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Automated determination of orotic acid, uracil and pseudouridine in urine by high-performance liquid chromatography with column switching

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ABSTRACT

A column-switching high-performance liquid chromatographic method, requiring no sample preparation apart from filtration, is described for quantification of urinary orotic acid, uracil and pseudouridine. The analyses were carried out using a reversed-phase octadecylsilane-bonded column for sample clean-up and a cation-exchange column for separation; 5–20 μl samples of urine were directly analysed, and more than 100 samples could be analysed consecutively. Each sample required only 30 min. Detection limits of these compounds were 5 pmol. Creatinine-related urinary uracil excretion was lowest in the newborn period (17.3 ± 14.4 μmol/g of creatinine). A patient with partial ornithine transcarbamylase deficiency and his mother usually excreted a high level of uracil during the period of normal orotic acid excretion and normal serum ammonia level.

INTRODUCTION

The measurement of urinary orotic acid and uracil, which are intermediates in pyrimidine biosynthesis, is important for screening inborn errors of metabolism, such as in the urea cycle [1] and in pyrimidine metabolism disorders [2,3]. Pseudouridine is a degradation product of transfer RNA, and its measurement might be useful as a marker for cancer and in cancer therapy evaluation [4]. There are a large number of procedures for measuring orotic acid. Colorimetric assay [5,6] are commonly used to measure total orotic acid (orotic acid plus orotidine) in urine, but removal of many urinary compounds interfering with the colour reaction [7–9] is necessary in order to achieve accuracy. Although isotope dilution analysis [10,11] is accurate, it is a rather complicated procedure, requiring expensive instruments. Several high-performance liquid chromatographic (HPLC)

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methods have been reported [1,12–15]. Recently, simple methods for measuring orotic acid [16], uracil [17] and orotic acid and orotidine [18] were reported. However, these HPLC methods for simultaneous determination of orotic acid and uracil require rather complicated sample preparation.

This paper describes an accurate quantitative method requiring no sample preparation, apart from filtration, for urinary orotic acid, uracil and pseudouridine. It used HPLC with an automated column-switching system to obtain preliminary reference values. The urinary pyrimidine excretion status in a patient with partial ornithine transcarbamylase (OTC) deficiency and in his mother was also evaluated.

EXPERIMENTAL

Chemicals and solutions

Analytical-grade sulphuric acid and acetonitrile were purchased from Wako (Tokyo, Japan). Deionized water was passed through a Milli-Q Labo (Nihon Millipore Kogyo, Yonezawa, Japan). Orotic acid was purchased from Sigma (St. Louis, MO, USA), uracil was from Wako, and pseudouridine was from Seikagaku Kougyou (Chiba, Japan). Other reference standards were analytical grade from Sigma.

Urine samples

Single voided urine samples were collected from healthy control subjects (newborns, $n = 14$; children aged 1–15 years, $n = 35$; adults, $n = 25$), and from a patient with partial OTC deficiency (liver OTC acitivity was 10% of control values) and his mother. These urine samples were frozen and stored at −20°C. Immediately prior to analysis, each sample was passed through a 0.45-μm Centricut filter (Kurabou, Osaka, Japan) to remove particulate matter.

The urinary creatinine level was measured by Jaffe's method by using an autoanalyser.

HPLC apparatus

Two Eyela PLC-5 liquid chromatographs (Tokyo Rikakikai, Tokyo, Japan), with pumps and detectors were used. An SC-15 computerized system controller (Tokyo Rikakikai) consisting of an electric valve, a gradient system and a KSP-600 autosampler (Kyowa Seimitsu, Tokyo, Japan) was used. An on-line ERC-3611 Erma degasser (Erma, Tokyo, Japan) was used for eluent delivery, and C-R4A Chromatopack integrator (Shimadzu, Kyoto, Japan) was used for data analysis. A Model 1040M photodiode-array spectrophotometric detector (Yokogawa, Tokyo, Japan) was used for peak identification and to establish peak purity.

HPLC procedure

A precisely measured 5–20 μl aliquot of each sample was applied to the first column (a reversed-phase ODS-C_{18} column: Develosil ODS-5, 150 mm × 6 mm I.D., particle size 5 μm, Nomura Chemical, Seto, Japan), and one fraction from the ODS column was delivered to the second column (cation-exchange column: MCI GEL CK08EH, H^+ form, 300 mm × 8 mm I.D., particle size 9 μm, Mitsubishi Kasei, Tokyo, Japan) by an automated column-switching system. The ODS column was eluted with 5 m*M* H_2SO_4 for 10 min, and was then washed with acetonitrile–water (50:50) for 10 min. Finally, it was equilibrated with 5 m*M* H_2SO_4 for 10 min. The cation-exchange column was eluted isocratically with 5 m*M* H_2SO_4. Both column temperatures were 30°C and both flow-rates were 0.8 ml/min. The two columns were connected for a period of 7 min (just before uracil was eluted) to 10 min (just after orotic acid was eluted) using an electric switching valve. The eluate from the cation-exchange column was continuously monitored at 254 and 280 nm (Fig. 1), and peaks were detected with a Model 1040M photodiode-array spectrophotometric detector in some samples. External standards were analysed at a rate of one per ten urine samples. Retention times and peak heights were recorded using the C-R4A Chromatopack integrator.

