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High-performance capillary electrophoresis of a fermentation-derived cyclic peptide analog, animal growth promoter[1]

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Abstract

We have developed HPCE methods for the analysis of sulfomycin (trivial name) and related compounds (code name, crude material=U82127=**I**), which is an animal growth promoter derived from a fermentation beer. The crude material, **I**, isolated from the fermentation consisted of more than 40 components which were not completely separated by conventional HPLC. Thus, as a complementary analysis method, we have optimized HPCE conditions for **I** using various capillaries including uncoated, coated, and packed using various buffers. The optimized HPCE conditions were obtained with an uncoated fused-silica capillary and a buffer that consisted of 30 m*M* Tris-tricine, 10 m*M* SDS, 10 m*M* NaCl and 20% MeOH, pH 8.0. Using these HPCE conditions, we were able to separate the one main component collected from the HPLC effluent into two or three components. In order to identify the main components of the fermentation product, an off-line HPLC–HPCE–MS analysis for **I** was performed. From the MALDI-TOF-MS results, the separated components collected from HPCE had different molecular masses. Four lots of **I** samples having different characteristics were also analyzed by HPCE to investigate lot-to-lot differences in peak profiles. The four lots of **I** were found to have very similar peak profiles. In this paper, **I** refers to the crude fermentation product and sulfomycin to the purified, major HPLC component of **I**.

Keywords: Sulfomycin

1. Introduction

HPLC is a widely established method for the analysis of a wide range of molecules, while HPCE is of increasing importance. Idei et al. [1] have described how one can decide which method is more advantageous in solving a given task. They indicated several points, e.g., resolution, selectivity, sensitivity, peak capacity, duration and cost of the analysis, solvent and chemical consumption, in order to choose a method [1]. However, both HPLC and CE can be used, as they complement each other for obtaining more information for a sample [1–3]. Although HPLC is a well established technique used for the separation of small peptides, its efficiency for larger peptides is not 100% successful [2]. A sample that showed one major peak in HPLC analysis was sometimes resolved into multiple peaks in HPCE separation, and the results could be confirmed by MS [2].

There are several books [4,5] and reviews [6] describing applications of HPCE. However, optimization of HPCE conditions, particularly for a complex mixture, often requires trial and error. To optimize the separation conditions, the effect of pH,

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temperature, voltage, buffer concentration and composition, and buffer modifiers, e.g., organic solvent, surfactant and inorganic ions should be considered [7–11]. Sometimes, Zn (2+) [11] or Cu (2+) [12] can be added to the buffer for complexation with amines, amino acids and peptides. For a better separation, 2D (2-dimensional, preseparation capillary/analytical capillary) [13], or 3D (size-exclusion chromatography/reversed-phase liquid chromatography/optically gated capillary zone electrophoresis) [14] methods were introduced.

Nissen et al. [15] have reviewed and evaluated the developments and state-of-the-art in CE–MS. Usually, CE has been coupled with on-line electrospray (ESI) mass spectrometric detection [16–22]. However, because the column effluent is continuously introduced into the ion source, it is limited to buffers that contain volatile species [23]. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF-MS) is a powerful analytical technique, capable of excellent sensitivity and tolerant of diverse conditions, but on-line coupling of CE/MALDI-TOF-MS has met with limited success [23]. On-line MS detection has not yet been achieved in micellar based electromigration (MECC) approaches, and this is an important challenge [15]. Because off-line coupling of CE with MALDI-TOF-MS has several advantages over on-line, such as minimizing contamination of the ion source with non-volatile CE buffers and allowing the conditions for both the separation and MS to be independently optimized [23], it has been used for the analysis of proteins and peptides [23–27].

Here, we have developed methods for sulfomycin crude material U82127, (**I**), of which the main component is a cyclic peptide analog having a molecular mass (M_r) of 1244, using HPCE techniques. Material **I** consisted of more than 40 components, and these were not completely separated by HPLC. We have tried various capillaries, e.g., uncoated fused-silica, coated silica (polyethylene glycol, C_{18}, glycerol, sulfonic, and fluorocarbon-coated), and packed (SC5 SCX and SC5 ODS) capillaries, to optimize the separation conditions for **I**.

Using the optimized conditions, achieved with an uncoated fused-silica capillary and 30 m*M* Tris-tricine, 10 m*M* SDS, 10 m*M* NaCl and 20% MeOH, pH 8.0, we separated the one main peak collected from HPLC into 2–3 peaks. To confirm if the resolved peaks from HPCE were just conformers or different compounds with different M_r, MALDI-TOF-MS analysis was used. We collected the two separated peaks from the HPCE eluent, and removed the salts and surfactant using solid-phase extraction (SPE) for the MS analysis. MS showed different peak profiles for the two CE components, suggesting these components were different molecules.

We have also analyzed four lots of **I** samples, with different characteristics, using the optimized conditions, to investigate the correlation between peak profile and stability. The peak profiles were dependent on the freshness of the run buffer. Because the samples of **I** had very similar peak profiles, it was difficult to compare any differences.

