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Purification of pneumocandins by preparative silica-gel high-performance liquid chromatography

A.E. Osawa[a], R. Sitrin[b], S.S. Lee[c,*]

[a]*Fusion Medical Technologies Inc., 1615 Plymouth St., Mountain View, CA 94043, USA*

[b]*Bioprocess Research and Development, Merck Research Laboratories, Merck and Co., Inc., West Point, PA 19486, USA*

[c]*Sterile Process Technology, Vaccine Technology and Engineering, Merck Manufacturing Division, Merck and Co., Inc., P.O. Box 4, WP29M-4, West Point, PA 19486, USA*

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Abstract

Pneumocandin B_0, a secondary metabolite produced by the fermentation of *Glarea lozoyensis gen.* et sp. nov., has been found to be a potent antifungal compound. Separation from its major impurity, the isomer pneumocandin C_0, proved to be intractable by reversed-phase chromatography or crystallization and could only be performed by normal-phase liquid chromatography. The scale up of the chromatography from an analytical scale column to a ~6 l bed was conducted to produce large quantities of high purity pneumocandin B_0. The challenges of relatively low productivity due to long elution times and to poor product solubility in the mobile phase were addressed. We speculated that the loading per injection was ultimately limited by nonideal retention mechanisms which led to significant peak broadening at very high loading.

Keywords: Pneumocandins; Antifungal agents; Antibiotics; Cyclic peptides; Secondary metabolite

1. Introduction

One of the most challenging tasks in isolating secondary metabolites from fermentation broths is the removal of numerous structural analogs of the desired product formed by the host organism. The similar physical and chemical properties of these analogs render the development of the purification process highly difficult. Pneumocandin B_0 is a potent antifungal agent produced as a recently discovered secondary metabolite by the fermentation of *Zalerion arboricola* [1], later re-classified as *Glarea lozoyensis gen.* et sp. nov. [2]. Small-scale isolation procedures involving solvent extraction and liquid chromatography were described previously [3,4]. The structure of pneumocandin B_0 and its analogs, all comprised of a cyclic hexapeptide to which a dimethylmyristic acid tail is attached, are illustrated in Fig. 1. Pneumocandin B_0 is the product of interest, with a molecular weight of 1069 Daltons. The analogs A_0, B_0, C_0 and D_0 differ only in the substitution of the proline residue at the upper left of the figure. A key impurity coproduced by the fermentation is the isomer pneumocandin C_0 [5], which differs from B_0 only by a single carbon shift of a hydroxyl group. Crystallization and reversed-phase

*Corresponding author.

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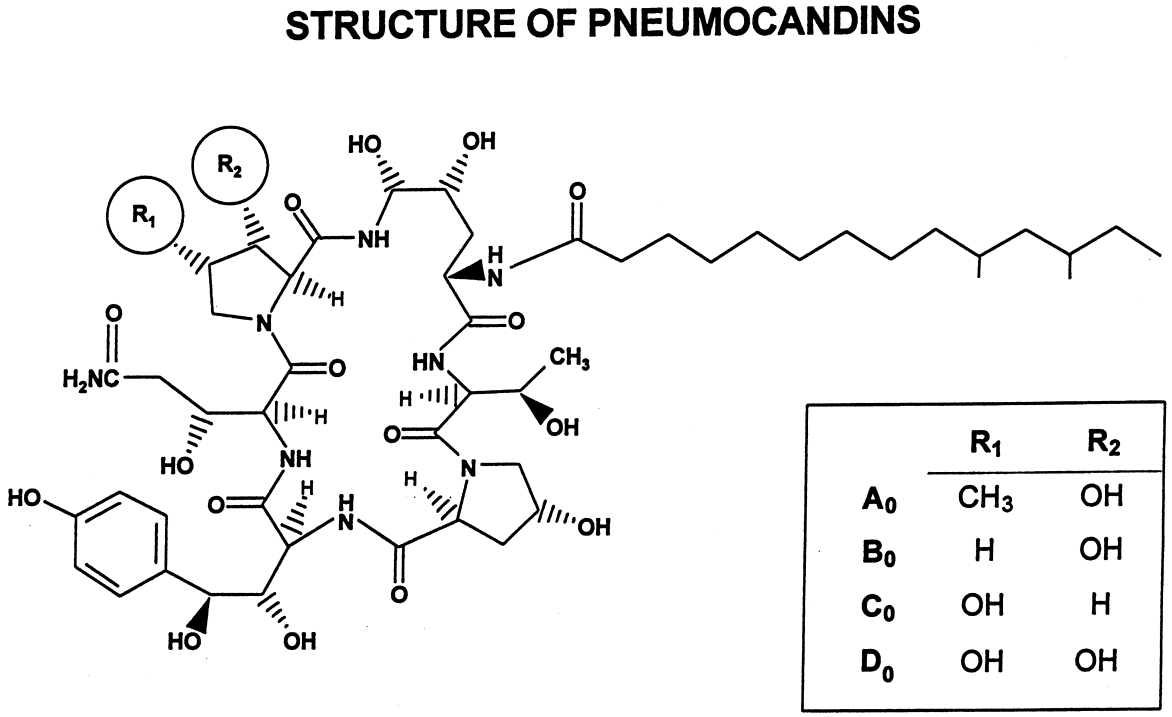

