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Efficient purification of paclitaxel from yews using high-performance displacement chromatography technique

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ABSTRACT

Paclitaxel was purified using high-performance displacement chromatography (HPDC) technique, but not by the mechanism of HPDC. On small scale, paclitaxel was extracted with methanol from dry needles of *Taxus canadensis* and was enriched by extracting with chloroform after removing water-soluble hydrophilic components and hexane-soluble hydrophobic components. Then, 93–99% purity of paclitaxel was obtained using the HPDC technique. On large scale, taxanes were enriched by solvent partitioning between acetic acid/MeOH/H_2O and hexane and extracted with CH_2Cl_2. Taxanes except paclitaxel were further removed by extracting with methanol–water–trifluoroacetic acid (1.0:98.9:0.1, v/v/v). Applying HPDC technique to water-insoluble substances is problematic as this method requires a highly aqueous solvent system. In order to overcome this incompatibility, a system was set up where paclitaxel, although in low concentration, was extracted by methanol–water–trifluoroacetic acid (10.0:89.9:0.1, v/v/v). Recycling the extracting solvent to ensure minimal volume, the extracted paclitaxel was adsorbed on a C_{18} trap column. A C_{18} column of 4.6 mm internal diameter was then connected to the trap column. The HPDC technique was thus carried out using an isocratic acetonitrile–water–trifluoroacetic acid (30.0:69.9:0.1, v/v/v) mobile phase consisting of a displacer cetylpyridinium trifluoroacetate (3 mg/mL). Paclitaxel was co-eluted with the displacer and spontaneously crystallized. The crystal (114 mg) showed 99.4% purity and only 10% of paclitaxel in the starting crude extract was lost during the enrichment/purification processes. This large scale purification method was successfully applied to purify paclitaxel from Chinese yew in small scale, suggesting general applicability of the method. This is the first report of purifying a water-insoluble natural product using HPDC technique.

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1. Introduction

Yew trees, taxonomically classified under the genus *Taxus* (the family Taxaceae), represent an important source of biologically active secondary metabolites, especially tetracyclic taxane diterpenoids or taxoids. Paclitaxel (taxol A, taxol®), a highly oxygenated taxoid amide, originally isolated in 1971 from the bark of the Pacific yew tree *Taxus brevifolia* [1], is a powerful therapeutic drug for cancer chemotherapy [2]. Particularly, this molecule and its synthetic analogue docetaxel (Taxotere) [3,4] exhibit strong antitumor activity against ovarian and breast cancers [5,6] and additional exciting clinical uses are anticipated [7–10]. The chemical structures of paclitaxel and docetaxel are shown in Fig. 1. Over the past two decades, important biological activity, limited supplies, and unique structural framework of paclitaxel have attracted the attention of biologists, medicinal chemists, and synthetic chemists.

Worldwide efforts had been made to overcome the supply problem, due to the supply by isolation from the bark of the slow-growing yew trees is limited <0.01 wt% of paclitaxel [11,12]. Numerous studies have shown that paclitaxel and related taxanes are found in variety of yews such as *Taxus brevifolia*, *T. baccata*, *T. canadensis*, *T. celebica*, *T. cuspidata*, *T. floridana*, *T. globosa*, and *T. wallichiana*. All these species contain paclitaxel in very limited amounts of 0.004–0.05 wt% of dried needles [13]. The low abundance of paclitaxel makes the production of the drug very costly. Meanwhile, large-scale utilization of yew barks as the primary source of paclitaxel for research and clinical purposes is not environmentally sustainable since bark stripping leads to the

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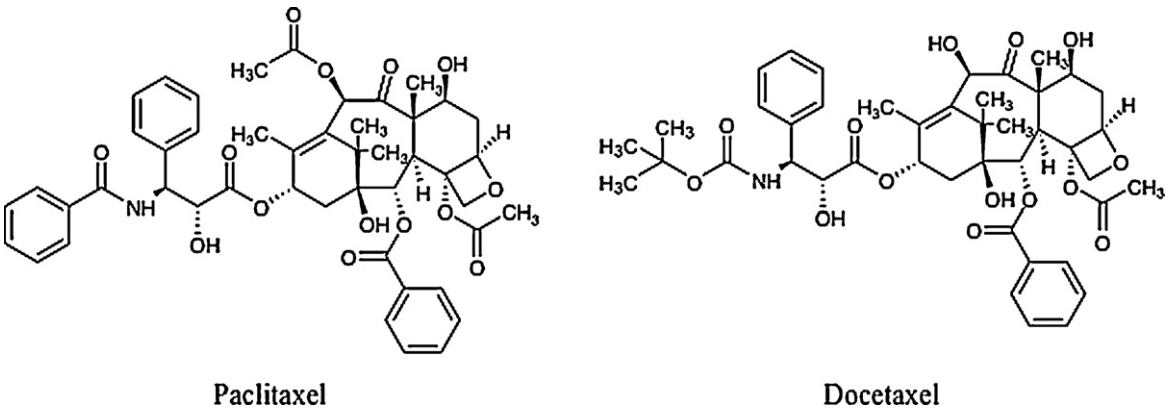

Fig. 1. Chemical structures of paclitaxel (Taxol®) and docetaxel (Taxotere®). The chemical formula of paclitaxel is $C_{47}H_{51}NO_{14}$ (monoisotopic molecular mass = 853.3310 Da). The chemical formula of docetaxel is $C_{43}H_{53}NO_{14}$ (monoisotopic molecular mass = 807.8792 Da).

destruction of scarce plant material. As a result, over the past decades, renewable biomass of yew such as needles, twigs, and/or stems has been recognized as a valuable source of paclitaxel and its several semi-synthetic precursors having the same core skeleton. Numerous attempts to develop an economical and reliable alternative method for paclitaxel and docetaxel production have also been made, including chemical and semisynthesis [14,15], total-synthesis [16,17], and the cell culture [18–20].

