Liquid-Chromatographic Profiling of Endogenous Fluorescent Substances in Sera and Urine of Uremic and Normal Subjects

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Endogenous fluorescent substances increase in serum during uremia. We have used "high-performance" liquid chromatography to profile these fluorescent substances in both uremic and normal body fluids. The fluorescence excitation and emission maxima we used were 322 and 415 nm, respectively. Of the numerous fluorescent substances found in uremic body fluids and in normal urine, some were also detectable in normal serum, but at relatively weak fluorescence intensities.

Additional Keyphrases: hemodialysis · hemofiltration

Several investigators have reported that an endogenous fluorescent substance or substances increase in the blood of patients with chronic uremia (1–9). This fluorescence is considered to be associated with albumin and is a source of error in fluorometric assay of creatine kinase BB isoenzyme (1, 2, 5, 6). Schwertner (9), using gel chromatography and thin-layer chromatography, isolated from uremic body fluids and normal urine a highly water-soluble, strongly fluorescent substance having an emission maximum of 415 ± 5 nm.

We report here the use of "high-performance" liquid chromatography (HPLC) to profile these fluorescent substances in both uremic and normal body fluids.

Materials and Methods

Subjects and Samples

We selected for this study five patients with chronic renal failure who had been maintained on hemodialysis or hemofiltration for more than six months. They excreted about 400–900 mL of urine per day. Three healthy subjects were used as normal controls.

Uremic ultrafiltrates were obtained by in vivo isolated ultrafiltration through a cuprophan-membrane artificial kidney. Sera obtained from healthy subjects were ultrafiltered through a Centriflo CF-25 (Amicon Corp., Lexington, MA 02173), which has a nominal molecular mass cutoff of about 25 000 daltons, to obtain normal ultrafiltrates.

Hemodialysis and hemofiltration were performed with cuprophan- and polyacrylonitrile-membrane dialyzers, respectively. The spent hemodialysates, about 3 L, were collected during the first 60 min of treatment. Hemofiltration was performed by the post-dilution technique. The total amount of hemofiltrate collected was about 20 L.

We used freshly voided urine, to avoid the risk of inadequate collection and of changes during storage. The urine was ultrafiltered through a Centriflo CF-25.

Sample Treatment

We condensed and desalted the samples as reported elsewhere (10). In brief, we pumped them through a 0.8 × 10 cm LiChroprep RP-18 (Merck, Darmstadt, F.R.G.) column. After washing with trifluoroacetic acid/water (1/99 by vol), we eluted the samples with n-propanol/trifluoroacetic acid/water (60/1/30 by vol). The collected eluent was then lyophilized. Amino acids and guanidine compounds could also be removed from the samples (10, 11).

Analytical Procedure

We used a Model LC-3A HPLC system (Shimadzu, Kyoto, Japan), which included a Model SIL-1A injector, a Model GRE-2B gradient former, and a Model RF-500LC spectrofluorometer equipped with a 12-µL flow-through cell, and followed the method described previously (10). The column was a 0.46 × 25 cm LiChrosorb RP-18 (particle size, 5 µm; Merck). The elution conditions were as follows: solvent A, acetonitrile/heptanefluorobutyrlic acid/water (10/0.1/89.9 by vol); solvent B, acetonitrile/heptanefluorobutyrlic acid/water (50/0.1/49.9 by vol); temperature, ambient; flow-rate, 1.5 mL/min. The eluent was solvent A isocratically for 30 min, then a linear gradient from solvent A to solvent B over a period of 100 min. The spectrofluorometer was set for 322 nm excitation and 415 nm emission wavelengths (band width, 10 nm) (9). The sensitivity setting of the spectrofluorometer was a fourfold attenuation of full-scale sensitivity.

All reagents were obtained from Nakarai, Kyoto, Japan, and were of analytical or HPLC grade.

Results

As Figure 1 shows, many fluorescent substances are present in ultrafiltrates of serum from uremic patients. More than 50 fluorescent peaks could be resolved, some of which were strongly fluorescent. Some of these fluorescent peaks could be detected in ultrafiltrates of serum from normal subjects, but the relative fluorescent intensities of the peaks were weak.

Several analyses revealed that the numbers assigned to peaks in Figures 1–3 correspond with each other.

Fig. 1. Typical chromatograms of fluorescent components in ultrafiltrates, representing an original sample volume of 20 mL, from uremic patients (-----) or from normal subjects (-----).

