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CORRELATION OF UROKINASE ACTIVITY FROM BIOPOTENCY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAYS

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SUMMARY

A simpler, less expensive, and faster high-performance liquid chromatographic method was shown to be an alternative to urokinase potency determinations by the Ploug method. Post-elution recovery of the low-molecular-weight form was 104 ± 2.4% as determined by the Ploug method. Two analysts reported relative standard deviations of 1.6% and 1.1% based on peak height determination of eight replicate injections of a single sample of low-molecular-weight material. Linearity at the same wavelength for low- and high-molecular-weight forms was 0.9999 and 0.9992, respectively, for peak height *versus* potency.

INTRODUCTION

The proteolytic enzyme urokinase, which catalyzes the conversion of plasminogen to plasmin, appears in two active forms with molecular weights of approximately 54 000 (S_2) and 32 000 (S_1). The current testing procedures for the bulk material are labor- and capital-intensive, and do not discriminate between the two components.

The use of a TSK G3000SW column has been suggested for the separation of the high- and low-molecular-weight forms of urokinase[1]. This paper describes a detailed investigation of the separation, particularly regarding correlation with activity determinations made by the Ploug falling ball assay method[2].

MATERIALS AND METHODS

Materials

Sodium chloride, sodium azide, and potassium phosphate monobasic were reagent grade and used as received. Standard low-molecular-weight urokinase was obtained as a lyophilized powder (Abbott Labs., North Chicago, IL, U.S.A.) and reconstituted with distilled water. High-molecular-weight urokinase was purified by repetitive gel filtration chromatography on Sephadex G75 (Pharmacia, Piscataway, NJ, U.S.A.). Fractions containing purified high-molecular-weight urokinase, ob-

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tained by cation-exchange chromatography, were repetitively chromatographed to increase their purity and confirmed by chromatography of the final product. Blue dextran, ovalbumin, bovine serum albumin and α-chymotrypsinogen A molecular weight standards (Sigma, St. Louis, MO, U.S.A.) were used as received.

Measurement of urokinase activity

Urokinase activity was determined by a modified Ploug falling ball method[2] using urokinase standard (Abbott Labs.) verified against a WHO standard. In the modified method used here, thrombin, plasma and excess plasminogen are mixed at 0°C until clotting is complete. Urokinase is added and the sample incubated at 37°C. The time required for a small ball to drop through the dissolving clot is monitored and urokinase potency calculated via comparison with standard material of known potency.

High-performance liquid chromatography (HPLC)

A TSK G3000SW column (30 cm × 7.5 mm I.D.) was used (Alltech Assoc., Deerfield, IL, U.S.A.). The mobile phase consisted of 0.1 *M* potassium dihydrogen phosphate adjusted to pH 6.0 with 10 *M* sodium hydroxide, 0.1 *M* sodium chloride, and 0.005 *M* sodium azide to prevent microbial growth. The flow-rate at ambient column temperature was 0.5 ml/min with typical column pressures of 200–300 p.s.i. The chromatographic system consisted of a Beckman 112 pump, 210 injector, and 165 variable-wavelength detector set at 280 nm. A Shimadzu C-R3A integrator was used to obtain peak area data; height measurements were made by the integrator or manually. All potency values were calculated on an external standard basis from replicate injections of a single standard of known potency.

Post-column recovery of urokinase

Better resolution of the two forms is obtained with smaller injection volumes. To give enhanced sensitivity for Ploug determinations, however, 200-μl injections were made when fractions were collected. To calculate the percent potency recovered, 200-μl aliquots of the samples were diluted to 5 ml in mobile phase and submitted for the Ploug potency assay. These values were compared with the potency values obtained after injection of 200 μl of material, collection of the appropriate peak (approximately 0.5–1 ml), and dilution of the eluate to 5 ml prior to submission for the Ploug assay.

RESULTS AND DISCUSSION

Although superior results have been reported with relatively acidic mobile phases for urokinase separation[1], optimum resolution was found in this laboratory at a pH of 6.0. A typical chromatogram of material containing both low- and high-molecular-weight forms is seen in Fig. 1.