Traditional processes of isolation and purification of paclitaxel from yew tree and plant cell cultures involve generally multiple steps of liquid–liquid partitioning, enrichment, purification by liquid chromatography, such as; low-pressure liquid chromatography [21–24], and preparative high-performance liquid chromatography (HPLC) [25,26], industrial preparative liquid chromatography [27], counter current chromatography [28–30], and simulated moving-bed chromatography [31]. In addition, these separation procedures adopted loose decent quantities of paclitaxel and also are bothered by interfering substances such as cephalomannine and 10-deacetyl-7-*epi*-paclitaxel, which are difficult to separate from paclitaxel [22,29,30]. Therefore, conventional purification methods require several steps of purification with poor recovery where most will be confined mainly to small-scale chromatography, resulting in low selectivity, recovery and high production cost. Even the recent method of converting several taxanes to a common primary amine is still confronted with problems, as after conversion to paclitaxel by benzoylation it undergoes three major purification steps; an extraction and isolation of the crude taxanes from dried powder of yew biomass, further isolation and purification of the primary taxane constituents to over 95% purity, and the conversion of the primary taxanes to paclitaxel followed by the final purification [32].

Recently, we have reported a one-step HPDC purification of two hydrophilic isoquinoline alkaloids palmatine and its derivative *d,l*-tetrahydropalmatine from the crude extract of the root bark of the African anti-malarial medicinal plant *Enantia chlorantha* [33]. Purification of some hydrophilic natural products has been a challenge in natural product chemistry. Normal phase column chromatography is powerful to purify substances that are soluble in organic solvents, but not to purify some hydrophilic compounds. Similarly, reverse phase column chromatography including HPDC faces challenges to purify some hydrophilic compounds due to their low affinity to the nonpolar stationary phase.

Some water-insoluble hydrophobic natural products are another challenge to be purified using reverse phase HPDC technique. The sample dissolved in organic solvents must be injected in a very small volume in comparison to the void volume of the column. This way, the sample's organic solvent would allow the sample to bind to the resin. In our present work, we used the HPDC technique to purify a water-insoluble paclitaxel that was extracted from *T. canadensis*. In contrast to the mechanism of HPDC, in which a displacer elutes out after the elution of the target ingredients [34], paclitaxel was co-eluted with the displacer. After eluting off the column, paclitaxel crystallized spontaneously, while the displacer remained in solution, producing paclitaxel crystals of 99.4% purity.

2. Materials and methods

2.1. Plant material

Paclitaxel was extracted with methanol (MeOH) from dry needles of *T. canadensis*. The crude extract was used for developing the purification methods in small scale. For large scale paclitaxel purification, a crude extract of *T. canadensis* in 50–60% MeOH/H_2O was purchased from Paxis Pharmaceutical Inc. Paclitaxel was enriched to 0.158 mg/mL in the extracted solution based on its HPLC profile. In addition, paclitaxel was also extracted with MeOH from dry needles of cultivated Chinese yew *Taxus media* and purified using the HPDC technique in order to demonstrate the applicability of the method to other yews. The dry needles were supplied by Shaanxi Taxus Co. Ltd., China.

2.2. Reagents

HPLC-grade trifluoroacetic acid (TFA), acetonitrile, MeOH, hexane, CH_2Cl_2, ammonium hydroxide, acetic acid and water were purchased from Baker (Phillipsburgh, NJ, USA). Cetylpyridinium chloride was supplied by Sigma–Aldrich (St. Louis, MO, USA). Other solvents used were of analytical grade. Solvents used for the HPDC techniques and HPLC were filtered through 0.22-μm Millipore filter and degassed by sparging with helium.

2.3. Apparatus

The HPDC techniques were carried out using a Waters HPLC Millennium system [33], which is composed of two Model 303 pumps for the carrier and displacer solution, a stirrer (Caframo, BDC 3030 Model) and a heating Fisher Scientific Isotemp apparatus, a Waters 2996 photodiode array detector (210–400 nm), and a Waters Fraction Collector II (Waters, Milford, MA, USA). A Shiseido Capcell Pak C_{18} (AQ S-5 μm) column (250 mm × 4.6 mm) was used in the HPDC technique on both small and large scale purifications. A Shiseido C_{18} AQ column (50 mm × 20 mm, 20 μm) was used as a trap column on large scale purification. Both Shiseido columns were purchased from JM Science, Grand Island, NY, USA.

The purity of paclitaxel in the collected fractions or the crude extract of *T. canadensis* was analyzed by a Waters liquid chromatography system, which is composed of a Waters 717 autosampler, a Waters 600 model pump, and a Waters 996 photodiode array detector (200–500 nm). The analyses were carried out with a Waters Symmetry Shield RP C_{18} column (50 mm × 4.6 mm, 3.5 μm) from Waters protected by a Waters Symmetry Shield C_{18} sentry guard column (20 mm × 3.9 mm, 3.5 μm) at room temperature. Instrument management and data acquisition were performed using Waters Empower software. The mobile phase consisted of solvent A (0.1%, v/v, TFA in H_2O) and solvent B (0.1%, v/v, TFA in acetonitrile) at a flow-rate of 2 mL/min and a gradient of acetonitrile concentration from 0 to 90% in 10 or 12 min.

The nuclear magnetic resonance (NMR) spectra were measured in $CDCl_3$ at 27 °C on a Bruker Avance 500 (Bruker, Karlsruhe, Germany) spectrometer operating at 500 MHz (^1H) with $CDCl_3$ solvent as internal reference. High resolution electrospray ionization mass spectrometry (ESI–MS/MS) was performed on a Micromass Waters Q-TOF Global mass spectrometer (Micromass, Manchester, UK), a hybrid quadrupole time-of-flight mass spectrometer equipped with a NanoLockSpray Source for accurate mass determination and a NanoFlow™ ESI ion source operated in positive ion mode (+ESI). An explosion-proof IEC Centrifuge, Model EXD was used to collect the taxane precipitates once the hydrophilic and hydrophobic components were removed.

