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Short Communication

Determination of orotic acid in urine

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Abstract

Orotic acid was separated from other urinary constituents by ion-pair formation with tetrabutylammonium, and isocratic elution from a reversed-phase column. Absorbance at 280 nm was recorded for quantitation. Owing to the better column characteristics the separations are somewhat faster, and the sensitivity of the method is higher than those of analogous methods using anion-exchange columns. The method was used for the determination of orotic acid in human urine, in urine of rats with portacaval shunts and in small (30 μl) urine samples from sparse fur mice. Shunted rats excreted *ca.* 100% more orotic acid per 24 h than sham-operated controls, in spite of their considerably lower body weight. Excessive orotic acid in urine indicates a conditional deficiency of ornithine. Sparse fur mice are congenitally hyperammonemic because of a defective hepatic ornithine carbamoyltransferase. Determination of orotic acid in the urine is a suitable method to identify those animals among litter mates which have the hereditary enzyme defect.

1. Introduction

Excessive urinary excretion of orotic acid has been suggested to be evidence for a conditional deficiency of ornithine or arginine [1]. For this and other reasons, determination of orotic acid in urine is of diagnostic importance. In normal human urine only small amounts of orotic acid are found [2], and thus selective and sensitive methods are required.

The early colorimetric methods are non-selective [3]. In recent years chromatographic methods have been described which promised to improve sensitivity and selectivity and, by automation, allowed more convenient procedure (see refs. 4–6 and references therein).

In our attempt to use a more recent version [7] of the method of Ferrari *et al.* [5] for the determination of orotic acid in small (30 μl) rodent urine samples, we observed losses of orotic acid during the prepurification step with Sep-Pak C_{18} cartridges. Moreover, in contrast to Ferrari *et al.*, we were not able to remove by the

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described procedure traces of a material which strongly absorbed around 240 nm (presumably peptides). Even if we coupled two columns each 250 mm long the orotic acid concentrations obtained by this method were well above the reported values [8].

Although the losses by adsorption to the reversed-phase material during prepurification may be negligible if 500-μl urine samples are used, as was suggested by the authors of the procedure, we nevertheless made an attempt to circumvent the mentioned shortcomings of the method.

The separation of orotic acid on an anion-exchange column [4] was well reproducible, but owing to the column characteristics and the slow elution, the peaks were broad, and the sensitivity of the method correspondingly low. In order to retain the selectivity of anion-exchange chromatography, but improve the sensitivity, we attempted to use ion-pair formation of orotic acid with tetrabutylammonium as counterion, and separation on a reversed-phase column.

2. Experimental

2.1. Chemicals

Common laboratory chemicals were from E. Merck (Darmstadt, Germany). Acetonitrile RS for HPLC was from Farmitalia (Carlo Erba, Milano, Italy), tetrabutylammoniumdihydrogenphosphate (97% pure) from Aldrich (Steinheim, Germany), and orotic acid monohydrate (98% pure) from Janssen Chimica (Beerse, Belgium). Stock solutions of orotic acid (2 m*M* in distilled water) were prepared and stored frozen until use; thawing and freezing was avoided. Dilutions were prepared in water when needed. Amberlite CG 120 II, 200–400 mesh was from Serva (Heidelberg, Germany). It was equilibrated with 2 *M* NaOH over night, washed with distilled water, followed by 2 *M* HCl, 50% acetone and 50% methanol, until the washing fluid was colorless. The resin was stored under 2 *M* HCl in a refrigerator.

Water for HPLC and for the preparation of reagent solutions was prepared by distillation of tap water over H_3PO_4.

2.2. Prepurification

For prepurification, chromatography on a strong cation exchanger (Amberlite CG 120 II, H^+-form) was used essentially as described by Harris and Oberholzer [3]. The column-bed volume was 0.4 ml; urine volumes may vary from 30–500 μl. Elution of neutral and acidic components of urine was achieved with 0.85 ml of water. In the case of 500-μl urine samples the eluate was brought to pH 7.0–7.5 by mixing with 150 μl of a 0.1 *M* solution of tetrabutylammoniumdihydrogenphosphate in 0.1 *M* borate buffer pH 8.0, and if required, by further addition of 10 μl 10 *M* NaOH. In the case of smaller urine volumes the volume of borate buffer was proportionately smaller. By using smaller Amberlite columns, the method can be miniaturized, if routinely small urine volumes are analyzed.

2.3. Chromatography

Fifty-μl aliquots of the prepurified urine samples were applied (automatically) onto the columns. For routine determinations a Varian Vista 5500 liquid chromatograph, equipped with a Model 9090 Autosampler was used.

