Journal of Chromatography, 188 (1980) 383–390

CHROM. 12,410

ASSAY METHOD FOR UROKINASE ACTIVITY BY CAPILLARY-TUBE ISOTACHOPHORESIS USING A SYNTHETIC SUBSTRATE

KAZUO KATOH and HIROSHI MIYAZAKI*

Research Laboratories of Pharmaceutical Division, Nippon Kayaku Co., 3-31 Shimo, Kita-ku, Tokyo 115 (Japan)

(First received March 23rd, 1979; revised manuscript received September 20th, 1979)

SUMMARY

Urokinase activity was determined by capillary-tube isotachophoresis using N-α-acetyl-L-lysine methyl ester as a synthetic substrate. The resulting N-α-acetyl-L-lysine was separated and detected isotachophoretically using methanolic potassium hydroxide solution adjusted to pH 5.32 by adding α-ketoglutaric acid as a leading electrolyte and methanolic betaine hydrochloride solution as a terminating electrolyte.

The enzymatic reaction was stopped by addition of tannic acid and the resulting supernatant solution was injected into an isotachophoretic analyser. Linearity was observed at urokinase activities in the range 1–30 I.U. The urokinase activities in commercial products determined by the method were in good agreement with those determined by Walton's modified plate method.

The coefficient of variation of the method was less than 3.4%.

INTRODUCTION

The physiological role of urokinase is to convert plasminogen into plasmin, and the resulting plasmin lyses the clots of fibrin[1,2]. Conventional assay methods such as the test-tube method[3] and the fibrin plate method[4] are based on this mechanism. However, these two methods have the disadvantage that the analytical error may be increased owing to the subjective judgement of the end-point by the analyst.

On the other hand, spectrophotometric methods based on esterase activity of urokinase have been reported by various investigators. Of these synthetic substrates, N-α-acetyl-L-lysine methyl ester is the most specific substrate for the determination of urokinase activity, and the assay methods using this substrate were developed by Sherry *et al.*[5] and Hamberg and Savolainen[6]. However, these methods require tedious and time-consuming procedures for the determination of the reaction products.

Capillary-tube isotachophoresis[7,8] has been used as a rapid and specific method for the simultaneous qualitative and quantitative analyses of a mixture of peptides[9] and amino acids[10].

* To whom correspondence should be addressed.

This paper describes the isotachophoresis of N-α-acetyl-L-lysine, the separation of which has been thought to be difficult, and a rapid method for the determination of urokinase activity by isotachophoresis.

EXPERIMENTALS

Reagents

N-α-Acetyl-L-lysine methyl ester monohydrochloride and N-α-acetyl-L-lysine monohydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). Tannic acid and tris(hydroxymethyl)aminomethane (Tris) were purchased from E. Merck (Darmstadt, G.F.R.). Potassium hydroxide, α-ketoglutaric acid, 2-amino-2-methyl-1,3-propanediol (Ammediol) tetra-*n*-butyl-ammonium chloride and betaine hydrochloride were purchased from Tokyo Kasei Co. (Tokyo, Japan). Barium hydroxide was of extra-pure reagent grade and purchased from Wako (Osaka, Japan). Methanol was of reagent grade and purchased from Kanto Chemical Co. (Tokyo, Japan). Absolute ethanol was purchased from Amakasu Chemical Co. (Tokyo, Japan). Gelatin was obtained from Difco Labs. (Detroit, Mich., U.S.A.).

The substrate solution for enzymatic reactions was prepared by dissolving 5.5 mg of N-α-acetyl-L-lysine methyl ester monohydrochloride in 10 ml of 0.01 *M* phosphate buffer containing 0.01% of gelatin (buffer solution A).

Measurement of urokinase activity using plasminogen

The modified method of Walton[4] was used as a representative method with plasminogen as a substrate. The content of protein in urokinase solution was determined by the Folin method[12].

Incubation procedure for determination of urokinase activity

Urokinase preparations were dissolved in 6 ml of 0.06 *M* Tris–hydrochloric acid buffer (pH 7.2) containing 0.09 *M* sodium chloride and 0.1% of gelatin.

