Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1098 (2005) 55–65
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Influence of mobile phase composition and thermodynamics on the normal phase chromatography of echinocandins

David J. Roush *, Lisa Y. Hwang [1], Firoz D. Antia [2]

Merck & Co., Inc., BioProcess R&D, BioPurification Development Group, P.O. Box 2000, RY805S-100, Rahway, NJ 07065, USA

Received 18 November 2004; received in revised form 11 August 2005; accepted 15 August 2005
Available online 31 August 2005

Abstract

In the normal phase preparative HPLC of fermentation derived echinocandins, resolution of key impurities from the product of interest, pneumocandin B_o, is accomplished using a ternary ethyl acetate/methanol/water mobile phase with silica gel as the sorbent. In this work, previous characterization of the system is extended to define the impact and role of water content on the separation efficiency and retention of pneumocandin B_o. Experimental results indicate that column efficiency, measured using both the product of interest and small molecule tracers (compounds used for pulse tests), is good despite the use of an irregular silica and unusually high levels (greater than 6%) of water in the mobile phase. In contrast to column efficiency measurements using small molecules (MEK and toluene), measurements performed with the product itself indicate improved efficiency with increasing water content of the mobile phase. Building on these results, a scale-up/scale-down protocol was developed based on measurements of column efficiency using theoretical plate counts determined with pneumocandin B_o. Since the solubility of pneumocandin B_o in the ternary mobile phase is relatively low, a higher strength solvent with higher levels of methanol and water is employed for dissolution of the crude product at concentrations of up to 40 g/L. The mismatch between the high strength solvent used for the feed introduction and the mobile phase has the potential to affect column performance. The impact of this mismatch using plate count measurements with the product at both analytical and semi-preparative scales was found not to be significant. Finally, a van't Hoff analysis was performed to characterize the thermodynamics of adsorption of pneumocandin B_o on silica. The analysis shows that the adsorption process for pneumocandin B_o on silica in the ternary solvent system is endothermic ($\Delta H_{ads} > 0$), implying that the adsorption is entropically driven. Results from an overall water balance across the column indicate significant enrichment of adsorbed water on the silica surface. These results further emphasize the importance of selective partitioning of water between the bulk mobile phase and the silica as a dominant factor in controlling retention.

Keywords: Pneumocandin; Echinocandin; Normal phase HPLC; Ternary mobile phase; Irregular silica; Preparative HPLC; van't Hoff

1. Introduction

Echinocandins are a class of naturally occurring lipopeptides with antifungal activity. The structures of three closely related members of the subclass known as pneumocandins are given in Fig. 1. They are cyclic hexapeptides with multiple hydroxyl groups and a hydrophobic dimethyl myristate tail connected via an amide bond to the α-amino group of the (hydroxylated) ornithine residue. The three compounds in the figure differ from each other only in subtle modifications to the side chain of the proline residue adjacent to the "ornithine". Indeed, pneumocandins B_o and C_o differ only in the position of the hydroxyl group on this side chain, whereas pneumocandin A_o has an additional methyl group [1].

The large-scale isolation of pneumocandin B_o from cultures of the fungus *Glarea lozoyensis* [2] remains a technical challenge despite intense research efforts directed at controlling the distribution and amounts of various analogues produced by the organism during fermentation [3–8]. The organism can produce, in addition to the desired compound, 20 or more echinocandins including pneumocandins A_o and C_o [9,10]. Furthermore, these lipopeptides have unique solubility properties; they are essentially insoluble in water and most pure solvents, but dissolve in alcohols and some aqueous/organic solvent mixtures [11].

* Corresponding author. Tel.: +1 732 594 3204; fax: +1 732 594 4973.
E-mail address: david_roush@merck.com (D.J. Roush).
[1] Present address: Department of Chemical Engineering, Stauffer III, 381 North-South Mall, Stanford University, Stanford, CA 94305-5025, USA.
[2] Present address: Merck & Co., Inc., Chemical Engineering R&D, P.O. Box 2000, RY818-B310, Rahway, NJ 07065, USA.

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.08.042

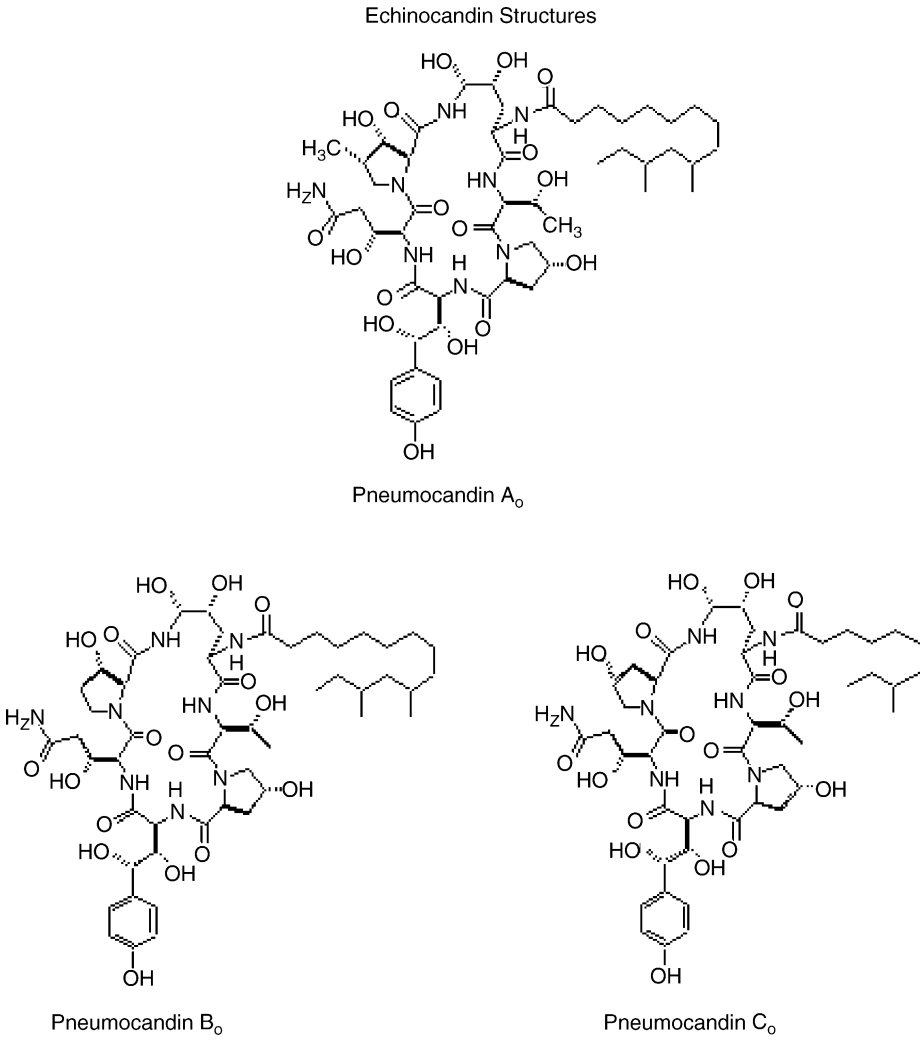

Fig. 1. Structures of three pertinent pneumocandins including the product of interest, pneumocandin B_o are presented. Analogue impurity pneumocandin A_o represents the addition of a methyl group at the 4 position on the hydroxy-proline functional group in the polar hexapeptide core. Pneumocandin C_o is a positional isomer of the product representing a shift in the hydroxyl group from the 3 position (pneumocandin B_o) to the 4 position (pneumocandin C_o) of the same hydroxy-proline group. For more details on pneumocandin B_o structure and details, see references [1–6].

