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Separation of leucinostatins by capillary zone electrophoresis

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ABSTRACT

A capillary zone electrophoretic method was used to separate some leucinostatins, the nonapeptides obtained by submerged cultures of *Paecilomyces marquandii* or *Paecilomyces lilacinus*. These compounds are of pharmaceutical interest for their remarkable antibiotic, cytotoxic and phytotoxic activities. The proposed method allows the separation of leucinostatins from very complex mixtures and shows high efficiency, good resolution and a very short analysis time.

INTRODUCTION

Recently we reported the isolation and structural elucidation of several components of a new family of peptides, named leucinostatins followed by alphabetical letters (Fig. 1), produced by submerged cultures of *Paecilomyces marquandii* [1–6]. Leucinostatins A, B and D have been independently found by Japanese workers in the cultural broth of *Paecilomyces lilacinus* A-257 [7,8]. These peculiar nonapeptides show very interesting antibiotic, cytotoxic and phytotoxic properties.

As the separation of these compounds presented some difficulties because of their very similar R_F values (Leucinostatin F could in fact only be obtained by preparative thin-layer chromatography [6], we tried to improve their separation by applying capillary zone electrophoresis (CZE), as this technique has already been successfully applied in protein and peptide analyses [9–15] and to charged and/or uncharged molecules [16]. This paper describes the use of CZE to separate leucinostatins in very complex mixtures with excellent efficiency, high resolution and a short analysis time.

EXPERIMENTAL

The leucinostatins were kindly provided by the Institute of Medicinal Chemistry and Pharmaceutical Techniques of Perugia University. Other reagents and solvents were supplied by Carlo Erba.

A Bio-Rad Labs. (Richmond, CA, USA) HPE 100 apparatus equipped with a UV detector with a deuterium lamp (190–380 nm) was used. The apparatus was equipped with a power supply able to deliver up to 12 kV. Sampling and electrophoresis were controlled by a microprocessor. Separation of the leucinostatins was performed in a Bio-Rad Labs. Model 148-3002 HPE capillary cartridge (20 cm × 0.025 mm, coated).

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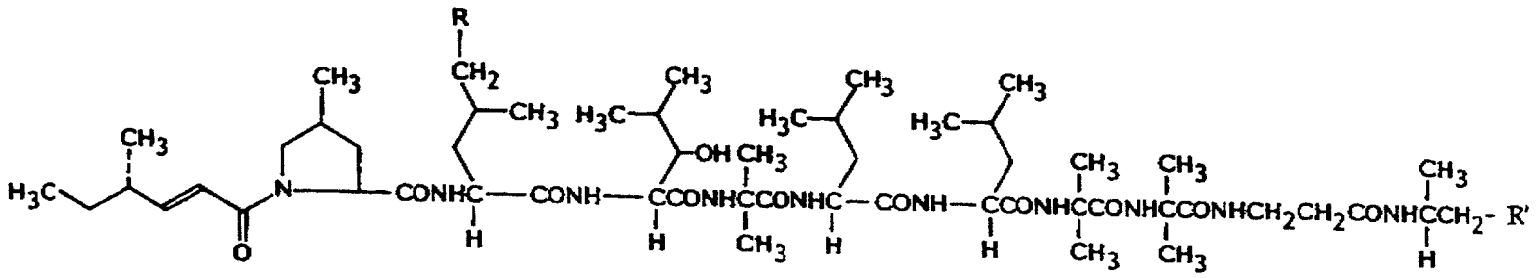

Leucinostatin A	R = $CH(OH)CH_2COCH_2CH_3$	R' = $N(CH_3)_2$
Leucinostatin B	R = $CH(OH)CH_2COCH_2CH_3$	R' = $NHCH_3$
Leucinostatin C	R = $CH(OH)CH_2COCH_2CH_3$	R' = NH_2
Leucinostatin D	R = H	R' = $N(CH_3)_2$
Leucinostatin F	R = H	R' = $NHCH_3$
Leucinostatin H	R = H	R' = $NO(CH_3)_2$
Leucinostatin K	R = $CH(OH)CH_2COCH_2CH_3$	R' = $NO(CH_3)_2$

Fig. 1. Structures of leucinostatins. Et = Ethyl; Me = methyl.

The electrophoretic experiments were carried out at 8 kV (constant) and 15 μA. Sampling was performed by using the electrophoretic application method at 7 kV for 6 s. The capillary was filled with the background electrolyte (BGE) of 0.1 *M* phosphate buffer (pH 2.5) by using a 100-μl Hamilton microsyringe. Electropherograms were recorded with an LKB Model 2210 line recorder at a chart speed of 10 mm/min. UV detection was carried out at 206 nm.

