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Development of High Performance Liquid Chromatography for Paclitaxel Purification from Plant Cell Cultures

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Abstract Paclitaxel can be produced in high yield and with a high degree of purity from plant cell cultures of *Taxus chinensis*. The complete purification method was systematically established and described. This method was an efficient procedure for the purification of paclitaxel from crude paclitaxel, consisting of reverse-phase chromatography, followed by a normal-phase chromatography. The two-stage HPLC purification scheme serves as an effective and economical approach for resolving paclitaxel from complex mixtures of taxoids, with high purity (>99%) and low impurities (<0.1%). The process is readily scalable to a pilot plant and eventually to a production environment where multikilogram quantities of material are expected to be produced. The process has been optimized to minimize solvent usage, complexity, and operating costs.

Key words: Paclitaxel, plant cell culture, *Taxus chinensis*, purification, high performance liquid chromatography

Paclitaxel (Taxol) production in plant cell and tissue cultures has the potential to provide sufficient quantities of paclitaxel for chemotherapeutic use. Cell culture offers the potential advantage of availability and reliable production by using a renewable resource [6] whereas bark stripping methods lead to the destruction of scarce plant material. In addition, chemical synthesis of paclitaxel is very complex with a very low yield. A tissue culture process is also more adaptable to increase for the demand of paclitaxel [13, 14].

Not much information is currently available, with regards to the procedures which are directly scalable to commercial operations, for isolation and purification of paclitaxel. A report by Witherup *et al.* [16] details a process for isolation of paclitaxel from *Taxus brevifolia* needles. The crude paclitaxel resulting from the low resolution chromatography contains only 4% paclitaxel on a weight basis, necessitating an excessive number of HPLC that runs to effect final purification of paclitaxel. The low resolution chromatography steps require such large volumes of solvent and large amounts of chromatographic media that the process is impractical for a scale-up consideration. According to the paper by Cardellina II [1], normal phase chromatography on a CN-bonded phase column, using hexane/IPA as the eluant, is used for the final purification of paclitaxel extracted from *Taxus brevifolia* bark. By the approach taken by Witherup *et al.* [16], excessive solvent usage makes the process impractical for a scale-up to commercial operations. A different approach to reach paclitaxel purification was reported by Vanhaelen-Fastri *et al.* [15] in which high speed countercurrent chromatography (HSCCC) was investigated as an alternative to HPLC for the final separation of paclitaxel from related compounds. Several other papers [2, 3, 4, 7, 10, 11, 12, 18] deal with the isolation of paclitaxel from dried plant materials as part of investigations into the identity of paclitaxel constituents. These isolation schemes generally follow the procedures described above and include an initial extraction, a partition step to remove polar/water soluble constituents, a partition step to remove waxy substances, and one or more chromatography steps (silica gel, Sephadex LH-20, Celite) to separate paclitaxel from interfering substituents. The resulting crude paclitaxel products typically containing 1% to 4% paclitaxel on a weight basis and final separation of paclitaxel is accomplished by using an open column chromatography, HPLC, or a combination of the two methods. The problem inherent in all of the published procedures for paclitaxel purification is the large amounts of solvent required. The high solvent usage is dictated, in part, by the reliance on large open column chromatographic techniques for pre-purification of paclitaxel to the point where a suitable material exists for process scale HPLC. The size of these chromatographic separation systems is governed in a large part by the degree of paclitaxel purity in the feedstock material. In the early stages of prepurification, paclitaxel typically represents

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only 1-2% of the material undergoing chromatographic separation. Therefore, the size of the chromatographic system is large relative to the amount of paclitaxel obtained.

Several considerations dictated the approach to be used for the final purification of paclitaxel from other taxoids. First, it was recognized early in the research that the taxoid profile of the cell culture extracts as determined by HPLC was significantly different from that obtained from the plant tissue. Major taxoid constituents which existed in cell culture extracts did not occur at all or in a very low concentration in plant materials [9]. This raised the question and possibility that the impurity profile of paclitaxel purified from cell cultures could be substantially different from that currently obtained from bark, and this difference could present regulatory hurdles for its acceptance as a bulk drug substance. One of the goals established for developing the final purification scheme, therefore, was to eliminate these impurities from the final product. The second consideration in choosing a final purification scheme was to develop a process which could be adapted to a variety of existing pilot-plant facilities without introducing new technologies or investing heavily in a new capital equipment.