Moreover, it is difficult, if not impossible, to purify them by crystallization.

The present work describes the further characterization of the normal phase high performance liquid chromatography (HPLC) step for purification of pneumocandin B_o, extending previous research that demonstrated the feasibility of pneumocandin purification by HPLC [12,13]. Typical preparative separations by normal phase HPLC are performed with bare silica, either spherical or irregular, often employing a binary mobile phase blend of a relatively polar and a relatively non-polar compound. Previous research presented in the literature [14] indicates the feasibility of using a ternary solvent system for chromatographic purification for both liquid–solid (LSC) and liquid–liquid (LLC) chromatography. Water is usually added to the mobile phase at low levels (<1%) to eliminate variations in the water content of the solvents and to de-activate highly polar binding sites on the silica surface [15]. However, balancing the solvent polarity, selectivity, and retention afforded by the mobile phase via the use of a ternary solvent system for preparative normal phase separations has only seen limited utilization [16]. In particular, the use of high levels of water – up to about 7% in the mobile phase – for controlling the selectivity in the normal-phase pneumocandin purification, as is used in this case, is unusual.

The goal of these experimental studies was to develop a set of tools to enable accurate scale-down of an existing purification process. These tools, including the use of plate count measurements derived from product adsorption studies, were used to gain a better scientific understanding of the contributions of solvent composition of the load solution on the retention behavior of pneumocandin B_o on silica in the normal phase mode.

The effect of water on the compound adsorption and separation efficiency in normal phase chromatography, in particular in a ternary mobile phase with high water content, is of considerable interest. Although a significant amount of literature exists on the physical chemistry of water–silica interactions including the impact of the types of silanol groups on the kinetics and thermodynamics of water adsorption to silica [17–19], few conclusive

experimental studies have been presented in the literature that extend this research to a normal phase chromatography application. One exception is the research of C.A. Fung Kee Fung and M. Burke who evaluated the interactions of water with silanol groups and modified silica surfaces via differential scanning calorimetry [20]; while interesting, their study does not provide guidance for actual chromatographic operations. Previous work in the ternary ethyl acetate (EtOAc)/methanol (MeOH)/water system [13] indicated that column efficiency, as determined using a relatively small compound – methyl ethyl ketone (MEK) – as the tracer is essentially constant with increasing water content over the typical operating range, corresponding to reduced velocities in the range of 6–16.

The previous work is extended here to examine the effect of mobile phase composition on the column efficiency using pneumocandin B_o itself as the tracer. Results presented here for the pneumocandin B_o system indicate the opposite trend is true—column efficiency increases with increasing water content. This observation provides insight into the role of water in mediating the interaction of pneumocandin B_o with the silica surface.

The ternary feed solvent is enriched in methanol (MeOH) and water relative to the mobile phase to increase the solubility of pneumocandin B_o versus the mobile phase eluent. However, since the load solvent has a higher solvent strength than the mobile phase, the change in conditions from the high to low strength across the eluting band could be anticipated to increase bandspreading, and therefore lower column efficiency. The relationship of the impact of feed volume on resolution in preparative chromatography has been discussed with significant rigor previously in the literature [21]. The tradeoff between potential loss of column efficiency due to the solvent mismatch versus the increased productivity from higher column loadings is also examined through column efficiency measurements using the product, pneumocandin B_o.

Finally, to gain a better understanding of the binding mechanisms of pneumocandin B_o to the sorbent, a van't Hoff analysis [22] has been performed to determine the thermodynamic driving forces for binding (enthalpic, entropic or a combination of the two). The extent of selective water adsorption on the silica sorbent resulting in an enhanced partitioning of pneumocandin B_o was also examined. Two possible explanations exist—direct interaction of the pneumocandin B_o with the silanol groups present on the silica surface or indirect interaction between the silanol groups and pneumocandin B_o mediated by waters of hydration for both the silanol groups and pneumocandin B_o. The goal of the experiments was to help to discern the role of water in the adsorption of pneumocandin B_o to silica. Changes in retention factor as a function of temperature and mobile phase composition have been measured. Enthalpies and entropies of adsorption are observed to be a function of water content in the mobile phase. In addition, an understanding of the interaction of water with the stationary phase is provided by calculating a mass balance to determine the partitioning of water between the bulk solvent and the sorbent surface. These data combined with results from other researchers presented in the literature [17,18] support the concept of a multi-layered water structure surrounding the silica surface in this ternary solvent system that controls the retention of pneumocandin B_o.

2. Experimental

2.1. Materials and methods

All experimental studies employed irregular silica from Amicon (Cherry Hill, MA) designated Grade 631, Si-60 of nominal particle size 20 μm. Analytical scale columns (4.6 mm × 250 mm) were obtained prepacked from Amicon (Beverly, MA). All solvents employed in the ternary mobile phase (ethyl acetate, methanol and water) were HPLC grade from Fisher Scientific (Pittsburgh, PA) or EM Science (Gibbstown, NJ). Mobile phases employed for the preparative scale chromatography were prepared from solvents of equivalent purity. Volumetric compositions of solutions are designated as volumes prior to mixing and do not account for non-ideal mixing effects. For example, an EtOAc/MeOH/H_2O solution designated as 84/9/7 was prepared by adding 84 volumes of EtOAc, 9 volumes of MeOH and 7 volumes of H_2O.

Tracer solutions for both analytical and preparative scale were prepared by adding 2.5 vol% methyl ethyl ketone (MEK; GR grade from EM Science) and 0.5 vol% toluene (ACS grade from Fisher Scientific) to the ternary mobile phase.

Feedstock for the preparative separation experiments was derived from an isolated solid intermediate containing the product of interest, pneumocandin B_o, at 89.6 wt.% purity, in addition to more than 10 other structurally related (analogue) impurities. The structure of the product and two other marker analogue impurities is presented in Fig. 1. The feed to the preparative scale HPLC experiments contained the product at a concentration ranging from 4 to 5 g/L.

2.2. Equipment

Analytical scale experiments were performed on a Hewlett Packard HP-1100 HPLC system composed of a quaternary pump, column thermostat and variable wavelength detector with detection monitored at 278 nm. The data analyses were performed using ChemStation Software Rev. A.05.04 and a Windows 95 (Microsoft: Eugene, OR) operating system. All mobile phases were prepared via blending of solvents off-line to HPLC operations. Injections for tracer experiments were performed at volumes from 5 to 20 μL resulting in resin loadings for product streams in the range 10–12.5 mg/L-resin. Analyses of fractions from preparative HPLC runs were performed with the HP-1100 system.

All preparative scale experiments were conducted on a custom Biotage HPLC system with a 60 mm I.D. (0.5 m length) Prochrom (Indianapolis, IN) dynamic axial compression (DAC) column. The preparative column, 60 mm I.D., was packed with 390 g of the Amicon silica, mixed with approximately 1.1 L of 87/9/7 mobile phase. The slurry was stirred until homogeneous and poured into the empty column. The column was closed and compressed by pressurizing to 40 bar resulting in a packed column length of 270 mm.

All samples for column efficiency measurements for the preparative scale experiments were manually injected via a syringe with injection volumes of 5 mL followed by 80 mL of load solvent to completely clear the injection loop for a total injection volume equivalent to 11% of the column volume.

The software used for data analysis was Bio-GRAMS/386™ for Microsoft Windows Version 3.03 Level 1.

Water content of samples was determined by a coulometric Karl Fischer (KF) titration. These measurements were performed on a Metrohm 684 KF Coulometer from Brinkmann Instruments, Inc. (Westbury, NY).