2. Experimental

2.1. Reagents and materials

Reagents were of reagent grade or better and used as received. DMF (N,N-dimethyl-formamide), sodium hydroxide, ethylene glycol, *o*-phosphoric acid and HPLC-grade acetonitrile were from Fisher Scientific (Pittsburgh, PA, USA). Tricine, Brij 35, TFA, zinc chloride and SDS were from Sigma (St. Louis, MO, USA). Ultra-pure Tris was from ICN (Irvine, CA, USA). HPLC-grade methanol and C_{18} coated material were from J.T. Baker (Philipsburg, NJ, USA). Polystyrene divinylbenzene (PS-DVB) copolymer was from Supelco Corporation (Bellefonte, PA, USA). Electrophoresis solutions were prepared with water from a Corning Glass Works (Corning, NY, USA) Megapure MG-1 purification system. Sulfomycin (purified **I** by HPLC), and four lots of **I** samples were from Pharmacia and Upjohn (Kalamazoo, MI, USA). These were dissolved in DMF at a concentration of 10 mg/ml and 10-fold diluted with methanol.

2.2. Capillary electrophoresis

CZE separations were performed on a SpectroVision Electropherograph (Groton Detector Technology, Concord, MA, USA) operating at 20–25 kV. Detection was provided by a Spectro 100 UV–Vis detector (Spectra-Physics, San Jose, CA, USA) at

210 nm. For data processing, a Macintosh Classic computer with Dynamax MacIntegrator I (Rainin Instrument Co., Woburn, MA, USA) was used. Several kinds of capillaries were tested. The uncoated fused-silica capillary (75 μm I.D.×80 cm, 56 cm to detector) was from Polymicro Technologies (Phoenix, AZ, USA). CZE SepTM-100/C_{18}, CZE SepTM-200/Glycerol and CZE SepTM-300/Sulfonic Silica column (75 μm I.D.×70 cm, 49 cm to detector) were from Phenomenex (Torrance, CA, USA). H250 (C_{18} coated capillary) and H150 (C_{18} coated capillary; 50 μm I.D.×100 cm, 65 cm to detector) were from Supelco (Bellefonte, PA, USA). μSIL-WAX (75 μm I.D.×70 cm, 49 cm to detector) and fluorocarbon-coated (FC; 75 μm I.D.×75 cm, 50 cm to detector) columns were from J&W Scientific (Folsom, CA, USA). Packed capillaries, SC5 SCX and SC5 ODS1 (75 μm I.D.×85 cm, 30 cm packed) were from Phase Separations (Deeside, UK).

Initially, a rough optimization of the CE buffer was performed by changing the pH of the buffer and investigating the effects of SDS and MeOH with an uncoated capillary. More precise optimization of the buffer was performed by changing the concentration and composition of the roughly optimized buffer, which was Tris-tricine, MeOH, SDS, NaCl. For the C_{18} coated capillary, 0.01% Brij 35 was used instead of SDS, and for the FC coated capillary, FC surfactant from J&W Scientific (Folsom, CA, USA) was added.

The uncoated capillary was treated before use by flushing with 0.1 *M* NaOH followed by HPLC grade water, then run buffer. CZE SepTM-100/C_{18} column was flushed with 0.5 *M* Brij, and CZE SepTM-200/Glycerol and CZE SepTM-300/Sulfonic Silica columns were washed with HPLC grade water between runs. μSIL-WAX column was flushed with 20 m*M* *o*-phosphoric acid solution. FC column was regenerated by flushing with water and acetone three times, and equilibrated with run buffer overnight after drying. Packed capillaries, SC5 SCX and SC5 ODS1 were washed by electrophoretic elution of run buffer.

2.3. Chromatographic conditions

HPLC was performed with a Gilson Model 232 System (Gilson Medical Electronics, Middleton, WI, USA), consisting of two Gilson Model 302 pumps, a Gilson Model 811B dynamic mixer, and a Gilson Model 115 variable wavelength UV detector set to 230 nm (0.1 AUFS). Data were acquired on a Dell 386 computer (Dell Computer, Austin, TX, USA) using Gilson Model 715 HPLC controller, version 1.20, software. For separations of **I** and sulfomycin, a Nova-Pak C_{18} column, 150×3.9 mm ID, 4 μm, 60 Å (Waters, Milford, MA, USA) was used.

Two mobile phases were prepared for gradient elution RP-HPLC. The first was of 0.1 % trifluoroacetic acid (TFA) (Aldrich/Sigma, Milwaukee, WI, USA) in water (A) and the second of 0.1% TFA in acetonitrile (B). These solutions were filtered through a 0.45 μm Durapore membrane (Millipore, Bedford, MA, USA) and then degassed under vacuum. Gradient elution employed: time (min)/%B; 0–0.5/25; 0.5–30/50. The main peak of **I**, sulfomycin, was collected and concentrated using a SpeedVac Concentrator (room temperature) from Savant Instruments (Farmingdale, NY, USA).

2.4. Optimization of solid-phase extraction (SPE)

An SPE method was used to remove salts and SDS from the CE fractions. Because sulfomycin is hydrophobic, a C_{18} packing material (40 μm, from J.T. Baker, Phillpsburg, NJ, USA) and 12% cross-linked PS-DVB copolymer resin (16–18 μm, Supelco, Bellefonte, PA, USA) were chosen. In pure water or 10 or 20% methanol solution, both materials did not settle and floated on the surface or adhered to the tube. We were able to precipitate both materials using a 20% acetonitrile aqueous solution. Before treating the main peak isolated by HPLC, we tested the extraction ability of the two materials using standard sulfomycin.