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Figure 2 shows that both normal and uremic urine contain a large number of fluorescent substances. Most of the strong peaks observed in uremic ultrafiltrates are also observed in profiles of both normal and uremic urine. Some strong peaks seen in profiles of both normal and uremic urine are absent or scarce in uremic ultrafiltrates. We observed some variations in the elution profiles of uremic urine from patient to patient. Except for the different relative fluorescent intensities, the elution profiles of uremic urine resembled those of uremic ultrafiltrates.

There were many more fluorescent substances in the hemofiltrates than in the hemodialysates (Figure 3), probably because of the differences of membrane pore size used and of total amounts of fluid volume.

**Discussion**

Recently, Schwertner (9), using gel chromatography and thin-layer chromatography, found only one strongly fluorescent substance in uremic body fluids and in normal urine, but suggested that "methods with greater resolution, such as high-performance liquid chromatography, may produce different results." Our results clearly demonstrate that numerous endogenous fluorescent substances are present in uremic body fluids and in normal urine.

The solvent system used for HPLC analysis was initially intended to separate medium-sized peptides (10). Therefore, at least the fluorescent peaks eluting after 100 min are probably peptidic substances.

Our results also showed that fluorescent substances can be separated without alcohol extraction (9). In addition, the technique of batch adsorption of fluorescent substances in a large amount of body fluids to octadecaisy1-silica particles is a very efficient first step in the concentration and desalting of samples. Therefore, any body fluid—including urine, which contains larger numbers of constituents than do other body fluids—can be analyzed for endogenous fluorescent substances. Moreover, the solvent system used is volatile, which facilitates further direct investigation of separated substances.

Uremic urine, obtained from dialyzed patients whose creatinine clearance was <3 mL/min, contained larger amounts of fluorescent substances. Such results are consistent with the important role of residual renal function in the elimination of metabolic wastes.

We could not elucidate the origin or physiological role of the fluorescent substances, but several authors (2-7) have reported that the endogenous fluorescence is associated with albumin. It is well known that the binding of drugs to albumin is diminished in uremia (12). Depner et al. (13) extracted an inhibitor responsible for impaired drug binding from uremic body fluids, and reported that a crude preparation of a binding inhibitor has fluorescence, with excitation and emission maxima at 340 and 420 nm, respectively. Therefore, it is possible that one or more of the fluorescent substances detected in this study are binding inhibitors. This issue requires further investigation.

The identification of the individual peaks remains a major problem and is now in progress in our laboratory.
Immunoadsorption to Improve Gas Chromatography/High-Resolution Mass Spectrometry of Estradiol-17β in Plasma

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We describe a new, highly selective procedure for the determination of estradiol-17β in plasma. Samples are extracted with a micro-cellulose-coupled antiserum to estradiol-17β. Conversion of the extracted steroid to the bistrimethylsilyl) ether is followed by gas chromatography/high-resolution mass spectrometry with selected ion monitoring. Precise quantification is achieved through the use of [1H3]estradiol-17β as internal standard.

Additional Keyphrases: steroids · radioassay

Analytical procedures incorporating mass-spectrometric detection are widely advocated as reference techniques to assess the performance of routine assays of steroid hormones in biological fluids (1–3). Typically, the reference procedures incorporate solvent extraction and chromatographic fractionation prior to combined gas chromatography–mass spectrometry (GC-MS). The specificity of the overall procedure (and therefore the accuracy of the quantitative data) is dependent on the selectivity of the sample fractionation and of the instrumental analysis. Thus, ideally, the various stages in the analytical procedure should complement one another in achieving unequivocal identification and reliable quantification of the analyte. We have, for example, recently described a procedure for the determination of estradiol-17β in saliva (4), in which a micro-scale anion-exchange chromatography column is used with sequential elution in reversed-phase, straight-phase, and ion-exchange modes (5). We therefore characterize estradiol-17β on the basis of polarity (judged by solvent extraction and chromatography), acidity, volatility (during gas chromatography), and mass spectrometric properties.

Here we describe an alternative approach for establishing fractionation procedures that complement the specificity of GC-MS detection. Extraction of estradiol-17β from plasma by using a solid-phase-coupled antiserum rapidly and specifically fractionates samples before the GC-MS procedure. To enhance precision, we use a deuterium-labeled internal standard.

Solid-phase-coupled antisera have been used previously in other contexts for extraction by immunoadsorption. Dyas et al. (6), for example, developed a highly specific radioimmunoassay for ethynyl estradiol in plasma by incorporating an initial immunoadsorption step. Similarly, Glencross et al. (7), using an antiserum coupled to Sepharose, extracted estradiol-17β from large volumes of bovine blood and milk for subsequent radioimmunoassay.

References