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# Note

## Determination of orotic acid in urine and serum by isotachophoretic analysis

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Orotic acid is present in only trace amounts in normal human urine, and greatly increased excretion of orotic acid has been observed in congenital orotic aciduria [1]. Orotic acid excretion also increases during drug treatment [2] and in the state of hyperammonemia, such as a disorder in the urea cycle, *e.g.* ornithine transcarbamylase deficiency [3], arginase deficiency [4], ammonium salt load [5], Reye's syndrome [6] and arginine deficiency [7]. Slight increases have been reported during human pregnancy [8], following premature birth [9], in cases of deficiencies of purine-nucleoside phosphorylase [10] or phosphoribosyl pyrophosphate synthetase [11] and some combined immunodeficiency disorders [12].

This compound has been determined by an isotope-dilution method [1], enzymic UV spectrophotometry [13], anion-exchange column chromatography [14], a colorimetric method [15] and high-performance liquid chromatography [16]. UV detection with isotachophoresis has been found to be useful as a screening test for detecting orotic acid [17].

This paper describes a new analytical approach for the analysis of orotic acid in urine and serum by using an isotachophoretic analyser without UV detection.

## EXPERIMENTAL

### *Materials*

Orotic acid was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were analytical grade.

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*Apparatus*

The capillary apparatus was an IP-3A isotachophoretic analyser from Shimadzu Seisakusho (Kyoto, Japan). The determination of orotic acid in urine and serum was carried out in a capillary tube (20 cm × 0.5 mm I.D.) maintained at 20°C. The migration current was 100 μA. The leading electrolyte was 0.01 *M* hydrochloric acid and β-alanine (pH 3.1) containing 0.001 *M* cupric chloride. The terminal electrolyte was 0.01 *M* glutamic acid.

*Assay of urine and serum*

A 2-ml volume of each urine sample was adjusted to pH 9.0 with 2 *M* ammonium hydroxide and applied to a column containing 5 ml of Diaion SA ($HCOO^-$ form; anion exchanger, 100 mesh; Mitubishi Kasei, Tokyo, Japan). The column was washed with 50 ml of water and 30 ml of 10% formic acid, and eluted with 50 ml of 0.2 *M* hydrochloric acid. The eluate was evaporated to dryness under reduced pressure. An aliquot of the residue was dissolved in 1 ml of water, and 5–10 μl of that solution were subjected to isotachophoretic analysis.

A four-fold volume of 1.25% sulphosalicylic acid was added to rat serum and centrifuged at 2000 *g* for 15 min. The supernatant was adjusted to pH 9.0 with 2 *M* ammonium hydrochloride and applied to a column containing 5 ml of Diaion SA ($HCOO^-$ form) and treated as described for urine.

## RESULTS AND DISCUSSION

We were able to detect authentic orotic acid by isotachophoresis, with 0.01 *M* hydrochloric acid and β-alanine (pH 3.1) as leading electrolyte and 0.01 *M* glutamic acid as terminal electrolyte. We also found that orotic acid was eluted by the method described in Experimental. Fig. 1A shows an isotachopherogram of authentic orotic acid. Fig. 1B and C show isotachopherograms of normal human urine alone (B) and mixed with orotic acid (C). Under these conditions, authentic orotic acid and other metabolites in urine were detected in the same zone.

Therefore, the analytical conditions necessary for detecting orotic acid without interference from other metabolites in urine were examined. Cupric chloride has been used to change potential gradients [18], and we obtained good results when we added cupric chloride to the leading electrolyte (Fig. 2). Cupric chloride changed the effective mobility of orotic acid. Fig. 2A shows an isotachopherogram of authentic orotic acid. The effective mobility of orotic acid was slightly changed, but those of the metabolites in normal human urine were almost the same (Fig. 2B). Moreover, in their mixture, orotic acid was detected as a different zone (Fig. 2C).

Fig. 3 shows the influence of cupric chloride on the zone length of orotic acid. Maximum changes of zone length were obtained with 0.0003 *M* cupric chloride. Over that concentration, the curve becomes a plateau.