Separations were achieved at room temperature on a column (250 mm × 4.6 mm I.D.) filled with Merck Superspher RP 18 endcapped (4-μm particle size) (Bischoff, Leonberg, Germany). The guard column (20 mm × 3 mm I.D.) was filled with Pellicular ODS (Whatman, Clifton, NJ, USA). The eluent consisted of a 5 m*M* solution of tetrabutylammoniumdihydrogenphosphate, which was adjusted with NaOH to pH 7.4, and contained 20 ml acetonitrile per litre; elution mode: isocratic; flow-rate: 0.8 ml min^{-1}. In order to avoid interference with urinary constituents which migrate slower than orotic acid, samples were applied automatically (using a Varian Autosampler 9090) only every 75 min. Quantitative evaluation was achieved by recording the absorbance at 280 nm. Signals were

processed by a Milton Roy CI-10 integrator (LDC, Paris, France).

Since Amberlite, in contrast with Sep Pak C_{18} material, does not retain lipophilic components of the urine, the column was washed after *ca.* 50 urine samples. By pumping a 7:3 mixture of eluent and acetonitrile for 30 min, the contaminants were removed. After re-equilibration for *ca.* 30 min, and determining the elution time of an orotic acid standard, the separation of urine samples may be recommenced.

2.4. Urine samples

(a) Human urine was collected from nine healthy adult volunteers (7 males, 2 females) free of medication. The urines were stored at −20°C until analyses were performed.

(b) Twenty-four hour rat urines were from Sprague–Dawley rats with portacaval shunts [9]. Sham-operated animals served as controls. Four weeks after shunting twenty-four hour urine samples were collected using metabolic cages. The glass vessels contained 6 ml of ethanol. (Ethanol was used to avoid hydrolytic liberation of ammonia from glutamine, since the same urines were used for ammonia and amino acid determinations. For exclusive determination of orotic acid 5 ml of 1 *M* HCl is preferable as bactericide.)

(c) A few drops of mouse urine were collected on Parafilm. (The animals urinate due to an anxiogenic effect of immobilization by a firm grip.) Storage at −20°C until analysis.

Ammonia in rat plasma was determined using an ammonia electrode (Model 95-12; Orion Research, Cambridge, MA, USA). For more details of the method, see ref. [10].

Creatinine was determined in 10-μl urine samples by a miniaturized version of the procedure of Loken [11].

3. Results

It is known from previous experience [3], and it was confirmed in the present work, that the recovery of orotic acid after chromatography even of only 30-μl urine samples or of small quantities (1–15 nmol) of standard orotic acid solutions on Amberlite CG 120 columns is $\geq 94\%$ (data not shown).

Since the material of the guard column (Pellicular ODS) tended to retain small quantities of orotic acid, similar to the Sep Pak C_{18} cartridges (Waters, Milford, MA, USA), it was necessary to run several urine samples in order to occupy the orotic acid binding sites. Once this was achieved, no losses of orotic acid were observed. This was verified by comparison of peak heights and areas, respectively, obtained from 1 nmol orotic acid samples, with those of urine samples with and without orotic acid addition. Peaks were strictly additive (recovery $>98\%$).

In Fig. 1, chromatograms of a rat urine are shown before and after spiking with 5 nmol orotic acid per 500 μl of urine. The retention

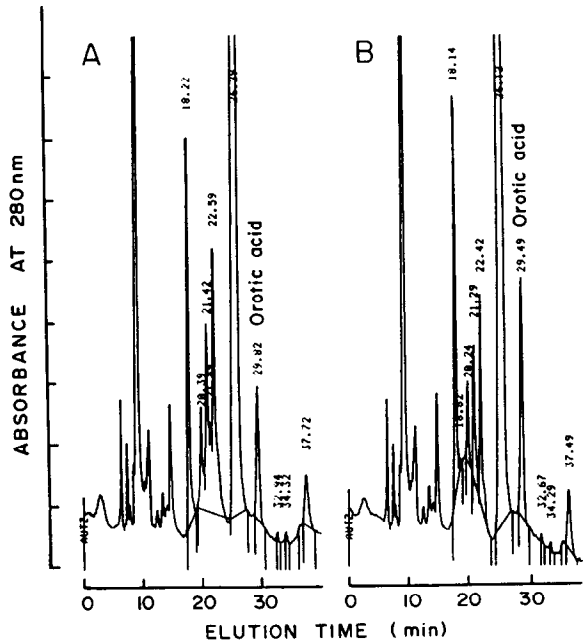

Fig. 1. Conditions: 500-μl urine samples were prepurified by chromatography on Amberlite (total volume after neutralisation 1.5 ml); 50-μl aliquots were separated within a large series of analogous samples isocratically, as described in the methods section, without washing the columns in between the individual samples. Absorbance (arbitrary units) was recorded at 280 nm (AU/mV 0.02). In the case of chromatogram B 500 μl urine was spiked with 2 nmol orotic acid before prepurification.

time of orotic acid decreased gradually with prolonged use of the column. Since, however, the retention time of other urine constituents diminished concomitantly, and peak width decreased at the same time, separations were not much affected. Nevertheless, for unambiguous peak identification it is advisable to run from time to time samples spiked with orotic acid.

