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RAPID METHOD FOR THE DETERMINATION OF UROKINASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TETSUYA SOMENO, KAZUO KATOH, KUNIHIRO NIIJIMA and HIROSHI MIYAZAKI*
Research Laboratories of the Pharmaceutical Division, Nippon Kayaku Co., 3-31 Shimo, Kita-ku, Tokyo 115 (Japan)
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SUMMARY

A simple, rapid and reproducible high-performance liquid chromatographic method for the quantitation of urokinase was developed by the separation of two types of urokinase with molecular weights of 54,000 and 32,000 in its crude solutions containing various proteins as impurities. TSK-GEL 3000 SW column chromatography with 0.2 *M* phosphate buffer solution of pH 3.0 as the mobile phase at room temperature was used.

The ratios of the two types of urokinase determined by the method were in good agreement with those obtained by Sephadex G-100 gel chromatography followed by Walton's modified plate method.

INTRODUCTION

Urokinase, one of the proteolytic enzymes found in human urine, catalyses the conversion of plasminogen to plasmin[1,2]. Therefore, conventional assays for urokinase were generally performed, such as a test-tube method[3], a fibrin plate method[4] and a two-stage method[5]. In addition, there were a number of simple and convenient assay methods for urokinase activities involving the use of synthetic substrates[6–8].

It has been shown that there are two active types of urokinase with apparent molecular weights of 54,000 for the high-molecular-weight type (H-UK) and 32,000 for the low-molecular-weight type (L-UK)[9–11]. The relative specific activities of H-UK to L-UK appear to differ according to the assay method used. Those obtained by the Chandler's loop method[12], which is considered to reflect the activities *in vivo*, suggested that H-UK may be more potent than L-UK against fibrinolytic activity *in vivo*. Consequently, it is essential to determine simultaneously the individual and total activities of these urokinase components in samples of interest.

Sephadex G-100 gel chromatography[13] and sodium dodecyl sulphate poly-

* To whom correspondence should be addressed.

acrylamide gel electrophoresis (SDS-PAGE)[14] have been used for the separation of the two components from urokinase solution. However, these methods require tedious and time-consuming operations, and their reproducibility is poor, because the amount of urokinase has to be determined in each separated fraction or zone by bioassay or densitometry.

Recently, high-performance liquid chromatography (HPLC) has been applied to the separation of proteins[15]. This paper described a simple, rapid and reproducible method for the simultaneous determination of the total and individual activities of H-UK and L-UK in a sample solution by HPLC.

MATERIALS AND METHODS

Materials

Sodium dihydrogen phosphate and phosphoric acid were purchased from Kanto Chemical Co. (Tokyo, Japan) and insulin from Fluka (Buchs, Switzerland). Standard H-UK (86,600 I.U./mg protein) and L-UK (192,300 I.U./mg protein) solutions were purified in our laboratories according to the modified method of White *et al.*[13]. The uniformity of the standard urokinase was confirmed by SDS-PAGE[14], PAGE at pH 4.0 (ref. 16) and Sephadex G-100 gel chromatographic analyses. Various urokinase solutions with different specific activities were prepared from urine in our laboratories.

Measurement of urokinase activity using plasminogen

Urokinase activity was determined by the modified fibrin plate method of Walton using a WHO[19] standard. The amount of protein was determined by the Folin–Lowry method[17] using bovine serum albumin as a standard protein.

Sephadex G-100 gel chromatography of urokinase solutions

Sephadex G-100 gel column (142 × 2.0 cm I.D.) chromatography was carried out using 0.025 *M* phosphate buffer solution of pH 7.5 containing 1.5% of sodium chloride as the eluent at 2°. The flow-rate of the eluate was maintained at 4.08 ml/h. The urokinase activity in each fraction (1.84 ml per tube) collected was determined by Walton's modified plate method.

