*Journal of Chromatography*, 566 (1991) 327–332
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5811

# Automated high-performance liquid chromatographic assay of enzymatic activities

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ABSTRACT

High-performance liquid chromatography (HPLC) is a powerful technique which enables a reliable and quantitative determination of enzyme activities. The purpose of the work reported here was to develop an automatic assay of enzymatic activity. Using an automatic sample processor and injector, a program was developed which allows the complete automation of each step of analysis (calibration, enzymatic reaction, HPLC determination). This program can be adapted to different experimental requirements as each step can be performed independently and each input (time, volume, number of standards) is made by answering questions asked by instrument. Using this approach both kinetic and single-point determinations can be carried out, and in the latter case different samples can be analysed sequentially. This paper reports the automated analysis of trypsin.

## INTRODUCTION

The most commonly used techniques for enzyme assays are ultraviolet (UV) and visible spectrophotometry and potentiometry. However, owing to its separating power and versatility, high-performance liquid chromatography (HPLC) represents an interesting alternative [1,2]. An important characteristic of this method is that both the substrate and product can be measured simultaneously, thus eliminating interference from competing secondary reactions.

The strategy used to design an HPLC enzymatic assay is based on the following steps: (1) the development of a chromatographic procedure for the separation of the substrate, product and, if possible, an internal standard; (2) the assessment of the range of linearity between the substrate and product concentrations and the response of the detector; and (3) the study of the reaction and stopping conditions for the HPLC assay. Based on these criteria, several enzymes have been assayed in this laboratory including chymotrypsin [3], NAD-kinase [4] and urokinase [5].

0378-4347/91/$03.50 

The purpose of the work reported here was the development of an automatic method of determination of enzymatic activity. A software program, suitable for various exprimental conditions and which allows complete automation of all the steps involved, has been developed. Using this approach both kinetic and single-point determinations can be carried out, and different samples or kinetics can be analysed sequentially. An example of the automated HPLC assay of trypsin using a microbore column is presented in this paper.

## EXPERIMENTAL

### *Materials*

Trypsin (Type XIII), benzoyl-L-arginine (BA) and benzoyl-L-arginine ethyl ester hydrochloride (BAEE · HCl) were obtained from Sigma (St. Louis, MO, U.S.A.). 7-Hydroxy-4-methylcoumarin (internal standard) was from Boehringer (Mannheim, Germany). Acetonitrile was of HPLC grade (J. T. Baker, Deventer, The Netherlands). HPLC-grade water was from Elgastat Ultra High Quality (Elga, U.K.) and was filtered through a 0.45-$\mu$m membrane (Type HA, Millipore, Bedford, MA, U.S.A.).

### *Chromatographic conditions*

The HPLC system consisted of a Model 510 pump and an Model 680 automated controller (Waters Assoc., Milford, MA, U.S.A.) together with an HP 1040A photodiode array detector (Hewlett-Packard, Waldbronn, Germany). The acquisition of UV spectra was automatic at the apex, both inflexion points and at the base of all peaks (200–400 nm, step 2 nm).

For each automation step, consisting of the injection of the samples, a Model 232 automatic sample processor and injector (ASPI) with a Model 401 dilutor (Gilson Medical Electronics, Villiers-le-Bel, France) was used. The system was equipped with a Rheodyne 7010 injector and thermostated Gilson racks.

The column used was a $\mu$Bondapak $C_{18}$ (300 mm × 2 mm I.D., 10 $\mu$m; Waters Assoc.). The eluent was 10 m*M* sodium dihydrogenphosphate buffer, pH 2.8–acetonitrile (70:30, v/v) at flow-rate of 0.4 ml/min. The UV detection was at 230 nm.

### *Automation*

All the calibration and enzyme reaction steps have been automated as follows (Fig. 1).

