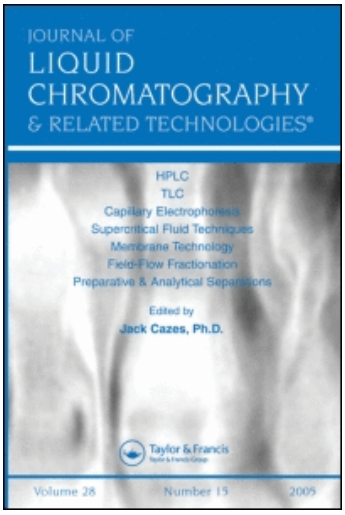

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Urinary Uric Acid Determination by Reversed-Phase High Pressure Liquid Chromatography

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URINARY URIC ACID DETERMINATION BY REVERSED-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY.

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ABSTRACT

Reversed-phase high pressure liquid chromatography with UV detection was proven to be a powerful method for the separation and quantitation of urinary uric acid. We have compared three different treatments for urine samples previous chromatographic injection: alkaline methanol extraction, ethylacetate extraction and centrifugation. It was also studied storage conditions for urine samples.
Our findings show that the method has high specificity and reproducibility for urinary uric acid. Samples are stables and require only centrifugation previous injection to the chromatograph.

INTRODUCTION

Uric acid is a major end-product of exogenous and endogenous purine metabolism. In man, it is derived from purine-nucleoside-phosphorilase substratum. The products, guanine and hypoxanthine are transformed in xanthine by the action of guanase and xanthine oxidase respectively. Also, the transformation of xanthine in uric acid is catalized by xanthine oxidase.

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Uric acid excretion pattern is modified in several diseases-gout, leukemia-. A decreased renal excretion can be induced by drugs or dietary factors. (1)

Analytical methods for clinical determinations of uric acid are based either in colorimetric reduction with phosphotungstic acid (2) or in enzymatic oxidation to allantoin. (3)

Although the first method is satisfactory for routine analysis, its usefulness is limited because the presence of other reduc ing substances, specially in urine samples.

To increase the specificity in the determination of the uric acid, the use of the enzymatic method is adequated. This method is bases in UV absorption measurements at 294 nm, before and after incubation of the sample with enzyme uricase. In this way, interferences are reduced, but the procedure is sensible to uricase inhibitors. Although the specificity of this procedure is high, it has poor reproducibility.

To improve the reproducibility of the enzymatic method, it has been proposed another procedure by Kageyama. (4) This procedure combines the action of uricase on uric acid to produce allantoin, with later formation of a colored compound -3,5-diacetil-1,4-dihydrolutidine- and measured at 410 nm. Fluorometry can be used to measure this compound. (5) A modification of this procedure has been proposed by Trivedi and collaborators. (6)

Electrochemical methods have been developed for the determination of uric acid, but these methods have not received wide attention. (7)

Verghessen and col. (8) have proposed the use of isotachophoresis for the determination of uric acid in serum. This procedure is less influenced by certain metabolites.

In last years, the use of chromatographic techniques for the analysis of uric acid in biological fluids has been

increased, especially high pressure liquid chromatography -HPLC-. (9-19)

Slaunwhite and col. (10) have compared colorimetric, enzymatic and HPLC methods for serum uric acid. They have concluded that the chromatographic method is worthy candidate for consideration as a reference method. Pachla and Kissinger have proposed HPLC as a selected method. (17)

It was found excellent correlation between gas chromatography and HPLC, although HPLC has the advantage that the sample requires simpler procedures for prepurification, no prior derivatization and has greater sensibility than gas chromatography. (14)

In general, for determinations of uric acid in urine and serum samples by HPLC, ionic-exchange columns have been used. (9-13,17,18)

Although less sensibility than electrochemical detection, UV detectors offer the advantage of greater adaptability to analysis of other biological compounds.

This paper deals with the use of reversed-phase liquid chromatography for the quantitative determination of urinary uric acid. Different methods for the treatment of urine samples, previous injection in the liquid chromatograph, have been proposed. (13,21) These methods include centrifugation and extraction procedures. We have compared three different procedures to select an appropiate method for a quantitative viewpoint.

