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DETERMINATION OF URINARY OROTATE EXCRETION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new reversed-phase high-performance liquid chromatographic procedure for the determination of urinary orotate excretion is described. It is a selective, sensitive and rapid method, suitable for the differentiation of inherited metabolic diseases with abnormal orotate metabolism.

INTRODUCTION

Orotate, an intermediate in pyrimidine biosynthesis, is a component of all cells. Its concentration in body fluids is higher in inherited metabolic diseases, such as orotic aciduria, lysinuric protein intolerance, and in some inborn errors of urea synthesis [1]. Measurement of orotate excretion in timed urine collections, following an oral protein load, allows heterozygotes with ornithine carbamoyl transferase (OCT) deficiency to be recognized [2].

Owing to its significance in clinical biochemistry, several methods have been developed to measure orotate concentration in biological samples. Microbiological assays [3–5] are rather cumbersome and lack specificity. Enzymic spectrophotometric [6,7] and polarographic [8] methods are too insensitive to measure orotate levels in normal urine. Colorimetric assays [9–12] are simple and widely used to detect abnormal orotate concentrations in urine, but a number of urinary compounds interfere with the colour reaction and the analytical accuracy is reduced at the low orotate concentration found in normal urine. Removing these interfering molecules is a necessary step in the development of a reliable and sensitive colorimetric procedure [13,14].

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The anion-exchange column chromatographic assays [15,16] are time-consuming. Labelled orotate was used to develop radiochemical methods [17–19] that are specific and highly sensitive, but they involve the use of radioactive compounds. A stable-isotope dilution analysis has been reported by Jakobs et al. [20]. This method requires the laboratory synthesis of [$^{15}N_2$]orotic acid and the availability of a gas chromatographic–mass spectrometric (GC–MS) system equipped with a chemical ionization device. Owing to its sensitivity and specificity GC has also been used to measure various urinary pyrimidines, including orotate [21].

The high-performance liquid chromatographic (HPLC) methods are rapid and sensitive. However, they need time-consuming sample preparation [22] or give unsatisfactory resolution of the orotate peak [23–26].

This paper reports a new method for the determination of orotate in urine by means of reversed-phase HPLC. It is rapid, easy to perform and gives good sensitivity and reproducibility, which makes it suitable for clinical purposes.

EXPERIMENTAL

Chemicals

Acetonitrile (LiChrosolv grade) and hydrochloric acid (Selectipure grade) were purchased from Merck (Darmstadt, F.R.G.). Water was obtained from a Milli-Q Waters purification system (Millipore, Milford, MA, U.S.A.). All solutions were passed through a 0.45-μm Millipore filter and degassed before use. Anhydrous orotic acid was obtained from Fluka (Buchs, Switzerland), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Pierce (Rockford, IL, U.S.A.)

HPLC apparatus

HPLC determinations were carried out with a Waters Assoc. (Milford, MA, U.S.A.) system composed of two Model 510 pumps, a Model U6K injector, a Model 441 absorbance detector and a Baseline 810 chromatography workstation.

GC–MS apparatus

The GC–MS apparatus used was a Kratos Model MS25FRA mass spectrometer (Manchester, U.K.), equipped with a Carlo Erba MFC500 gas chromatograph (Milan, Italy) and a DS90 data analysis system (Data General, Westboro, MA, U.S.A.)

Sample preparation for HPLC

A 500-μl urine sample, filtered through a 0.22-μm Millex-GS filter, was passed through a Sep-Pak C_{18} cartridge (Waters) activated with 2 ml of acetonitrile–water (60:40, v/v) and 5 ml of water. The cartridge was washed twice with 0.5

ml of water. The eluates were pooled and acidified to pH 1.5–2.0 with 37% hydrochloric acid. A 25-μl sample was injected into the HPLC apparatus.

