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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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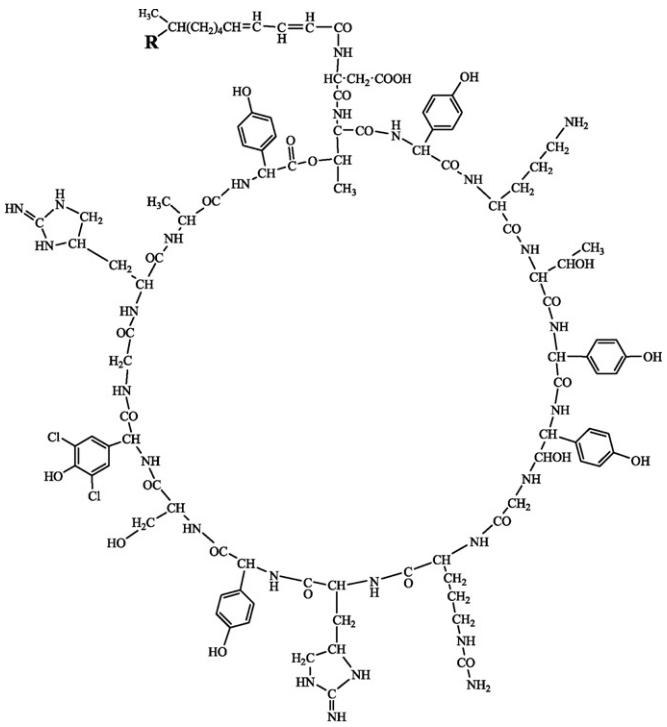

Fig. 1. Structural formulae of the main compounds of enramycin-A and -B.

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.

Table 1
Two-phase solvent system ratio of ethyl acetate/*n*-butanol/hexane/water (V/V/V) for enramycin.

Ratio (V/V/V)	Concentration of TFA in water (%)	Partition coefficient (*K*)		Separation factor (α)
		Enramycin-A	Enramycin-B	
Ethyl acetate/*n*-butanol/hexane/water[a]				
50/0/0/50	0	0	0	0
40/10/0/50	0	0	0.1 ± 0.1	0
30/20/0/50	0	0.1 ± 0.1	0.2 ± 0.1	1.4 ± 0.3
20/30/0/50	0	0.5 ± 0.2	0.8 ± 0.5	1.7 ± 0.3
10/40/0/50	0	1.2 ± 0.5	2.1 ± 0.6	1.9 ± 0.3
50/0/0/50	0.1	0	0	0
40/10/0/50	0.1	0.1 ± 0.1	0.1 ± 0.1	1.8 ± 0.1
30/20/0/50	0.1	3.5 ± 0.8	8.9 ± 2.2	2.6 ± 0.6
20/30/0/50	0.1	17.4 ± 1.0	69.9 ± 7.9	4.0 ± 0.2
10/40/0/50	0.1	26.9 ± 5.1	64.3 ± 11.6	2.4 ± 0.2
50/0/0/50	0.01	0	0	0
40/10/0/50	0.01	0	0	0
30/20/0/50	0.01	0.4 ± 0.1	0.8 ± 0.4	2.3 ± 0.8
20/30/0/50	0.01	3.0 ± 0.6	9.8 ± 3.7	3.2 ± 0.6
10/40/0/50	0.01	4.7 ± 0.4	14.2 ± 2.6	3.0 ± 0.4
20/30/1/50	0.01	1.6 ± 0.2	4.1 ± 0.9	2.6 ± 0.2
20/30/5/50	0.01	0.3 ± 0.1	0.6 ± 0.1	2.0 ± 0.1
20/30/10/50	0.01	0	0	0
0/50/0/50	0.05	9.3 ± 0.8	30.1 ± 0.3	3.4 ± 0.2
0/49/1/50	0.05	5.5 ± 0.3	15.4 ± 1.1	2.8 ± 0.1
0/48/2/50	0.05	4.6 ± 0.1	13.3 ± 0.8	2.9 ± 0.1
0/47/3/50	0.05	3.3 ± 1.2	9.8 ± 1.2	3.0 ± 0.2
0/45/5/50	0.05	1.6 ± 0.1	4.8 ± 1.1	3.0 ± 0.5
0/43/7/50	0.05	0.6 ± 0.1	1.4 ± 0.1	2.2 ± 0.1
0/40/10/50	0.05	0.2 ± 0.1	0.4 ± 0.1	2.1 ± 0.1
				(SD, *n* = 3)

[a] Two-phase solvent system.