To two empty 1.5 ml microcentrifuge tubes (Brinkmann Instruments, Westbury, NY, USA), 1 μl of 1 mg/ml sulfomycin was added, respectively. A small amount of C_{18} or PS-DVB material was added to each tube, respectively. To each tube, 50 μl of 20% acetonitrile–water was added and the tubes were Vortex mixed. After centrifuging, the supernatant was discarded. The SPE materials were washed four times with 20% acetonitrile–water using the procedure described above. After removing the final wash solution, 10 μl of methanol was added in order to dissolve extracted sulfomycin. The tubes were

Vortex mixed and centrifuged, and the solution was moved into a smaller tube for CE.

2.5. Off-line HPLC–HPCE–MS analysis

Sulfomycin was separated by HPLC, and one main peak was collected in a 1.5 ml microcentrifuge tube. The collected components were concentrated using a SpeedVac Concentrator and the residue reconstituted with 20 μl methanol. The concentrated sample was analyzed with the optimized HPCE conditions. The two resolved main peaks in the HPCE were collected in 1.5 ml microcentrifuge tubes, respectively. To remove the salts and surfactant in the CE buffer, an SPE method with PS-DVB copolymer and 20% ACN was used. After extraction by the PS-DVB copolymer, the remaining solution was decanted. The same procedure was repeated five more times and the pH of the decanted solution was tested each time. The extracted sample was removed from the support and dissolved in methanol. The solvent was evaporated using a SpeedVac Concentrator for MS. MALDI-TOF-MS analyses, Fig. 6C–D, were performed by Quality Control Biochemicals (Hopkinton, MA, USA). The dried samples were brought up in 1 ml of ACN–water (50:50, v/v). A 1 μl volume of each sample was spotted in 1 μl of the α-cyano-4 hydroxycinnamic acid matrix. The samples were read on a Voyager RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA). MALDI-TOF-MS analyses, Fig. 6A–B, were performed at PerSeptive Biosystems also on a Voyager RP BioSpectrometry Workstation, now using a 2,5-dihydroxybenzoic acid matrix, with the same volumes as above. A nitrogen laser operating at 337 nm was used to ionize the samples. Ions were accelerated with a potential of 30 kV.

3. Results and discussion

3.1. Optimization of analysis methods for I using HPCE

Material **I** consisted of >40 components and a main component of **I**, sulfomycin, was a modified cyclic peptide analog (Fig. 1). Because HPLC often cannot resolve compounds of very similar structures, especially those of high M_r, we developed a complementary HPCE method. Since suitable HPCE conditions were not available, initially a rough optimization of the buffer was performed by changing pH and investigating the effects of SDS and MeOH with an uncoated capillary. Because **I** is a very hydrophobic compound, methanol was added to the buffer as a modifier. Material **I** could not be separated without 10 m*M* SDS, throughout a range of pH 3–11. This may be a form of SDS-modified CZE, rather than MECC, since the SDS concentration was below the critical micelle concentration (cmc). More precise optimization of the buffer was performed by changing the concentration and composition of the roughly optimized components, Tris-tricine, MeOH, SDS and NaCl. Finally, one obtained optimized conditions, which consisted of 30 m*M* Tris-tricine, 10 m*M* SDS, 10 m*M* NaCl and 20% MeOH.

Several coated or packed capillaries were studied, using the optimized conditions, as a starting point. Following the instruction manual, 0.01% Brij 35 was used instead of SDS for the C_{18} coated capillary, and FC surfactant from J&W Scientific was added for the FC coated capillary. For the packed capillaries, more than 50% MeOH or ACN was used. Most capillaries

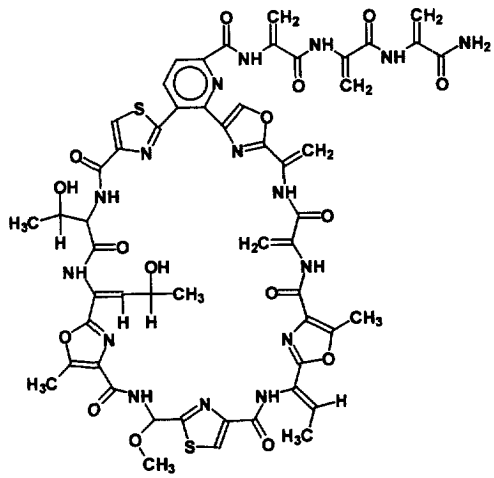

Fig. 1. Structure of the main component of sulfomycin.

were not useful for the separation of this complex sample. Particularly, in the isocratic capillary electrochromatography (CEC) mode, **I** could not be eluted at all or eluted together with the solvent.

The electropherogram obtained using a C_{18} coated capillary with a buffer of 30 m*M* Tris-tricine, 0.01% Brij 35, 10 m*M* NaCl and 20% MeOH, pH 8.0, showed quite good resolution (data not shown). However, baseline separation was not achieved and reproducibility, especially column-to-column, was not quite acceptable, for capillaries made by either Phenomenex or Supelco.

Electropherograms were also obtained using an FC coated capillary with a buffer of 30 m*M* Tris-tricine, 10 m*M* SDS, 20% MeOH and 0.05% FC-N (neutral oligomeric FC surfactant), pH 8.0 (data not shown). When 10 mg/ml **I** was injected, spikes appeared, while when 1 mg/ml **I** was injected, one main peak and several smaller peaks were resolved. Electropherograms suggested that injection of high concentrations of **I** caused precipitation during the separation. When 10 mg/ml **I** was injected with the same buffer used with the FC capillary, but now with an uncoated capillary, the same peak profile was obtained (data not shown). These results indicated that the spikes were caused by the FC-N surfactant and not the FC capillary.

