A Large Scale Separation of Taxanes from the Bark Extract of *Taxus yunnanesis* and ^{1}H- and ^{13}C-NMR Assignments for 7-*epi*-10-Deacetyltaxol

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A large-scale separation of paclitaxel from semi-purified bark extract of *Taxus yunnanesis* was investigated. The chromatographic behavior of paclitaxel and two close eluting analogues, cephalomannine and 7-*epi*-10-deacetyltaxol were systematically studied on a C_{18} bonded phase column with different mobile phase in reverse phase mode. According to the notably different selectivity of the methanol and acetonitrile with water in the mobile phase and the most important requirement of capacity in preparative chromatography, the optimum suitably mobile phase used in a large-scale isolation of paclitaxel and 7-*epi*-10-deacetyltaxol on a preparative C_{18} column was given. Cephalomannine was eliminated by ozonolysis and after then separated throughout a normal phase silica column. The whole large-scale process for high purity paclitaxel from the bark extract of *Taxus yunnanesis* consisted of a preliminary purification with Biotage FLASH 150i system based on a prepacked normal phase silica cartridge followed by using a C_{18} Nova-pak™ column in Waters PrepLC™ 4000 preparative HPLC system. The structure of 7-*epi*-10-deacetyltaxol was elucidated by 2D NMR technologies of TOCSY, DQF-COSY, HMQC and HMBC, *etc*.

Keywords Separation, taxanes, paclitaxel, cephalomannine, 7-*epi*-10-deacyltaxol, *Taxus yunnanesis*, ^{1}H- and ^{13}C-NMR, 2D NMR

Paciltaxel (**I**) (Fig. 1), a complex diterpene amide first isolated from the bark of the western yew tree, *Taxus brevifolia*, has been the subject of intensive research due to its unique cancer chemotherapeutic properties.[1,2] Since **I** occurs with a series of closely related taxanes in various species of *Taxus*,[3] considerable work has been directed to devising effective methods of analysis and purification. General isolations of **I** and other taxanes from the bark or needle extract of *Taxus brevifolia* have been reported[4-12] by using C_{18}, PFT, C_8, Alltech Adsorbo HS C_{18}, and Zorbax SW-Taxane packed columns. The most difficult process came up from separating its closely eluting analogs, *i. e.*, 7-*epi*-10-deacetyltaxol (**II**) and cephalomannine (**III**) with suitable mobile phases. A known difficult step in the purification is the separation of **I** and **III**[13] which differs from **I** only in the amide portion of the molecule. Ozone oxidation of **III**[14] in the presence of **I** and **II** is a viable initial step to ease the separation of the two similar compounds. So the really particularly difficult step is the separation of **I** and **II**. Steven[15] focused on the separation of **I** from the closely eluting analog **II**, and reported a new method utilizing a commercially available pentafluorophenyl (PFP) column to give a base-resolution of **I**, **II** and **III**. But few preparative methods for large-scale separating **I** from **II** have been reported so far. Until now there are few details published too for large-scale purification for high purity **I** from bark extract of *Taxus yunnanesis*. Unfortunately, the content of **II** from bark extract of *Taxus*

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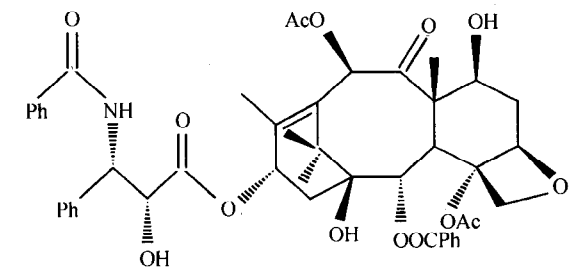
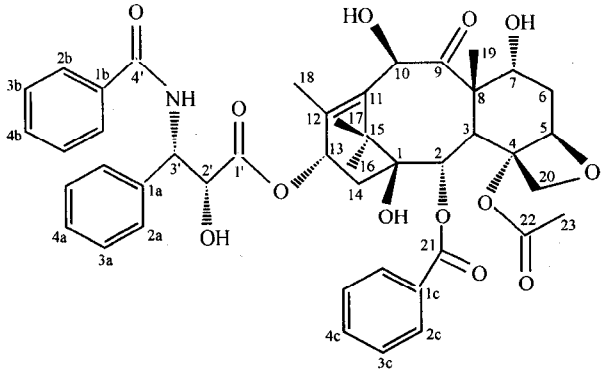
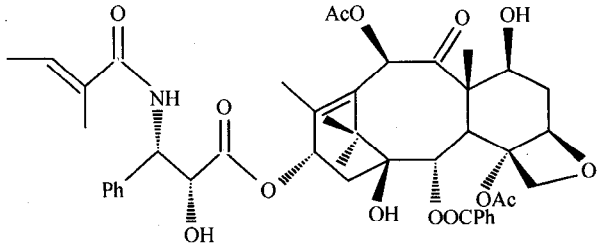