Fig. 1. Structure of pneumocandins.

chromatography had been unable to isolate B_0 from C_0. Thus, a large scale normal-phase liquid chromatography step using a tertiary mobile phase was developed for the large-scale production of the antibiotic pneumocandin B_0 at high purity.

2. Experimental

2.1. Analytical HPLC assay

For analytical quantitation, a 250 mm×4.6 mm I.D. LiChroCART silica-gel column packed with 3 μm LiChrospher Si 60 (E. Merck, Darmstadt, Germany) was used with a mobile phase consisting of ethyl acetate (EtOAc), methanol (MeOH), and water at a ratio of 86/7/7. This composition is a modification of the one proposed by Schwartz et al. [1], replacing 5% aqueous acetic acid with water, and was found to give somewhat better resolution of minor components while simplifying mobile phase formulation. Solvents were HPLC grade from Fisher (Pittsburgh, PA) and were delivered by a Rainin HP pump (Woburn, MA). Column effluent was monitored at 278 nm with a Knauer Model 87 variable wavelength detector. A Dynamax AI-1 autosampler was used to inject 100 μl samples for assay. HPLC system control and data acquisition were performed with Rainin Dynamax software. Typical results are listed in Table 1.

Table 1
HPLC assay retention times for pneumocandin analogs

Column	Mobile phase	Flow-rate (ml/min)	Retention time (min)			k'		
			A_0	B_0	C_0	A_0	B_0	C_0
LiChroCART	EtOAc/MeOH/H_2O (86/7/7)	1.15	17.6	19.6	24.3	9.7	11	14

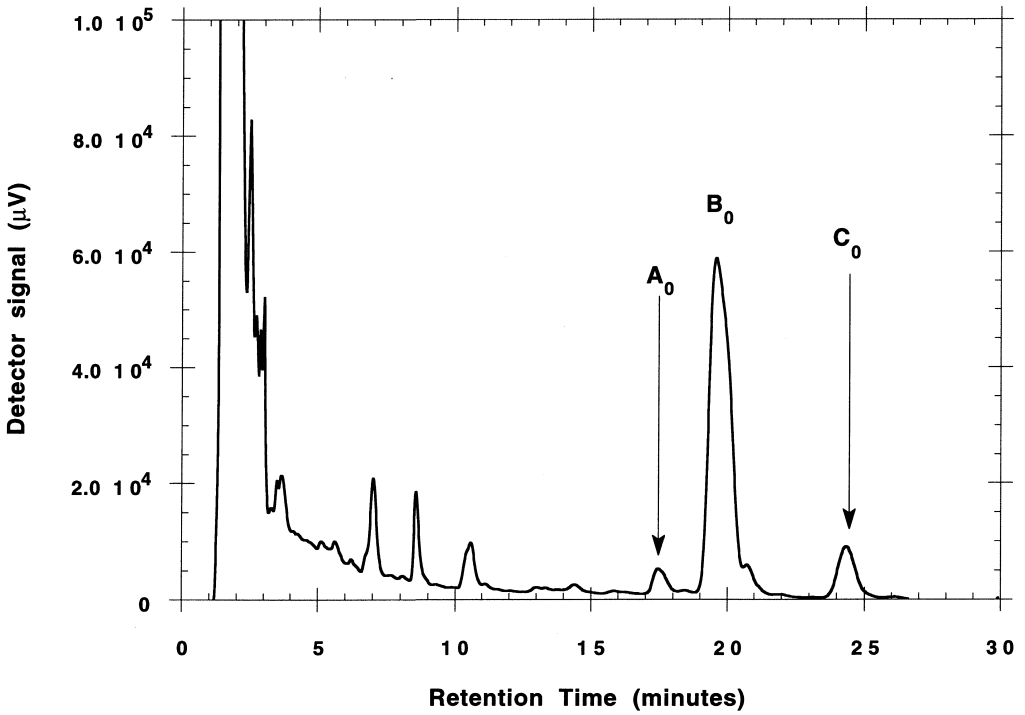

Fig. 2. A typical analytical HPLC chromatogram for pneumocandins using silica gel. Column: Lichrocart (3 μm, Si_6O); Mobile phase: EtOAc/MeOH/H_2O=86/7/7; flow-rate: 1.5 ml/min; column pressure: 1200–2000 psig.

