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Note

Isotachophoretic analysis for the determination of urinary γ-carboxyglutamic acid

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γ-Carboxyglutamic acid (Gla) has been found in the vitamin K-dependent blood coagulation factors [1], in other serum proteins [2,3] in osteocalcin [4], a vitamin K-dependent protein synthesized in bone and certain pathologically calcified tissues such as renal stone [5] and atherosclerotic plaque [6]. Both free Gla and bound Gla are found in human urine [7], and its excretion has been related to the degradative turnover of Gla-containing proteins [8]. In fact, increased levels of urinary Gla have been found in osteoporosis [9] and a variety of subcutaneous calcification disorders [10].

These results indicate that the accurate determination of Gla is important in the investigation of Gla-containing proteins and in attempts to clarify the function of vitamin K. The method elaborated by Fernlund [7] consists of the concentration of Gla by an ion-exchange resin using an automated amino acid analyser. Gundberg et al. [11] described an isotope dilution procedure that employs selective elution of Gla from an anion-exchange column and fluorescent detection with *o*-phthalaldehyde. More recently, Gla in the presence of acetaldehyde was converted into a proline derivative, determined by colorimetric procedure [12] using a secondary amine in an alkaline medium. A high-performance liquid chromatographic method [13] and a radioimmunoassay [14] were developed for the determination of Gla.

The isotachophoretic method [15–17] presented here is the first description of the use of isotachophoresis in the quantitative determination of Gla in human urine.

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EXPERIMENTAL

Standard Gla was obtained from Calbiochem-Behring. All other chemicals used were of analytical-reagent grade. The samples from normal subjects were obtained from laboratory personnel.

Sample preparation

Aliquots (2 ml) of each urine sample were applied to a column containing 5 ml of Diaion SK-1 (H^+ cation exchanger, 100 mesh; Mitsubishi Kasei, Tokyo, Japan) and washed with 50 ml of water. The washing was dried under reduced pressure. The residue was dissolved with 2 ml of water, applied to a column containing 5 ml of Diaion SA-100 ($HCOO^-$ form) washed with 5 ml each of water and 10% formic acid, and then eluted with 15 ml of 10% formic acid. The eluate of 10% formic acid was evaporated to dryness under reduced pressure. The residue was dissolved 1 ml of pure water, and an aliquot of the solution was analysed by using an amino acid analyser and an isotachophoretic analyser.

A 0.5-ml volume of the solution containing Gla was adjusted to 3 *M* hydrochloric acid by the addition of concentrated hydrochloric acid, and then hydrolysed in boiling water for 60 min. The solution was evaporated to dryness under reduced pressure, dissolved in 0.5 ml of pure water and then analysed using an isotachophoretic analyser.

Apparatus

The capillary apparatus used was a Shimadzu IP-3A isotachophoretic analyser (Shimadzu Seisakusho, Kyoto, Japan). The separations were carried out in a capillary tube (20 cm×0.2 mm I.D.) maintained at a constant temperature of 20°C. The final migration current was 10 μA. The detector cell was 0.05 mm×0.2 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* caproic acid.

RESULTS AND DISCUSSION

The aim of this experiment was to devise a new method for the detection of urinary Gla by isotachophoresis. Isotachophoretic analyses of standard Gla, a mixture of Gla and glutamic acid (Glu), and the hydrolysates of standard Gla are shown in Fig. 1. Fig. 1B shows that standard Gla and Glu were well separated under the experimental conditions used. Standard Gla in 2 *M* hydrochloric acid analysed after heating at 60°C for 5 min, but Gla was not hydrolysed (Fig. 1C). However, standard Gla was completely hydrolysed to Glu when it was heated at 100°C for 30 min in 6 *M* hydrochloric acid (Fig. 1D). These results indicate that Gla is converted quantitatively into Glu by treatment with strong acid.

The slope of the calibration curve drawn by plotting the step length versus the concentration of Gla was linear over the concentration range 0–5 nmol.

The fraction containing Gla fractionated by the procedure described in Experimental was analysed by isotachophoresis. A zone that had the same potential

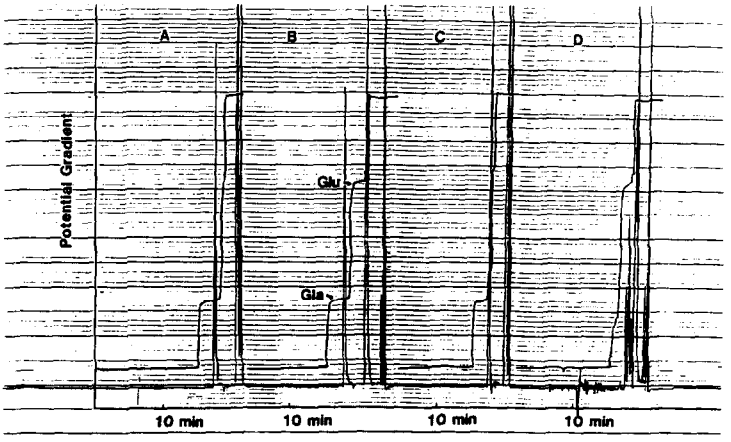

Fig. 1. Isotachophoresis of (A) standard Gla, (B) a mixture of Gla and Glu, (C) the hydrolysate of Gla (5 min at 60°C in 2 *M* hydrochloric acid) and (D) the hydrolysate of Gla (30 min at 100°C in 6 *M* hydrochloric acid). The leading electrolyte was 0.01 *M* hydrochloric acid and *β*-alanine (pH 3.1). The terminating electrolyte was 0.01 *M* sodium caproic acid.

