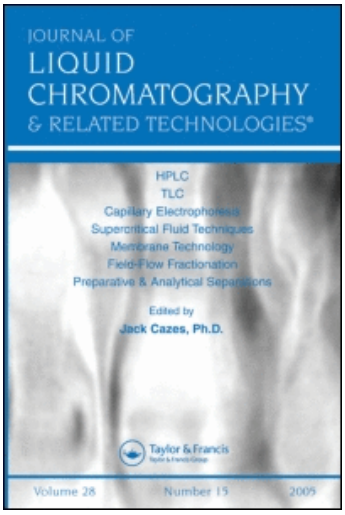

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A Facile Two-Column Chromatographic Process for Efficient Purification of Paclitaxel from Crude Extract

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Abstract: A facile, preparative low-pressure chromatographic process was developed for purification of paclitaxel from crude extract of *Taxus* spp. The process consisted of normal-phase and a reversed-phase chromatography without utilization of any liquid–liquid partition step. The normal-phase chromatography was designed to separate paclitaxel from a majority of unwanted compounds to obtain an initial purification. Silica gel and Al_2O_3 were compared as packing materials for this step. The result demonstrated that basic Al_2O_3 was better than silica gel to give a higher throughput. A further advantage of the Al_2O_3 was its ability of removing 10-deacetyl-7-epi-paclitaxel, a compound difficult to be separated from the paclitaxel in subsequent purification. The chromatographic fraction of Al_2O_3 was purified by a subsequent reversed-phase column chromatography, employing a novel uniform porous microsphere, named PST, as the packing material. Combination of the two steps of column chromatography and one step of crystallization was able to purify paclitaxel, from 0.46% to more than 98% with the total recovery of 71%. This integrated chromatographic procedure was reproducible, efficient, and simple to use.

Keywords: Crude extract of *Taxus* spp., paclitaxel, 10-deacetyl-7-epi-paclitaxel, preparative low-pressure chromatography, purification

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INTRODUCTION

Studies on paclitaxel (Taxol, a registered brand name of Bristol–Myers Squibb) around the world span more than three decades, since it was originally discovered from the bark of *Taxus brevifolia* tree by Wani et al.[1] From then on, paclitaxel, as an exceptionally promising anti-tumor agent, has been approved by the US Food and Drug Administration for the treatment of ovarian cancer (1992), breast cancer (1994), and Kaposi's sarcoma (1997). It also shows some strong indications of efficacy in the treatment of other cancers, including those of the colon and lung, as well as melanoma and lymphoma.[2] Paclitaxel has been recognized as one of the best cancer chemotherapeutic agents from plant secondary metabolites in two decades. Some factors precluding the availability of paclitaxel are its scarcity, low abundance in the tree (only approximately 1 g/10 kg of the bark), and difficulty of purification. As a consequence, total synthesis and semi-synthesis from natural taxoids as well as plant tissue culture have been explored.[3,4]

On the other hand, the methods of isolation and purification of paclitaxel have been described in numerous literatures, even though few of those are directly scalable to commercial operation. Most of the procedures involve multi-steps of liquid–liquid partitioning, low-resolution chromatography, and preparative high-performance liquid chromatography (HPLC). A report by Cardellina, II[5] described a three-step partition protocol using hexane, carbon tetrachloride, and chloroform, giving a recovery of 70% paclitaxel from *Taxus brevifolia* bark. As with the approach taken by Castor et al.,[6] excessive solvent usage and low recovery make the process tedious and impractical for scale-up.

In the subsequent preparative chromatographic procedures, commercially available media such as bonded silica with cyano-, phenyl-, octadecyl-, and so on, suffer from insufficient selectivity among closely eluting analogues. As a result, several steps of low-resolution chromatography or recycle chromatography were employed to achieve the desired purification.[7,8] In order to remove the closely related taxane and get a high purity of paclitaxel, HPLC with a proprietary bonded material is necessary and effective.[9,10] However, these columns suffer from low loadability, column plugging and deterioration, high-pressure operation, and costly scale-up.[11]

Therefore, the objective of this study was to develop a facile and effective chromatographic process for purification of paclitaxel from the crude extract of *Taxus* spp. in order to eliminate the hazardous solvents and the expense associated with high-pressure equipment. The process consisted of two steps of preparative column chromatography. One was a normal-phase column (NPC) chromatography with high loadability and limited resolution. The other was reversed-phase column (RPC) chromatography with high resolution and speed of separation. The NPC chromatography was employed during the initial purification stage, instead of multi-step

liquid–liquid partition, to enhance the recovery and remove some related taxanes that co-elute with paclitaxel on RPC. The RPC chromatography employed a novel uniform microsphere, named PST, as the packing material, to ensure an improved resolution and a higher recovery. Both columns were able to run at low-operating pressure and high flow rate to ensure an economical process.

