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Short communication

High-performance liquid chromatographic determination of orotic acid as its methyl derivative in human urine

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Abstract

Described is a method for the determination of orotic acid as its methyl ester in human urine. The method involves the use of solid-phase extraction to isolate pyrimidines from urine and derivatization with methanol and sulfuric acid, followed by isocratic high-performance liquid chromatography on a reversed-phase C_{18} column with UV absorbance detection. The assay is shown to be sufficiently sensitive for use in clinical investigations where elevated orotic acid excretion is suspected.

Keywords: Orotic acid

1. Introduction

Orotic acid (OA), a metabolite of aspartic acid, is an intermediate in the de novo synthesis of pyrimidines. The measurement of urinary orotate is of principal interest in hereditary orotic aciduria, lysinuric protein intolerance and in some inborn errors of urea synthesis. OA excretion is also elevated by ammonia intoxication, during feeding of diets high in protein and in patients treated with allopurinol or 6-azauridine [1].

Metabolic and genetic disorders associated with high levels of endogenous OA appear to pose an increased risk of cancer development, because an excess of OA in the diet promotes the carcinogenic process in different organs, especially in the liver [2].

OA is a very important substance but difficult to analyze in biological samples: reversed-phase chromatographic procedures are difficult to set up because OA has a pK_a value which may be the lowest among endogenous carboxylic acids [3]; ion-pair chromatography [4] results in inconsistent resolution values; ion-exchange [5] gives rise to long retention times, and dual columns ''switching technique'' [6] is cumbersome.

In our high-performance liquid chromatography (HPLC) system, OA is methylated before analysis: methyl ester (MeOA) formation increases relative retention by a factor of 3 (OA=1.2±0.1 min; MeOA=3.35±0.18 min) in reversed-phase chromatography with a mobile phase containing 5% acetonitrile.

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2. Experimental

2.1. Chemicals

Orotic acid (6-carboxy-2,4-dihydroxypyrimidine), orotic acid methyl ester and triethanolamine were purchased from Sigma (St. Louis, MO, USA); acetonitrile, methanol, perchloric acid, sulfuric acid, sodium sulfate and water (HPLC-grade) were from Merck (Darmstadt, Germany).

2.2. Sample preparation and extraction

A C_{18} microcolumn (Sep-Pak, Waters, Milford, MA, USA) was activated with 5 ml of methanol and 2 ml of water. Urine samples (2 ml) were slowly eluted through the cartridge, the eluate was collected, combined with 1 ml washing with water and then dried in a rotary evaporator at 55°C under vacuum.

2.3. Pre-column derivatization

Sulfuric acid (0.1 *M*) in methanol (0.5 ml) was added to the dried sample and derivatization to methyl orotate was accomplished in a sealed vial at 80°C for 20 min with continuous agitation. After cooling, the acidic derivatized sample was diluted 10:1 with the mobile phase and 100 μl were injected into the HPLC system.

2.4. Chromatography

The HPLC system (Beckman, San Ramon, CA, USA) consisted of a solvent module, a programmable UV absorbance detector, an Altex 210A injection device fitted with a 100 μl loop and a signal processor (Beckman System Gold).

The samples were eluted isocratically from a Spherisorb ODS 2 column (150×4.6 mm I.D., 5 μm