2.3. Calculation of diffusion coefficient for pneumocandin B_o

In order to convert the retention data acquired for pneumocandin B_0 to a dimensionless form suitable for comparison with the previous data from small molecule tracer experiments, a diffusion constant for pneumocandin B_0 is required, D_{B_0}. No explicit experimental data were obtained to determine D_{B_0} as part of this study. Instead, an estimate was made based on published data by other researchers. Since pneumocandin B_0 is a hexapeptide coupled to an aliphatic tail, two classes of compounds were used to bracket the range of diffusion coefficients—complex organics (the fluorescent dyes oxonine and pyronine) and peptide fragments. An estimate for D_{B_0} of 1.9×10^{-6} cm^2 s^{-1} was determined based on literature data [27–29] in order to perform a van Deemter analysis (reference Appendix A for more details).

2.4. Determination of column void volume

The column void volume was determined for each analytical column employed in the experiments using the MEK/toluene tracer solution under unretained conditions in isocratic mode. Experiments were performed using a mixture of two mobile phases: mobile phase A composed of 87/9/7 (EtOAc/MeOH/water) and mobile phase B composed of 0/96/7. A mixture of mobile phase A and B was used to reach a given isocratic mobile phase composition. As the fraction of the stronger elution solvent (eluent B) was increased, the retention time of the tracer compounds decreased. The fraction of eluent B was increased stepwise until the retention times of the tracers were no longer a function of increasing eluent B composition—the retention times at that point were then employed to determine the void or unretained volume of the column. For simplicity, any potential change in Vo (void volume) with changes in eluent composition and temperature was ignored. For the two columns employed in the study, the corresponding void volumes and associated void fractions were 3.27 mL (0.787) and 3.30 mL (0.794), respectively. The most direct comparison from the literature is for C_{18} derivatized silica (13 μm particle size) packed in dynamic axial compression columns [30]. Void fractions reported [30] ranged from 0.65 to 0.9 depending on column length (3–22 cm) and packing pressure (up to 900 N/cm^2).

2.5. Thermodynamic parameters—van't Hoff analyses

The binding mechanism of pneumocandin B_0 onto the sorbent can be characterized by studying the effect of temperature change on the retention factor of pneumocandin B_0. This can be related to the Gibb's free energy of adsorption as shown in Eqs. (1)–(4).

$$\Delta G_{ads} = \Delta H - T\Delta S = -RT \ln K \quad (1)$$

(spontaneous when $\Delta G < 0$)

$$\ln K = \frac{-\Delta G_{ads}}{RT} \quad (2)$$

$$\ln k' = \ln \phi + \ln K \quad (3)$$

$$\ln k' = \left(\frac{-\Delta H}{R}\right)\left(\frac{1}{T}\right) + \left[\ln \phi + \frac{\Delta S}{R}\right] \quad (4)$$

where ΔH is the enthalpy of adsorption, ΔS the entropy of adsorption, T the absolute temperature (K), K the equilibrium constant for adsorption, k' the retention factor, and ϕ is the phase ratio or the ratio of the effective stationary phase area to the mobile phase volume in the column. A van't Hoff plot, i.e., $\ln k'$ versus $1/T$, gives a slope of $(-\Delta H/R)$ and a y-intercept of $(\ln \phi + \Delta S/R)$ [22].

3. Results

3.1. Effect of mobile phase composition on column performance—analytical scale

The key goal of mobile phase selection and optimization is to maximize the overall productivity of the chromatographic purification. However, due to the unique interplay of solubility, retention and selectivity, a ternary mobile phase consisting of EtOAc, MeOH and H_2O is required for the normal-phase chromatography of pneumocandins [1,13]. The results of previous experiments on the same system [13] performed to evaluate the impact of changes in mobile phase composition, indicated that the alcohol level in the mobile phase controls the retentivity of the compound while the water level controls the resolution of the key impurities.

In order to assess the impact of mobile phase composition on column performance, initial experiments were performed to determine column efficiency using a small molecule tracer, methyl ethyl ketone (MEK). Results from these experiments are presented in Fig. 2 as a plot of reduced plate height (h) versus reduced velocity (ν) [23,24] also known as van Deemter curves [25,26]. The effect of water on separation efficiency is almost insignificant over the typical large scale HPLC operating range corresponding to reduced velocities in the range of 6–16 (corresponding to flowrates of 1000–2400 mL/min for the 150 mm I.D. column used in that study). However, the effect of water becomes more pronounced at higher velocities (e.g. reduced velocity of 22) as reported previously [13].

Subsequently, studies were performed to evaluate the effect of changes in mobile phase composition on column efficiency using the product, pneumocandin B_0, as the marker compound.

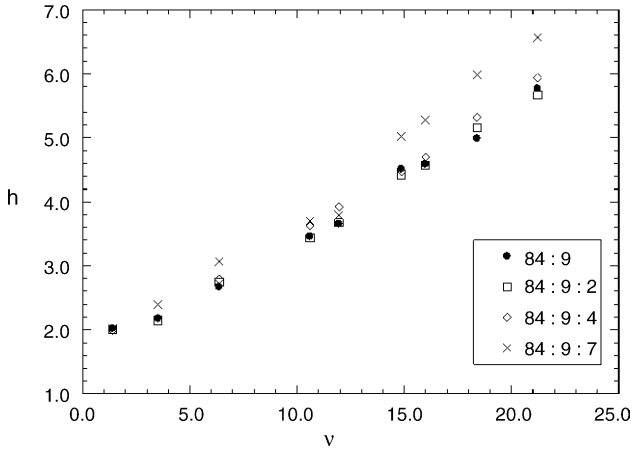

Fig. 2. Reduced plate height (h) vs. reduced velocity (ν) over a range of mobile phase compositions from anhydrous (84/9) to the base case composition of 84/9/7. All column efficiency measurements were performed using an analytical scale column of nominal dimensions 4.6 mm × 250 mm using an MEK/toluene tracer [13].

Control experiments were performed to ensure that equilibrium partitioning/adsorption was occurring in this large molecule system by measuring the effect of flowrate on retention factor, k', over a range of solvent compositions (87/9/7 to 87/9/1). These experiments were designed to employ a constant ratio of EtOAc to MeOH while only varying the water content. These results indicate that k' is independent of flowrate over a range of linear velocities from 72 to 1080 cm/h for a given water content (Fig. 3) indicating that equilibrium is achieved under these operating conditions. k' of pneumocandin Bo does depend strongly on water content (Fig. 4) declining as water content is increased—from an average of 43 (87/9/1) to 6 (87/9/7).

Experimental results from the column efficiency experiments performed with pneumocandin B_o are summarized in the van Deemter plots presented in Fig. 5A. Although van Deemter

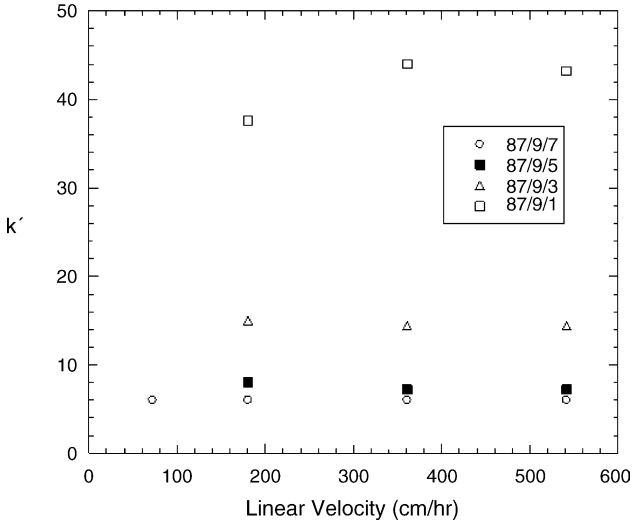

Fig. 3. Plot of retention factor k' vs. linear velocity over a range of mobile phase compositions from 87/9/7 to 87/9/1 illustrating the insensitivity of k' to linear velocity supporting the idea of approximating adsorption equilibrium in this HPLC application.