EXPERIMENTAL

Materials and Reagents

Paclitaxel standard (>98.5%) was purchased from Sigma (MO, USA). The crude extract of *Taxus* spp. (with about 0.46% content of paclitaxel) and 10-deacetyl-7-epi-paclitaxel standard (>98%) were cordially donated by Hande Inc. (Yunnan, China). Basic Al_2O_3 chromatographic supports (50–70 μm) were supplied by Shanghai Chemical Works (Shanghai, China). Silica gel (50–70 μm) was purchased from Qingdao Ocean Chemical Works (Qingdao, China). HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific (Leics, UK). HPLC grade water was prepared by Millipore Milli-RO 3 PLUS module coupled to a Milli-Q RG system (Millipore, Bedford, USA). All other chemicals were of analytical grade.

Apparatus

All chromatographic runs were carried out on an ÄKTA Purifier 100 chromatography system (GE Healthcare, Uppsala, Sweden), composed of a model P-900 pump, a model UV-900 UV-monitor, UNICORN™ software system control, and Type C chromatographic system (GE Healthcare, Uppsala, Sweden). The rotary vacuum evaporator was obtained from Kelong Company (Beijing, China). The HPLC system was Agilent 1100 equipped with a binary pump, an online vacuum degasser, a diode-array detector, an auto-sampler, and a thermostatted column compartment under Agilent ChemStation software (Agilent Technologies, USA). The HPLC Curosil-PFP column (250 mm × 4.5 mm ID, 5 μm) was obtained from Phenomenex (Torrance, USA). ESI-MS was performed on a Finnigan LCQ DecaXP ion-trap mass spectrometry (Thermo Finnigan, San Jose, CA, USA). HR16/10 column (100 mm × 16 mm ID) was obtained from GE Healthcare (Uppsala, Sweden). The glass column (350 mm × 25 mm ID), used in normal-phase chromatography, was purchased from Beijing Chemical Reagents Company (Beijing, China).

Preparation of PST

The PST microsphere was prepared by combining a microporous glass membrane emulsification technique and suspension polymerization process. A mixture of styrene monomer, divinyl benzene crosslinker, diluent (dodecyl alcohol and heptane), and dissolved benzoyl peroxide initiator was used as the dispersed phase (oil phase), and water containing dissolved poly-(vinyl alcohol) stabilizer, was used as the continuous phase (aqueous phase). The oil phase was continuously forced by nitrogen gas pressure through the porous membrane into the aqueous phase to form the uniform droplet. The simple membrane emulsification system was shown in Figure 1. The detailed membrane emulsification process was available elsewhere.[12]

The emulsion obtained was transferred to a four-neck glass separatory flask equipped with a semicircular anchor-type blade, a condenser, and a nitrogen inlet nozzle. After the emulsion was purged with nitrogen gas for 1 hr, the nozzle was lifted above the surface of the emulsion and the temperature was increased to 75°C for the polymerization. The polymerization was carried out for 10 hr under a nitrogen atmosphere.

After polymerization, polymer microspheres were removed from the suspension by centrifugation, washed with ethyl alcohol, and dried under vacuum.

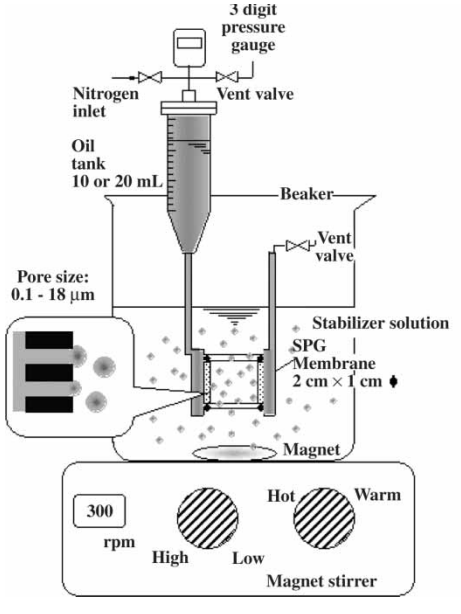

Figure 1. Simple membrane emulsification system.

