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An approach to on-line electrospray mass spectrometric detection of polypeptide antibiotics of enramycin for high-speed counter-current chromatographic separation

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ABSTRACT

In the field of pharmaceutical and biomedical analysis of peptides, a rapid on-line detection and identification for a methodology have been required for the discovery of new biological active products. In this study, a high-speed counter-current chromatography with electrospray mass spectrometry (HSCCC/ESI-MS) was developed for the on-line detection and purification of polypeptide antibiotics of enramycin-A and -B. The analytes were purified on HSCCC model CCC-1000 (multi-layer coil planet centrifuge) with a volatile solvent of two-phase system composed of *n*-butanol/hexane/0.05% aqueous trifluoroacetic acid solution (43/7/50, V/V/V), and detected on an LCMS-2010EV quadrupole mass spectrometer fitted with an ESI source system in positive ionization following scan mode (*m*/*z* 100–2000). The HSCCC/ESI-MS peaks indicated that enramycin-A (major *m*/*z* 786 $[M+3H]^{3+}$ and minor *m*/*z* 1179 $[M+2H]^{2+}$) and enramycin-B (major *m*/*z* 791 $[M+3H]^{3+}$ and minor *m*/*z* 1185 $[M+2H]^{2+}$) have the peak resolution value of 2.9 from 15 mg of loaded enramycin powder. The HSCCC collected amounts of the peak fractions were additionally 4.3 mg (enramycin-A), and 5.9 mg (enramycin-B), respectively. These purified substances were analyzed by LC/ESI-MS with scan positive mode. Based on the LC/ESI-MS chromatograms and spectra of the fractions, enramycin-A and -B were estimated to be over 95% purity. The overall results indicate that this approach of HSCCC/ESI-MS is a powerful technique for the purification and identification of bioactive peptides.

1. Introduction

Peptides are an important group of compounds that play a significant role in physiological processes, molecular biology, antibiotic effects and clinical research. For the pharmaceutical techniques, the separation, detection and purification of peptides are considered to be the most important processes than others [1,2]. The principle of chromatographic separation is based on the interaction of the solutes with the solid support and the mobile phase. The HPLC separation mode has been used for the pharmaceutical and/or biomedical analysis of various peptides. Specially, the HPLC separations coupled with mass spectrometry (LC/MS) are an important tool for the analysis of various peptides. The recent LC/MS methods (nano-, multi-dimensional LC, UPLC with MS and/or MS/MS) are advantaged for the separation and detection, but awkward to use for the large-scale purification of various peptides. Recently, Azevedo et al. [3] discussed that simple liquid–liquid extraction technique was more useful for the purification of bio-molecules, such as monoclonal antibodies, growth factors and hormones with an easy scale-up and continuous operation mode than chromatographic methods. However, the advances in chromatographic method with MS detector will continue to be the corner stone for the identification of various peptides. Thus, the efficient, useful, automatic, simple and possible large-scale chromatographic techniques with MS would be needed for the separation, detection and purification of peptides in pharmaceutical researches.

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support, and has been widely used in preparative separation and purification of natural products [4]. In addition, it was reviewed that HSCCC was useful to separate and purify various components from antibiotic complexes [5,6]. Recent study was reported that HSCCC system was efficiently applied to separate the biopolymers (peptide, nucleic acid, and protein) [7]. Moreover, HSCCC with electrospray ionization MS (HSCCC/ESI-MS) was applied

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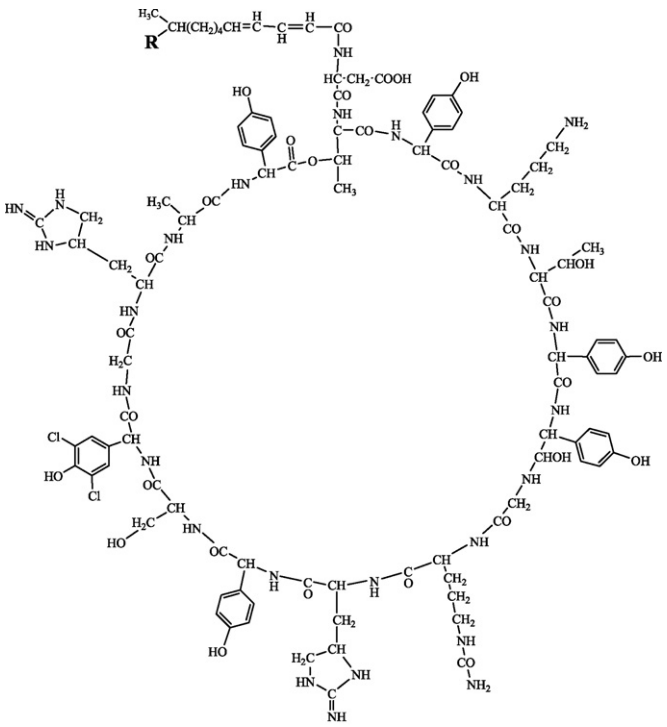

Fig. 1. Structural formulae of the main compounds of enramycin-A and -B.

