Simultaneous Liquid Chromatography of 5-Fluorouracil, Uridine, Hypoxanthine, Xanthine, Uric Acid, Allopurinol, and Oxipurinol in Plasma

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A reversed-phase "high-pressure" liquid-chromatographic method is described for simultaneous analysis for 5-fluorouracil, uridine, hypoxanthine, xanthine, uric acid, allopurinol, and oxipurinol. Separation was optimal with phosphate buffer (50 mmol/L, pH 4.60) as eluent. A simple acid extraction procedure yielded quantitative recoveries and permitted adequate separation from interfering peaks. Compounds were identified by their retention times, absorbance ratios, co-solvent with standards, and enzymatic shifts. With a computerized integrator we quantitated these compounds in widely varying concentrations with a single injection. The limit of sensitivity was 0.1 μmol/L for the compounds studied. This method was applied to determine mean values for those compounds in normal human plasma. They are (in μmol/L): uracil acid 278 (SD 55), hypoxanthine 0.46 (SD 0.21), xanthine 0.40 (SD 0.27), and uridine 4.50 (SD 0.70). Erythrocytes and platelets can continue to release hypoxanthine and xanthine into plasma or serum after a blood specimen has been drawn. We believe this explains the higher values previously reported for hypoxanthine and xanthine in serum.

Oxipurinol, an active metabolite of allopurinol and inhibitor of xanthine oxidase (EC 1.2.3.2) (1) and orotidine-5'-phosphate decarboxylase (EC 4.1.1.23) (2), decreases the toxicity of 5-fluorouracil for some animals and cultured cells (3, 4). Two clinical trials underway at this institution are directed at determining whether therapy with allopurinol alters the pharmacokinetics and toxicity of 5-fluorouracil in cancer patients. As part of these studies, we wished to simultaneously monitor 5-fluorouracil, uridine, hypoxanthine, xanthine, uric acid, allopurinol, and oxipurinol in a large number of clinical specimens of blood.

Many liquid-chromatographic procedures have been reported for various one of these compounds: 5-fluorouracil (5, 6); uridine, hypoxanthine, and xanthine (7-9); uric acid (10, 11); and allopurinol and oxipurinol (12, 13). We report here a technique for simultaneously measuring all of them. Plasma and serum samples were analyzed by this method to establish normal reference intervals for the endogenous purine metabolites, hypoxanthine, xanthine, and uric acid, and the pyrimidine metabolite, uridine. We also compared concentrations of these substances in plasma and serum as a function of duration of contact with the formed elements of blood.

Materials and Methods

Equipments

The liquid-chromatographic system (Waters Associates Inc., Milford, MA 01757) we used consisted of two Model 500A solvent-delivery pumps, a Model U6K sample injector, a Model 440 dual-wavelength ultraviolet detector (operated at 254 and 280 nm), a Model 660 solvent programmer, and a Model 730 Data Module dual-recording integrator capable of printing retention times of chromatographic peaks on chart paper and calculating peak areas from the 254-nm detector. The 2.9 mm × 25 cm reversed-phase column was of µBondapak C18 (Waters Associates).

Reagents

The eluent was 50 mmol/L potassium dihydrogen phosphate, pH 4.50 to 4.70.

5-Fluorouracil standard was obtained as a 500-mg injectable solution (Roche, Inc., Nutley, NJ 07110). Standards of hypoxanthine, xanthine, uric acid, uridine, allopurinol, and oxipurinol were purchased from Sigma Chemical Co., St. Louis, MO 63178.

Stock standard solutions were prepared in a concentration of 1 mmol/L in distilled water. These were diluted 100-fold with eluent to make 10 μmol/L working standards. 5-Fluorouracil and uridine were water-soluble, but the others required addition of a drop or two of 1 mol/L NaOH to dissolve. We also prepared a mixed standard consisting of 10 μmol of each compound per liter for use in the daily calibration run. All standards were apportioned and kept frozen until use.