Urokinases with different specific activities were prepared as solutions containing 0.025 *M* phosphate buffer (pH 7.5) containing 1.5% of sodium chloride.

A 100-μl volume of the substrate solution and an aliquot of the sample solution containing urokinase activities in the range 1–10 I.U. were placed in a test-tube and the total volume of the mixture was made up to 130 μl with buffer solution A. The test-tube was shaken gently for 60 min at 37.5°. After 20 μl of a 0.2% solution of tannic acid in buffer solution A had been added to the reaction mixture, the solution was cooled for 5 min in an ice-bath to stop completely the reaction and the precipitate was removed by centrifugation. An aliquot of the resulting supernatant was injected into the isotachophoretic analyser.

Determination of minimum amount of tannic acid for cessation of the enzyme reaction

A 10-μl volume of urokinase in the buffer solution A (1000 I.U./ml), 100 μl of the substrate solution and 20 μl of buffer solution A were used as the incubation mixture for determination of urokinase activity. After the first enzymatic reaction for 30 min at 37.5°, 18 μl of the solutions with various concentrations of tannic acid were added to the reaction mixture and the solution was cooled for 5 min in an

ice-bath. The solution was incubated again for 30 min at 37.5° and finally 2 μl of 2.0% tannic acid solution in water were added to stop completely the reaction.

Isotachophoretic conditions for determination of urokinase activity

The isotachophoresis was carried out with a Shimadzu isotachophoretic analyser, Model IP-1B and PGD-1, equipped with a capillary of length 150 mm and I.D. 0.57 mm.

As the leading and terminating electrolytes for isotachophoresis, 0.01 *N* methanolic potassium hydroxide solution adjusted to pH 5.32 by adding α-ketoglutaric acid and 0.01 *M* methanolic betaine hydrochloride solution respectively, were used after deaeration with an aspirator. The starting voltage was 2.2 kV and the final voltage was 5.1 kV, maintaining a migration current of 50 μA at 25°.

RESULTS AND DISCUSSION

N-α-Acetyl-L-lysine methyl ester exhibits basicity due to the α-amino group of lysine and its pK_a value is 10.8. However, the basicity of N-α-acetyl-L-lysine obtained by incubation of the substrate with urokinase decreases owing to the influence of the carboxyl group at the α-position in the molecule, and the pK_a value of the resulting product is 3.5.

Isotachophoretic conditions for the migration of this product towards the anode in alkaline solution of pH 8.0, 8.5, 9.0, 9.5 and 10.0 were investigated by adding ammediol or Tris solution as the leading electrolyte and aqueous solutions of β-alanine(pH 10.9), phenol (pH 10.0), γ-aminobutyric acid (pH 9.7), δ-aminocaproic acid (pH 9.4) and ω-aminocapric acid (pH 9.8), their pH values being adjusted with barium hydroxide solution, as the terminating electrolyte. However, the product did not migrate towards the anode under these conditions. Therefore, the isotachophoretic conditions for the migration of this compound as a cation were investigated. Potassium and barium ions have generally been used as the leading electrolytes for the migration of amino acids as cations.

Kopwillem *et al.*[15] reported the separation of basic amino acids such as lysine, arginine and histidine using an aqueous barium solution adjusted to pH 9.9 by adding valine as a leading electrolyte and Tris buffer adjusted to pH 8.0 with hydrochloric acid as the terminating electrolyte. Although the isotachophoresis of N-α-acetyl-L-lysine and its methyl ester was carried out under the above conditions, these compounds in acidic solution were tried again using aqueous potassium hydroxide solution adjusted to pH 3.0, 4.0, 5.0 and 6.0 by adding α-ketoglutaric acid, cacodylic acid or caproic acid as the leading electrolyte, and Tris–hydrochloric acid buffer (pH 8.0) as the terminating electrolyte.

N-α-Acetyl-L-lysine was not detected because its mobility was lower than that of the terminating ion under the above condition. As shown in Fig. 1, only its methyl ester migrated when aqueous potassium hydroxide solution adjusted to pH 5.4 by adding cacodylic acid and aqueous tetra-*n*-butylammonium chloride solution were used as the leading and terminating electrolytes, respectively.