Separate acetonitrile–water (1:1, v/v) solutions of leucinostatin A, B, D, F, H and K (1 mg/ml) were prepared. The samples for analysis were obtained by transferring 1 ml of each solution into a 30-ml volumetric flask and diluting to volume with the same solvent mixture.

N-Methylation by phase-transfer catalysis of leucinostatin A was applied to give the methylammonium salt [Fig. 1, R′ = $N^+(CH_3)_3$]. The reaction was carried out by using an equivalent amount of iodomethane in dry dichloromethane in the presence of powdered potassium hydroxide and tetrabutylammonium bromide as catalyst with stirring at room temperature.

RESULTS AND DISCUSSION

Several electrolyte systems were tested in order to optimize the separation conditions. As the leucinostatins considered have very similar structures they therefore need specific methods for their separation. The best conditions were realized using phosphate buffer (pH 2.5). With this BGE all leucinostatins moved as cations with a relative high velocity.

Working standard solutions were prepared with leucinostatins in order to verify the specificity of the proposed method. Fig. 2 shows the electropherogram of a mixture containing leucinostatin A, B, D and K. Whereas leucinostatin D and K are well separated, A and B are unresolved. The separation of the latter two leucinostatins is possible only when the methylammonium salt of leucinostatin A is formed (Fig. 3).

The electropherogram of a mixture of several leucinostatins (Fig. 4) shows partial overlapping of the peaks of leucinostatin F and K; however this partial separation can be considered satisfactory for the detection of such a complex mixture of compounds.

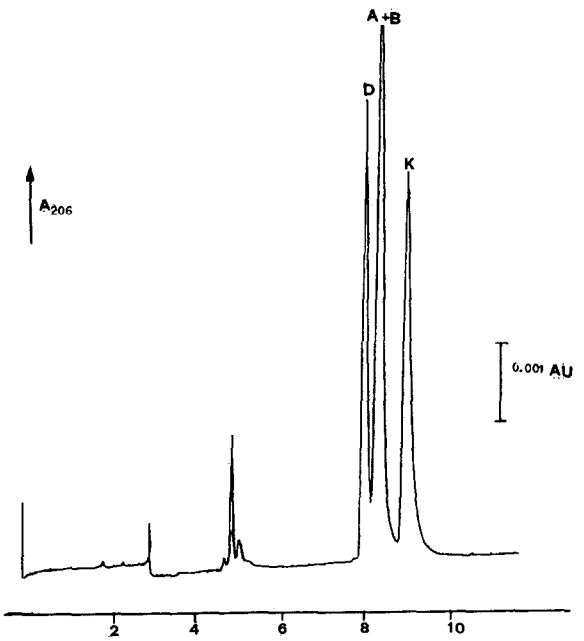

Fig. 2. Electropherogram of the separation of leucinostatin A, B, D and K. BGE: 0.1 *M* phosphate buffer (pH 2.5). Sampling: electrophoresis at 7 kV for 6 s. The mixture contained $3.33 \cdot 10^{-2}$ mg/ml of each leucinostatin. Electrophoretic experiment: 8 kV; 15 μA. Time scale in min.

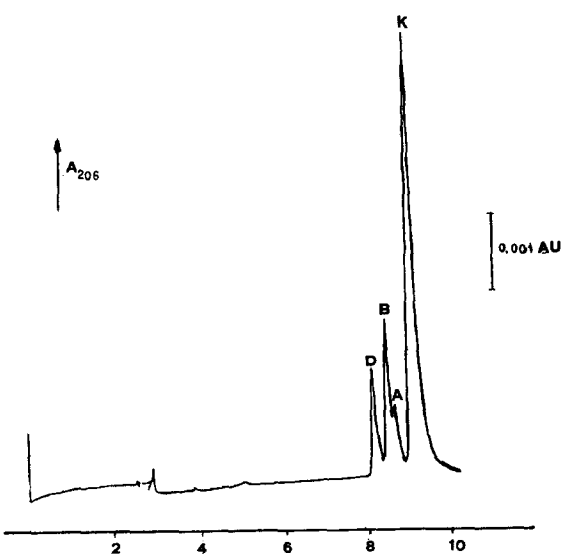

Fig. 3. Electropherogram of the separation of leucinostatin A (methylammonium salt), B, D and K. Conditions as in Fig. 2.

