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Quantification of urinary uric acid in the presence of thymol and thimerosal by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method was developed as an alternative to automated enzymatic analysis of uric acid in human urine preserved with thymol and/or thimerosal. Uric acid (t_R=10 min) and creatinine (t_R=5 min) were separated and quantified during isocratic elution (0.025 *M* acetate buffer, pH 4.5) from a μBondapak C_{18} column. The uric-acid peak was identified chemically by incubating urine samples with uricase. The thymol/thimerosal peak appeared at 31 min during the washing step and did not interfere with the analysis. We validated the high-performance liquid chromatographic method for linearity, precision and accuracy, and the results were found to be excellent.

Keywords: Uric acid; Thymol; Thimerosal; Creatinine

1. Introduction

Excess urinary uric acid secretion is a key factor in the potential development of renal stones. Uric acid crystals also can act as a nidus for calcium oxalate and calcium phosphate stones [1]. Related investigations by this laboratory have shown that space flight crew members have an increased risk of developing calcium oxalate, calcium phosphate and uric acid stones [2]. Urinary components that contribute to stone-forming potential, including uric acid, before, during and after each Space Shuttle mission are routinely analyzed.

Because refrigerated or frozen storage space is limited aboard spacecraft, urine samples often are stored at room temperature during flight with addition of thymol (0.05%) and/or thimerosal (0.1%) as preservatives. The use of thimerosal, but not thymol, interferes with automated means of analysing uric acid with instruments such as a Beckman Synchron CX5. (The addition of HCl to stabilize urine samples also interferes with this analysis). We sought to develop an HPLC method as an alternative, to circumvent this interference in urine samples. We also used the HPLC method to investigate the mechanism by which thimerosal interferes with the analysis of uric acid in the Beckman system.

Numerous HPLC methods have been developed for quantifying uric acid and other metabolites in plasma and urine [3–8]. Most of those methods were not designed for routine clinical analyses in urine samples. For routine assessments of uric acid and creatinine, fully automated assays are superior to HPLC methods in terms of speed, simplicity and quality control. However, the HPLC method described here is a novel application for urine analysis

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of uric acid, intended for situations in which enzymatic methods cannot be used.

2. Experimental

2.1. Equipment

The HPLC system used is a modification of that described by Arín et al. [6] for analyzing sheep urine. The HPLC system consisted of two Model 510 pumps, a Model 440 UV detector, a Model 712 WISP autosampler and a Maxima 820 system controller (all from Waters Associates, Milford, MA, USA). The analytical column was a μBondapak C_{18} column (300×3.9 mm) with a Guard-Pak C_{18} guard column. All chromatograms were printed with a NEC Pinwriter P6. Selected chromatograms were transferred from the Maxima as ASCII files to SigmaPlot for presentation.

2.2. Chemicals and reagents

Creatinine, uric acid, uricase, thymol and thimerosal were purchased from Sigma (St. Louis, MO, USA). Methanol (Burdick and Jackson brand) was purchased from Baxter Scientific (Waukegan, IL, USA). Sodium acetate and acetic acid were obtained from Fisher Scientific (Fairlawn, NJ, USA). Lyphochek urine controls were purchased from Bio-Rad (Anaheim, CA, USA). All water was processed by a Milli-Q plus system. HPLC solvents were filtered through 0.2 μm bottle-top filters purchased from Costar (Cambridge, MA, USA). The Millex-LCR_4 and Acrodisk LC13PVDF (0.45 μm) sample filters were purchased from Millipore (Bedford, MA, USA) and Gelman (Ann Arbor, MI, USA), respectively.

2.3. Standards solutions

The uric acid stock solution (1 mg/ml) was prepared by dissolving uric acid in 0.1 *M* NaOH. The creatinine stock solution (1 mg/ml) was prepared by dissolving creatinine in water.

For working standards, creatinine (20, 100, 200 and 300 μg/ml) and uric acid (5, 25, 50 and 75 μg/ml) were prepared by proper dilution of the stock solution with water. For the standard addition study, uric acid or creatinine were dissolved in 1 *M* NaOH or water to make 10 mg/ml solutions. Thymol and thimerosal solutions (50 mg/ml) were prepared by dissolving the compounds in methanol or water, respectively. All stock solutions were kept at 2–4°C for up to three days.