High-performance liquid chromatography

A TSK-GEL 3000 SW packed column (60 cm × 7.5 mm I.D.) was purchased from Toyo Soda (Tokyo, Japan). The separation of the two types of urokinase was carried out with a Hitachi Model 635 liquid chromatograph equipped with a syringe loading injector with a 50-μl sample loop (Rheodyne, U.S.A.) at room temperature. The eluent flow-rate was 0.5 ml/min, the detector wavelength 280 nm and the detector sensitivity 0.02 a.u.f.s.

Assay procedure for determination of urokinase activity by HPLC

To 100 μl of urokinase solutions were added 40 μl of an internal standard solution (insulin; 1 mg/ml of the eluent buffer solution) and 60 μl of the same buffer solution. The mixture was shaken briefly by hand and an aliquot of 50 μl was immediately injected into the chromatograph. The peak heights of H-UK, L-UK and the

internal standard on the chromatogram were measured and the activity of each urokinase was calculated from its peak-height ratio to the internal standard on the calibration graph.

RESULTS AND DISCUSSION

HPLC has generally been applied to the analysis of proteins by the use of neutral aqueous buffer solutions[15]. The separation of H-UK and L-UK was examined in neutral phosphate buffer solution. However, the two types of urokinase could not be separated, because only the H-UK peak was substantially broadened in the neutral solution. Therefore, various buffer solutions with wide ranges of pH were investigated for improving the separation of H-UK and L-UK by HPLC. Fig. 1 shows the influence of pH on the resolution of H-UK and L-UK in the pH range 2–6. The resolution (R_s) between two peaks, according to Snyder and Kirkland[18], is given by $R_s = 2(t_{R_1} - t_{R_2})/w_1 + w_2$, where t_{R_1} and t_{R_2} are the retention times of H-UK and L-UK, respectively, and w_1 and w_2 represent the peak widths of H-UK and L-UK, respectively, in units of time. If R_s is greater than 1.0, complete separation of both peaks should be obtained. At pH above 3.5, a good separation was not obtained owing to broadening of the H-UK peak. An excellent resolution ($R_s = 1.0$) was obtained at pH below 3.

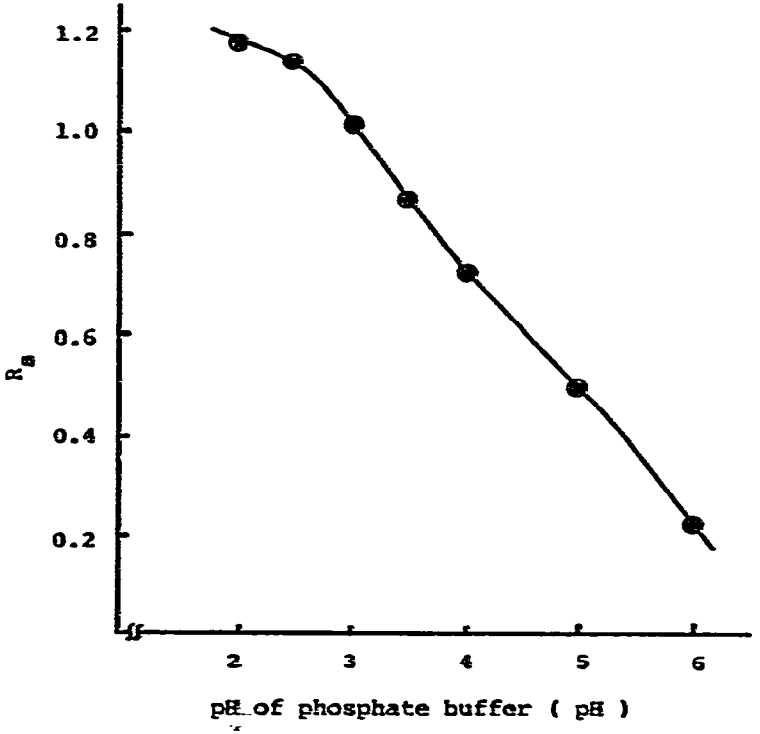


Fig. 1. Effect of pH on the resolution (R_s) of the two types of urokinase by HPLC. R_s is represented by the equation: R_s = interval between the two urokinase peaks/mean band width of the two urokinase peaks.