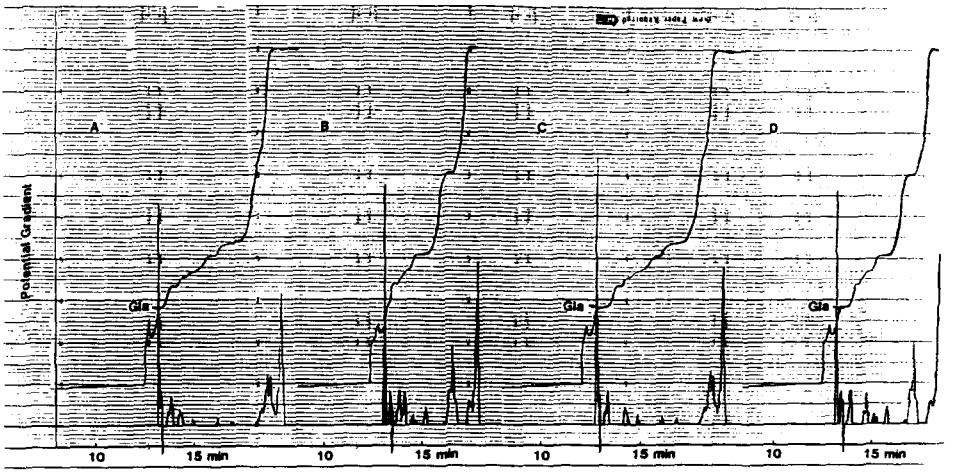

Fig. 2. Isotachophoresis of (A) the fraction containing Gla from human urine, (B) the hydrolysate of A, (C) A plus Gla, and (D) B plus Gla.

gradient as standard Gla was detected in the urine of a normal human (Fig. 2). The zone of Gla in Fig. 2A and that of standard Gla were made to overlap by addition of standard Gla to the zone of Gla in the urine sample, resulting in an elongation of the zone Gla in the urine sample (Fig. 2C).

The urine sample containing Gla was hydrolysed, and an aliquot of the sample was analysed by isotachophoresis (Fig. 2B). As the result, the zone of Gla in the hydrolysate was shorter than the zone of Gla in the non-hydrolysate, and the zone of Glu was detected as the new zone but the zone that had a potential gradient the same as standard Gla did not completely disappear.

These results indicate that the zone containing Gla in the urine sample is the mixture zone containing Gla and any other compounds. Therefore, the determi-

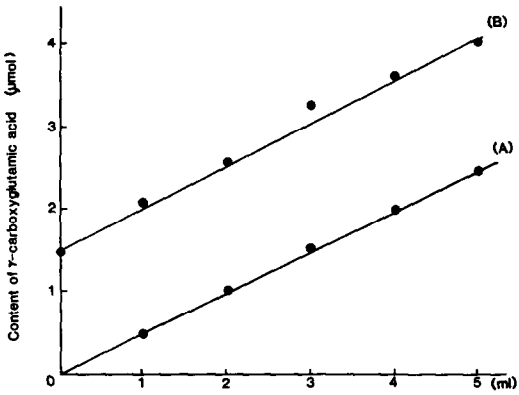

Fig. 3. Isotachophoretic detection of standard Gla in the presence of different amounts of concentrated urine. (A) The fraction containing Gla from human urine; (B) A plus Gla.

nation of Gla in the urine sample was carried out by subtracting the zone that had a potential gradient the same as Gla in the hydrolysate from the zone of Gla in non-hydrolysate.

In order to check the recovery of Gla in the Diaion SA-100 concentration step, an internal standard of authentic Gla (1.5 μmol) was added to the different amounts of the normal human urine before chromatography on Diaion SA-100. The same urine without any addition of Gla was processed in parallel. The results indicate that the slopes of the calibration graphs of both were linear, and that the recovery of the added Gla ranged from 95 to 99%. (Fig. 3).

The results for the determination of Gla in normal human urine using isotachophoresis and amino acid analysis are compared in Table I. It can be seen that the two methods correlate well, and the values for Gla in the normal human urine (Table II) are in the normal range reported by Fernlund [7] and Pecci and Cavallini [12].

The isotachophoretic method presented here is simpler than that using amino acid analysis and more sensitive than that using colorimetry. The results indicate that the method should be very useful, and is suitable for the quantitative estimation of Gla in urine.

TABLE I

COMPARISON OF THE VALUES OF URINARY Gla DETERMINED BY ISOTACHOPHORETIC AND AUTOMATED AMINO ACID ANALYSER

Subject	Gla content (nmol/ml)	
	Isotachophoretic analyser	Amino acid analyser
1	232	215
2	363	355
3	375	366
4	156	140
5	197	190

TABLE II

DETERMINATION OF Gla IN NORMAL HUMAN URINE

Subject	Concentration	
	nmol/ml	nmol/mg of creatinine
Male		
1	385	134.7
2	215	196.7
3	355	171.2
4	366	127.6
5	140	168.7
Female		
1	110	86.8
2	188	198.3
3	305	169.7
4	140	218.7
5	232	25.6

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