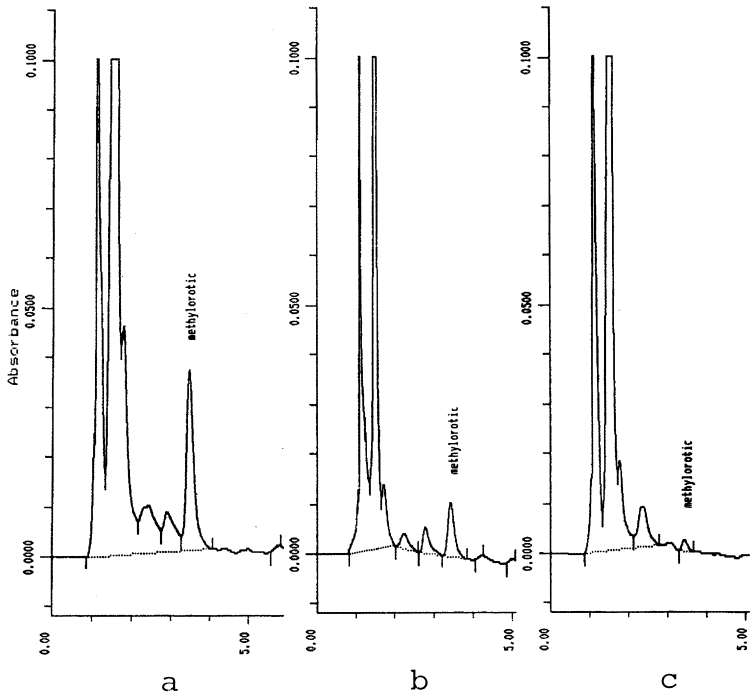

Fig. 1. Chromatograms of (a) urine sample containing 57.6 μ*M* OA from a female carrier for OTC deficiency; (b) urine from a formula-fed neonate, containing 12.6 μ*M* OA; (c) urine from a normal adult, containing 2.1 μ*M* OA. Chromatographic conditions: column, Spherisorb ODS2; mobile phase, 0.1 *M* triethanolamine, pH 3.0 adjusted with perchloric acid–acetonitrile (95:5, v/v); flow-rate, 1.5 ml/min. The results were recorderd at 280 nm and 0.1 a.u.f.s.

particle size) (Phase Separations, Deeside, UK) with a mixture of 95% (v/v) triethanolamine (0.1 *M*) adjusted to pH 3.0 with concentrated perchloric acid and 5% acetonitrile.

The mobile phase was filtered through a 0.2 μm Nylon filter and pumped at 1.5 ml/min. Detection wavelength was set at 280 nm (0.1 or 0.05 absorption units full-scale). Using this isocratic system a run time of 15 min was required to ensure that all UV absorbing components were eluted prior to the next injection.

3. Results

Derivatization of OA with methanol–H_2SO_4 gives a quantitative yield of MeOA [7]. The MeOA concentration was linearly related to peak area between 2 and 200 μ*M*. The correlation coefficient determined by linear regression analysis ($n=10$) was $r=0.9985$.

Analytical recovery was studied by adding 100 μl of appropriate OA solutions to 900 μl of a urine sample to obtain the following OA concentrations: 2.5, 5, 25 and 50 μ*M*. Each sample was analyzed ten times. Under these conditions the mean analytical recovery was 89.2% (S.D. 4.5%; $n=40$). The lowest detectable concentration (signal-to-noise ratio of 3:1) was approx. 0.3 μ*M* (30 p*M*/100 μl injection) and the quantitation limit in urine samples was 1.8 μ*M* (0.3 μg/ml) with a relative standard deviation less than 10% for a 100 μl sample volume.