HPLC procedure

Two coupled LiChrospher 100 RP-18 (125 mm×4 mm I.D., 5 μm particle size, Merck, Darmstadt, F.R.G.) columns were used. The mobile phase was 3.2 m*M* hydrochloric acid at a flow-rate of 1.0 ml/min. Chromatographic separation was carried out in 5 min at room temperature, and absorbance was monitored at 280 nm. The columns were then washed for 3 min with acetonitrile–8 m*M* hydrochloric acid (60:40, v/v)) and equilibrated for 7 min with the mobile phase.

Sample processing for mass spectrometry

After HPLC separation, the fraction containing the material to be identified was recovered and freeze-dried. The residue was silylated with adequate volumes of BSTFA and TMCS, allowed to react at 60°C for 30 min, and 1 μl was analysed by GC–MS.

GC–MS procedure

A capillary WCOT fused-silica column (25 m×0.32 mm I.D.) coated with CP-Sil 5 (Chrompack, Middelburg, The Netherlands) was used under the following conditions: oven temperature programme, 10°C/min from 100 to 200°C; injector port temperature, 250°C; carrier gas, helium at a flow-rate of 1.0 ml/min; reactant gas for chemical ionization analysis, ammonia (0.0002 Torr).

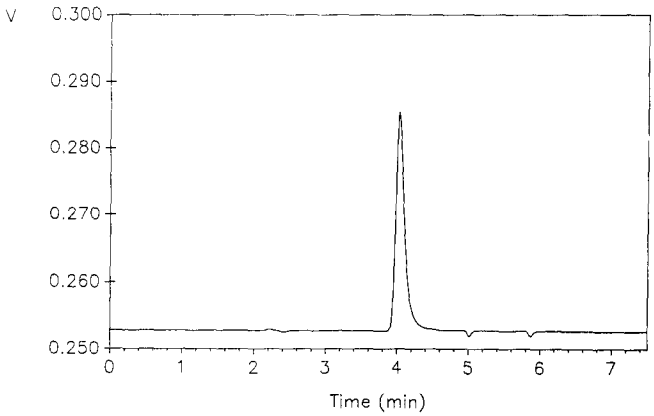

Fig. 1. Chromatogram of a 10 μg/ml standard solution of orotate.

RESULTS AND DISCUSSION

The chromatographic profile of a solution of pure orotate (10 μg/ml) and of a sample of pooled human urine, processed as described above, are shown in Figs. 1 and 2, respectively. Fig. 3 shows the profile obtained when an orotate solution was added to the same urine sample.

The chromatographic peak of urinary orotate was well resolved. Its retention time of 4.06 ± 0.022 min (mean $\pm$ S.D., $n = 10$) was identical with that of standard orotate (4.08 ± 0.013 min).

The structural identity between orotate and the corresponding urinary compound was confirmed by comparing their absorbance ratios (A_{280}/A_{254}) (data not shown), their GC profiles (Fig. 4) and ammonia chemical ionization mass

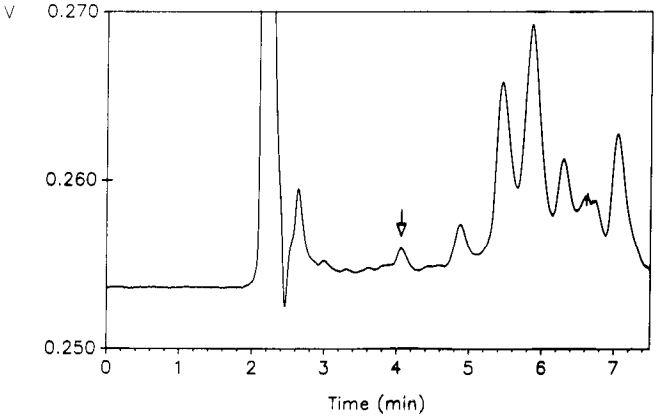

Fig. 2. Chromatogram of a pooled urine sample from six healthy volunteers. The orotate peak is marked with an arrow.