2.4. Stability test of enramycin in 0.05% aqueous TFA

The reports indicated that the physical stability of enramycin in low pH was very important problem for a few hours [10,11]. Therefore, we investigated the stability of enramycin in HSCCC mobile phase solvents for separation times. The 10 μg/mL of enramycin standard was prepared by diluting an aliquot of the stock solution in 0.05% aqueous TFA (HSCCC mobile phase). Then, this solvent was stored at room temperature (same condition for HSCCC separation). For stability and decomposed investigation, the time-dependent change of enramycin-A and -B was determined by LC/ESI-MS with SIM mode. In a minute or less after preparation of enramycin standard in 0.05% aqueous TFA, this solution (20 μL) was transferred into separate test tubes and evaporated to dryness at 30 °C. Next, we tried to this preparation of enramycin standard every half hour (n = 14) for 7 h. These samples were adjusted with 20 μL of water/methanol (50/50, V/V) and measured by LC/ESI-MS.

2.5. HSCCC/ESI-MS system

High-speed counter-current chromatography (HSCCC) was performed using an HSCCC model CCC-1000 (multi-layer coil planet centrifuge, Pharma-Tech Research Corp., Baltimore Maryland, USA) with a 7.6 cm orbital radius that produces a synchronous type-J planetary motion with a maximum speed of 1000 rpm. This centrifuge was equipped with three column holders and three multi-layer coiled columns. Each multi-layer coiled column on the holder consists of nine coiled layers of 1.6 mm i. d. polytetrafluoroethylene (PTFE) tubing with capacity of about 120 mL. All three columns are connected in series to provide a total capacity of about 350 mL. The beta values of the coil range from 0.5 at the internal terminal to 0.75 at the external terminal. The separated effluent from 'tail-' outlet of the coil-column was transferred and divided using splitter valve (Low-pressure MicroSplitter Valve, GL Science Co., Tokyo, Japan) to fraction collector and a quadrupole MS detector with an ESI source (2010EV, Shimadzu Co., Kyoto, Japan) (Fig. 2). For ESI-MS detection, a microsplitter valve was used to obtain a flow-rate of 0.2 mL/min. The MS equipment and conditions were an above LC/MS analysis of enramycin. In data analysis state, the acquired chromatogram was shown using total ion monitoring mode ranged from m/z 100–2000 in fragment table.

2.6. HSCCC/ESI-MS conditions for the purification of enramycin-A and -B

The volatile solvent of two-phase system composed of *n*-butanol/hexane/0.05% aqueous TFA solution (43/7/50, V/V/V) at room temperature was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking three separate times, and followed by inverting the vessel and manipulating its stopcock. Phases were separated before use. First, the column was entirely filled with the upper stationary phase. Secondly, a 15 mg of dried enramycin was dissolved in 2 mL of each phase. Finally, these supernatant was then loaded into the column. The column was rotated at 950 rpm, while the lower mobile phase was pumped into the head of the column at a flow-rate of 1.0 mL/min using an HPLC pump (L-6000 pump, Hitachi Co., Tokyo, Japan). The effluent from the outlet of the column was monitored using ESI-MS, and fractionated into test tubes at 1 min/tube using a fraction collector (Model 2128 Fraction Collector, Bio-Rad Laboratories, Inc., NY, USA). These HSCCC fractions were analyzed at extracted ions of m/z 786 for enramycin-A, and m/z 791 for enramycin-B from total ion chromatogram (TIC: m/z 100–2000) in ESI-MS with scan mode. Based on the extracted ion chromatogram, we calculated the peak resolution (R_s) of enramycin-A and -B.