A typical analytical chromatogram is shown in Fig. 2.

2.2. Preparative scale HPLC

A Prochrom LC 150 column (Indianapolis, IN) of axial compression design, 15 cm ID, was used for the preparative scale liquid chromatography runs. It was typically packed with 3.0–3.8 kg Matrex silica (Amicon, Danvers, MA; 10–20 μm, 60 Å), resulting in a bed volume of 6.0–7.5 l. Kromasil silica (Eka-Nobel, Bohus, Sweden; 10–20 μm, 100 Å) was also used. The column was slurry-packed in a mobile phase using axial compression at ~75 bar hydraulic pressure. Solvent delivery, detection, and injection were performed using a Dorr-Oliver Peak Performer system. Unless specifically stated, a standard flow-rate of 2000 ml/min was used, resulting in a back pressure of ~850 psi. The injection volume, typically 600 ml, was restricted to less than 15% of the bed volume, and fed to the column at 300 ml/min. UV detection at 278 nm was employed for on-line product monitoring. The mobile phase used was EtOAc/MeOH/H_2O=84/9/7. A mixture of EtOAc/MeOH/H_2O with lower EtOAc content was used to dissolve the feed for loading. This solvent system had much higher solubility for pneumocandins and no deleterious effect on the separation provided that the feed volume was kept at a small fraction of the bed volume. Fractions ranging in size from 900 ml to 3600 ml were collected and assayed for pneumocandins by analytical HPLC assay (see preceding section). The Prochrom column and control system are situated in our Natural Product Isolation Pilot Plant (Rahway, NJ).

2.3. Heated preparative HPLC studies

For preparative runs at elevated temperatures, the mobile phase was pressurized to 25–30 psig and heated to a temperature of 68°C as it entered the pump, resulting in temperatures of 55°C and 50°C at the column inlet and exit, respectively. A recycle line was established to enable preheating of the system

with minimal solvent consumption. The column and all lines and valves were well-insulated. Hot feed was prepared in the loading solvent at 55°C and injected immediately after preparation. Preparative scale runs using heated mobile phase were performed using a special mixture of pneumocandins B_0 and C_0 at a ratio of about 3:1. The effluent was cooled to 30–35°C for operator safety by running it through a short coil immersed in ice water at the exit of the column. Fractions of 950 ml and 3600 ml were collected and assayed by analytical HPLC.

2.4. Breakthrough curve studies

Breakthrough curve studies were performed using solutions of pneumocandin B_0 in EtOAc/MeOH/H_2O=84/9/7, the large-scale HPLC mobile phase, to determine adsorption isotherms for pneumocandin B_0. A pneumocandin B_0 solution of known concentration was pumped through an analytical column pre-equilibrated with the mobile phase. A plateau in concentration at the column outlet was reached within 5 column volumes. After 80 ml (~20 column volumes) of loading solution had passed through the column, 100 ml of fresh mobile phase containing no pneumocandin B_0 was washed through the column and the retained solute was eluted. Analytical scale columns packed with either Eka-Nobel Kromasil (5 μm/100 Å) or Amicon Matrex silica (5 μm/60 Å) were used. Flow-rates were varied from 0.5 ml/min to 2.0 ml/min, temperature was set at either 25°C or 55°C, and the concentration of the solutions ranged from 0.8 g/l to 4.0 g/l. A detector wavelength of 286 nm was chosen, as absorbance at this wavelength is linear to over 4.0 g/l.

3. Results and discussion

3.1. Issues in the purification of pneumocandin B_0

Resolution by reversed-phase HPLC was found to be poor between pneumocandins A_0 and B_0 and nonexistent between B_0 and C_0. Retention on reversed-phase columns is apparently dominated by the interaction of the stationary phase with the aliphatic tail, which is identical for all analogs. Only by the use of a normal-phase support was any separation between B_0 and C_0 achieved, and separation of B_0 and C_0 remained particularly difficult given their minor structural difference. Prior to chromatography, 5–15% of the pneumocandins were present as C_0. The target levels for A_0 and C_0 after chromatography were 5% and 2.5% of the pneumocandins, respectively, as subsequent steps result in further purification.