Fig. 2 shows the retention times of H-UK and L-UK relative to the internal standard in the buffer solutions with various pH ranges. The mobility of H-UK was influenced more than that of L-UK by the pH of the solutions. Consequently, the retention time of H-UK in a neutral solution is longer than would be expected from its molecular weight. Actually, it is well known that basic proteins such as lysozyme are eluted more slowly than expected under neutral conditions owing to their adsorption on TSK-GEL 3000 SW[15].

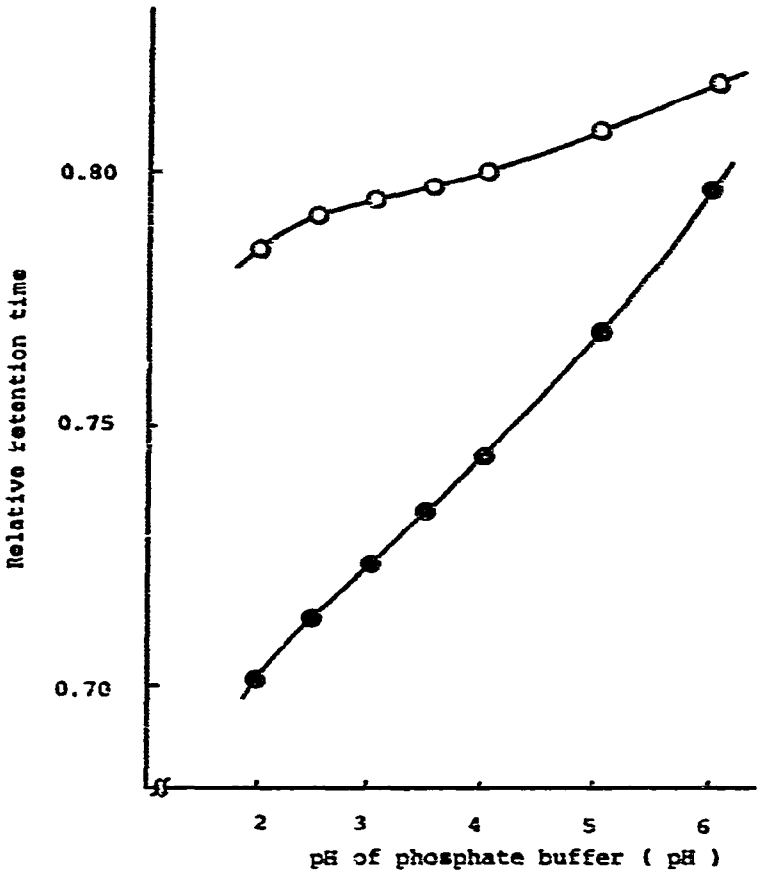


Fig. 2. Effect of pH on the retention times of H-UK (●) and L-UK (○) relative to the internal standard.

The solutes adsorbed on TSK-GEL 3000 SW were eluted by addition of sodium sulphate or sodium chloride solution. However, when 0.2 *M* phosphate buffer solution of pH 3.0 was used, adsorption on this gel did not occur without the addition of salts. On the other hand, the use of this gel is desirable in the pH range 3–8. Therefore, 0.2 *M* phosphate buffer solution of pH 3.0 was used as the mobile phase.

The mean recoveries and standard deviations of urokinase activity obtained by this TSK-GEL 3000 SW column chromatography were 94.5 ± 7.2% for H-UK and 100.4 ± 8.4% for L-UK.