*Calibration.* Increasing amounts of stock solutions (up to three compounds and one internal standard) were dispensed into vials by means of the ASPI, then mixed and diluted to prepare the calibration points. For the trypsin assay 10–60 $\mu$l of BAEE · HCl (1.4 m*M*) and BA (0.2 m*M*) were used. A 10-$\mu$l volume of internal standard (5.6 m*M*) was added to each tube and the sample was diluted to 0.5 ml with water. Automatic injections of 5 $\mu$l were made for each vial.

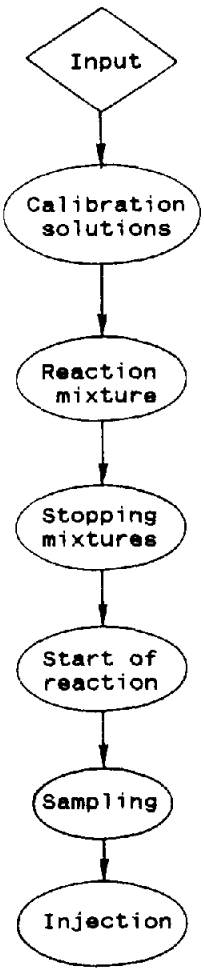


Fig. 1. Flow diagram of steps in the automated enzyme assay.

*Assay of enzyme activity.* Using the ASPI, 200 μl of substrate (16 m*M*) and 800 μl of 50 m*M* Tris–HCl buffer (pH 8.0) were mixed. A 5–25 μl volume of enzyme solution (protein 3 mg/ml) was then added to start the reaction (25°C).

For each determination of the kinetics, one reaction mixture was prepared and at fixed intervals aliquots of 10 μl were acidified by a 50-fold dilution in the stopping mixture (10 μl of internal standard and 20 m*M* HCl, pH 3.1). Automatic replicate injections were made. The experimental volumes were transferred by a mobile arm equipped with a needle.

The HPLC data were acquired and evaluated using the sequence program of the photodiode array detector.

## RESULTS AND DISCUSSION

The Gilson Model 232 autosampler combined with software developed in this laboratory allowed the sequence for enzymatic assay to be performed automatically, as shown in Fig. 1. The process variables (volume, time, number of samples) were input manually. For the kinetic study of the enzyme acivity, the vials were placed sequentially in the thermostated enzyme activity racks (Fig. 2).

Fig. 3 shows the isocratic elution and the peak spectra of the substrate, product and internal standard in the automatic assay of trypsin activity. Using a microbore column it was possible to obtain a high sensitivity; linear relationships between peak area and the amount of injected standards were in the range 50–1000 pmol for BAEE ($r$ = 0.999) and 10–300 pmol for BA ($r$ = 1.000).

Tripsyn yielded a significant amount of BA during the reaction, sufficient for the quantitative determination of enzyme activity (Fig. 4). The kinetics were linear within a 10-min incubation time when trypsin ranged between 1 and 10 $\mu$g (Fig. 5). Reproducible results (S.D. 1.9%) were obtained in the assay of the specific activity of various amounts of enzyme. The automatic HPLC data (see Fig. 5) were in good agreement with those obtained by the non-automated HPLC procedure ($\pm$ 1.2%) and the titrimetic methods ($\pm$ 1.5%).

With minor modifications of the program it is possible to fully automate a series of kinetic (about 10–20) or single-point assays of enzyme activity (50–100 samples) for sequential analysis.

In conclusion, the enzymatic assay described here adds further advantages to the HPLC techniques. The advantages are the result of the use of simple comput-