NPC and RPC Chromatography Purification

Basic Al_2O_3 Adsorption Chromatography

The activated basic Al_2O_3 was packed in a 250 mm × 25 mm ID column as slurry in chloroform and equilibrated with chloroform. A 2.0 g crude extraction was dissolved in the appropriate amount of chloroform and then loaded onto the column, followed by chloroform wash to remove non-absorbed impurities at flow-rate 3 CVs/hr. Then, isocratic elution was performed with chloroform/methanol (97 : 3, v/v) at 3 CVs/hr. The eluent was detected at UV 227 nm. The collected fraction of paclitaxel was evaporated with a rotary vacuum evaporator at 40–45°C. The residue was re-suspended in methanol for HPLC assay.

Silica Gel Adsorption Chromatography

The silica gel was packed in a 250 mm × 25 mm ID column as slurry in chloroform and equilibrated with chloroform. A 2.0 g crude extraction was dissolved in an appropriate amount of chloroform and then loaded onto the column, followed by chloroform wash to remove non-absorbed impurities at flow-rate 1.2 CVs/hr. Then, isocratic elution was performed with chloroform/methanol (96:4, v/v) at 1.2 CVs/hr. The eluent was detected at UV 227 nm. The collected fraction of paclitaxel was evaporated with a rotary vacuum evaporator at 40–45°C. The residue was re-suspended in methanol for HPLC assay.

PST Reversed-Phased Chromatography

A 90 mm × 16 mm ID column was slurry packed with PST medium and was washed with a sufficient amount of methanol. The column performed the chromatographic work on the ÄKTA Purifier 100 workstation. After washing, the column was equilibrated with 70% methanol aqueous wash solvent, a 6.3 mg paclitaxel fraction of initial purification was dissolved in a small amount of the wash solvent, and then injected into the column. First, the column was washed with enough washing solvent at 17 CVs/hr. Then, isocratic elution was performed with 80% aqueous methanol at about 10 CVs/hr and the elution fractions of the desired product were collected with UV 270 nm monitoring. The collected fraction of paclitaxel was filtered for HPLC assay. Finally, the used column was regenerated with a sufficient amount of methanol.

Crystallization of Paclitaxel

Fractions of the target product from the PST reversed-phase chromatography were crystallized out directly. The fractions were allowed to stand at the

ambient temperature for about 8 hr and the needle crystals of paclitaxel were collected. Then the crystals were re-suspended in methanol and filtered for HPLC assay and NMR structure determination.

Analytical Method

HPLC Analysis

In this study, HPLC was used to analyze the collected fractions from the purification steps. A Curosil-PFP column and a gradient elution were used in this assay. The method employed a linear gradient from 40/60 ACN/water (v/v) at the start time to 52/48 ACN/water (v/v) in 25 min followed by a 5 min hold time, then followed by return to the starting composition and equilibrated for 10 min prior to the next injection. Injection volumes were 20 μL sample and effluent was monitored at 227 nm with a photo diode-array detector. The column was maintained at ambient temperature. Paclitaxel fractions were quantitated by comparing the average peak response of the sample to that of the standard. Paclitaxel and 10-deacetyl-7-epi-paclitaxel were identified by their relative retention time and, in some cases, by spiking with the reference standard.

Structure Determination

The collected fraction from silica gel chromatography corresponding to 10-deacetyl-7-epi-paclitaxel was directly injected to the mass spectrometer. A spray voltage of 4.5 kV was employed and the temperature of the heated transfer capillary was set to 275°C. The mass spectrometer was scanned from $m/z = 500$ to 1000 in positive ion mode.

The ^{1}H and ^{13}C NMR spectra were recorded at 20°C in $CDCl_3$ using Bruker DPX300 spectrometer.

RESULTS AND DISCUSSION

Initial NP Chromatography

In the crude extract of *Taxus* spp., a large amount of non-polar components and polar impurities including many taxanes were found. In order to obtain high-purity paclitaxel with chromatography methods, adsorption onto silica gel or Al_2O_3 was found to be very useful to initially clean up the very crude feed in natural product preparative isolation.

