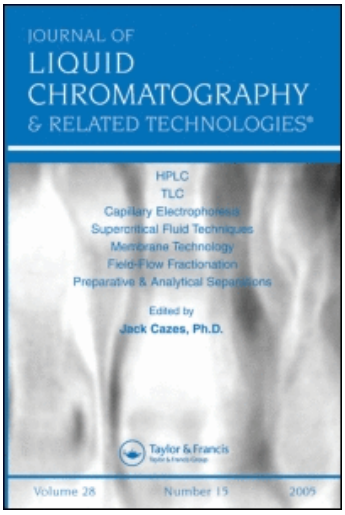

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Assay of Arginine-Esterase Activities by Reversed Phase High Performance Liquid Chromatography

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ASSAY OF ARGININE-ESTERASE ACTIVITIES BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Application of a high performance liquid chromatographic technique to assay of arginine-esterase activities is presented. Enzyme reaction was carried out with benzoyl-L-arginine ethylester as a substrate and analysis was performed on a reversed phase chromatographic system using a μ Bondapak C_{18} or a Radial-PAK A column and buffered aqueous methanol as the mobile phase. The enzyme activities were determined by the peak height of cleaved product (benzoyl-L-arginine). The minimum detection limit for benzoyl-L-arginine was 0.02 nM on each column. The generality of this method was demonstrated by its application to determination of plasmin activity, and so it might be suitable for both kinetic studies and routine assays of plasmin-like esterases.

INTRODUCTION

Several methods are available at present for the determination of plasmin (EC 3.4.21.7)-like esterases such as kallikrein (EC 3.4.21.8), trypsin (EC 3.4.21.4) and other arginine-esterases using various arginine derivatives such as benzoyl-L-arginine ethylester (BAEE) and tosyl-L-arginine methylester (TAME) as substrates.

In these assays, enzyme activities are measured by following the increase in the optical density due to the hydrolyzed product (1,2), or by direct electrometric titration of the carboxyl groups liberated (3,4), by colorimetric determination of hydroxamate-ferric complex (5,6) or the color developed by chromotropic acid reagent (7,8), and also by following the oxidation of alcohol liberated with alcohol dehydrogenase (9).

Recently a new sensitive method for the rapid determination of arginine-esterases using high performance liquid chromatography (HPLC) to separative determination of hydrolyzed product of TAME or BAEE has been reported by Matsumoto et al. (10). However, under the conditions used in their experiments the substrates were retained on the column, and the column condition became worse by repeated injection of incubation mixture.

We now report a rapid and sensitive method for the assay of plasmin using BAEE as a substrate, the product(s) of the reaction being analyzed by reversed phase system of HPLC. This method, as demonstrated by its successful application to plasmin (11) and kallikrein (12) in our laboratory, appears to be of wide utility.

MATERIALS AND METHODS

Reagents

Benzoyl-L-arginine (BA) and BAEE·HCl were purchased from Protein Research Foundation, Osaka, Japan. Human plasmin (25 CU/vial) was obtained from AB KABI, Stockholm, Sweden. High-purity methanol was of Katayama Chemical Industry Co., Ltd., Osaka, Japan. All other chemicals were of analytical grade.

Chromatographic system

Analyses were carried out on a liquid chromatograph consisting of a solvent delivery system (Model

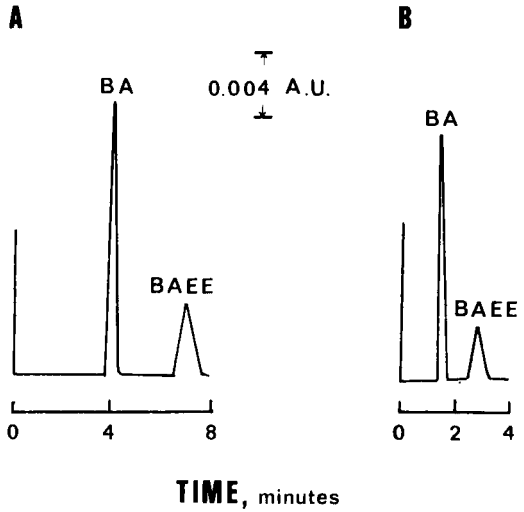

FIGURE 1

Chromatograms of a standard mixture of BA and BAEE (12.5 nM each).
Column: A) μ Bondapak C_{18} (4 mm ID x 30 cm)
B) Radial-PAK A (8 mm ID x 10 cm)
Solvent: A) 0.01 M ammonium acetate, pH 6.8/methanol (50 : 50, v/v)
B) 0.02 M sodium sulfate and acetic acid, pH 4.6/methanol (20 : 80, v/v)
Flow rate: A) 1.0 ml/min. B) 4.0 ml/min.