Fig. 2 shows the electropherograms obtained using optimized HPCE conditions for **I** (A) and sulfomycin (B). However, the main peaks No. 14 (Fig. 2A) and No. 12 (Fig. 2B) still contained more than one component, which was confirmed by subsequent MS. Sometimes the peaks were separated depending on the buffer freshness (see below). The main peaks in Fig. 2A and Fig. 2B (No. 14 and No. 12) have identical migration times, though numbered differently because of the data collection system. We emphasize that CE peaks 1′ and 2′, (Fig. 4) were isolated from runs without buffer replenishment, which offer poorer resolutions than seen with replacement of buffer for every run (see below). In view of the results with run-to-run buffer replenishment, peaks 1′ and 2′ in these earlier assays and fraction isolations were not pure, single species. It is questionable if the fully resolved peaks seen with buffer replenishment, as below, could have been separately collected, without overlap of nearby peaks (Fig. 7B).

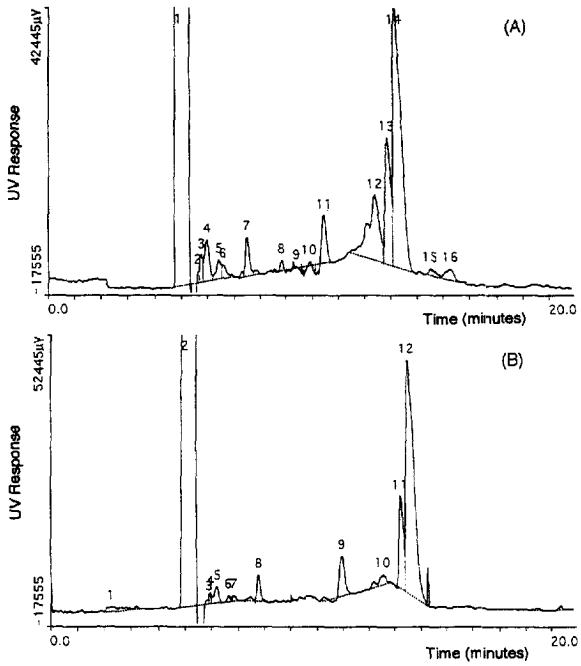

Fig. 2. Electropherograms obtained using optimized HPCE conditions: capillary: uncoated fused silica, 70 cm×50 μm (45 cm to detector); buffer: 30 m*M* Tris-tricine, 10 m*M* SDS, 20% MeOH, 10 m*M* NaCl, pH 8.0; applied voltage: 20 kV; detection: UV at 210 nm; sample: **I** (A) and sulfomycin (B); concentration: 1 mg/ml in DMF–MeOH (1:9); injection: hydrodynamic injection for 20 s.

3.2. *Off-line HPLC–HPCE–MS analysis*

Using the optimized conditions, we were able to separate one main peak of sulfomycin collected from the HPLC effluent (Fig. 3) into 2 (sometimes 3) peaks (Fig. 4). The ability to reproducibly resolve this HPLC peak into several component CE peaks was a result of how well the HPCE conditions were optimized and especially the buffer freshness. It was never possible to resolve the HPLC peak into separate species using HPLC (Fig. 3). Broad peaks in Fig. 4 likely resulted from less-than-optimal HPCE conditions and more than a single species. To confirm whether the separated HPCE peaks were conformers or different compounds having different M_r, MALDI-TOF-MS analysis was performed. For this, the two peaks from HPCE were separately collected. Because the HPCE buffer contained high

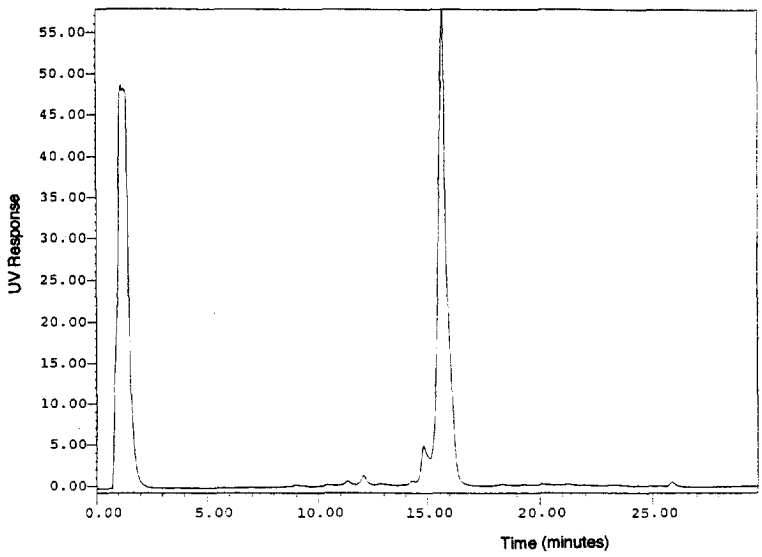

Fig. 3. HPLC separation of sulfomycin: column: Nova-Pak C_{18} (150×3.9 mm I.D.); mobile phase: 0.1% TFA in H_2O (A) and 0.1% TFA in ACN (B); gradient elution: time (min)/%B; 0–0.5/25; 0.5–30/50; detection: UV at 230 nm; sample: sulfomycin; concentration: 1 mg/ml in DMF–MeOH (1:9); injection volume: 25 μl.

concentrations of salts and SDS, this sample was not compatible with MS. To remove the salts and SDS, microdialysis or microcentrifugation proved most useful, but the amount of the collected sample was too small. A SPE method was considered, in order to avoid loss of the sample during removal of salts and SDS. Because sulfomycin was very hydrophobic, hydrophobic SPE materials, a C_{18} coated material, 40 μm, and a 12% crosslinked polystyrene divinylbenzene (PS-DVB) copolymer resin, 16–18 μm were tried. Before treating the actual samples, we tested the extraction ability of the SPE materials using standard sulfomycin (Section 2).