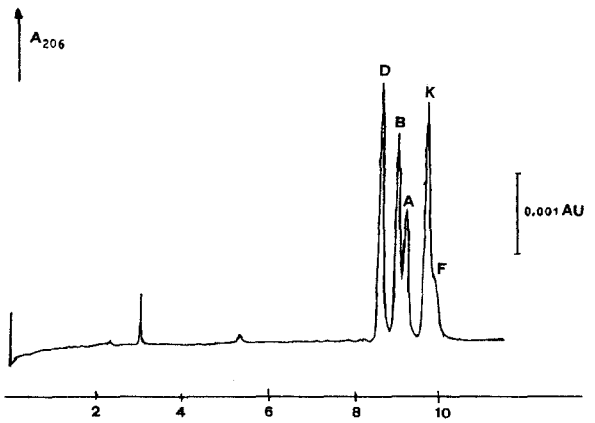

Fig. 4. Electropherogram obtained from a mixture of leucinostatin D, B, A (methylammonium salt), K and F. Conditions as in Fig. 2.

Using the same working conditions, a good separation of leucinostatin A, D, H and K was achieved (Fig. 5).

Additionally the proposed CZE method allows the purity control of leucinostatins extracted from culture broth. The electropherogram for to a crude sample of leucinostatin B (Fig. 6) shows very clearly that other compounds are also present. The latter

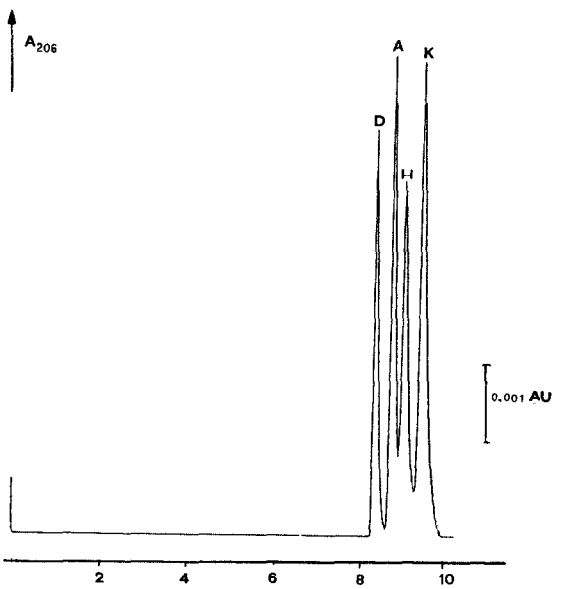

Fig. 5. Separation of leucinostatin D, A, H and K. Conditions as in Fig. 2.

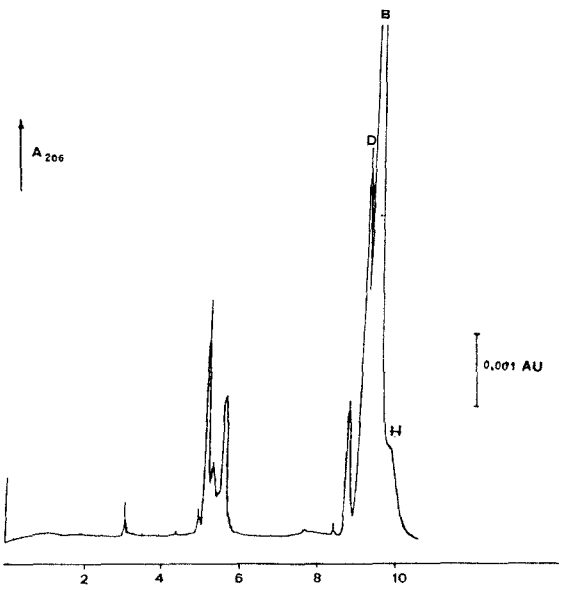

Fig. 6. CZE analysis of a crude leucinostatin B sample. Leucinostatin D and H are also present. Conditions as in Fig. 2.

were characterized by enrichment with leucinostatin standards. In this way it was possible to identify leucinostatins D and H; the peak at 8.8 min is probably due to an unknown leucinostatin, whereas the remaining peaks between 3 and 6 min are not leucinostatins.

The results demonstrate CZE is a sensitive, specific and rapid method which allows the identification, with good resolution, of leucinostatin A, B, D, H, K and partially F in only 10 min. The possibility of distinguishing between leucinostatin A and B using their ammonium salt formation probably would not be easy in a crude sample where other leucinostatin ammonium salts could form.

Leucinostatins have been shown to be very toxic [17] and as these compounds are produced by a microorganism which may also grow on stored foodstuffs [18], the detection of these peptides is very important.

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