Recovery and Purification of Paclitaxel Using Low-Pressure Liquid Chromatography

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An efficient and economical low-pressure liquid chromatography process has been developed for paclitaxel recovery and purification directly from plant-tissue culture (PTC) broth. PTC broth is first diluted with ethanol to ensure paclitaxel dissolution and then passed through a column packed with a high-capacity polystyrene divinyl-benzene sorbent. A step increase in ethanol concentration in the mobile phase (ethanol:water) is used to concentrate and compress the taxane bands to as high as 29-fold of influent concentrations (about 1 mg/L). A recycle technique is then used to separate the concentrated paclitaxel band from other taxane bands, achieving 95% purity with more than 90% recovery and 99% purity with more than 80% recovery. In this process, the same low-pressure columns are used to capture, concentrate and purify paclitaxel. Theoretical predictions agree closely with the stepwise elution and recycle chromatography data. After validation, simulations are used to explore various design and operating alternatives. Analysis of the alternatives shows that the process cost can be further reduced by using higher feed concentration, larger loading volume, smaller particle size, and optimal gradient and recycle strategies.

Introduction

Paclitaxel is recognized as one of the best cancer drugs that has emerged in the last 15 years (Joyce, 1993; Junod, 1992; Kingston, 1991). Until the recent introduction of semisynthesis, paclitaxel has been isolated from the bark of the Pacific yew by solvent extraction, followed by chromatographic purification of the extract (Schiff and Horowitz, 1980; Cragg and Snader, 1991). Because of the limited supply of the bark, this approach cannot assure a steady supply of paclitaxel in the long term. It is obvious that alternative technologies need to be developed (Ketchum and Gibson, 1993).

Plant-tissue culture (PTC) technologies are currently among the most promising alternatives for large-scale paclitaxel production (Christen et al., 1991; Ketchum and Gibson, 1993, 1995, 1996; Farina and Hanck, 1994; Fett-Neto, 1992, 1993, 1994; Gibson et al., 1993, 1995; Srinivasan et al., 1995; Ketchum et al., 1995). PTC has unique advantages compared to extraction from bark or needles of yew trees. The production rate of paclitaxel in plant-tissue culture is higher than that in growing bark or needles. The production under well-controlled conditions in bioreactors is more consistent than in cultivated trees, which can be affected by season, weather, water, soil, and other environmental conditions. Increasing the scale of production in bioreactors is also easier than increasing the number of cultivated trees. More importantly, the isolation and purification of paclitaxel from plant-tissue culture broth is relatively easy compared to that from bark or needle extracts. For these reasons, it is important to develop an efficient and cost-effective process for the recovery and purification of paclitaxel from PTC broth.

Most paclitaxel separation processes reported in the literature use solvent extraction followed by high-performance liquid chromatograph (HPLC) purification (Kingston et al., 1982; Witherup et al., 1990; Cardellina, 1990, 1991; Harvey, 1991; Caster and Tyler, 1993; Wickremesinhe and Arteca, 1993; Nair, 1994; Rao, 1995). Typical solvents used in solvent extraction are methylene chloride, methanol, ethanol, and chloroform. Several different solvents are usually used in a recovery and purification process. Solvent-exchange steps are tedious, energy intensive, and costly. Reducing the hazardous-solvent usage can translate into significant savings and environmental benefits. In addition, eliminating the expense associated with high-pressure equipment and the waste due

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to solvent exchange can make the process more economical. Therefore, this study focuses on the development of a simple and effective process in which only one environmentally benign solvent is needed and low-pressure liquid chromatography (LPLC) is used both for recovery and for purification.

The major challenges associated with paclitaxel recovery and purification from PTC broth include the low concentration of paclitaxel (typically from 20 mg/L to 150 mg/L) (Fett-Neto et al., 1992; Ketchum and Gibson, 1993; Bringi et al., 1995), high impurity concentrations (> 6 g/L), and a large number of structurally similar taxanes present in the broth (Witherup et al., 1989; Cardellina, 1991; Harvey, 1991). Elution involving a step change in solvent concentration (stepwise elution) provides a potential solution to the low-concentration problem (Snyder et al., 1979; Yamamoto et al., 1988, 1990, 1992; Kim et al., 1992). Compared to linear and geometrical gradients, stepwise elution can save solvent and can compress an elution band to a much higher concentration. Stepwise elution is also much easier to operate because only two solvent compositions are needed in the entire process. Moreover, nonlinear isotherm and dispersion effects due to mass transfer are suppressed significantly in stepwise elution (Ma et al., 1996b).

For the structurally similar taxane impurities, conventional low-pressure chromatography methods often can achieve good separation if a sufficiently long column is used. However, yield of pure product is usually low because of severe band spreading due to mass-transfer limitations (Au, 1995). Recycle chromatography allows a sample to be passed over a column several times without the need for a long column or reinjection of the sample (Wankat, 1986). This results in a high-resolution, increased efficiency and low-pressure drop (Nakamura et al., 1973; Martin et al., 1976; Bailly and Tondeur, 1982; Seidel-Morgenstern and Guiochon, 1993; Heuer et al., 1995). Basically, there are two recycle strategies: closed-loop (Bombaugh et al., 1969; Biesenberger et al., 1971) and alternative pumping recycle (Duvdevani et al., 1971). In closed-loop recycle, the detector outlet connects with a pump inlet (Figure 1a). In alternative pumping recycle, a sample is passed alternatively through two columns and the pump is not included in the circuit (Figure 1b). The latter eliminates any band spreading due to mixing in the pump, but it consumes more solvent than the closed-loop approach (Martin et al., 1976). This strategy is adopted here in order to avoid serious mixing problems in low-pressure pumps. Both recycle strategies can have different arrangements, such as "simple recycle," "recycle with shaving," and "recycle with mixing" (Wankat, 1986; Crary et al., 1989; Seidel-Morgenstern and Guiochon, 1993; Guiochon et al., 1994). The "simple recycle" procedure, in which the whole sample is recycled, is used in this study for simplicity and accuracy because of the low concentrations of taxanes in PTC broth.