I Paclitaxel



II 7-*epi*-10-Deacetyltaxol



III Cephalomannine

Fig. 1 Structures of compounds **I**, **II** and **III**.

yunnanesis (as shown in Fig. 2A) is much higher than other *Taxus*. According to the general isolation of bark extract from other *Taxus spp*. biomass, the purification for high purity **I** from *Taxus yunnanesis* barks poses the additional problem showed as Fig. 2A in which there is relatively large amount of the closely eluting analog, 7-*epi*-10-deacetyltaxol (**II**). It is a very difficult step for clearly separating **II** and **III** to give the high purity **I** at the same time, and thus it is necessary to develop a more efficient process. In this article the chromatographic behavior of **I**, **II** and **III** was studied in details based on a C_{18} bonded phase column with different mobile phase in reverse phase mode. The new method which also can give a base-resolution of **I**, **II** and **III** without resorting to specialty column but a C_{18} column that is the most commonly used in analysis and large-scale preparative separation. According to the results of the analytic tests on a C_{18} column with different mobile phases in isocratic mode, we have developed a large-scale isolation of high purity **I** from the bark extract of *Taxus yunnanesis*, which consisted of a preliminary purification with Biotage FLASH 150i system based on a prepacked normal phase silica cartridge followed by the use of C_{18} Nova-pak™ column in Waters PrepLC™ 4000 preparative HPLC system. Cephalomannine was eliminated by ozonolysis and then after completely separated by using Biotage FLASH 150i system.[16] In the course of using preparative LC, isocratic elution was used in both normal-phase method and reverse-phase mode, which made the recycle of mobile phase possible through simple distillation under reduced pressure. It drastically reduced the consumption of expensive HPLC grade solvents and also prevented the drainage of the waste mobile phase from polluting the environment.

Experimental

Materials

The bark extract of *Taxus yunnanesis* (**I** ≥ 1%) was purchased from Yunnan Bada Bio-tech Co., Ltd. Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific Worldwide (Shanghai Representative Office, China). Ethyl acetate and *n*-hexane were of analytical grade which were purchased from Shanghai Chemicals Factory. All solvents were filtered through a 0.2 μm Nelon 66 filter before being used. Water was distilled twice then filtered through a 0.2 μm Nelon 66 filter.

Apparatus and columns

Normal phase preparative chromatography system

Biotage FLASH 150i™ consisted of an electropolished 304 stainless-steel solvent reservoir having 37-liter capacity, a Sample Injection Module (SIM) having 1000 mL size, an AM-190 air manifold, a FLASH 150i radial compression module and a prepacked flash cartridge

which is prepacked with 2.5 kg KP-Sil™ silica (150 mm × 300 mm, 10 μm).

Preparative high performance liquid chromatography system

Waters PrepLC™ 4000 high performance preparative chromatography system consisted of a solvent delivery unit, a standard 3725I injector (a 10 mL loop), a waters 2487 Dual λ Absorbance Detector with an NEC-586 computer (Millennium^{32} software), a Nova-pak™ C_{18} column (50 mm × 300 mm, 6 μm) and a Symmetry™ C_{18} column (19 mm × 300 mm, 7 μm).

Analytical high performance liquid chromatography system

Beckman analytical HPLC system consisted of two 110B solvent delivery modules, a 168 Photodiode array detector with a Digital PII computer (Gold System software), a Phenomenex analytical C_{18} column marked as LUNA™(2) (4.6 mm × 150 mm, 5 μm).

Methods

Method of ozonolysis

30 g of semi-purified bark extract of *Taxus yunnanesis* were dissolvedin 300 mL of MeOH/CH_2Cl_2(1:2) and treated with ozone in an oxygen stream at the flow rate of 100 mL/min at room temperature. HPLC analysis showed the reaction was complete after 3 h. The ozonized solution was purged with N_2 and then the solvent was removed under vacuum.

