Journal of Chromatography, 308 (1984) 398—401
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2063

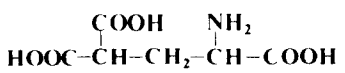
Letter to the Editor

An improved method for the determination of γ-carboxyglutamic acid in human urine by high-performance liquid chromatography

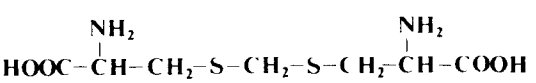
Sir,

γ-Carboxyglutamic acid (Gla, Fig. 1) has been found as a constituent of several vitamin K-dependent, calcium-binding proteins, and free Gla has been demonstrated to be excreted in urine [1]. Since urinary Gla is a final degradation product of Gla-containing proteins [2], its measurement supplies useful clinical information regarding bone metabolism and the blood coagulation systems [3]. Recently, we developed a rapid and sensitive high-performance liquid chromatographic (HPLC) method for the determination of Gla using pre-column derivatization with *o*-phthalaldehyde (OPA) and a silica-based anion-exchange column [4]. During the course of assay of urinary Gla by this method, the need for a suitable internal standard which reacts with OPA to form a fluorescent product but which is not present in biological samples became evident. It was also desirable to minimize the time-dependent decomposition of the OPA derivative of Gla in urine, so that the procedure could be made suitable for routine analysis using an autosampler. The purpose of this communication is to report an improved method which solves these problems.

The present method consists of an alkaline pretreatment of urine samples and an internal standardization method using djenkolic acid (Fig. 1), as described below. A 0.5-ml aliquot of human urine was mixed with an equal volume of 5 *M* potassium hydroxide and the mixture was hydrolyzed at 110°C for 24 h as described previously [5]. The hydrolysate was ice-cooled, spiked



γ-Carboxyglutamic Acid (Gla)



Djenkolic Acid

Fig. 1. Structures of γ-carboxyglutamic acid and djenkolic acid.

0378-4347/84/$03.00

with 0.1 ml of djenkolic acid (Tokyo Kasei Industries, Tokyo, Japan) in 0.01 *M* potassium hydroxide (0.1 mg/ml), and then neutralized by dropwise addition of 70% perchloric acid. After standing for 30 min on ice, the solution was centrifuged at 10,000 *g* for 5 min to remove a potassium perchlorate precipitate and the supernatant was mixed with about 30 mg of charcoal to decolourize it. The mixture was filtered through a 0.45-μm filter (Cat. No. E-251, Gelman Science Japan Ltd., Tokyo, Japan) and the filtrate was analyzed by HPLC according to the method described previously [4] using identical conditions, except that the solvent flow-rate was set at 1.5 ml/min instead of 2.0 ml/min.

In our previous work, urine was directly derivatized with OPA-reagent and then analyzed by HPLC [4]. As shown in Fig. 2, the fluorescence of OPA-Gla in urine was decreased by 32% at 3 h after the derivatization. In contrast, when the urine was hydrolyzed in alkali as described above and then derivatized with OPA, the fluorescence of OPA-Gla decreased by only 6% within 3 h. The same result was also obtained with Gla in bone hydrolyzate. These results suggest that the rapid decomposition of OPA-Gla in intact urine is probably caused by peptides or proteins in the urine. The alkaline hydrolysis releases Gla from protein-bound forms, and free Gla is very stable under the conditions used [6]. Thus, pretreatment of urine samples by alkaline hydrolysis was adopted in the standard procedure.

