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Note

Determination of hypoxanthine, xanthine, and uric acid in biological fluids by ion-pair high-performance liquid chromatography

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While numerous methods have been described in the literature[1–6] for the determination of uric acid in biological fluids, few procedures are available which can readily quantify the intermediate metabolites of the purine salvage pathway (Fig. 1), without employing arduous and time consuming techniques. Because of chemical structural similarities of hypoxanthine, xanthine, and uric acid, interferences between two or more of these compounds have been a major problem in the past.

Recent studies involving the use of high-performance liquid chromatography (HPLC) have alleviated many of these difficulties. In this report, we describe a simple and specific method incorporating the use of reversed-phase liquid chromato-

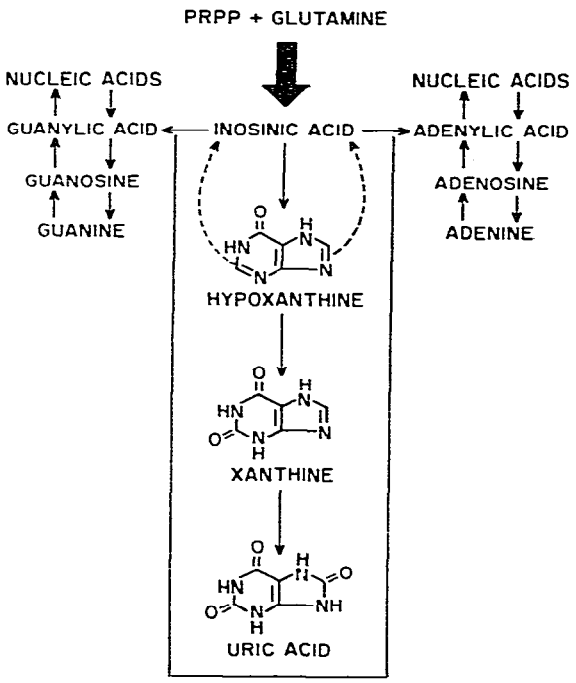

Fig. 1. Scheme of the purine salvage pathway for the biosynthesis of purine bases, nucleosides and nucleotides in eukaryotic systems. PRPP=5-Phosphoribosyl-pyrophosphate.

graphy and paired-ion chromatography for separating these closely related compounds. The method is capable of quantifying these oxypurines at levels as low as 5 ng on column. Analysis time is 6 min per sample. No pre-treatment other than deproteinization of sample is required. Because of the simplicity of the method, its application may be directly utilized for various types of basic research projects, as well as for routine clinical determinations in the laboratory.

EXPERIMENTAL*

The method was developed using a Waters Model ALC/GPC-204 liquid chromatograph. The system consisted of two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a 280nm UV detector, a Houston Instrument Omni-Scribe A5000 dual pen recorder, and a Columbia Scientific Industries Supergrator-3 integrator.

Reagents

Analytical grade sodium acetate, trihydrated (Mallinckrodt, St. Louis, Mo., U.S.A.) mixed with PIC - B7 reagent (1-heptane sulfonic acid, Waters Assoc., Milford, Mass., U.S.A.) was used as the mobile phase. Uric acid (99%), hypoxanthine, and xanthine (98%) (Aldrich, Milwaukee, Wisc., U.S.A.) were used to prepare all working standards.

Procedure

A 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc.) was used to chromatograph uric acid and its metabolic intermediates in standard solutions and experimental specimens. The mobile phase consisted of 0.005 *M* 1-heptane sulfonic acid mixed with an equal volume of 0.01 *M* sodium acetate. The pH of the solution was 4.00. The mixed mobile phase was isocratically pumped through the column at a flow-rate of 1.5 ml/min. Column pressures ranged between 1200–1500 p.s.i. All separations were performed at ambient temperatures. A 5-μl volume of a 10% solution of neutralized perchloric acid extract from biological fluids was introduced onto the column through a continuous flow loop injector. Peak areas were measured by an on-line computing integrator.

RESULTS AND DISCUSSION

The separation of nucleotides, nucleosides, purines and analogues of purines during the last few years by HPLC has immensely facilitated analytical work essential for studying the effects of various pathologic states. The utilization of methods capable of detecting and quantifying these compounds may suggest metabolic errors or alterations occurring within the pathway. Such occurrences have been observed in the purine salvage system of mammalian erythrocytic malaria parsites[7]. Similarly, human subjects with Lesch–Nyhan syndrome have also demonstrated abnormal metabolic profiles, as observed in their urinary purine excretion patterns[8].

* The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

Our primary interest for developing a method for quantifying the oxypurines shown in Fig. 1 was to establish the metabolic profiles of these compounds in plasmodia during the various intra-erythrocytic stages of the parasite. The method described in this paper is simple and specific for measuring the concentrations of hypoxantine, xanthine and uric acid. Nucleotides, nucleosides and free base purines, which were present in our samples did not interfere with the separation. Allopurinol, an analog of hypoxanthine, was also separated by the method.