Analytical recovery

The analytical recovery was studied by adding a 100-μl sample of the standard mixture solution to 900 μl of a pre-analysed urine sample to obtain the following concentrations: 10, 25, 50 and 100 nmol/ml. Each sample was analysed five times.

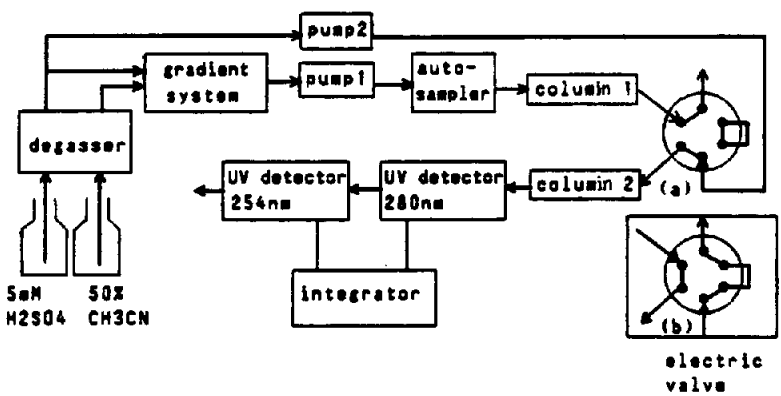

Fig. 1. Black diagram of the dual-column HPLC system: column 1, Develosil ODS-5 (150 mm × 6 mm I.D.); eluent, 5 m*M* H_2SO_4 and 50% acetonitrile; column 2, MCI GEL CK08EH, H^+ form (300 mm × 8 mm I.D.); eluent, 5 m*M* H_2SO_4. (a) 0–7 and 10–30 min, the two columns are separated; (b) 7–10 min, the two columns are connected.

RESULTS AND DISCUSSION

Separation of orotic acid, pseudouridine and uracil, as well as orotidine, uridine and thymine, was achieved using the first column (ODS-C_{18}), as shown in Fig. 2. Orotic acid, pseudouridine and uracil were eluted at retention times of 7–10 min. The three compounds were then applied to the second column (cation-exchange column) by the column-switching system, and were well separated as shown in Fig. 3a. The chromatographic profile of the urine samples from a patient with OTC deficiency and from a healthy adult had three major peaks, corresponding to the standard mixture of orotic acid, pseudouridine and uracil (retention times and absorption ratio at 280/254 nm), and no other significant peaks (Fig. 3b and c). A photodiode-array spectrophotometric detector revealed that the absorption spectra of the three peaks in these chromatograms matched those of standard orotic acid, pseudouridine and uracil (data not shown).

The retention times (mean ± S.D., n = 5, in series) of orotic acid, pseudouri-

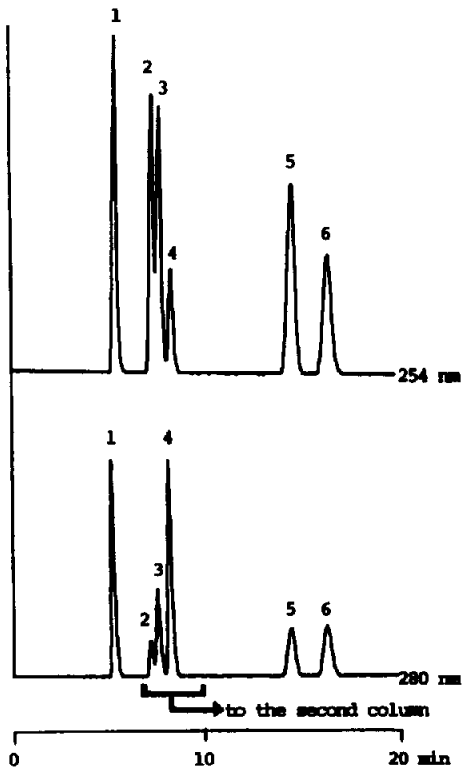

Fig. 2. First-column chromatogram of the standard mixture divided into six pyrimidine derivatives (concentration 100 nmol/ml). Detection wavelength: (upper) 254 nm at 0.04 a.u.f.s.; (lower) 280 nm at 0.04 a.u.f.s. Peaks: 1 = orotidine; 2 = uracil; 3 = pseudouridine; 4 = orotic acid; 5 = uridine; 6 = thymine.