There were several major obstacles in improving the productivity of the preparative normal-phase HPLC process. The retention times of the components were highly sensitive to slight changes in the mobile phase composition. They were also relatively long—the value for the capacity factor k' using the 84/9/7 mobile phase being approximately 10 and 14 for the desired product and the last impurity, respectively. Moreover, loading per injection was limited by a low solubility in the mobile phase. Further limitations to the productivity might be due to suspected nonidealities in product retention mechanisms (see later sections).

3.2. Optimization of the mobile phase

Ethyl acetate–alcohol–water and chloroform–methanol-based mobile phases were first found to be successful for the separation of pneumocandin-like molecules on silica gel by Mizuno et al. [6] and Satoi et al. [7] for the isolation of aculeacins. Some typical proportions were CH_2Cl_2MeOH=10/1 and EtOAc/*n*-BuOH/H_2O=2/10/1. Resolution of pneumocandins by silica-gel thin-layer chromatography employing aqueous mixtures of chlorohydrocarbon and alcohol such as CH_2Cl_2/MeOH/5% acetic acid=72.5/27.5/10 and CH_2Cl_2/MeOH/H_2O=80/20/2 were reported by Schwartz et al. [1,3,4]. These mobile phases translated well to an analytical HPLC column packed with underivatized silica. Operational issues pertaining to the limitation of use of chlorinated solvents in production settings led to efforts in optimizing an ethyl acetate-based solvent system for pneumocandin separations, and the composition EtOAc/MeOH/5% aqueous acetic acid=85/10/5 was found to give good separation between pneumocandins B_0 and C_0 on silica. Further studies indicated that acetic acid was not essential for maintaining the stability of the compound or the separation.

The high water and methanol content of these mobile phases may indicate that a partition-type separation mechanism is at work. However, partition chromatography systems, such as centrifugal counter-current chromatography, are not typically suitable for large-scale production.

Separation between analogs was enhanced by increasing the relative proportion of EtOAc. The interaction between the stationary phase and the hexapeptide heads of the analogs was presumably enhanced by decreasing the polarity of the mobile phase. This, however, also decreased the solubility for the pneumocandins and lengthened the residence time of the solutes on the column. At EtOAc concentrations near 88%, the separation grew highly sensitive to fluctuations in mobile phase composition, and EtOAc concentrations greater than 88% resulted in phase separation. Raising the relative methanol concentration increased the solubility of B_0 in the mobile phase, but B_0/C_0 resolution deteriorated rapidly. Optimization of the mobile phase composition by a factorial experiment design is the subject of another paper.

With these considerations in mind, mobile phase compositions centering around the ratio 85/10/5 were screened. For analytical separations, a composition of EtOAc/MeOH/H_2O=86/7/7 was adopted as it gave somewhat better resolution between minor components. For preparative scale work, the objective was to increase solubility and reduce retention time without compromising resolution, and decreasing the EtOAc to a composition of 84/9/7 was found to be effective. A typical chromatogram is shown in Fig. 3, which is the result of a single injection of 12.5 g B_0 (1.7 g B_0/l bed; 2.5 g pneumocandins/l bed). Sandwiched by A_0 and C_0, pneumocandin B_0 starts to elute off the column after about 14.5 min, and the last impurity (D_0) begins eluting after about 20 min. In the pilot plant, great care was taken in preparing and storing the mobile phase because of the sensitivity of the retention profile to its composition.