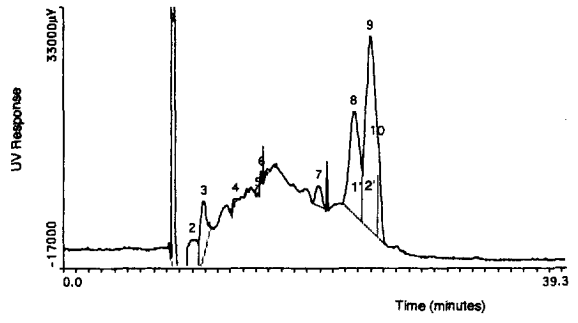

Fig. 4. HPCE separation of a collected main component of sulfomycin first separated by HPLC: capillary: uncoated fused silica, 92 cm×75 μm (60 cm to detector); buffer: 30 m*M* Tris-tricine, 10 m*M* SDS, 20% MeOH, 10 m*M* NaCl, pH 8.0; applied voltage: 25 kV; detection: UV at 210 nm; injection: hydrodynamic injection for 30 s. Sample preparation is described in Section 2.

The electropherograms, Fig. 5, show the SPE results obtained. The upper electropherogram (Fig. 5A) represents the control, a direct injection of 0.1 mg/ml sulfomycin without SPE. In Fig. 5B, sulfomycin was recovered >80% for each main peak (4) with the PS-DVB. The C_{18} coated material showed poor extracting ability for sulfomycin, (Fig. 5C).

To extract the real sample collected from the HPCE buffer, PS-DVB resin was used with the same procedures as for standard sulfomycin. A blank MS analysis was also performed, where the HPCE buffer (same volume as for real sample) without sulfomycin present, was analyzed alongside the original, HPLC purified (not HPCE isolated), control sample. After extracting the sample, SPE resin was washed with 20% acetonitrile–water mixture five times. The pH of the wash solution was measured each time with pH paper. Initially, the pH of the CE solution was 8.0, but after washing the resin 3 times, wash solution became neutral. Samples were recovered from the SPE resin with 10 μl of methanol. The

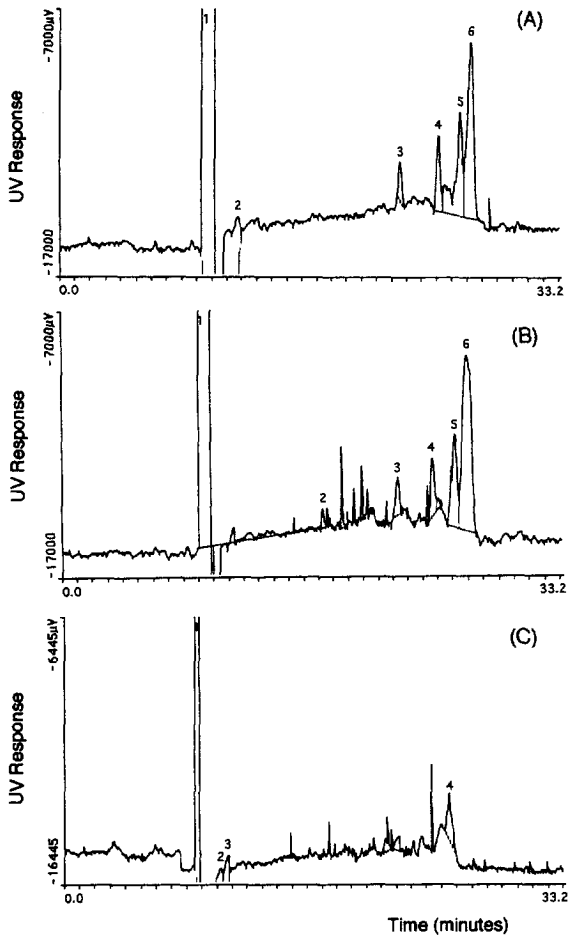

Fig. 5. Removal of salts and SDS from HPCE buffer by SPE method: capillary: uncoated fused silica, 92 cm×75 μm (60 cm to detector); buffer: 30 m*M* Tris-tricine, 10 m*M* SDS, 20% MeOH, 10 m*M* NaCl, pH 8.0; applied voltage: 25 kV; detection: UV at 210 nm; sample: sulfomycin; concentration: 0.1 mg/ml in MeOH; injection: hydrodynamic injection for 1 min. SPE materials: no extraction; control standard (A), polystyrene divinylbenzene (B) and C_{18} coated material (C).

extracted samples were again analyzed with HPCE, but it was not possible to observe the peaks because the concentration was too low. These extracts were immediately subjected to MALDI-TOF-MS.