Thus, urokinase activity could be determined indirectly by measuring the residual amounts of this substrate in the incubation mixture[13]. A disadvantage of this method is the relatively low sensitivity: at least 4 I.U. of urokinase are required.

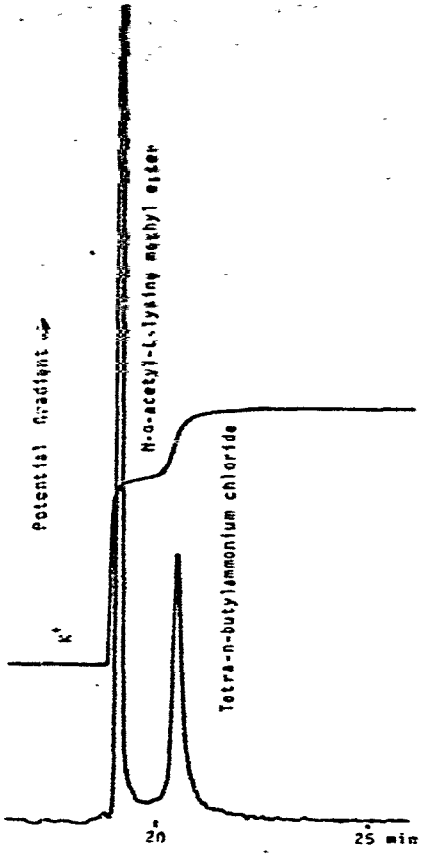

Fig. 1. Isotachopherogram of N-α-acetyl-L-lysine methyl ester.

Therefore, it is necessary to determine directly the N-α-acetyl-L-lysine in order to enhance the sensitivity of isotachophoresis. However, it was very difficult to make this compound migrate in an aqueous solution. Consequently, the use of organic solvents[14] was tried in order to make the lysine analogues migrate. Contrary to our expectations, the lysine analogues were not detected when methanolic potassium hydroxide solution adjusted to pH 3.0–6.0 was used as the leading electrolyte and methanolic Tris solution (pH 8.0) as the terminating electrolyte. This suggests that the mobilities of the ions of interest may be equal to or lower than that of the terminating ion. Thus, it was found that migration of N-α-acetyl-L-lysine was observed on the isotachopherogram when 0.01 *N* methanolic potassium hydroxide solution adjusted to pH 5.32 by adding α-ketoglutaric acid was used as the leading electrolyte and 0.01 *M* methanolic betaine hydrochloride solution as the terminating electrolyte.

Fig. 2a shows the isotachophoretic separation of N-α-acetyl-L-lysine and its methyl ester. Fig. 2b shows a representative isotachopherogram of the incubation mixture for the assay of urokinase activity. The potential unit (P.U.) values[11] of the two compounds were found to be 0.256 and 0.121, respectively. Betaine hydrochloride was very useful as a terminating ion in organic solvents, especially absolute ethanol.