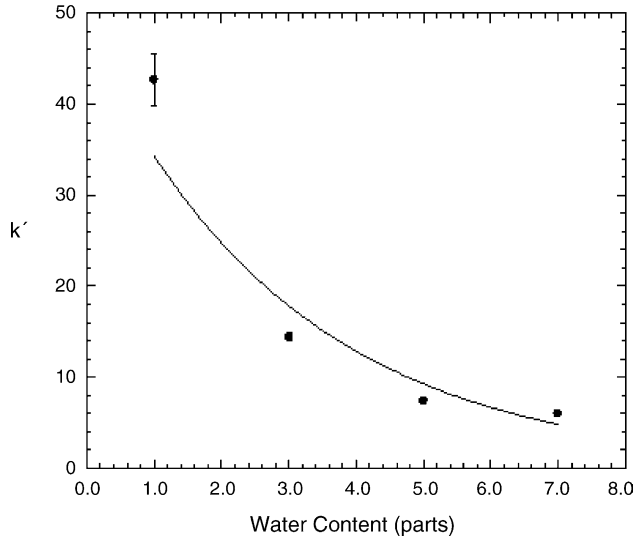

Fig. 4. Plot of retention factor, k', for pneumocandin B_o as a function of water content in the mobile phase. Mobile phase compositions (EtOAc/MeOH/H_2O) were 87/9/7, 87/9/5, 87/9/3 and 87/9/1. An exponential function (plotted in the figure) provides the best least squares fit of the data. Note error bars from replicate experiments are provided but are less than the size of the datum point with the exception of the 1.0 parts water case.

curves are typically characterized by three or more terms, at the high reduced velocities examined in this study and often employed in large scale HPLC operations, they are often adequately represented by a linear model. Indeed, the data presented in Fig. 5A did show a linear proportionality between the reduced plate height and reduced velocity for all water contents examined. The results for pneumocandin B_o show that increasing water content actually improves column efficiency. This is contrary to the observation for small molecules at high reduced velocity (Fig. 2) that water has a deleterious effect on column performance. For example, increasing the water content from one part (87/9/1) to seven parts (87/9/7) at a reduced velocity of 189 leads to a more than a three-fold reduction in reduced plate height (65–19). Another way of interpreting the data is to examine the change in reduced plate height as a function of water content in the mobile phase for a series of fixed reduced velocities as presented in Fig. 5B. It is clear from this alternate presentation of the data that by merely increasing the water content of the mobile phase while operating at the same reduced velocity, reduced plate height can be reduced by more than two-fold (ν of 126).

3.2. Evaluation of column efficiency using pneumocandin B_o at semi-prep scale

Semi-prep experiments were performed with a 0.76 L column (60 mm I.D. × 270 mm L) and the results were compared with those obtained with the 4.1 mL analytical column (4.6 mm I.D. × 250 mm L). The results plotted in Fig. 6 show actual plate counts as a function of linear velocity. The close correspondence between the curves measured at different scales suggests that there is practically no difference in the column packing quality at the semi-prep and analytical scales. This confirms earlier litera-

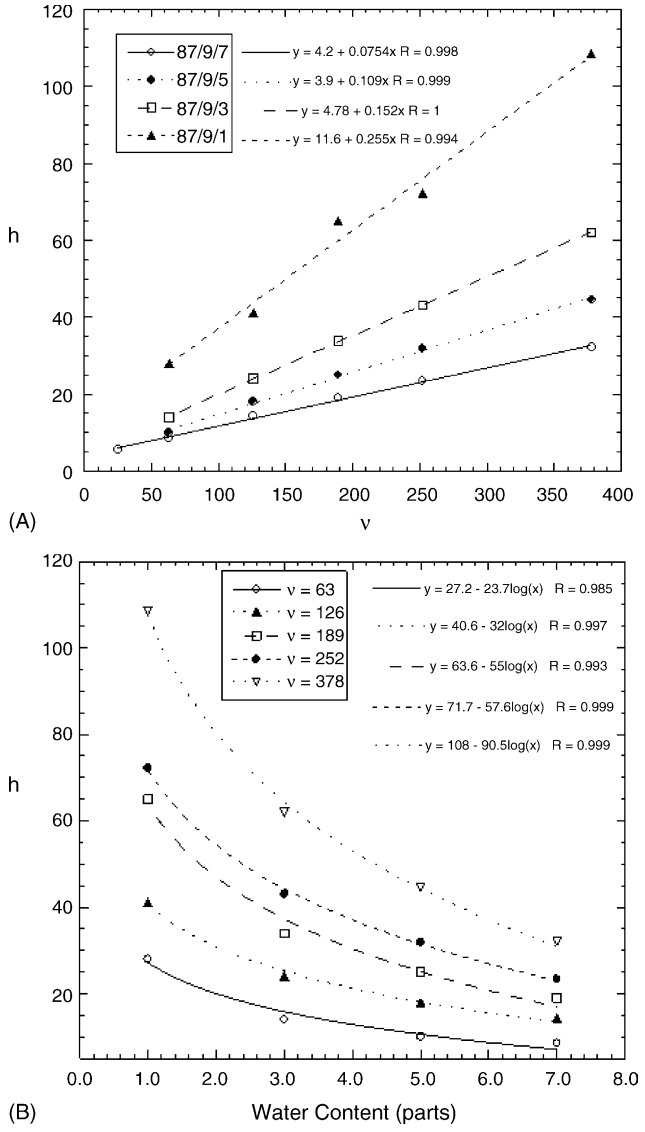

Fig. 5. (A) The effect of reduced velocity (ν) on column efficiency as measured by reduced plate height (h), for pneumocandin B_o with varying water content from one to seven parts (analytical scale column). A linear least squares fit of the data results in $R > 0.994$ for all cases. (B) Reduced plate height as a function of water content (parts) for a range of fixed reduced velocity (ν) for pneumocandin B_o. Note a logarithmic relationship exists between plate height and water content.

ture results regarding the consistent scale-up of chromatographic performance using dynamic axial compression columns [31] and appears to hold in this case even for a relatively inexpensive irregular silica packed at the relatively low compression pressure of 40 bar. In our experience, plate counts in dynamic axial compression columns remain consistent even at larger scales (ex. 300 mm I.D.).