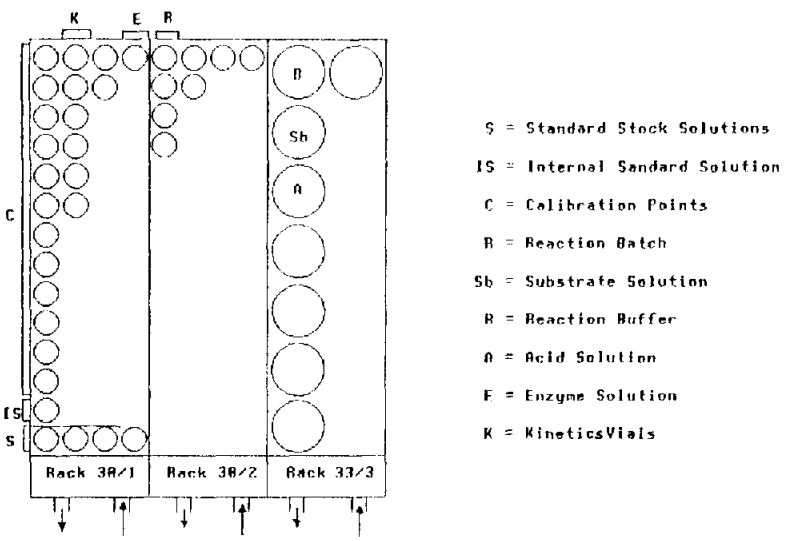


Fig. 2. Design of the automatic apparatus for the enzyme assay.

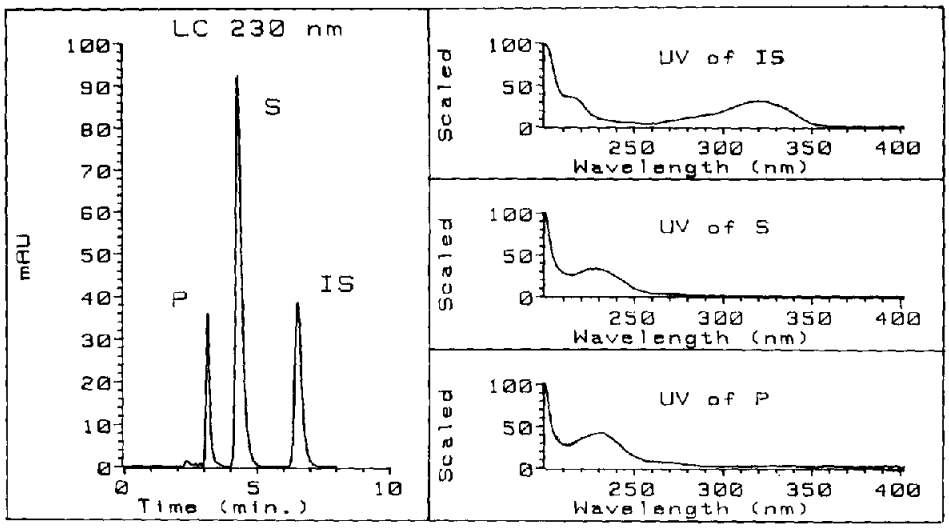


Fig. 3. (A) Typical chromatogram and ultraviolet spectra of (B) 7-hydroxy-4-methylcoumarin (IS), (C) benzoyl-L-arginine ethyl ester (S) and (D) benzoyl-L-arginine (P). Column: μBondapak $C_{18}$. Eluent, 1 m*M* sodium dihydrogenphosphate pH 2.8–acetonitrile (70:30, v/v). Flow-rate, 0.4 ml/min. Detection, 230 nm.

