Journal of Chromatography, 223 (1981) 182—187
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 799

Note

Determination of γ-carboxyglutamic acid excretion in urine

RAMADASAN KUTTAN*, NANCY WILSON and RUGGERO TENNI*

Laboratories of Connective Tissue Research, Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030 (U.S.A.)

(Received October 10th, 1980)

The presence of γ-carboxyglutamic acid (GLA) in several calcium-binding proteins has been documented by many investigators [1, 2] and free GLA has been demonstrated to be excreted in urine [3] in quantities easily measurable with an amino acid analyzer.

Since the urinary GLA is a product of degradation of GLA-containing proteins, its measurement may be relevant to the study of metabolic diseases affecting bone metabolism and blood clotting mechanisms.

This paper describes a simple method suitable for the routine measurement of GLA in several urine specimens. The method affords a recovery of added GLA greater than 95% and has been used to follow the daily excretion of GLA in a quadriplegic patient.

EXPERIMENTAL

Materials

Pure GLA (monoammonium salt) was purchased from Calbiochem (San Diego, CA, U.S.A.); Dowex 50-X2 (H^+, 200—400 mesh) was purchased from BioRad Labs. (Richmond, CA, U.S.A.). The resin was washed with five bed volumes of 6 *N* hydrochloric acid and then washed with water to neutrality before use. Anion-exchange resin AG 1-X4 (Cl^-) was also purchased from BioRad Labs. It was converted to the OH^- form by stirring it with 1 *N* sodium hydroxide solution, and after removal of excess alkali was converted to the $HCOO^-$ form using 1 *M* formic acid.

*Present address: Istituto di Chimica Biologica, Universita di Pavia, Pavia, Italy.

0378-4347/81/0000—0000/$02.50

Sample preparation

Urine was collected daily from a 17-year old male, quadriplegic because of traumatic injury in the cervical portion of the spinal cord. Urine collections (24-h) were started 17 days after the accident and continued for nearly 70 days. Urine samples collected in periods of 5—7 days were pooled and the total volume of each pool was recorded. These urine samples were immediately frozen until analyzed. Urine samples (24-h) were also collected from normal children of both sexes (average age 15 years) and immediately frozen until analyzed.

GLA determination was done on aliquots of these urine samples after passing through either an anion-exchange resin or a cation-exchange resin, as described below, followed by analysis of the effluent with the amino acid analyzer.

Quantitation of GLA using an anion-exchange resin

The method of Fernlund [3] was suitably modified to estimate GLA in smaller volumes of urine. Urine samples were centrifuged at 3000 *g* for 15 min in a refrigerated centrifuge and 10 ml of the clear supernatant were applied on an AG 1-X4 (200—400 mesh) $HCOO^-$ column (10.5 × 0.6 cm). The column was then washed with 10 ml water. GLA was eluted with 88% formic acid—water (1:10). The first 2.5 ml of formic acid eluate were discarded and the next 8 ml were collected. The eluate was dried in a desiccator containing sodium hydroxide pellets. The residue was dissolved in 1 ml of the diluting buffer (0.2 *M* sodium citrate buffer, pH 2.2). After centrifugation at 3000 *g*, 15 min, 0.3 ml of the supernatant was mixed with 60 μl of 4 *M* sodium chloride solution and 0.24 ml of diluting buffer. A 0.5-ml aliquot of this sample (which corresponds to 2.5 ml of urine) was injected into an automatic amino acid analyzer.

