Liquid-Chromatographic Determination of Inosine, Xanthine, and Hypoxanthine in Uremic Patients Receiving Hemodialysis Treatment

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We used a liquid-chromatographic method to measure xanthine, hypoxanthine, and inosine in plasma of uremic patients before and after hemodialysis. The concentrations of all three were higher than in normal subjects. After dialysis treatment, the values were significantly lower than before dialysis, but still above normal.

Patients with chronic renal failure tend to retain the products of metabolism. It has often been suggested that symptoms of uremia are related to the accumulation of some of these substances (1–2). However, it has not yet been established whether clinical uremia is a result of the presence of a toxin (or toxins) or the result of a combination of chemical derangements, including dehydration or overhydration, acidemia, starvation, trauma, and (or) anemia.

Special attention has been directed to the measurement of numerous compounds that accumulate in uremic blood and to the elucidation of their possible toxicity. In addition to the well-recognized alterations in ura and other nitrogenous products, in electrolytes, and in hydrogen ion concentration, various other compounds—aliphatic amines, phenol, and some purines—are present in abnormal amounts in the serum (3–4). These are known to be toxic both to experimental animals and to humans (5–6).

Purine compounds are important biological components, a measure of their importance being the severe abnormalities exhibited by patients with defects in purine metabolism. Little is known, however, about changes in the concentration of purines in uremic blood before and after hemodialysis. Attempts to correct the uremic syndrome by performing conventional hemodialysis are only partly successful, perhaps in part because of inadequate removal of purine compounds.

In the present investigation, a "high-performance" liquid-chromatographic method is described and used for estimating inosine, xanthine, and hypoxanthine in blood of uremic patients who are undergoing hemodialysis.

Materials and Methods

Reagents and Standards

All chemicals used were of analytical grade. Uric acid, inosine, xanthine, hypoxanthine, purine-nucleoside phosphorylase (EC 2.4.2.1), and xanthine oxidase (EC 1.1.3.22) were all from Sigma Chemical Co., St. Louis, MO.

Blood-Sample Treatment

Twenty control samples were collected from personnel in our institute, ages 25 to 46 years. Pathological samples were obtained from 20 patients with end-stage renal disease who had been maintained on twice-weekly hemodialysis procedure of 4-h duration for an average period of 18 months (range eight to 26). Their ages ranged from 28 to 55 years (mean 46.2). Protein intake was 1.2 g per kilogram of body weight per day. Each specimen from patients was taken before and after a session of hemodialysis. The pre- and post-dialysis mean values for blood urea nitrogen were respectively 800 mg/L and 320 mg/L.

Analytical Procedure

We used the following procedure to isolate inosine, xanthine, and hypoxanthine from the plasma. Venous blood was sampled from controls and patients into chilled (in crushed ice) heparinized tubes and centrifuged at 2500 x g for 15 min. Plasma was then transferred to chilled test tubes and 200-μL aliquots were immediately placed in an Amicon MPS-1 micropartition system with a YMB membrane and centrifuged (4°C, 2500 x g, 15 min). Twenty-microliter portions of the resulting filtrates were then injected directly onto the chromatographic column.

"High-performance" liquid chromatography. The chromatograph used was a Perkin-Elmer Series 3B equipped with dual pumps capable of generating a gradient elution and with a 0.4 x 12.5 cm reversed-phase (HS-5C18) column from Perkin Elmer. Chromatograms were recorded by monitoring absorbance at 254 nm with a LC 85 UV detector (Perkin-Elmer) and for data-processing we used an LCI-100 integrator (Perkin-Elmer). Mobile phase: eluent A was KH2PO4 (10 mg/L) and the pH was adjusted to 5.8 with NaOH. Eluent B was methanol/water (80/20 by vol). For elution we used a 15-min linear gradient from 0 to 100% of eluent B. The flow rate was 1 mL/min. Column temperature was ambient. The absorbance range was 0.008 A full scale.

Analytical Variables

Recovery. Five pooled plasma samples were supplemented with three different standard solutions of inosine, hypoxanthine, and xanthine. We analyzed in quadruplicate (n = 4) each plasma specimen containing each of the three different concentrations of added standard. The analytical recovery of each compound is given in Table 1.

| Table 1. Analytical Recovery of Inosine, Hypoxanthine, and Xanthine Added to Plasma |
|-----------------------------------------|---------|------|
| **Added** | **Recovered** | **Recovery, %** |
| **Inosine** | **μmol/L** |        |
| 1.20     | 1.19       | 99.6  |
| 8.40     | 8.32       | 98.1  |
| 21.50    | 21.19      | 98.6  |
| **Hypoxanthine** | **Mean (SD) 99.1 (0.5)** |
| 1.29     | 1.27       | 98.6  |
| 6.46     | 6.26       | 96.9  |
| 25.81    | 25.06      | 97.1  |
| **Xanthine** | **Mean (SD) 97.5 (0.92)** |
| 1.50     | 1.48       | 98.6  |
| 5.14     | 5.06       | 98.4  |
| 18.86    | 18.48      | 97.9  |

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**Precision.** To determine between-run precision, we analyzed plasma controls on 10 different days. For a plasma control containing 2.5 μmol of inosine, 4.3 μmol of xanthine, and 3.2 μmol of hypoxanthine per liter, the CVs as determined from 10 assays performed over a 10-day period were 6.1%, 5%, and 7.2%, respectively.