Process scale HPLC is a logical choice for purifying production scale quantities of paclitaxel since the selectivity and high resolving capabilities of HPLC have long been exploited in the analysis of paclitaxel and other taxoids. A number of different chromatographic media have been investigated using both normal-phase and reverse-phase HPLC systems. In general, these systems have been optimized for separating taxoid components extracted from bark or needles of *Taxus* plants. Due to the differences which exist between the taxoid patterns extracted from culture systems and that which is present in bark and needle extracts, much of the published information is not directly relevant to our separation needs. As a part of the current development effort, a series of selectivity studies and optimization experiments were conducted to reach a process scale HPLC separation system that is optimized for cell culture extracts.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

Suspension cells originated from *Taxus chinensis* were maintained under darkness at 24°C with shaking at 150 rpm. Suspension cells were cultured in a modified Gamborg's B5 medium supplemented with 30 g/l sucrose, 10 μM of NAA (naphthalene acetic acid), 0.2 μM of BA (6-benzylamino purine), 1 g/l of casein hydrolysate, and 1 g/l of 2-[N-morpholino] ethanesulfonic acid (MES). Cell cultures were transferred to a fresh medium every two weeks. In a prolonged culture for production, 1% and 2% maltose were added to the culture medium on day 7 and day 21, respectively, and 4 μM of $AgNO_3$ was added on the initiation of culture as an elicitor [5]. After the culture, biomass was recovered with a decanter (Westfalia, CA150 Clarifying Decanter) and high-speed centrifuge (α-Laval, BTPX 205GD-35CDEFP).

Analysis of Paclitaxel and Taxoids

A Hewlett-Packard 1090 HPLC was used for all analytical characterizations of intermediate or finished products. An HPLC system with Econosil C18 column (Alltech, 4.6× 250 mm, d_p=5 μm) was used to achieve quantitative analysis. The elution was performed by gradient condition from the mixture of acetonitrile and water mixture from 20:80 to 92:8 within 50 min (flow rate=1.0 ml/min). Injection volumes were of 10 μl and effluent was monitored at 227 nm (paclitaxel) or 255 nm (internal standard) with the photo diode-array detector. Purity determinations of intermediate and finished products were made using an internal standard assay to compare the paclitaxel content of the test material to the paclitaxel content of the reference paclitaxel. An internal standard solution containing 1,000 μg/ml of n-propyl paraben in methanol was mixed in equal portions with accurately prepared stock solutions (~1,000 μg/ml) of test and reference samples. These solutions were analyzed on the HPLC system. Response ratios were calculated as the ratio of the peak areas for paclitaxel and internal standard and the ratios were corrected to reflect a sample concentration of 1,000 μg/ml. Purity values were calculated by comparing the response ratio determined for the test sample to that obtained for the reference paclitaxel. Results for the HPLC system was averaged to reach a final purity value. Concentrations for taxoids other than paclitaxel were estimated by reference to the paclitaxel peak area [8].

Starting cultures (suspension cultures) were homogeneously taken and extracted overnight in a methyl-t-butyl ether (MtBE) with 1% cetypyridinium chloride (CPC) solution. After an extraction, the supernatant was loaded on the LC-NH_2 SPE column (Supelco Co.) and eluted with the MtBE and methanol mixture (85:15). Eluant was mixed well and dried in the speed vacuum evaporator. Dried residue was redissolved in methanol and used for the quantitative analysis of paclitaxel.