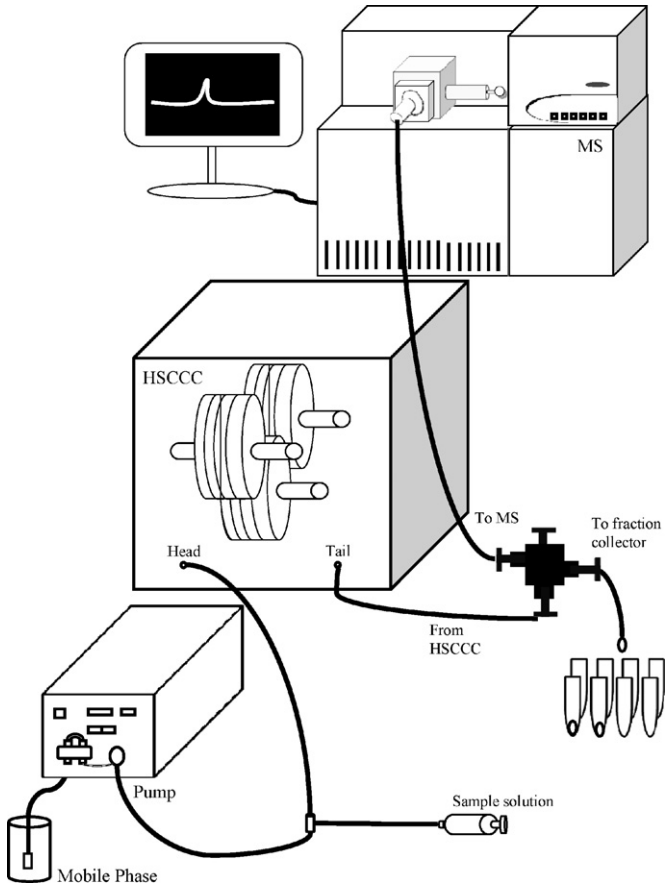

Fig. 2. Schematic diagram of the HSCCC/ESI-MS system. HSCCC instrument was a multi-layer coil planet centrifuge model CCC-1000 (Pharma-Tech. Research Co., USA) coupled to an LCMS-2010EV system (Shimadzu Co., Japan) and a Model 2128 Fraction Collector fraction collector (Bio-Rad Laboratories, Inc., USA) using an low-pressure MicroSplitter Valve (GL Science Co., Japan).

2.7. Identification of purified enramycin-A and -B using LC/ESI-MS

An above LC/MS condition for the determination of enramycin-A and -B was modified to use the MS scan mode for identification of purified compounds. The ESI-MS scan modes were monitored from *m*/*z* 100–2000.

3. Results and discussion

3.1. LC/ESI-MS analysis for the evaluation of K values

Polypeptide antibiotic of enramycin usually contains several components, which have different structures and are mixed together proportionally. These compounds could be separated on TSK-GEL ODS 100 V column due to their polarity difference; hence, reversed-phase LC was used for the analysis of major components such as enramycin-A and -B. These main peaks could be identified as enramycin-A and -B types using ESI-MS detection. LC/ESI-MS TIC chromatogram and mass spectra of enramycin-A and -B are shown in Fig. 3. As shown in ESI-MS scan mode (Fig. 3(B) and (C)), the ions of *m*/*z* 1179 $[M+2H]^{2+}$, 786 $[M+3H]^{3+}$ and 590 $[M+4H]^{4+}$ for enramycin-A, and *m*/*z* 1185 $[M+2H]^{2+}$, 791 $[M+3H]^{3+}$ and 593 $[M+4H]^{4+}$ for enramycin-B were detected in the ESI-MS spectra, respectively. The triply charged ions of the enramycin gave higher ESI responses than the corresponding others charged ions. Therefore, the triply charged ions were used for the evaluation of *K* values to desire a volatile solvent of two-phase system of HSCCC.