The extracted samples (2) dissolved in methanol were evaporated to dryness. MS results showed quite different spectral (m/z) profiles for the two samples, Fig. 6C,D. One sample contained an expected compound having M_r of 1244 (Fig. 6C), the correct M_r for sulfomycin, but the other sample contained compounds with M_r values of 1201, 978 and 962 (Fig. 6D). It was not clear if the unexpected compounds were fragments of the main component (M_r 1244) or definitely different compounds. It was possible that species were being formed during the CE separation and collection. Perhaps sample concentration, solvent evaporation to dryness, and simple CE isolation caused fragmentation of what was originally a single species of M_r 1244. The CE isolation and preconcentration steps were very mild, without excess heat or harsh solvents. More importantly, the presence of several TOF-MS peaks from both isolated CE fractions agreed with the results eventually obtained, as below, showing several major peaks in CE using fully optimized, reproducible conditions, especially buffer replenishment. These four peaks, described below, were completely consistent with the 3–4 peaks observed in the CE isolation and subsequent TOF-MS. Though one cannot eliminate the possibility that various peaks observed via TOF-MS analysis of the CE isolates resulted from degradation of what was a single species, the overall results are not consistent. MALDI-TOF-MS analysis of the intact sample, after an initial purification by preparative RP-HPLC, also showed several MS peaks present, rather than just one at M_r 1244 (Fig. 6A).

In Fig. 6D, from the second HPCE peak isolated and concentrated, there was a peak at M_r 978, which may have been the Na adduct of the peak at M_r 954 in Fig. 6A, which was an authentic standard of **I** (purified by HPLC). Because these two spectra were obtained at different times, with different instruments, matrices, and operators, though with similar MALDI operating conditions, it is not unexpected that these (slight) differences would arise. Spectra for Fig. 6A,B were both performed at PerSeptive Biosystems, while those for Fig. 6C,D (two CE fractions) were performed at Quality Biochemicals.

The peak at M_r 962 in Fig. 6D did not appear in Fig. 6A, which may have been due to its suppression in the HPLC purified sample (Fig. 6A) and/or fragmentation of the 978 m/z peak in Fig. 6D (loss of oxygen or NH_2). The peak at about M_r 1200 in Fig. 6A agreed in its M_r value with that observed in Fig. 6D, though more difficult to observe (Fig. 6A) because of the differences in sensitivity under which these two spectra were obtained. It is apparent from

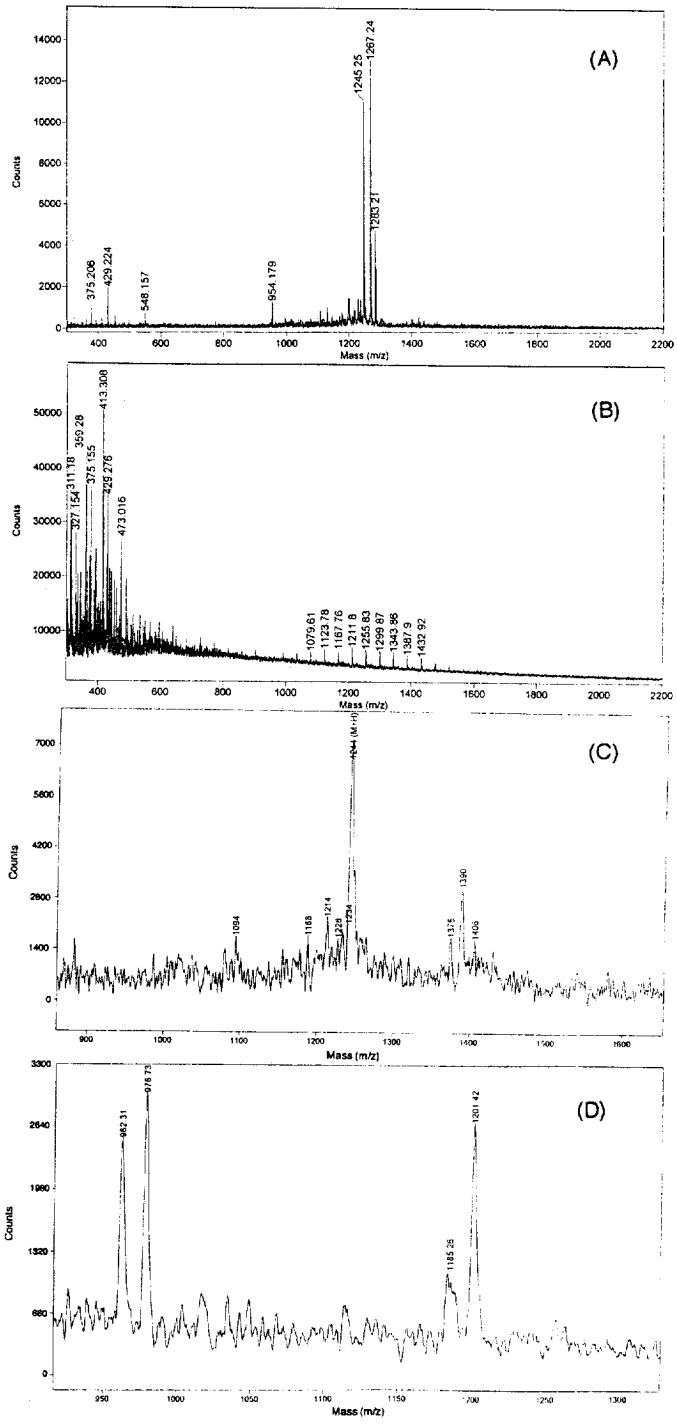

Fig. 6. MALDI-TOF-MS analysis of sulfomycin: I standard after HPLC purification (A), MS spectra of blank CE–solid-phase extraction (B), MS spectra of two main peaks, the first (C) and second eluted main peak (D), by HPCE separation. Sample preparation and MS conditions are described in Section 2.