The goal of this work is to develop a LPLC process for the recovery and purification of paclitaxel from PTC broth (Figure 2). An efficient model-based design approach (Figure 3) is used to reduce a significant amount of trial and error, which is usually needed in process development and scale-up. In this approach, a high-capacity sorbent and an environmentally benign solvent are first selected. Adsorption isotherms and intraparticle diffusivities of paclitaxel and closely related taxanes are then estimated from small-scale bench experiments, which have been reported elsewhere (Ma et al., 1996b). These intrinsic parameters are used in simulations based on a detailed rate model, VERSE (versatile reaction separation), to generate promising designs. The basic assumptions and the general equations for this model can be found in Berninger et al. (1991) and Ma et al. (1996a). In this model, detailed mass-transfer mechanisms [axial dispersion, film mass transfer, pore diffusion, surface diffusion, or parallel diffusion (Ma

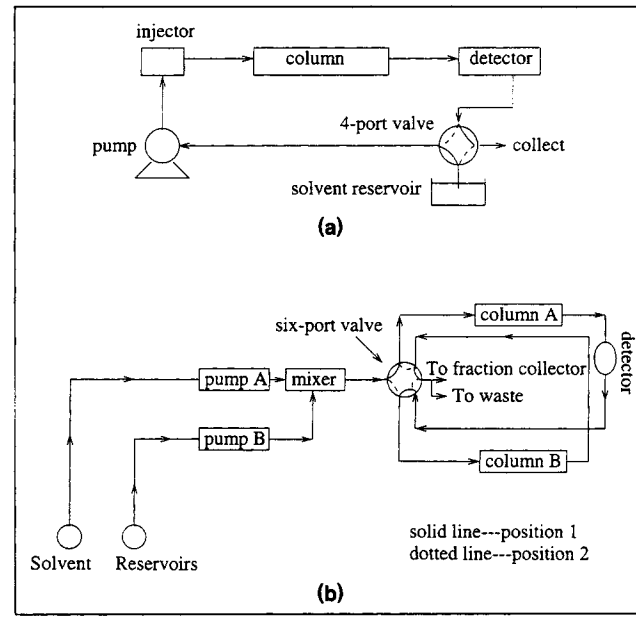

Figure 1. (a) Closed-loop recycling apparatus; (b) recycle low-pressure liquid chromatography system.

For 2-column experiments (experiment B-2), the feed is pumped through column B, column A, and detector. For 3-column experiments (experiment B-3), the feed is pumped through column A, detector, column B, column A, and detector. For 4-column experiments (experiment B-4 and experiment R-2), the feed is pumped through column B, column A, detector, column B, column A, and detector.

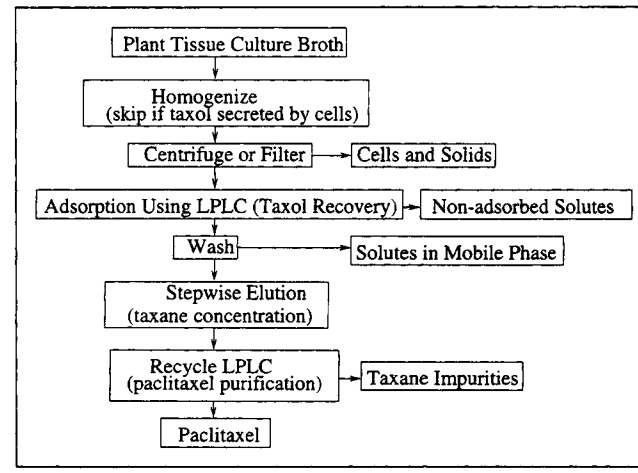

Figure 2. Proposed separation process for paclitaxel plant-tissue culture broth.

Low-pressure liquid chromatography is used for taxane recovery and purification.

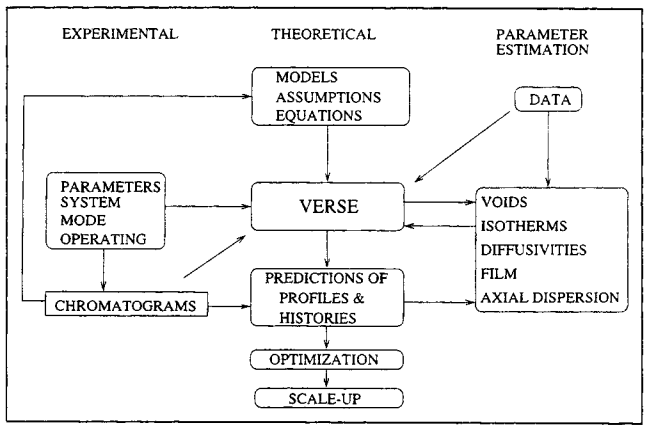

Figure 3. Model-based design approach.

et al., 1996a)] have been considered. Bench-scale experiments under different operating conditions are then carried out to validate the parameters and simulations. After validation, the simulations are used to explore various design and operating alternatives. Cost analysis of the alternatives is then used to identify design and operating conditions that can give high product purity, high yield, and low separation cost.

Our results show that the strategy of combining stepwise elution and recycle in hydrophobic LPLC can be used effectively to achieve high yield (> 90%) and high paclitaxel purity (~ 95%). In this LPLC process, the same column is used for both the capture and the separation of paclitaxel from closely related impurities. VERSE simulations of paclitaxel and cephalomannine effluent histories are in close agreement with the data for binary mixtures and PTC broth. Simulation results suggest several means to save solvent and reduce labor and equipment costs.

Experimental Studies

Materials

HPLC-grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ). HPLC-grade tetrahydrofuran (THF) was obtained from Sigma Chemical Co. (St. Louis, MO). Pure ethanol was purchased from McCormick Distilling Co. (Weston, MA). Ethanol was degassed prior to use by sonicating in an ultrasonic bath (Fairlawn, NJ). Distilled deionized water (DDW) was obtained through the use of a Milli—Q™ system by Millipore (Bedford, MA). All solvents used were filtered through 0.2 μm Nylon 66 filters that were purchased from Alltech (Deerfield, IL). Blue dextran was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium nitrate was purchased from Mallinckrodt Chemical Co. (Paris, KY).

A taxane kit, which consisted of paclitaxel (MW 854), cephalomannine (MW 832), 10-deacetyltaxol (MW812), 7-epi-10-deacetylbaccatin (MW812), baccatin III (MW 587), and 10-deacetyl baccatin III (MW 545), was generously provided by the National Cancer Institute (NCI). A binary mixture of paclitaxel and cephalomannine (7:3.2 w/w), purified from needle extract, was a gift from Dr. Ching-Jer Chang of the Dept. of Pharmacy, Purdue University (West Lafayette, IN). Additional paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO). A lypholized sample of plant-tissue culture broth from *Taxus cuspidata* (Gibson et al., 1995) was a gift of Dr. Ray Ketchum of the U.S. Department of Agriculture (U.S.D.A., Ithaca, NY) and Dr. M. Shuler of Cornell University (Ithaca, NY).

The polystyrene divinyl-benzene copolymer resin used in all LPLC columns was graciously provided by Dow Chemical Co. (Midland, MI). This sorbent has essentially two types of pores: large pores to allow for effective mass transfer and smaller pores, taking up about 20–30% of the total pore volume, to provide sufficient capacity for small molecules (MW < 1,000).

Instrumentation

HPLC System. An HPLC system was used for the analysis of fractions collected from LPLC experiments. The system consists of two pumps (Waters 510), a tunable single-wavelength detector (Waters 486), and an injector (Waters U6K). A Waters Millenium 2010 software operated in a Windows environment was used for data collection. A Waters Nova-Pak C-18 column (60 Å, 4 μm, 3.9 mm × 150 mm) was used in the HPLC system for analysis.