2.4. Urine samples

Urine samples were obtained from test subjects, filtered and diluted 1:10 (v/v) before being injected into the HPLC system. Urine was preserved by adding 0.05% thymol, 0.1% thimerosal, or both before analysis.

2.5. Chromatography

Urinary creatinine and uric acid were separated and quantified under isocratic conditions. The elution conditions for each 45 min sample run were as follows:

1. Analytical run (0–15 min); sodium acetate, 0.25 *M*, pH 4.5 (Pump A).
2. Washing run (15–30 min); from 0 to 100% 0.1 *M* acetic acid in methanol (Pump B).
3. (30–35 min); from 100% to 0% 0.1 *M* acetic acid in methanol (Pump B).
4. Re-equilibration (35–45 min); sodium acetate, 0.25 *M*, pH 4.5 (Pump A).

Analyses were carried out at a flow-rate of 1.0 ml/min with UV detection at 254 nm. Alternative detection systems, using UV detection at 235 nm [4,5], 210 nm [7], or diode array spectra at 210 nm, 234 nm and 290 nm [8], are possible. All injection volumes were 20 μl. All separations were conducted at ambient temperature.

2.6. Method validation

The HPLC method was validated in two ways. The first involved spiking 1 ml urine samples with known amounts of uric acid or creatinine (10 mg/ml, in 1 *M* NaOH or water, respectively) and then calculating the recovery of these compounds. The second method, chemical identification, involved

adding 100 μl of water or uricase (0.125 U) to 1 ml of urine, incubating the mixtures at 37°C for 60 min and transferring them to WISP vials, where they were maintained at room temperature before injection. The disappearance of the expected peak for uric acid in the uricase-treated sample thus was identified chemically as being the uric acid peak.

2.7. Correlation study

The correlation study involved grouping urine samples and commercial control urine (Bio-Rad) into two sets of tubes, one set for HPLC analysis and the other for analysis on a Beckman Synchron CX5 analyzer.

2.8. Thimerosal and inhibition of uricase

The interference of thimerosal with uric acid analysis on the Synchron was verified by testing four sets of urine samples, each containing 0, 0.01, 0.1 or 0.5% thimerosal. In order to assess whether this interference resulted from the thimerosal inhibiting uricase, we investigated the inhibition of uricase by HPLC as follows: The reaction mixture contained 100 μg of uric acid, 1 mg of thimerosal and 25 mU of uricase in 1 ml of either 0.01 *M* phosphate buffer (pH 8.6) or 0.01 *M* NaOH (pH 12.2). Two vials were used for each experiment, one without uricase (vial 1) and the other with uricase (vial 2). The 45-min run time served as the incubation time. Immediately after this experiment, unpreserved reaction mixtures (i.e., those without thimerosal) were examined using the same procedure. Tests were conducted at ambient temperature and with ambient oxygen in the reaction mixture.

3. Results and discussion

3.1. HPLC method

Creatinine and uric acid were separated and quantified through elution under isocratic conditions (0.025 *M* acetate buffer, pH 4.5) from a μBondapak C_{18} column. Fig. 1B illustrates a whole-urine profile from a 45-min run. The creatinine peak (t_R=5 min), uric acid peak (t_R=10 min) and other unidentified