2.4. Purification of paclitaxel from T. canadensis on small scale

The dry needles (10 g) of *T. canadensis* were ground to powder using a Waring blender. Paclitaxel was extracted from the powder with 500 mL (5 × 100 mL) of MeOH. The methanol extracts were combined and concentrated down to 2.5 mL. Addition of H_2O (47.5 mL) to the methanol extract precipitated paclitaxel, while the solvent containing water-soluble hydrophilic components was discarded. The precipitate was washed with hexane 3-times to remove hexane-soluble hydrophobic components (Hexane was removed by centrifugation) and paclitaxel was extracted from the remaining precipitate with chloroform (3 × 50 mL). The chloroform extracts were combined and concentrated under nitrogen. The residue (100 mg), in which paclitaxel was enriched, was dissolved in 1 mL of acetonitrile–H_2O–TFA (50.0:49.9:0.1, v/v/v) and was injected onto a Shiseido Capcell Pak C_{18} (AQ S-5 μm) column (250 mm × 4.6 mm) equilibrated with 0.1% TFA/H_2O. Then, cetylpyridinium·TFA (6 mg/mL in acetonitrile–H_2O–TFA (40.0:59.9:0.1, v/v/v) was applied to the column at a flow rate of 0.5 mL/min. The eluent was collected every minute and analyzed by an analytical HPLC.

2.5. Preparation of T. canadensis extract and enrichment of taxanes on large scale

Extraction of 100 mg of paclitaxel from *T. canadensis* (285 μg/g dried needles) [13] required over 350 g of dried needles. Therefore, for practicality reason, instead of starting from dried needles of *T. canadensis*, the paclitaxel extract in 50–60% MeOH/H_2O was purchased from Paxis Pharmaceutical Inc. for large scale purification. The extract (800 mL) containing 126 mg of paclitaxel was mixed with 100 mL of glacial acetic acid and 400 mL of saturated NaCl/H_2O, and was partitioned with hexane (400 mL). The hexane layer, which contained most of the chlorophylls and nonpolar components, was discarded. The acetic acid/MeOH/H_2O layer contained taxanes and was filtered for use in the next step. The layer between hexane and aqueous MeOH–acetic acid contained some amount of the desired materials and was thus extracted with MeOH/H_2O/acetic acid (47.5:47.5:5.0, v/v/v; 300 mL) three times. The solutions containing taxanes were then combined. Taxanes were extracted by solvent–solvent partitioning with CH_2Cl_2 (3 × 600 mL). The CH_2Cl_2 solution was further treated with ammonium hydroxide (100 mL), filtered and dried over Na_2SO_4. The resulting extract (oil) was dissolved in glacial acetic acid (50 mL). Hexane (450 mL) was added to precipitate out the taxanes. After filtration, taxanes were redissolved in CH_2Cl_2 (100 mL), followed by another filtration. Hexane (900 mL) was added once more to the CH_2Cl_2 solution to re-precipitate the taxanes, which were further dried over Na_2SO_4, giving a yield of 1.5 g of the taxane-enriched material.

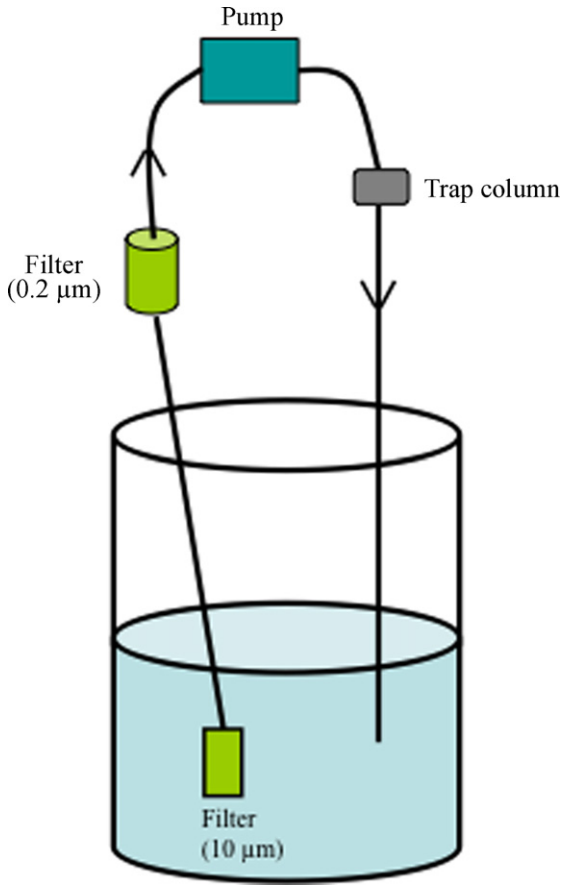

Fig. 2. A schematic diagram to extract and trap hydrophilic taxanes and paclitaxel. The enriched hydrophilic taxanes or paclitaxel residues are slowly extracted in MeOH/H_2O/TFA (1.0:98.9:0.1% or 10.0:89.9:0.1%, v/v/v, respectively) and trapped in the trap column. After the trap column, the extracting solvent is recycled. Two filters protect the pump and the trap column.