Fig. 3 shows a chromatogram obtained by HPLC of 50 μl of a standard mixture containing purified H-UK (1470 I.U.), L-UK (2500 I.U.) and insulin (10 μg). The retention times relative to insulin were 0.726 ± 0.009 for H-UK and 0.795 ± 0.006 for L-UK.

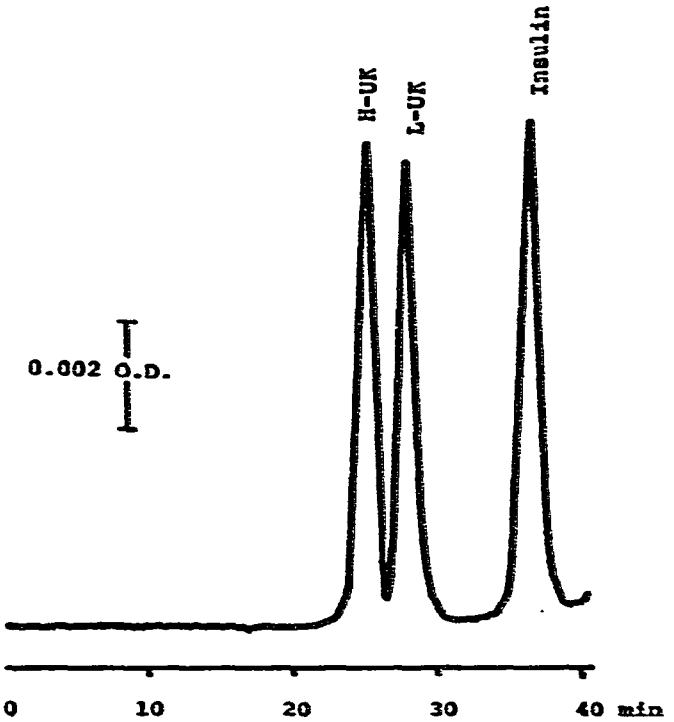


Fig. 3. Representative chromatogram obtained by HPLC of 50 μl of a standard mixture containing H-UK (1470 I.U.), L-UK (2500 I.U.) and internal standard (10 μg).

The limits of determination of urokinase by the present method were 3000 I.U./ml for H-UK and 4500 I.U./ml for L-UK. Fig. 4 shows the calibration graphs for the determination of H-UK and L-UK using insulin as the internal standard. It can be seen that there is a linear relationship between the activity of urokinase in the range 3000–50,000 I.U./ml and the peak-height ratio. The following equations were obtained by the method of least-squares: $y = 29{,}259x + 193$ (I.U./ml) and $r = 0.9993$ for H-UK, and $y = 49{,}659x + 342$ (I.U./ml) and $r = 0.9996$ for L-UK, where y is the activity of urokinase (I.U./ml) and x is the peak-height ratio.

Total activities of the samples can be calculated by summation of the activities of H-UK and L-UK. The percentage of H-UK or L-UK in a sample was obtained by dividing the activity of the individual urokinase by the total activity. An advantage of the proposed method is the rapid determination of the total and individual activities