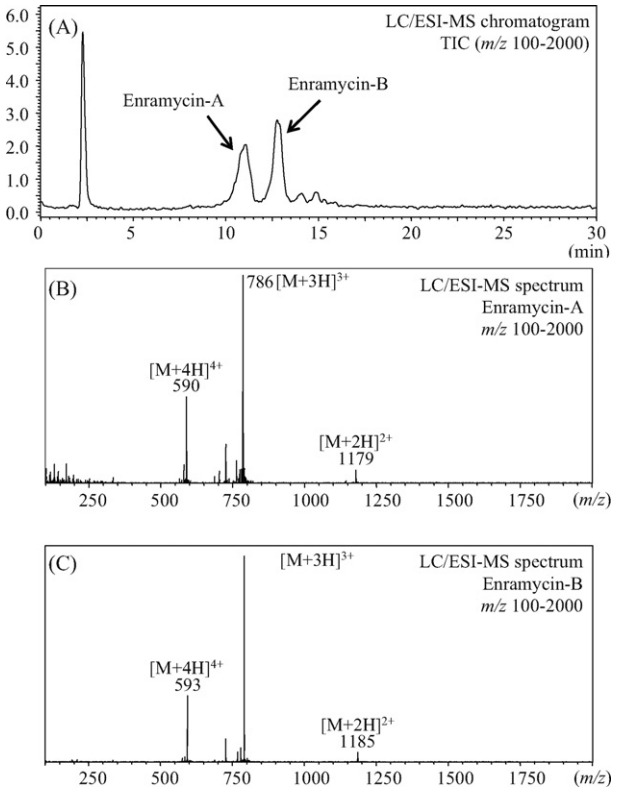

Fig. 3. LC/ESI-MS chromatogram and spectra of enramycin. (A) TIC chromatogram (*m*/*z* 100–2000) in ESI-positive mode. (B) MS spectrum of enramycin-A ranged from *m*/*z* 100–2000 in ESI-positive mode. (C) MS spectrum of enramycin-B ranged from *m*/*z* 100–2000 in ESI-positive mode.

To achieve successful HSCCC separation of the components, the volatile solvent of two-phase system should satisfy the following requirements [4].

Requirement (1): The settling time of the two-phase solvent system with samples should be shorter than 30 s.
Requirement (2): The *K* value should be close to about 1.0.
Requirement (3): These α values between target compounds should be greater than 1.5.
Requirement (4): The two-phase solvents are nearly equal volumes of each phase.

We investigated the *K* values of enramycin-A and -B in the volatile solvents two-phase systems which satisfy the Requirement (1) and (4). Based on the volatile solvents of two-phase system of ethyl acetate/*n*-butanol/hexane/aqueous TFA, *K* and α values of enramycin-A and -B were shown in Table 1. The volatile solvent of two-phase system of the *n*-butanol/hexane/aqueous TFA was closed to Requirement (2). We investigated the optimal concentration of TFA aqueous compositions for the satisfactory reproducible *K* values of enramycin-A and -B. The α value of enramycin-A and -B in the *n*-butanol/hexane/0.05% aqueous TFA solution was useful to separate these compounds according to Requirement (3). Finally, the ratio of the *n*-butanol/hexane/0.05% aqueous TFA solution at 43/7/50 (V/V/V) is selected for the HSCCC separation of enramycin-A and -B.

3.2. Stability test of enramycin in 0.05% aqueous TFA solution

Enramycin was unstable compound in low pH for the storage period [10,11]. Thus, it is needed to investigate the physical stability of enramycin in HSCCC solvent (*n*-butanol/hexane/0.05% aqueous

TFA solution). Ten μg/mL solutions of enramycin were prepared by diluting stability solutions (0.05% aqueous TFA), and was storage at room temperature (same condition of HSCCC separation) for the keeping times (7 h). These solutions were measured by LC/ESI-MS with SIM mode. The relative standard deviations of SIM responses (every half hour for analytical times, $n = 14$) of enramycin-A and -B were 2.1% and 2.8%, respectively. It indicates that enramycin-A and -B in 0.05% aqueous TFA solutions are undecomposed for HSCCC separation time (7 h).

