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DETERMINATION OF UROKINASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH RADIOISOTOPE DETECTION

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SUMMARY

A specific method for the determination of two types of urokinase was developed, involving high-performance liquid chromatography (HPLC) with radioisotope detection using [^{3}H]diisopropyl fluorophosphate (DFP) as a pre-labelling reagent. The serine moiety located in the active site of urokinase was reacted selectively with [^{3}H]DFP to form [^{3}H]DFP–urokinase adduct. Two types of urokinase were determined by monitoring the radioactivity of each peak separated by means of HPLC. The total radioactivity of the two peaks was in good agreement with the urokinase activity obtained by a modified fibrin plate method.

INTRODUCTION

Urokinase exists in human urine and catalyses the conversion of plasminogen to plasmin[1,2]. The resulting plasmin lyses the clots of fibrin. 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)[3–5]. Lormeau *et al.*[6] reported that the fibrinolytic activity of H-UK was more potent than that of L-UK in the physiological blood level of plasminogen.

Sephadex G-100 gel chromatography[7] and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE)[8] have been used as conventional methods for the determination of two types of urokinase. These methods have the disadvantage that they require tedious and time-consuming procedures.

In a previous paper[9], we reported a method for the determination of urokinase activity by HPLC using a UV detector. However, this method could not be applied to determine directly the activity of H-UK and L-UK in a commercially available preparation and crude urokinase solution owing to disturbance of the other proteins that were contained as a stabilizer in the preparation or impurities in human urine. Hence it was necessary to develop a highly specific method for the detection of urokinase.

Landmann and Markwardt[10] showed that diisopropyl fluorophosphate (DFP) was more reactive to urokinase than that of the other irreversible protease inhibitors. SDS–PAGE using [^{3}H]DFP has been used for the determination of two types of

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urokinase by Soberano *et al.*[11]. However, this method has the disadvantage that it requires a skilful operator and a tedious operation. Consequently, this method would be unsuitable for quantitative analysis owing to the poor reproducibility of extraction from the zones of [^{3}H]DFP–urokinase on the gel.

This paper describes with a rapid and specific method for the determination of two types of urokinase in samples containing a large amount of other proteins by HPLC with radioisotope detection (RID) using [^{3}H]DFP as a pre-labelling reagent.

EXPERIMENTAL

Chemicals and reagents

[^{3}H]DFP (1 Ci/mmol, 500 μCi/ml) solution and Aquasol® 2 were purchased from New England Nuclear (MA, U.S.A.). Glutarylglycylarginine-4-methylcoumaryl-7-amide (Glt–Gly–Arg–MCA) was purchased from the Protein Research Foundation (Osaka, Japan). The standard H-UK (124,208 I.U./mg) and L-UK (209,160 I.U./mg) were prepared in our laboratories. The homogeneity of each urokinase was confirmed by means of SDS-PAGE and Sephadex G-100 gel chromatography.

Apparatus

A Shimadzu Model LC-2 HPLC instrument equipped with a sample injector (Rheodyne, Berkeley, CA, U.S.A.) was employed. A TSK-GEL 3000 SW column (60 × 7.5 mm I.D.) with a mean diameter of 10 μm (Toyo Soda, Tokyo, Japan) was used for the separation of [^{3}H]DFP–H-UK and [^{3}H]DFP–L-UK. A Berthold HPLC radioactivity monitor (LB 503) was used for the measurement of the radioactivity in the eluate. Liquid scintillator was delivered by an Altex (Berkeley, CA, U.S.A.) Model 110A pump.

Measurement of urokinase activity

The urokinase activity was determined by a modification of the fibrin plate method of Walton[12] using a WHO standard[13]. The time course of inactivation of urokinase with DFP was investigated by measuring the residual activity of the reaction mixture using Glt–Gly–Arg–MCA[14] as a substrate.

Preparation of samples

To 100 μl of urokinase solution (1000–25,000 I.U./ml) in a phosphate buffer solution (pH 7.3–8.3) was added 10 μl of [^{3}H]DFP solution (final concentration $4.5 \cdot 10^{-5}$ *M*). The mixture was vigorously shaken for 120 min at 37°C. An aliquot of 50 μl of the reaction mixture was injected into the HPLC system using a 50-μl Rheodyne loop.