Fig. 6B (CE and SPE blank), that the peaks in Fig. 6C,D were not due to the CE buffer, perhaps owing to incomplete removal of salts and/or SDS. The blank sample MS spectrum using exactly the same CE buffer and volume and isolation (SPE) procedures, shown in Fig. 6B, showed a series (very high sensitivity) of homologous (perhaps SPE polymer derived) peaks, mostly of higher M_r than those in Fig. 6C,D. None of these had the same M_r values for the peaks observed in Fig. 6C,D. These series of peaks in Fig. 6B were of such low intensity (y-axis scale) that they did not appear in Fig. 6C,D, in comparison with the sample peaks from sulfomycin.

There were two additional, higher M_r peaks in Fig. 6A, at 1267 and 1283 m/z values, which most likely arose from the Na and K adducts of the 1244 peak. Thus, it appeared that: (1) the method of HPCE isolation, extraction from buffer, elution from SPE support, preconcentration and other steps used to collect and MS analyze the two HPCE peaks did not lead to the formation of new peaks (species) not found, by and large, in the original, intact, HPLC purified sample before HPCE manipulations and (2) none of the blank MS peaks appeared in the MS spectra for the HPLC or HPCE purified samples (and vice-versa), suggesting that such species were not artifacts of the overall steps used to isolate and collect the HPCE fractions for MS analysis. It can be finally concluded that the various species observed by HPCE and off-line MALDI-TOF-MS analysis were more than likely present in the original, HPLC purified sample, and were not formed during the analytical methods used here to separate, collect, and then identify.

To obtain more information about the unexpected compounds, further analysis, e.g., using HPCE–electrospray (ESI)–MS will be required. It will become necessary to modify the current CE buffer, so that it is compatible with ESI sample requirements, which usually avoid SDS and nonvolatile salts. Simple CE–ESI-MS will not suffice to identify the structures of the four peaks in the final, optimized CE separations, Fig. 7. That will require CE–ESI-MS–MS methods, together with interpretation of the fragments generated and how they relate to their structures. From the optimized HPCE conditions below, it appears that there are (at least) four compounds present in the original HPLC peak. Most likely, these HPCE peaks correspond to the four m/z species in the MS.

3.3. Effect of buffer replenishment

Four lots of **I**, with different degrees of purification, were analyzed by HPCE to investigate lot-to-lot differences. The peak profiles were dependent on the freshness of the buffer. Electropherograms obtained with a fresh buffer showed very sharp and well resolved peaks. Subsequent electropherograms with the same buffer showed different profiles (Fig. 7A). Electropherograms obtained by further injections of the same sample with the same buffer became simplified, by a merging of the eluting peaks. This might have been caused by the small buffer reservoir used in this work (about 1.5 ml), and the use of a concentrated sample (1 mg/ml). These samples of **I** had similar peak profiles, and no lot-to-lot differences were observed (data not shown). We obtained more reproducible electropherograms by changing the buffer for every injection (Fig. 7B). This suggested that buffer replenishment, as well as washing the capillary between runs, were important for obtaining reproducible data. We believe that the four main peaks observed with these HPCE conditions (Fig. 7) are different species having different M_r and mass spectra.

4. Conclusion

We have optimized analytical conditions for **I** using HPCE. HPCE as well as HPLC did not provide completely satisfactory results. HPLC separations showed a number of resolved peaks, including tiny amounts of numerous components, but the method could not separate the main peak(s). The HPCE method could separate the main components, but could not, at the same time, show a peak profile for the minor components. This was due to the limited sample loading and narrow I.D. detection window in HPCE. It has not been possible to determine the structures of the resolved main peaks by HPCE without knowing their M_r and fragmentation patterns. To obtain such information, it will first be necessary to analyze the sample using HPCE–ESI-MS. Because the sulfomycin sample was never