3.3. HSCCC/ESI-MS separation, detection and purification of enramycin-A and -B

This advanced HSCCC method can be useful for obtaining various peptides on the large-scale up such as industrial levels [14]. Therefore, we have developed an efficient, effective and possible large-scale HSCCC method with ESI-MS for the separation, detection and purification of two components of polypeptide antibiotics (enramycin) using the volatile solvents two-phase system. To achieve successful HSCCC/ESI-MS method for the peptides, the HSCCC/ESI-MS system should satisfy the following requirements.

Requirement (5): The two-phase solvent has a volatile ability for ESI-MS.
Requirement (6): The HSCCC mobile phase could be bifurcated into fraction collector and ESI-MS detector using simple splitter valve (Fig. 2).

Thus, we investigated that the two-phase volatile solvent (Table 1), and splitter were studied regarding to Requirements (5) and (6). For Requirement (5), the elution of non-polar solvents such as hexane is potential influence on the signal-noise of MS scan mode [8]. In the HSCCC chromatogram, the solvent composition of the first elution area from start to solvent front (at about 120 min) is upper stationary phase containing *n*-hexane, that of the next second area from 120 to 210 min is a two-phase solvent mixture both upper stationary and lower mobile phases, and that of the final area after 210 min is lower mobile phase (not containing hexane) (Fig. 4). However, the MS responses of enramycin-A and -B were detected by extracted ion mode, and have the stability ionization. Thus, this volatile non-polar solvent would be useful to HSCCC/ESI-MS. On the other hand, various non-polar and volatile solvents would be investigated to the applicability evaluation of ESI-MS for future work. For Requirement (6), recent studies were reported that normal flow Y- or T-splitter, and PEEK pipes with different diameter and length were used to control the split ratio manually and improved the stability of the effluent into the MS detector [8,9]. However, these approaches are primitive techniques that the stability and continuous micro flow-rate would be calibrated by simple splitter valve. Based on the HSCCC method for separating these polypeptide antibiotics, we tried to apply the micro splitter valve for LC/MS analysis with adjusting to a flow-rate of 0.2 mL/min for ESI. This microsplitter valve was useful to calibrate the microflow-rate of mobile phase (0.2 mL/min) for the ESI-MS [15].

Earlier experimental trials were focused on HSCCC/MS operated with various ionization techniques and modes [16–18]. On the other hand, in this study, first preliminary experiment of HSCCC/MS is the representative research for the efficient, useful, and easy techniques regarding to bioactive peptides. Thus, the positive scan mode (wide spectrum ranged from m/z 100–2000) was used for the validated detection and identification of various peptides in the HSCCC/ESI-MS system. Therefore, an efficient and effective HSCCC/ESI-MS with positive scan mode is developed for the separation and purification of polypeptide antibiotics (enramycin-A and -B) using the simple solvent system of *n*-butanol/hexane/0.05% aqueous TFA solution (43/7/50, V/V/V). We

