Serum Uric Acid Determined by Reversed-Phase Liquid Chromatography with Spectrophotometric Detection

Ernest J. Kiser, George F. Johnson, and David L. Witte

We describe a method for determining uric acid in serum by reversed-phase liquid chromatography with spectrophotometric detection at 280 nm. Serum, 100 μl, is mixed with 100 μl of a solution containing, per liter, 70 ml of acetonitrile in sodium acetate (20 mmol/liter, pH 4.0) and 500 mg of the internal standard, adenine. The mixture is allowed to stand in an ice bath for 3 min, then centrifuged. A 7.5-μl portion of the supernate is chromatographed on a "μBondpak C₁₈" column, with a 35 ml/liter solution of acetonitrile in sodium acetate (10 mmol/liter, pH 4.0) as the mobile phase. For 10 runs of duplicates, the within-run CV was 1.2% and the day-to-day CV (10 days) was 2.5% for a uric acid concentration of 53 mg/liter. Sera from 100 patients were analyzed for uric acid by the proposed method, a continuous-flow (SMA 12/60) method, and a uricase method; mean values for the 100 sera were 64, 71, and 64 mg/liter, respectively. Correlations were as follows: r = 0.987 for proposed method vs. SMA 12/60 and r = 0.997 for proposed method vs. uricase method. The proposed method is sensitive and specific and we think it will be useful for evaluating other uric acid methods for interference and specificity.

Additional Keyphrases: suggested reference method - intermethod comparison

The most common automated method for uric acid determination in use today depends on the ability of uric acid to reduce phosphotungstate to "tungsten blue," a procedure that can give falsely high results in the presence of certain drugs or of abnormal concentrations of endogenous metabolites. The enzyme uricase (urate oxidase, EC 1.7.3.3) offers greater specificity.

More recently, a specific procedure based on anion-exchange liquid chromatography and electrochemical detection has been developed (1, 2). We present in this paper a new, alternative, high-performance liquid-chromatographic procedure, involving a bonded alky stationary phase and spectrophotometric detection. The method was derived from a specific procedure for serum theophylline that is used in many laboratories (3). Our method has been extensively compared to an automated uricase procedure and we have used it to investigate further the specificity of the automated phosphotunge-

state method. We believe the specificity, reproducibility, and simplicity of our new chromatographic method is such that it deserves consideration as a reference method for serum uric acid determination.

Methods and Materials

Apparatus

We used a Model ALC/GPC 204 Liquid Chromatograph (Waters Associates, Inc., Milford, Mass. 01757) fitted with a reversed-phase "μBondpak C₁₈" column and equipped with an ultraviolet detector for the chromatography and detection at 280 nm of uric acid. We used a discrete analyzer (ABA-100; Abbott Diagnostics, South Pasadena, Calif. 91030) for the enzymatic determination of uric acid, and a continuous-flow analyzer (SMA 12/60; Technicon Corp., Tarrytown, N. Y. 10591) to determine uric acid by the phosphotungstate method.

Reagents

Mobile phase (35 ml of acetonitrile in pH 4.0, 10 mmol/liter sodium acetate buffer.) Prepare the mobile phase by dissolving 2.722 g of sodium acetate trihydrate (J.T. Baker Chemical Co., Phillipsburg, N.J. 08865) in two liters of distilled water, and electrometrically adjusting the pH of this solution to 4.0 with glacial acetic acid. Next, dilute 70 ml of acetonitrile (Mallinkrodt Inc., St. Louis, Mo. 63147), in a 2-liter volumetric flask, to volume with the buffer. Filter the resulting solution through a 0.45-μm (average pore size) filter, to remove particulates and to aid in degassing the solution.

