Serum samples, dialysis fluids, and hemofiltrates were obtained from patients undergoing chronic hemodialysis or hemofiltration treatment. The dialysis fluids were collected during the first 30 min of hemodialysis treatment; the hemofiltrates were pooled from a 2-h treatment period. In both methods a Cuprophan membrane was used; dialyzing-fluid flow rates were about 500 mL/min and ultrafiltration flow rates were 1.5 L/h. Urine samples and normal serum samples were from individuals without renal disease. Average creatinine values for persons with chronic renal failure and for normal individuals were 98 (SD 35) and 8.5 (SD 0.05) mg/L, respectively.

We added 10 mL of methanol to 2 mL of serum, urine, hemofiltrate, or dialyzer, shook the mixture for 15 min on a shaker, then centrifuged. The alcohol-aqueous phase was removed and evaporated at room temperature in an air current. The residue, reconstituted in water, was applied to the gel-filtration columns; reconstituted in methanol, it was applied to thin-layer chromatographic plates after recrystallization.

To determine the relative molecular mass of the fluorescent compound, we used a glass column (38 × 2 cm i.d.) packed with 200–400 mesh Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA 94894). Blue Dextran, Mₐ 2 × 10⁶, was used to determine the void volume of the column bed, and vitamin B₁₂, NADH, riboflavin phosphate, and glutathione were used as molecular mass calibrators. We layered the extract from 2 mL of biological fluid on the packed column and eluted with a pH 7.5 phosphate buffer containing, per liter, 0.14 mol of NaCl, 1.55 mmol of K₂HPO₄, and 8 mmol of Na₂HPO₄. Effluent fractions of 4 mL were collected and analyzed by recording the fluorescence emission spectrum from 300–540 nm at an excitation wavelength of 322 nm (Hitachi/Perkin-Elmer MPF-214 fluorescence spectrophotometer; Perkin-Elmer Corp., Norwalk, CT 06856). Excitation and emission slit widths were 5 and 12 nm, respectively.

The major fluorescent compound found in serum extracts was also found in hemofiltrates and in dialysis fluids. It had an elution volume of 112 mL and excitation and emission maxima of 322 ± 4 nm and 415 ± 5 nm, respectively. The fluorescent species was also present in urine of normal individuals. The fluorescence intensity of the substance from urine was >10-fold that of serum (Table 1) and that in hemofiltrates about half of that serum—about fourfold that of dialysis fluids. The fluorescent substance in serum of renal-disease patients was about 80-fold that in serum of normal individuals. Another fluorescent species—with an elution volume of 129 mL and excitation and emission maxima of 360 and 460 nm, respectively—was also found; its intensities were <0.1 that of the major fluorescent species. We might add that the major fluorescent species cannot be viewed on the polyacrylamide gels used in this study, however, the fluorescence can be readily followed on Sephadex columns under long-wavelength ultraviolet light.

Elution volumes of Blue Dextran, vitamin B₁₂, NADH, riboflavin phosphate, and glutathione were 48, 75, 91, 116, and 90 mL, respectively. There were some differences in the elution volume of different standards relative to their molecular mass, e.g., with glutathione. This lack of linearity is apparently a common characteristic of compounds of lower molecular mass and of non-homologous species. Thus we can only conclude that the molecular mass of the fluorescent compound is probably <1000.

Serum, urine, and hemofiltrate extracts were also analyzed by thin-layer chromatography on silica gel, further to characterize the chromatographic properties of the fluorescent unknown. The substance from each of the three fluids showed similar Rₚ values in each of three separate chromatographic solvents. The Rₚ values with methanol, methanol/chloroform (2/1), and ethanol were 0.76, 0.65, and 0.44, respectively. The fluorescent species was best resolved from other constituents with ethanol as developing solvent. We also analyzed the fluorescent material, extracted from several TLC plates, by gel-filtration chromatography. Its elution behavior of the substance corresponded to the previously determined elution patterns of the substance in extracts of biological fluid.

Evidently the fluorescent substance found in increased amounts in serum of patients with chronic renal disease is also present in hemofiltrates and in dialysis fluids, as well as in urine of normal individuals. Its relative molecular mass is estimated to be <1000. Its concentration in serum of patients with chronic renal disease is 80 times greater than that of serum from normal individuals.

The fluorescence in such serum samples can be readily visualized under long-wavelength ultraviolet light and can be differentiated from normal samples in most cases where the creatinine concentrations exceed 50 mg/L. Because the urine samples were obtained from individuals not taking drugs, we conclude that the unidentified fluorescent substance is not a drug or drug metabolite.

References

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Race-Related Differences in Reference Intervals for Creatine Kinase

To the Editor:

We compliment Lott and Stang for their review of creatine kinase (EC 2.7.3.2) and lactate dehydrogenase (EC 1.1.27) (1). However, we wish to bring to your attention an omission that we believe is all too frequently overlooked, relative to normal values for creatine kinase.