2.6. Extraction of taxanes excluding paclitaxel on large scale

The taxane-enriched material (1.5 g) was dissolved in MeOH (100 mL) in the presence of celite (90 g) and was dried on the surface of celite with a rotary evaporator. This maximized the surface area of the taxanes for extraction. Here, we define hydrophilic taxanes as those that specifically exclude paclitaxel compared with overall taxanes that include paclitaxel. Hydrophilic taxanes were extracted with MeOH/H_2O/TFA (1.0:98.9:0.1, v/v/v; 1000 mL) while stirring at 35 °C. This solution was then passed through a C_{18} reverse-phase Vydac column (250 mm × 20 mm) at a continuous 10 mL/min flow-rate trapping the taxanes. The solvent composition was aqueous enough to trap hydrophilic taxanes in the reverse phase column and also enough to extract hydrophilic taxanes at low concentrations. The solvent consumption was minimized by recycling the solvent after trapping the extracted hydrophilic taxanes in the trap column (Fig. 2). Most of hydrophilic taxanes were extracted in 6–7 h. Two filters were used to prevent the celite-taxane suspension from

entering into the pump and the trap column. The fist was a 10 μm solvent inlet filter, which removes most of the celite-taxane suspension and can handle the flow rate of 10 mL/min. The second filter is a 0.2 μm Whatman Schleicher & Schuell, Pplycap^{TM} AS, Aqueous solution filter device, Polycap 36 AS which was in place to remove a residual amount of the fine precipitates of hydrophilic taxanes that were produced upon stirring. Its large 400 cm^2 filtration area easily handled the 10 mL/min flow rate.

2.7. Purification of paclitaxel on large scale

After the extraction of hydrophilic taxanes, paclitaxel in the remaining residue was dissolved in MeOH (100 mL) in the presence of celite (90 g) and was dried on the surface of celite with a rotary evaporator. This maximizes the surface area of paclitaxel to be extracted. Paclitaxel was extracted with 1000 mL of MeOH–H_2O–TFA (10.0:89.9:0.1, v/v/v) while stirring at 35 °C, and was trapped onto a Shiseido C_{18} AQ column (50 mm × 20 mm, 20 μm) protected by a Waters RP_{18} guard column (10 mm × 19 mm, 10 μm) at continuous 10 mL/min flow-rate. The solvent composition was aqueous enough to trap paclitaxel in the reverse phase column and also enough to extract paclitaxel at low concentration. The columns were pre-equilibrated with MeOH/H_2O/TFA (10.0:89.9:0.1, v/v/v). The solvent consumption was minimized by recycling the solvent after trapping the extracted paclitaxel in the trap column (Fig. 2). Paclitaxel was quantitatively extracted in 72 h. The pump and the trap column were protected by the same two filters (10 μm and 0.2 μm pore sizes) as described above.

After extraction and trapping of paclitaxel, a Shiseido Capcell Pak C_{18} (AQ S-5 μm) column (250 mm × 4.6 mm) pre-equilibrated with the mobile phase acetonitrile/H_2O/TFA (30.0:69.9:0.1, v/v/v) was connected to the trap column. Then, cetylpyridinium·TFA (3 mg/mL in acetonitrile/H_2O/TFA (30.0:69.9:0.1, v/v/v)) was applied to the columns at a flow rate of 1.0 mL/min. The eluent was fractionally collected every 20 min. The columns were regenerated by washing with MeOH/acetonitrile/H_2O (1:1:1, v/v/v), followed by another washing with acetonitrile/H_2O/TFA (95.0:4.9:0.1, v/v/v). The guard and trap columns were then re-equilibrated with MeOH/H_2O/TFA (10.0:89.9:0.1, v/v/v), and the column (250 mm × 4.6 mm) was re-equilibrated with acetonitrile/H_2O/TFA (30.0:69.9:0.1, v/v/v).

3. Results and discussion

3.1. Purification of paclitaxel on small scale

Fig. 3 shows the HPLC chromatogram of the crude extract with detection at 227 nm.

Paclitaxel was then enriched by removing the hydrophilic and hydrophobic components, followed by extraction with chloroform. Fig. 4A shows the HPLC chromatogram of the enriched paclitaxel with detection at 227 nm. The HPDC technique was applied to 100 mg of the enriched paclitaxel dissolved in 0.5 mL acetonitrile as described in Section 2. Fig. 4B shows the chromatogram at 227 nm. Due to the high concentration of acetonitrile (40%) the majority of the components, which were more hydrophilic than paclitaxel, eluted after the injection peak and conveniently before cetylpyridinium·TFA eluted. Paclitaxel was distinct from those hydrophilic components as paclitaxel itself was co-eluted with the displacer at around 80 min. The fraction at 80 min, after drying and re-dissolving in 100 μL of acetonitrile was analyzed (10 μL injection) by an analytical HPLC (Fig. 4C) and showed that paclitaxel was highly enriched. Fortunately, shortly after eluting off the column, paclitaxel spontaneously crystallized whereas the displacer and the minor impurities remained in solution. The paclitaxel crystals were 93–99% pure based on the HPLC chromatogram with detection at 227 nm (Fig. 4D). In HPDC, the displacer must elute after paclitaxel. In fact, paclitaxel eluted off the column before the displacer cetylpyridinium·TFA when no or low concentrations of acetonitrile were in the mobile phase; however, the tailings of the trains of the impurities, which are more hydrophilic than paclitaxel, overlapped to paclitaxel such that we could not get high purity of paclitaxel. We then discovered that higher concentrations of acetonitrile in the mobile phase improved the purity of paclitaxel, even though it no longer fits to the mechanism of HPDC. Although the scale-up raised a few technical challenges as addressed below, this small scale experiment encouraged us to purify paclitaxel on larger scale.