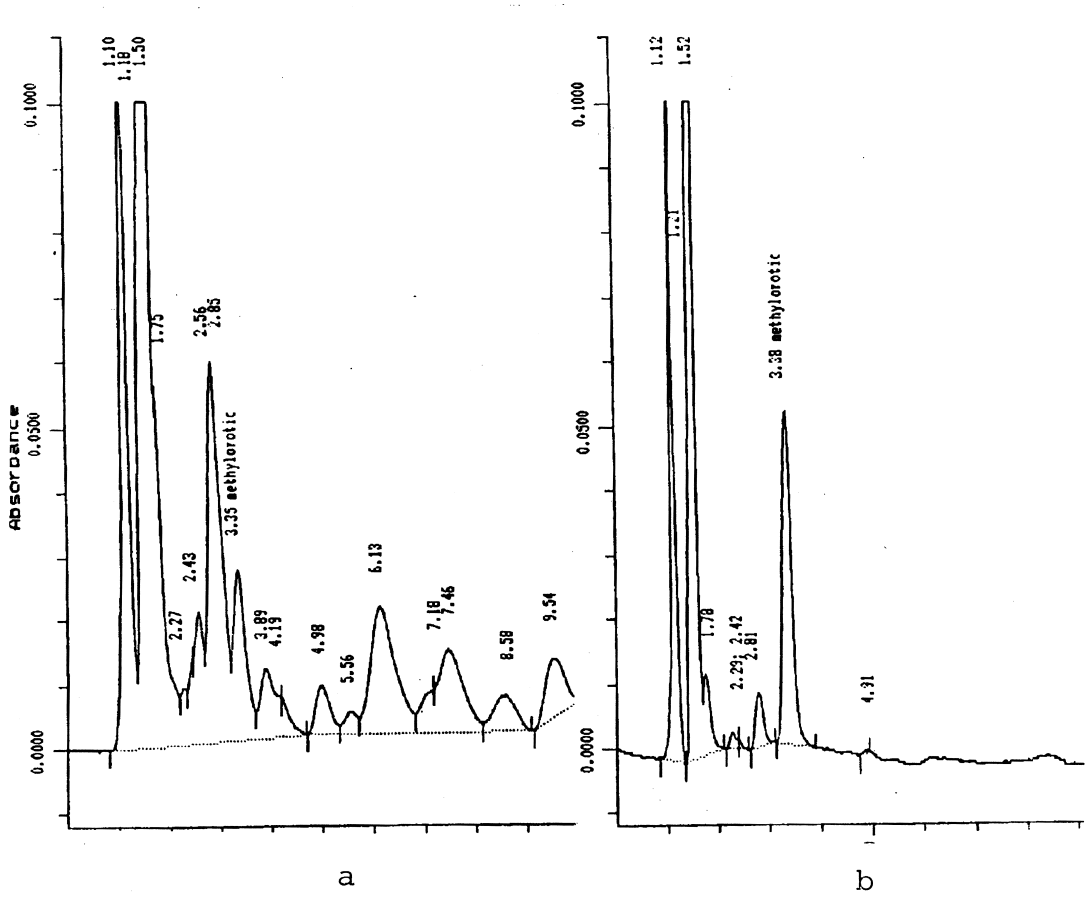

Fig. 2. Chromatograms of a sample of urine spiked with OA (70 μ*M*) after (a) direct derivatization of urine and (b) Sep-Pak C_{18} prefractionation. Separation was achieved using a Spherisorb ODS2 column and the isocratic HPLC system described in Section 2.4. The results were recorded at 280 nm over 10 min at a flow-rate of 1.5 ml/min and 0.1 a.u.f.s.

Our method has been tested in a clinical study of samples of urine from normal adults ($n=12$), neonates (age 16–20 days) fed with maternal milk ($n=44$) or cow's milk-based formula ($n=11$) and a female obligate carrier for ornithine carbamyl transferase (OTC; EC 2.1.3.3) deficiency (Fig. 1).

The mean value of OA in the normal adult urine was 4.2 ± 3.0 μM/g creatinine, in agreement with that of Ohba et al. [4] and Seiler et al. [6].

Urine samples from breast-fed infants contained 11.6 ± 9.1 μM/g creatinine (trace 25 μM), whereas in formula-fed infants OA excretion was 21.1 ± 12.0 μM/g creatinine (trace 46 μM) and appeared related to high content of OA in cow milk (100 mg/l) compared to trace amounts in human milk [8].

4. Discussion

Our procedure combines a solid-phase purification step with an efficient OA derivatization which permits a reliable separation of its methyl ester by reversed-phase chromatography.

Urinary OA, not retained on a C_{18} cartridge, was separated from most of the low-polarity compounds that could interfere with the methylation reaction: the efficiency of this step is shown in Fig. 2, where a sample of urine was analyzed before and after solid-phase extraction.

Derivatization was carried-out under mild conditions because the high acidity of the active hydrogen of OA required a low-strength alkylating agent. Samples injected on to the column contain 0.01 M sulfuric acid, a concentration insufficient to reduce the performance of the chromatographic system.

Furthermore, MeOA is less polar than the starting acid, it has superior stability and its use results in increased detection sensitivity. Molar absorption at 280 nm (ε_{280}) was 12.500 for MeOA and 9.150 for OA.

In summary, the method described can be used for rapid screening of urea cycle disorders and for testing the effect of different nutrients on OA metabolism and excretion.

References

[1] S.W. Brusilow, E. Hauser, J. Chromatogr. 439 (1989) 388–391.
[2] S. Vasudevan, E. Laconi, P.M. Rao, S. Rajalakshmi, D.S. Sarma, Carcinogenesis 15 (1994) 2497–2500.
[3] P. Banditt, J. Chromatogr. B 660 (1994) 176–179.
[4] S. Ohba, M. Kobayashi, T. Katoh, J. Chromatogr. 568 (1991) 325–332.
[5] M.T. McCann, M.M. Thompson, I.C. Gueron, M. Tuchman, Clin. Chem. 41 (1995) 739–743.
[6] N. Seiler, C. Grauffel, G. Therrien, S. Sarhan, B. Knoedgen, J. Chromatogr. B 653 (1994) 87–91.
[7] A. Akane, S. Fukushima, K. Matsubara, S. Takahashi, H. Shiono, J. Chromatogr. 529 (1990) 155–160.
[8] P.O. Okonkovo, J.E. Kinsella, Am. J. Clin. Nutr. 22 (1969) 532–539.