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Note

Quantitative analysis of cystathionine and perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the urine of a patient with cystathioninuria using isotachophoresis

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We have reported in previous papers [1—3] that the following unusual sulphur-containing amino acids have been excreted in the urine of a cystathioninuric patient: S-(3-hydroxy-3-carboxy-*n*-propyl)cysteine, S-(β-carboxymethyl)homocysteine, S-(2-hydroxy-2-carboxyethyl)homocysteine, perhydro-1,4-thiazepine-3,5-dicarboxylic acid, cystathionine sulphoxide and N-acetylcystathionine. After that, it was reported that cystathionine was oxidized with snake venom L-amino acid oxidase [4]. But the biosynthesis and physiological roles of these compounds in vivo are as yet not entirely understood.

All sulphur-containing amino acids described above, except for perhydro-1,4-thiazepine-3,5-dicarboxylic acid, could be easily determined with an amino acid analyser, but the extinction coefficient of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the ninhydrin reaction, because it only has an imino group in its structure, was too low for this compound to be determined by an amino acid analyser.

Therefore, a new method for determining urinary perhydro-1,4-thiazepine-3,5-dicarboxylic acid was devised using isotachophoresis [5—10]. The determination of cystathionine in rat tissue using isotachophoresis has been reported in a previous paper [11]. The determination of cystathionine in the urine of a cystathioninuric patient has also been achieved using this method.

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MATERIALS AND METHODS

Cystathionine was obtained from Sigma. Authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid was synthesized as reported in previous papers [4, 12]. All other chemicals used were analytical grade. The urine samples from normal humans were obtained from laboratory personnel. The sample from a patient with cystathioninuria was obtained from an elder sister reported in a previous paper [1].

A 2-ml volume of each urine sample was applied to a column containing 5 ml of Diaion SK-1 (H^+ cation exchanger, mesh 100; Mitsubishi Kasei, Tokyo, Japan), washed with 50 ml of water and eluted with 30 ml of 2 *M* ammonium hydroxide. The eluate was evaporated to dryness under reduced pressure. An aliquot of the residue was hydrolysed in 6 *M* hydrochloric acid, and the hydrolysate was evaporated to dryness under reduced pressure. The two residues of non-hydrolysate and hydrolysate were analysed by isotachophoresis for the determination of cystathionine.

The determination of cystathionine using an amino acid analyser was carried out by directly analysing the urine of a cystathioninuric patient. The effluent plus 50 ml of water from the Diaion SK-1 (H^+) column described for the analysis of cystathionine was adjusted to pH 9.0 with 2 *M* ammonium hydroxide and applied to a column containing 5 ml of Diaion SA (OH^- anion exchanger, mesh 100; Mitsubishi Kasei) washed with 30 ml of 2 *M* acetic acid and eluted with 30 ml of 0.5 *M* hydrochloric acid. The eluate was evaporated to dryness under reduced pressure. The identification of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the residue was carried out by comparing its chromatographic behaviour with that of an authentic sample. An aliquot of the residue was analysed by an isotachophoretic analyser.

Instrumentation

Assay conditions for perhydro-1,4-thiazepine-3,5-dicarboxylic acid. The capillary apparatus used was a Shimadzu IP-1B isotachophoretic analyser (Shimadzu Seisakusho, Kyoto, Japan). The separations were carried out in a capillary tube, 20 cm × 0.5 mm I.D., maintained at 20°C. The migration current was 100 μA. The detector cell was 0.05 cm × 0.5 mm I.D., the chart speed was 10 mm/min. The leading electrolyte consisted of 0.01 *M* hydrochloric acid and β-alanine (pH 3.1). The terminating electrolyte was 0.01 *M* glutamic acid.