Because samples were frozen before analysis, we have studied the influence of the storage on urinary uric acid determinations

MATERIALS and METHODS

Apparatus.-

A Varian High Pressure Liquid Chromatograph Model 8500, coupled with a VariChrom variable wavelength Spectrophotometer

(8 ul cell) and a Varian 9176 Recorder were used for chromatographic determinations. Samples were introduced with a 10 ul syringe (Hamilton), through a Varian AeroGraph High Pressure Septumless Liquid Chromatography Injector.
Urine samples were centrifuged with a Beckman Model TJ-6 Centrifuge, coupled with a refrigeration unit.
pH measurements of urine samples were carried out with a Selection 2000 Ion Analyzer (Beckman Inst., Inc.) using a Beckman Laboratory Combination pH Electrode # 39504.
UV spectra were carried out in a Beckman Model 26 Spectrophotometer using 1 cm quarz cells.

Reagents and Solvents.-

All reagents used were Analytical Reagent Grade, Uric acid -99%, for biochemical purpose-, purchased from E. Merck (Darmstadt, G.F.R.), was used without further purification. It was checked by UV spectrophotometry. Spectra were obtained in 0.1N NaOH solutions.
Absorption coefficient at 294 nm (log E= 4.1) is similar to the value found in the literature. (20)
Water was purified with a Milli-RO 15 reagent-grade water system (Millipore Corp.) and glass-redestiled.

Chromatographic Conditions.-

A prepacked MicroPack CH-10 stainless steel column (250x2 mm i.d. -2594 theoretical plates-) purchased from Varian was used.
K_2HPO_4 0.1M Buffer solution (pH 2.3) was used as mobile phase at an elution rate of 30 ml/h. It was filtered and degassed by vacuum.
UV detector was set at 294 and 222 nm alternatively. An slit of 16 and a sensitivity of 0.5 was used. Chart speed was 1 cm/min.

Urine Samples.-

Normal urine specimens were obtained from laboratory personnel. First void urine samples were used. Samples were stored at -10°C if not analyzed inmediatly.

Calibration Curves.-

A calibration curve was prepared by appropiate dilutions of a stock solution (500 mg/l in 0.1N NaOH). Peak height and peak area were used.

Sample Treatment.-

It was used three differents urine sample treatments:

a) Centrifugation.- Urine samples were centrifuged 15 min. at 3000 rpm. The supernatant is injected directly in the chromatograph.

b) Alkaline methanol extraction.- This procedure was proposed by Milner and Perkins. (13)

c) Ethylacetate extraction.- This procedure was proposed by Molnar and Horvath. (21)

RESULTS and DISCUSSION

The chromatographic procedure was optimized in order to obtain a rapid analysis of uric acid.

Peaks in the chromatogram were monitored by spectrophotometry at 294 nm. At this wavelength many possible interferences can be avoided. Due to the possibility of simultaneous analysis of other substances present in urine samples in addition to uric acid, it was also monitored at 222 nm. Typical chromatograms of standard uric acid and urine sample at 222 and 294 nm are shown in the Figure-1-.

Standard solutions of uric acid in the range of 0.05-1.0 ug were injected to the chromatograph. The retention time

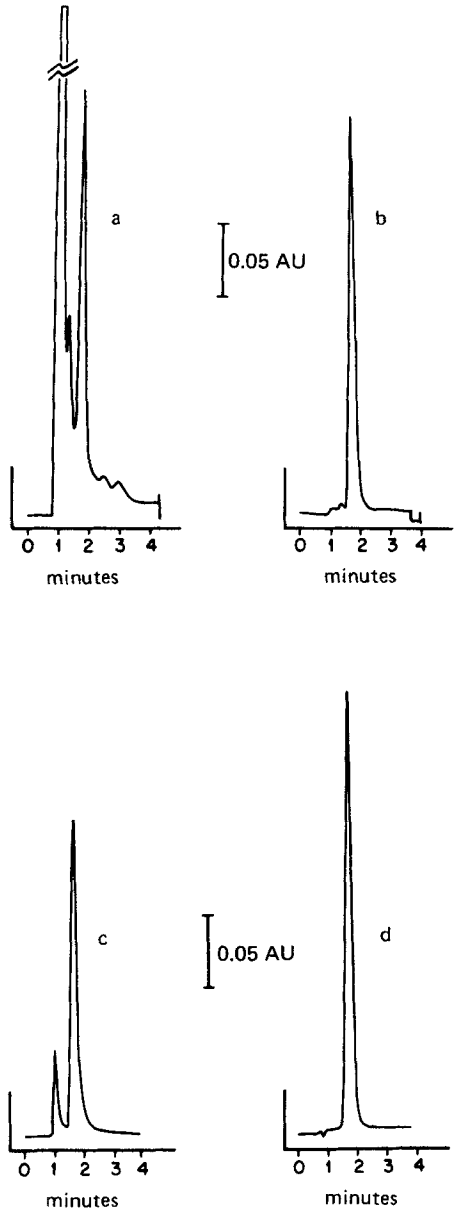


FIGURE -1- Reversed-Phase HPLC Chromatograms.
a)Urine sample (1 ul); b)UA standard. Detector at 222 nm.
c)Urine sample (0.5 ul);d)UA standard.Detector at 294 nm.

for the uric acid was 102 ± 4 seg at the chromatographic conditions used.