Other chemicals used in these experiments included acetonitrile and methanol from Waters Associates, 1,1,2-trichlorofluoroethane (Freon) from the Wholesale Supply Co., Los Angeles, CA 90038; tricaprylyl tertiary amine (Alamine 336) from General Mills Chemicals Inc., Kankakee, IL; perchloric acid from Fisher Scientific Co., Fair Lawn, NJ 07410; and xanthine oxidase and catalase (EC 1.11.1.6) from Calbiochem, San Diego, CA 92112.

Sample Preparation

Plasma or serum, 0.5 mL, was deproteinized with one-tenth volume of 4.4 mol/L perchloric acid, and the excess acid was neutralized with 1 mL of Alamine/Freon (4.9 g/20 mL) as described by Khym (14). The pH of the final aqueous phase was about 4.60.

All the blood donors had no history of liver or renal abnormalities and their plasma or serum did not appear hemolytic, lipemic, or icteric.

Blood was obtained from these donors at unselected times, and the plasma was obtained by anticoagulating the blood with disodium ethylenediaminetetraacetate and centrifuging at 1000 × g for 10 min. Platelet-rich plasma was prepared by centrifuging whole blood at 150 × g for 15 min to remove other formed elements. To prepare plasma samples containing only erythrocytes or leukocytes, we resuspended the cell pellet recovered from the 150 × g centrifugation in isotonic saline and separated these cells on a Ficoll/Hypaque gradient (14). Washed erythrocytes or leukocytes were then added back to separate plasma samples from which all formed elements had been removed by centrifugation, in proportions corresponding to their respective concentrations in whole blood.

Plasma depleted of nucleosides and bases was prepared by stirring 10 mL of plasma with 0.5 g of activated charcoal for 30 min at room temperature. Sera from cancer patients undergoing treatment with both 5-fluorouracil and allopurinol were obtained before and at various times during constant intravenous infusion of 5-fluorouracil.

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Chromatographic Procedures

The compounds of interest were separated under isocratic conditions with the phosphate buffer as eluent, at a flow rate of 1.5 mL/min, continued for 15 min. Aqueous acetonitrile (600 mL/L) was used to purge the column periodically and at the end of each day, to prolong the useful life of the column and ensure a steady baseline.

A standard volume (20 μL) of each standard or sample was injected into the column and the sensitivity was set at 0.01 A full scale. The mixed standard was run daily and a three-point calibration curve was constructed weekly by injecting 10, 20, and 40 μL of mixed standard.

Results

Chromatographic Conditions

We measured how long the compounds in the mixed standard were retained on the C18 column as a function of the concentration and pH of the phosphate buffer. Varying the concentration from 12.5 to 50 mmol/L (keeping the pH at 4.60) revealed little concentration dependency for most compounds, except for hypoxanthine and uridine, which merged as a single peak at the lower concentration but were separated at the higher. However, further increase in eluent concentration up to 100 mmol/L only slightly improved their separation. Most compounds except uric acid were also insensitive to changes in pH over the range from 3.50 to 6.50. Uric acid eluted earlier in higher pH eluent, before 5-fluorouracil, and later in lower pH eluent, between 5-fluorouracil and hypoxanthine. The separation was best with the phosphate buffer concentration and pH specified under Reagents.

Compound Identification

We identified each compound under study as unambiguously as possible on the basis of retention time, peak height ratio at 254 nm vs that at 280 nm, and co-elution with known pure standard. Hypoxanthine and xanthine were also identified by the enzymatic peak-shift techniques as described elsewhere (7-9).