Assay conditions for cystathionine. The leading electrolyte consisted of 0.01 *M* hydrochloric acid and 2-amino-2-methyl-1,3-propanediol (amediol) containing 5% polyvinyl alcohol (pH 8.9). The terminal electrolyte was 0.01 *M* γ-aminobutyric acid and barium hydroxide (pH 10.9). The other conditions were the same as for the analysis of perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

RESULTS AND DISCUSSION

Determination of cystathionine

The described procedure was used to determine cystathionine in the urine of a cystathioninuric patient. Authentic cystathionine gave a sharp zone under the

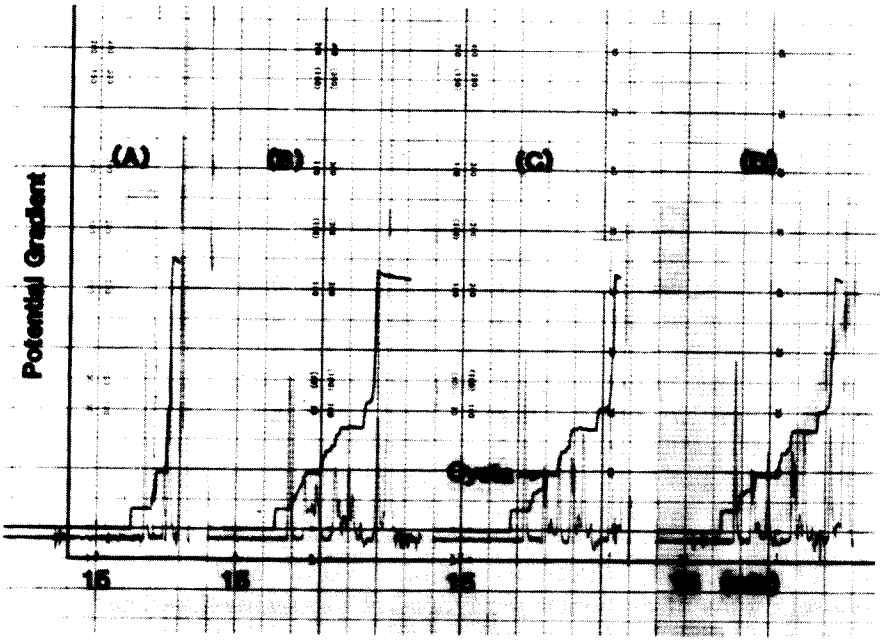

Fig. 1. Isotachophoretic runs of (A) authentic cystathionine, (B) non-hydrolysed urine and (C) hydrolysed urine of a cystathioninuric patient, and (D) hydrolysed urine as in C plus authentic cystathionine. Analytical conditions are described under Materials and methods.

described analytical conditions (Fig. 1A). A zone that had the same potential gradient as authentic cystathionine was not detected in normal human urine, but it was detected in the urine sample of a cystathioninuric patient (Fig. 1B). The sample of Fig. 1B was hydrolysed in 6 *M* hydrochloric acid for 24 h. An aliquot of the hydrolysate (Fig. 1C) was analysed by the isotachophoretic analyser just at the point where the zone of cystathionine in the hydrolysate was shorter than the zone of cystathionine in the non-hydrolysate as shown in Fig. 1C. The zone of cystathionine in Fig. 1C and that of authentic cysta-

TABLE I

COMPARISON OF CYSTATHIONINE CONCENTRATION IN THE URINE OF A CYSTATHIONINURIC PATIENT AS DETERMINED BY ISOTACHOPHORETIC AND AMINO ACID ANALYSERS

Values are expressed in μmol/ml.

Sample	Isotachophoretic analyser		Amino acid analyser
	Non-hydrolysed	Hydrolysed	
1	4.26	2.84	2.89
2	4.33	2.84	2.91
3	4.25	2.41	2.86
4	4.31	2.84	2.93
5	4.26	2.83	2.86
Mean ± S.E.	4.28 ± 0.032	2.75 ± 0.17	2.89 ± 0.028

thionine were made to overlap by adding authentic cystathionine to the urine sample; this resulted in an elongation of the cystathionine zone in the urine sample as shown in Fig. 1D. The recovery of authentic cystathionine during hydrolysis was 93—96% (n = 5).