Quantitation of GLA using a cation-exchange resin

An aliquot of the urine sample was acidified with 6 *N* hydrochlorid acid to pH 3.0. The urine was centrifuged at 3000 *g* for 15 min in a refrigerated centrifuge and 5 ml of the urine were applied on a 3.5 × 0.6 cm column of Dowex 50-X2 (H^+, 200—400 mesh). The eluate was collected; the column was washed with additional 5 ml of water which were added to the first eluate. The combined eluates were made alkaline (pH 10.0) by adding a few drops of 2.5 *N* sodium hydroxide (pH paper, ColorpHast). This solution was dried in a desiccator containing sodium hydroxide pellets and concentrated sulfuric acid. In order to remove completely the ammonia which interferes in the GLA determination, 1 ml of water was added twice to the residue and this was dried again. Finally 1 ml of 1 *M* acetic acid was added to neutralize the alkali and dried again. The residue was dissolved in 1 ml of diluting buffer (0.2 *M* sodium citrate buffer, pH 2.2). After centrifugation at 3000 *g* for 15 min, 0.3 ml of the supernatant was mixed with 60 μl of 4 *M* sodium chloride solution and 0.24 ml diluting buffer. A 0.5-ml aliquot of this sample (which represents 1.25 ml of urine) was injected into an automatic amino acid analyzer. Completion of the GLA elution from the cation-exchange column was determined by eluting the column with 5 ml of 1 *N* ammonium hydroxide, which elutes all retained amino acids. After complete removal of ammonia, this eluate was analyzed for GLA with the amino acid analyzer.

Amino acid analysis

An automatic amino acid analyzer (Beckman model 121) with automatic digital integrator (Infotronics) was used to analyze GLA in these urine samples. A satisfactory program is as follows. Beckman PA-28 resin (column 56 × 0.9 cm, temperature 51°C) was first regenerated with 0.2 *M* sodium hydroxide (1—40 min), and then equilibrated with 0.2 *M* sodium citrate buffer, pH 2.2 (40—80 min). Buffer flow-rate was maintained at 50 ml/h. The sample was injected between 80 and 86 min. The column was eluted with the same buffer for 110 min (86—196 min), after which 0.2 *M* sodium citrate buffer, pH 3.20 was started. Ninhydrin started at 159 min, flow-rate 25 ml/h. The GLA elutes at 190—200 min, i.e., 109 min after injection.

Color factor determination

The color yield of GLA is low compared to other amino acids [4] and hence an authentic GLA has been used as standard. A solution of GLA (5 μmole/ml) was made in diluting buffer. A 10—30 μl aliquot of this solution was mixed with 60 μl of 4 *M* sodium chloride solution and 510—530 μl of diluting buffer. A 500-μl aliquot of this solution (41.6—125 nmole GLA) was injected into the automatic amino acid analyzer. The color factor was calculated by dividing the amount of the GLA applied to the column by the area corresponding to the peak of GLA.

Recovery of GLA

GLA (0.25—0.5 μmole) was added to 5 ml of the urine sample and the urine was processed with the cation-exchange resin method. The same urine without any addition of GLA was processed in parallel. The amount of GLA was calculated in both cases using the color factor for pure GLA and the percentage recovery was calculated.

Other methods

Creatinine was determined by the Jaffe reaction [5]. Acid hydrolysis of GLA was done by heating the samples of urinary GLA containing the fraction with 6 *N* hydrochloric acid, at 110°C for 24 h.

RESULTS

Fig. 1 shows the elution profile of urinary GLA on the amino acid analyzer after prior purification by cation-exchange resin. Fig. 1A shows the analysis of the fraction not retained on Dowex 50-X2 H^+ column and Fig. 1B shows the analysis of the subsequent fraction eluted with 1 *N* ammonia. Clearly, all the GLA present in the urine passes through the Dowex 50-X2 H^+ column and none is retained. Acid hydrolysis of GLA obtained from Dowex 50-X2 completely destroyed it (Fig. 1C) due to its conversion to glutamic acid (not shown) [2].

The position of standard GLA in the amino acid analyzer effluent is shown in Fig. 2A. GLA from urine prepared by the cation-exchange resin appears in identical position (Fig. 2B). Mixing authentic GLA with the urinary GLA prepared by cation-exchange resin produces a single, augmented peak (Fig. 2C).