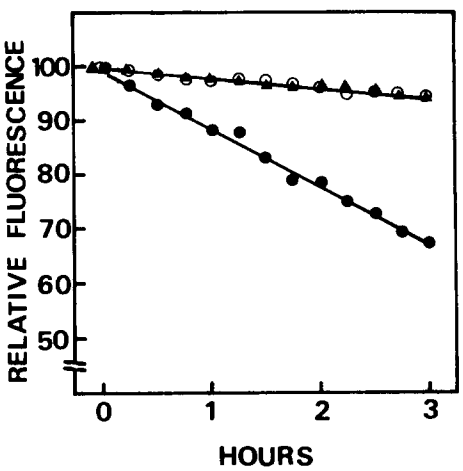


Fig. 2. Kinetics of the fluorescence decay of OPA-Gla in various samples at room temperature. (•), Human urine; (○), an alkaline hydrolyzate of the human urine; (▲), an alkaline hydrolyzate of rat bone. These samples were derivatized with OPA and analyzed by the HPLC method as described in the text.

Fig. 3 shows chromatograms of urine before and after the alkaline hydrolysis. Under the HPLC conditions used, Gla is eluted last of all the protein-constituent amino acids because it has the most acidic nature [4]. The alkaline hydrolysis resulted in the disappearance of peptide peaks and no peak was observed later than that of Gla in the alkaline hydrolyzate of urine. It is noteworthy that the hydrolysis improves the sharpness of the Gla peak. In this method, djenkolic acid was used as an internal standard for the analysis of

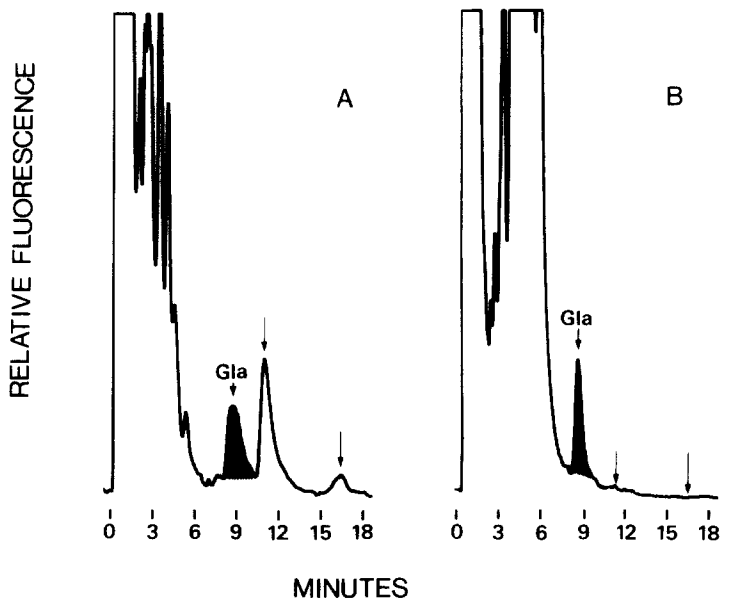


Fig. 3 Chromatograms of (A) human urine and (B) an alkaline hydrolyzate of the urine The arrows represent peptide peaks which disappeared after the alkaline hydrolysis.

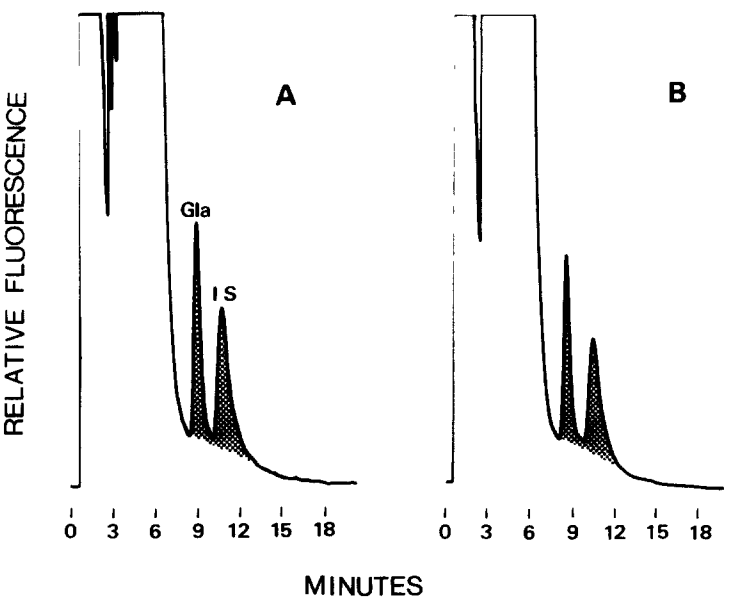


Fig. 4. Chromatograms of an alkaline hydrolyzate of human urine at (A) 5 min and (B) 13 h after fluorescence derivatization.