Recycle LPLC System. A recycle LPLC system is designed using a Pharmacia (Piscataway, NJ) Fast Protein Liquid Chromatography (FPLC) system. The system consists of two pumps (Pharmacia P-500), a liquid chromatography controller (Pharmacia LCC-500), an injection valve (Pharmacia MV-7), and a fraction collector (Pharmacia Frac-100). Data monitoring and collection were handled using a photodiode array detector (Waters 990) and accompanying data-acquisition software. The recycle is realized by replacing the sample loop in the MV-7 injection valve with a column. The principle is the same as the alternative pumping recycle using a six-port, two-position valve (Duvdevani et al., 1971). In this configuration (Figure 1b), when flow takes place along the solid lines, the system is said to be in position 1 (loading position in MV-7 valve); when the valve is switched such that flow occurs along the dotted lines, the system is said to be in position 2 (injection position in MV-7 valve). When a band of interest has left one of the columns and entered the second one, the valve is switched so that the position of the two columns with respect to the flow direction is exchanged. Recycle can be continued in this way. Because only one detector is used in this system, the feed is loaded to either Column A or Column B such that the detector is always downstream from the last column.

The columns used in this system were Omni low-pressure borosilicate glass columns purchased from Alltech (Deerfield, IL). The columns were fitted with one fixed-end and one adjustable-end fitting, allowing for different column lengths. All columns were slurry packed with a polystyrene divinyl-benzene copolymer resin.

Methods

HPLC Assay. In this study, HPLC is used to analyze the collected fractions from the purification step. In this assay, a Waters Nova-Pak C-18 column and a premixed mobile phase of H_2O:CH_3CN:THF(58:33:9 v/v), at a flow rate of 1.0 mL/min, were used (Au, 1995). The sample injection volume was 10 μL. The chromatograms were examined at a wavelength of 227 nm using the single-wavelength detector (Waters

486), which is more sensitive than the photodiode-array detector. All solvents were degassed for approximately 15 min prior to analysis, and the column was washed using acetonitrile after analysis.

LPLC Column Preparation. The resin particles as received are spherical and have a large diameter (> 600 μm). The resin was chopped and sieved to obtain different particle sizes. The particles became irregular-shaped after the chopping. The irregular-shaped particles resulted in higher pressure drop than spherical particles of the same size. A slurry technique was used to pack the particles into a LPLC glass column. Once a column was filled with settled sorbent particles, it was attached to the LPLC system to continue the packing process. Ethanol and DDW were alternately pumped through the column. The column pressure drop was maintained at a constant value (10 atm) while the flow rate was adjusted accordingly. The bed eventually reached a stable height and the adjustable plunger was pushed to the top of the packing. Three columns (A, B and C) were prepared by this method (see Table 2).

Column porosities were determined from the retention times of 0.1-mL pulses of blue dextran (0.3–0.5 g/L in DDW) and sodium nitrate (2–3 g/L in DDW). DDW was used as the mobile phase and the flow rate was 0.2 mL/min. The column HETP (height equivalent to theoretical plate) was calculated based on the sodium nitrate peaks.

LPLC Experiments: Binary Mixture of Paclitaxel and Cephalomannine. Four experiments utilizing this binary mixture were carried out in this study. Two samples (I and II) and three columns (A, B and C) were used (Tables 1 and 2). The samples were made immediately before the elution experiments to avoid possible degradation. Generally, the columns were preequilibrated using the solvent with the same percentage of ethanol as the selected sample. After sample loading, the columns were immediately eluted using 100% ethanol. The effluent was monitored using a PDA detector. For nonrecycle experiments (experiments B-1 and B-2), fractions were collected when cephalomannine began to be washed out. For recycle experiments (experiments B-3 and B-4), the MV-7 valve was used as the same function as the six-port valve in Figure 1b. Usually, the valve position was not changed until both of the paclitaxel and cephalomannine bands had left one of the columns and entered the second one. But if the two bands spread more than one column, part of them (either paclitaxel or cephalomannine band) would be washed out of the recycle system. Here, the mass balance of cephalomannine must be maintained because its feed concentration is much lower than paclitaxel (Table 1). Repeated loss due to washing out will result in detection difficulties. Therefore, in order to avoid loss of cephalomannine, the valve position was changed when the front of the cephalomannine band reached the exit of the second column. However, a portion of paclitaxel still remained in the first column. This valve switch caused this portion of paclitaxel to be washed out from the recycle system. In order to have accurate accounting of paclitaxel recovery and purity, this paclitaxel portion was collected and analyzed. The elution profiles of paclitaxel and cephalomannine were constructed according to the HPLC analysis of the fractions collected in the experiments. The details of these experiments are as follows:

Table 1. HPLC Assay of Loading Samples

Sample	Ethanol	Paclitaxel	Cephalomannine
Sample I	30%	1.08 mg/L	0.34 mg/L
Sample II	30%	1.94 mg/L	0.63 mg/L
Real broth	30%	1.01 mg/L	0.48 mg/L

Table 2. Column Characterization

Column	Particle Size (Radius)	Dimension	ϵ_b	ϵ_p	HETP
A	40 μm	7.7 cm × 1 cm ID	0.393	0.736	0.033
B	40 μm	7.8 cm × 1 cm ID	0.473	0.769	0.079
C	40 μm	5.2 cm × 1 cm ID	0.466	0.771	0.053

Experiment B-1 (one-column experiment): loading sample I into column C for 50 min at a flow rate of 0.2 mL/min. Eighteen 4-min fractions were collected starting at 30 min after step-up to 100% ethanol.

Experiment B-2 (two-column experiment): loading sample II into column B for 141 min at a flow rate of 0.2 mL/min. Column B was connected with column A through the MV-7 valve, which was at position 2. The flow direction was column B → column A → detector. Twenty-four 5-min fractions were collected starting at 69 min after step-up to 100% ethanol.

Experiment B-3 (three-column experiment): loading sample II into column A for 142 min at a flow rate of 0.2 mL/min. Column A was connected with column B through the MV-7 valve, and the valve was at position 1. At 118 min after step-up, the valve was switched to position 2. Recycle began at that moment. The flow direction was column A → detector → column B → column A → detector. Twenty-eight 5-min fractions were collected starting at 180 min after step-up to 100% ethanol.

Experiment B-4 (four-column experiment): loading sample II into column B for 317-min valve, and the valve was at position 2. At 130 min after step-up, the valve was switched to position 1. Recycle began at that moment. After 83 min, the valve was switched back to position 2. The flow direction was column B → column A → detector → column B → column A → detector. Fraction collection began at 69 min after the last valve switch.