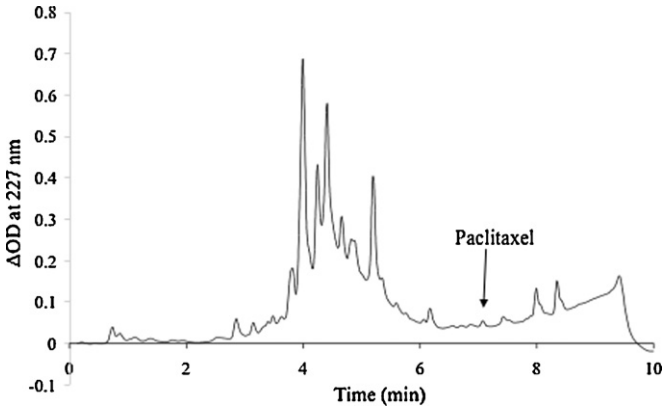

Fig. 3. The HPLC chromatogram of the crude extract with detection at 227 nm (the absorbance maximum of paclitaxel). Taxanes were extracted from dry leaves of *Taxus canadensis* by MeOH. The separation was carried out on Waters Symmetry Shield RP C_{18} column (50 mm × 4.6 mm, 3.5 μm) with acetonitrile gradient in 0.1% TFA from 0 to 90% in 10 min.

3.2. Enrichment of taxanes on large scale

In the previous article [33], we developed a one-step purification of water-soluble palmatine from the crude extract by HPDC. Since palmatine is a major component (~65% of the dry material) in the crude extract of African medicinal plant *E. chlorantha*, we obtained palmatine at >98% purity. The loss of palmatine in HPDC was minimal. Also the high loading capacity of the column in HPDC mode, which is 50–100-fold higher than that of elution mode, allowed us to purify 68 mg of palmatine from 100 mg of the fine dried powder of the crude extract by using a small column (250 mm × 4.6 mm) [33].

The taxanes are distinct from palmatine. First, they have low solubility in water making it difficult for use of HPDC technique, which works in aqueous or highly aqueous mobile phase [35]. Aqueous or highly aqueous mobile phase provides high binding energy between the sample molecules and the reverse phase stationary phase and high sample loading capacity of the column. Due to the low solubility, a large volume of water or highly aqueous solvent system is required to dissolve the taxanes, resulting in a massive sample injection volume onto a small HPLC column. The second distinct difference is that the taxanes are a minor component in the crude extract from any source of yews, limiting the amounts of taxanes to be purified using HPDC technique. Although Paxis Pharmaceutical Inc. enriched paclitaxel by removing some of the hydrophilic components through adding H_2O to the MeOH extract, paclitaxel was still a minor component as shown in the HPLC chromatogram of the company's product (Fig. 5A). This problem was bypassed by enriching the taxanes through the removals of water- and hexane-soluble components and by further enriching them with CH_2Cl_2 extraction as described

