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MEASUREMENT OF URINARY PYRIMIDINE BASES AND NUCLEOSIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid procedure for the isolation, separation, identification and measurement of urinary pyrimidine bases and nucleosides by high-performance liquid chromatography (HPLC) is presented. The initial isolation of these compounds from urine was accomplished with small disposable ion-exchange columns. HPLC was performed on a silica gel column with a mobile phase composed of methylene chloride, methanol and 1 *M* aqueous ammonium formate buffer. Peaks were recorded at both 254 nm and 280 nm and the response ratio was used in conjunction with the elution volume for compound identification. The minimum detectable amount (signal-to-noise ratio = 2) ranged from 0.2 ng for uracil to 2.2 ng for cytidine. Linearity and recovery for thymine, uracil, uridine, pseudouridine, orotic acid and orotidine added to urine was demonstrated over almost a 10^3 concentration range. The potential application of this method for the study of inborn errors in the urea cycle is discussed.

INTRODUCTION

The ability to quantitatively measure the urinary excretion of certain pyrimi-

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dines is essential in the study and treatment of patients with primary or secondary hyperammonemia. The sequence of reactions leading to the formation of pyrimidines by the orotate pathway is the major source of pyrimidine components for DNA and RNA synthesis. This pathway is initiated by the enzyme carbamyl phosphate synthetase. When the urea cycle is either overloaded as a result of secondary hyperammonemia or is blocked as a result of an enzyme deficiency in the cycle itself, the excess carbamyl phosphate is available for extramitochondrial carbamyl aspartate synthetase. This is then shunted into the pyrimidine biosynthetic pathway resulting in increased urinary excretion of uracil, uridine, and orotic acid [1—3]. The availability of rapid quantitative procedures for measuring these urinary pyrimidines is useful, not only for research purposes, but also for monitoring the effectiveness of various forms of dietary therapy being used to treat patients with hyperammonemia [4].

A number of analytical methods have been used in the past to measure various urinary pyrimidines. Paper and ion-exchange chromatography combined with the measurement of UV absorption have been used for the determination of pseudouridine in normal urine [5, 6] and elevated levels of orotic acid, uracil and uridine in urine from patients with hyperammonemia [7]. Gas chromatography has been used for measuring urinary orotic acid in patients with ornithine transcarbamylase deficiency and orotic acid and orotidine in patients treated with azauridine [8]. Various colorimetric procedures have also been used for the measurement of orotic acid [9—11]. More recently various high-performance liquid chromatographic (HPLC) procedures have been reported for the separation of a number of pyrimidines and their nucleosides [12—14].

We describe here a simple procedure for the rapid separation and quantitation of a number of urinary pyrimidines using HPLC. These procedures were designed specifically for the study of urinary pyrimidines in patients with primary hyperammonemia.

EXPERIMENTAL

Instrumentation

The HPLC equipment used consisted of a Waters Assoc. (Milford, Mass., U.S.A.) Model M-6000A pump, a Model UK6 injector and a Model 440 dual wavelength, two-channel UV monitor. The UV monitor was used to simultaneously record chromatograms at both 254 nm and 280 nm on a Houston Instruments (Austin, Texas, U.S.A.) Model 5211-5 Omniscribe two-pen recorder.

Materials

All organic solvents used were distilled in glass (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Standard compounds were purchased from Sigma (St. Louis, Mo., U.S.A.) except for 2'-O-methylcytidine which was purchased from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Ion-exchange resins were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Isolation of pyrimidines from urine

A small disposable mixed-bed ion-exchange column was used to isolate a fraction containing neutral pyrimidines and another containing acidic pyrimidines from urine. The column was prepared by adding a slurry containing equal volumes of both AG 50W-X8 hydrogen form and AG 1-X8 acetate form (both 100—200 mesh) ion-exchange resins to a 5.75-in. disposable transfer pipette with a plug of glass-wool in its tip until a bed height of 1 cm was obtained. The transfer pipette was silylated before packing with 5% dimethyldichlorosilane in toluene. This was found useful for preventing the adhesion of the resin to the walls. The column was washed with 2 ml of 2 *M* acetic acid and 2 ml of distilled deionized water before use.

A 200-μl urine sample was applied to the column and collected in a screw-cap test tube. The column was eluted with two 1.0-ml portions of distilled deionized water. This fraction contained the "neutral" pyrimidines (uracil, uridine, pseudouridine, thymine, thymidine, and 5-hydroxymethyluracil) and could be used directly for HPLC analysis if elevated amounts of these pyrimidines are present. For the measurement of normal levels of uracil and pseudouridine further purification is required.