LPLC Experiments: Real Broth. The lypholized dry mass (6.443 g) obtained from 1 L of filtered PTC broth was stored in a freezer until use. Based on HPLC assay of the broth, this PTC broth sample represents the worst-case scenario for separation (i.e., typical broth has a paclitaxel concentration from 20 to 150 mg/L). The broth was reconstituted by measuring out 1 L of filtered DDW in an Erlenmeyer flask and adding the lypholized sample. Once reconstituted, 1 L of ethanol was added and the broth was sonicated. The pH was measured and adjusted by adding drops of acetic acid to attain a pH of roughly 5.5. Maintaining the PTC broth at a slightly acidic pH is important in order to avoid degradation (Au, 1995). A portion of the broth containing 50% ethanol was diluted with filtered DDW to obtain 30% ethanol in broth. Before loading, the broth was filtered in order to remove a small amount of insoluble materials.

The basic steps and considerations are the same as the binary mixture experiments except a 2-h washing step (using 100% water) is added before step-up to 100% ethanol. Be-

cause of the low taxane concentration in real broth and low flow rate used in this process, the loading times should be long enough to maintain certain productivities. The details of the experiments are described below.

Experiment R-1 (one-column experiment): loading real broth into column A for 400 min at a flow rate of 0.5 mL/min. Ninety fractions of 1-mL samples were collected starting at 30 min after step-up to 100% ethanol.

Experiment R-2 (four-column experiment): loading real broth into column B for 540 min at a flow rate of 0.2 mL/min. Column B was connected with column A through the MV-7 valve, and the valve was at position 2. At 135 min after step-up, the valve was switched to position 1. Recycle began at that moment. After 85 min, the valve was switched back to position 2. The flow direction was column B → column A → column B → column A. Fractions collection began at 60 min after the last valve switch.

Two additional real-broth experiments have been obtained and reported elsewhere (Ma et al., 1996b). They are not included in this article to avoid duplication.

Results and Discussion

HPLC assay

Several HPLC assays have been reported in the literature. The literature studies use either gradient elution methods, which require long equilibration time, or specialty columns, which are costly. Therefore, an alternative needs to be considered. The HPLC assay developed in this study gives good resolution of various taxanes. The peaks of three key taxanes (paclitaxel, cephalomannine, and 7-epi-10-deacetylpaclitaxel) can be totally separated from each other. This simple isocratic method requires short analysis time and it can analyze taxanes with very low concentrations (0.2 mg/L). A single column can be used for more than 500 injections in this method.

Besides the single-wavelength detector, the PDA detector, which was used to monitor effluent histories for LPLC experiments, was also used to confirm the identities of the taxane peaks. UV/vis spectra of paclitaxel and cephalomannine standards obtained from the PDA detector were used to confirm the identities of the paclitaxel and cephalomannine peaks and to ensure that there were no impurities eluting or coeluting with them. In order to quantify eluted component concentrations, a series of taxane standard solutions in pure ethanol were analyzed using the HPLC assay. The obtained calibration curves (peak area vs. concentration) at 227 nm show a good linear fit (Au, 1995). According to these curves, we obtained the concentrations of paclitaxel and cephalomannine in the different loading samples (Table 1). This table shows that the concentration of paclitaxel in the loading sample of PTC broth is very low (1.01 mg/L).

LPLC column characterization

All columns were packed and characterized as described in the section of LPLC column preparation. The properties of these columns are shown in Table 2. The similarity of their properties show that the packing procedure is reproducible. Compared to those packed with larger sorbent particles (73-μm radius) (Au, 1995), these columns have lower HETP values. As expected, the column efficiency is increased when smaller sorbent particles are used. Because the glass columns with adjustable end fitting have a pressure limit of 1.5 MPa, particles smaller than 40 μm in radius cannot be used with the LPLC equipment.

Binary mixture experiments

Because only a small amount of PTC broth was available, simulations were first used to identify the design and operation conditions that are required for high purity and high yield using recycle LPLC. A binary mixture of paclitaxel and cephalomannine was then tested to confirm the simulation results. According to the HPLC chromatogram of the PTC broth (Figure 4), paclitaxel and cephalomannine are the two major taxanes in the PTC broth, which has little 7-epi-10-deacetylpaclitaxel and 10-deacetylpaclitaxel. Therefore, it appears reasonable to use paclitaxel and cephalomannine as the model compounds in the binary mixture study.

The LPLC elution profiles based on the HPLC assay of the collected fractions for experiments B-1 and B-2 are presented in Figures 5a and 5b. These figures show that paclitaxel was concentrated from 2.8-fold to 3.7-fold of its initial concentration when the loading volume increased from 10 mL (experiment B-1) to 28.2 mL (experiment B-2). In fact, the stepwise elution mode can compress paclitaxel to a higher concentration when a larger volume of sample is loaded. These figures also show that increasing column length can improve the separation. Because of the pressure limit of the glass column, column length cannot be further increased. In order to achieve better separation under the column length and pressure-drop limit, recycle was used. Here the alternative pumping recycle technique was used in order to avoid serious mixing in the low-pressure pumps. The results of experiments B-3 and B-4 are shown in Figures 5c and 5d. The figures indicate that recycle technique can improve the separation and the resolution of paclitaxel and cephalomannine increases with the cycle times. The four-column experiment (experiment B-4) can get nearly base-line separation. The figures also indicate that paclitaxel and cephalomannine bands do not broaden very much with increasing cycle times. The larger loading volume in experiment B-4 resulted in the more

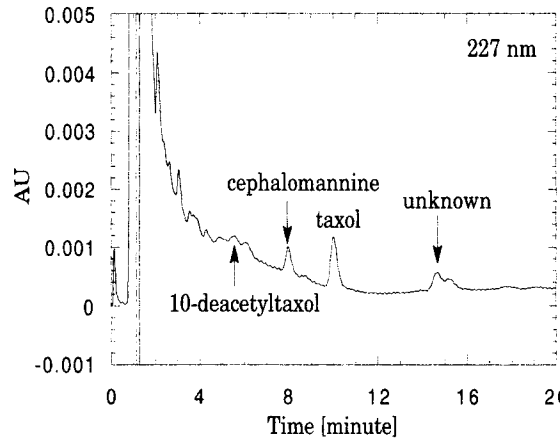

Figure 4. HPLC chromatogram of plant-tissue culture broth.