Peak height and peak area were linearly increasing with the amount of urine ($r^2 = 0.99$) if 30–500 μl samples were prepurified by cation-exchange column chromatography, and separated under the conditions described in the Experimental section. Quantitative evaluation of the chromatograms by peak height and peak area determination gave results of approximately the same precision. The reproducibility of the procedure was tested by analyzing ten 30-μl aliquots of a urine pool from sparse fur mice; S.D. was 4.6 percent of the mean value (3.75 nmol orotic acid per 30 μl urine). Furthermore, seven orotic acid standard solutions ranging from 0.3–6.6 μM (15–333 pmol per 50 μl sample volume) were analyzed in duplicate. The mean S.D. of the duplicate determinations over this concentration range was 6.2%. Fifteen picomol orotic acid is considered the lowest amount per 50-μl sample volume that may be determined with acceptable accuracy by our method.

With 4.9 ± 3.6 μmol orotic acid per g creatinine, the mean value of the human urines was in excellent agreement with that of Ohba *et al.* [8], who used a reversed-phase column for preseparation, and then switched automatically to a cation-exchange column [6].

Mean orotic acid excretion per 24-h period by six rats (weighing 440 ± 40 g four weeks after sham operation) was 0.4 ± 0.1 μmol. Rats with portacaval shunts (with the same starting weight of 200 g, but weighing only 270 ± 50 g four weeks after shunting) excreted 0.9 ± 0.3 μmol orotic acid per 24-h period. Due to the enhanced excretion of creatinine by the shunted rats (42 ± 8 mg per 24 h) compared with sham operated animals (20 ± 3 mg per 24 h), the amount of orotic acid per mg creatinine was the same in both groups (shunted : 22 ± 8 μmol g^{-1}; sham operated: 21 ± 2 μmol g^{-1}. The considerably greater variation of the values of the shunted rats is most probably due to variations of hepatic metabolic activity of the individual animals). Plasma ammonia concentrations of the sham operated rats were 0.10 ± 0.01 mM, and 0.3 ± 0.1 mM for the rats with portacaval shunts, in good agreement with published data [12].

The method was also applied to the determination of orotic acid in small (30 μl) urine samples from a colony of sparse fur mice [13], with the aim to identify among litter mates those animals with a hereditary defect of ornithine carbamoyltransferase. The X-chromosomal sparse fur (spf) mice excreted 3300 ± 2900 nmol orotic acid per mg creatinine; their normal litter mates only 127 ± 20 nmol per mg creatinine (mean values $\pm$ S.D., $n = 8$).

4. Discussion

Our procedure combines a known prepurification step which yields nearly quantitative recovery [3] with the separation of the ion pairs formed by acids with tetrabutylammonium. We found purification on Amberlite not more cumbersome than filtration [4] but more efficient in removing potentially interfering urine constituents. Owing to superior column characteristics, the separations of the ion pairs were more complete, faster, and the detection sensitivity superior than analogous separations on an anion-exchange column [4].

Elevation of plasma ammonia concentrations favours the enhanced formation of carbamoyl phosphate. If ornithine is not available in sufficient quantities, or if urea formation is impaired due to a limited ornithine carbamoyltransferase activity, orotic acid is formed in excess and excreted. This is considered a marker of a functional deficit of ornithine [1].

In our experiment, rats with portacaval shunt excreted per 24 h about twice as much orotic acid than their sham-operated counterparts, although their body weight was lower by *ca.* 40%. This finding could be interpreted as a conditional deficiency of ornithine due to the shunt. However, based on creatinine excretion, there was no

difference in orotic acid excretion between the two groups. This example demonstrates a difficulty in the evaluation of data of urinary constituents. If different metabolic or nutritional states are compared, values based on creatinine excretion may be misleading.

In conclusion, our procedure seems to fulfil the criteria of selectivity, sensitivity, reproducibility, and convenience of a method suited for the routine assay of orotic acid in urine.

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