HPLC–radioisotope detection conditions

A 0.2 *M* sodium phosphate solution (pH 3.0) was used as a mobile phase and its flow-rate was maintained at 0.5 ml/min. The eluate from the column was mixed automatically with the liquid scintillator delivered at a flow-rate of 3.5 ml/min. The radioactivity of the resulting mixture was measured by a radioactivity monitor set at a sensitivity of $3 \cdot 10^3$ cpm, a time constant of 3 sec and chart speed of 20 cm/h.

Inactivation of urokinase with [^{3}H]DFP

To 1 ml of H-UK (25,000 I.U./ml) or L-UK (25,000 I.U./ml) solution (0.1 *M* sodium phosphate buffer solution, pH 7.5, containing 1.5% sodium chloride) were added 100 μl of [^{3}H]DFP (final concentration $4.5 \cdot 10^{-5}$ *M*) and the reaction mixture was incubated at 37°C. The residual urokinase activity of the reaction mixture was measured at intervals of 10–20 min using Glt–Gly–Arg–MCA as a substrate.

Determination of optimum derivatization conditions

To 1 ml of H-UK or L-UK solution (25,000 IU/ml) with various pH values containing 0.1 *M* sodium phosphate and 1.5% sodium chloride were added 100 μl of the [^{3}H]DFP solution. After the mixture had been shaken well for 120 min at 37°C, an aliquot of 50 μl of the reaction mixture was injected into the HPLC column. The percentage of resulting [^{3}H]DFP–urokinase was plotted graphically. The influence of reagent concentration on the formation of [^{3}H]DFP–urokinase was also investigated by adding various amounts of [^{3}H]DFP. After incubation for 120 min at 37°C, the reaction mixture was subjected to HPLC–RID. Thus, the optimum concentration of [^{3}H]DFP was determined to be $4.5 \cdot 10^{-5}$ *M* by adding various concentrations of [^{3}H]DFP to the H-UK or L-UK solution at pH 7.5.

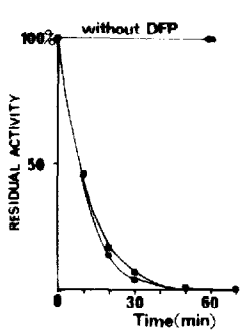
RESULTS AND DISCUSSION

In a previous paper, we reported that the separation efficiency between H-UK and L-UK on a TSK-GEL 3000 SW column was improved considerably by decreasing the pH of the buffer solution. Thus, 0.2 *M* sodium phosphate solution at pH 3.0 was used as an effluent solution for this separation.

Two techniques were considered for the selective detection of urokinase activity using an on-line system: a pre-labelled technique using a radioactive inhibitor of urokinase and a post-column technique using a fluorescent substrate such as Glt–Gly–Arg–MCA. A pre-labelling technique using an irreversible radioactive inhibitor may be more suitable than a post-column technique using a substrate for this assay, because the derivatization for the pre-labelling may be less restrictive with respect to the mobile phase conditions (pH, salt concentration, temperature). Therefore, an attempt at the specific detection of urokinase was made by utilizing protease inhibitors as pre-labelling reagents. Although a number of inhibitors against urokinase have been reported, reversible inhibitors could not be used for this purpose because the complex of urokinase with a reversible inhibitor such as α-N-benzylsulphonyl-*p*-aminophenylalanine in acidic solution was extremely liable to dissociate during HPLC analysis. Only irreversible inhibitors labelled with a radioisotope seems to be suitable for the determination of urokinase by HPLC–RID.

Landmann and Markwardt[10] reported the inactivation of urokinase by irreversible inhibitors such as DFP, tosyllysine chloromethyl ketone and *p*-nitrophenyl *p'*-guanidinobenzoate at a concentration of $2.5 \cdot 10^{-5}$ *M* and pH 7.5 at 25°C. In this study, DFP was found to be the most sensitive to urokinase. Therefore, DFP was chosen as a radioactive pre-labelling reagent for urokinase. DFP gave a stable complex by the formation of a covalent linkage with a hydroxyl group of an active serine residue in urokinase, and the resulting complex did not dissociate in acidic solution.