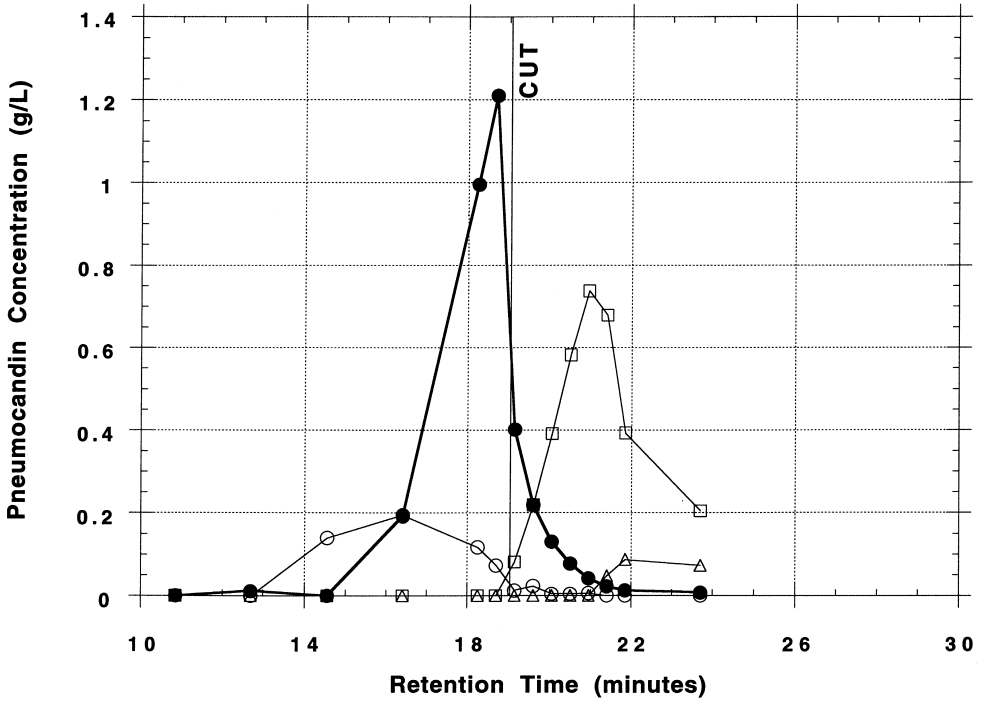

Fig. 3. A typical preparative normal phase HPLC chromatogram for pneumocandins separation. Column: Prochrom LC150 (Amicon 15 μm/60 Å); mobile phase: EtOAc/MeOH/H_2O=84/9/7; flow-rate: 2 l/min; column pressure: ~850 psig; column loading: 2.5 g pneumocandins/l bed; temperature: ambient. Legend: Pneumocandin A_0 (– ○ –), B_0 (– ● –), C_0 (– □ –), D_0 (– △ –).

3.3. 'Boxcar' injections

The lengthy retention times of the pneumocandin analogs were exploited in order to improve the productivity of the preparative HPLC process. A second sample was injected just prior to the expected elution of the first sample. Termed a 'boxcar' injection scheme, as several such staggered injections would result in a train-like concatenation of product peaks, a set of three boxcar injections (each 11.9 g B_0, or 1.6 g B_0/l bed) and another set of four boxcar injections (each 10.0 g B_0, or 1.3 g B_0/l bed) were performed.

Based on results presented in the preceding section, a 25 min injection interval was chosen for the 'boxcar' injections. About 18 fractions were taken per injection and assayed by analytical HPLC. The results were tabulated and the optimal pooling of B_0 rich cuts was determined to obtain less than 2.5% of the pneumocandins as C_0. The profiles of the pneumocandins in these runs are given in Figs. 4 and 5, respectively. As illustrated in Figs. 4 and 5, each set of peaks in the boxcar train displays the expected characteristic profile, the first cuts having a high presence of A_0 and the later ones rich in C_0 and D_0. For later injections in the boxcar series, the A_0 peak co-eluted with the late arriving D_0 from the previous injection, making their removal even more efficient.

Selected fractions rich in pneumocandin B_0 were then pooled, concentrated under reduced pressure to an aqueous slurry, which was vacuum-dried and stored at 2–8°C awaiting further processing. The composition of the solid was typically 89% B_0, 2% C_0, 5% A_0 and 0% D_0, with the remainder as unidentified minors. The yield of B_0 was typically 80–85%.

3.4. Heated mobile phase studies

The poor solubility of pneumocandin B_0 at ambient temperature limited the loading of solute onto the column. The solubility of pneumocandin B_0 in