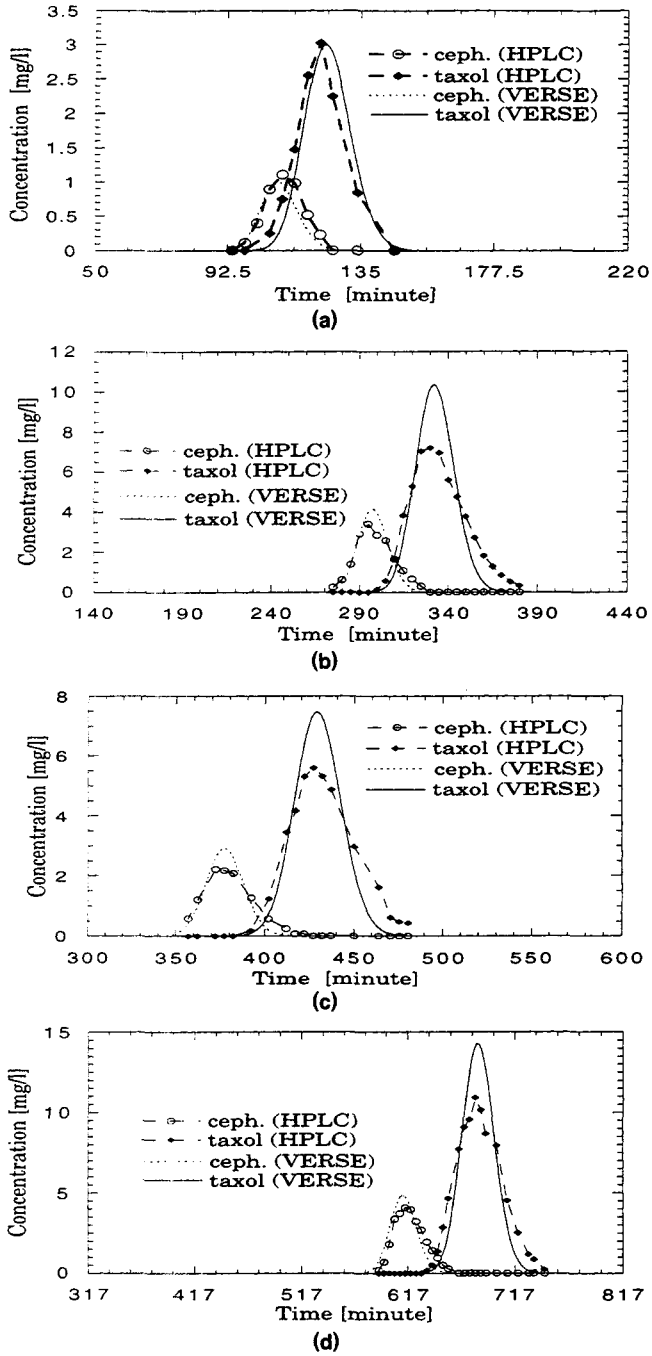

Figure 5. VERSE simulation vs. HPLC assay developed profiles for binary experiments.

(a) Experiment B-1, loading sample I (paclitaxel: 1.08 mg/L) into column C for 50 min at a flow rate of 0.2 mL/min "ceph." is the short form of cephalomannine. (b) Experiment B-2, loading sample II (paclitaxel: 1.94 mg/L) into column B for 141 min at a flow rate of 0.2 mL/min. Column B was connected with column A through the MV-7 valve, which was at position 2. (c) Experiment B-3, loading sample II (paclitaxel: 1.94 mg/L) into column A for 142 min at a flow rate of 0.2 mL/min. Column A was connected with column B through the MV-7 valve, and the valve was at position 1. At 118 min after step-up, the valve was switched to position 2. Recycle began at that moment. (d) Experiment B-4, loading sample II (paclitaxel: 1.94 mg/L) into column B for 317 min at a flow rate of 0.2 mL/min. Column B was connected with column A through the MV-7 valve, and the valve was at position 2. At 130 min after step-up, the valve was switched to position 1. Recycle began at that moment. After 83 min, the valve was switched back to position 2.

concentrated peaks in Figure 5d although the sample was recycled more times.

According to the elution profiles of experiments B-2 and B-3 (Figures 5b and 5c), the retention times of cephalomannine and paclitaxel in column A are 83 and 95 min, respectively. Because there is no significant difference between columns A and B, the retention times are assumed to be the same for both columns. From the elution profiles of experiments B-3 and B-4, it is easy to find that the sample bands spread slightly beyond the length of one column and both of the paclitaxel and cephalomannine bandwidths are less than one column length. Therefore, only a small portion of paclitaxel was washed out during each valve switch. In all recycle experiments, the first band (cephalomannine) did not reach the last band (paclitaxel) of the former cycle because the sample bandwidth is less than the length of the two columns. Therefore, there is no contamination between different cycles.

Real-broth experiments

The binary-mixture experiments demonstrated the ability to achieve nearly complete separation of paclitaxel and cephalomannine using a four-column recycle (Figure 5d). These results suggest that the same strategy (loading, ethanol stepwise elution, and recycle) could be used for recovery and purification of paclitaxel from PTC broth. Since a large number of nonadsorbing and low-affinity impurities are present in PTC broth, it is necessary to add a water washing step before the 100% ethanol elution.

To prevent any taxane degradation, all the collected fractions were analyzed immediately after each experiment. It was found later that the samples remained fairly intact over extended periods (several months) if they were stored in a freezer. According to periodic HPLC analysis, there was no evidence of degradation after two months of storage in a freezer and only slight degradation after seven months of storage. A representative HPLC chromatogram of these fractions is shown in Figure 6. In order to identify taxanes and to know their concentrations in the collected fractions, a number of taxane standards (paclitaxel, cephalomannine, 10-deacetylpaclitaxel, 7-epi-10-deacetylpaclitaxel, baccatin III,

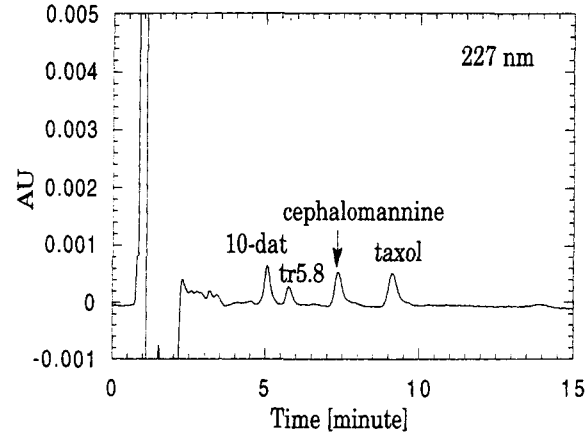

Figure 6. HPLC chromatogram of the fraction at 1,001 min for experiment R-2.

"10-dat" is the short form of 10-deacetylpaclitaxel.

and 10-deacetylbaccatin III) were analyzed using the developed HPLC procedure, and their retention times were noted. Because of the potential of slight variability in mobile-phase composition, taxane standards were run for each batch of mobile phase. Also, some fractions were analyzed using PDA detector and spiked using standard taxanes to ensure correct taxane identification. The elution profiles constructed from the HPLC assay provide quantitative results for evaluating the strategy and, more specifically, illustrate the effect of recycle technique on the separation of paclitaxel in real broth.

Analysis of the fractions collected in experiment R-1 reveals that there are eight major components within the elution window of paclitaxel. Three of these compounds were identified as paclitaxel, cephalomannine, and 10-deacetylpaclitaxel by comparison of retention times to those of the standards. The five remaining components did not correspond to any of the taxane standards that were assayed (including 7-epi-10-deacetylpacilitaxel, baccatin III, and 10-deacetylbaccatin III). They are listed by their retention times in the HPLC assay, in which paclitaxel retention time is 9.4 min. They are tr2.9 (whose retention time is 2.9 min), tr4.1, tr5.8, tr14.0, and tr16.4, respectively (Figure 6). Because of the inability to identify all the components appearing on the HPLC chromatograms, it is impossible to quantify the concentrations of these components accurately. An approximation can be made by assuming that all components have the same extinction coefficient as paclitaxel, and then the concentrations of these unidentified impurities can be estimated. From the area profiles of these eight components (Figure 7a), it is evident that the paclitaxel peak still overlaps significantly with the impurities in this single-column experiment.