Preparation of Crude Paclitaxel for HPLC Purification

The schematics to purify crude paclitaxel by HPLC is shown in Fig. 1. The method to produce crude paclitaxel from the biomass of *Taxus chinensis* cultures includes the steps of (i) methanol extraction of biomass from *Taxus* genus plant to obtain a crude extract, (ii) MtBE extraction of crude extract from (i), (iii) mix with equal volume of hexane and extract with CPC, and (iv) two precipitations of crude extract from extraction to obtain a crude paclitaxel (purity: ~25%, w/w) for HPLC purification. An RP-HPLC profile of crude paclitaxel is shown in Fig. 2. As can be

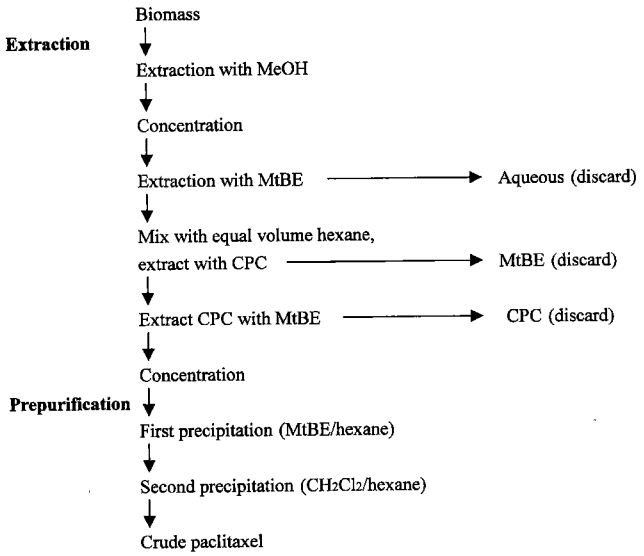

Fig. 1. Process schematic of extraction and prepurification for HPLC purification.

seen in the chromatogram, paclitaxel is identified as a major taxoid component of the mixture and constitutes >25% of the total sample on a weight basis. Many other peaks in the chromatogram have been tentatively identified as taxoids based on their UV spectra, similar to the UV spectra of paclitaxel. Identified components, which are also known as constituents of the *Taxus* plant tissue, include cephalomannine, 7-epi-taxol, and 10-deacetyltaxol. In addition to these known components, a series of taxoids which have not been reported as yet to occur in plant tissues have also been identified in crude paclitaxel. These include taxol C, 10-deacetyltaxol C, N-methyl taxol C, and taxcuitine [9], in which taxoids are very similar in structure to paclitaxel and they have nearly an equal bioactivity

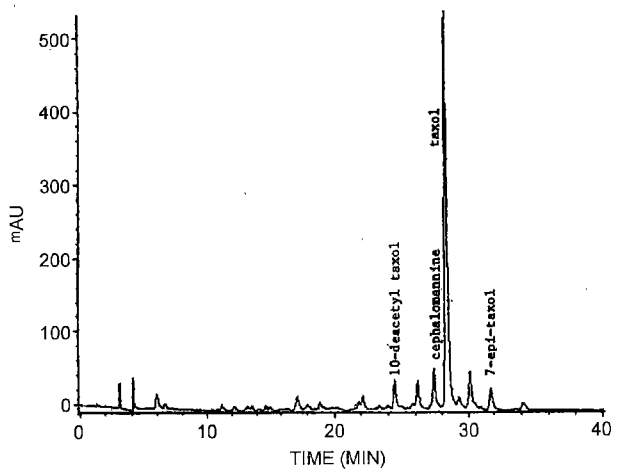

Fig. 2. HPLC chromatogram of crude paclitaxel from biomass for HPLC purification.

as paclitaxel in tubulin binding assays and cytotoxicity screens. In addition to these characterized compounds, a number of uncharacterized peaks have been observed in the HPLC profile. The concentration of taxoids other than paclitaxel is estimated to be 10- 15% on a weight basis (as determined by peak area comparisons of suspected taxoid peaks to peak areas of known amounts of paclitaxel). The nature of the unaccounted mass of crude paclitaxel (60-65%) has not been determined but it is currently under investigation.

RESULTS AND DISCUSSION

The goal of the HPLC purification is to achieve high purity (>99%) of paclitaxel. Allowable impurities include cephalomannine (<0.3%) and paclitaxel degradation products 10-deacetyltaxol and 7-epi-taxol (total concentration <0.1%). The total peak area of all other UV absorbing peaks in the HPLC profile must not exceed 0.1% of the paclitaxel peak area [8]. In developing a HPLC purification process, there was much hope that a single chromatographic system would be sufficient to obtain pure paclitaxel in high yield. No such system was found and this is due in part to the complex taxoid profile of crude paclitaxel and also in part to the rigorous nature of product specifications. A two-step HPLC purification was adopted by using a reverse-phase separation on C18 as the first step and normal-phase separation on CN-bonded phase silica as the second step.