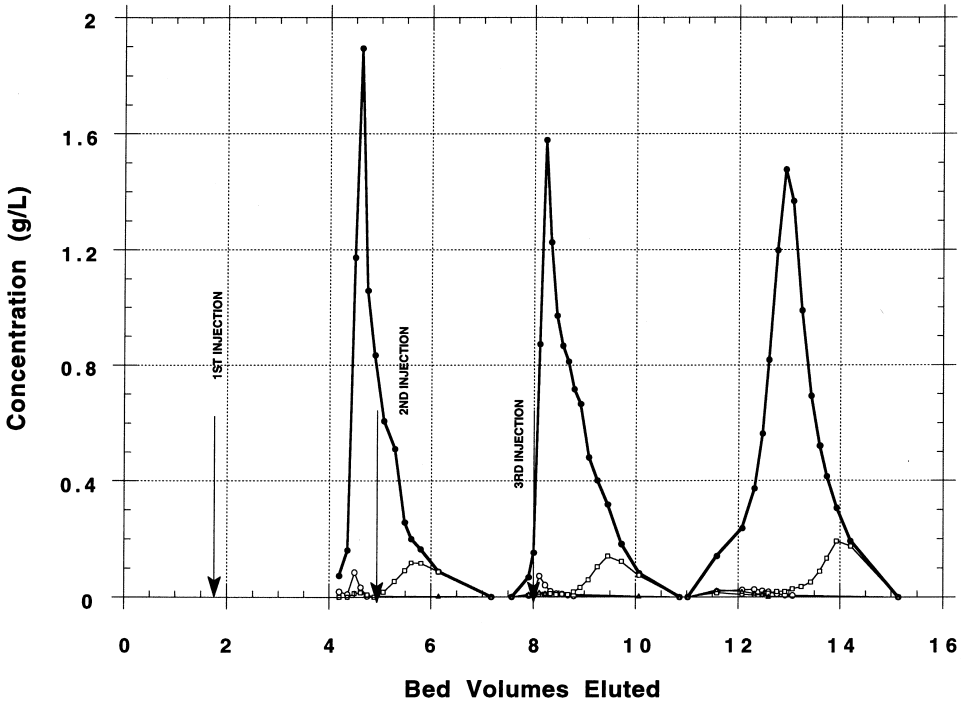

Fig. 4. A set of three boxcar preparative normal phase HPLC runs for pneumocandins separation. Column: Prochrom LC 150 (Amicon 15 μm/60 Å); mobile phase: EtOAc/MeOH/H_2O=84/9/7; flow-rate: 2 l/min; column pressure: ~850 psig; column loading: 2.5 g pneumocandins/l bed (1.6 g B_0/l bed); temperature: ambient. Legend: Pneumocandin A_0 (– ○ –), B_0 (– ● –), C_0 (– □ –), D_0 (– △ –).

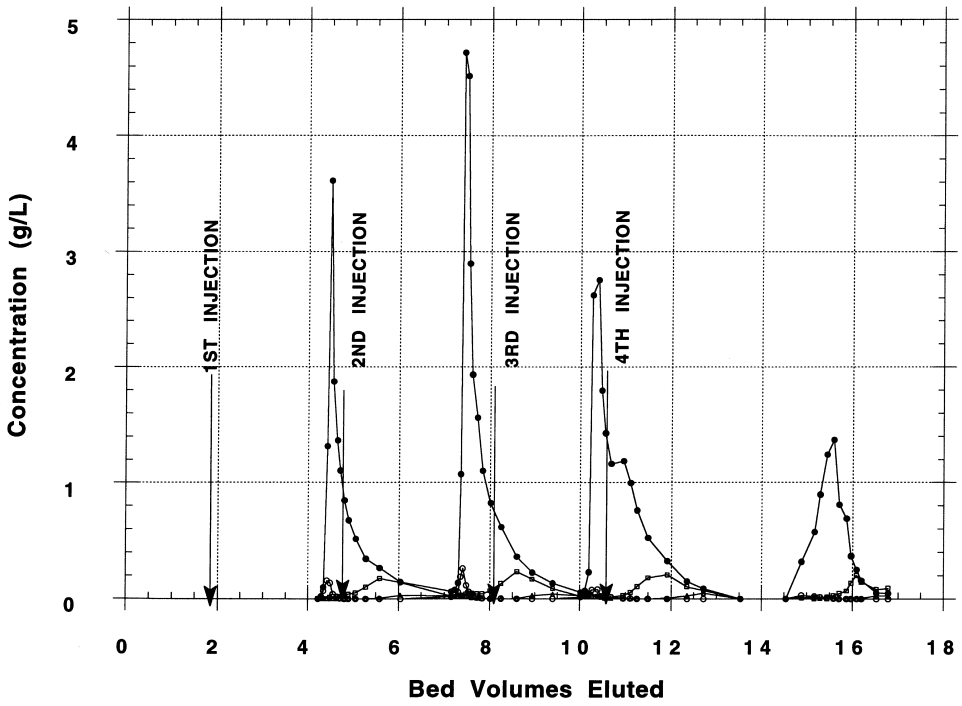

Fig. 5. A set of four boxcar preparative normal phase HPLC chromatogram for pneumocandins separation. Column: Prochrom LC15O (Amicon 15 μm/60 Å); mobile phase: EtOAc/MeOH/H_2O=84/9/7; flow-rate: 2 l/min; column pressure: ~850 psig; column loading: 2.5 g pneumocandins/l bed 1.3 g B_0/l bed; temperature: ambient. Legend: Pneumocandin A_0 (– ○ –), B_0 (– ● –), C_0 (– □ –), D_0 (– △ –).

the EtOAc/MeOH/H_2O=84/9/7 mobile phase is 4.3 g/l at 25°C and 30 g/l at 45°C, without any product degradation. By decreasing the EtOAc content further, a solubility of 100 g/l at 45°C was obtained.