Uric acid stock standard (1 g/liter). We prepared this by dissolving 500.0 mg of uric acid (National Bureau of Standards, Standard Reference Material No. 913) in a 500-ml volumetric flask with a warm lithium carbonate solution [300.0 mg of lithium carbonate (Mallinkrodt) in 75 ml of warm distilled water (60 °C), filtered before use]. The uric acid stock standard was heated for 1 h under hot running water with frequent mixing, then allowed to cool to room temperature. The mixture was diluted to volume with distilled water and was left to stand for 6 h at room temperature. The resulting clear solution was stored at 5 °C in a polypropylene bottle. From this stock solution, standard uric acid solutions were prepared by diluting with distilled water to give

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concentrations of 20, 50, 100, 150, and 200 mg/liter. The uric acid stock standard is stable at 4 °C for at least one month.

Adenine internal standard (0.5 g/liter). Fifty milligrams of synthetic adenine (lot no. 510266; Calbiochem, San Diego, Calif. 92112) was placed in a 100-ml volumetric flask. The adenine was dissolved in a sodium acetate buffer (20 mmol/liter, pH 4.0) containing 70 ml of acetonitrile per liter. This buffer was prepared by dissolving 2.722 g of sodium acetate trihydrate in 1 liter of distilled water and adjusting the pH to 4.0 with glacial acetic acid. Acetonitrile, 70 ml in a 1-liter volumetric flask, was diluted to volume with the pH 4.0 buffer, and filtered through a 0.45-µm filter to remove particulate matter before adding the buffered solvent to the adenine.

Chromatographic Procedure

Dispense 100 µl of each diluted standard, control, and sample into individual microcentrifuge tubes. Next, add 100 µl of the adenine internal standard to all the tubes. Vortex-mix the contents of the tubes for 20 s and place the tubes in an ice bath for 3 min to promote protein precipitation. Centrifuge all tubes for 2 min at full speed, and inject 7.5 µl of the supernatant fluid from each tube into the chromatograph with a 10-µl syringe. Operate the chromatograph under the following conditions: solvent flow rate, 2.5 ml/min; attenuation, 0.1 A full scale; and wavelength, 280 nm. For all samples and standards measure the heights of peaks having the retention times of uric acid and adenine, and calculate the ratio (peak height of uric acid/peak height of adenine). Construct a linear standard curve from the peak-height ratios of the standards and determine sample concentration by reference to this curve. Each run included a five-point standard curve and duplicates of three control sera.

Uricase Method

The enzymatic procedure used is based on the system of Haeckel (4), the reaction scheme being as follows:

\[
\text{uricase} \quad \text{Uric acid} \rightarrow \text{H}_2\text{O}_2 + \text{allantoin}
\]

\[
\text{H}_2\text{O}_2 + \text{ethanol} \rightarrow 2 \text{ H}_2\text{O} + \text{acetaldehyde}
\]

\[
\text{Acetaldehyde} + \text{NAD}^+ \rightarrow \text{NADH} + \text{H}^+ + \text{acetate}
\]

The reagents used are commercially available from Smith Kline Instruments, Inc., Sunnyvale, Calif. 94086. Their procedure for first revolution reading (FRR) on the ABA-100 is as follows:

<table>
<thead>
<tr>
<th>Filter</th>
<th>380/340</th>
<th>FRR</th>
<th>On</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubator</td>
<td>37 °C</td>
<td>Syringe plate</td>
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</tr>
<tr>
<td>Mode</td>
<td>Rate</td>
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<td>25 µl</td>
</tr>
<tr>
<td>Direction</td>
<td>Down</td>
<td>Analysis time</td>
<td>10 min</td>
</tr>
</tbody>
</table>

The uric acid concentrations of unknowns were calculated from the unweighted linear least squares fit obtained with five standards included on each carousel. Standards were the same as those used for the present chromatographic method.

Continuous-Flow Method

In the continuous-flow (SMA 12/60) procedure uric acid is removed from serum by dialysis. The uric acid quantitatively reduces phosphotungstic acid complex in the presence of hydroxylamine to a phosphotungstic acid complex, which is colorimetrically monitored at 660 nm (7). The SMA 12/60 was calibrated with commercial reference sera ("Hyland Q-Pak"; Hyland Div. Traverol Labs., Inc., Costa Mesa, Calif. 92626). Sera with uric acid concentrations exceeding 120 mg/liter were diluted threefold with de-ionized water and re-analyzed.

