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Note

Improved high-performance liquid chromatographic assay for trypsin

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The assay of arginine esterases, using benzoyl-L-arginine ethyl ester (BAEE) and tosyl-L-arginine ethyl ester (TAME) as substrates, has been carried out by colorimetric[1], spectrophotometric[2] and titrimetric[3] methods. High-performance liquid chromatography (HPLC) has been also introduced[4] to the analysis of plasmin and kallicrein as models for arginine esterases. This method is based on the separation of BAEE and benzoyl-L-arginine (BA) on a reversed-phase column, followed by their determination using an external standard. However, the suggested chromatographic conditions were found to be unsuitable for routine analysis of trypsin.

We have developed an improved reversed-phase HPLC assay using 0.01 *M* phosphate buffer (pH 2.7)–acetonitrile as eluent and adenosine as the internal standard.

EXPERIMENTAL

Materials

Benzoyl-L-arginine ethyl ester hydrochloride (BAEE · HCl), benzoyl-L-arginine (BA) and trypsin (Type XIII) were obtained from Sigma (St. Louis, MO, U.S.A.). Adenosine was from Boehringer (Mannheim, F.R.G.). Acetonitrile was of HPLC grade (Chromasolv; Riedel de Haën, Hannover, F.R.G.). Water was distilled from glass apparatus and filtered through a 0.45-μm membrane (Type HA, Millipore). All other chemicals were of analytical grade.

Chromatographic conditions

The HPLC system consisted of a Model 6000 A pump, equipped with a Model U6K universal injector, a Model 440 ultraviolet detector and a Model 730 Data Module (Waters Assoc., Milford, MA, U.S.A.). Separations were accomplished on μBondapak C_{18} from Waters Assoc. The eluent was 0.01 *M* sodium dihydrogenphosphate buffer, pH 2.7–acetonitrile (70:30) at a flow-rate of 1 ml/min. The column effluent was monitored by UV absorption at 254 nm (0.05 a.u.f.s.).

Calibration curves

Into individual test-tubes (1 ml) were pipetted known volumes (20–200 μl) of BAEE · HCl (1.82 m*M*) and BA (0.73 m*M*) aqueous solutions. After addition of the

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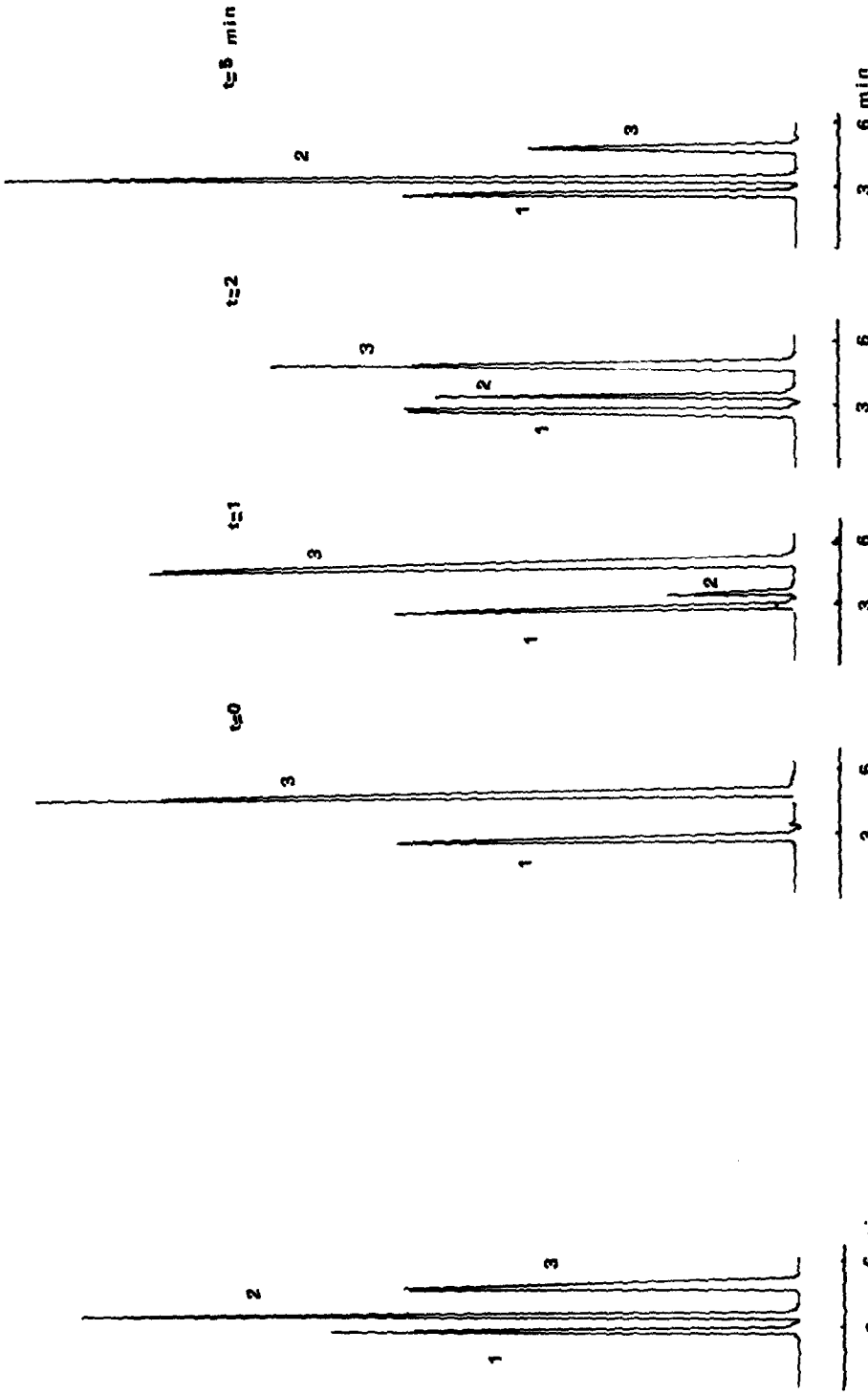