Heated runs on analytical scale columns were first conducted to estimate appropriate loading levels. Touching band separation was achieved at 2.5 g pneumocandins/l bed loading, with overlapping but distinct peaks seen at 7.2 g pneumocandins/l bed. Fig. 6 depicts a preparative run performed at the same loading as the case shown in Fig. 3, but with a heated mobile phase on a 6 l bed of normal-phase silica (Amicon, ~15 μm, 60 Å) in the Prochrom unit. A flow-rate of 2.0 l/min (3 bed volumes/min) and an injection volume of 0.6 l (10% bed volume) were used. Besides a slight broadening of the B_0 peak, and earlier elution of A_0 peak, there are otherwise no significant effects due to the heating of the mobile phase.

A higher loading run was then performed using 600 ml of feed solution at 75 g/l pneumocandins in loading solution at 55°C on a 6 l bed of normal-phase silica (7.5 g/l bed loading). Results are shown in Fig. 7, in which the lines delineating the 'cut' point are determined by analytical HPLC assays of the fractions giving a pneumocandin C_0 concentration of less than 2.5% of the pneumocandins. The resulting yield for these injections was about 75%, slightly lower than the 80–85% observed for the unheated mobile phase yet at about three times its productivity per injection.

3.5. Limitations to productivity

As expected, resolution between B_0 and C_0 decreased with increasing column loading of pneumocandins. Exacerbating the situation were suspected nonidealities in the apparent adsorption of the pneumocandins onto the stationary phase, accompanied by possible partitioning behaviors. We speculated that the water–methanol rich mobile phase may have

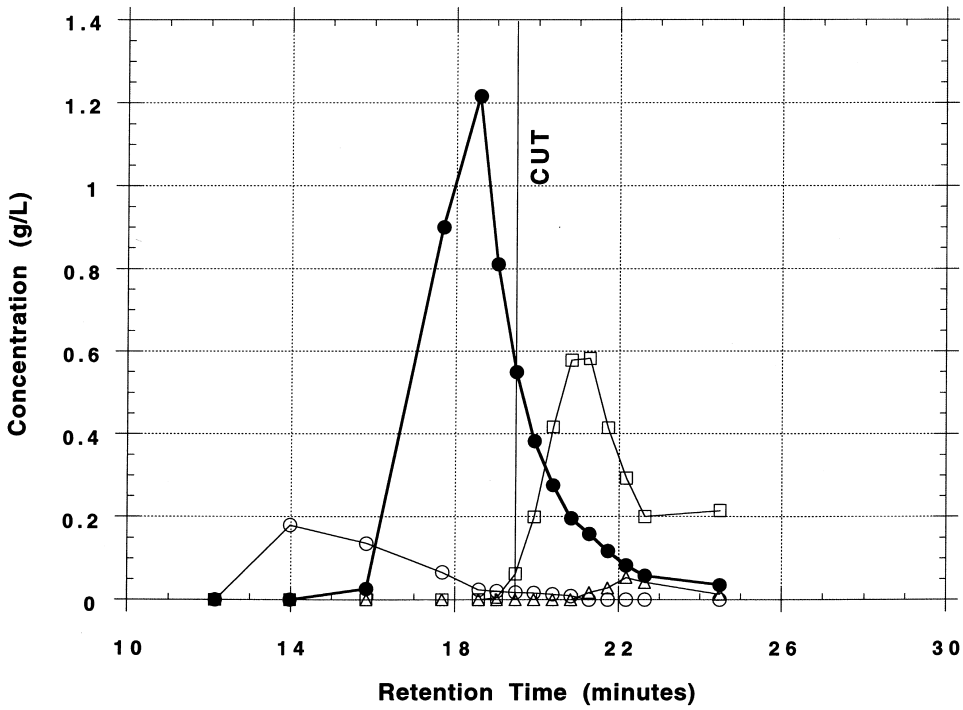

Fig. 6. A preparative normal phase HPLC chromatogram for pneumocandins separation at an elevated temperature. Column: Prochrom LC150 (Amicon 15 μm/60 Å), 6 l bed; mobile phase: EtOAc/MeOH/H_2O=84/9/7; flow-rate: 2 l/min; column pressure: ~850 psig; column loading: 2.5 g pneumocandins/l bed and 600 ml injection; temperature: 55°C. Legend: Pneumocandin A_0 (– ○ –), B_0 (– ● –), C_0 (– □ –), D_0 (– △ –).

formed a tightly held polar solvent layer on the silica surface. Partition of pneumocandins between the bulk mobile phase and this layer, or even multiple layers, may be a critical step in this separation.