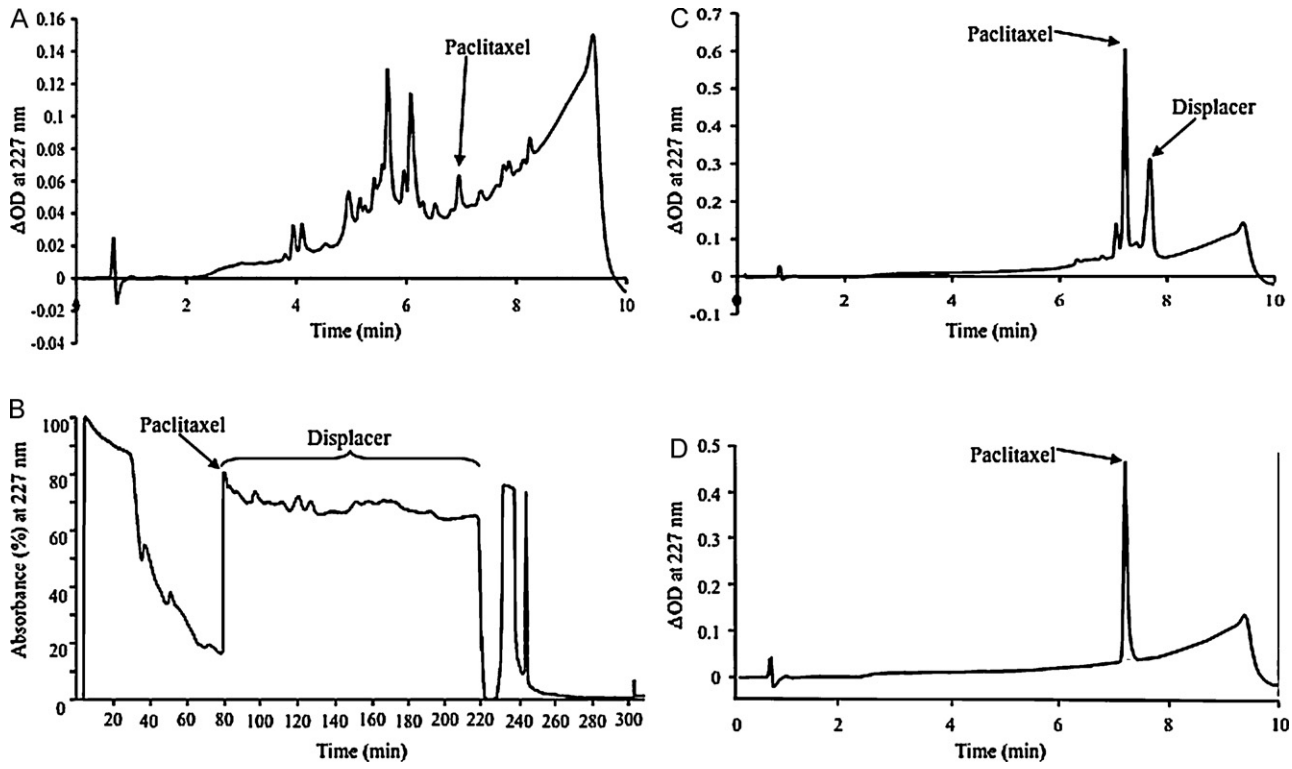

Fig. 4. (A) The HPLC chromatogram of the enriched taxanes with detection at 227 nm. Taxanes were enriched by removing hydrophilic and hydrophobic components with water and hexane, respectively, followed by extraction with chloroform. (B) The chromatogram with detection at 227 nm using the HPDC technique. The HPDC technique (cetylpyridinium·TFA displacer (6 mg/mL in 40% acetronitrile/H_2O containing 0.1% TFA); a Shiseido Capcell Pak C_{18} column (250 mm × 4.6 mm); 0.5 mL/min flow rate) was applied to the enriched taxanes in Fig. 4A. Paclitaxel was co-eluted with the displacer cetylpyridinium·TFA at around 80 min. (C) The HPLC chromatogram of the fraction collected at 80 min. Although paclitaxel spontaneously crystallized after coming out from the HPLC column, the entire fraction at 80 min was dried and dissolved in 500 μL of MeOH in order to investigate the components coming out of the column. The eluted cetylpyridinium·TFA is labeled as "Displacer" in the chromatogram. (D) The HPLC chromatogram of the purified paclitaxel crystal. The separations in (A), (C) and (D) were carried out on Waters Symmetry Shield RP C_{18} column (50 mm × 4.6 mm, 3.5 μm) with acetonitrile gradient in 0.1% TFA from 0 to 90% in 10 min.

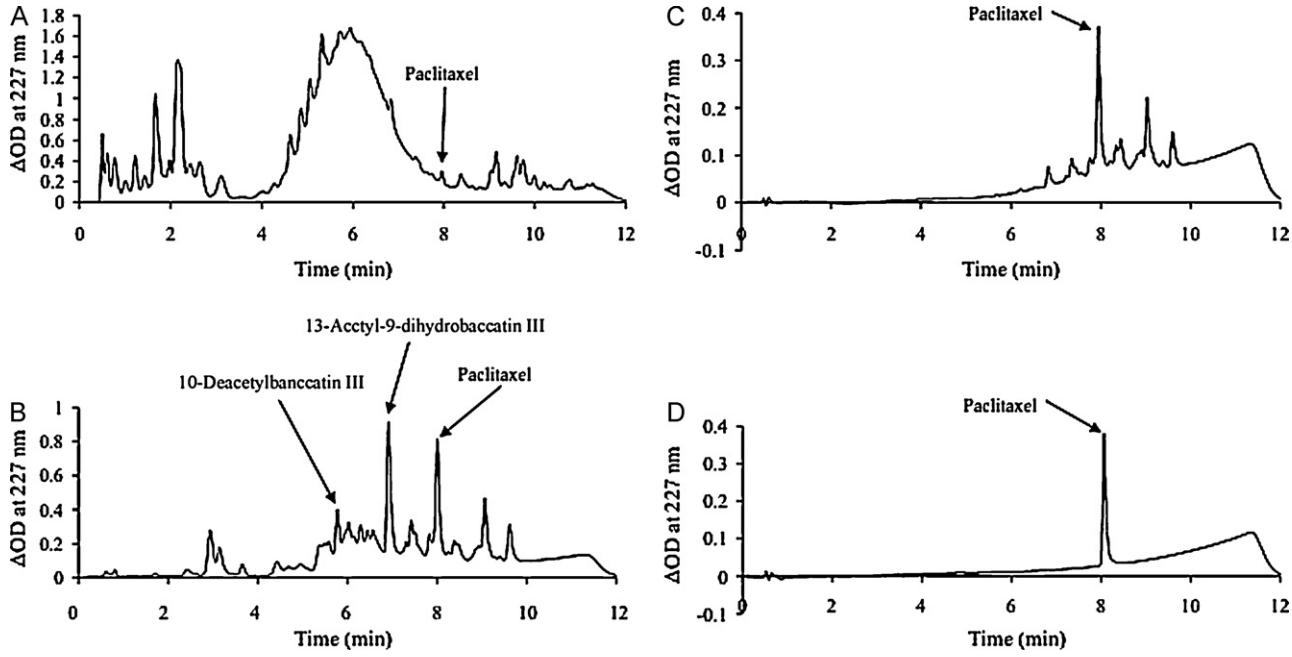

Fig. 5. (A) Paclitaxel extract in 50–60% MeOH/H_2O was purchased from Paxis pharmaceutical Inc. as a starting material. The HPLC chromatogram is shown, where paclitaxel peak is labeled. (B) Taxanes were enriched by removing water- and hexane-soluble components and further extraction with CH_2Cl_2. The HPLC chromatogram of the enriched taxanes is shown, where a few taxanes, 10-deacetylbaccatin III, 13-acetyl-9-dihycrobaccatin III and paclitaxel are labeled. (C) The HPLC chromatogram of the enriched paclitaxel after removing hydrophilic taxanes with MeOH/H_2O/TFA (1.0:98.9:0.1, v/v/v). (D) The HPLC chromatogram of paclitaxel purified by the HPDC technique (cetylpyridinium·TFA displacer (3 mg/mL in 30% acetronitrile/H_2O containing 0.1% TFA); a Waters RP_{18} guard column (10 mm × 19 mm)—a Shiseido C_{18} AQ trap column (50 mm × 20 mm)—a Shiseido Capcell Pak C_{18} column (250 mm × 4.6 mm); 1.0 mL/min flow rate). Paclitaxel is spontaneously crystallized after eluting out of the HPLC column and is washed with water. The separations in (A)–(D) were carried out on Waters Symmetry Shield RP C_{18} column (50 mm × 4.6 mm, 3.5 μm) with acetonitrile gradient in 0.1% TFA from 0 to 90% in 12 min.

