et al. (4), finding a linear correlation between the relative fluorescent intensity and the creatinine concentration in serum from patients with chronic renal failure, concluded that deterioration of renal function is accompanied by an increase in the unidentified fluorescent substances.

We have separated and identified some naturally fluorescent compounds in uremic serum and hemodialysate, by use of reversed-phase “high-performance” liquid chromatography (HPLC) with fluorescence detection. We also used a unique enzymatic peak-shift identification procedure.

Materials and Methods

Materials

Apparatus. The mobile phase was pumped through the apparatus with an SP8700 solvent-delivery system (Spectra-Physics, Santa Clara, CA 95051). Samples were injected via a syringe loading valve fitted with a 100-µL sample loop (Model 7130; Rheodyne Inc., Berkeley, CA 94710) onto a 3.9 × 300 mm stainless-steel column containing 7 µm Zorbax ODS (Du Pont Co., Wilmington, DE 19898) packing material. The compounds of interest were detected with an Amino filter fluorometer (American Instrument Co., Silver Spring, MD 20910) equipped with a GE germicidal lamp (Model J4-7126), a Wratten 2C emission filter (peak wavelength 405 nm, approximate bandwidth 10 nm), and an Amino J4-7469 excitation filter (peak wavelength 295.4 nm, bandwidth at 50% of maximum transmission 9.2 nm). All chromatograms, retention times, and peak areas were recorded with an SP4100 computing integrator (Spectra-Physics).

Water was purified by passage through a Milli-R04 system, followed by a final cleanup through a Milli-Q system (Millipore Corp., Bedford, MA 01730). Proteins ($M_r$,
> 10 000) were removed from serum samples by ultrafiltration with a Model 1EA XX42 013 10 ultrafiltration system (Millipore Corp.). Solvents and samples were filtered through 0.20-μm (av. pore-size) Nalgene filter units (Sybron/Nalge, Rochester, NY 14602) before analysis.

Reagents. All water used was "HPLC" grade, prepared as described previously. HPLC-grade methanol was obtained from Burdick and Jackson Laboratories, Muskegon, MI 49442. Glacial acetic acid and concentrated ammonium hydroxide were purchased from Fisher Scientific, Fair Lawn, NJ 07410. Tryptophan was from Eastman Organic Chemicals, Rochester, NY 14650, and 5-hydroxyindole-3-acetic acid, kynurenic acid, indoxyl sulfate, and aryl sulfatase (EC 3.1.6.1; type H-2, from Helix pomatia) were from Sigma Chemical Co., St. Louis, MO 63178. Ammonium acetate buffer (12.5 mmol/L, pH 4.0) was prepared by diluting 1.50 g of glacial acetic acid to approximately 1.9 L with HPLC-grade water, adjusting the pH to 4.0 with ammonium hydroxide, and then diluting to 2 L with more HPLC-grade water.

Preparation of standards. Standard solutions were prepared by dissolving weighed amounts of the known compounds in unused hemodialysate. The solutions were stored at 4°C and were kept for no longer than three days (we detected no significant decrease in peak heights or areas within this time).

Preparation of samples. We obtained serum and hemodialysate samples from Divine Providence Hospital, Williamsport, PA. The patients from whom the samples were obtained were not under treatment with salicylates or other drugs during the collection of blood. The hemodialysate samples were placed on ice immediately after collection; serum samples were placed on ice after blood centrifugation. For longer storage (several days), samples were placed in a -25°C freezer until use. For quantitative studies, samples were not frozen but kept at 4°C and used within two days after collection. Serum samples were ultrafiltered and hemodialysate samples were filtered through the 0.20-μm filters before analysis.

Procedures

Chromographic conditions. This separation required the use of a binary gradient composed of ammonium acetate buffer (12.5 mmol/L, pH 4.0) and methanol. These two solutions were filtered through 0.20-μm filters and degassed by bubbling with helium before use. The flow rate was 1.5 mL/min. Gradient conditions were as follows:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Buffer</th>
<th>% Methanol</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
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<tr>
<td>5</td>
<td>100</td>
<td>0</td>
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<td>15</td>
<td>80</td>
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<tr>
<td>35</td>
<td>0</td>
<td>100</td>
</tr>
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The column was operated at room temperature and the detector was set on the 0 → 3 range. The SP4100 computing integrator was programmed to have an attenuation of 4 and a chart speed of 0.5 cm/min.