The column was next washed with two 1.0-ml portions of 10 *M* formic acid. This fraction was dried under a stream of nitrogen at 60°, redissolved in 1.0 ml of concentrated ammonium hydroxide, heated 10 min at 60° and again dried under a stream of nitrogen at 60°. The residue was dissolved in 200 μl of 20% 1.0 *M* ammonium formate buffer in methanol for injection into the HPLC column.

During recovery studies with orotidine, it was observed that when the sample from 10 *M* formic acid was dried at 60° under a stream of nitrogen, the recoveries were usually no more than 50%. It was also noted that a peak was present in the chromatograms of these samples with a retention time nearly identical to that of orotic acid but with a 254 nm to 280 nm ratio of 1.51. This was identical to the ratio for orotidine. Treatment of these samples with ammonia resulted in the disappearance of the new peak and markedly improved recoveries for orotidine. It appears that the new peak was a formate ester of orotidine. We have, therefore, incorporated the ammonolysis step into the isolation procedure in order to convert any formate ester back into the original orotidine.

Further purification of the "neutral" fraction collected from the mixed-bed ion-exchange column was accomplished in an anion-exchange column. The column was constructed as described above except that it was filled to a bed height of 0.5 cm with AG 1-XB (200—400 mesh) ion-exchange resin in the hydroxyl form. Concentrated ammonium hydroxide (200 μl) was used to adjust the previous collection to pH 11.0. This was applied to the column. The column was washed with two 1.0-ml portions of distilled deionized water and the pyrimidines eluted with two 1.0-ml portions of 1.0 *M* acetic acid. This fraction was dried at 60° under a stream of nitrogen and dissolved in 200 μl of 90% methanol for injection into the HPLC column.

High-performance liquid chromatography

Chromatography was performed in a Brownlee Labs. column (25 cm × 4.6

mm I.D.) packed with LiChrosorb SI-100, 5 μm average diameter silica gel particles. The mobile phase used for chromatography of the "neutral" fraction materials was composed of methylene chloride—methanol—1.0 *M* ammonium formate buffer pH 3.0 (75:22:3, v/v/v). This was designated mobile phase A. A solvent composed of the same components in a volume ratio of 64:30:6 was used for chromatography of the orotic acid containing fraction. This was designated mobile phase B. The flow-rate used was 2.0 ml/min (1900 p.s.i.).

RESULTS

Fig. 1 shows the separation achieved when a standard mixture of pyrimidines

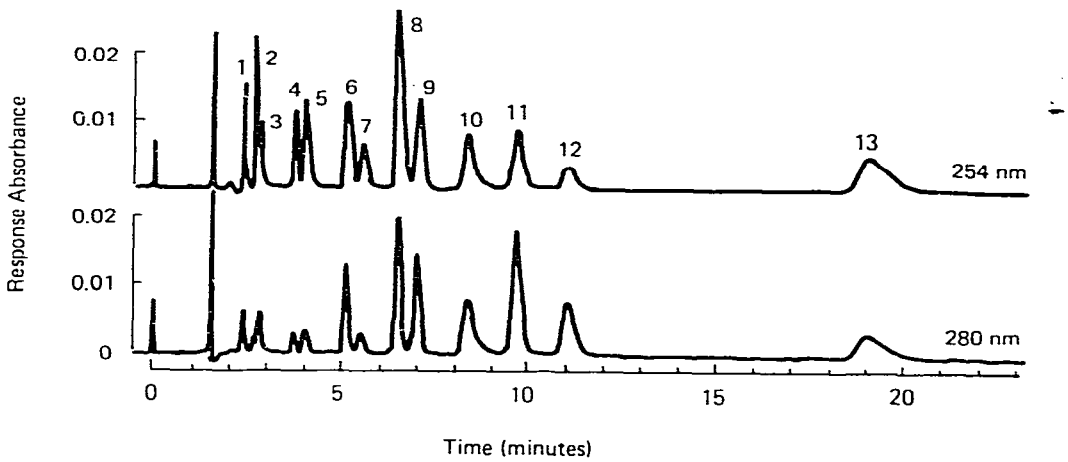

Fig. 1. Separation of a standard mixture of biologically interesting pyrimidines. Column, LiChrosorb SI-100 (25 cm × 4.6 mm I.D.). Mobile phase, methylene chloride—methanol—ammonium formate buffer (75:22:3). Flow-rate, 2 ml/min. 1, Thymine; 2, uracil; 3, thymidine; 4, 5-hydroxymethyluracil; 5, uridine; 6, 3-methylcytosine; 7, pseudouridine; 8, cytosine; 9, deoxycytidine; 10, 2′-O-methylcytidine; 11, cytidine; 12, orotic acid; 13, orotidine.

known to occur in biological systems was chromatographed with mobile phase A. Good separation of most of these pyrimidines could be achieved in less than 10 min. Orotic acid, however, eluted at 11 min and orotidine at 19 min. Mobile phase B was therefore developed to shorten the elution time and increase the sensitivity for orotic acid and orotidine. Fig. 2 demonstrates their separation with this solvent. Under these conditions they both elute completely in less than 8 min.