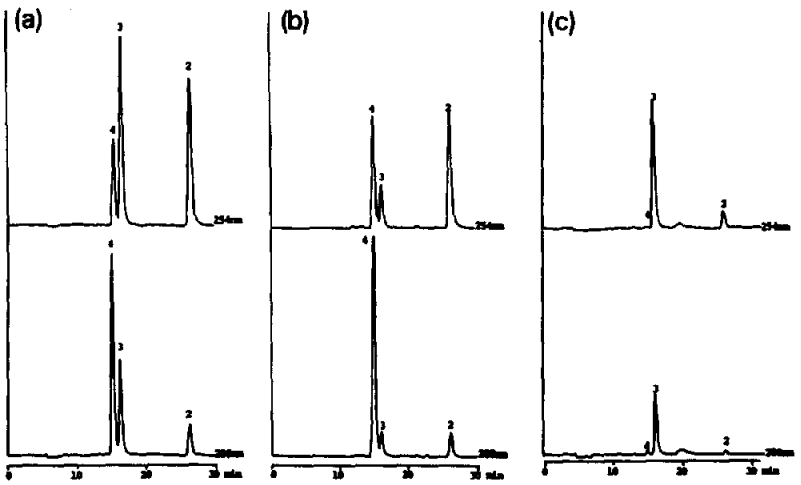

Fig. 3. Second-column chromatogram (one part from the first colum). Detection wavelength: (upper) 254 nm; (lower) 280 nm. (a) Standard mixture (concentration 100 nmol/ml) at 0.02 a.u.f.s.; (b) 5-μl urine sample from a patient with OTC deficiency at 0.16 a.u.f.s.; (c) 5-μl urine sample from a healthy person at 0.01 a.u.f.s. Peak numbers as in Fig. 2.

dine and uracil were 16.33 ± 0.17, 17.37 ± 0.17 and 25.33 ± 0.18 min, respectively. Although the retention times varied slightly from day to day because of slight changes in the column conditions, there were no significant variations over a short period (analysis time for 100 samples).

Calibration curves for orotic acid, pseudouridine and uracil were obtained by processing aliquots of an aqueous standard mixture solution at different concentrations (1, 10, 50, 100, 500 and 1000 nmol/ml). The relationships between standard concentrations and peak heights were linear in the concentration range 1–1000 nmol/ml. The correlation coefficients for the three compounds under study (r, obtained from five measurements) were 0.9991, 0.9997 and 0.9994, respectively. Detection limits were 5 pmol per 5 μl injected.

Table I shows the recovery of standard compounds added to urine. Orotic acid and uracil recoveries were between 95.4 and 107.4%. Because the endogenous pseudouridine level was high (331.3 nmol/ml), pseudouridine recovery was not accurate at a lower added concentration (10 nmol/ml). The coefficient of variation was 0.5–2.1% for peak heights.

Preliminary reference values for the urine of healthy newborns, children and adults are given in Table II. These values are similar to those given in previous reports, which were based on the following procedures: enzymic and colorimetric procedures [9], the monoclonal antibody procedure [4] and HPLC [1,17]. Orotic acid values in our study were slightly lower than those obtained with the colorimetric procedure [7], which measured total orotic acid. Recently, creatinine-related uracil excretion was reported as being unrelated to age [17], but our results

TABLE I

RECOVERY AND COEFFICIENTS OF VARIATION FOR OROTIC ACID (OA), PSEUDOURIDINE (PS) AND URACIL (URA) ADDED TO A URINE SAMPLE

Added standard (nmol/ml)	Recovery (n = 5) (%)			Coefficient of variation (%)		
	OA	PS	URA	OA	PS	URA
10	96.1	89.8[a]	107.4	2.1	0.9	1.0
25	95.4	98.5	105.4	1.1	1.1	1.2
50	97.2	96.9	101.9	0.5	0.8	0.7
100	96.4	94.2	98.1	0.8	0.7	1.0

[a] Pseudouridine recovery was not accurate at 10 nmol/ml added, because the endogenous level was high (331.3 nmol/ml).

show that it is lowest in the newborn period. Further study of uracil reference values, including the newborn period, is necessary.

The absorption ratio at 280 to 254 nm of each urine sample peak was identical with the standard ratio for orotic acid, pseudouridine and uracil, and their values

TABLE II

URINARY OROTIC ACID, URACIL AND PSEUDOURIDINE IN CONTROLS, AND IN A PATIENT WITH PARTIAL OTC DEFICIENCY AND IN HIS MOTHER

Values in parentheses are the ranges.