Figure 2 shows the profile of Al_2O_3 chromatography to isolate paclitaxel from the crude extract of *Taxus* spp. The fractions corresponding to paclitaxel

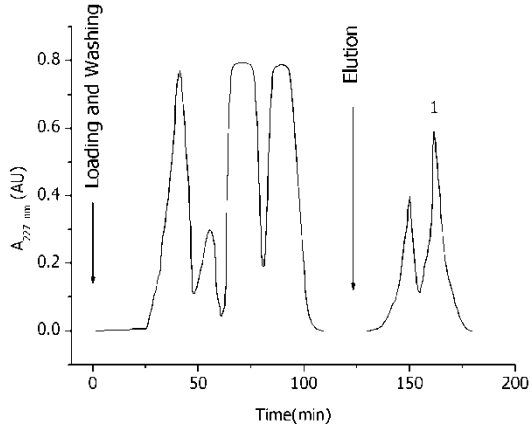

Figure 2. The profile of chromatographic purification of paclitaxel from crude extract by Al_2O_3 column. Chromatographic condition: column size (250 mm × 25 mm ID), sample loading: 2 g, washing solvent: $CHCl_3$, eluant: 3/97 $CH_3OH/CHCl_3$ (v/v), washing and elution rate: 3 CVs/hr, UV 227 nm. Peak 1 represented paclitaxel.

were analyzed by HPLC, the result of which is shown in Figure 4. It can be seen from this figure that paclitaxel could be isolated from many related compounds in the crude extract, which included a large quantity of impurities such as oils, waxes, and other highly hydrophobic components. Owing to their higher hydrophobicities, these impurities were eluted before the paclitaxel fraction. Paclitaxel of greater than 24% purity could be obtained after this step of Al_2O_3 chromatography, with a recovery of more than 97%. The purification factor was about 53.

Figure 3 showed the chromatographic result using another medium, silica gel. The fraction containing paclitaxel was analyzed by HPLC, the result of which is shown in Figure 4, with the HPLC assay result of Al_2O_3 chromatography. The comparison of HPLC assay results demonstrated that the silica gel chromatography gave a purity of 19%, much less than that obtained on the Al_2O_3 column and the purification factor of 42, less than the Al_2O_3 chromatography, although this recovery was almost similar. Furthermore, the Al_2O_3 chromatography took much less time to complete than the silica chromatography, as shown in a shorter period of elution in Figure 2, than that in Figure 3 for the same amount of crude extract separation.

An important feature of Al_2O_3 chromatography, however, was its ability to remove a fraction of impurity, named 10-deacetyl-7-epi-paclitaxel, from paclitaxel, thus facilitating the further purification process. One of the major challenges associated with paclitaxel recovery and purification from crude extract was the separation of paclitaxel from structurally similar taxanes, including cephalomannine and 10-deacetyl-7-epi-paclitaxel.

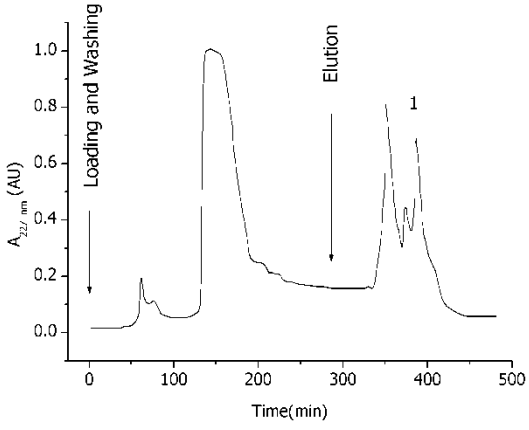

Figure 3. The profile of chromatographic purification of paclitaxel from crude extract by silica gel column. Chromatographic conditions: column size (250 mm × 25 mm ID), sample loading: 2 g, washing solvent: $CHCl_3$, eluant: 4/96 $CH_3OH/CHCl_3$ (v/v), washing and elution rate: 1.2 CVs/hr, UV 227 nm. Peak 1 represented paclitaxel.