Figure 1 depicts a chromatogram of the mixed standard. Chromatograms of normal plasma and plasma from a cancer patient being treated concurrently with 5-fluorouracil and allopurinol are shown in Figure 2. The most prominent peak in plasma is uric acid, which at the sensitivity setting used produced a full-scale peak. Hypoxanthine, uridine, and xanthine are sufficiently separated to allow peak-area calculation with the Data Module, and also for manual peak-height measurement. Hypoxanthine has a peak height ratio of 10 at 254 nm vs 280 nm and is not detectable at 280 nm in concentrations <1 μmol/L, at which concentration its peak at 254 nm is still readily measurable. 5-Fluorouracil appears as a sharp peak, which can be measured easily. An interfering peak near it raises the baseline and causes some variation in peak area as calculated by the Data Module, and this interfering substance cannot be eliminated by changing buffer concentration or pH, or both. Oxpurinol, the major metabolite found during allopurinol treatment, ranges from 10 to 100 μmol/L in the plasma of patients who are receiving 300 mg of allopurinol every 8 h. A small interfering peak appears at the position of oxipurinol in some normal samples, but it corresponds to <2 μmol of oxipurinol per liter.

Guanine, which has a retention time of 7.30 min, is not resolved from hypoxanthine under our chromatographic conditions. However, it produces a fivefold larger peak than does an equimolar concentration of hypoxanthine at 280 nm, and as little as 0.2 μmol/L is still detectable. No guanine was detected in normal plasma samples after hypoxanthine had been removed by treatment with xanthine oxidase. Thymine, which can be detected in normal plasma and serum, elutes at 9.05 min and is completely separated from the two neighboring peaks, those corresponding to xanthine and oxipurinol.

Quantitative Analysis

A three-point calibration standard curve, plotted weekly for two months, was reproducible and linear over the concentrations of interest. The day-to-day variation of mixed standard calculated by the Data Module was within 95% confidence limits for all compounds. The concentrations of the compounds in unknown specimens were determined either from the standard calibration curve or calculated from the mixed standard by comparing peak area—or peak height at 280 nm in the case of 5-fluorouracil.

Analytical Recoveries

Stock standards were added to charcoal-treated blank plasma to give final concentrations of 400 μmol/L for uric acid and oxipurinol and 40 μmol/L for 5-fluorouracil, hypoxanthine, uridine, xanthine, and allopurinol. This solution was further diluted 10- and 100-fold with the treated plasma, which had been previously tested and found to contain only 3.50 μmol of urate per liter, with no detectable hypoxanthine, uridine, or xanthine.

We analyzed five samples of each plasma specimen containing each of three different concentrations of the added standards; the analytical recovery of each compound is given in Table 1. The only compounds for which the average recovery was significantly <100% was allopurinol, and this is probably attributable to the broader peak of this compound, which elutes late under isocratic conditions.

Comparison of Serum and Plasma

We compared concentrations in serum and plasma and...
found substantial concentration differences for hypoxanthine and xanthine, but not for uric acid and uridine. Specifically, the concentrations of hypoxanthine and xanthine in plasma samples separated immediately from the formed elements of blood was 0.46 (SD 0.21) and 0.40 (SD 0.27) μmol/L, respectively. In serum separated from the clot after 1 h at room temperature, the concentration of hypoxanthine had increased to 3.20 (SD 1.40) μmol/L, a sevenfold increase over plasma. The concentration of xanthine had also increased to 1.40 (SD 0.60) μmol/L, an increase of 3.5-fold over the value for plasma. When heparinized blood was left at room temperature for 1 h, and the plasma then separated for analysis, a similar increase in these two compounds was also observed. The concentration of uric acid in plasma was 276 (SD 55) μmol/L and of uridine 4.50 (SD 1.70) μmol/L.

Figure 3 shows the changes in hypoxanthine concentration with time in serum and plasma. Whereas the concentration in plasma separated without delay remained stable over 24 h, the concentration in serum remaining in contact with formed elements increased exponentially from 0.25 μmol/L initially to 220 μmol/L at 24 h. Changes in xanthine concentration followed the same pattern but were less marked (data not shown). We investigated the source of the hypoxanthine appearing in the serum, using plasma containing erythrocytes, leukocytes, or platelets, respectively, in concentrations corresponding to that in normal whole blood. Figure 3 indicates that the erythrocytes and platelets are the main contributors of hypoxanthine, whereas the leukocytes release <5% of the total appearing in the serum.