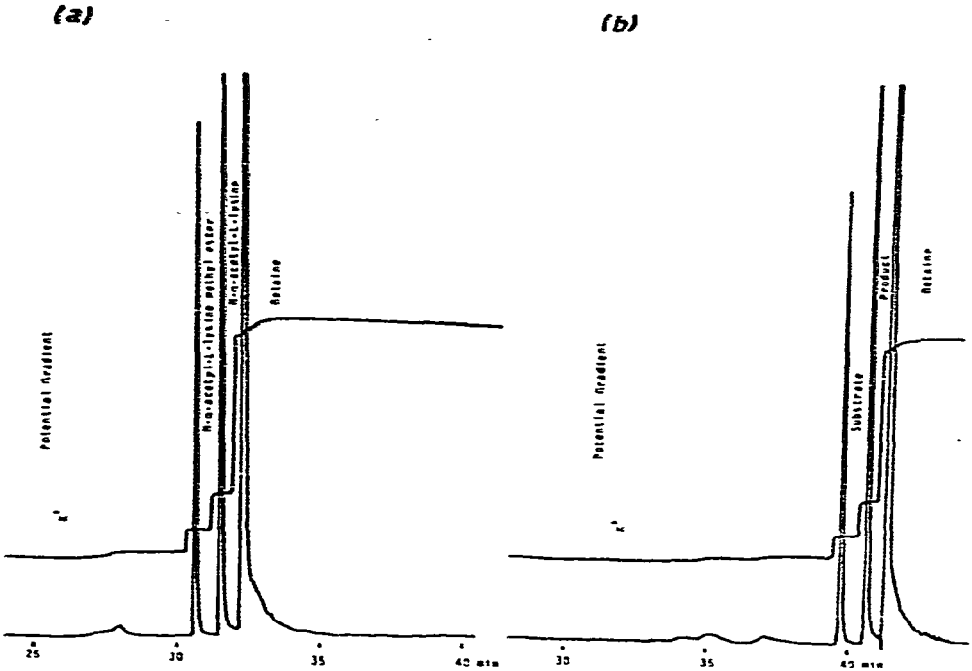

Fig. 2. (a) Isotachophoretic separation of N-α-acetyl-L-lysine and its methyl ester and (b) representative isotachopherogram of the reaction mixture. A 10-μl volume of urokinase solution (690 LU./ml of 0.025 *M* phosphate buffer containing 1.5% of sodium chloride buffer, pH 7.5) was used.

As shown in Fig. 3, the calibration graph for N-α-acetyl-L-lysine was a straight line.

The presence of large amounts of inorganic salts in the incubation mixture is undesirable in isotachophoresis because of the time required for migration of these inorganic ions because the mobilities of the salts were greater than that of the

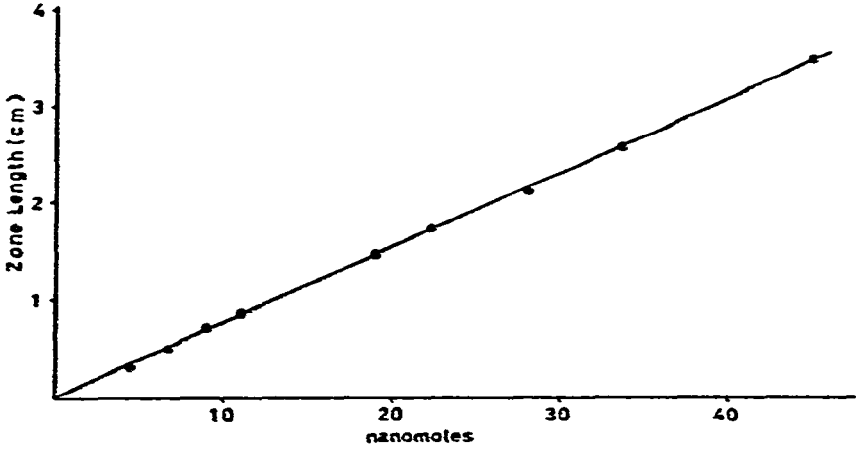

Fig. 3. Calibration graph for quantification of N-α-acetyl-L-lysine.

product. Therefore, the concentration of the buffer in the incubation mixture should be kept as low as possible, and phosphate buffer solution containing 0.1% of gelatin, which assists in the stabilization of urokinase, can be used as the incubation mixture. The optimum concentration of the substrate for this assay was then investigated.

Fig. 4a shows the titration curve for substrate solutions in the concentration range $4.27 \cdot 10^{-4}$–$3.43 \cdot 10^{-2}$ *M*. The results indicate that the enzymatic reaction attains a maximum at a concentration of $1.4 \cdot 10^{-2}$ *M* and subsequently 100 μl of $2.22 \cdot 10^{-2}$ *M* substrate solution were used in the experiment. Fig. 4b shows the zone length of N-α-acetyl-L-lysine produced at various pH values. The optimum pH was 7.5. Fig. 4c shows the relationship between the zone length of N-α-acetyl-L-lysine and the incubation time. Good linearity was obtained from 0.5 to 60 min. The incubation time was subsequently fixed at 60 min.