Table I lists the capacity factors (k') measured for the 13 biological pyrimidines chromatographed with mobile phase A. Orotic acid and orotidine gave k' values of 3.5 and 5.3, respectively, when chromatographed with mobile phase B. Table I also lists response ratios between 254 nm and 280 nm. This ratio is a useful identifying characteristic for each pyrimidine and can be used together with the retention time for peak identification and to detect the presence of interfering compounds in the peak. The presence of an overlapping UV absorbing peak normally will change the ratio significantly. The 254 nm to

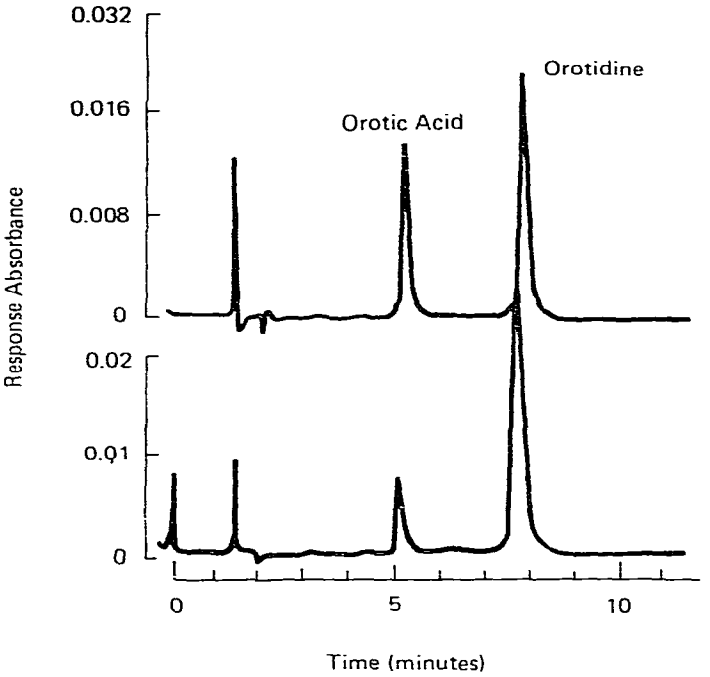

Fig. 2. Separation of orotic acid (0.2 μg) and orotidine (1.0 μg) standards. Column, LiChrosorb SI-100 (25 cm × 4.6 mm I.D.). Mobile phase, methylene chloride—methanol—ammonium formate buffer (64:30:6). Flow-rate, 2 ml/min.

TABLE I

CAPACITY FACTORS (k') AND 254 nm/280 nm RESPONSE RATIOS MEASURED WITH MOBILE PHASE A

No.	Compound	k'	254 nm/280 nm
1	Thymine	0.66	2.50
2	Uracil	0.88	9.10
3	Thymidine	0.98	1.70
4	5-Hydroxymethyluracil	1.59	3.70
5	Uridine	1.79	3.60
6	3-Methylcytosine	2.56	1.00
7	Pseudouridine	2.83	2.40
8	Cytosine	3.50	1.30
9	Deoxycytidine	3.86	0.96
10	2'-O-Methylcytidine	4.77	1.00
11	Cytidine	5.67	0.98
12	Orotic acid	6.61	0.51
13	Orotidine	13.10	1.50

280 nm ratios for orotic acid and orotidine are the same in mobile phases A and B.

The percent recovery for various pyrimidine standards added to urine are presented in Table II. These data were calculated by comparison of the peak

TABLE II

RECOVERY OF PYRIMIDINE STANDARDS ADDED TO NORMAL URINE

S = 8.9, 23.1, 5.45.

Amount added (μg/mg)	Percent recovery					
	Thymine	Uracil	Uridine	Pseudouridine	Orotate	Orotidine
1.6	108.7	118.1	97.2	*	**	**
8.0	101.4	96.2	96.4	*	91.3	99.6
40.0	101.9	95.8	100.6	100.8	93.3	90.8
200.0	96.1	89.8	92.6	92.8	94.3	100.5
1,000.0	97.1	88.7	94.1	95.4	89.3	95.9
Mean	101.0	99.2	96.2	98.8	92.1	96.7

*Recovery of pseudouridine was not measured at the 1.6 and 8.0 μg/ml addition levels because of the much higher endogenous level in urine.