Fig. 1. Typical chromatogram of adenosine (1), BA (2) and BAEE (3). See text for the chromatographic conditions.

Fig. 2. HPLC of the reaction mixture after different incubation times. See text for chromatographic conditions.

internal standard (180 μl of 0.05 m*M* adenosine in water), replicate injections of 10 μl were made for each sample.

Stability of BAEE · HCl solutions

5 m*M* BAEE · HCl solutions were kept at 25°C for 1 h at pH 8 or 2.6. At 10-min intervals, 50-μl aliquots were diluted to 500 μl in the internal standard solution (0.022 m*M* adenosine in 0.004 m*M* hydrochloric acid), and 10-μl aliquots were repetitively injected in the course of 24 h.

Assay of trypsin activity

HPLC Method. 2.0–5 m*M* BAEE · HCl in 50 m*M* Tris–HCl buffer pH 8 was equilibrated at 25°C, then the reaction was started by the addition of trypsin (2.5, 5.0, 7.5 μg). At 2-min intervals, 50 μl were acidified by a ten-fold dilution in the internal standard solution (0.022 m*M* in 0.004 m*M* hydrochloric acid pH 2.6), and replicate injections of 10 μl were made.

Titrimetric method. Trypsin activity was measured at 25°C and pH 8 by automatic titration of the hydrogen ions produced during the hydrolysis of BAEE with an Autotitrator TTT80 equipped with an autoburette ABU 80 (Radiometer, Copenhagen, Denmark). The titration was carried out with 10 m*M* sodium hydroxide in a nitrogen atmosphere. The reaction mixture was 2.0–5.0 m*M* BAEE · HCl in 20 m*M* calcium chloride pH 8 or in 1.5 m*M* borate buffer containing 20 m*M* calcium chloride pH 8 according to the Fédération Internationale Pharmaceutique (FIP) method[5]. The reaction was started by the addition of 5–15 μl of trypsin solution (5 mg/ml in 1 m*M* hydrochloric acid) and the titration was automatically recorded by a Radiometer Rec 80 Servograph.

RESULTS AND DISCUSSION

Various columns for reversed-phase HPLC were examined for the separation of the assay mixture, such as RP-18 Speri 5 (100 mm × 4.6 mm) (Kontron, Zürich, Switzerland), Bio-Sil ODS-5S (250 mm × 4 mm) (Bio-Rad Labs., Richmond, CA, U.S.A.) and μBondapak C_{18} (Waters Assoc.). The last column gave the most satisfactory separation of the peaks using sodium dihydrogenphosphate buffer pH 2.7–acetonitrile (70:30) as eluent. A flow-rate of 1 ml/min was optimal since separations could be obtained in less than 6 min (Fig. 1).

Standard samples were analyzed according to the procedure described under *Calibration curves* and the detector response for both BAEE and BA was linear for a concentration range of 0.1–8 nmol ($r = 0.9996$) and 0.05–3.2 nmol ($r = 0.9995$), respectively. The experimental incubation showed (Fig. 2) significant formation of BA during the reaction. In a control incubation, in which the enzyme solution was not added, a small amount of BA was formed at pH 8 (0.002 μmol/min), while at pH 2.6 no hydrolysis was detected within 24 h. The activity of trypsin was calculated from the enzymatically obtained BA using the equation

$$\text{specific activity } (\mu\text{mol/min} \cdot \text{mg}) = \frac{a - b}{c}$$