Good linearity was observed for peak height and peak area for both wavelengths. Figure-2-.

We have found that the ratio between slopes for peak height at 294 nm respect to 222 nm, is similar to the ratio for peak area at the same wavelengths, 2.27 and 2.29 respectively. This confirm the possibility to used either 294 or 222 nm and peak height or peak area for the quantitation of uric acid in urine samples.

To confirm the identification of the uric acid, besides the retention time, we have used the stopped-flow UV scanning technique proposed by Krstulovic and col. (22,23) Due to the instrumentation used, the UV scanning was done manually. Figure-3-

The treatment of urine samples previous HPLC analysis is necessary to avoid interferences and extend the life of

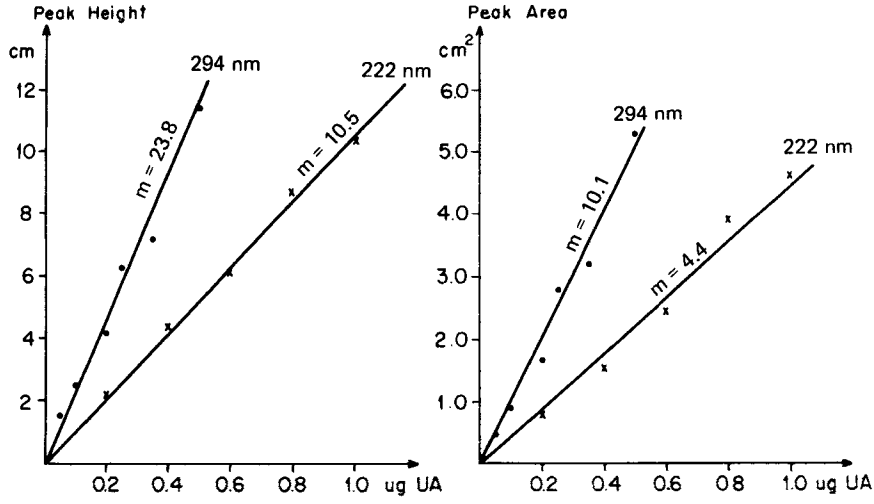


FIGURE -2- Uric Acid Calibration Curves.
Column: Micropack CH-10. Mobile phase: K_2HPO_4 0.1M Buffer.
Elution rate: 30 ml/h.

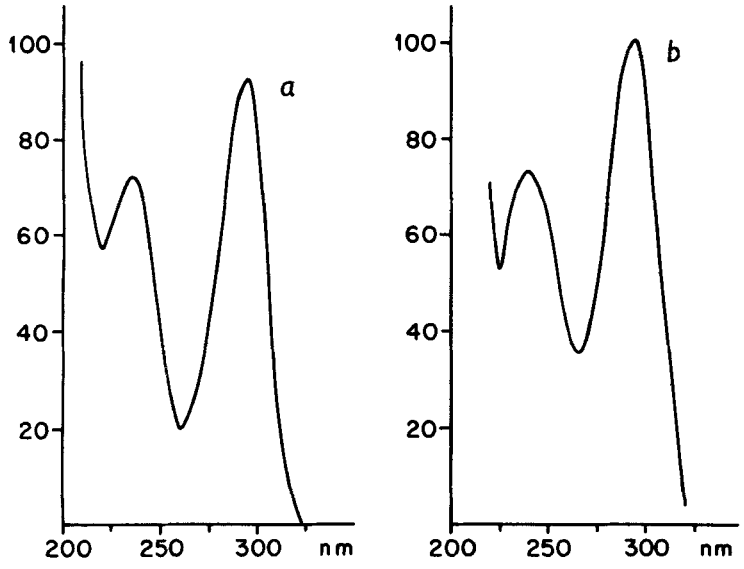
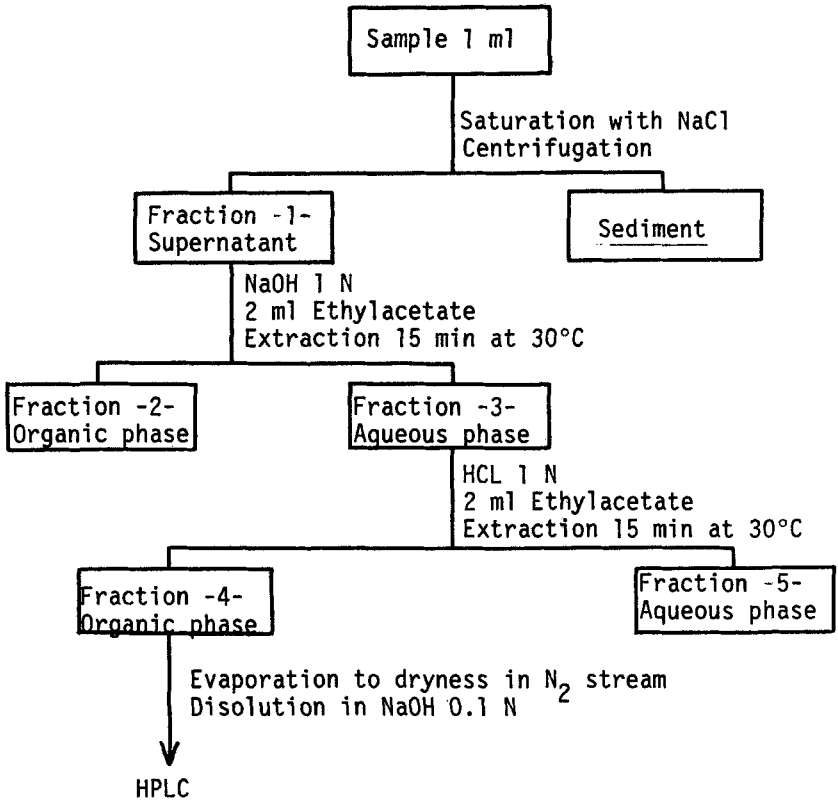