The results for the determination of cystathionine in the urine of the cystathioninuric patient using an isotachophoretic analyser and an amino acid analyser are compared in Table I. The values of cystathionine in non-hydrolysed urine determined using isotachophoresis were higher than the values obtained using the amino acid analyser. On the other hand, the values of cystathionine in the hydrolysed urine agreed well with the values obtained with the amino acid analyser.

These results indicate that the zone with the same potential gradient as authentic cystathionine in non-hydrolysed urine samples comprises several zones, but the cystathionine zone in the hydrolysed urine is a single zone. Therefore, the method presented here is also applicable for the determination of cystathionine in urine as reported previously [11].

Determination of perhydro-1,4-thiazepine-3,5-dicarboxylic acid

Isotachophoretic runs of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid, normal human urine, and perhydro-1,4-thiazepine-3,5-dicarboxylic acid added to normal urine are shown in Fig. 2A—C, respectively. The results indicate that perhydro-1,4-thiazepine-3,5-dicarboxylic acid is absent in normal human urine (Fig. 2B and Table II).

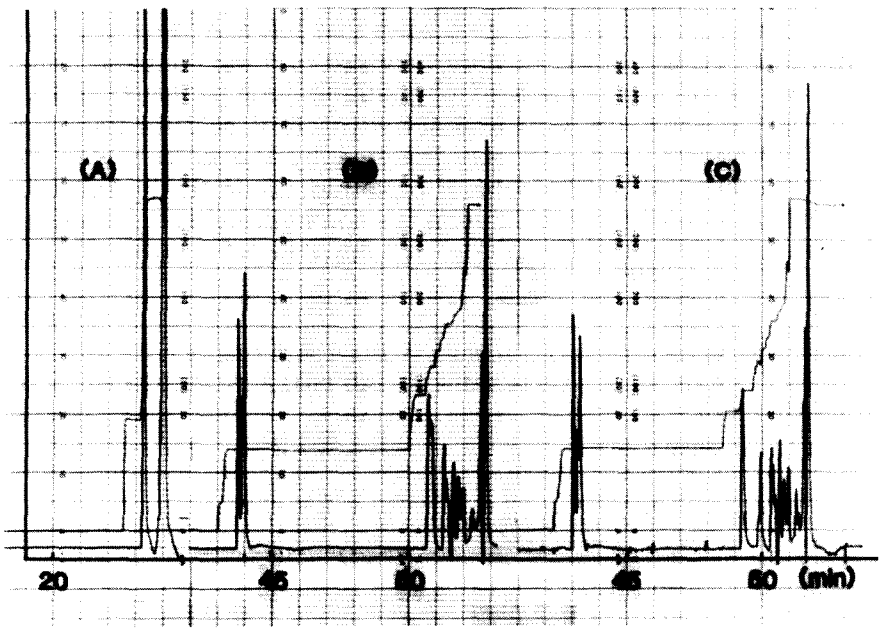

Fig. 2. Isotachophoretic runs of (A) authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid, (B) normal urine, and (C) normal urine plus authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

TABLE II

DETERMINATION OF PERHYDRO-1,4-THIAZEPINE-3,5-DICARBOXYLIC ACID IN THE URINE OF A CYSTATHIONINURIC PATIENT AND IN NORMAL HUMAN URINE

Values are expressed in μmol/ml.

Sample	Cystathioninuric patient	Normal human urine
1	0.97	N.D.*
2	0.91	N.D.
3	0.94	N.D.
4	0.93	N.D.
5	0.95	N.D.
Mean ± S.E.	0.94 ± 0.02	

*N.D., not detectable.

Isotachophoretic runs of urine samples of a cystathioninuric patient are shown in Fig. 3. The zone that had the same potential gradient as the zone of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid was made to overlap by adding authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid to the urine sample, resulting in an elongation of the zone of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the urine sample, as shown in Fig. 3.