Results

Chromatograms

Figure 1a shows a chromatogram of uric acid with the internal standard adenine present. Under our chromatographic conditions, the retention time for uric acid is 1.8 min; for adenine, 2.7 min. We saw no chromatographic peaks from the sera investigated that had the same retention time as adenine. Formaldehyde could not be used as a preservative for uric acid standards. When it is present in uric acid standards, the peak height of uric acid is diminished and a second peak appears with a retention time of 2.1 min (Figure 1b). Uric acid in a serum matrix (Figure 1c) has the same retention time as does uric acid in the standards. When 8 ml of a pooled serum with a uric acid concentration of 27 mg/liter was treated with 0.1 ml of uricase (17 U/liter), left at room temperature for 48 h, and then analyzed by our procedure, no chromatographic peak with the retention time of uric acid was detected (Figure 1d).

Other Analytical Variables

Analytical recovery studies. When stock uric acid (1 g/liter) was added to two separate diluted sera to increase the uric acid concentration by 50 mg/liter, and the sera analyzed, analytical recoveries were 100 and 104%.

Standard curves and linearity. Standard curves, run on a daily basis, exhibited a correlation coefficient of at least 0.9995 for peak-height ratios of adenine/uric acid vs. uric acid concentration. New standards were prepared for each run, and these same standards were run by both the automated uricase method and the present method. Standards were analyzed before samples and controls, in ascending concentration. The present method demonstrated a linear relation between instrument response and uric acid concentration to 500 mg/liter. The average absorbance of a 100 mg/liter standard taken through the procedure was 0.08 A.

Precision and drift. Three controls were run, each in duplicate, for both the present and the uricase method on each of the 10 days that samples were run. The controls were Ortho Abnormal Serum, Hyland Reference
comparison between the present method (y) and the SMA 12/60 method (x). The unweighted linear regression line for these data was $y = 0.874x + 2.2$ mg/liter, with a correlation coefficient ($r$) of 0.987. The mean uric acid concentration by the present method was 64.0 mg/liter, 71.0 mg/liter by the SMA 12/60 procedure. Because in the SMA 12/60 procedure samples with a uric acid concentration exceeding 120 mg/liter must be diluted and re-analyzed, we compared results by the present and SMA 12/60 procedures by using the 84 specimens that did not have such concentrations. The unweighted linear regression line for these data [present method (y) vs. SMA 12/60 (x)] was $y = 0.967x - 2.1$ mg/liter, with $r = 0.996$. The mean uric acid concentration by the present method for these 84 samples was 48.5 mg/liter, 52.5 mg/liter by the SMA 12/60 method. The mean for the 16 specimens with uric acid exceeding 120 mg/liter was 146.1 mg/liter for the present method, 168.9 mg/liter for SMA 12/60 method.

Figure 3 shows the results of the comparison between the present method (y) and an automated uricase procedure (x). The unweighted linear regression line for these data was $y = 1.015x - 0.2$ mg/liter, with $r = 0.997$. Mean values were 64.0 mg/liter for the present method, 63.5 mg/liter for the uricase procedure. Means for the 84 samples that had values of 120 mg/liter or less by the continuous-flow procedure were 48.5 mg/liter for the present method and 48.3 mg/liter for the uricase method; for the 16 samples with continuous-flow values greater than 120 mg/liter, the respective means were 146.1 and 143.7 mg/liter.

**Interference Studies**

**Allopurinol and oxipurinol.** Allopurinol and oxipurinol (1H-pyrazolo[3,4-d]pyrimidine-4,6-diol), when added to serum specimens to give concentrations of 50 mg/liter each, produced no observable change in the apparent uric acid concentrations by any of the three methods. In the present procedure, uric acid, oxipurinol, allopurinol, and adenine chromatograph with retention times of 1.8, 2.3, 2.5, and 2.7 min, respectively. At the