The HPLC system was selected based on studies conducted with various chromatographic media in combination with various mobile phase combinations. Chromatographic media that was selected for this survey included C18, C8, phenyl, and CN packings run under reverse-phase conditions, and silica, CN, and alumina packings run under normal-phase conditions. Solvents used for reverse-phase HPLC included methanol/water and acetonitrile/water mixtures. Solvents selected for normal-phase operations included ethanol/hexane, ethyl acetate/hexane, and methylene chloride/hexane. The packings evaluated were, for the most part, 10 μm diameter and were packed in 4.6×250 mm columns. Isocratic solvent conditions were established for each column and fractions were collected under isocratic conditions.

As mentioned above, the selectivity survey failed to identify a single chromatographic system which resulted in paclitaxel meeting product specifications. The selectivity surveys revealed that C18 packings from various manufacturers were similar in terms of the nature and concentration of impurities present in the paclitaxel produced under simulated process conditions (Table 1). The primary contaminants detected included cephalomannine, an allowed contaminant in a final product up to a concentration of 0.3%, and two unknown taxoid component (labeled unknowns A and B) which should preferably not exist in the final product. In

Table 1. Results of selectivity comparisons of C18 packings. Impurities detected in recovered paclitaxel.

Column	Manufacturer	Packing size	Mobile phase	Plate count[a]	Impurity concentration in recovered paclitaxel[b]		
					Cepha.	A	B
Kromasil C18	Eka Nobel	10 μm	$MeOH/H_2O$	10112	10.6%	2.7%	0.2%
Kromasil C18	Eka Nobel	10 μm	CH_3CN/H_2O	22732	-	-	0.8%
Impaq C18	PQ Corp.	10 μm	$MeOH/H_2O$	8528	1.4%	2.1%	-
Impaq C18	PQ Corp.	10 μm	CH_3CN/H_2O	17956	-	-	0.8%
Lichrosphere C18	EM	10 μm	$MeOH/H_2O$	5824	2.2%	2.2%	-
Lichrosphere C18	EM	10 μm	CH_3CN/H_2O	10980	-	-	1.0%
Lichrosphere C18(E)	EM	10 μm	$MeOH/H_2O$	10665	0.4%	2.3%	-
Lichrosphere C18(E)	EM	10 μm	CH_3CN/H_2O	16324	0.1%	-	0.2%
Whatman C18	Whatman	10 μm	$MeOH/H_2O$	8392	0.1%	2.4%	1.2%
Whatman C18	Whatman	10 μm	CH_3CN/H_2O	10664	-	-	0.3%
YMC-C18AQ	YMC Corp.	10 μm	$MeOH/H_2O$	33856	-	1.9%	-
YMC-C18AQ	YMC Corp.	10 μm	CH_3CN/H_2O	50176	-	-	0.1%

[a]Plate count calculated by half-height method.
[b]Concentrations expressed relative to paclitaxel. Composition of starting material: Cephalomannine=10.3%, unknown A=2.3%, and unknown B=1.2%.

addition to these major contaminants, several uncharacterized taxoids were detected in trace amounts.

A reverse-phase separation on a C18 column using methanol/water as the mobile phase was selected as the first step in the two-step process based on several criteria. First, C18 packings are available from several manufacturers in large quantities, and due to the high volume of use they have achieved both in process scale HPLC applications and analytical applications, where lot-to-lot reproducibility is generally favorable. Second, these packings typically afford excellent physical and chemical stability (except at extremes of pH) and tend to be among the least reactive chromatographic media in terms of their chemical interaction with sample components. Third, procedures for packing media into stable HPLC beds have been optimized for C18 packings and a few technical hurdles need to be addressed. Finally, due to the high surface area and high carbon load of C18 packings, a sample throughout should be high on this packing. Among the various suitable C18 packings identified in the studies, Lichrosphere C18E (EM Separations) was selected for further process development work.