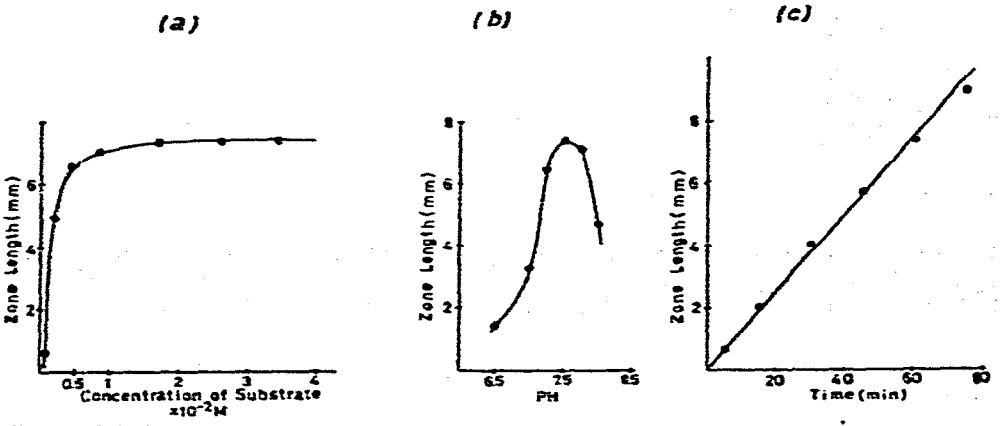

Fig. 4. (a) Subtrate titration curve; (b) relationship between zone length and pH of the reaction mixture; and (c) relationship between zone length and incubation time. A 10-μl volume of urokinase solution (1000 I.U./ml of buffer A) was used in each experiment. Each zone length was obtained by subtracting that in a blank run without urokinase from that of the incubation mixture.

In general, strong acids such as trichloroacetic or perchloric acid have been used as the terminator of the enzymatic reaction. However, these acids are unsuitable for isotachophoretic analysis because a long period is required for the migration of large amounts of these acids. In contrast, it was found that tannic acid is useful not only as a terminator but also as deproteinizing agent for enzymatic mixtures.

Fig. 5 shows the minimum amount of tannic acid required for terminating the reaction. The enzymatic reaction could be stopped completely by adding more than a 30-fold excess of tannic acid over the amount of protein in the urokinase solution.

Fig. 6 shows the calibration graph for the determination of urokinase, constructed by subtracting the zone length in a blank run without urokinase from that of the incubation mixture. A straight line was obtained in the range from 1 to 30 I.U. Then, the urokinase activities in commercially available preparations and active ingredients with various specific activities were determined by the present method and Walton's modified plate method.

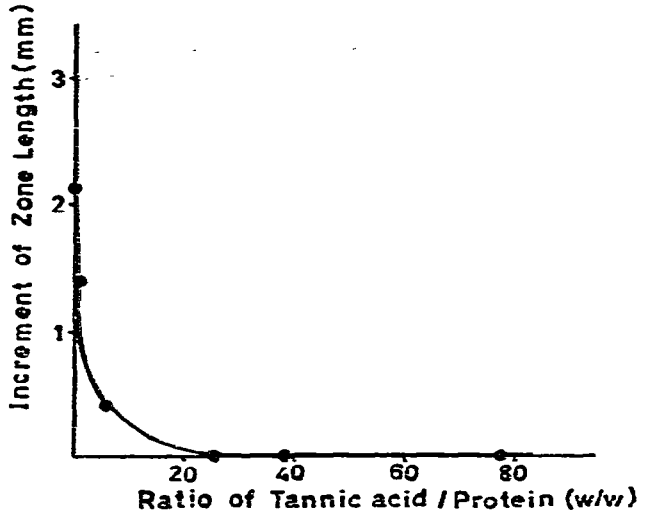

Fig. 5. Minimum amount of tannic acid for terminating the enzymatic reaction. The minimum amount was obtained when there was no increase in the zone length in the first and second incubations.