Method of normal phase preparative liquid chromatography

100 g of ozonized semi-purified bark extract coated directly onto 200 g silica was loaded in the SIM of Biotage FLASH 150I™ chromatography system. The cartridge was eluted by isocratic elution with ethyl acetate: *n*-hexane (4:3) mobile phase. The column effluents were collected into fraction bottles. Fractions of approximately 2 L were collected and these were monitored *in-situ* by UV absorbance at 254 nm. The column fractions were concentrated under reduced pressure ($\leqslant 1 \times 10^4$ Pa), then the vapors were led into a vertical tube-type condenser, where they were distillated by chilled water and ice mixture at 0℃ and the condense can be reused after slightly correcting the polarity equal to that of the original mobile phase. Soon after, the fractions were collected in groups based on the HPLC analysis for purity and composition. The groups of the fractions which were measured with the 95 + % level of purity for **I** were used for the next step with preparative HPLC.

Method of reverse phase preparative chromato-graphy

The samples dissolved in a 1:1 mixture of acetonitrile and water was filtered through a 0.2 μm Nylon filter and introduced to the column by a manual inlet valve on the front of the pump and washed with a selected mobile phase. The detector was set at 227 nm. The flow rate for the Nova-pak™ C_{18} column (50 mm × 300 mm, 6 μm) was 50 mL per min, for Symmetry™ C_{18} column (19 mm × 300 mm, 7 μm) 10 mL per min.

Method of analytical high performance liquid chromatography

The mobile phase for the C_{18} column of Phenomenex LUNA™ (2) (4.6 mm × 150 mm, 5 μm) was used with a 3:3:4 mixture of acetonitile, methanol and water. The flow rate was 1.0 mL/min. For purity determinations using the photodiode array detector having absorbance measurement over the entire 190—600 nm wavelength, the data were collected both at 227 nm and 254 nm.

Other methods

Thin-layer chromatography was carried out using silica gel HF-60, 254 + 366 (EM Science/Fisher) and systems consisting of ethyl acetate/hexane. Visualization was by a UV-lamp and by charring with 1 mol/L H_2SO_4. Ozonolysis was carried out using the ozonizer made by Ozone Research and Equipment Company, Shanghai, China. The following instruments were used to record the spectra and described here: UV, CVI, AS240 with 2-meter optical fiber probe; FT-IR, Nicolet Avatar 360; Mass spectra (FAB) were obtained on a QUATTRO spectrometer using a cesium gun operated at 15 keV of energy; NMR was obtained on Varian Inova-600 spectrometer.

Results and discussion

Operation sequence and the result of each step

Step 1: First purification in the normal-phase liquid chromatography system

The large-scale process for high purity paclitaxel was carried out in four steps. In the first step, the raw bark extract of *Taxus yunnanesis* ($\mathbf{I} \geqslant 1\%$) containing the components shown in Fig. 2A was purified by the FLASH 150i normal phase chromatographic system, which gave a semi-purified bark extract having 60 + %