3.3. Effect of load solvent composition on column efficiency

Since a solvent mismatch exists between the higher strength solvent used to prepare the HPLC feed and the mobile phase, control experiments were performed to assess this effect on both an analytical and preparative scale. Due to limitations in the analytical system configuration, the volume of injection was

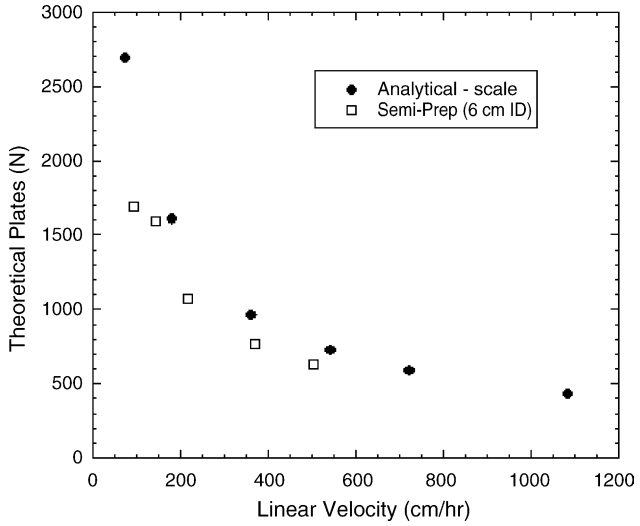

Fig. 6. The effect of linear velocity on column performance as number of theoretical plates (N) at analytical and semi-prep (60 mm I.D.) scales using pneumocandin B_o as a tracer. Note comparable performance over the range of linear velocities that could be achieved a semi-prep scale.

limited to 0.2% of the column volume. Hence, the impact of the solvent mismatch at a larger volume injection (e.g., 10 vol% injected in a prep run) could not be determined at the analytical scale. For the 0.2 vol% case, the measured plate counts differed by only 2% over the range of parameters explored (data not shown).

The experiment was then performed at the semi-prep scale (60 mm I.D.). The feed to the experiments was purified pneumocandin B_o at a product concentration of 3.6 g/L in two different higher strength feed solvents, C and D. The two feed solvents evaluated had higher pneumocandin B_o solubility and elution strength versus the ternary mobile phase by increasing the methanol content of the ternary solution. Note that the volume of injection for the semi-prep scale isolation was typically 11% of the column volume. The two feed solvents, C and D, were tested at nominal flowrates of 90 and 200 mL/min.

The results of these experiments (Fig. 7) indicate that for the final feed solvent chosen, solvent C, column efficiency (and eventually preparative chromatography) was not greatly affected by the use of feed solvent up to 11% of the column volume versus the eluent control (87/9/7). A least squares linear regression of the data indicates a linear relationship between h and reduced velocity. Chromatography performance was comparable to previously reported [13].

3.4. Thermodynamics of pneumocandin B_o adsorption on silica

Experiments were performed at analytical scale to determine the thermodynamic driving forces for adsorption of pneumocandin B_o on silica including the potential impact of water content in the eluent. Temperatures ranging from 10 to 60 °C were tested for the 87/9/7, 87/9/5, 87/9/3, and 87/9/1 mobile phases. The loading solution for all of the temperature experiments was the same 4 g/L solution of pneumocandin B_o in 87/9/7 mobile

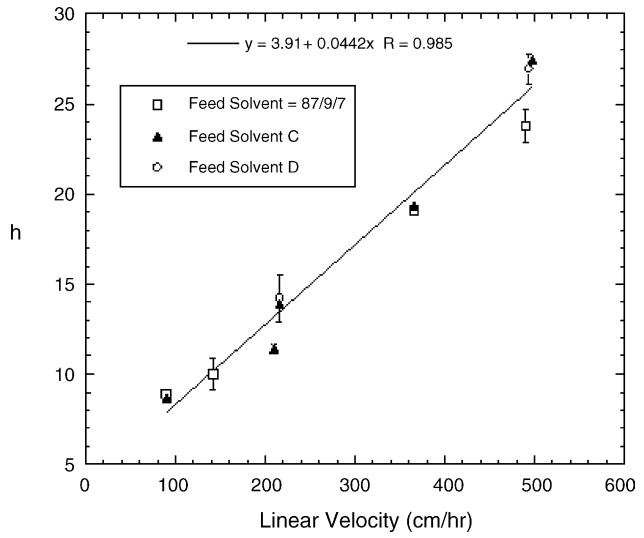

Fig. 7. The effect of solvent mismatch between feed solvent and mobile phase on column performance as determined by reduced plate height (h) as a function of linear velocity. Experiments were performed at analytical and semi-prep (60 mm I.D.) scales using pneumocandin B_o as a tracer. Feed solvent 87/9/7 is the control while feed solvents C and D are higher eluent strength. Error bars are presented from replicate experiments. A linear least squares fit of all the data is presented in the figure confirming the insensitivity of the reduced plate height to solvent composition of the feed.

phase used in the preparative scale experiments. Triplicates of every injection were run at a flowrate of 1 mL/min. The column thermostat maintained the temperature within ±0.5 °C of the setpoint temperature for each run.

A van't Hoff analysis of the data for each mobile phase composition was performed and is plotted in Fig. 8 along with the results of a linear least squares regression analysis. A summary of the enthalpy of adsorption, ΔH_{ads}, for each of the mobile phase compositions is provided in Table 1. For all of

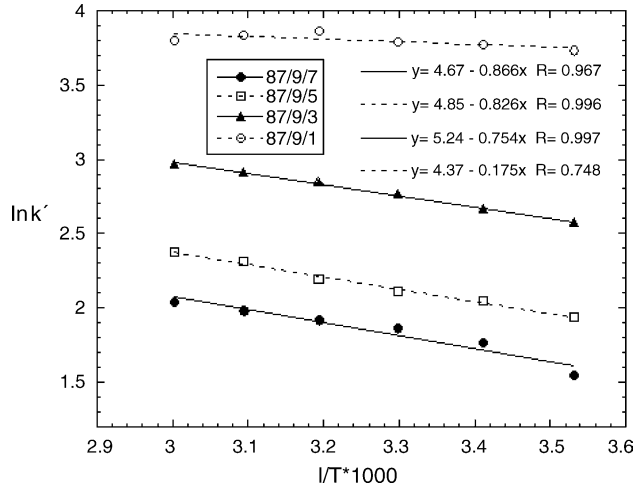

Fig. 8. A van't Hoff analysis of retention of pneumocandin B_o over a range of temperature from 10 to 60 °C and a range of water content from one to seven parts. Note that the slopes of all the linear least squares fits of the data over the range of water content are negative indicating a positive ΔH_{ads} or an endothermic process. The intercepts of all of the least squares fits are positive indicating an entropically driven adsorption process.

Table 1
Summary of results from van't Hoff analysis of retention data for pneumocandin B_o over a range of water content

Mobile phase composition (EtOAc/MeOH/water)	ΔH_{ads} (kcal/mol)	$\ln(\phi) + \Delta S_{ads}/R$
87/9/7	1.72	4.67
87/9/5	1.64	4.85
87/9/3	1.50	5.24
87/9/1	0.35	4.37

the mobile phases, the slope of the van't Hoff plot was negative indicating a positive or unfavorable ΔH_{ads}. The enthalpy of adsorption was observed to increase and become more thermodynamically unfavorable with increasing water content in the mobile phase. Specifically, ΔH_{ads} was observed to increase as the water content in the mobile phase increased from one to seven volumes.

3.5. Determination of potential water enrichment in stationary phase

Water balance experiments were performed to determine the extent (if any) of preferential water adsorption on the silica surface as a function of increased water content in the mobile phase. These experiments were driven by the desire to further understand this effect of water on the binding of pneumocandin B_o.

The differences in the level of adsorbed water on the silica for the two mobile phases, 87/9/7 and 87/9/1, versus an anhydrous column were determined by performing a water balance calculation on an analytical column packed with the same silica employed for the prep scale runs. First, the system and column were flushed with ethyl acetate for several hours to remove any water. The system and column were then equilibrated with the mobile phase for a minimum of 1 h at 1.0 mL/min or the equivalent of 15 column volumes. Once equilibrated, the flow was switched from the ternary mobile phase to neat MeOH, and the eluent was collected in three consecutive 40 mL aliquots (or the equivalent of 10 column volumes per aliquot) starting immediately after the flow was switched.