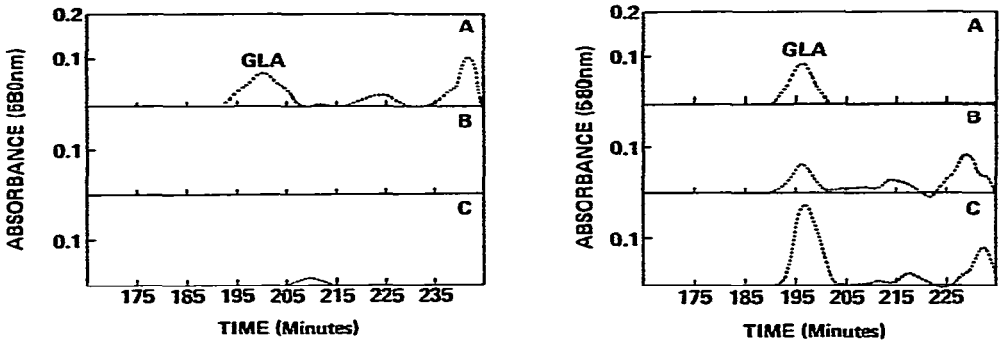

Fig. 1. GLA analysis in the urine after initial passage through a cation-exchange resin. (A) Pass through fraction; (B) ammonia eluate; (C) pass through fraction after acid hydrolysis.

Fig. 2. Elution pattern in the amino acid analyzer of (A) authentic GLA; (B) urinary GLA prepared by cation-exchange column; (C) urinary GLA mixed with authentic GLA.

TABLE I

RECOVERY OF γ-CARBOXYGLUTAMATE FROM URINE

	GLA (nmole)	
	Experiment 1	Experiment 2
Amount of GLA in 5 ml urine	222	222
Amount of externally added GLA to urine	250	500
Total GLA recovered	452	718
Recovery (%)	95.6	99.4

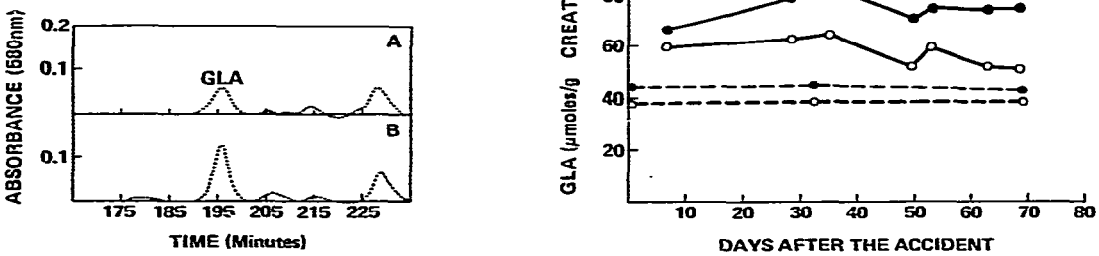

Fig. 3. Comparison of amino acid analyzer pattern of urinary GLA containing fraction prepared using (A) cation-exchange resin, and (B) anion-exchange resin.

Fig. 4. Urinary GLA in quadriplegic patient as measured by cationic procedure (●——●) and anionic procedure (○——○) . The dotted line shows the mean of the values for age-matched controls measured by cationic procedure (●- - - - -●) and by anionic procedure (○- - - - -○). The statistics of the values are as follows. Cationic method: average for paraplegic patient 69.2 ± 8.05 μmole/g creatinine, and controls 44.4 ± 17.8 μmole/g creatinine; $p < 0.01$. Anionic method: average paraplegic patient 50.7 ± 8.9 μmole/g creatinine, and controls 37.5 ± 8.9 μmole/g creatinine; $p < 0.015$.

Fig. 3 compares the amino acid analyzer profile of GLA prepared either by cation-exchange resin (Fig. 3A) or by anion-exchange resin (Fig. 3B). As seen in the figure both profiles are identical; the greater amount of GLA in the lower tracing reflects the larger size of GLA sample applied to the column (see Experimental).

Table I shows the percentage recovery of γ-carboxyglutamate from the urine by this procedure; 97.5% of the added γ-carboxyglutamate was recovered by this method.

Fig. 4 shows the pattern of excretion of GLA in one quadriplegic patient using the present method and the old one based on the use of the anion-exchange resin. The pattern of excretion is essentially the same when measured by the two methods. The mean daily excretion of GLA in this patient was found to be higher as compared to the age-matched controls when estimations were done by both procedures. However, it was found that the values are about 20—30% higher when measured by the new method.