**Measurement of orotic acid and orotidine at the 1.6 μg/ml level is not accurate due to the presence of small interfering peaks.

heights obtained from standards of known concentrations to peak heights obtained from injection of the urine extracts. The endogenous levels of uracil and pseudouridine in the normal urine were found to be 7.0 and 56.2 mg/ml respectively. Because of the potential effect on the accuracy of the measurements resulting from these high endogenous levels, the percent recovery of pseudouridine at 1.6 and 8.0 μg/ml was not measured. The high percent recovery (118%) for uracil at the 1.6 μg/ml level also appears to be a result of the high endogenous level of uracil in the urine.

Fig. 3 illustrates the pyrimidines found in the neutral fraction isolated from a normal urine specimen. Uracil and pseudouridine are the only pyrimidines in this fraction at levels detectable in our system. Table III shows the uracil and pseudouridine levels measured in six normal urines. The normal excretion of uracil ranged from 8.8 to 14.0 μg/ml creatinine with a mean of 12.2 μg/ml, and the normal excretion of pseudouridine ranged from 73 to 174 μg/ml with a mean of 108.5 μg/ml.

The reproducibility of the measurement of endogenous uracil and pseudouridine in a normal urine was determined by performing the isolation and measurement procedure on 10 separate portions of the same normal urine sample. The mean concentrations measured for uracil and pseudouridine were 14.9 mg/ml and 191 mg/ml of urine respectively, with relative percent standard deviations of 11.1 and 9.6 respectively.

DISCUSSION

The HPLC separation of the 13 pyrimidines studied here is rapidly and reliably accomplished on a LiChrosorb SI-100 column that provides high efficiencies of about 11,000 theoretical plates for all the compounds tested. This efficiency permits the use of relatively short analysis times while maintaining good resolution between the pyrimidines being measured and any interfering

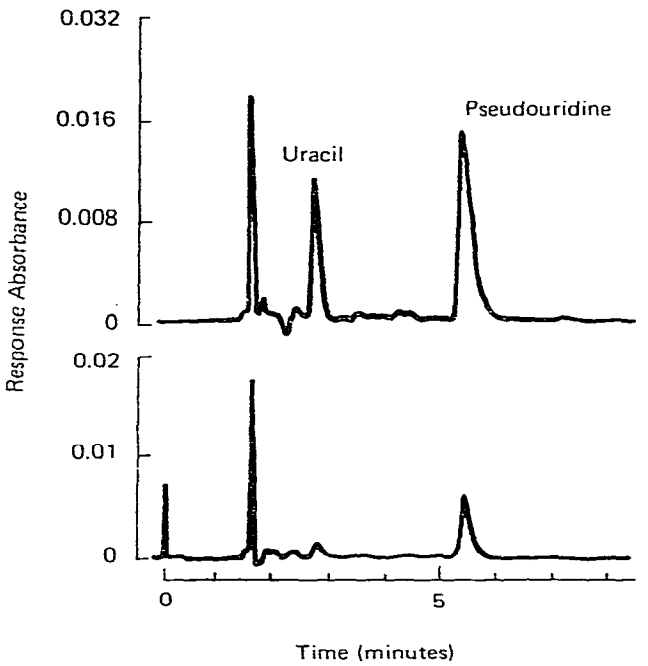

Fig. 3. Separation of pyrimidines from the "neutral" fraction of normal urine.

TABLE III

URACIL AND PSEUDOURIDINE LEVELS IN NORMAL URINES (μg/mg CREATININE)

	Uracil	Pseudouridine
	13.3	95.0
	14.0	124.0
	10.8	92.0
	12.6	73.0
	8.8	93.0
	13.5	174.0
Mean	12.2 (S.D. ± 2.0)	108.5 (S.D. ± 36.0)

compounds that may be present in urine samples. The ion-exchange procedures used for isolation of the pyrimidines from urine are fast and easy to use and provide good recoveries and suitable clean-up for HPLC analysis.

We have not attempted the isolation and analysis of basic pyrimidines (compounds 6 and 8—11 in Fig. 1) from urine samples but it is proposed that this could be accomplished with the use of small cation-exchange columns similar to those used for the neutral and acidic pyrimidines. It also appears feasible that these procedures could be applied to the study of pyrimidines in other biological fluids such as serum, cerebrospinal fluid, amniotic fluid, tissue extracts, nucleic acid hydrolysates, etc.

A rapid quantitative procedure for urinary pyrimidines is an alternative to

blood ammonia assays in studies of the effects of metabolic blocks in the urea cycle. Direct measurement of blood ammonia levels is subject to a number of technical pitfalls [15] and requires a considerable volume of venous blood from an infant. Increased urinary pyrimidine excretion is a reflection of the hyperammonemia and can be used for both diagnostic purposes and for the routine monitoring of patients undergoing dietary or α-ketoacid therapy. This rapid HPLC procedure is currently being used to measure pyrimidine levels in patients with various blocks in the urea cycle and will soon be routinely used for dietary monitoring.

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