The radioactivity of the [^{3}H]DFP–urokinase complex could be measured di-



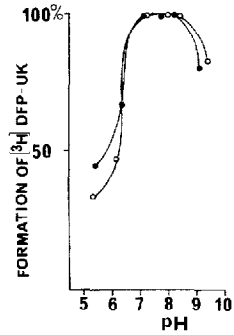
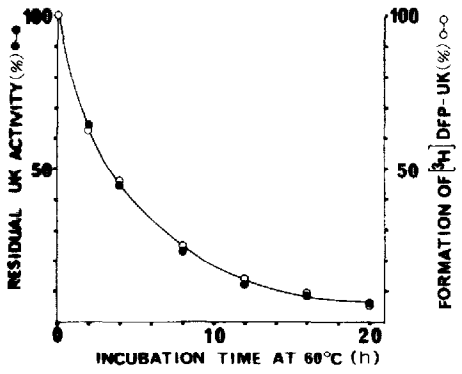


Fig. 1. Time course of inactivation of H-UK (●) and L-UK (■) with DFP ($4.5 \cdot 10^{-5}$ *M*) in sodium phosphate buffer (pH 7.5) containing 1.5% NaCl at 37°C.

Fig. 2. pH dependence of [^{3}H]DFP–urokinase adducts formed by the reaction of H-UK (●) and L-UK (○) with [^{3}H]DFP ($4.5 \cdot 10^{-5}$ *M*) for 120 min at 37°C.

rectly by using a glass scintillator as a flow cell counting system. However, the reproducibility of this measurement was very poor owing to adsorption of the complex on the glass scintillator. Although other solid flow cells such as an anthracene scintillator were tried, the reproducibility could not be improved. Thus, a homogeneous counting system was used by mixing a liquid scintillator with the eluate.

The reactivity of H-UK and L-UK with [^{3}H]DFP was investigated by measuring the residual activity of urokinase in an incubation mixture. Fig. 1 shows the time course of the residual activities of H-UK and L-UK. When the urokinase solution



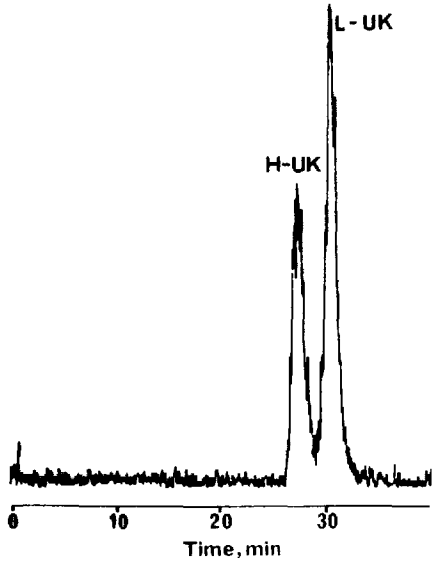


Fig. 3. Relationship between the residual urokinase activity and the percentage of the total peak area on the radiochromatogram of [^{3}H]DFP–urokinase adducts formed from the same sample.

Fig. 4. Representative radiochromatogram of the reaction products obtained by the incubation of a standard mixture containing H-UK (9000 I.U./ml) and L-UK (10,400 I.U./ml) with [^{3}H]DFP.

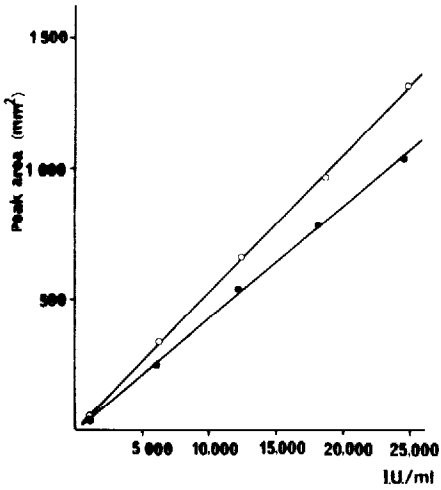


Fig. 5. Calibration graphs for H-UK (●) and L-UK (○).

without addition of [^{3}H]DFP was kept at 37°C for 2 h, the loss of urokinase activity in this solution was negligible, which indicates that the formation of the [^{3}H]DFP–urokinase complex had proceeded to completion.