### Table 1. Precision of Present and Automated Uricase Methods for Uric Acid *

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<thead>
<tr>
<th></th>
<th>Present</th>
<th>Uricase</th>
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<tbody>
<tr>
<td><strong>Pooled serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mg/liter)</td>
<td>53</td>
<td>50</td>
</tr>
<tr>
<td>Within-run CV, %</td>
<td>1.19</td>
<td>1.11</td>
</tr>
<tr>
<td>Day-to-day CV, %</td>
<td>2.48</td>
<td>2.55</td>
</tr>
<tr>
<td><strong>Control I</strong></td>
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<td></td>
</tr>
<tr>
<td>Mean (mg/liter)</td>
<td>93</td>
<td>87</td>
</tr>
<tr>
<td>Within-run CV, %</td>
<td>1.29</td>
<td>0.68</td>
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<tr>
<td>Day-to-day CV, %</td>
<td>2.49</td>
<td>3.14</td>
</tr>
<tr>
<td><strong>Control II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mg/liter)</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>Within-run CV, %</td>
<td>1.24</td>
<td>1.90</td>
</tr>
<tr>
<td>Day-to-day CV, %</td>
<td>1.83</td>
<td>3.40</td>
</tr>
</tbody>
</table>

* 10 runs of duplicate samples.
+ Ortho Abnormal II Control Serum.
* Hyland Reference Serum.

Serum, and a serum pool. Within-run and day-to-day coefficients of variation for each control were calculated for both methods by one-way analysis of variance (Table 1). The serum pool was analyzed at the beginning and end of the patients’ samples, to assess drift. The within-run variation calculated for this control did not significantly exceed that observed for the other controls. A maximum of 16 sera were analyzed at one time.

**Method-Comparison Studies**

One hundred samples of sera were obtained that had been collected over a two-week period, analyzed previously by the SMA 12/60 method, and frozen. We analyzed these samples over a 10-day period, 10 samples analyzed daily by the present and the automated uricase methods. The range of uric acid concentrations selected for assay was as wide as possible. Figure 2 shows the
detector attenuation used in the present procedure, neither allopurinol nor oxipurinol was detectable. The precursors of uric acid, hypoxanthine and xanthine, chromatograph with retention times of 1.9 and 2.1 min, respectively. Neither compound interferes with the present uric acid determination, although there is overlap in retention times between uric acid and hypoxanthine. At 280 nm, in our solvent system, the absorbance of hypoxanthine is only 0.1 that of uric acid at the same concentration.

Acetaminophen and salicylate. Acetaminophen interference was investigated by adding this drug to a pooled serum to obtain a concentration of 500 mg/liter. Uric acid concentrations were measured in these specimens by all three procedures. For the present and uricase procedures we saw no change in the uric acid concentration of this serum pool. However, the continuous-flow procedure showed an increase of 25 mg/liter in apparent uric acid concentration. This interference has been previously documented for the SMA 12/60 uric acid method (5). In an overdosed patient with an acetaminophen concentration of 200 mg/liter, similar results were obtained: uric acid concentrations of 61, 33, and 35 mg/liter were determined by the SMA 12/60, present, and uricase methods, respectively. Acetaminophen has a longer retention time than either uric acid or adenine and only appears in the chromatograms at concentrations >50 mg/liter under our chromatographic conditions. Salicylate has an even longer retention time than acetaminophen and does not interfere.

Theophylline. Samples from 10 patients with an average serum theophylline concentration of 17.7 mg/liter were assayed for uric acid by the three methods. The mean uric acid values obtained were 58, 62, and 57 mg/liter by the present, continuous-flow, and uricase methods, respectively. Theophylline and its major metabolites do not interfere because of their longer retention times than that of either adenine or uric acid in our chromatographic procedure (3).