Several methods of removal of cephalomannine have been reported,[5,13,14] but none of the published methods removes another closely eluting taxane, named 10-deacetyl-7-epi-paclitaxel, from the desired fraction, other than HPLC with a proprietary packing material. According to Steven et al.,[15] 10-deacetyl-7-epi-paclitaxel was the impurity most difficult to be removed

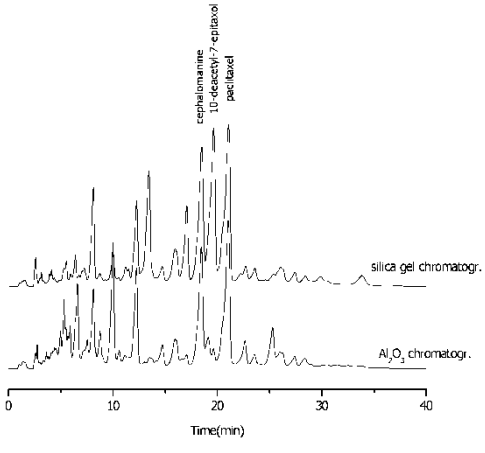

Figure 4. HPLC analysis of chromatographic fractions corresponding to peak 1 in Figures 2 and 3. Chromatographic conditions: Curosil PFP column (250 mm × 4.6 mm, ID 5 μm), with a linear gradient from 40/60 ACN/water (v/v) at the start time to 52/48 ACN/water (v/v) in 25 min followed by a 5 min hold time.

from paclitaxel. In our result, as in Figure 4, the fraction from the silica column contained a peak of 10-deacetyl-7-epi-paclitaxel. However, the peak of 10-deacetyl-7-epi-paclitaxel almost disappeared in the fraction from Al_2O_3 chromatography. To further verify the peak of 10-deacetyl-7-epi-paclitaxel, the peak fraction was collected and submitted to ESI-MS analysis. The ESI mass spectrum was shown in Figure 5. The ESI mass spectrum of 10-deacetyl-7-epi-paclitaxel were identical to those previously observed by Blay et al.[16]

The removal of 10-deacetyl-7-epi-paclitaxel in the initial separation step by Al_2O_3 column chromatography was of great benefit. Normal-phase Al_2O_3 chromatography is a relatively cheap process, offering several advantages such as the easily obtained low-cost chromatography medium- and low-operating pressure, ensuring a fast separation. The crude extract of *Taxus* spp. was difficult to be dissolved in solvents for reversed-phase chromatography but was highly soluble in the solvent of normal-phase chromatography. The comparison of the two normal-phase chromatography columns revealed the advantage of Al_2O_3 over the silica, a phenomenon seldom reported in the literature. As a result, Al_2O_3 chromatography was chosen for initial separation of paclitaxel from the crude extract of *Taxus* spp.

Subsequent RP Chromatography

A large amount of less polar impurities were removed during the initial Al_2O_3 chromatographic separation, but taxane analogues, which were more polar than paclitaxel with structures similar to paclitaxel, still existed and were difficult to remove on the NPC. In order to improve the purification resolution

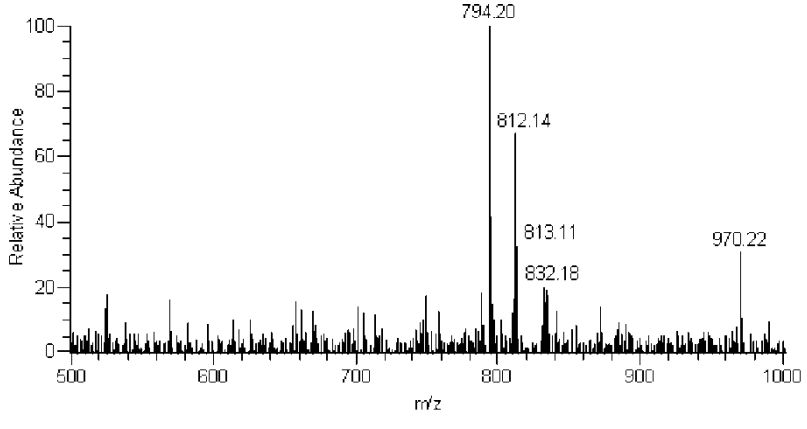

Figure 5. ESI-MS spectrum of the peak fraction of 10-deacetyl-7-epi-paclitaxel in Figure 4.

and the recovery, we developed another low-pressure chromatography process using a novel porous medium. The PST microsphere is a kind of styrene-divinylbenzene polymer resin without extra bonded groups, which was prepared by combining a microporous glass membrane emulsification technique and suspension polymerization process. Due mainly to its larger particle size than conventional HPLC media and uniform size distribution with a large specific surface, the PST column could be run at a pressure much lower than that of HPLC, yet giving a high resolution and speed of separation.