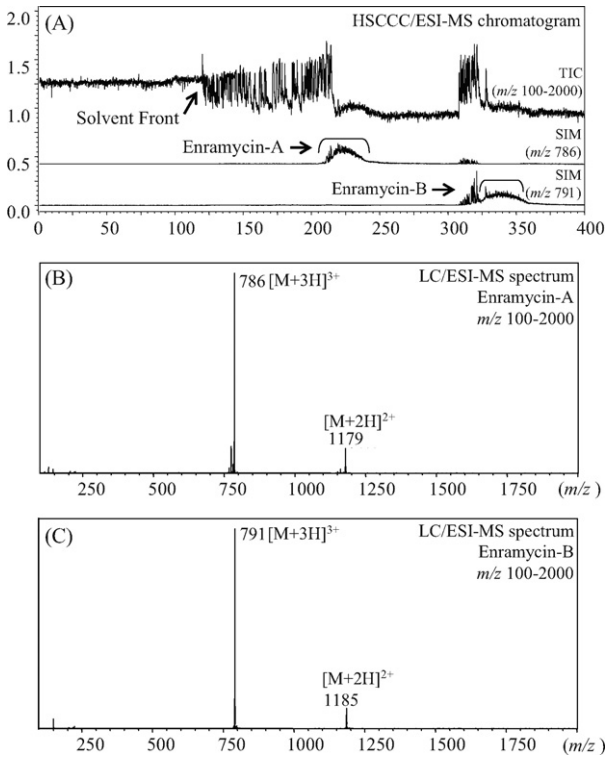

Fig. 4. HSCCC/ESI-MS chromatogram and spectra of enramycin. (A) TIC (m/z 100–2000) and extracted ion's (m/z 786 for enramycin-A, and m/z 791 for enramycin-B) chromatograms in ESI-positive mode. (B) MS spectrum of enramycin-A ranged from m/z 100–2000 in ESI-positive mode. (C) MS spectrum of enramycin-B ranged from m/z 100–2000 in ESI-positive mode.

used 15 mg of enramycin powder in the above-mentioned HSCCC system. Fig. 4 shows the TIC and SIM ions (m/z 786 for enramycin-A, and m/z 791 for enramycin-B) chromatograms by HSCCC/ESI-MS with positive mode. As shown in ESI-MS scan mode (Fig. 4(B) and (C)), these ions of m/z 1179 $[M+2H]^{2+}$ and 786 $[M+3H]^{3+}$ for enramycin-A, and m/z 1185 $[M+2H]^{2+}$ and 791 $[M+3H]^{3+}$ for enramycin-B are detected in the ESI-MS spectra, respectively. These charged ions of the enramycin were similar to LC/ESI-MS spectra (Fig. 3(B) and (C)). Therefore, these charged ions were used to identify these polypeptide enramycin using HSCCC/ESI-MS.

The retention of the stationary phase was 54.0%. The total separation time was about 7 h, and the total elution volume was 400 mL for HSCCC system. The HSCCC fractionated effluent peaks of enramycin-A and -B indicated that the R_S value was 2.9. Based on this HSCCC/ESI-MS chromatogram, the collected fractions were combined into two pooled fractions such as enramycin-A and -B. The amounts of the peak fractions were 4.3 mg (enramycin-A), and 5.9 mg (enramycin-B). These results showed that very useful and efficient purification method for polypeptide enramycin was indicated using HSCCC/ESI-MS.

3.4. Identification and evaluation of purified enramycin-A and -B by LC/MS

The HSCCC fractions of enramycin-A and -B were analyzed using LC/ESI-MS with scan mode. The respective TIC (m/z 100–2000) chromatograms of these fractions are shown in Fig. 5. Moreover, these MS spectra of the fractions from TIC chromatograms were expected to be enramycin-A and -B, respectively (Fig. 5(B) and (D)). The purities were estimated to be over 95% (100% by abundance of 230 nm from retention time: 5–30 min). Based on MS spectra of