Prompted by the reports of Meltzer and colleagues (2–4), we undertook to check the validity of their statement that black men and women have normally higher total creatine kinase values than white men and women.

We conclude from assays (5) performed on sera from 36 black men and 33 black women that there is indeed a statistically significant difference in the

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Table 1. Relative Fluorescent Intensities of the Fluorescent Species in Various Biological Fluids after Molecular-Exclusion Chromatography

<table>
<thead>
<tr>
<th>Rel. fluorescence a (and SD)</th>
<th>Biological fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>805 (110)</td>
</tr>
<tr>
<td>Serum, renal-disease patients</td>
<td>80 (30)</td>
</tr>
<tr>
<td>Hemofiltrate</td>
<td>44 (21)</td>
</tr>
<tr>
<td>Dialysis fluid</td>
<td>11 (5)</td>
</tr>
<tr>
<td>Normal serum</td>
<td>1 (0.2)</td>
</tr>
</tbody>
</table>

* Individual fluorescent values depend on many factors, such as dialysis methods, frequencies of dialysis, and severity of disease.
reference intervals. For black men the interval was 20–143 U/L, for black women 18–80 U/L. The reference interval for white men was 0–80 U/L and for white women 6–50 U/L.

Physicians and laboratory personnel should be aware of race-related differences. Unfortunately, manufacturers of commercial creatine kinase kits do not indicate race as a variable when they list their normal values.

References

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Improved Extraction of Valproic Acid from Serum before Chromatography

To the Editor:

We read with interest the Letter by Freeman and Rawal (Clin. Chem. 26, 674–5, 1980) on this topic. A similar extraction procedure was described by Dijkhuis and Vervolet (1), and in an international inter-laboratory study (2) most respondents (approximately 60%), particularly in Europe, used this type of procedure. Our experience with direct extraction of valproic acid serum dichloromethane brought to light several problems:

* In spite of careful mixing, an emulsion formed, which was difficult to break.

* Although the syringe was filled with air, which was expelled when the needle had passed through the aqueous phase into the lower organic layer, the needles often became blocked.

* The detector (flame ionization) became contaminated with a carbon deposit due to incomplete combustion of the chlorinated hydrocarbon solvent, leading to increased baseline noise and loss of sensitivity. The seriousness of this problem may be related to detector design (we use Pye Series 104 chromatographs) but the baseline noise in the chromatogram published by Freeman and Rawal is probably evidence of this trouble. This problem is also reported in the instruction manual for the Perkin Elmer F 11.

These practical difficulties led to the investigation of solvents with density lower than that of water and of different acidification procedures. The following method is the result of these investigations.

Serum (100 µL) is acidified with hydrochloric acid (1 mol/L, 100 µL) containing cyclohexane carboxylic acid (10 µg, internal standard for valproic acid) and 3,3-dimethyl-4-methylacinnimide (10 µg, internal standard for ethosuximide). Ethyl acetate (200 µL) is added and the stopped tubes are mixed by inversion for 1 min. The tubes are centrifuged (5 min, 1000 rpm) to separate the aqueous and organic phases. An aliquot of the organic (upper) layer (2–3 µL) is withdrawn for chromatography. The extraction can be performed in the glass vials used with the Fye automatic liquid injection system, and we have regularly used automatic injection for valproic acid and ethosuximide analysis over the past two years with batches of up to 60 samples.

Chromatographic analysis is performed on a glass column (1.5 m, 2 mm i.d.) packed with 3% SP 1000 on Gas Chrom Q 80–100 mesh at a temperature of 175 °C with a carrier gas (nitrogen) flow rate of 30 mL/min. Ethosuximide is also determined quantitatively in this procedure. Figure 1 shows a chromatogram of an extract of serum containing valproic acid (47 µg/mL) and ethosuximide (50 µg/mL).

The results of intra-laboratory studies of reproducibility over a six-month period, with use of a horse-serum control pool are presented in Table 1. The results obtained by this method in an inter-laboratory quality assessment scheme [BARTS CONTROL (3)] have not shown any evidence of consistent bias (systematic error of proportional or additive nature).

Table 1. Imprecision of Simultaneous Valproic Acid/ Ethosuximide Assay

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean (%)</th>
<th>SD (%)</th>
<th>CV (%)</th>
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</thead>
<tbody>
<tr>
<td>Valproic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>within-batch</td>
<td>26</td>
<td>47.6</td>
<td>2.1</td>
<td>4.4</td>
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<tr>
<td>between-batch</td>
<td>26</td>
<td>47.3</td>
<td>2.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>within-batch</td>
<td>26</td>
<td>50.0</td>
<td>1.6</td>
<td>3.2</td>
</tr>
<tr>
<td>between-batch</td>
<td>26</td>
<td>49.8</td>
<td>1.9</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Derived from difference between first and last control sample in batch.

References

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