Having selected a reverse-phase separation on C18 as the first step in the multi-modal chromatographic separation, the choice of the second system was dictated by optimizing the separation of paclitaxel from contaminants eluting with paclitaxel in the first system. Among the chromatographic systems evaluated, the normal-phase system utilizing CN packing and ethanol/hexane as the mobile phase appeared to be the best. In the selectivity studies, CN packings from various manufacturers were found to exhibit very different selectivity characteristics. The spherical 10 μm packing from EM Separations (Lichrosphere CN) provided the best overall resolution of sample components and it was selected for a further scale-up work. This packing was found to give excellent separation of paclitaxel degradation products 7-epi-taxol and 10-deacetyltaxol and it was strongly felt that this ability to resolve paclitaxel from its degradation products may be very useful in the final stage of purification.

HPLC Stage 1

Conditions for reverse-phase chromatography are shown in Table 2. Although analytical size column hardware has been used in this development work, the packing employed can be used in commercial operations. The packing chosen for a scale-up is a 10 μm spherical packing having a pore diameter of 100 Å, a surface area of 350 m^2/ml, a carbon loading of 21.5% along with the fact that it has been fully end-capped to minimize sample interactions with the support material. Packing from other manufacturers having similar specifications could prove suitable for production scale applications.

Due to the virtual insolubility of crude paclitaxel in water and only a slight solubility in the mobile phase, sample introduction techniques became a major focus of the development work. A series of experiments were conducted to evaluate sample solubility in various potential solvents and the effects those solvents had on the chromatographic performance (e.g., peak symmetry and plate counts). Solvents evaluated include methanol, ethanol, ethyl acetate, acetone, dimethyl sulfoxide (DMSO), and methylene chloride. The procedure recommended in Table 2, dissolving the sample in dimethyl sulfoxide and adding water immediately before injection, was far superior to the other conditions tested.

The capacity of the stationary phase for crude paclitaxel was determined to be >19 mg/ml of the packed bed volume for 4.0×250 mm columns. This capacity was determined in two ways. First, a series of injections were made using increasing amounts of crude paclitaxel in a fixed volume of injection solvent, and then chromatographic performance criteria (i.e., peak symmetry and plate counts) were compared.

Table 2. Conditions for stage 1 and stage 2 preparative HPLC fractionation.

Stage	Conditions
HPLC system 1:	
Column	Lichrosphere C18(E), 4.0×250 mm, d_p=10 μm (Cat. #50848, EM Separations)
Mobile phase	65% MeOH in Water
Flow rate	1 mL/min
Detector	227 nm (path length=1 mm)
Sample	Dissolve 50 mg crude paclitaxel in 128 μl water immediately before injection
Retention time of paclitaxel	15 min
Column flush cycle (MeOH)	10 min
Equilibration time	5 min
HPLC system 2 :	
Column	Lichrosphere CN, 4.0×250 mm, d_p=10 mm (Cat. #50845, EM Separations)
Mobile phase	40% CH_2Cl_2+1% EtOH in Hexane
Flow rate	1 mL/min
Detector	227 nm (path length=1 mm, att.=2.56)
Retention time of paclitaxel	15 min
Column flush cycle	none
Time between injections	20 min

Second, the paclitaxel peak was collected and the concentration of impurities were determined for each sample. The results (Table 3) indicate that although chromatographic performance somewhat decreased and the concentration of impurities increased with each increasing sample load, there were no dramatic changes in the chromatographic profiles indicative of the column overload. For the highest load tested (i.e., 60 mg crude paclitaxel on a 4.0×250 mm column), the level of impurities observed in the paclitaxel fraction were within the resolving capacities of the second HPLC system.

Surprisingly, there did not appear to be any degradation of chromatographic performance due to limitations of paclitaxel solubility in the mobile phase. However, under conditions of high sample load, paclitaxel fractions collected from the HPLC quickly developed precipitates. Although it may be possible to increase sample load beyond the level investigated in this study, paclitaxel solubility in the mobile phase may quickly become limited. Based on column capacities which were determined in this study, projected capacities for 2.54 cm, 7.62 cm, and 20.32 cm columns are 2.42 g, 21.8 g, and 154.9 g of crude paclitaxel/injection, respectively, assuming the linear scale-up.