A Karl Fischer Coulometer was used to measure the amount of water in the original mobile phase flowing through the system and in the eluent collected. The baseline amount of water in the pure MeOH was also measured. From these values, a water balance was calculated for the 87/9/7 and 87/9/1 mobile phase experiments to determine the amount of water that was bound to the silica. These results were then converted to a surface coverage (molecules water/m^2 silica and mg water/m^2 silica) in order to determine the extent of the water coverage (expressed in terms of number of water layers) for both cases. Table 2 provides a summary of the water balance performed for the two mobile phases with different levels of water. The results indicate multiple layers of coverage for the 87/9/7 case and a substantial enrichment in adsorbed water for the 87/9/7 case versus the 87/9/1 mobile phase case.

Table 2
Summary of results from water enrichment experiments for the two mobile phase compositions—87/9/7 and 89/7/1

Experimental			Calculated	
Mobile phase composition (EtOAc/MeOH/water)	Vol (mL) H_2O retained in Col. (mL)	Adsorbed water (mg H_2O/m^2 silica)	Surface coverage on silica (molecules H_2O/m^2)	Estimated number of water layers
87/9/7	0.21	0.90	3.0×10^{19}	2–15
87/9/1	0.01	0.04	1.4×10^{18}	0.1–0.7

Parameters used in the calculations include silica surface area [53] of 600 m^2/g, packing density of 0.51 g/mL. Estimated number of layers based on data presented by Zhuravlev [15].

4. Discussion

4.1. Effect of mobile phase on column efficiency—analytical scale

The experimental results from the column efficiency experiments performed with pneumocandin B_o are contrary to the expectation that water would have a deleterious effect on column performance; increasing water content actually improved separation efficiency. For example, increasing the water content from one part (87/9/1) to seven parts (87/9/7) at a reduced velocity of 189 led to a more than a three-fold reduction in reduced plate height (65–19). It is important to note that there is a marked difference in measured reduced plate heights with pneumocandin B_o versus small molecule tracers. Previous experimental results [13] using an MEK/toluene tracer demonstrated that reduced plate heights in the range of 2–6 (range of reduced velocity from 1 to 24) could be achieved under similar operating conditions. The larger reduced plate heights observed for pneumocandin B_o are thus attributable to the change in tracer molecules, specifically lower diffusivity of pneumocandin B_o versus small molecules, as opposed to column packing quality. Experimental results for reduced plate height versus reduced velocity obtained for the pneumocandin B_o experiments are consistent with predicted values from the literature [32,33]. These results combined with the results from the water balance experiments (Section 3.5) support the hypothesis that water plays an important beneficial role in mediating the interactions between pneumocandin B_o and the silica sorbent.

4.2. Evaluation of column efficiency using pneumocandin B_o at semi-prep scale

Preparative chromatography scaleup, described in detail in a recent review article [34], should ideally involve matching column efficiency (reduced plate height) as scale is increased for a fixed reduced velocity. From a productivity perspective, preservation of the functional relationship between reduced plate height and reduced velocity is equally as important. Although column efficiency tests using small molecule tracers provide a good estimation of gross column packing efficiency, the ultimate goal is to match retention performance for the product of interest. Another approach that is attempted is to match the minimum number of plates (N) required to achieve a separation over a range of scales.

The results clearly indicate that performance can be measured over a range of scales from analytical to semi-prep using purified compound as a tracer. Note that a slight decrease in column performance upon scale-up can be expected partly due to differences in packing efficiency and injection method. Differences in injection method (syringe versus pump) could impact the distribution of the sample and the injection profile resulting in a change in column efficiency as a function of scale-up. Another important consideration is the operating range that is accessible. A significantly larger range is usually available in the scale-down system versus a typical semi-prep system. Although it may not always be feasible to perform column efficiency experiments with purified product, these research results demonstrate the feasibility and benefit of such an approach using minimum number of theoretical plates as a scale-up parameter.

4.3. Effect of load solvent composition on column efficiency

The unusual solubility profile of pneumocandin B_o necessitated the use of a different and higher eluent strength load solvent than was employed for the mobile phase. No significant difference in column efficiency could be experimentally discerned even with a 11 vol% injection of the stronger elution solvent. These results indicate the loss of column efficiency due to mismatch of load and elution solvents may not be detrimental.

4.4. Thermodynamics of adsorption

Experimental results from van't Hoff analyses of retention of pneumocandin B_o as a function of temperature suggest that adsorption is endothermic or enthalpically unfavorable ($\Delta H_{ads} > 0$) implying the process is entropically driven. Since the core of pneumocandin B_o is composed of a cyclic hexapeptide with multiple exposed hydroxyl groups, the potential exists for a significant amount of water of hydration to be associated with the hydroxyl groups, and these could interact with the silica surface of the adsorbent. The analogy could be made to the binding of a protein to a surface resulting in the release of waters of hydration [35] which has previously been determined to be entropically driven [36,37].

Another important point is the influence of mobile phase composition on the thermodynamic driving forces governing adsorption. The results of this study indicate that the enthalpy of adsorption increases with increasing water content. Although there is very little data available on the thermodynamics of

adsorption to silica for natural products to act as a comparison, a recent review paper provides some information on the interaction energy between bound waters and silica. Zhuravlev [17] estimates that the interaction energy between a surface silanol group and water that is physically adsorbed (at surface coverage less than a monolayer) can be up to 62 kcal/mol. If this value is compared to the relatively low ΔH_{ads} for pneumocandin B_o (shown in Table 1), it is unlikely that pneumocandin B_o is dislodging water molecules associated with the surface of the silica but more likely partitioning within a hydration shell close to the surface of the silica.

Information on the impact of mobile phase composition on thermodynamics has been presented for chiral chromatography employing modified silica stationary phases [38,39] and for PEO on reversed phase silica stationary phases [40]. Kazuaki et al. [38] present the argument that a hydration shell surrounding a compound present in a highly aqueous mobile phase is more ordered in the mobile phase versus the stationary phase so that there is an increase in entropy when the compound adsorbs to the stationary phase [38]. For another system where water controls adsorption, Record et al. [39] have suggested that a change in enthalpy associated with adsorption of a protein to a stationary phase results from changes in activities of the waters of hydration that are released from the stationary phase into the bulk. Cho et al. have also recently reported a similar entropically driven process for the RP-HPLC separation of high molecular weight PEO on reversed phase resins [40]. The results of these other researchers are consistent with the observations and proposed mechanism of water mediating the retention of pneumocandin B_o on silica in normal phase chromatography.

4.5. Determination of stationary phase water enrichment

Experimental results indicate a 20-fold higher level of bound water to the silica surface for the high water content mobile phase (87/9/7) versus the low water case (87/9/1). This result taken with the other data presented on the impact of water content in the mobile phase on the thermodynamics of adsorption confirms the role of water in mediating the retention of pneumocandin B_o on silica.