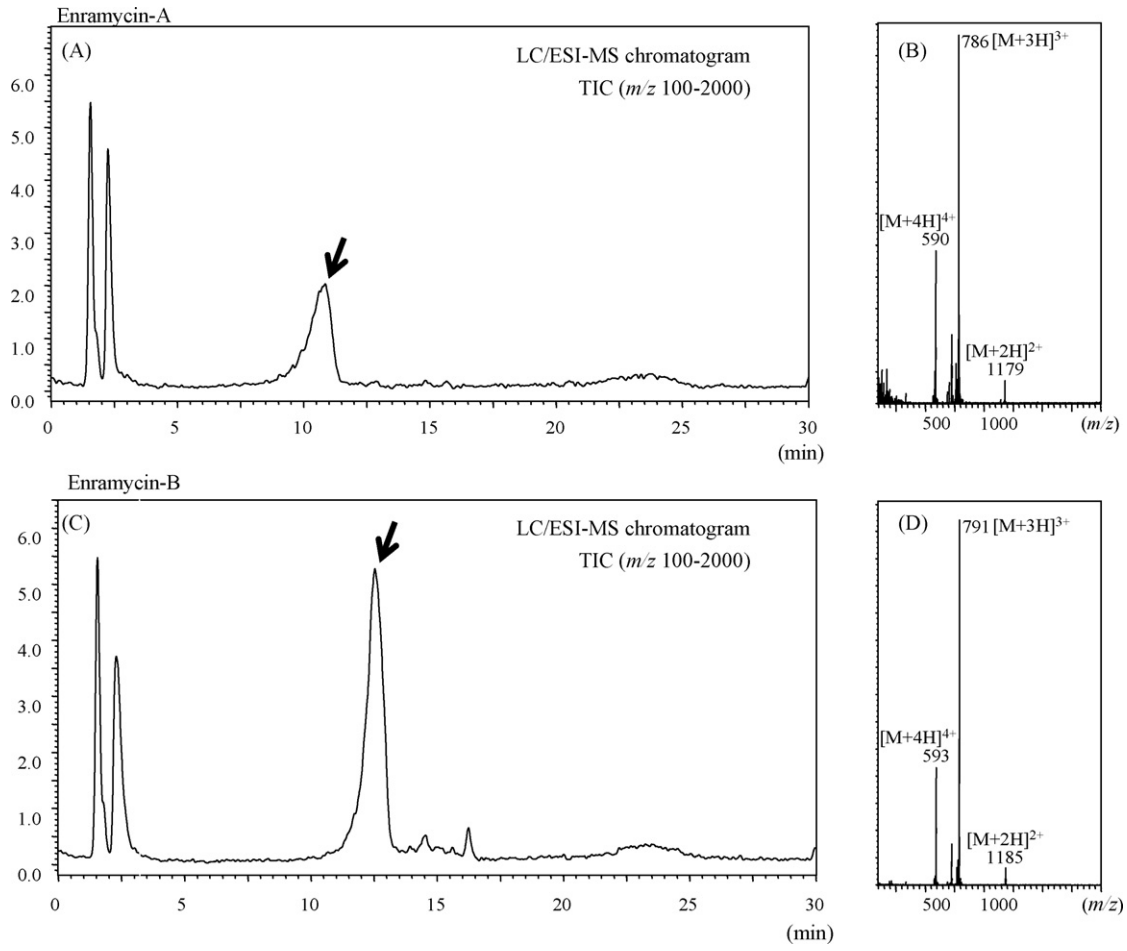

Fig. 5. LC/ESI-MS chromatograms and spectra of HSCCC fractions of enramycin-A and -B. (A) LC/ESI-MS (*m*/*z* 100–2000) chromatogram of enramycin-A by the HSCCC/ESI-MS purification. (B) MS spectrum (*m*/*z* 100–2000) of enramycin-A by the HSCCC/ESI-MS purification. (C) LC/ESI-MS (*m*/*z* 100–2000) chromatogram of enramycin-B by the HSCCC/ESI-MS purification. (D) MS spectrum (*m*/*z* 100–2000) of enramycin-B by the HSCCC/ESI-MS purification.

enramycin-A and -B, the identification of these peptides is able to use these informational factors for monitoring these peptides.

4. Conclusion

In conclusion, we were able to purify enramycin-A and -B using HSCCC/ESI-MS with the volatile solvents two-phase system composed of *n*-butanol/hexane/0.05% aqueous TFA solution (43/7/50, V/V/V). The overall results indicate that this approach of HSCCC/ESI-MS separation, purification and identification is a powerful technique for the development of analytical and proteomic methods regarding to the bioactive peptides.

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