In order to get better separation of paclitaxel from the impurities in real broth, a recycle scheme, experiment R-2, was carried out as described in the section on LPLC experiments. Analysis of the fractions collected in this experiment shows that there are only four major components left within the paclitaxel elution window (Figure 7b). Recycle greatly improved the separation of paclitaxel from the impurities. A comparison of experiment R-2 with experiment B-4 (both are four-column recycle) shows that the nontaxane impurities in real broth have no significant effect on paclitaxel and cephalomannine effluent histories.

Based on the HPLC assay, the recovery and compression factors of paclitaxel were calculated (Table 3). This LPLC process with stepwise elution and simple recycle achieved significant peak compression and 91% paclitaxel recovery at 95% purity. The compression factor is defined as the ratio of the maximum effluent concentration to the feed concentration of the solute. The recovery percentage is calculated from the ratio of the amount recovered (at 95% paclitaxel purity) to the amount loaded.

VERSE simulations and design considerations

Input Data. In VERSE simulations, experimental conditions must be defined and estimations of mass-transfer parameters and adsorption isotherm parameters must be provided. The experimental conditions include loading volume, wash time, stepwise elution conditions, number of components, and feed concentrations of the components. In addition, experimentally determined column characteristics, which include length, particle size, and porosity, must also be provided. The linear reverse-phase modulator isotherms were measured and reported elsewhere (Au, 1995; Ma et al., 1996b). The following isotherm equations correlate the equilibrium data closely.

$$Q_{\text{Paclitaxel}} = 2{,}852.3 \cdot \exp(-5.3757\phi)C$$

$$Q_{\text{Cephalomannine}} = 2{,}150.3 \cdot \exp(-5.4184\phi)C.$$

Here Q has the units of mg per mL solid adsorbent.

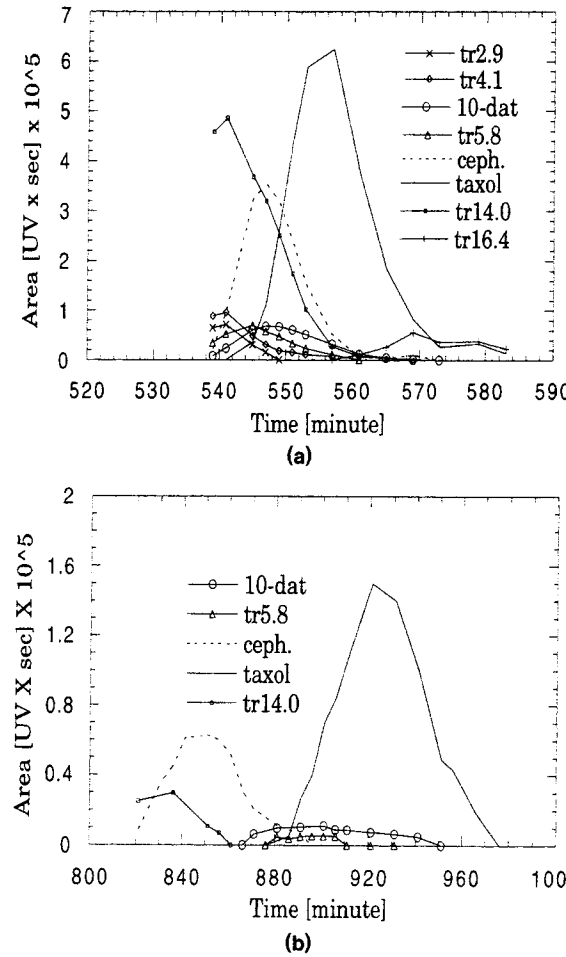

Figure 7. Area profiles of paclitaxel and the major structurally similar impurities within paclitaxel elution window.

(a) Experiment R-1, loading real broth (paclitaxel: 1.01 mg/L) into the column A for 400 min at a flow rate of 0.5 mL/min. (b) Experiment R-2, loading real broth (paclitaxel: 1.01 mg/L) into the column B for 540 min at a flow rate of 0.2 mL/min. Column B was connected with column A through the MV-7 valve, and the valve was at position 2. At 135 min after step-up, the valve was switched to position 1. Recycle began at that moment. After 85 min, the valve was switched back to position 2.

Table 3. Percent Recovery and Compression Factor of Paclitaxel

Experiment	Compression Factor	Paclitaxel Recovery (Purity = 95%)
R-1	29	30
R-2	7	91

Table 4. VERSE Simulation Parameters and Dimensionless Groups used in Experiment B-2

System Parameters	Length 15.5 cm	ID 10 mm	Particle Radius 40 μm	ϵ_b 0.433	ϵ_p 0.753
Numerical parameters	N_a 100	N_p 1	n_a 4	n_p 4	atol 0.001
Mass-transfer parameters	E_b 1.0147×10^{-2}	k_f 0.15661	D_z 2.46×10^{-4}	D_p 1.31×10^{-4}	— —
Dimensionless group	*Re* 3.802×10^{-3}	*Pe* 1796.6	*Bi* 6.3505	*Nf* 8107.4	*Np* 324.98

In the system studied, paclitaxel and cephalomannine are present in PTC broth at very low concentrations (typically a few mg per liter) and the sorbent has a high capacity. The preceding linear reverse-phase modulator isotherms were used for the following simulations.

Table 4 shows the parameters used in the simulation of experiment B-2. Because the structures of paclitaxel and cephalomannine are similar, their Brownian diffusivities and intraparticle diffusivities are taken to be the same. The same numerical parameters, isotherms and diffusivities are used in the simulations of all other experiments. The system and operating parameters are different for each experiment. They are reported in the section of LPLC experiments.

It has been shown in a previous study (Ma et al., 1996a) that both pore and surface diffusion mechanisms give similar spreading in linear isotherm systems. For this reason, surface-diffusion effects (if significant) can be lumped into pore-diffusion effects and the pore-diffusion model should be adequate for the simulations in this work. Because of the large particle size (80 μm) in this LPLC process, mass-transfer effects cannot be neglected. Using the moment analysis reported by Ma et al. (1996a), we found that peak spreading is due to both intraparticle diffusion and film diffusion in all the systems studied here.

VERSE Simulations of Experiments. The VERSE simulations are compared with the effluent histories that are constructed from the HPLC assay in Figures 5 and 8. In the simulation of the recycle experiments, extra column dispersion effect is neglected. The function of recycle is therefore the same as increasing the column length. The simulation results for the single-column experiment B-1 are in close agreement with the data (Figure 5a). For multiple-column experiments (Figures 5b through 5d), the simulated peaks are slightly broader than the data. This small discrepancy could be caused by ignoring extra column dispersion in the simulations.