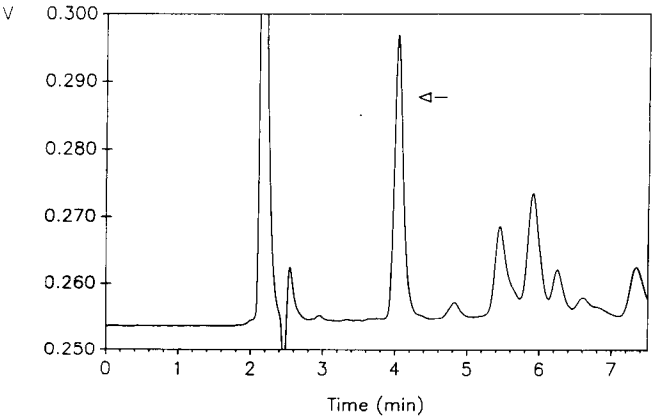

Fig. 3. Chromatogram of the same pooled urine sample as in Fig. 2, with added orotate solution. The orotate peak is marked with an arrow.

spectra (Fig. 5), after collection of the corresponding HPLC fraction. The GC–MS analyses of these fractions did not show any interfering substance in urine samples from healthy volunteers. The calibration curve was obtained by processing aliquots of aqueous orotate solutions at different concentrations (0.1, 0.25, 0.5, 1, 10, 25, 75 and 100 μg/ml). The regression equation (obtained from ten measurements) is $y = 1.471 \cdot 10^{-2}x + 3.432 \cdot 10^{-3}$, where y is the orotate concentration in the diluted sample (μg/ml) and x is peak area/1000. The rela-

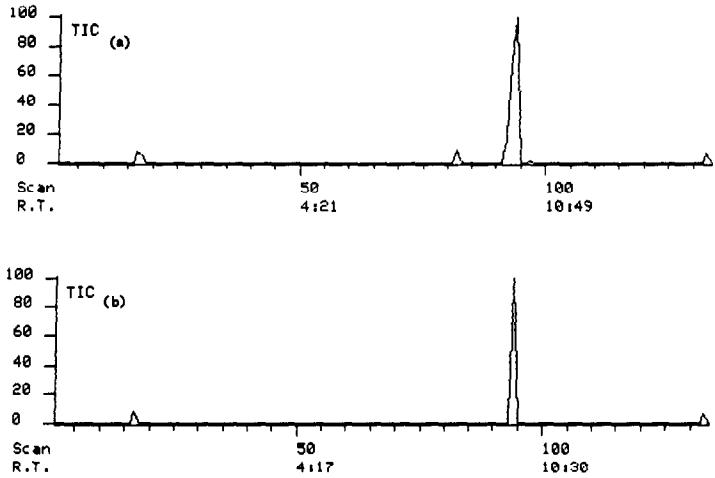

Fig. 4. GC profiles of (a) the HPLC fraction containing the orotate peak and (b) an orotate solution (chemical ionization, NH_3 at $2 \cdot 10^{-4}$ Torr).

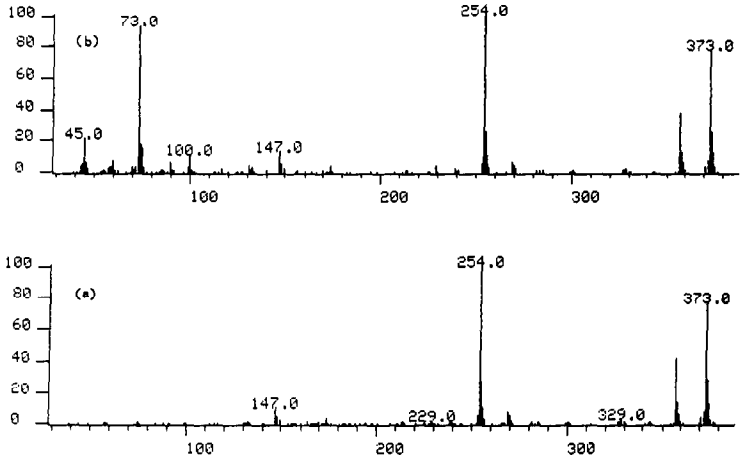

Fig. 5. Mass spectra of (a) pure orotate and (b) the orotate contained in the corresponding HPLC fraction containing the orotate peak (chemical ionization, NH_3 at $2 \cdot 10^{-4}$ Torr).