From a series of chromatograms depicted below, the applicability of the method is demonstrated. Fig. 2 represents the separation of a standard solution, containing uric acid, hypoxanthine, xanthine and allopurinol. Linearity was observed for all concentrations of the oxypurines used in this study (25 ng–1 μg). Correlation coefficients for uric acid, hypoxanthine, and xanthine were 0.999, 0.979 and 0.996, respectively. Based on these initial separations, extracts from malaria parasites (*P. knowlesi*) were chromatographed. The chromatogram is shown in Fig. 3. For a series of samples containing two or more of these compounds, the separations were excellent.

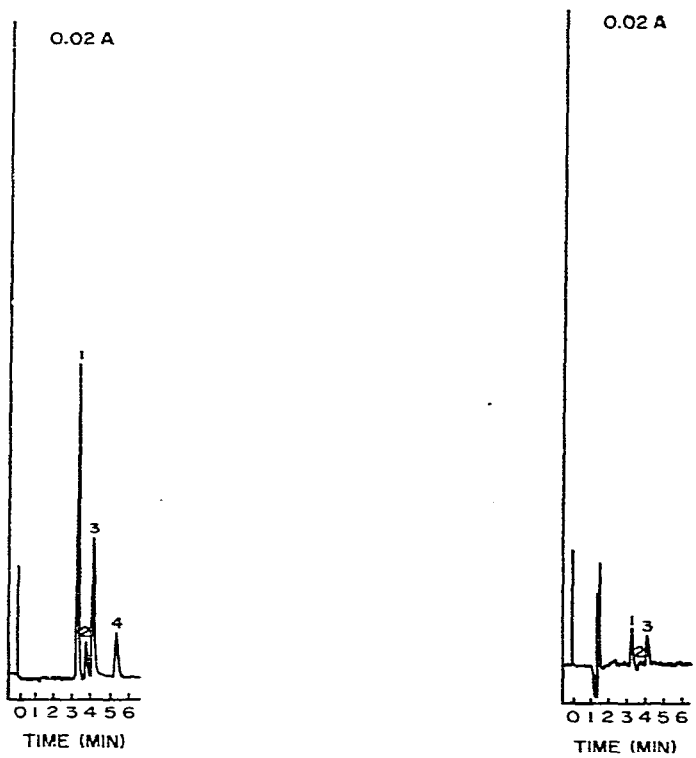

Fig. 2. Chromatogram of a standard solution containing; (1) uric acid (25 ng), (2) hypoxanthine (40 ng), (3) xanthine (40 ng), and (4) allopurinol (25 ng). Column: 30 cm × 3.9 mm μBondapak C_{18}. Mobile phase: 50% 0.005 *M* 1-heptane sulfonic acid and 50% 0.01 *M* sodium acetate, pH 4.00. Flow-rate: 1.5 ml/min. Column temperature: Ambient.

Fig. 3. Chromatogram of a neutralized perchloric acid extract of *P. knowlesi*, isolated from monkey blood. Peaks: 1 = uric acid; 2 = hypoxanthine; 3 = xanthine. Sample volume: 5 μl. Detection wavelength: 280 nm.

Analyzing a series of spiked pooled extract samples containing various amounts of hypoxanthine, xanthine and uric acid, the accuracy and precision of the method are demonstrated. The results shown in Table I summarize the data collected from this study.

TABLE I

PRECISION AND ACCURACY OF AN ION-PAIR HPLC METHOD FOR QUANTIFYING HYPOXANTHINE, XANTHINE, AND URIC ACID IN SPIKED POOLED PLASMODIA EXTRACTS

Compound	*Sample (ng/sample)*				*Mean ± S.D. (n = 8)*
	1	*2*	*3*	*4*	
Uric Acid					
Before addition	8.70	8.35	8.19	8.56	8.45 ± 0.22
Amount added	10.00	15.00	20.00	25.00	
Amount found	17.29	22.01	27.20	32.25	
Percent recovered	92.50	94.30	96.50	96.10	95.85 ± 1.05
Xanthine					
Before addition	23.16	23.00	23.77	23.24	23.29 ± 0.33
Amount added	10.00	15.00	20.00	25.00	
Amount found	30.10	35.26	40.92	45.44	
Percent recovered	90.80	92.80	93.50	94.20	92.82 ± 1.46
Hypoxanthine					
Before addition	12.56	11.99	12.31	12.17	12.25 ± 0.24
Amount added	10.00	15.00	20.00	25.00	
Amount found	20.34	24.10	29.43	34.08	
Percent recovered	90.20	89.30	91.10	91.70	90.57 ± 1.05

Because of the advantages of excellent resolution and short analysis time, the analytical method described in this report offers a convenient alternative to the conventional methodologies currently employed.

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