The flow-rate had a substantial impact on the capacity factor, k', indicating the presence of a kinetic or dynamic limitation to adsorption. All analogs appeared to share parallel dependencies of k' with flow-rate. It is difficult to determine whether this limitation is due to a steric hindrance of the solute into the pores of the silica or due to some finite equilibration time required for partitioning with the silica and/or a silica-bound phase.

Another possible nonideality leading to poor resolution is multilayer adsorption of the pnuemocandins. With their hydrophilic heads and hydrophobic tails, the pneumocandins resemble surfactant-type molecules, such as the *n*-alkyl substituted polyethylene glycols C_8E_6, $C_{12}E_5$ and $C_{12}E_8$, for which multilayer adsorption on silica is a commonly acknowledged phenomenon [8,9]. Further experimentation is required to confirm this hypothesis.

4. Conclusions

The preparative HPLC process to resolve very minor differences between molecules has been demonstrated in the separation of pneumocandin B_0 from pneumocandin C_0 on normal-phase silica. The low productivity of the separation, due to long retention times and low solubility in the mobile phase, was alleviated by judicious use of staggered injections and by heating the mobile phase. We postulate that the limitations to loading ultimately lie in the retention mechanism. Nonidealities, such as kinetic limitations, partitioning effects, and multilayer adsorption, may have contributed to inadequate resolution at higher loading of solute per injection. As a result, future studies to improve productivity must

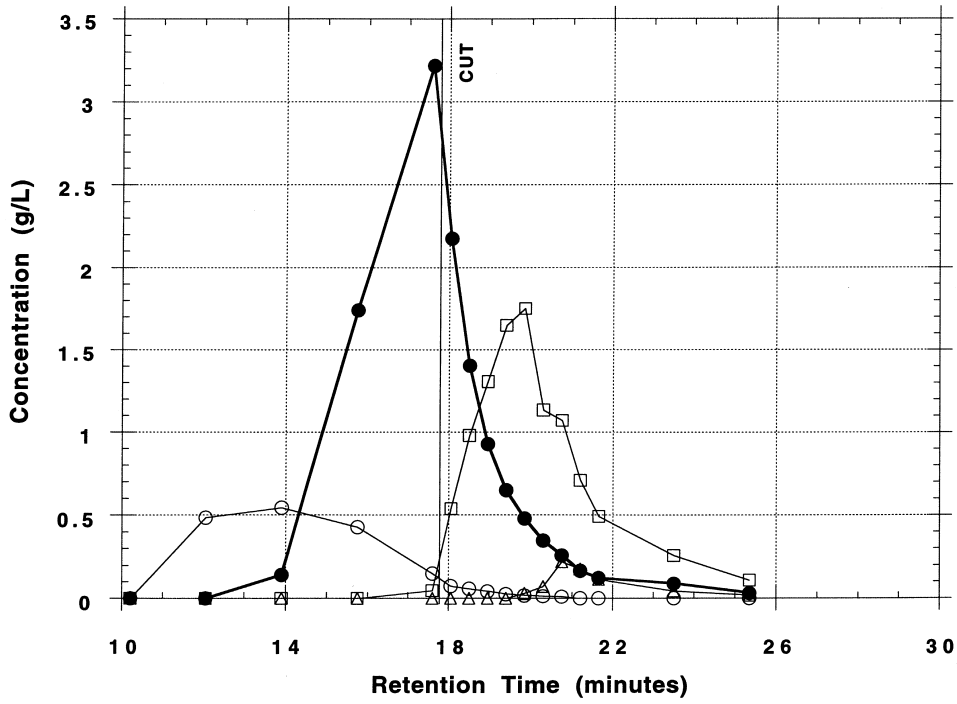

Fig. 7. A preparative normal phase HPLC chromatogram for pneumocandins separation at an elevated temperature and higher loading. Column: Prochrom LC150 (Amicon 15 μm/60 Å), 6 l bed; mobile phase: EtOAc/MeOH/H_2O=84/9/7; flow-rate: 2 l/min, column pressure: ~850 psig; column loading: 7.5 g pneumocandins/l bed; temperature: 55°C. Legend: Pneumocandin A_0 (– ○ –), B_0 (– ● –), C_0 (– □ –), D_0 (– △ –).

focus on the shortening of cycle time rather than increasing the mass of solute per run.

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