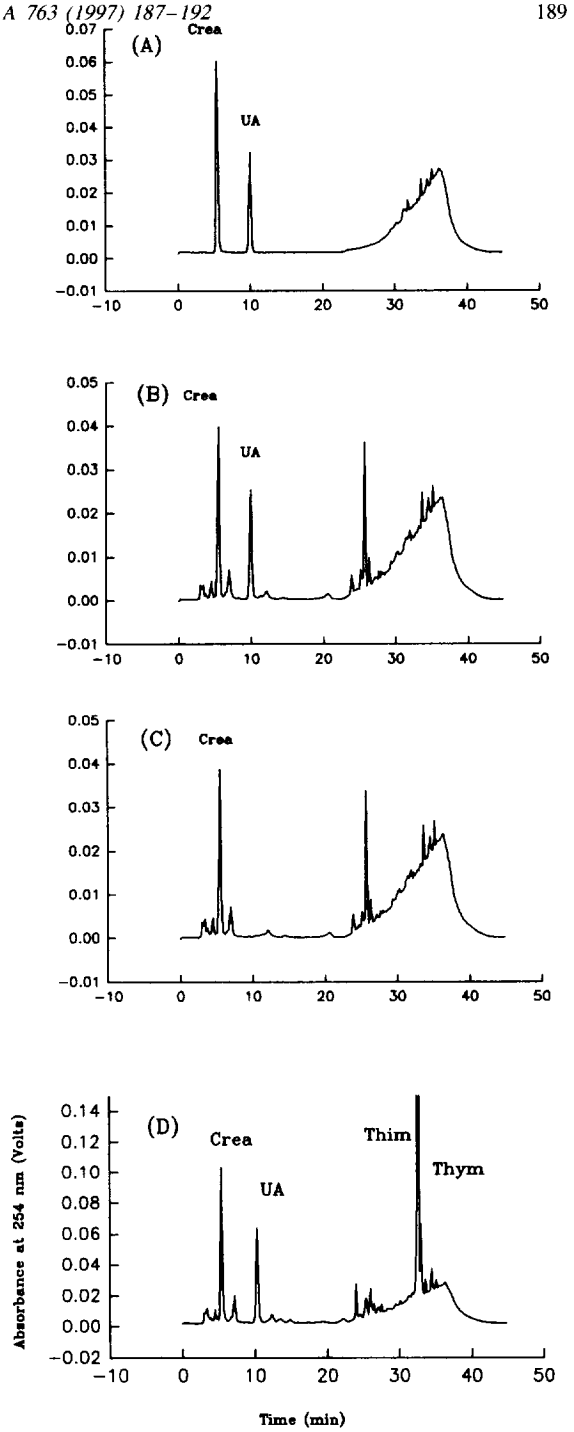

Fig. 1. Typical chromatograms. Panel A, chromatogram of a standard solution of creatinine (Crea, 100 μg/ml) plus uric acid (UA, 25 μg/ml). Panel B, chromatogram of urine without uricase; Panel C, urine with 0.125 U/ml uricase; Panel D, chromatogram of urine preserved with 0.05% thymol (Thym) and 0.1% thimerosal (Thim).

peaks separated within the first 15 min. The next 30 min were used for column clean-up (0.1 *M* acetic acid in methanol) and re-equilibration for the next sample run. No "carry-over" peaks were found over twenty consecutive sample runs in 16 h. However, a large late peak (OD>1) appeared occasionally at the washing step and required an extra 10–20 min for equilibration.

Because the actual separation of uric acid and creatinine from other urinary metabolites takes place under isocratic conditions, the flow-rate could be adjusted between 0.8 and 1.0 ml/min to achieve optimal resolution. In general, chromatograms in which the retention time for creatinine was 5 min and that of uric acid was 10 min had good resolution. Interestingly, a doublet uric acid peak appeared when the standard was in 0.1 *M* potassium phosphate (pH 8.6), but not for uric acid in 0.01 *M* potassium phosphate, 0.01 *M* NaOH, or 0.1 *M* NaOH.

3.2. Calibration

Within the linear range of the detector, the calibration graph was linear from 20 to 300 μg/ml for creatinine and from 5 to 75 μg/ml for uric acid. Both creatinine and uric acid had correlation coefficients of 0.999 ($y=6.0+24.7x$ for creatinine and $y=-32.8+10.7x$ for uric acid); y=peak area (mV s), x=concentration (μg/ml). The linear range covered the normal physiological ranges of creatinine (500–1000 μg/ml) and uric acid (125–375 μg/ml) after a 1:10 (v/v) dilution.