The standard curve, drawn by plotting zone length against different concentra-

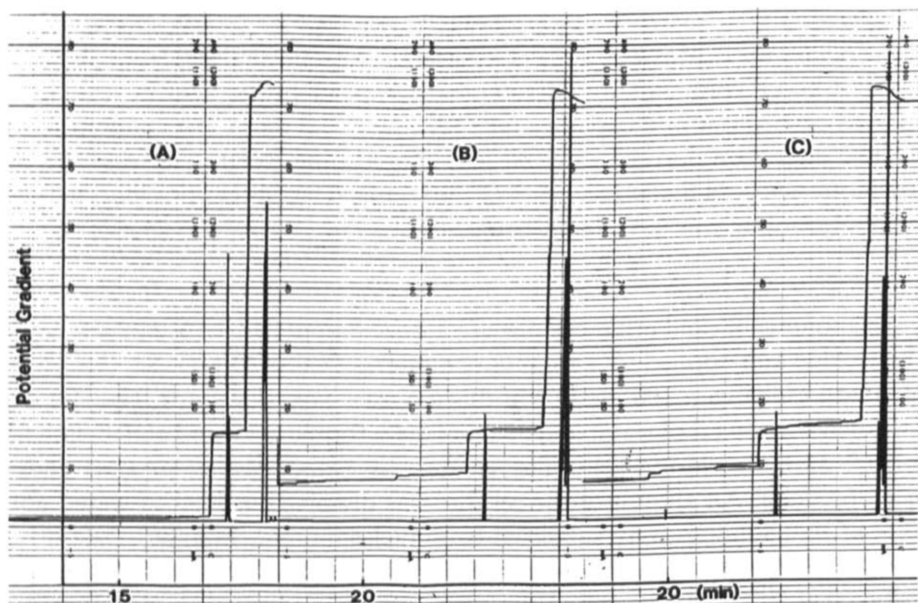


Fig. 1. Isotachopherogram of 5 µl of 5 m*M* orotic acid (A), 5 µl of normal human urine treated as described (B) and a mixture of A and B (C) The leading electrolyte was 0.01 *M* hydrochloric acid and β-alanine (pH 3 1). The terminal electrolyte was 0.01 *M* glutamic acid. The migration current was 100 µA The chart speed was 10 mm/min.

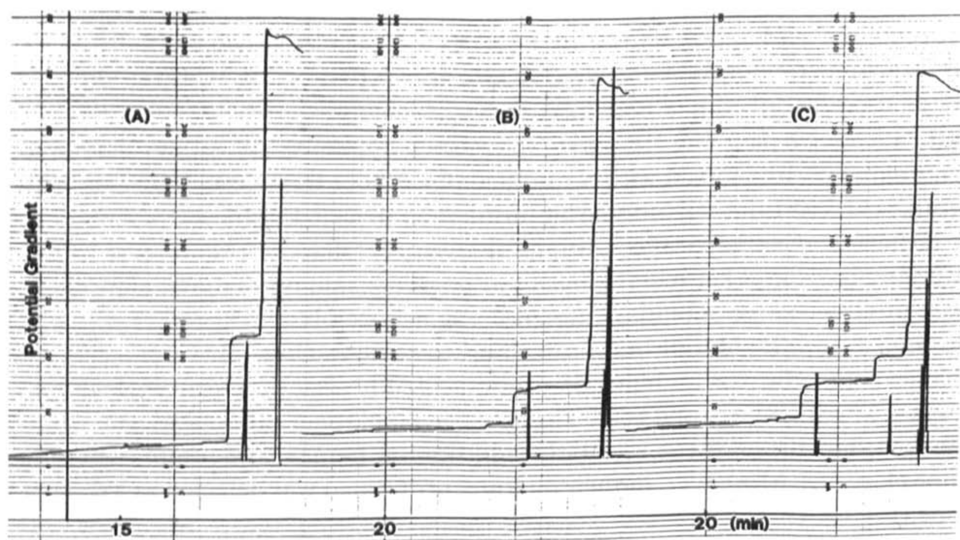


Fig. 2. Isotachopherograms of 5 µl of 5 m*M* orotic acid (A), 5 µl of normal human urine treated as described (B) and a mixture of A and B (C) The leading electrolyte was 0 01 *M* hydrochloric acid and β-alanine (pH 3.1) containing 0.001 *M* cupric chloride The terminal electrolyte was 0 01 *M* glutamic acid The migration current was 100 µA. The chart speed was 10 mm/min.

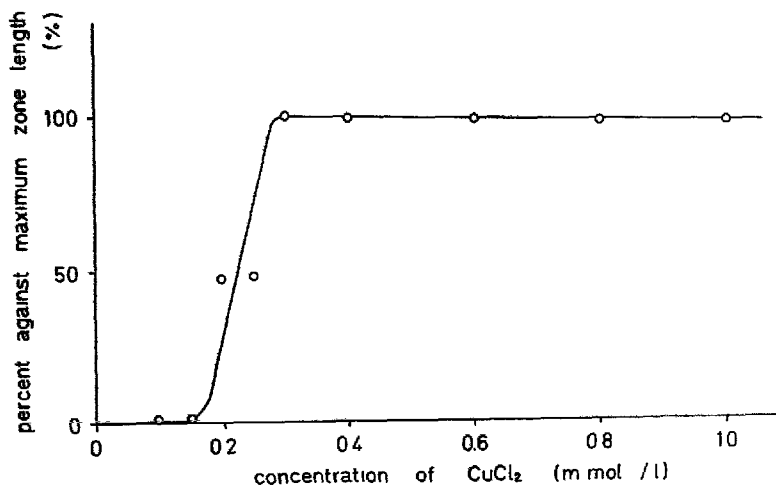


Fig. 3. Influence of cupric chloride on the zone length of orotic acid.

tions of authentic orotic acid under the conditions described, showed a good linear relationship from 0 to 50 nmol.