for continuous MS data acquisition, and 'target'-guided fractionation of small molecular such as polyphenolic compounds [8], and coumarins [9]. An HSCCC/ESI-MS technique to separate and purity the bioactive peptides, however, has not been reported. Here we report the development of an HSCCC/ESI-MS method for the separation and purification of polypeptide antibiotics of enramycin. Enramycin as a model mixture is a linear-ring peptide antibiotic produced by *Streptomyces fungicidicus* that was first isolated in 1967 [10], and consists of two major components named enramycin-A and -B (Fig. 1). It is highly stable in dry form and in aqueous solution with a pH 3.5–7.5. Enramycin has an absorption maximum near 230 nm, but the intensity is not entirely sufficient for evaluation [11]. Thus, the MS is better than UV detector for the identification and determination of enramycin. On the other hand, the separation of enramycin-A and -B was reported to use HPLC on Nucleosil $5C_{18}$ with 0.05 mol/L NaH_2PO_4/acetonitrile (65/35, V/V) [12]. Castiglione et al. [13] suggested that the determination of the structure and conformation of enramycin is an important first step in addressing the problem of how its antimicrobial action is exerted. They reported the complete ^1H NMR resonance assignments together with the spatial structure of enramycin-A determined by molecular modeling calculations [13]. Based on this result, the preparative fractionation and/or purification of enramycin-A and -B from a crude drug formulation are important experiments for the conformation of 3D structure using NMR.

In this study, an HSCCC/ESI-MS method was developed for the separation, detection and purification of polypeptide antibiotics of enramycin-A and -B from pharmaceutical material. This HSCCC/ESI-MS approach indicates the simple, rapid, useful and methodologies for the separation, detection and purification of bioactive peptides.

2. Experimental

2.1. Reagents and standard solution

Enramycin was supplied by Takeda Pharm. Co, Ltd. (Osaka, Japan), and the structural formulas of its main components are shown in Fig. 1. Stock solution (1.0 mg/mL) was prepared by dissolving the appropriate amount of standard in water/methanol (50/50, V/V). The standard solutions (10 μg/mL) were prepared by diluting an aliquot of the stock solution with water/methanol (50/50, V/V) for the determination of the partition coefficient (K) of enramycin.

HPLC-grade water, *n*-butanol, methanol, acetonitrile, hexane, ethyl acetate, formic acid (FA; 99%, LC/MS-grade) and trifluoroacetic acid (TFA; 98%) were obtained from Wako Chemical Co., Inc. (Osaka, Japan). Purified water was obtained from a Milli-Q purifying system (Millipore, Bedford, MA. USA).

2.2. LC/MS equipment and conditions for the determination of enramycin

LC/MS was performed using an LCMS-2010EV system (Shimadzu Co., Kyoto, Japan) that was coupled to a quadrupole mass spectrometer fitted with an ESI source. LC separation was performed using a TSK-GEL ODS 100 V column (2.0 mm × 150 mm, 3 μm: Tosoh Co., Tokyo, Japan). The mobile phase consisted of 0.1% aqueous FA (Solvent A) and 0.1% FA in acetonitrile (Solvent B). The LC linear gradient was as follows: 20% Solvent B at 0 min, 60% Solvent B at 30 min, 100% Solvent B at 30.1 min, 100% Solvent B at 35.0 min, and 20% Solvent B at 35.1 min with a flow-rate of 0.2 mL/min. The injection volume was 10 μL. The column temperature was 40 °C. The mass spectrometer was operated with an electrospray source in positive ionization and single ion monitoring (enramycin-A, $[M+3H]^{3+}$ m/z 786; enramycin-B, $[M+3H]^{3+}$ m/z 791) modes for analytical state. The ESI source conditions were: nebulizer gas rate of 0.18 L/min, CDL temperature of 230 °C, block temperature of 200 °C, probe voltage of +1.5 kV, interface temperature of 250 °C, and 1-s event time, respectively, and were obtained from a nitrogen source (N_2 Supplier Model 24S, Anest Iwata Co., Yokohama, Japan). In data analysis state, the acquired chromatogram was shown using TIC mode in fragment table. This LC/MS system was operated using LC/MS solution ver. 3.41–324.

2.3. Evaluation of the K and separation factor (α) values of enramycin-A and -B

In this study, we developed more sensitive evaluation of K and α values using the low dose of two-phase solvent system and high sensitive LC/ESI-MS with SIM mode than others. First, 1 mL of standard solution (10 μg/mL) in test tube was evaporated to dryness at 30 °C. These residues were added to the two mutually equilibrated solvent phases (0.5 mL each; see Table 1) in a test tube, and mixed to equilibrate. After settling, equal volumes of the upper and lower phases (50 μL each) were transferred into separate test tubes and evaporated to dryness at 30 °C. Then, the samples were adjusted with 50 μL of water/methanol (50/50, V/V) and measured by LC/ESI-MS with SIM mode. Each phase was assessed by LC/ESI-MS and the area of each SIM peak (m/z 786 for enramycin-A, and m/z 791 for enramycin-B) was used to determine the K values for the each component. The K value was calculated as "K = (SIM response of enramycin-A or -B in upper phase solvent)/(SIM response of enramycin-A or -B in lower phase solvent)" [4]. The α value was calculated as "$\alpha = K_m/K_n$, $K_m > K_n$" for enramycin-A and -B.