Discussion

Hypoxanthine, xanthine, and uric acid have been studied by spectrophotometric (15), enzymic (16–18), and oxygen consumption (19) techniques, but these are not suited to simultaneous analyses for all the compounds in which we are interested. Thin-layer chromatography can be used to measure multiple compounds, but the procedure is usually more time consuming due to the requirement of long elution time to extract compounds from absorbent (20). A gas–liquid chromatographic method has been described for the separation and quantitation of all the desired compounds (21), but the requirement for extensive sample prepurification and derivatization severely limits its use for routine analysis of these polar compounds in blood. Our method offers the ad-
vantages of speed and sensitivity, and it is especially effective for the quantitation of many polar compounds in biological fluids. Thus we believe it to be the method of choice. It is readily adapted to clinical analysis of large numbers of specimens. The simple extraction procedure yielded almost quantitative recoveries of all the compounds of interest, as judged from the dilution recovery study; the standard curve for all these compounds was linear; and results were reproducible over a 100-fold difference in concentration, as evaluated during two months.

The concentration of uric acid in serum and plasma is much greater than that of the other compounds, and at the sensitivity setting required for detection of other bases and nucleosides it produces a broad peak that usually is superimposed on other peaks in the chromatogram, making quantitation of both uric acid and the other compounds difficult. Many investigators have preferred to elute uric acid and other interfering substances near the void volume by using a phosphate buffer of higher pH (5.50 or greater) in analysis for nucleosides and bases by liquid chromatography (7-9). Other investigators (9) have used a fluorescent detector to differentiate uric acid from the other substances that absorb ultraviolet light but are not fluorescent. In our procedure, uric acid can be separated from the other substances by decreasing the pH of the phosphate buffer to 4.60. This also allows quantitation of 5-fluorouracil and hypoxanthine, peaks that otherwise would be covered by the broad peak of uric acid.

Hypoxanthine, guanine, xanthine, thymine, and uridine were generally poorly separated when low concentration of phosphate eluent was used (7-9). Recently, Putterman et al. (22) reported simultaneous measurement of hypoxanthine, xanthine, uric acid, allopurinol, and oxipurinol by liquid chromatography with use of ammonium phosphate (50 mmol/L, pH 4.50) as eluent. They found that hypoxanthine, guanine, and uridine were not separated well on the C 18 µBondapak column, nor were xanthine, thymine, and thymidylic acid, or oxipurinol and adenyl acid. They used a separate column of Sephadex G-10 to separate the nucleosides and nucleotides from the bases. We have found slightly better separation with 50 mmol/L potassium dihydrogen phosphate as an eluent than with ammonium phosphate. Its pH is easily maintained at 4.60 ± 0.10, which results in a more consistent retention time. With our technique most compounds plus 5-fluorouracil can be separated without an additional pre-column separation being necessary. However, hypoxanthine and guanine are not separated in this eluent. Nevertheless, hypoxanthine absorbs so weakly at 280 nm that no visible peak is detected at concentrations below 1 µmol/L, and guanine, which produces a fivefold larger peak than an equimolar amount of hypoxanthine, can be detected at a lower limit of 0.2 µmol/L. Thus we can estimate the extent of guanine contamination in a sample containing less than 1 µmol of hypoxanthine per liter by injecting a larger volume (100 µL) of extract and noting the degree of distortion of the 254/280 nm peak height ratio for hypoxanthine. Guanine contamination was also looked for by using xanthine oxidase to remove hypoxanthine. We detected no guanine in any sample by either technique.

Thymine was completely separated from the peaks corresponding to xanthine and oxipurinol. Adenyl and thymidylic acids were not investigated because their intracellular location and low concentration in plasma did not lead us to expect them to interfere with the compounds assayed (23). Other nucleosides and bases were found by other investigators (7-9, 22) not to interfere with measurement of the compounds of