After elution of the paclitaxel, it is necessary to regenerate the column in order to remove any strongly retained components. The regeneration process reduces the potential for column fouling, helps to increase column lifetime, and reduces the likelihood of impurity carryover to the next injection cycle. It seemed that a 10 min wash with methanol was found to be sufficient. Re-equilibration of the column to initial conditions required 5 min.

A study of the stability of C18 packing to repeated injections of the crude paclitaxel was performed. This study utilized 2 mm ID columns (250 mm length) in order to minimize the quantity of crude paclitaxel that is required. A series of 51 injections of 8 mg of crude paclitaxel was made onto the HPLC column. Periodically, during this series of injections, the paclitaxel fraction was collected and assayed for impurities to determine whether or not the system performance would degrade over time. The analytical results for the recovered paclitaxel fractions do not show any evidence of system deterioration (Table 4). The preparative HPLC chromatograms remained consistent throughout the study.

Table 3. Concentration of impurities in recovered paclitaxel as a function of sample load for stage 1 HPLC (column dimensions: 4.0×250 mm).

Sample load (mg/injection)	N[b]	A[c]	Impurities, % of paclitaxel[a]	
			Cephalomannine	Unknown
1.83	5824	1.05	1.36	4.32
7.00	-	-	1.93	5.51
8.74	-	-	1.90	4.13
12.50	-	-	1.96	3.91
16.13	-	-	1.90	2.84
24.03	-	-	1.90	4.00
37.75	4178	1.11	3.73	2.54
53.55	3933	1.25	6.03	2.66

[a]Composition of crude paclitaxel: cephalomannine=11.87%, unknown A= 5.48%.
[b]Plate count calculated by half-height method (plates/meter).
[c]Peak symmetry.

HPLC Stage 2

Conditions for normal-phase chromatography on CN bonded phase columns are shown in Table 2. The packing chosen

Table 4. Lifetime study on stage 1 HPLC impurities detected in recovered paclitaxel (column: 2.0×250 mm).

Injection number	Concentration of UV absorbing impurities (%)
1	3.88
2	3.54
10	4.53
15	5.46
24	5.19
31	5.61
37	3.72
44	4.63

for a scale-up is a 10 μm spherical packing having a pore size of 100 Å and a surface area of 350 m^2/ml (Lichrosphere 100 CN, EM Separations). As mentioned earlier, CN packings from different manufacturers were found to vary significantly in their ability to resolve taxoids. An initial concern with this packing was whether lot-to-lot variability would preclude its use as a reproducible chromatographic system. Two lots of the packing material from the same manufacturer were tested and it was discovered that, although slight selectivity differences do exist for different lots of this material, an overall chromatographic performance was acceptable for both lots and performance was superior to that obtained with other suppliers. By working closely with the manufacturer, it is possible to adequately control lot-to-lot variability for this application. For example, this may require supplying the manufacturer with a standard to be used for evaluating the packing suitability for this application.

As with the reverse-phase system, conditions for sample introduction onto the HPLC column quickly became of paramount importance during attempts to increase the sample load. As with the reverse-phase system, paclitaxel exhibits virtually no solubility in the weak elution solvent (in this case, hexane), and hence a study was undertaken to choose an injection solvent in which paclitaxel would be soluble, but unfortunately, the solvent had a minimal impact on its chromatographic performance. Solvents evaluated include ethanol, methylene chloride, methanol, acetone, dimethyl sulfoxide, ethyl acetate, and methyl t-butyl ether. Ethyl acetate was found to be preferable due to the high solubility of paclitaxel in this solvent and the minimal effect on the chromatographic performance. Attempts were then made to determine column capacity. With the initial solvent system chosen for scale-up (ethanol/hexane, 17/83), it was quickly determined that capacity was limited by paclitaxel solubility in the mobile phase. At capacities higher than 0.6 mg paclitaxel/ml of the packed bed volume (i.e., 2 mg injection on a 4.0×250 mm column), severe peak asymmetry was observed and this had a very harsh impact on the peak resolution. Attempts were made to find an organic modifier for the mobile phase which would strongly improve paclitaxel solubility and maintain chromatographic resolution as well. Unfortunately, all organic modifiers which improved paclitaxel solubility (methylene chloride, methyl-t-butyl ether, trichloro ethylene) also had an effect of reducing chromatographic resolution compared to the ethanol/hexane system. The mobile phase chosen for scale-up (ethanol/methylene chloride/hexane, 1/40/59) represents the best compromise between sample capacity and resolution. With this system, a capacity of 3.8 mg paclitaxel/ml of column volume (i.e., 12 mg on a 4.0×250 mm column) could be achieved before solubility limitations were shown in the peak skewing.