3.3. Method validation

The intra-assay relative standard deviation (R.S.D.) was less than 2% and the mean recovery was 103% (Table 1). The uric acid peak also was identified chemically by incubating urine samples with uricase; the expected uric acid peak (Fig. 1B) was completely eliminated by uricase treatment (Fig. 1C). The expected end-product, allantoin, could not be identified in this system, probably because of its low absorbance at 254 nm.

3.4. Comparison with the results obtained using the Beckman Synchron analyzer

The use of the Beckman synchron analyzer for measurement of uric acid and creatinine was based on enzymatic [9] and colorimetric [10] methods, respectively. Creatinine and uric acid measured with the HPLC system correlated well with measurements from the Synchron (Fig. 2). The regression line and correlation coefficient for uric acid were $y=2.3+0.94x$, $r=0.9805$; those for creatinine were $y=-7.9+0.88x$, $r=0.9747$. There is no significant difference between the uric acid assays by HPLC and Synchron CX5. However, for the creatinine assay, the HPLC values were significantly higher than the colorimetric values ($p<0.001$, paired t-test). This is probably due to differences in methodology.

3.5. Thimerosal and inhibition of uricase

As shown in Table 2, thimerosal (in amounts as small as 0.01%) significantly decreased the amounts of uric acid measured with the Synchron, compared to those in untreated control samples. Further decreases were noted in urine containing 0.1% thimerosal, but the extent of the decrease levelled off at 0.5% thimerosal.

Table 1
Accuracy for the simultaneous determination of uric acid and creatinine in 1 ml of urine

Analyte	Amount added (μl)		Expected concentration	Amount found	S.D. ($n=4$)	R.S.D.	Recovery
	Uric acid (10 mg/ml)	Creatinine (10 mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(%)	(%)
Uric acid	0	0	0.40	0.40	0.004	1.00	–
	10	40	0.47	0.50	0.002	0.40	106
	50	200	0.72	0.72	0.006	0.83	100
Creatinine	0	0	1.49	1.49	0.018	1.21	–
	10	40	1.80	1.82	0.007	0.38	101
	50	200	2.79	2.93	0.010	0.34	105

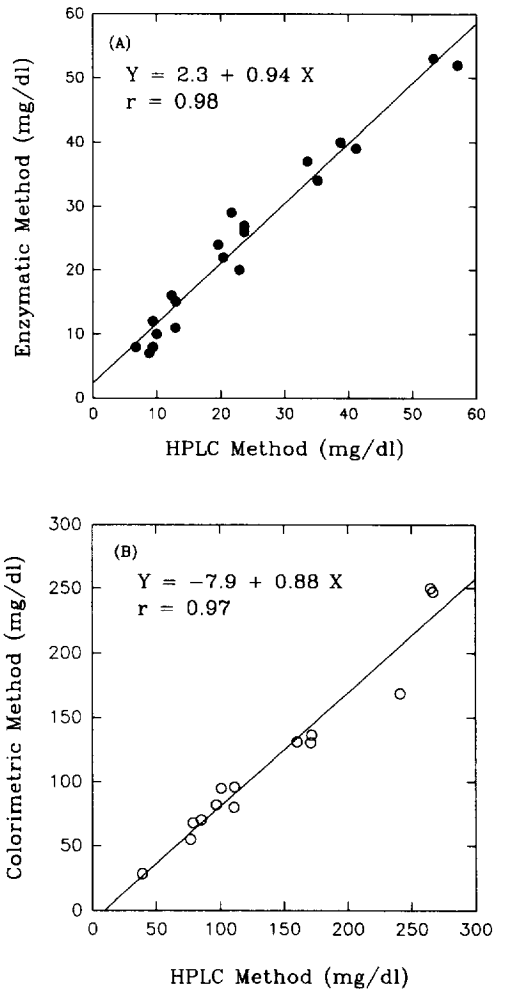

Fig. 2. Correlation of Beckman Synchron CX5 (enzymatic or colorimetric) vs. HPLC analysis of uric acid (A) and creatinine (B).