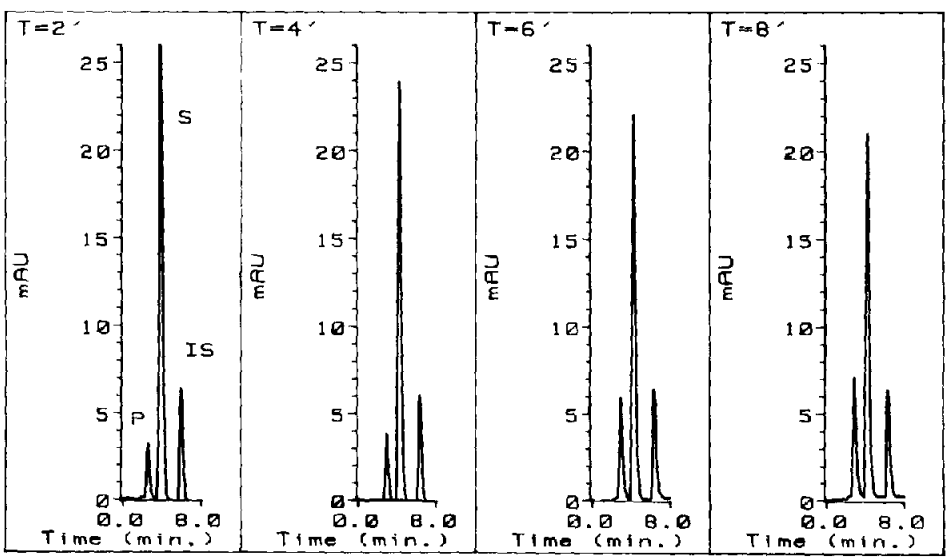


Fig. 4. Chromatograms of the reaction mixture at various incubation times (T). See Fig. 3 for chromatographic conditions.

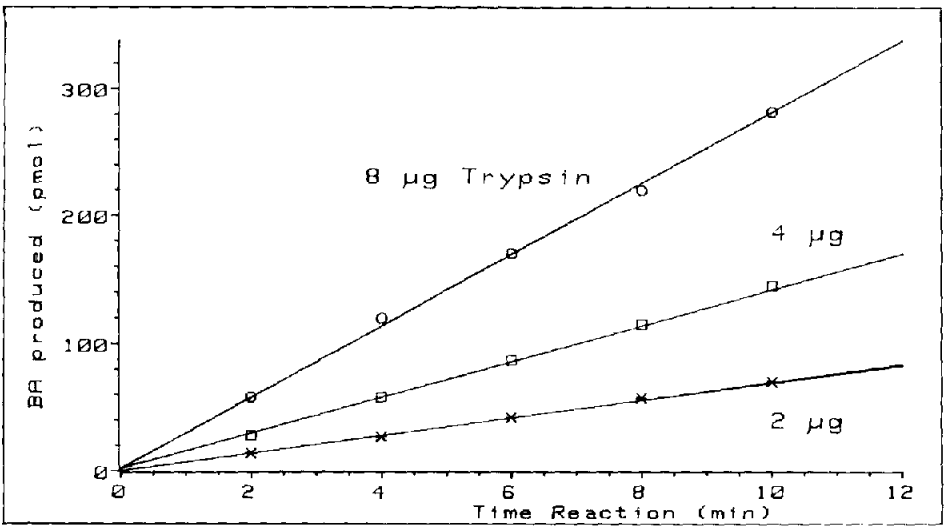


Fig. 5. Picomoles of benzoyl-L-arginine (BA) produced as a function of the amount of enzyme.

er software and an automated device. These advantages can be summarized as follows: (1) the software is suitable for various experimental requirements: (2) the introduction of experimental parameters is simple to perform; (3) there are less human errors: (4) continuous routine assays and kinetic studies are possible; and (5) the methods are time-saving (more assays are possible per unit time).

It is also possible to connect the automated HPLC method of enzyme assay to the FADIA program [6,7], software which allows the calculation of enzyme activities using the ratio of substrate/product instead of an internal standard.

## REFERENCES

1 E. F. Rossomando, *High Performance Liquid Chromatography in Enzymatic Analysis*, Wiley, New York, 1987.
2 P. R. Brown and A. M. Krstulovic, *Anal. Biochem.*, 99 (1979) 1.
3 P. G. Pietta, P. L. Mauri and M. Pace, *J. Chromatogr.*, 411 (1987) 498.
4 M. Pace, P. L. Mauri, C. Gardana and P. G. Pietta, *J. Chromatogr.*, 476 (1989) 487.
5 P. G. Pietta, P. L. Mauri and M. Pace, *Chromatographia*, 24 (1987) 439.
6 P. L. Mauri, M. Bitonti, M. Pace, P. G. Pietta and D. Agnellini, *Comput. Appl. Biosci.*, 6 (1990) 395.
7 M. Pace, P. L. Mauri, P. G. Pietta and D. Agnellini, *Anal. Biochem.*, 176 (1989) 437.