tionship between orotate concentration and peak area was linear between 0.1 and 100 μg/ml.

The standard error of the slope was $7.881 \cdot 10^{-6}$. The 95% confidence limits were $1.473 \cdot 10^{-2}$ and $1.469 \cdot 10^{-2}$ and the correlation coefficient (r) was 0.9999. The signal-to-noise ratio at an orotate concentration of 0.1 μg/ml was 12.5.

The analytical recovery was studied by adding 50 μl of orotate solution to 450 μl of a pooled urine sample (a mixture of six individual urine collections) to obtain the following concentrations: 0.2, 0.5, 2.0, 10.0, 20.0 and 50.0 μg/ml, in addition to the orotate already contained in the urine pool (0.46 μg/ml). Each sample was then processed as described in Experimental and analysed ten times. The recovery of added orotate ranged from 97.2 to 105.6% for the concentration range 0.2–50.0 μg/ml (Table I).

The day-to-day precision (C.V.) at the 10 μg/ml level was 0.52% ($n=50$). All the measurements were carried out consecutively within ninety days.

TABLE I

RECOVERY OF OROTATE ADDED TO POOLED URINE SAMPLES ($n=10$)

Orotate added (μg/ml)	Recovery (%)	Coefficient of variation (%)	
		Area	Retention time
0.00	–	2.06	0.72
0.20	100.1	1.59	0.69
0.50	99.3	1.32	0.52
2.00	105.2	1.08	0.70
10.00	97.8	0.84	0.67
20.00	97.2	0.55	0.55
50.00	98.7	0.58	0.53

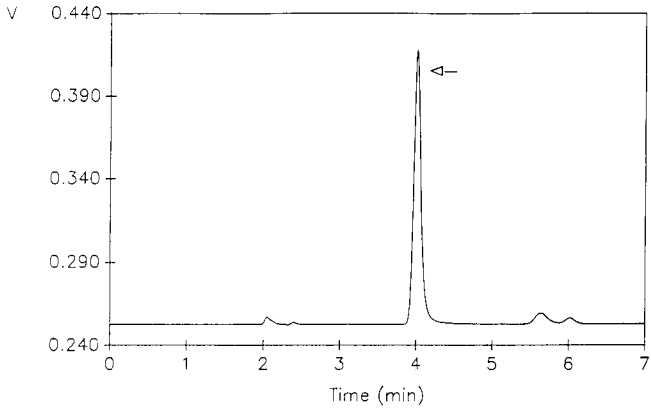

Fig. 6. Chromatogram of a urine sample from a patient with ornithine carbamoyl transferase (OCT) deficiency. The orotate peak is marked with an arrow.

The clinical utility of our method was evaluated with a sample from a two-year-old girl, with hyperammonemia, and normal plasma and urine amino acid concentrations. The HPLC analysis of her urine sample (Fig. 6) revealed an abnormal orotate excretion of 6860 μmol/g of creatinine (reference values, 0.7–3.9 μmol/g of creatinine [16]), and the diagnosis of ornithine carbamoyl transferase (OCT) deficiency was confirmed by liver enzyme assay (liver OCT activity, 18% of control values).

This method is sensitive and precise. The HPLC procedure is rapid (15 min from injection to injection) and sample preparation is quick and easy. The method is suitable for use in the clinical laboratory for the diagnosis of inherited metabolic diseases and abnormal orotate metabolism.

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