General chromatographic procedure. The column was equilibrated with the ammonium acetate buffer for 20 min at a flow of 1.5 mL/min before each analysis. For the peak identification studies, we injected 100 μL of filtered uremic serum or dialysate and used the gradient conditions specified above. We then determined retention times for the following compounds: indican, 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, tryptophan, 5-hydroxytryptamine, indole-3-lactic acid, indole-3-acetic acid, indole-3-acetamide, tryptamine, kynurenic acid, and indole. The peaks from the uremic sample were compared with those from the known compounds and likely matches were proposed. We then added to the uremic sample each of the known compounds, one at a time, and inspected the chromatograms corresponding to each injection to see if a new peak had appeared or an existing peak enlarged. In this way we determined the probable identities of several compounds in the uremic samples.

Several compounds that we observed frequently in the uremic samples were chosen for a quantitative study. We prepared standard solutions of 2.5 to 120 mg/L concentration by dissolving weighed amounts of the compounds in unused hemodialysate. The limits of detection were determined by successively diluting each standard until the signal-to-noise ratio was about 2.

Enzymic peak shift. We also used type H-2 aryl sulfatase (10 kilo-units/mL) to convert indican (3-indoxyl sulfate) into 3-hydroxyindole. After preparing a blank (2.0 mL of water mixed with 0.1 mL of sulfatase) and incubating for 1 h at 25°C, we removed the enzyme by ultrafiltration and chromatographed an aliquot of the solution to determine whether there were any possible interferences from the enzyme solution. We then mixed 2.0 mL of standard indican solution (50 μmol/L) with 0.10 mL of water and treated this similarly, recording the retention time and area for the indican peak. Finally, we mixed 2.0 mL of the standard indican solution with 0.10 mL of the sulfatase solution and allowed the mixture to react for 1 h at 25°C. After ultrafiltration and chromatography, we recorded the retention time and area for the new peak (3-hydroxyindole). At this point, we repeated the above procedure, using 2.0 mL of hemodialysate in place of the indican solution. A decrease in the indican peak and the appearance of the 3-hydroxyindole peak were observed in the resulting chromatogram.

Because the ultrafiltration steps were very time-consuming, we adopted an alternative procedure to remove the

![Fig. 1. Chromatograms of uremic and normal serum](image-url)
enzyme: addition of 0.10 mL of trichloroacetic acid (1.0 mg/L) and centrifugation. This was much more convenient and gave results identical to those obtained using ultrafiltration.

**Results and Discussion**

As Figure 1 shows, there is a striking difference between the amounts of fluorescent substances present in uremic serum and those in normal serum. We have tentatively identified four of these compounds as indican, tryptophan, kynurenic acid, and 5-hydroxyindole-3-acetic acid by co-chromatography with authentic standards. Results of the enzymic peak-shift procedure (Figure 2) confirm the identification of indican.

The concentrations of these indole compounds varied from patient to patient and from treatment to treatment. Indican was the easiest to detect and was usually present in uremic serum at approximately 30 μmol/L. Concentrations of kynurenic acid, tryptophan, and 5-hydroxyindole-3-acetic acid were more variable, ranging from the limit of detection (respectively, 5.3, 4.9, and 2.6 μmol/L) to approximately 50 μmol/L. We calculated that a liter of normal serum contains less than 0.1 μmol of indican, 5.3 μmol of kynurenic acid, 4.9 μmol of tryptophan, and 2.6 μmol of 5-hydroxyindole-3-acetic acid.

The earlier studies, which have reported unknown fluorescent substances in uremic bio-fluids, cite emission wave-length maxima ranging from 340 to 430 nm (1, 2, 4). We found the emission maxima for indican and 5-hydroxyindole-3-acetic acid to be 390 and 342 nm, respectively. The emission maxima for kynurenic acid and tryptophan are reported to be 405-435 and 365 nm, respectively (5). These data, of course, do not provide sufficient evidence for positive identification, but they do suggest that the compounds we found could be the same as several of the unidentified species reported earlier (1-4).

It was also reported that the unknown substances are bound to albumin (1-3, 6). We did not investigate this possibility because hemodialysate does not contain proteins and the serum samples were protein-filtered.

A previous study of the accumulation of serum indolic metabolites in uremia involved cation-exchange chromatography (7). Although the authors were not clear on their method of identification, they reported finding tryptophan, indole-3-acetic acid, 5-hydroxyindole-3-acetic acid, and, in some cases, 5-hydroxytryptophol and N-acetylttryptophan. The separation time for the cation-exchange method was 50 min. The analysis time with our method is about 30 min, and indican can also be detected.

Vladutiu et al. (1) suggested that there is a need for a method to distinguish between chronic renal failure and acute renal failure. Because indican is present and easily detected in both serum and hemodialysate from patients with chronic renal failure, we suggest that it may prove useful as a clinical marker for chronic renal disease.

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