**Sample Handling**

Purines are quickly released from erythrocytes, leukocytes, and platelets into plasma or serum after a blood specimen is drawn (7–8). To minimize this we have studied the changes in purine concentration in samples from four healthy controls during 5 h. After blood collection, 2-mL aliquots were withdrawn at the following intervals: 0, 0.5, 1, and 5 h. At these intervals, plasma was immediately separated by centrifugation and analyzed. A similar assay was performed in an ice bath.

We have observed that purine concentration (especially that of hypoxanthine) increases with time. After 1 h of blood storage at room temperature, hypoxanthine concentrations in plasma have already doubled. The increase was significantly lower when samples were stored in an ice bath instead of being left at room temperature. For this reason we placed blood specimens in chilled tubes and centrifuged them within 5 min, in a refrigerated centrifuge. Immediately after centrifugation we submitted them to ultrafiltration and analysis.

**Results**

Figure 1 shows a chromatogram of a standard solution containing, per liter, 10 μmol each of inosine, xanthine, and hypoxanthine and 20 μmol of uric acid.

Calibration curves were prepared by treating the standard mixtures in the same way as plasma samples. Absorbances of inosine, xanthine, and hypoxanthine are linearly related to concentration of injected standard over a wide range, as is shown in Figure 2.

The minimum amount of inosine, xanthine, and hypoxanthine that could be reliably distinguished from zero (signal/noise ratio >2) was 0.1 μmol/L.

Figure 3 shows the chromatogram for a uremic patient before and after dialysis. There were similarities among chromatograms for uremic patients, but it is difficult to present one that might be considered “typical” of the uremic state.

Chromatographic peaks for inosine, xanthine, and hypoxanthine were identified by confirming that (a) their retention time were identical with those of the standards; (b) the peaks co-migrated with simultaneously injected standard; (c) their ultraviolet spectral analysis and absorbance ratios (as determined by use of the stop-flow technique over the
range 220–320 nm) were identical with those of the standards; and (d) the peaks attributed to inosine, xanthine, and hypoxanthine disappeared in samples incubated with purine nucleoside phosphorylase and xanthine oxidase, respectively. Criterion d was used only during the initial evaluation of the method. Thereafter, peaks were simply identified by retention time and absorbance ratios.

The concentration of uric acid in plasma is much greater than that of the other compounds, and at the sensitivity setting we used it produced a full-scale peak (retention time 2.08 min), which eluted with other interfering substances near the void volume (Figure 3). Hypoxanthine (retention time 4.72 min) and xanthine (retention time 4.81 min) are sufficiently resolved to allow peak-area calculation with the LCI 100 integrator. Retention times of all compounds remained constant for up to three months of continuous column use. Thereafter the column deteriorated, with a decrease in retention times and loss of resolution.

Table 2 summarizes results of the analyses. Inosine, xanthine, and hypoxanthine concentrations were significantly higher in the hemodialysis group than in control group, but were lower after dialysis than before.

**Discussion**

In the past, the search for uremic toxins has been very selective, aimed at studying the concentration of single metabolic constituents in blood. Because of the interrelated nature of the metabolites associated with the uremic syndrome and the lack of chemical tests for identification and quantification, much of the information gathered to date is at best controversial.

High concentrations of certain metabolites have been shown to inhibit enzymes, and it has been suggested that others cause metabolic cycles to take secondary pathways (9–10).

To our knowledge, no studies have been available regarding the quantitative determination of inosine, xanthine, and hypoxanthine in plasma of patients with chronic renal failure before and after dialysis treatment.

We used high-performance liquid chromatography to obtain a good separation of inosine, xanthine, and hypoxanthine in plasma. Our method is similar to that of previously reported assays incorporating reversed-phase liquid-chromatographic separation and ultraviolet detection of various purine nucleosides and bases (11–12). Use of an ultrafiltration system to deproteinize plasma simplifies sample preparation without interfering with analyte recoveries. The normal concentrations of inosine and hypoxanthine in plasma reported here agree with values reported by other authors. For xanthine, our results are similar to those of Boullie et al. (7) and Harmsen et al. (13), but Wung and Howell (8) and Simmonds and Harkness (14) reported lower values. Normally occurring values for xanthine reported in the literature vary widely. Using our detection method, we found values for inosine, xanthine, and hypoxanthine in patients respectively about 13, six, and nine times higher than in controls. It remains difficult to evaluate whether the purine compounds we determined in plasma of dialysed uremics exert a toxic effect. However, in evaluating them as uremic toxins, one should take in account the possibility of increased susceptibility of the uremic organism due to the changed pathophysiology as well as of the collective toxicity of these compounds.

**Table 2. Concentrations (µmol/L) of Inosine, Hypoxanthine, and Xanthine (Mean and SD) in Plasma of Controls and Hemodialysis Patients before and after Dialysis**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 20)</th>
<th>Hemodialysis patients (n = 20)</th>
<th>Before</th>
<th>After</th>
<th>P values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inosine</td>
<td>0.56 (0.21)</td>
<td>0.56 (0.21)</td>
<td>7.01 (1.50)</td>
<td>3.92 (0.66)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1.22 (0.30)</td>
<td>1.22 (0.30)</td>
<td>10.40 (2.00)</td>
<td>4.80 (0.81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Xanthine</td>
<td>1.42 (0.28)</td>
<td>1.42 (0.28)</td>
<td>9.05 (1.12)</td>
<td>3.96 (0.38)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Derived from Student's t-test when patients before dialysis are compared with normal.

**References**