Due to lack of available starting material (i.e., paclitaxel purified through the stage 1 HPLC system), it was not possible to conduct an extensive column lifetime study. System suitability tests were conducted by making a series of injections to HPLC system 1 with paclitaxel material (paclitaxel purity: 81%) and collecting the paclitaxel peak. The injections were made at a sample load of 8 mg (column: 4.0×250 mm) with a total run time of 20 min. No column regeneration was completed between injections. The material obtained met product specifications and showed consistent impurity profiles for each injection cycle.

Table 5. Results of the HPLC analysis of recovered paclitaxel from stage 1 and stage 2 HPLC.

Contents	Stage 1	Stage 2
Paclitaxel content	81.0% (w/w)	99.5%
Cephalomannine	3.2%	<0.1%
Unknown B	0.9%	<0.1%
10-Deacetyltaxol	1.3%	<0.1%
Unknown A	<1.0%	-

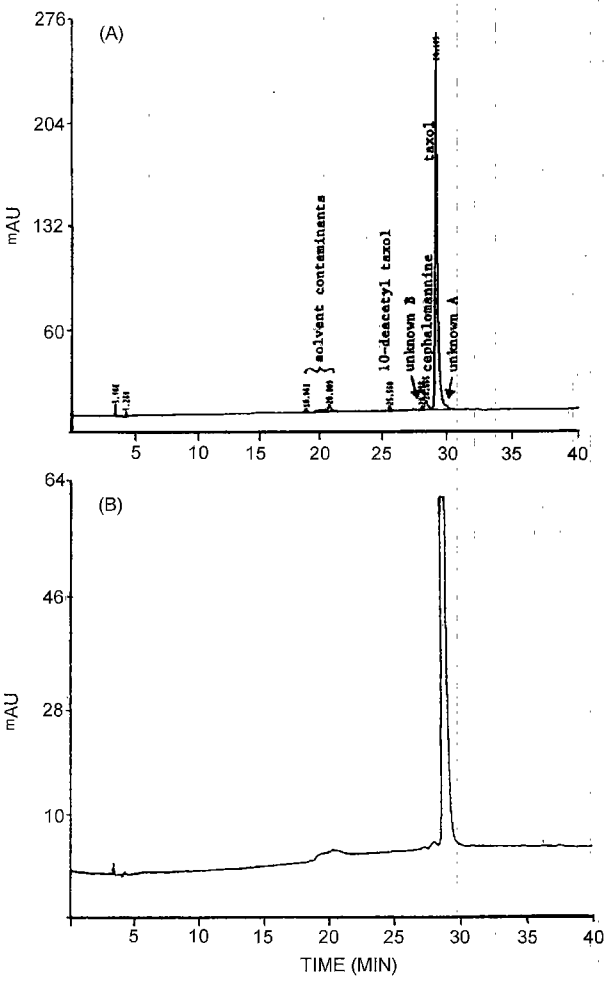

Fig. 3. HPLC chromatograms of paclitaxel recovered after stage 1 and stage 2 preparative HPLC.

Analytical characteristics of the paclitaxel obtained after stage 1 and stage 2 HPLC are shown in Table 5 and Fig. 3. As discussed previously, the final product met the specifications for purified paclitaxel. The intermediate product (after stage 1 HPLC) had an overall paclitaxel purity of 81% with a cephalomannine content of 3.2% and two additional impurities which were detected by analytical HPLC at an estimated total concentration of 2–3%. The two-stage HPLC purification scheme serves as an effective and economical approach for resolving paclitaxel from complex mixtures of taxoids. The process is readily scalable to a pilot plant and eventually to a production environment where multikilogram quantities of material are expected to be produced. As much as possible, the process has been optimized to minimize solvent usage, complexity, and operating costs.

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