Molecular weight determinations were made using as calibration standards dextran blue (void volume marker), bovine serum albumin, ovalbumin, and α-chymotrypsinogen A. Good precision was found for this measurement based on eight replicate injections of a single sample (molecular weight 30 600 ± 1.3%). G3000SW columns which had been calibrated using this mixture, however, gave broad, mis-

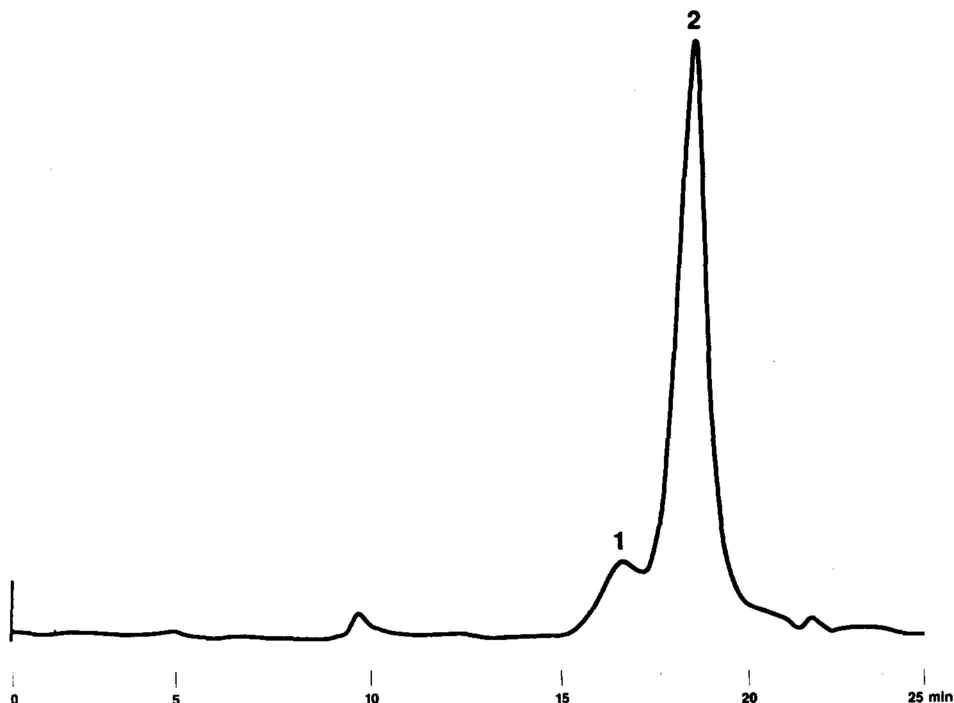

Fig. 1. Typical chromatogram of low- and high-molecular-weight urokinase; approximately 125 000 IU/ml S_1 (2) and 21 000 IU/ml S_2 (1).

shapen peaks for injections of pure S_2 material. Suspecting adsorption of α-chymotrypsinogen A which might degrade the S_2 as it passed through the column[3], a calibration solution without the suspect component was injected onto a fresh column. No S_2 degradation was seen thereafter. A suitable substitute for the α-chymotrypsinogen A could not be found, leaving only two points for subsequent molecular weight calibration curves.

The linearity of potency recovery was demonstrated by injecting solutions containing from 0 to 120 000 IU/ml S_1 urokinase and comparing pre- and post-elution potency values measured by a Ploug U-5 assay. Correlation coefficients of experimental *versus* theoretical potencies were 0.9987 and 0.9972, respectively, indicating little difference is seen before and after chromatography. Two analysts also tested the precision of urokinase recovery by making replicate injections of a single sample of material containing approximately 45 000 IU/mL S_1. The results of these precision studies are shown in Table I, along with mean recovery values for eight lots of material containing 30 000–50 000 IU/mL S_1. Biological activity was also maintained in samples of material purified by HPLC, lyophilized, and then reconstituted.

The effect of variations in the mobile phase was examined by systematically changing pH, buffer strength, ionic strength and column temperature while maintaining other variables constant. Ionic strength and column temperature had essentially no effect on peak resolution or assay results, while optimal resolution was seen at pH 6.0 and a buffer strength of 0.1 *M*.

TABLE I

PRECISION OF POST-ELUTION POTENCY RECOVERY

	Analyst 1		*Analyst 2*	
	Single lot	*8 lots*	*Single lot*	*8 lots*
Mean (% recovery)	104	96	110	104
Rel. std. dev. (%)	2.4	6.9	1.2	4.0

The linearity of the UV detector response at 280 nm to low-molecular-weight urokinase from 0 to 200 000 IU/ml was determined. A correlation of 0.9984 was seen for peak area measurements, and 0.9999 for peak height determinations. The response from 0 to 300 000 IU/ml was examined for the S_2 form. Peak area *versus* potency gave a correlation of 0.9976, while peak height measurements yielded a value of 0.9992.