FIGURE -3- Uric Acid UV Spectra.
a) Normal UV Spectrum. b) Stopped-Flow UV Scanning.

the column. The most simple procedure is the centrifugation of the sample. In this way, particles are separed from it.
In the procedure of Milner and Perkins (13), the sample is extracted with alkaline methanol (pH 8.0), centrifuged and the supernatant is filtered, evaporated to dryness in N_2 stream and redissolved in 0.1N NaOH. Results are shown in the Table-1-.

In the Table-2- are shown comparative results of uric acid determinations using centrifugation and alkaline methanol extraction procedures. When we have used the alkaline methanol extraction method, the uric acid determined is approximately 92% of the urinary uric acid. The remain uric acid probably is in the precipitate as it is shown in the Table-1-.

The scheme of the sample treatment proposed by Molnar and Horvath (21) to extract urinary acids is the following:



According with this procedure, the uric acid is determined in the Fraction -4-, in the organic phase after acidic ethylacetate extraction.

Results obtained when this procedure is applied to standard solutions and urine samples are shown in the Table-3-. The Fraction-4- represents only 2% of total uric acid presents in the urine sample. During the extraction with ethylacetate after acidification, in the aqueous phase (Fraction -5-) appeared a white precipitate, which had high concentration of uric acid. This can explain the low concentration of uric acid found in the Fraction -4-.

We have used the method of standard addition to check for chemical interferences in the quantitation of urinary

TABLE -1-

Uric Acid Determination Following the Alkaline Methanol Extraction Procedure.

	Supernatant	Precipitate
	ug UA / ml of urine	
Standard Solution (1000 ug UA/ml)	980 (98%) (n = 2)	no
Sample -I-	855 (92.4%) (n = 2)	70 (7.6%)
Sample -II-	906 (92.4%) (n = 2)	75 (7.6%)

TABLE -2-

Comparation of Centrifugation and Alkaline Methanol Extraction Methods in the Determination of Uric Acid in Urine Samples.

	Centrifugation Method	Alkaline Methanol Extraction Method
	ug UA / ml of Urine (S.D.;n)	
Sample -III-	810 (5;4)	770 (95.1%) (29;4)
Sample -IV-	450 (16;4)	420 (93.3%) (19;4)
Sample -V-	280 (42;5)	240 (85.7%) (15;3)
Sample -VI-	830 (36;4)	720 (86.7%) (6;5)
Sample -VII-	800 (28;4)	740 (92.5%) (32;4)
Sample -VIII-	350 (12;4)	330 (94.3%) (42;4)

TABLE -3-

Determination of Uric Acid in the Different Fractions after Ethylacetate Extraction Procedure.