Table 2
Effect of thimerosal on urinary uric acid measurement by the Beckman Synchron CX5

Thimerosal concentration (%)	Uric acid concentration (Mean±SEM, mg/dl)	Inhibition (%)
0	75.2±0.28	–
0.01	71.6±0.35[a]	4.7
0.1	67.7±0.80[a]	10.0
0.5	67.2±0.32[a]	10.6

[a] $p<0.05$, $n=4$. Statistical evaluation was performed by an analysis of variance with repeated measures and comparison of all groups vs. control group (without thimerosal).

The Synchron uses a timed-end-point method to measure uric acid [9]. Uric acid is oxidized by uricase to produce allantoin and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulfonate (DCHBS), in a reaction catalyzed by peroxidase, to produce a colored product, quinoneimine, which has a strong absorbance at 520 nm:

$$\text{Uric acid} + O_2 + H_2O \overset{\text{Uricase}}{\rightarrow} \text{Allantoin} + H_2O_2 + CO_2 \quad (1)$$

$$H_2O_2 + \text{4-AAP} + \text{DCHBS} \overset{\text{Peroxidase}}{\rightarrow} \text{Quinoneimine} + H_2O \quad (2)$$

HPLC results indicate that thimerosal slightly inhibited the rate of reaction 1, defined as the extent of decrease in uric acid per min in the reaction mixture. At pH 8.6, the reaction rate during the first 45 min was slower in the mixture containing 0.1% thimerosal (−1.17 μg/min) than in the control without thimerosal (−1.33 μg/min). In a separate experiment conducted at pH 12.2, the difference in reaction rate was −1.22 (no thimerosal) vs. −0.79 μg/min (with thimerosal). This slight inhibition of uricase activity by thimerosal may be responsible for the lesser amounts of uric acid measured by the Synchron. Whether thimerosal inhibits peroxidase activity in reaction 2 is unknown.

3.6. Stability of uric acid in urine

We also experienced interference with enzymatic measurements of uric acid in urine to which HCl had been added. As shown in Table 3, concentrations of uric acid in acidified urine samples were much lower than in non-acidified urine samples. The low levels in the acidified urine were not a result of interference in the enzymatic reaction, since the HPLC method, which does not involve a chemical reaction, also produced comparable results. This finding implies that uric acid is not stable under acidic conditions.

In the presence of thymol and thimerosal, concentrations of uric acid, as measured by the Synchron, were lower than those determined by the HPLC system. Our HPLC method produced uric acid levels that were comparable to those of non-preserved urine, as measured by either technique. A

Table 3
Effect of preservatives on uric acid stability and analysis (units are mg/dl)

Sample number[a]	None		Acidified		Thymol + thimerosal	
	Enzymatic	HPLC	Enzymatic	HPLC	Enzymatic	HPLC
1	39	41	10	10	33	42
2	34	35	8	9	30	46
3	20	23	8	7	16	23
4	29	22	7	9	18	21
5	53	53	11	13	50	59
Control 1[b]	(15)	(13)				
Control 2	(22)	(20)				
Mean±SEM	35±5	35±6	9±1[c]	10±1[c]	29±6[c]	38±7

[a]Five individual urine samples are represented by sample numbers 1–5.
[b]Controls 1 and 2 are the commercially available Bio-Rad urine controls.
[c]$p<0.05$ (paired t-test) compared with non-preserved urine, corresponding to the same method.

typical chromatogram of a preserved urine sample is shown in Fig. 1D. The thymol and thimerosal peaks appeared at 31 min, during the washing step. No interference peaks were present near the peaks for uric acid and creatinine in non-preserved urine (chromatogram not shown).

4. Conclusions

We developed an HPLC assay to separate and quantify uric acid in thymol- and thimerosal-preserved urine. This method is suitable for simultaneous determination of uric acid and creatinine in human urine. Urinary uric acid and creatinine concentrations measured with this HPLC method correlated well with levels measured with a traditional automated enzymatic method and a colorimetric method, respectively. We demonstrated that thimerosal slightly inhibits uricase activity, an essential enzyme in the Beckman Synchron CX5 analyzer.

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