urinary Gla. This novel amino acid has been found so far only in djenkolic beans (*Pithecolobium lobatum*) and causes so-called djenkol poisoning when the beans are eaten [7]. As shown in Fig. 4, the internal standard was eluted 2 min later than Gla, and there is no interference of endogenous materials in the hydrolyzed urine with the internal standard peak, as described above. As shown in Fig. 5, the fluorescence of the OPA derivative of the internal standard

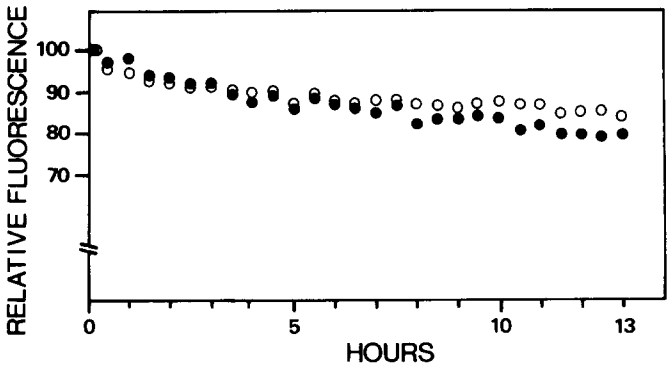


Fig. 5. Kinetics of the fluorescence decay of (•) derivatized Gla and (○) the internal standard in an alkaline hydrolyzate of human urine at room temperature. The urine sample was prepared as described in the text and analyzed by the standard HPLC method.

decayed slowly, at almost the same rate as that of Gla in a hydrolyzate of urine. During 13 h after the derivatization, the coefficient of variation of the peak area ratio of Gla/internal standard in the sample was found to be 4.5% (n = 27). These data indicate that this internal standard is suitable for the precise and reproducible analysis of urinary Gla using our HPLC method.

The present communication has demonstrated that a simple and specific procedure consisting of alkaline pretreatment and internal standardization facilitates the automated determination of urinary Gla by means of the HPLC method described previously [4]. This internal standardization method is also applicable in the analysis of Gla in protein samples and other biological tissues. We believe that the technique provides a useful tool in clinical and biochemical studies requiring the analysis of Gla.

ACKNOWLEDGEMENT

The authors thank Dr. M. Tomoeda of Eisai Company, for valuable assistance and discussions.

Department of Drug Metabolism and Pharmacokinetics, Tsukuba Research Laboratories, Eisai Company Ltd., Tokodai, Toyosato-machi, Tsukuba-gun, Ibaragi (Japan)

MANABU KUWADA*
KOUICHI KATAYAMA

1 J.W. Suttie (Editor), Vitamin K Metabolism and Vitamin K-Dependent Proteins, University Park Press, Baltimore, MD, 1979, p. 592.
2 D.V. Shah, J.K. Tews, A.E. Harper and J.W. Suttie, Biochim. Biophys. Acta, 539 (1978) 209.
3 J.B. Lian, L.M. Pachman, C.M. Gundberg and M.C. Maryjowski, Arthritis Rheum., 25 (1982) 1094.
4 M. Kuwada and K. Katayama, Anal. Biochem., 131 (1983) 173.
5 M. Kuwada and K. Katayama, Anal. Biochem., 117 (1981) 259.
6 P.V. Hauschka, Anal. Biochem., 80 (1977) 212.
7 A.G. van Veen and A.J. Hyman, Rec. Trav. Chim. Pays-Bas, 54 (1935) 493.

(First received September 7th, 1983; revised manuscript received January 6th, 1984)