Design Considerations. Since the simulations prove reliable in predicting the data for real broth, we used the simulations to explore various design alternatives. In this study, paclitaxel is the major product of interest. Its separation cost can be reduced if design and operating conditions are optimized. Usually, an economic analysis is used to minimize the cost per unit weight of product (Guiochon et al., 1994). To analyze the cost in large-scale production, we use the parameters for large-scale processes (production scale is about 80 g of paclitaxel/d) to study the effects of different design and operating conditions. The same sorbent and mobile phase are used in this large-scale process. To optimize this process, several parameters, such as particle size, column length, sample size, flow rate, gradient strategy, and recycle procedure, should be considered.

As shown in Figure 9a and 9b, it is evident that peak dispersion will decrease with decreasing particle size from 400 μm to 200 μm. In Figure 9a, column dimension is 100 cm in diameter and 100 cm in length, sample (in 30% ethanol) loading is 200 min at a flow rate of 10 L/min, stepwise elution is from 30 to 100% ethanol, and paclitaxel concentration in the loading sample is 20 mg/L. To obtain the same recovery and purity as in Figure 9a, more cycle times are needed for Figure 9b. Therefore, solvent consumption in Figure 9a is less than that in Figure 9b.

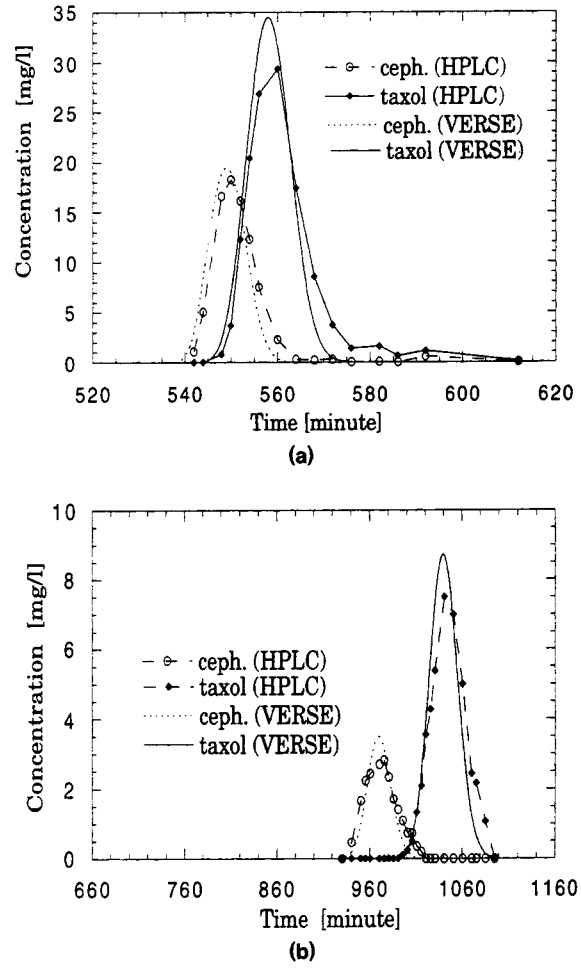

Figure 8. VERSE simulation profiles vs. HPLC assay-developed profiles.

(a) For experiment R-1, the details of this experiment are shown in the caption of Figure 7a. (b) For experiment R-2, the details of this experiment are shown in the caption of Figure 7b.

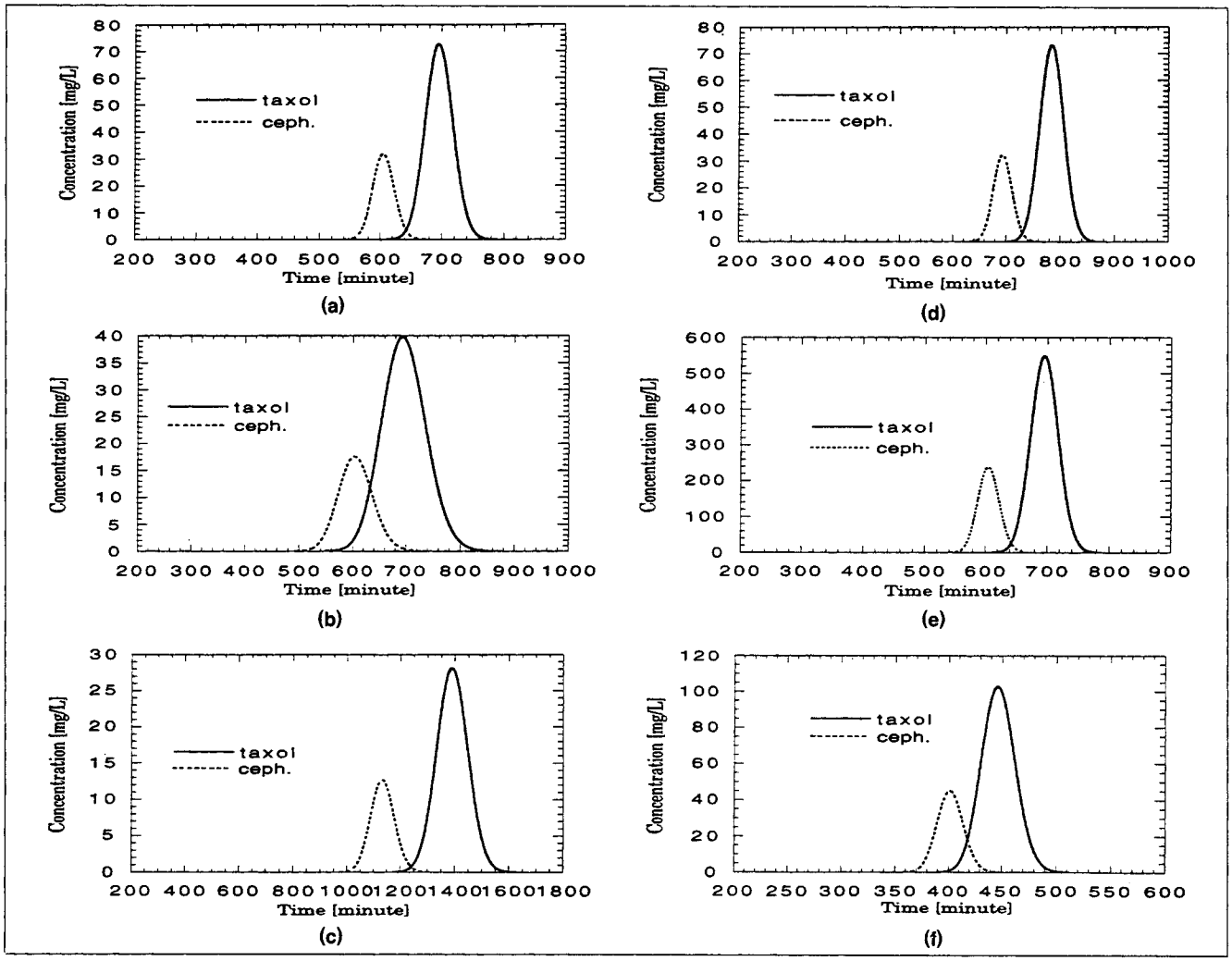

Figure 9. VERSE simulations of effects of design and operating parameters.