6000 A, Waters Associates, Milford, Mass.), an injector (Model U 6K, Waters Associates), a reversed phase analytical column (μ Bondapak C_{18} or Radial-PAK A, each from Waters Associates), an absorbance detector (Model 440, Waters Associates) operated at 254 nm, and a 10 mV chart recorder (Type 056, Hitachi, Co., Ltd., Tokyo, Japan). The solvent systems were as indicated in Figure 1. The chromatographic system was operated at room temperature and the flow rates for μ Bondapak C_{18} and Radial-PAK A columns were set at 1.0 ml/min. and 4.0 ml/min., respectively.

Procedure

Enzyme activity was measured by the following procedure. Fifty µl of enzyme solution was added to 250 µl of BAEE solution (10 µM/ml in 0.05 M Tris-HCl buffer to a final volume of 400 µl. The resulting mixture was incubated at 37°C for 30 minutes. After incubation, the reaction was stopped by the addition of 100 µl of 10 % (w/v) trichloroacetic acid, and the solution was filtrated through a 0.45 µm filter (Millipore Corp. Bedford, Mass.). Five µl of the filtrate was then injected into the chromatograph.

RESULTS AND DISCUSSION

Chromatograms of BA and BAEE standards were shown in Figure 1 A and B. These solute peaks were completely resolved and were symmetrical. The minimum detection limits on each column were 0.02 nM for BA and 0.1 nM for BAEE. The apparent differences in elution profiles and recoveries of the two materials were not observed on both columns, but the retention times of them on µ Bondapak C_{18} were shortened within 3.0 minutes by using a Radial-PAK A column. Therefore, the following procedures were carried out on the Radial-PAK A column.

A series of standard samples containing 0 to 5 µM each of BA and BAEE per ml were prepared and 5 µl aliquots were injected. Chromatographic results showed that the peak height versus concentration plots were linear up to 25 nM for each solute (Figure 2). These standard curves were utilized as a calibration curve of enzyme activities.

Practical enzyme assay was done according to the procedure described in MATERIALS AND METHODS. Enzyme activity was determined by measuring the peak height of BA. The column condition was checked by the determina-

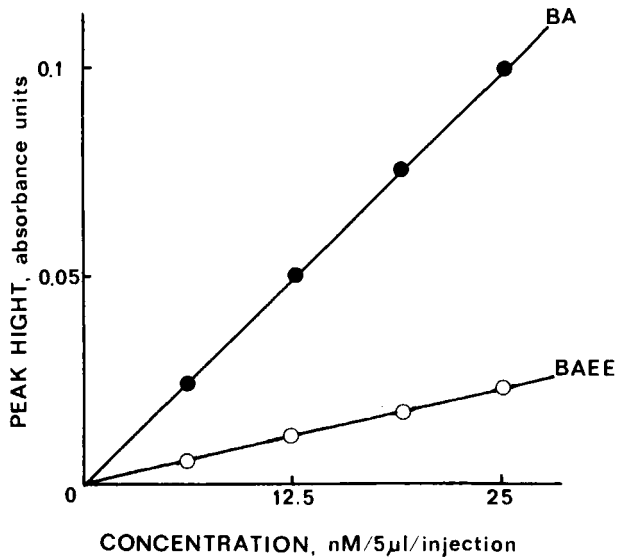

FIGURE 2

Plots of peak height as a function of analytical concentrations of BA and BAEE. Column: Radial-PAK A.

tion of the peak height ratio of BA and BAEE. Figure 3 shows the results of determination of plasmin activity. A good correlation of concentration of the cleaved product (BA) to plasmin activity in the range of 0.0015 - 0.06 CU of plasmin was obtained.

As results, this method might be useful not only for the microdetermination of plasmin, but for the routine assay of it. Therefore, it would be successfully applied to the determination of small amounts of other arginine-esterases in physiological or pathological investigations.

Finally, wide applications of a reversed phase HPLC to chemical assays of the other enzymes by the use of synthetic substrates with ultraviolet absorption are recommended.

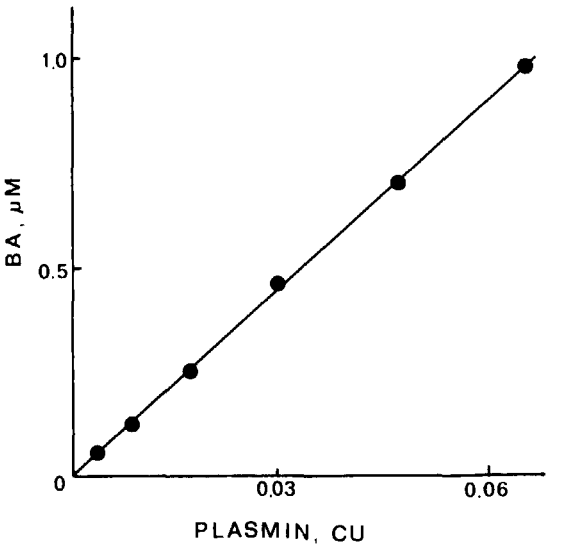

FIGURE 3

Assay of plasmin activity. Column: Radial-PAK A.

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