On the other hand, it seems that the incorporation of DFP into urokinase depends on the pH of the incubation mixture. Fig. 2 shows the effect of the pH of the reaction mixture on the formation of the [^{3}H]DFP–urokinase complex. The optimum pH was almost the same for complex formation with two types of urokinase. Thus, the samples should be adjusted to pH 7.3–8.3 by addition of sodium hydroxide

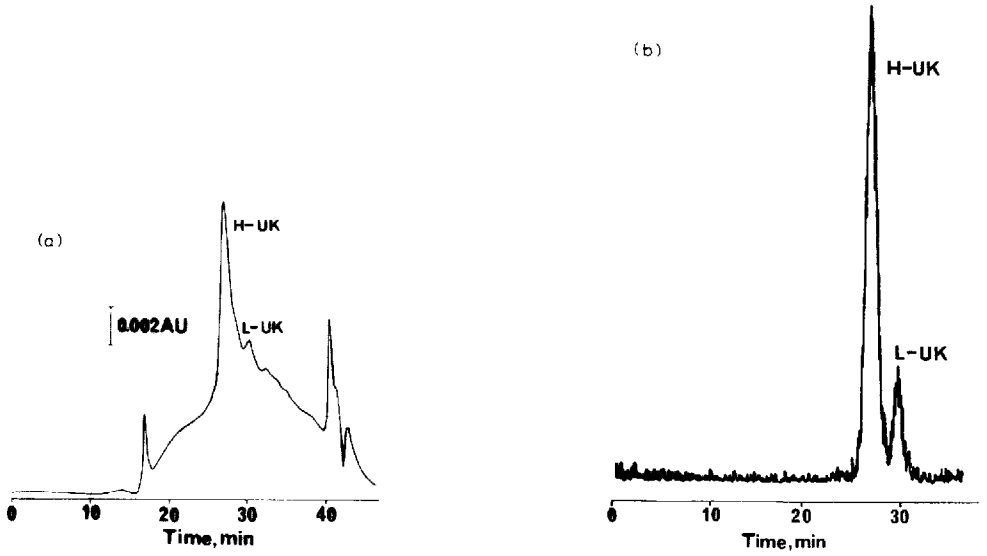


Fig. 6. (a) Typical chromatogram of a commercially available preparation of urokinase obtained by HPLC with UV detection. (b) Radiochromatogram of the same sample obtained by the present method using HPLC–RID.

solution or hydrochloric acid before the addition of [^{3}H]DFP.

The specificity of the reaction of DFP with urokinase was investigated by using a mixture of active and inactive urokinase, which was prepared by heating a known amount of highly purified urokinase at 60°C. Fig. 3 shows the time courses of the inactivation of urokinase, which were determined by the residual urokinase activity in a bioassay and the radioactivity of the peak on a radiochromatogram. The total radioactivity of the two peaks was in good agreement with the remaining activity of urokinase, indicating that DFP had reacted selectively with the hydroxyl group of serine located at an active site of urokinase.

Fig. 4 shows a representative chromatogram of the incubation mixture using a standard mixture of H-UK (9000 I.U./ml) and L-UK (10,400 I.U./ml).

Fig. 5 shows the calibration graphs for the determination of H-UK and L-UK constructed by measuring the peak areas on a radiochromatogram obtained using various known amounts of H-UK and L-UK solutions. A linear relationship was obtained between the urokinase activity in the range 1000–25,000 I.U./ml and the peak areas on the radiochromatogram. The standard deviations of the peak areas for 12,500 I.U./ml of H-UK and L-UK were less than 1.8% and 1.2%, respectively. The minimum detectable amount of H-UK and L-UK was 500 I.U./ml with a signal-to-noise ratio of 2.

Fig. 6a shows a representative HPLC trace of a commercially available urokinase preparation containing a large amount of protein as a stabilizer, obtained using a UV detector. Fig. 6b shows a radiochromatogram of the same sample obtained under the same HPLC conditions. It can be seen that with the UV detector it was difficult to determine H-UK and L-UK owing to interferences from the stabilizers, whereas the proposed method using radioactivity detection was completely free from these interferences.

The total activity of the solution was calculated to be 6150 I.U. per vial from the calibration graph. Using the fibrin plate method of Walton[12], the result was 6278 I.U. per vial. There is no statistically significant difference between these results.

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