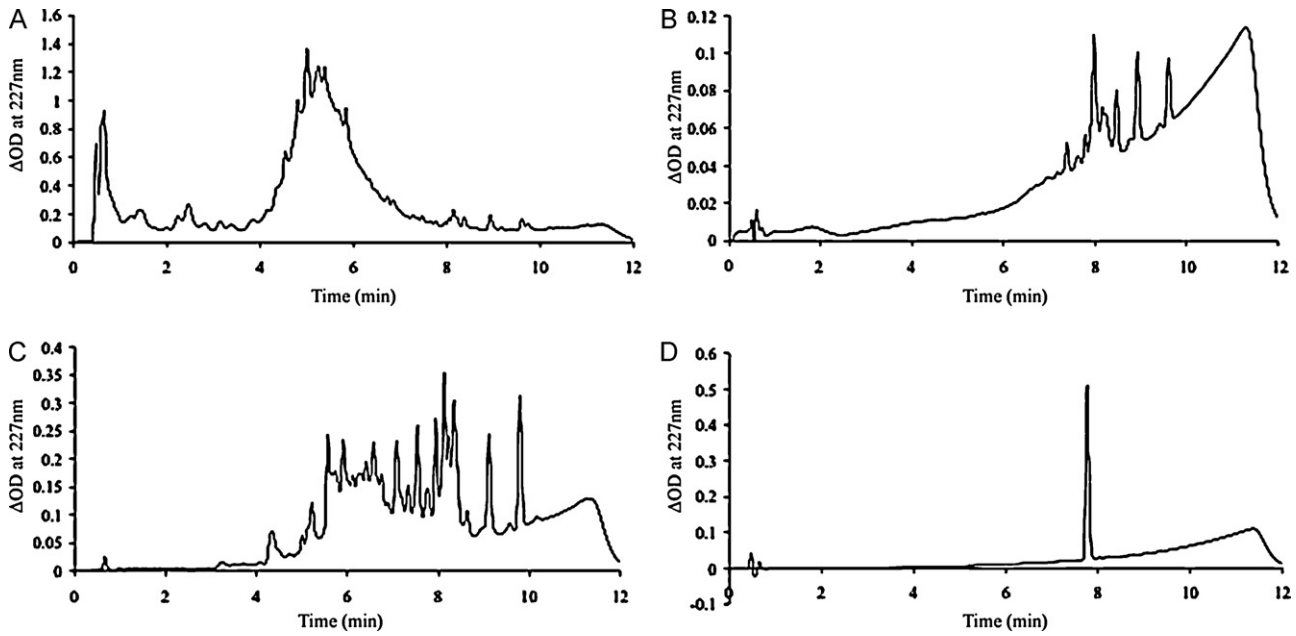

Fig. 6. (A) The HPLC chromatogram of the crude extract with detection at 227 nm. Paclitaxel was extracted from the ground dried needles of a Chinese yew *Taxus media* by MeOH. (B) The HPLC chromatogram of the enriched taxanes after removing hydrophilic and hydrophobic components by water and hexane, respectively and further extracting taxanes with CH_2Cl_2. (C) The HPLC chromatogram of the enriched paclitaxel after removing hydrophilic taxanes with MeOH/H_2O/TFA (1.0:98.9:0.1, v/v/v). (D) The HPLC chromatogram of the purified paclitaxel using the HPDC technique (the same as in Fig. 5(D)). Paclitaxel is spontaneously crystallized after eluted out of the HPLC column and is washed with water. The separations in (A)–(D) were carried out on Waters Symmetry Shield RP C_{18} column (50 mm × 4.6 mm, 3.5 μm) with acetonitrile gradient in 0.1% TFA from 0 to 90% in 12 min.

in Section 2. The filtered residue (1.5 g) contained paclitaxel at 8.4% purity (w/w). Fig. 5B shows the HPLC chromatogram of the enriched taxanes with detection at 227 nm. Some of the taxanes, 13-acetyl-9-dihydrobaccatin III, 10-deacetylbaccatin III and paclitaxel are labeled in Fig. 5B. Although the hydrophilic taxanes could be further enriched, the enrichment of the hydrophilic taxanes was not the objective of this study. Thus, no further enrichment of the hydrophilic taxanes was attempted.

3.3. Extraction of hydrophilic taxanes on large scale

As hydrophilic taxanes can be used for semi-synthesis of paclitaxel and docetaxel [32], they are separated from paclitaxel before the purification of paclitaxel. Hydrophilic taxanes are more hydrophilic than paclitaxel and are slightly soluble in MeOH/water/TFA (1.0:98.9:0.1, v/v/v), whereas paclitaxel is hardly soluble in this solvent. Using this difference in solubility, hydrophilic taxanes were extracted with MeOH/water/TFA (1.0:98.9:0.1, v/v/v). A large volume of MeOH/water/TFA (1.0:98.9:0.1, v/v/v) to dissolve hydrophilic taxanes was reduced as we recycled the extracting solvent after trapping hydrophilic taxanes in a reverse phase column as shown in Fig. 2. The extraction continued at a flow rate of 10 mL/min for 6–7 h, corresponding to usage of 3.6–4.2 L of MeOH/water/TFA (1.0:98.9:0.1, v/v/v). Fig. 5C shows the HPLC chromatogram of paclitaxel after extracting out hydrophilic taxanes. Most hydrophilic taxanes were extracted without paclitaxel. It should be noted that paclitaxel started slowly extracting after 6–7 h, in spite of its low solubility in MeOH/H_2O/TFA (1.0:98.9:0.1, v/v/v). This made it necessary to periodically monitor the output solvent by HPLC in order to minimize the loss of paclitaxel.