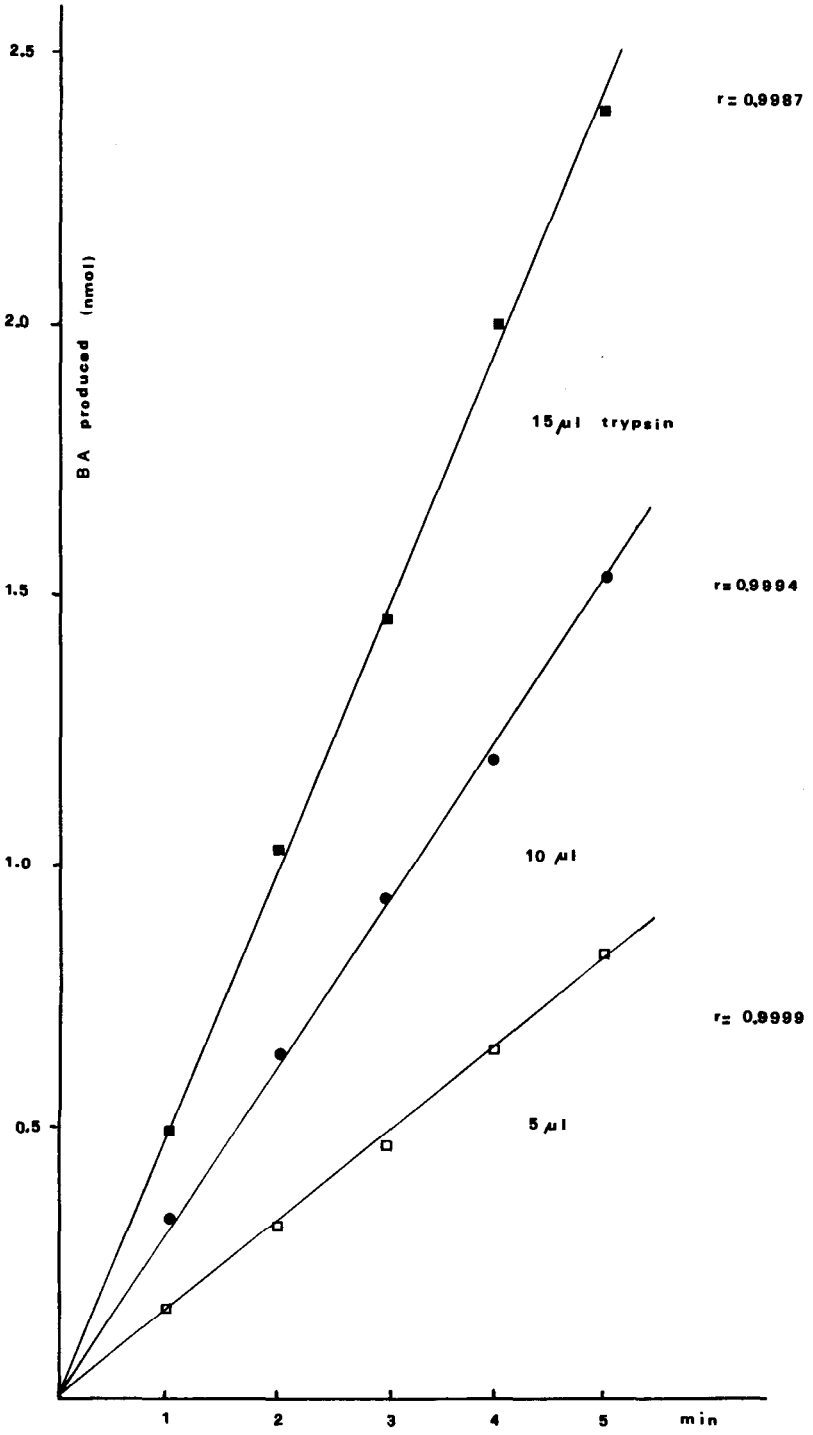

Fig. 3. Correlation of the amount of BA produced to the volume of trypsin employed.

where a = μmol/min of BA produced overall, b = μmol/min of BA produced by spontaneous hydrolysis and c = mg of trypsin.

A linear correlation between the amount of BA produced and the activity was observed in the range of 2–10 mg/ml of trypsin. Under these conditions the production of BA was proportional to the incubation time for the first 5 min (Fig. 3). The enzyme activity was then routinely obtained from a one-point determination, stopping the reaction after 2 min of incubation. According to this procedure, multiple determinations of commercial samples of trypsin gave reproducible values with a standard deviation of 2.4%.

To evaluate the HPLC assay, these values were compared with those obtained by the titrimetric method, which is the official procedure adopted by FIP[5]. The results obtained with the two methods were in good agreement, the differences being of the order of 1.6%.

In conclusion, the present HPLC method is reproducible, rapid and, due to the availability of the HPLC technique in analytical laboratories, represents a valuable alternative to the titrimetric methods.

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