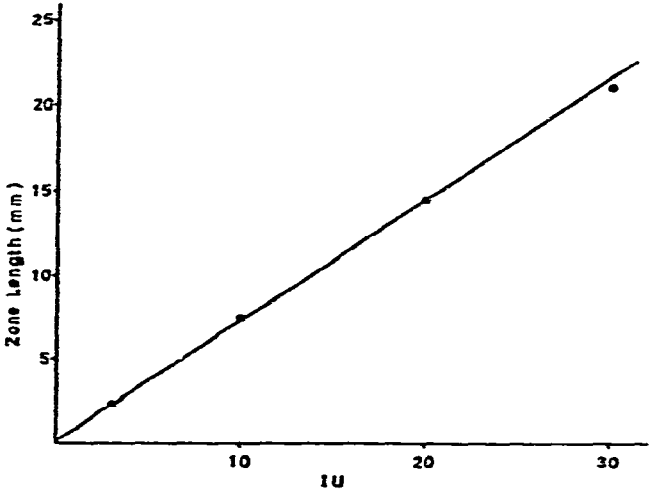

Fig. 6. Calibration graph for determination of urokinase activity.

Table I shows a comparison between the urokinase activities of the same samples determined by the two methods. Both results were in good agreement with each other.

The coefficient of variation of the isotachophoretic method was 3.4% ($n = 5$), indicating that it may be a convenient method for the determination of urokinase activity.

TABLE I

COMPARISON BETWEEN WALTON'S MODIFIED PLATE METHOD AND PRESENT METHOD FOR UROKINASE ACTIVITIES IN COMMERCIALLY AVAILABLE PREPARATIONS AND ACTIVE INGREDIENTS WITH VARIOUS SPECIFIC ACTIVITIES

Sample	*Sample No.*	*Specific activity (I.U./mg.)*	*Walton's modified method (I.U./vial)*	*Present method (I.U./vial)*
Commercially available preparation	1	—	6401	6240
	2	—	6483	6600
	3	—	6746	6960
	4	—	6528	6720
	5	—	6328	6360
Active ingredients	1	811	10,685	10,200
	2	14,350	36,167	34,560
	3	13,848	36,143	35,640
	4	15,890	28,765	28,200
	5	37,270	125,706	122,400

ACKNOWLEDGEMENTS

The authors are grateful to Dr. W. Tanaka, Research Laboratories, Nippon Kayaku Co., for his encouragement and useful suggestions throughout this work. They thank Mr. S. Arai and Mrs. T. Kyochi, Research Laboratories, Nippon Kayaku Co., for their cooperation in measuremening the urokinase activities by Walton's modified plate method.

REFERENCES

1 J. R. B. Wiliiams, *Brit. J. Exp. Pathol.*, 32 (1951) 530.
2 T. Astrup and I. Sterndorff, *Proc. Soc. Exp. Med.*, 81 (1952) 675.
3 J. Ploug, *Biochem. Biophys. Acta*, 24 (1957) 278.
4 P. L. Walton, *Clin. Chim. Acta*, 13 (1966) 680.
5 S. Sherry, N. Alkjaersig and A. P. Fletcher, *J. Lab. Clin. Med.*, 64 (1964) 145.
6 U. Hamberg and M.-L. Savolainen, *Acta Chem. Scand.*, 22 (1968) 1452.
7 H. Haglund, *Sci. Tools*, 17 (1970) 2.
8 F. M. Everaerts, J. L. Beckers and P. E. M. Verheggen, *Isotachophoresis—Theory, Instrumentation and Applications*, Elsevier, Amsterdam, Oxford, New York, 1976.
9 A. Kopwillem, F. Chillemi, A. B. Bosisio-Righetti and P. Righetti, *Protides Biol. Fluids, Proc. Colloq.*, 21 (1973) 657.
10 F. M. Everaerts and A. J. M. Vander Put, *J. Chromatogr.*, 52 (1970) 415.
11 H. Miyazaki and K. Katoh, *J. Chromatogr.*, 119 (1976) 369.
12 O. H. Lowry, J. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 67 (1959) 166.
13 H. Miyazaki, *Review Symposium on Analytical Isotachophoresis and Ion-Exchange Resin Chromatography, Sussex Univ., Brighton, Great Britain, 1976.*
14 J. L. Beckers and F. M. Everaerts, *J. Chromatogr.*, 51 (1970) 339.
15 A. Kopwillem, U. Moberg, G. W. Sjödahl, R. Lundin and H. Sievertsson, *Anal. Biochem.*, 67 (1975) 166.