3.4. Purification of paclitaxel on large scale

In order to selectively extract paclitaxel, the methanol content of the extraction solvent was increased to 10%. The increase of organic solvent content in the mobile phase has a disadvantage of having to decrease the loading capacity of paclitaxel on the column [35], but was required for a practical time scale of the extraction. The HPDC technology was applied as described in Section 2. Paclitaxel was co-eluted at 680–760 min with cetylpyridinium·TFA similar to that observed in the small scale purification (Section 3.1). Soon after eluted out from the column, paclitaxel spontaneously crystallized whereas cetylpyridinium·TFA remained in solution. The crystals were washed with water and dried *in vacuo*, resulting 114 mg of the dried crystal. The pH of the purified paclitaxel was neutral when it was dissolved in 90% MeOH/H_2O (v/v) and diluted with an excess of H_2O, confirming the lack of contamination with TFA. Its HPLC chromatogram showed 99.4% purity with no contamination of cetylpyridinium·TFA (Fig. 5D). The overall loss of paclitaxel in the enrichment/purification was 10%. Obviously, the elution time of paclitaxel was taking long and could be shortened by reducing the size of the trap column, increasing the concentration of cetylpyridinium·TFA, etc. However, the productivity was not the priority. Instead, minimum loss of paclitaxel during the enrichment/purification and the high purity of the end product were the emphasis. The purified paclitaxel was identified by high-resolution electrospray tandem mass spectrometry (HR–ESI–MS/MS), and 1H NMR (see Supplementary File). These data are in good agreement with those reported in the literature [36,37] and confirm that the purified compound is indeed paclitaxel.

ESI–MS/MS (CE = 9): *m/z* (% relative peak area): $[C_{47}H_{51}NO_{14} + H^+] = 854.3388$, found = 854.3406 (79); $[C_{47}H_{51}NO_{14} - C_2H_6O_3 + H^+] = 776.3071$, found = 776.3123 (11); $[C_{47}H_{51}NO_{14} - C_{14}H_{15}NO_4 + H^+] = 569.2387$, found = 569.2375 (100); $[C_{47}H_{51}NO_{14} - C_{16}H_{17}NO_5 + H^+] = 551.2281$, found = 551.2285 (52); $[C_{47}H_{51}NO_{14} - C_{18}H_{19}NO_6 + H^+] = 509.2175$, found = 509.2196 (30); $[C_{47}H_{51}NO_{14} - C_{31}H_{36}O_{10} + H^+] = 286.1079$, found = 286.1036 (98); $[C_{47}H_{51}NO_{14} - C_{31}H_{38}O_{11} + H^+] = 268.0974$, found = 268.0970 (14).

3.5. Purification of paclitaxel from Chinese yew on small scale

Paclitaxel has been extracted from several yews including *T. canadensis*. In order to demonstrate the generality of our

purification method using HPDC technique, we purified paclitaxel from another source, namely Chinese yew *Taxus media*. The same procedures were applied as those for *T. canadensis* with minor modifications in the enrichment of the taxanes. Briefly, paclitaxel was extracted from the dry needles by MeOH. The HPLC chromatogram of the crude extract is shown in Fig. 6A, which is, as expected, markedly different from Fig. 3 of the crude extract from *T. canadensis*. Nevertheless, the taxanes were enriched by following the purification procedure developed for *T. canadensis* on large scale (see Section 2) on smaller scale. The HPLC chromatogram of the enriched taxanes is shown in Fig. 6B. Hydrophilic taxanes were extracted in MeOH/H_2O/TFA (1.0:98.9:0.1, v/v/v) as outlined in Fig. 2. After the removal of hydrophilic taxanes, the HPLC chromatogram of the enriched paclitaxel is shown in Fig. 6C. Paclitaxel was then extracted in MeOH/H_2O/TFA (10.0:89.9:0.1, v/v/v) and purified using the HPDC technique in the same way for paclitaxel from *T. canadensis*. Soon after eluted out from the column, paclitaxel spontaneously crystallized whereas cetylpyridinium·TFA remained in solution. The crystals were washed with water and dried *in vacuo* through a lyophilizer. The pH of the purified paclitaxel was neutral when it was dissolved in 90% MeOH/H_2O (v/v) and diluted with an excess of H_2O, confirming no contamination of TFA. Its HPLC chromatogram showed 99.9% purity with no contamination of cetylpyridinium·TFA (Fig. 6D), suggesting that the method developed in this paper can purify paclitaxel from various yews. Furthermore, our purification method may be applicable to purify semi-synthetic paclitaxel and its analogs such as docetaxel. It may also lower the purification cost by reducing the size of the reverse phase C_{18} column used in the current industrial purification of paclitaxel. The structure of the purified paclitaxel was identified by HR–ESI–MS/MS, and ^{1}H NMR (see Supplementary File). These data are in good agreement with those reported in the literature [36,37], and confirm that the purified compound is paclitaxel.

ESI–MS/MS (CE = 9): *m/z* (% relative peak area): $[C_{47}H_{51}NO_{14} + H^+] = 854.3388$, found = 854.3380 (70); $[C_{47}H_{51}NO_{14} - C_2H_6O_3 + H^+] = 776.3071$, found = 776.3115 (10), $[C_{47}H_{51}NO_{14} - C_{14}H_{15}NO_4 + H^+] = 569.2387$, found = 569.2327 (100); $[C_{47}H_{51}NO_{14} - C_{16}H_{17}NO_5 + H^+] = 551.2281$, found = 551.2255 (53); $[C_{47}H_{51}NO_{14} - C_{18}H_{19}NO_6 + H^+] = 509.2175$, found = 509.2180 (30); $[C_{47}H_{51}NO_{14} - C_{31}H_{36}O_{10} + H^+] = 286.1079$, found = 286.1013 (74); $[C_{47}H_{51}NO_{14} - C_{31}H_{38}O_{11} + H^+] = 268.0974$, found = 268.0972 (10).

4. Conclusions

The HPDC technique was successfully applied to purify 114 mg of paclitaxel from *T. canadensis* with high purity of 99.4% on large scale. Only 10% of paclitaxel was lost throughout the enrichment and purification processes. Although paclitaxel was not purified by the mechanism of HPDC as it co-eluted with the displacer cetylpyridinium·TFA, we could take the advantages of HPDC technique such as high sample loading, high purity, and minimum loss of the sample. The method was successfully applied to purify paclitaxel from Chinese yew, suggesting the general applicability of the method to purify paclitaxel from various yews and possibly semi-synthetic paclitaxel.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.03.010.

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