Samples of normal rat urine and the urine of rats with orotic aciduria were subjected to isotachophoresis (Fig. 4). No zone corresponding to orotic acid was detected in the former (Fig. 4A), but a large zone was detected in the latter (Fig. 4B). It was necessary to identify this large zone as orotic acid by adding authentic

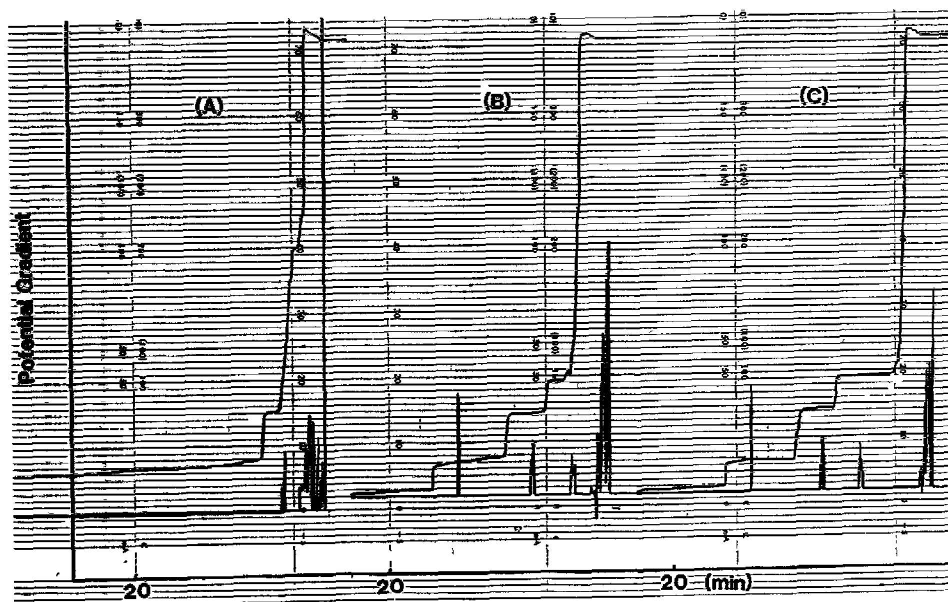


Fig. 4. Isotachopherograms of 5 $\mu$l of normal rat urine (A), 5 $\mu$l of urine from a rat fed with an arginine-deficient diet (B), both treated as described, and a mixture of B and 5 $\mu$l of 5 m$M$ orotic acid (C). Analytical conditions as in Fig. 2.

TABLE I

DETERMINATION OF OROTIC ACID IN NORMAL HUMAN URINE AND RAT URINE ($n = 5$)

| Sample | Orotic acid (μmol/ml) |
|---|---|
| Normal human urine | N.D.[a] |
| Normal human urine + orotic acid (1.0 μmol) | 1 00 ± 0 01 |
| Normal human urine + orotic acid (2.0 μmol) | 2 05 ± 0 03 |
| Normal human urine + orotic acid (2.5 μmol) | 2 52 ± 0.04 |
| Normal human urine + orotic acid (4.0 μmol) | 3.99 ± 0 01 |
| Normal human urine + orotic acid (5.0 μmol) | 5 10 ± 0 06 |
| Normal rat urine | N D[a] |
| Urine of arginine-deficient rat | 0 75 ± 0 05 |

[a] Not detectable.

orotic acid. Authentic orotic acid was added to the urine of rats with orotic aciduria, and the mixture was subjected to isotachophoresis. The zones were coincident (Fig. 4C).

Table I shows the determination of orotic acid in urine. Orotic acid was not detected in normal human or rat urine, although it was detected in the urine from rats fed an arginine-deficient diet. Table I also shows the recovery of various amounts of orotic acid added into the human urine. The recovery of orotic acid after treatment of Diaion SA was 99–104%.

Orotic acid is an intermediate metabolite in the biosyntehsis of pyrimidine nucleosides, and the pyrimidine pathway is related to the urea cycle. Increased excretion of orotic acid has been observed in the disorders of both the pyrimidine pathway and the urea cycle [1–12].

Orotic acid has been successfully separated and detected by isotachophoresis. In the previous methods, UV alkalinized buffer was used as the leading electrolyte and UV detection was found acceptable [17]. This new method can detect orotic acid in urine and serum by using an isotachophoretic analyser without UV detection. The pH selected for leading electrolyte was 3.1 in order to suppress the dissociation of organic acids, and we obtained a good separation of orotic acid by adding cupric chloride to the leading electrolyte. This isotachophoretic assay is a simple and useful method for screening disorders of the pyrimidine pathway and the urea cycle.

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