(a) For condition A, the column dimension is 100 cm in diameter and 100 cm in length. The same polystyrene divinyl-benzene sorbent as in earlier experiments is used, but its size is increased to be 200 μm in diameter. Paclitaxel concentration in the sample is 20 mg/L. This sample (in 30% ethanol) is first loaded for 200 min at a flow rate of 10 L/min, and then eluted using 100% ethanol. The same recycle strategy as in earlier experiments is applied. Two cycle times are used on this condition. (b) Condition B: particle size is changed to 400 μm. Other parameters are the same as condition A. (c) Condition C: step change from 30 to 80% ethanol. Other parameters are the same as condition A. (d) Condition D: linear gradient from 30 to 100% ethanol in 120 min. Other parameters are the same as condition A. (e) Condition E: paclitaxel concentration in the sample is increased to 150 mg/L. Other parameters are the same as condition A. (f) Condition F: no recycle is applied. Other parameters are the same as condition A.

Gradient conditions also have significant effects on the separation. The larger the step change in solvent concentration, the greater the peak compression and the shorter the cycle time (comparing Figure 9a with Figure 9c). However, the solubility of paclitaxel limits the loading ethanol concentration to be 30% or higher. A linear gradient from 30 to 100% ethanol in 2 h cannot improve the separation significantly (Figure 9d), and it requires a longer elution time and more solvent. Besides, it is difficult for the ethanol:water system to degas on-line. Therefore, the stepwise elution from 30 to 100% ethanol is the best strategy for this system.

Besides the parameters given earlier, paclitaxel concentration in the loading sample can have a significant effect on the production cost per gram of paclitaxel. As shown in Figures 9a and 9e, there is no significant effect on paclitaxel separation when paclitaxel concentration is increased from 20 to 150 mg/L. Because the equipment and labor costs per unit time are fixed, higher feed concentration and higher flow rate can increase production rate (amount of paclitaxel per unit time) and reduce the equipment and labor costs per gram of paclitaxel produced.

To obtain higher productivity at the same purity and to reduce solvent consumption, other recycle strategies, such as "recycling with mixing" can be used (Guiochon, 1994). However, the operation is more complicated than the simple recycle used in this work.

Cost analysis is very useful in further investigating the effects of different design and operation parameters on the process economy. To calculate total production cost per gram of paclitaxel, the following assumptions are made: (1) the equipment cost is \$500,000 and it can be used for 5 years; the sorbent cost is \$30/lb (\$66/kg) and it can last 2 years; labor cost is \$30/h; (2) the solvent (ethanol) is \$1 per liter; the used ethanol can be recycled by distillation; the net cost is 20% of

Table 5. Cost Distributions of the Process (Based on per Gram of Paclitaxel)*

Condition	Solvent ($)	Sorbent ($)	Equipment ($)	Labor ($)	Sum ($)
A	33 (70%)	1 (2%)	4 (9%)	9 (19%)	47 (100%)
B	100	2	8	18	128
C	69	2	8	18	97
D	35	1	5	9	50
E	5	~0	1	2	8
F	36	1	4	10	51

*The same recovery and purity as condition A are required for all other conditions. The six conditions are described in Figure 9.

its purchase cost; and (3) the feed (broth) and utility cost are excluded from the estimation. Based on these assumptions, the cost distributions are shown in Table 5. From this table, we can see that solvent is the major cost (more than 70%) of the recovery and purification process. This table also shows that the process cost can be further reduced by using smaller particle size, higher feed concentration, optimal gradient, and the recycle strategy.

Compared to the HPLC processes reported in the literature, this LPLC process eliminates the extraction step and needs only environmental benign solvent (ethanol). HPLC sorbents are about ten times more costly than LPLC sorbents (Taxane sorbent typical HPLC is about $5,000 per kilogram). HPLC equipment (columns and pumps) are also at least twice as costly as LPLC equipment. As shown in Figure 9f, the recovery of paclitaxel is about 10% less if no recycle is applied. The recycle LPLC process is better in terms of both yield and process cost. Overall the LPLC process developed in this study is more economical and environmentally benign than existing processes.

Conclusions

An effective HPLC assay for taxane analysis has been developed using a C-18 column and an isocratic mobile phase of H_2O:CH_3CN:THF(58:33:9). The advantages of this assay include the use of a column that is commonly available, long column life, rapid analysis time, and the ability to separate taxanes that typically closely elute or coelute with paclitaxel.

A polystyrene divinyl-benzene copolymer sorbent and an ethanol:water mobile phase have been identified as an effective system for paclitaxel recovery and purification. The results demonstrate the ability to use a stepwise elution for paclitaxel concentration. The recycle LPLC process using the scheme of loading, washing, stepwise elution and recycle has proved to be effective and economical for paclitaxel recovery and purification. The results of the real-broth experiments indicate that this technique can achieve higher paclitaxel purity and yield than the conventional solvent-extraction methods reported in the literature. The use of an ethanol:water mobile phase provides the additional advantages of being cost effective and environmentally benign compared to typical solvents used in the solvent-extraction schemes.

VERSE simulations based on the pore-diffusion model using a consistent set of diffusivities and isotherm parameters agree closely with experimental column effluent histories. After validation, the simulations are used to generate various design alternatives. Analysis of the alternatives shows that solvent consumption can be reduced by using a large step change in ethanol concentration, small particle size, large loading volume, and high paclitaxel concentration in feed. This model-based design approach significantly reduces the time and cost required in process development.

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Notation

Bi = Biot number
C = solute liquid-phase concentration
D_z = Brownian diffusivity
D_p = intraparticle diffusivity
E_b = axial dispersion coefficient
HETP = height equivalent to theoretical plate, cm
ID = column inter diameter
k_f = film mass-transfer constant
N_a = axial elements
N_{ap} = particle elements
Nf = dimensionless film mass-transfer coefficient
Np = dimensionless pore diffusion coefficient
PDA = photodiode array detector
Pe = bulk phase Péclet number
Q = solute solid-phase concentration
Re = Reynolds number
SMT = solvent movement theory
V = column volume
atol = absolute tolerance
ceph. = cephalomannine
n = column number
n_a = collocation points for axial
n_p = collocation points for particle
taxol = registered trademark with Bristol Myers Squibb
tr2.9 = component with retention time of 2.9 min
ϵ_b = interparticle void
ϵ_p = intraparticle porosity
ϕ = volume fraction of solvent

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