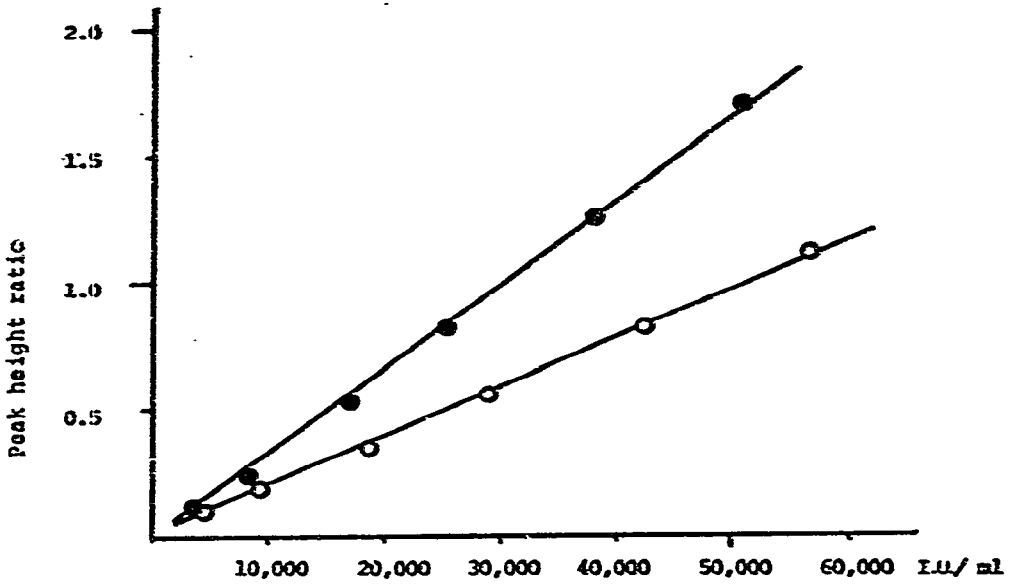
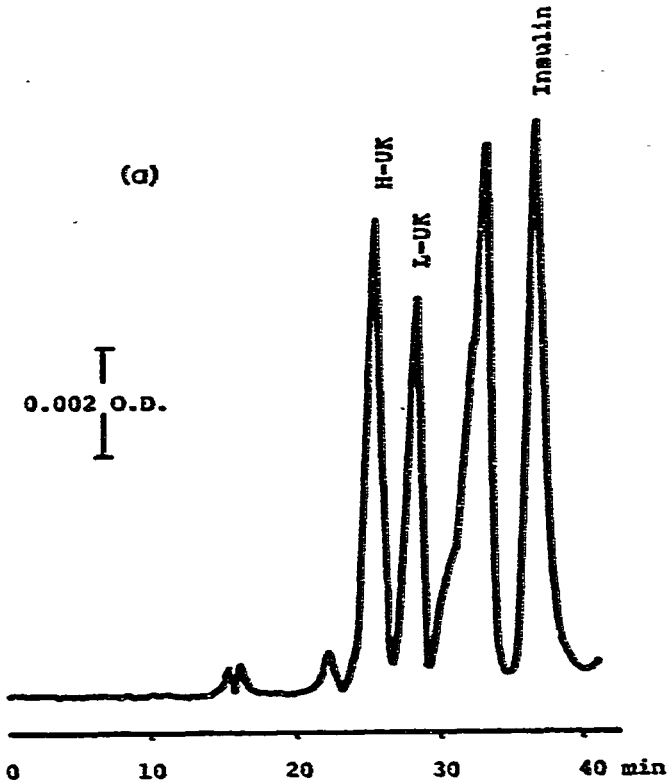


Fig. 4. Calibration graphs for H-UK (●) and L-UK (○).

of the two types of urokinase in the same sample using these reproducible calibration graphs. In contrast, in Sephadex G-100 gel chromatography followed by Walton's modified plate method, the standard urokinase for bioassay must be used in every measurement of activity in the separated fractions.

Fig. 5a shows a representative high-performance liquid chromatogram of urokinase solution with an activity that had previously been determined to be 65,300 I.U./ml by the plate method. In the proposed method, the activities of H-UK and L-UK were determined to be 26,000 and 37,300 I.U./ml, respectively, and the total activity of this sample solution was calculated to be 63,300 I.U./ml from the calibration graphs. Fig. 5b shows the Sephadex G-100 gel chromatogram of the same solution. The two chromatograms were similar. The total activity determined by the proposed method was in good agreement with that obtained by the plate method. The ratio of H-UK to the total activity was calculated to be 41.1%, which is in good agreement with that (40%) determined by the plate method after separation of H-UK and L-UK from the same sample by Sephadex G-100 gel chromatography.