### Table 1. Analytical Recoveries of Standards from Five Charcoal-Treated Plasma Specimens

<table>
<thead>
<tr>
<th>Added</th>
<th>Found *</th>
<th>Recovery, % (SD)</th>
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</thead>
<tbody>
<tr>
<td>5-Fluorouracil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>40.00 (2.00)</td>
<td>100 (5)</td>
</tr>
<tr>
<td>4</td>
<td>4.00 (0.20)</td>
<td>100 (5)</td>
</tr>
<tr>
<td>0.4</td>
<td>0.43 (0.07)</td>
<td>107 (10)</td>
</tr>
<tr>
<td>Uric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>440.00 (20)</td>
<td>110 (5)</td>
</tr>
<tr>
<td>40</td>
<td>42.00 (3.00)</td>
<td>105 (7)</td>
</tr>
<tr>
<td>4</td>
<td>3.90 (0.20)</td>
<td>98 (5)</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>37.00 (2.00)</td>
<td>93 (5)</td>
</tr>
<tr>
<td>4</td>
<td>4.10 (0.20)</td>
<td>102 (5)</td>
</tr>
<tr>
<td>0.4</td>
<td>0.40 (0.03)</td>
<td>100 (7)</td>
</tr>
<tr>
<td>Uridine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>40.00 (2.00)</td>
<td>100 (5)</td>
</tr>
<tr>
<td>4</td>
<td>4.20 (0.10)</td>
<td>105 (3)</td>
</tr>
<tr>
<td>0.4</td>
<td>0.38 (0.03)</td>
<td>95 (7)</td>
</tr>
<tr>
<td>Xanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>39.00 (2.00)</td>
<td>98 (5)</td>
</tr>
<tr>
<td>4</td>
<td>4.20 (0.10)</td>
<td>105 (3)</td>
</tr>
<tr>
<td>0.4</td>
<td>0.40 (0.03)</td>
<td>100 (7)</td>
</tr>
<tr>
<td>Oxipurinol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>370.00 (40)</td>
<td>93 (10)</td>
</tr>
<tr>
<td>40</td>
<td>38.00 (2.00)</td>
<td>96 (5)</td>
</tr>
<tr>
<td>4</td>
<td>0.40 (0.03)</td>
<td>100 (7)</td>
</tr>
<tr>
<td>Allopurinol</td>
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</tr>
<tr>
<td>40</td>
<td>38.00 (2.00)</td>
<td>95 (5)</td>
</tr>
<tr>
<td>4</td>
<td>3.80 (0.20)</td>
<td>95 (5)</td>
</tr>
<tr>
<td>0.4</td>
<td>0.35 (0.02)</td>
<td>89 (5)</td>
</tr>
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</table>

* Based on comparison with aqueous mixed standard that contained 10 µmol of each compound per liter.
interest, and we confirmed this in our preliminary study. In some normal samples we did observe a small peak appearing at the position of oxipurinol, but its size was negligible compared to the peak produced for samples from patients treated with allopurinol.

Most liquid-chromatographic methods for measurement of 5-fluorouracil have involved use of an organic solvent for the sample extraction, to obtain a "cleaner" chromatogram (5, 6). In the procedure reported here, without prior extraction with organic solvent, 5-fluorouracil appeared as a sharp peak, which could be easily detected. Although an unidentified peak appeared beside it, which raised the baseline for the 254-nm detector, this peak had a smaller absorbance at 280 nm than at 254 nm, where it did not interfere with 5-fluorouracil quantitation. Determination of 5-fluorouracil concentration by peak height measurement at 280 nm yielded a more consistent result than did integration of peak area at 254 nm.

The finding of large differences in the concentration of hypoxanthine and xanthine in serum and plasma freshly separated from the formed elements of blood emphasizes the importance of the timing of blood-sample preparation after venipuncture. Our results are consistent with those of Jørgensen et al. (18), who found a 1000-fold increase in hypoxanthine in serum left in contact with blood cells for 24 h. Our results also indicate that erythrocytes and platelets are the main source of the hypoxanthine and xanthine that appears in the serum. The rapid release of these oxidines by blood cells indicates that formed elements must be separated from plasma without delay for results to be reproducible and accurate. This probably accounts for the much higher concentrations of hypoxanthine and xanthine in serum that our investigators (22, 23) have reported.

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References