The profile of PST chromatography is shown in Figure 6 and the operation pressure did not exceed 0.05 MPa. The HPLC results showed that a paclitaxel content of 90.6% was obtainable from the initial purified fraction of the previous Al_2O_3 column. The recovery of paclitaxel exceeded 85% with the purification factor of 3.7. The profile of HPLC analysis was shown in Figure 7.

Total Results of Paclitaxel Purification

There were minor impurities left in the purified fraction of paclitaxel after the two chromatographic steps. Therefore, crystallization of paclitaxel from the fraction directly was performed. The final purity of paclitaxel was more than 98% after the single step of crystallization, with the recovery of 86%. Table 1 shows the total result of paclitaxel purification.

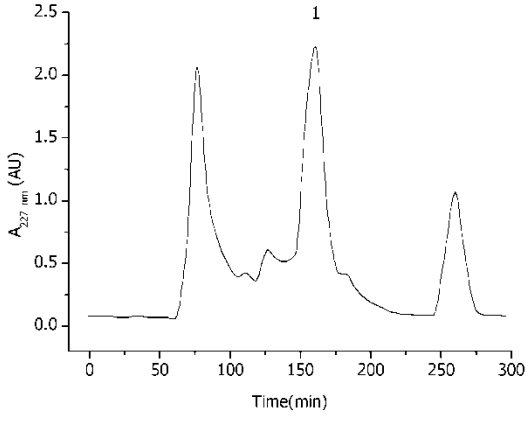

Figure 6. The profile of chromatographic purification of paclitaxel from initial chromatography of Al_2O_3 column by PST RPC chromatography. Chromatographic conditions: column size (90 mm × 16 mm ID), sample loading: 6.3 mg, washing solvent: 70% aqueous methanol, eluant: 80% aqueous methanol, washing flow-rate:17 CVs/hr, elution flow-rate:10 CVs/hr, UV 227 nm. Peak 1 represented paclitaxel.

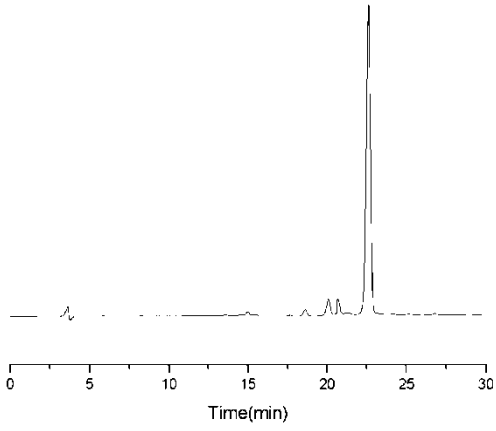

Figure 7. An HPLC analysis of the PST column chromatographic fractions corresponding to peak 1 in Figure 6. Chromatographic conditions as in Figure 4.

This process, consisting of the two chromatographic steps and one crystallization step, was reproducible, effective, and simple to operate without using any liquid–liquid partitioning method. Paclitaxel was purified from the initial 0.46% in the crude extract, to more than 98% in the final product, with the total recovery of 71%.

The absorbance spectrum of purified paclitaxel exhibited an absorption peak at 227 nm, which corresponds to the standard paclitaxel. The structure of the obtained paclitaxel was confirmed by NMR spectroscopy. These data were in agreement to those reported in the literature.[17]

CONCLUSION

In the present integrated chromatographic procedure, the initial Al_2O_3 normal-phase chromatography removed a large amount of impurities from the crude extract. Meanwhile, it also eliminated a taxane analogue, 10-deacetyl-7-epi-paclitaxel,

Table 1. The total result of paclitaxel purification

Steps	Total (mg)	Paclitaxel (mg)	Purity (%)	Recovery (%)
Crude extract	2,000	9.2	0.46	100
Al_2O_3 chromatography	36.7	8.93	24.3	97
RP chromatography	8.4	7.6	90.6	82.5
Crystallization	6.63	6.5	98	71

which was difficult to separate from the paclitaxel fraction by conventional methods. The initial NPC chromatography was beneficial for the subsequent RPC chromatography in this procedure. The novel polymer-based PST microsphere was an efficient RPC medium, providing excellent selectivity at low-operating pressure with high flow-rate. The fraction of PST column, eluted with 80% aqueous methanol, could be directly crystallized to give a purity of more than 98% and a total recovery of 71% for the whole purification process.

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