Discussion

Reversed-phase liquid chromatography with bonded alkyl phases has been extensively applied to the separation of essentially neutral molecular species. Recently, it has been realized that this chromatographic mode is also applicable to many substances that carry negative or positive charges at neutral pH; this flexibility greatly enhances the utility of reversed-phase chromatography in the clinical laboratory. Many different kinds of separations can be achieved with a single column merely by varying the composition of the mobile phase. Orcutt et al. (3) in their reversed-phase procedure for serum theophylline were able to separate this drug from its polar metabolites, which include 1-methyluric acid and 1,3-dimethyluric acid. In using this procedure, we observed an offscale peak that eluted just after the column void volume. This peak, which had the same retention time as uric acid, disappeared on incubating the serum with uricase. By decreasing the acetonitrile concentration in the mobile phase we were able to separate the uric acid from closely eluting compounds. Uric acid is detected in our procedure by its ultraviolet absorbance at 280 nm. In our solvent system, uric acid showed 98% of its peak absorbance at this wavelength. A serum uric acid concentration of 100 mg/liter would produce an absorbance of over 7.0 at 292 nm. Although greater sensitivity can be achieved with detectors such as the electrochemical detector (1, 2), the method we describe is easily adaptable to micro-scale samples.

The results shown in Figure 2 illustrate the high correlation between the present and continuous-flow methods, especially with those samples with uric acid values below 120 mg/liter ($r = 0.996$). The mean uric acid concentration of these samples by continuous-flow
analysis averaged 7.8% higher than the mean obtained by the present method. A portion of this difference can be explained by standardization differences between the two methods. The secondary reference material used for standardization of the SMA 12/60 method assayed 3.8% lower than its nominal assigned value by the present method. For those 16 samples shown in Figure 2 with SMA 12/60 values > 120 mg/liter, the mean SMA 12/60 results were 15.6% higher than the mean from the present method. This greater discrepancy for SMA 12/60 results above 120 mg/liter may be explained by errors in dilution or a significant change in matrix effects upon sample dilution, although samples that were carefully rediluted gave equivalent results, and results obtained for aqueous standards analyzed by the SMA 12/60 did not support a matrix effect. More probably, the larger discrepancy observed between the two methods for concentrations exceeding 120 mg/liter is attributable to interfering substances that are present as a result of uremia in these patients. All patients with SMA 12/60 uric acid values above 120 mg/liter had serum urea concentrations that were consistent with decreased renal function.

Comparison of the present method and the specific uricase procedure of Haeckel (4) indicated excellent agreement between the two procedures (Figure 3); the mean uric acid concentrations by the two procedures differed by 0.5 mg/liter. For the 16 samples with SMA 12/60 values greater than 120 mg/liter, the average difference was only 2.4 mg/liter. There was no statistically significant (P > 0.05) difference between the results obtained by the present and the uricase methods when either the sign test or Wilcoxon’s signed ranked test were applied to the paired data (6). The observed close agreement between the chromatographic and the enzymatic procedures for uric acid confers increased analytical validity on these procedures.

Many analytical determinations in the clinical laboratory are subject to recognized or possibly unrecognized interference from drugs and their metabolites that may be present in patients’ specimens. Comparison of one analytical technique with another based on a different physical principle serves to check not only for analyte specificity in the presence of endogenous substances but can yield useful information about drug interferences as well. Examination of the observed differences between the present and the uricase methods for uric acid in this study showed no absolute difference larger than expected from the analytical errors inherent in each method. Since the 100 samples analyzed included sera with hemolysis, lipemia, and bilirubinemia, interference from these substances, if present, must also be minimal compared to analytical errors in the uric acid procedures. Compounds chemically similar to uric acid were also tested for interferences with the chromatographic and uricase methods. Neither allopurinol nor its metabolite oxipurinol produced observable interference in either method. The precursors of uric acid, xanthine and hypoxanthine, also did not interfere. The presence of therapeutic concentrations of theophylline and presumably its metabolites caused no discrepancy between results on the same samples obtained by the present and uricase methods. The commonly prescribed analgesics, acetaminophen and salicylic acid, added to serum, also produced no increase in apparent uric acid concentration by either of these methods. Acetaminophen, however, at high concentrations showed the expected (5) interference with the SMA 12/60 method.

The method we describe in this paper is sensitive and specific, and exhibits good precision. It is especially valuable as a reference procedure for evaluating new uric acid methods for specificity and drug interference. Our method for determination of uric acid can be simply adapted for use in those laboratories now using reversed-phase chromatographic procedures for therapeutic drug monitoring.

References