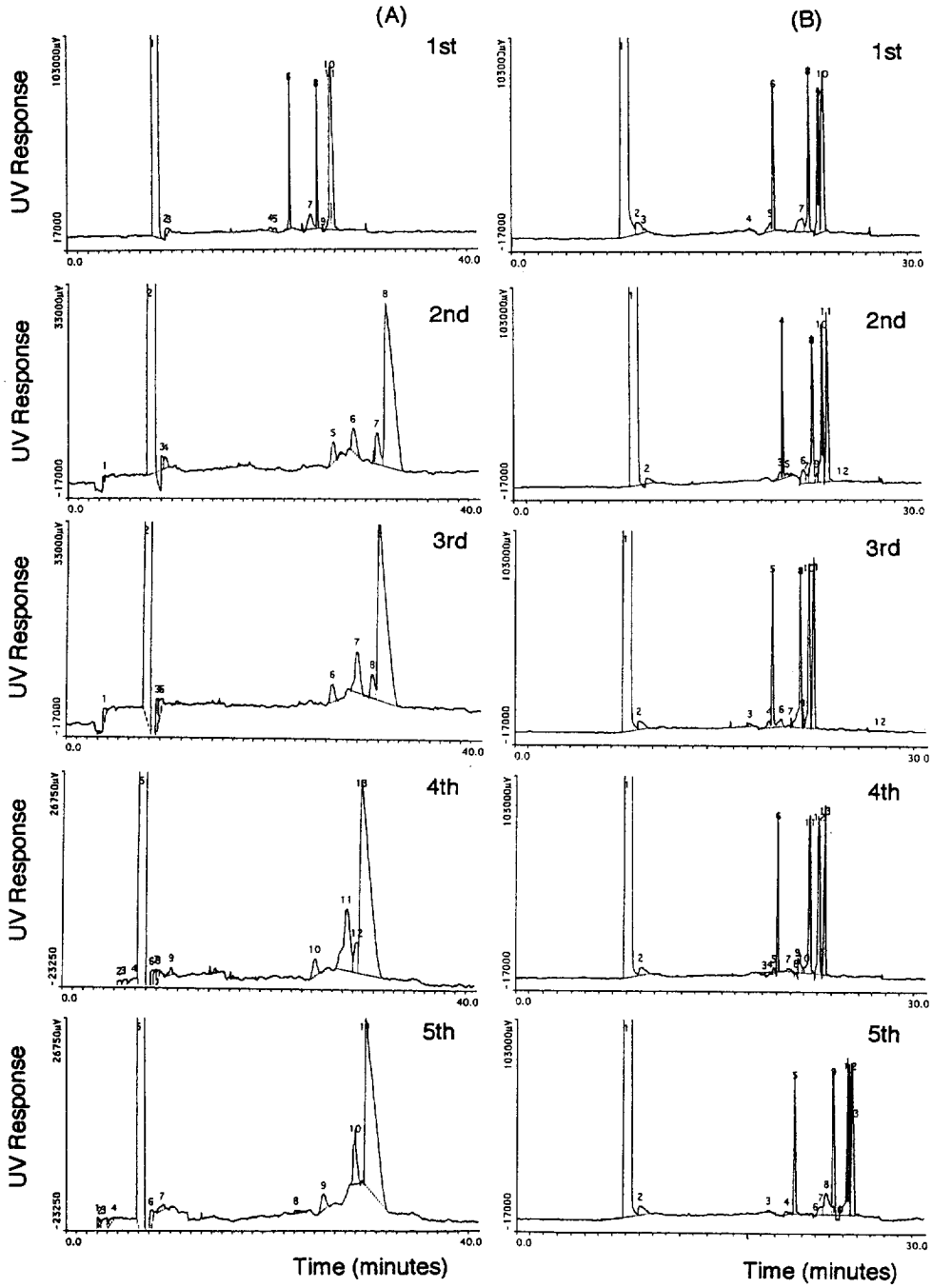

Fig. 7. Effect of buffer replenishment: run buffer was not changed during the whole analysis (A), changed for every injection (B). Capillary: uncoated fused silica, 79 cm×75 μm (56 cm to detector); buffer: 30 m*M* Tris-tricine, 10 m*M* SDS, 20% MeOH, 10 m*M* NaCl, pH 8.0; applied voltage: 25 kV; detection: UV at 210 nm; sample: lot No. 1; concentration: 1 mg/ml in DMF–MeOH (1:9); injection: hydrodynamic injection for 20 s.

resolved in HPCE without the use of 10 m*M* SDS, on-line ESI-MS experiments have not been performed. It is necessary to remove the SDS and other buffer salts from the eluent prior to ESI-MS analysis, and/or to modify the current eluent with a dilution buffer after the CE and prior to introduction into the ESI.

Usually, the importance of capillary washing has been emphasized in HPCE analyses to obtain reproducible data. Our results suggest that the run buffer replenishment, as well as column washing, are essential for reproducible data and resolutions. Particularly when a very complex sample is analyzed with a small buffer reservoir, it may prove necessary to change the buffer for every injection. Reasons for buffer replenishment have been discussed in the literature, and there are several possible causes ([4,5,28,29]). Changes in the buffer composition occur due to electrolytic reactions of the components. Due to the small volume in the reservoir, evaporation of solvents, even water, can lead to different concentrations that will change migrations and elution times. Electrokinetic injection conditions can selectively remove certain buffer ions, and concentrate others for subsequent injections, again leading to irreproducible results. Drastic changes in elution times for different runs on the same day, without buffer replenishment, can lead to vastly different migration times. Artifactual HPCE results can occur as a function of the sample concentration injected, and variations in such concentrations should be studied to ensure that observed peaks are real and not due to precipitation or complex formation.

5. Glossary (list of abbreviations)

CEC	capillary electrochromatography
CE	capillary electrophoresis
cmc	critical micelle concentration
ESI	electrospray ionization
FC	fluorocarbon coated capillary
HPCE	high-performance capillary electrophoresis (CE)
HPLC	high-performance liquid chromatography
M_r	molecular mass
MALDI	matrix assisted laser desorption ionization
MW_{ave}	weight average MW by MS
MS	mass spectrometry
PS-DVB	polystyrene-divinylbenzene
SDS	sodium dodecyl sulfate
SPE	solid-phase extraction
TOF-MS	time of flight mass spectrometry
U82127	**I**=the crude fermentation product mixture
Sulfomycin	the purified, major HPLC component of U82127
2D	two dimensional type separations, e.g., HPLC–HPCE (LC–CE)

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