Two analysts made eight replicate injections of the same material and calculated the potency based on injection of an external standard of known potency. The data are shown in Table II for both peak area and peak height calculations. Similarly, precision data for S_2 in a sample containing approximately 300 000 IU/ml S_1 are given. Linearity and precision data justify the use of peak heights for external standard calculations.

HPLC and Ploug U-4 assays were performed on eleven samples of material containing 50 000–300 000 IU/ml S_1. A paired t-test calculated with peak height assay values yielded t = 0.78 ($t_{0.1}$ = 1.363), indicating essentially no difference in the results obtained by the two methods.

Recovery of S_2 from spiked samples of S_1 (125 000 IU/ml) was demonstrated by comparison with an external standard of pure S_2. Table III contains experimental and calculated results. The limit of detection for S_2 is approximately 1% of S_1 on a potency–potency basis. The slightly high recovery values can be explained in several ways: (i) incomplete resolution of the S_1 and S_2 forms results in some over-estimation of peak heights, (ii) because only small quantities of purified S_2 were available, spikes were prepared by adding a few microliters of solution to S_1 standard, with less than optimal measurement of volumes, and (iii) the precision of measurement of S_2 was 7.2% at the highest level spiked (see Table II), thus all values reported are within the error of measurement.

TABLE II

PRECISION OF POTENCY DETERMINATIONS FOR S_1 AND S_2 BY HPLC

	S_1				*S_2 Peak height*
	Analyst 1		*Analyst 2*		
	Peak area	*Peak height*	*Peak area*	*Peak height*	
Mean (IU/ml)	251 800	217 675	244 000	212 000	20 067
Rel. std. dev. (%)	2.2	1.6	1.8	1.1	7.2

TABLE III

RECOVERY OF S_2 FROM S_1 (125 000 IU/ml)

IU/ml spiked	*IU/ml found*	*IU/ml recovered*	*% recovered*
0	3570	–	–
3914	7932	4362	111
7874	11 898	8328	106
11 590	16 460	12 890	111
15 214	21 021	17 451	115
		Mean	111

To test whether the method was stability-indicating, samples of S_1 material were subjected to refluxing for 3 h in acid, base and neutral aqueous solutions, 105°C for 1 week, and exposure to short-wavelength UV for 3 h. Quantitative recovery was found only for the sample exposed to UV light; in the other conditions, aggregate formation and low-molecular-weight fragments were observed with at best 2–3% recovery of intact S_1. Under refrigeration the sample solutions were stable for several months.

CONCLUSIONS

Besides the immediate consideration of providing a fast, simple assay for urokinase potency determinations, the method has potential value in other areas of biological testing. Post-chromatographic recovery of biological activity has long been a problem in enzyme purification[4]. Normal- and reversed-phase HPLC separations can require harsh conditions such as high pressures and high concentrations of organic modifiers which can induce degradation of the analyte. The conditions encountered in gravity-flow systems are less severe but these methods are expensive in terms of material and time. Long on-column residence times might also affect sensitive proteins. The system described here generates fairly low pressures (200–300 p.s.i. for a 30-cm analytical column) and uses a mobile phase containing no organic constituents. Residence time is under 20 min regardless of temperature, thus cold room conditions can be used with no penalty in throughput time if higher stability is achieved below ambient temperatures.

Compared to classical bioassays, more information can be obtained from an HPLC method since active constituents can be isolated and estimates of their relative amounts made through the use of standards. Although the initial cost of HPLC instrumentation is higher than that for bioassay equipment, long-term cost savings are typical of those seen for HPLC methods with their shorter analysis and hands-on time. The use of autosamplers can significantly reduce analyst time, further increasing the cost savings of this method over other means of activity determinations.

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REFERENCES

1 T. Someno, K. Katoh, K. Niijima and H. Miyazaki, *J. Chromatogr.*, 188 (1980) 185–192.
2 J. Ploug, *Biochim. Biophys. Acta,* 13 (1957) 278.
3 W. A. Gunzler, G. J. Steffens, F. Otting, G. Buse and L. Flohe, *Hoppe-Seyler's Z. Physiol. Chem.*, 363 (1982) 133–141.
4 F. Regnier, *Anal. Chem.*, 55 (1983) 1298A–1306A.