The urine sample described above contained a compound with the same R_F value as authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid on paper chromatography using *n*-butanol—acetic acid—water as mobile phase. The compound moved with the same mobility as synthetic perhydro-1,4-thiazepine-3,5-dicarboxylic acid on high-voltage paper electrophoresis, and gave a positive

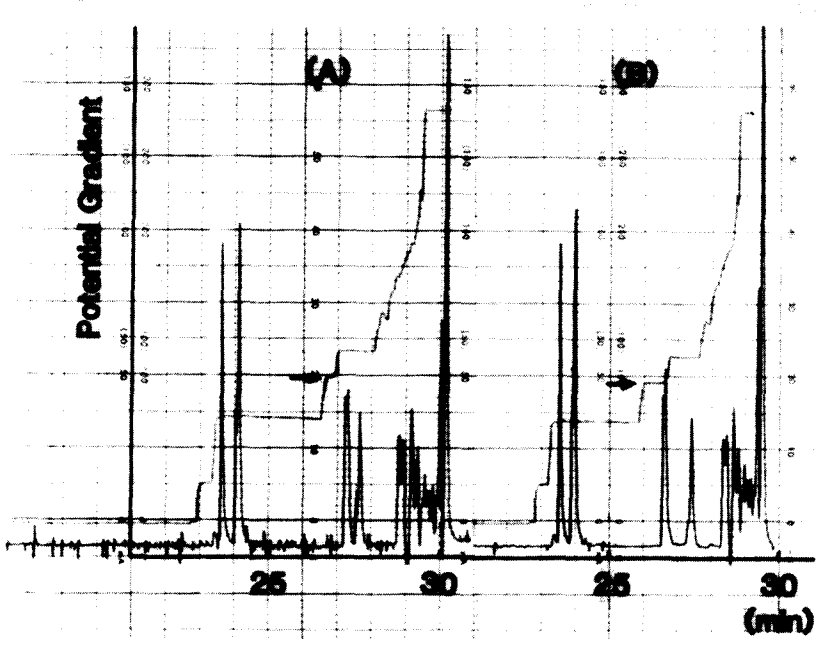

Fig. 3. Isotachophoretic runs of (A) urine of a cystathioninuric patient, and (B) the same urine plus authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

sulphur test [3]. These results indicate that the zone has the same potential gradient as the zone of authentic sample shown in Fig. 3A is perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

The slope of a standard curve drawn by plotting zone length against concentration of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid using isotachophoresis was linear from 0 to 50 nmol. It was possible to detect 1 nmol of perhydro-1,4-thiazepine-3,5-dicarboxylic acid using isotachophoresis.

The recovery of perhydro-1,4-thiazepine-3,5-dicarboxylic acid added to normal urine using the column chromatographic procedure described under Materials and methods was 92.3—103.1% (n = 5) and 92.6—95.3% (n = 5) in the urine of a cystathioninuric patient. Analyses of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid and aspartic acid were also carried out simultaneously by using the amino acid analyser. In this case, 0.5 nmol of aspartic acid could easily be detected, but not even 200 nmol of perhydro-1,4-thiazepine-3,5-dicarboxylic acid could be detected because of the lower colour value of the respective ninhydrin solution. When 500 nmol of this amino acid were analysed, it finally appeared as a small peak on the chart.

However, when the urine sample was analysed with the amino acid analyser, several compounds overlapped with perhydro-1,4-thiazepine-3,5-dicarboxylic acid and interfered with its analysis. It was thus impossible to determine this compound after the same column treatment as used in isotachophoretic analysis when using the amino acid analyser.

The isotachophoretic assay presented here was more simple and sensitive than that using the amino acid analyser. The results indicate that the method should be very useful, and can be adequately utilized for the quantitative estimation of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the urine of cystathioninuric patients.

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