Additional insight can be gained by reviewing data from other researchers [17,18] who have examined the impact of various features of silica, including the type (geminal, vicinal and isolated silanol) and density of silanol groups, on the thermodynamics of water adsorption to silica. Although these data were derived from a variety of different systems, as a whole they can be used to determine how the amount of water adsorbed to silica measured in this study compares with a predicted monolayer coverage. Zhuravlev [17] provided data for the average area occupied by an OH group for both vicinal and isolated silanol groups. Vicinal silanol groups had an average occupied surface area of 0.068 nm^2 per OH group whereas isolated silanol groups occupied from 0.45 to 6.67 nm^2 per OH group. If one assumes a one-to-one hydrogen bond interaction of water per OH group (from the silanol functional group), one can estimate the expected monolayer water coverage for a silica surface in mg-water/m^2-silica. Using these parameters, an estimated monolayer coverage ranges from 0.06 to 0.43 mg/m^2-silica, bracketing the estimate of 0.14 mg/m^2 reported by Muster et al. [18]. One can then use the estimate of 0.14 mg/m^2 to translate the data obtained for the ternary solvent system for pneumocandin B_o purification to number of layers of water coverage (data and calculated water layers presented in Table 2) for the two different mobile phase compositions. A comparison suggests that the low water mobile phase (87/9/1) has less than a monolayer of water on the silica surface. However, when the high water mobile phase (87/9/7) is employed, several layers of bound water (from 2 to 15) exist that are associated with the silica. This increase in the number of layers of water on the surface with increasing water content in the mobile phase helps to explain the large differences observed in retention of pneumocandin B_o.

Other researchers [41,42] have observed a similar phenomenon for the normal phase HPLC of carbohydrates using aminopropylsilane derivatized silica. Verhaar and Kuster [41] experimentally determined an enrichment of water on the sorbent relative to the stationary phase using a technique similar to the one described in this work. Verhaar and Kuster also observed an increase in water associated with the stationary phase when water content of the mobile phase was increased. Nikolov and Reilly [42] further extended this to demonstrate that the retention factor and elution order correlated with hydration number of the carbohydrates and was consistent with partition between "a water-enriched stagnant phase and a moving aqueous organic phase." Hansen [43] described the use of water content (2–90%) in the mobile phase to control polarity and partitioning onto silica over a wide range. Hansen further developed the concept of dynamically modified silica, using other additives including cetyltrimethylammonium (CTMA), to produce a dynamic reversed phase system [44,45]. Alpert [46] later extended the application of this concept to hydrophilic interaction chromatography for other applications including oligonucleotide and peptide separations where aqueous two-phase partitioning has been demonstrated.

The experimental data from this research coupled with the observations of previous researchers support the hypothesis that pneumocandin B_o retention is mediated by partitioning of pneumocandin B_o between a bulk mobile phase and a water-rich stationary phase. The results are also consistent with the conclusions of others [15,47] that there is a significant impact of small amounts of water in the mobile phase on retention of analytes in normal phase chromatography using bare silica as the adsorbent. As an example, Snyder et al. [15] described a decrease in retention factor for phenyl propanol on bare silica from 18 to 4 as the level of water was increased from 0 to 0.15% (100% of saturation) in a methylene chloride mobile phase. Accurate control of retention for pneumocandin B_o at low water content (ex. 87:9:1) was observed to be quite difficult (refer to Fig. 8). However, when water was increased to three volume fractions in the mobile phase (87:9:3), accurate control of retention was possible—consistent with the results of previous researchers [15,47] who emphasized that great care in the composition of the mobile phase is required for reproducible results at low water content.

5. Conclusions

Experimental results confirm that water mediates retention of pneumocandin B_o on silica in a positive fashion in this mobile phase system. Water enrichment on the silica surface with higher water content mobile phases has also been demonstrated. Retention of the product could be readily controlled by varying only the water content of the ternary mobile phase.

Experimental results indicate the feasibility of using column efficiency measurements with pure compound to ensure accurate scale-up. Although the proposed method requires the use of a small amount of purified product to determine column performance, this approach offers significant advantages over small molecule efficiency measurements that are more suitable for determination of gross packing irregularities. The technique of matching theoretical plates determined with the product of interest also allows for the potential to change column operating conditions (aspect ratio, linear velocity, etc.) upon scale-up while still achieving comparable separation.

Experimental results from this work also shed light on the thermodynamics of adsorption in a high water content mobile phase and potentially the role of water in normal phase chromatography. For this particular system, the adsorption process was entropically driven similar to observations of others in RP-HPLC, HIC [48] and protein IEX chromatography.

Acknowledgements

The authors wish to thank Dr. Kent Göklen, Mr. Joseph Nti-Gyabaah and Ms. Ellen Dahlgren for their contributions to the research efforts.

Appendix A. Calculation methodology for reduced plate height and reduced velocity

Determination of column efficiency for silica gel chromatography requires values for parameters which cannot be readily determined experimentally, including the diffusivity of the tracer in the mobile phase and the column void fraction.

As described previously [13], diffusion coefficients for the tracer solutions, methyl ethyl ketone (MEK) and toluene in the ternary mobile phase consisting of ethyl acetate, methanol and water were estimated using the Wilke–Chang equation [49–51] as described in Eq. (5).

$$D_{AB} = 7.4 \times 10^{-8}(\psi_B M_B)\frac{0.5T}{\mu V_A^{0.6}} \quad (5)$$

in which ψ_B is an association parameter for the solvent B, M_B the molar weight for solvent B, T the absolute temperature in Kelvin, μ the viscosity of the solution in centipoise, V_A the molar volume of the solute A in $cm^3\,g\,mol^{-1}$ as liquid at its normal boiling point. Application of literature data [49,52] to Eq. (5) yields diffusion coefficients of 3.17×10^{-5} and $3.40 \times 10^{-5}\,cm^2\,s^{-1}$ for toluene and MEK, respectively, in the ternary mobile phase solution.

Once the values of the diffusion coefficients for toluene and MEK were obtained, the reduced velocity, ν, could be calculated from the experimental data according to Eq. (6).

$$\nu = \frac{u d_p}{D_{AB}} \quad (6)$$

where u is the superficial velocity ($cm\,s^{-1}$) of the mobile phase, d_p the average particle size, and D_{AB} is the diffusion coefficient for MEK or toluene. Based on data provided by the manufacturer, a d_p of 18 μm or 1.8×10^{-3} cm was employed for the nominal 20 μm material. A void fraction of 0.79 was employed for the silica media based on experimental measurements (reference Section 2.4).

The starting point for an estimate of the diffusion coefficient of pneumocandin B_o is based on NMR diffusion measurements for a solvated peptide fragment of human growth hormone, hGH (9–19), in a mixed organic/aqueous system (d_3-TFE/D_2O) at 298.7 K [29]. The diffusion coefficients for hGH (9–19) ranged from 1.31 to $1.96 \times 10^{-6}\,cm^2\,s^{-1}$ depending on the molar ratio of TFE to D_2O. The viscosity of the solution of hGH (9–19) was also observed to change from 1.93 to 2.34×10^{-3} Pa s as the molar ratio of TFE to D_2O was varied. According to the authors this change in diffusion coefficient is attributable to change in the conformation of the peptide fragment from random coil to a more compact helical structure as the molar ratio of d_3-TFE/D_2O was varied. If one uses the mean diffusion coefficient (representing a mixture of conformations) and corrects these data for hGH (9–19) for viscosity to that of an aqueous system, the average diffusion coefficient for hGH (9–19) is $0.76 \times 10^{-6}\,cm^2\,s^{-1}$ or essentially the same as that reported for complex organic systems in water of $1 \times 10^{-6}\,cm^2\,s^{-1}$ [27]. One approach would therefore be to take an average of the diffusion coefficient for hGH and that for complex organic systems equivalent to $0.9 \times 10^{-6}\,cm^2\,s^{-1}$ to represent the diffusion coefficient for pneumocandin B_o in an aqueous system. However, pneumocandin B_o is actually present in a ternary solvent system of EtOAc/MeOH/H_2O so a correction for viscosity must be employed. Note that the viscosities for solvent mixtures employed during the experiments described in this manuscript are essentially constant at 0.47×10^{-3} Pa s (estimated range from 0.49×10^{-3} Pa s (87/9/7) to 0.45×10^{-3} Pa s (87/9/1)). Once a correction for viscosity is made to the estimated value of $0.9 \times 10^{-6}\,cm^2\,s^{-1}$ for pneumocandin B_o in the aqueous system, a reasonable estimate for D_{B_o} in the experimental solvent system is determined to be $1.9 \times 10^{-6}\,cm^2\,s^{-1}$.