	Fractions ug UA / ml of Urine (S.D.;n)				
	1	2	3	4	5
Standard Solution	1100 (10;4)	22 (12;5)	1060 (23;4)	15 (14;4)	400 (20;5)
Standard Solution	1090 (6;4)	54 (5;4)	1042 (10;4)	24 (8;4)	421 (6;4)
Sample -II-	991 (11;4)	94 (21;4)	- -	24 (2;4)	- -
Sample -III-	1090 (9;4)	46 (13;4)	1040 (18;4)	23 (3;4)	440 (12;4)
Sample -IV-	1080 (8;4)	31 (8;4)	1020 (15;4)	22 (14;4)	462 (9;4)

uric acid and find the best calibration curve. In this method, a number of equal aliquots of samples are taken and diluted, with the addition of increasing quantities of standard uric acid to a particular volume. These are then analyzed on the chromatograph.

Because the slope of the graph (m = 10.3) is the same as the slope of the normal calibration curve (m = 10.5) for simple standard solution, it can assume that chemical interference is negligible. Figure -4-.

To study the influence of storage conditions in the analysis of urinary uric acid, aliquots were kept in seven

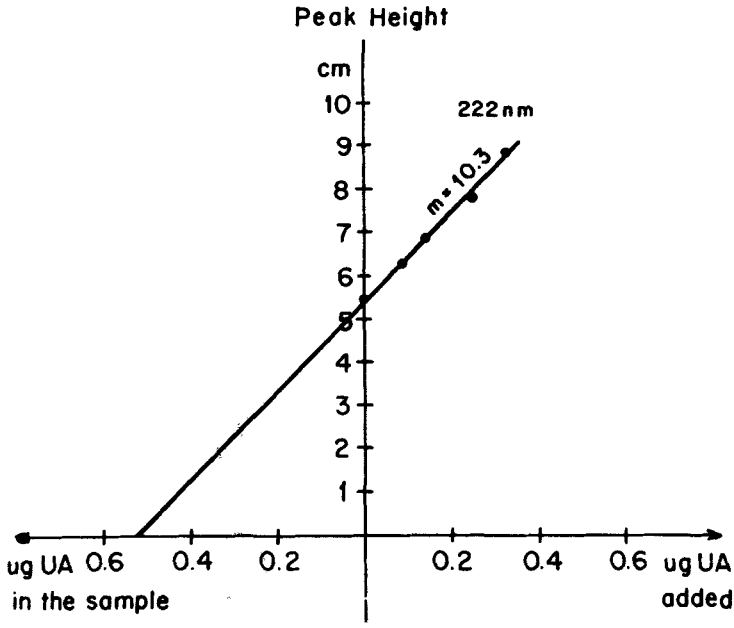


FIGURE -4- Standard Addition Method for Uric Acid in Urine.

polyethylene containers at -10°C. We have measured pH and uric acid concentration after definite periods of time and several freezing and defrosting cycles. Results are shown in the Table -4-.

From these results, we can observe the high stability of the uric acid in urine samples, after 65 days and 8 freezing and defrosting cycles. Also it is possible to observe the high reproducibility of the HPLC method. (x = 0.34 ug UA/ml of urine; n = 102;S.D. = 0.02).

We concluded that reversed-phase high pressure liquid chromatography with UV detection is a powerful method for the separation and quantitation of uric acid in urine samples. The method has high specificity and reproducibility; and samples are stables and require only centrifugation previous injection to the liquid chromatograph.

TABLE -4- UA mg/ml in Storaged Samples

Time of storage	Aliquot -1-	Aliquot -2-	Aliquot -3-	Aliquot -4-	Aliquot -5-	Aliquot -6-	Aliquot-7-
0 day	0.375 5.750.0150						
8 days	0.344 -1 0.016	0.364 -1 0.021					
16 days	0.344 5.652 0.005	0.344 -2 0.003					
29 days	0.314 5.723 0.011	0.354 5.743 0.012	0.334 5.791 0.010				
36 days	0.325 5.885 0.005	0.314 5.815 0.011	0.364 5.873 0.010	0.354 5.861 0.018			
58 days	0.344 5.737 0.022	0.374 5.767 0.018	0.354 5.765 0.009	0.354 5.773 0.009	0.344 5.742 0.009	0.354 5.751 0.008	
65 days	0.354 5.818 0.009	0.384 5.868 0.005	0.354 5.906 0.011	0.354 5.854 0.010	0.324 5.805 0.005	0.344 5.872 0.012	0.354 5.821 0.011
$\bar{x}$ UA n S.D.	0.34 30 0.019	0.35 24 0.025	0.35 16 0.013	0.35 12 0.020	0.33 8 0.010	0.35 8 0.013	0.35 4 0.011

Key

UA pH
n S.D.
defrosting

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