DISCUSSION

We have described a rapid method for the analysis of GLA in urine samples. The method takes advantage of the highly negative charge of GLA; under conditions in which most other amino acids are held on the Dowex 50-X2 H^+ column, GLA is not retained and flows through the column. Analysis of this effluent fraction on an automatic amino acid analyzer provides a reliable quantitation of GLA in urine.

This method was found to be simpler than the previously described one, based on the use of an anion-exchange column [3]. It eliminates the preparation of AG 1-X4 ($HCOO^-$), elution with water and formic acid, and collection of fractions. With our simpler method, several urine samples may be processed at the same time.

The only precaution to be exercised is to remove the ammonia from the samples. Ammonia has been known to interfere in the analysis of GLA [4]. Complete removal of ammonia may be achieved by repeatedly drying the sample in alkaline conditions. Final addition of 1.0 *M* acetic acid and further drying facilitate the adjustment of the pH of the sample with diluting buffer. The other salts present in urine at normal concentration do not interfere in the analysis.

GLA prepared by either procedure eluted from the amino acid analyzer column at the same time as a pure GLA standard. With this method, recovery of GLA added to urine was found to be 95% or higher, while with the anion-exchange column the recovery of GLA ranges from 85—89% [3]. Moreover, the pattern of urinary excretion of GLA measured in one patient by both procedures was comparable, even though values were slightly higher with the proposed method (about 20—30%).

GLA is present mainly in proteins which have a role in calcium binding such as those found in blood clotting [6], bone proteins [2,7], and that involved in pathological calcifications [8]. Hence a disturbance of any of these systems could produce an increase in GLA excretion. It is also known that GLA is not degraded in the body to a great extent [9]. An increased GLA excretion in

conditions such as osteoporosis, scleroderma, dermatomyositis and in patients treated with warfarin has been reported [10]. In the present paper we have studied a patient with quadriplegia and have found that the GLA excretion in this patient is as high as that reported in osteoporosis [10]. These values probably reflect the increased turnover of connective tissue components of the bone during paralysis and complete bed rest.

ACKNOWLEDGEMENTS

Supported by a grant from the Easter Seal Research Foundation of the National Easter Seal Society for Crippled Children and Adults (R-7821), and NASA contract 9-15478. The authors are thankful to Dr. J. Claus-Walker of the Departments of Rehabilitation and Physiology, Baylor College of Medicine, for providing the opportunity of studying the GLA urinary excretion of the quadriplegic patient; and to Dr. Nicola Di Ferrante and Dr. Daniela Tavella for their valuable help.

REFERENCES

1 J.W. Suttie (Editor), Vitamin K Metabolism and Vitamin K Dependent Proteins, University Park Press, Baltimore, MD, 1979.
2 P.U. Hauschika, J.B. Lian and P.M. Gallop, Proc. Nat. Acad. Sci. U.S., 72 (1975) 3925.
3 P. Fernlund, Clin. Chim. Acta, 72 (1976) 147.
4 D.A. Madar, R.A. Willis, K.A. Koehler and R.G. Hiskey, Anal. Biochem., 92 (1979) 466.
5 M. Jaffe, Anal. Biochem., 21 (1967) 98.
6 J. Stenflo, P. Fernlund, W. Egan and P. Roepstorff, Proc. Nat. Acad. Sci. U.S., 71 (1974) 2730.
7 P.A. Price, A.S. Otsuka, J.W. Poser, J. Kristaponis and N. Raman, Proc. Nat. Acad. Sci. U.S., 73 (1976) 1447.
8 J.B. Lian, M. Skinner, M.J. Glimcher and P.M. Gallop, Biochem. Biophys. Res. Commun., 71 (1976) 349.
9 D.V. Shah, J.K. Tews, A.E. Harper and J.W. Suttie, Biochim. Biophys. Acta, 539 (1978) 209.
10 R.J. Levy, J.B. Lian, C. Gundberg and P.M. Gallop, in J.W. Suttie (Editor), Vitamin K Metabolism and Vitamin K Dependent Proteins, University Park Press, Baltimore, MD, 1979, pp. 375—379.