Group or subject	Age	n	Concentration (mean ± S.D.) (μmol/g of creatinine)		
			Orotic acid	Uracil	Pseudouridine
Control (newborns)	5 days	14[a]	22.3 ± 9.9 (10.8–40.6)	17.3 ± 14.4 (trace–43.2)	1039.7 ± 387.9 (467.2–1794.7)
Control (children)	1–15 years	35[a]	8.3 ± 4.3 (2.5–21.3)	89.9 ± 59.8 (8.8–251.1)	306.2 ± 107.7 (152.4–616.1)
Control (adults)	16 years plus	25[a]	4.9 ± 1.8 (2.5–8.4)	56.2 ± 26.1 (14.9–140.0)	205.1 ± 31.3 (162.1–281.9)
Patient Y.M. (during hyperammonaemia)	2 years	3[b]	578.8 ± 556.4 (98.6–1358.7)	8122.1 ± 1009.1 (6786.3–9224.8)	1409.5 ± 176.8 (1197.1–1630.0)
Patient Y.M. (during non-hyperammonaemia)	3 years	38[b]	23.5 ± 12.9 (7.1–56.4)	451.2 ± 204.9 (221.1–1060.2)	563.9 ± 126.5 (288.8–777.3)
Mother of Y.M.	31 years	11[b]	14.9 ± 8.7 (2.6–30.8)	196.8 ± 74.0 (94.8–368.7)	297.9 ± 82.7 (184.8–451.1)

[a] Number of subjects as controls.

[b] Number of samples for patient Y.M. and for his mother.

(mean ± S.D., n = 9) were 1.907 ± 0.073, 0.297 ± 0.020, 0.116 ± 0.013, respectively. When the orotic acid value was low in concentrated urine, the ratio of orotic acid varied slightly owing to the presence of an interfering substance that was more absorbent at 254 nm than at 280 nm. However, when calculated at 280 nm, the value was within the normal range. This showed that automated sample extraction using the ODS column, followed by elimination of cationic substances and separation of the three compounds using the cation-exchange column, was satisfactory.

Orotic acid and uracil values in the urine of a patient with partial OTC deficiency were extremely high (98.6–1358.7 and 6786.3–9224.8 μmol/g of creatinine, respectively) during a period of hyperammonaemia. Although the urinary orotic acid level decreased to that observed in control children, the urinary uracil level during non-hyperammonaemia periods usually remained higher than the control values (Table II).

A protein loading test (1 g/kg) for the mother of a patient with partial OTC deficiency resulted in a very small rise in the serum ammonia value of 88.0 μg/dl (control, 56.3–74.2 μg/dl). However, a high excretion of orotic acid (93.1 μmol/g of creatinine) was observed compared with the control level (21.0–33.3 μmol/g of creatinine) (Fig. 4). This indicated that she was a carrier of OTC deficiency. Although her urinary uracil did not increase during the protein loading test period (the preloading value (206.8 μmol/g of creatinine) was the maximum value during the 6-h period following protein loading), her urinary uracil values were usually higher than the adult control values.

Chemical diagnosis of OTC deficiency has required a quantification of urinary orotic acid. However, in our patient with partial OTC deficiency, and in the heterozygous carrier (patient's mother), normal or slightly increased orotic acid excretion and a high urinary uracil value was sometimes observed, as in Van Gennip's patients [1]. Therefore the determination of both orotic aicd and uracil may be more reliable for the screening of OTC deficiency with normal ammonia levels, as well as for screening the carrier.

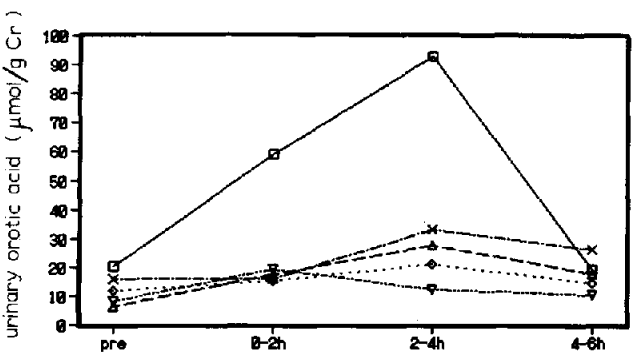

Fig. 4. Profile of urinary orotic acid level during each of the collection intervals over the 6 h after protein loading (1 g/kg weight): □, mother of a patient with partial OTC deficiency; other symbols, controls.

Although simple methods have been reported for measuring uracil [16], orotic acid [14,17] and orotic acid and orotidine [18] using HPLC with one column, previously reported methods for the simultaneous quantification of uracil and other pyrimidines require a complicated sample preparation step [1,12].

In our method, the total analysis time from urine sampling to the simultaneous calculation of the three pyrimidines is only 30 min. Moreover, more than 100 samples can be analysed consecutively. The automated measuring format also simplifies the technician's job. This method should prove useful in the screening of congenital metabolic diseases, such as pyrimidine metabolism disorders, inborn errors in the urea cycle and others.

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