Urokinase solutions with various specific activities prepared in our laboratories were analysed by the proposed method and the plate method after separation by Sephadex G-100 gel chromatography, and Table I compares the urokinase activities obtained. There were no statistically significant differences between the pairs of results obtained by the two methods on the same samples. The coefficient of variation of the proposed method was 3.8% ($n = 4$).



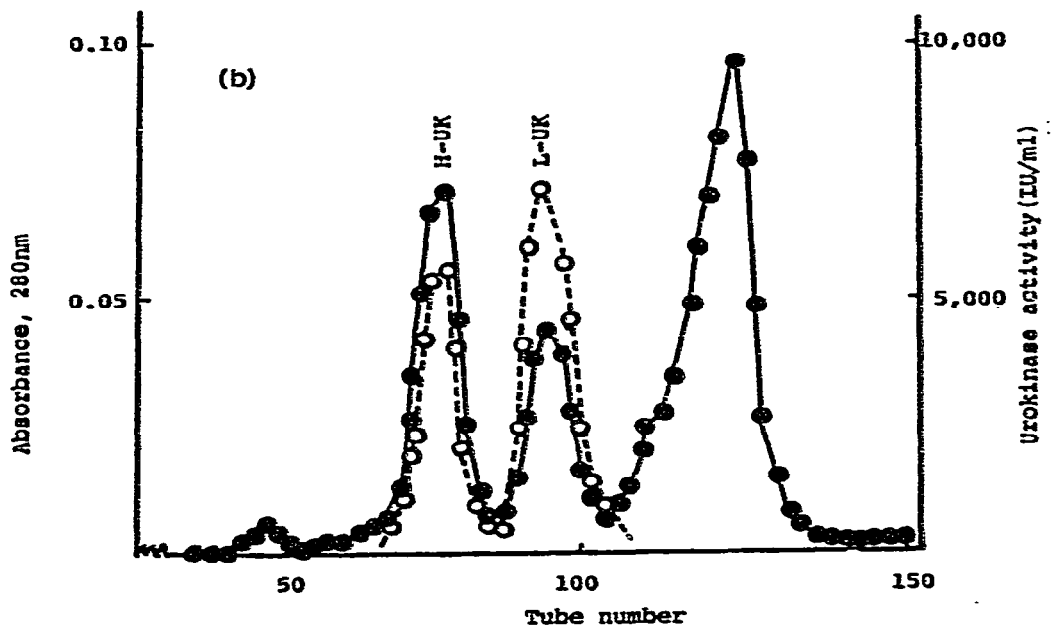


Fig. 5. (a) Typical chromatogram for determination of H-UK and L-UK in a sample by HPLC. (b) Chromatogram of the same urokinase solution obtained on Sephadex G-100. Approximately 150,000 I.U. were added to a column (142 × 2 cm I.D.) equilibrated with 0.025 *M* phosphate buffer containing 1.5% of sodium chloride (pH 7.5) at 2°. Each fraction of 1.84 ml was collected at a flow-rate of 4.08 ml/h. ●, Absorbance at 280 nm; ○, urokinase activity.

TABLE I

COMPARISON OF RESULTS FOR VARIOUS UROKINASE SOLUTIONS OBTAINED BY THE PROPOSED METHOD AND THE COMBINATION OF SEPHADEX G-100 GEL CHROMATOGRAPHY AND WALTON'S MODIFIED PLATE METHOD

A, Proposed method; B, Walton's modified plate method; C, Sephadex G-100 gel chromatography followed by Walton's modified plate method.

Sample No.	*Total activity (I.U./ml)*		*Ratio of H-UK to L-UK activity*	
	A	*B*	*A*	*C*
1	38,600	39,200	100:0	100:0
2	57,000	58,600	91:9	90:10
3	48,900	49,300	85:15	85:15
4	49,500	49,600	79:21	80:20
5	58,600	60,900	65:35	65:35
6	51,900	50,600	48:52	50:50
7	63,300	65,300	41:59	40:60

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