References

[1] R.E. Schwartz, D.F. Sesin, H. Joshua, K.E. Wilson, A.J. Kempf, K.E. Goklen, D. Kuehner, P. Gailliot, C. Gleason, R. White, E. Inamine, G. Bills, P. Salmon, L. Zitano, J. Antibiotics 45 (1992) 1853.
[2] G. Bills, G. Platas, F. Pelaez, P. Masurekar, Mycol. Res. 103 (2) (1999) 179.
[3] J.S. Tkacz, R.A. Giacobbe, R.L. Monaghan, J. Ind. Microbiol. 11 (1993) 95.
[4] N. Connors, L. Petersen, R. Hughes, K. Saini, R. Olewinski, P. Salmon, Appl. Microbiol. Biotech. 54 (2000) 814.

[5] L.A. Petersen, D.L. Hughes, R. Hughes, L. DeMichele, P. Salmon, N. Connors, J. Ind. Microbiol. Biotech. 26 (2001) 216.
[6] L. Petersen, R. Olewinski, P. Salmon, N. Connors, Appl. Microbiol. Biotechnol. 62 (2003) 263.
[7] D.J. Pollard, G. Hunt, T.F. Kirschner, P.M. Salmon, Bioprocess Biosyst. Eng. 24 (2002) 373.
[8] D.J. Pollard, R. Buccino, N.C. Connors, T.F. Kirschner, R.C. Olewinski, K. Saini, P.M. Salmon, Bioprocess Biosyst. Eng. 24 (2001) 13.
[9] O.D. Hensens, J.M. Liesch, D.L. Zink, J.L. Smith, C.F. Wichman, R.E. Schwartz, J. Antibiotics 45 (1992) 1875.
[10] A. Adeferati, O. Hensens, E.T.T. Jones, J. Tkacz, J. Antibiotics 45 (1992) 1953.
[11] R. Schwartz, P. Masurekar, R. White, in: J. Rippon, R. Fromtling (Eds.), Cutaneous Antifungal Agents, Marcel Dekker, New York, 1993, Chapter 27.
[12] A.E. Osawa, R. Sitrin, S.S. Lee, J. Chromatogr. A 831 (1999) 217.
[13] D.J. Roush, F.D. Antia, K.E. Goklen, J. Chromatogr. A 827 (1998) 373.
[14] J.F.K. Huber, M. Pawlowska, P. Markl, J. Chromatogr. 500 (1990) 257.
[15] L.R. Snyder, J.J. Kirkland, J.L. Gauch, Practical HPLC Method Development, second ed., Wiley, New York, NY, 1997, Chapter 6.
[16] P. Jandera, L. Petranek, M. Kucerova, J. Chromatogr. A 791 (1997) 1.
[17] L.T. Zhuravlev, Colloids Surf. A: Physicochem. Eng. Aspects 173 (2000) 1.
[18] T.H. Muster, C.A. Prestidge, R.A. Hayes, Colloids Surf. A: Physicochem. Eng. Aspects 176 (2001) 253.
[19] A. Fouzri, R. Dorbez-Sridi, M. Oumezzine, A. Missaoui, Biomol. Eng. 19 (2002) 207.
[20] C.A. Fung Kee Fung, M. Burke, J. Chromatogr. A 752 (1996) 41.
[21] A. Wehrli, U. Hermann, J.F.K. Huber, J. Chromatogr. 125 (1976) 59.
[22] T. Takeuchi, M. Masayoshi, D. Ishii, J. Chromatogr. 235 (1982) 309.
[23] J.J. van Deemter, F.J. Zuiderweg, A. Klinkenberg, Chem. Eng. Sci. 5 (1956) 271.
[24] J.C. Giddings, J. Chromatogr. 13 (1964) 301.
[25] G. Guichon, S. Shirazi, A. Katti, Fundamentals of Preparative and Nonlinear Chromatography, Academic Press, New York, NY, 1994, Chapter 6, p. 199.
[26] I. Schnoll-Bitai, J. Chromatogr. A 1084 (2005) 160.
[27] M. Pfenniger, G. Calzaferri, Chemphyschem 4 (2000) 211.
[28] Landolt-Bornstein, H. Borchens, H. Hausen, K. H. Hellwege, K. Schafer, E. Schmidt (Eds.), vol. 2, Sect. 5a, sixth ed., Springer, Berlin, 1969.
[29] S.A. Rogers-Sander, D. Vander Velde, Cynthia K. Larive, Fresenius J. Anal. Chem. 369 (2001) 308.
[30] B.J. Stanley, M. Sarker, G. Guichon, J. Chromatogr. A 741 (1996) 175.
[31] H. Colin, P. Hilaireau, J. de Tournemire, LC–GC 8 (1990) 302.
[32] F. Antia, C. Horvath, J. Chromatogr. 435 (1988) 1.
[33] Cs. Horvath, H.J. Lin, J. Chromatogr. 149 (1978) 43.
[34] G. Guichon, J. Chromatogr. A 965 (2002) 129.
[35] W. Norde, in: J.D. Andrade (Ed.), Surface and Interfacial Aspects of Biomedical Polymers, Plenum Press, New York, vol. 2, Chapter 8, p. 280.
[36] D.J. Roush, D.S. Gill, R.C. Willson, J. Chromatogr. A 653 (1993) 207.
[37] D.S. Gill, D.J. Roush, K.A. Shick, R.C. Willson, J. Chromatogr. A 715 (1995) 81.
[38] M. Kazuaki, T. Shoda, H. Kawabata, H. Matsukura, J. Liq. Chrom. Rel. Technol. 24 (2) (2001) 141.
[39] M.T. Record Jr., C.F. Anderson, T.M. Lohman, Q. Rev. Biophys. 11 (2) (1978) 103.
[40] D. Cho, S. Park, J. Hong, T. Chang, J. Chromatogr. A 986 (2003) 191.
[41] L.A.Th. Verhaar, B.F.M. Kuster, J. Chromatogr. 234 (1982) 57.
[42] Z.L. Nikolov, P.J. Reilly, J. Chromatogr. 325 (1985) 287.
[43] S.H. Hansen, J. Chromatogr. 212 (1981) 229.
[44] S.H. Hansen, P. Helboe, M. Thomsen, J. Chromatogr. 447 (1988) 182.
[45] S.H. Hansen, P. Helboe, M. Thomsen, J. Chromatogr. 544 (1991) 53.
[46] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[47] W.M. Niessen, J. van der Greef, Liquid Chromatography–Mass Spectrometry, Marcel Dekker, New York, 1992, Chapter 1, p. 7.
[48] A. Vailaya, Cs. Horvath, Ind. Eng. Res. 35 (1996) 2964.
[49] R. Bird, W. Stewart, E. Lightfoot, Transport Phenomena, J. Wiley and Sons, New York, 1960.
[50] C.R. Wilke, Chem. Eng. Prog. 45 (1949) 218.
[51] C.R. Wilke, P. Chang, AIChE J. 1 (1955) 264.
[52] Handbook of Chemistry and Physics, 63rd ed., CRC Press, 1982.
[53] Perry's Chem. Eng. Handbook, sixth ed., 1984, Chapter 16, p. 9.