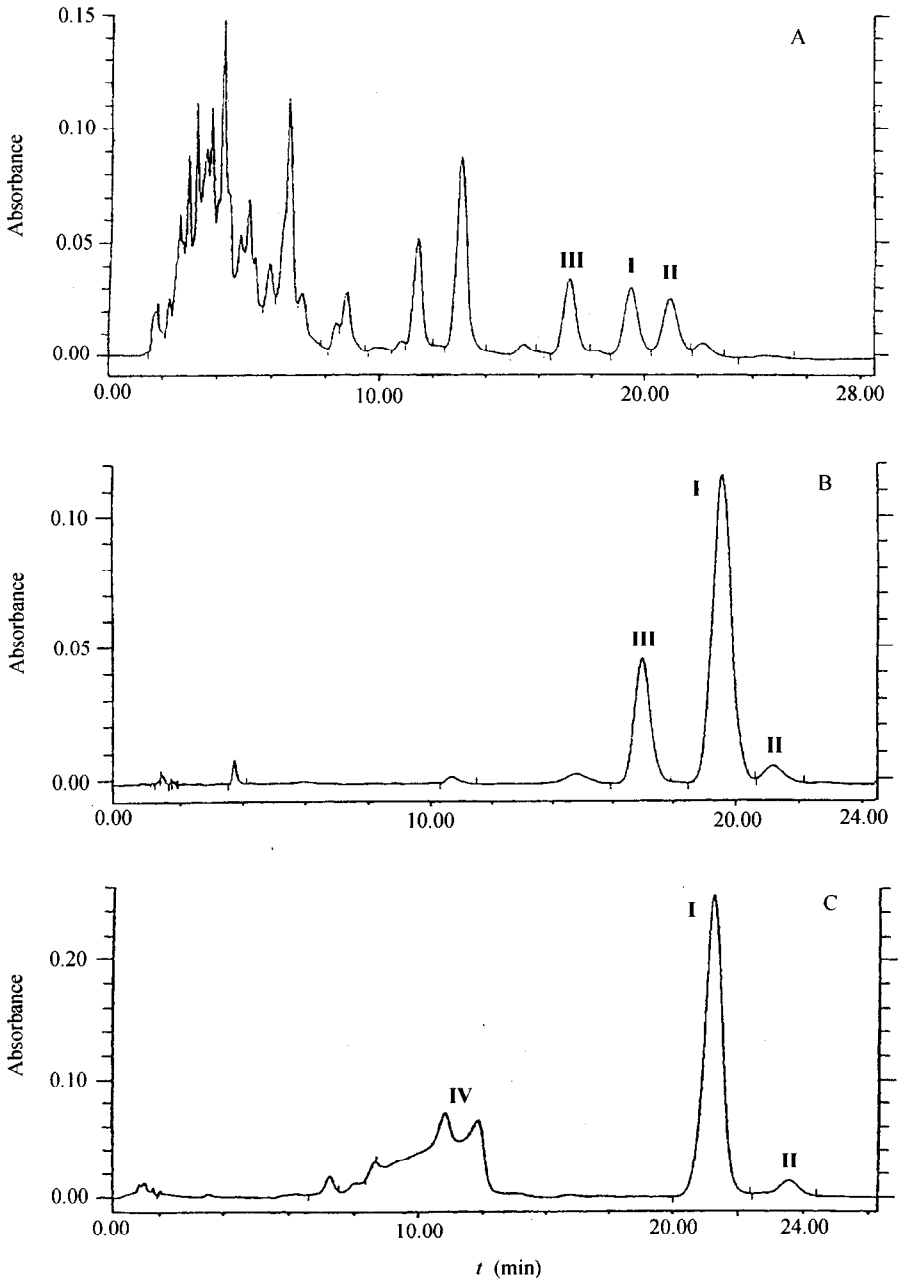


Fig. 2 Chromatograms of (A) raw bark extract of *Taxus yunnanesis*, (B) semi-purified bark extract of *Taxus yunnanesis* and (C) semi-purified bark extract of *Taxus yunnanesis* after ozonolysis. Mobile phase: 30:30:40 methanol:acetonitrile:water; Column: LUNA™ (2), 4.6 mm × 150 mm, 5 μm, C_{18}; flow rate: 1 mL/min. **I** = paclitaxel; **II** = 7-epi-10-deacetyltaxol; **III** = cephalomannine; **IV** = products of ozonolysis.

level of purity for **I** with recovery rate 90% in one run. The residual products having a low purity for **I** also can be re-purified in the next run with the raw bark extract or be collected together to purify in a special run. So the overall recovery of **I** was greater than 98% with the cycle operation.

Step 2: Ozonolysis

In the chromatogram of the product of the first step (as shown in Fig. 2B), three closely eluting taxanes were respectively eluted at the retention time 16.93 min (**III**, 28.527%), 19.51 min (**I**, 60.242%) and 21.18 min (**II**, 8.131%). In the second step, we used ozone to oxidize the tigloyl amide double bond of **III** in the semi-purified bark extract from Taxus yunnanesis, which resulted in more polarity compounds **IV** such as keto amide or hydrazone which eluted at the retention time of 10—12 min in the chromatogram as shown in Fig. 2C. The ozone also caused extensive bleaching of the sample, thus assisting further in the purification.

Step 3: Second purification in the normal-phase liquid chromatography system

In the third step, after running again the normal phase preparative liquid chromatography system of Biotage FLASH 150i™, we could gain 70 grams 95 + % level of purity for **I** from 100 grams ozonized semi-purified bark extract (**I** ≥ 60%) in a yield higher than 93%.

It is the use of FLASH 150i cartridges made from FDA approved that improve both the performance and reproducibility of separation. The use of Biotage's patented radial compression technology reduces the interstitial (void volume) within the packed bed. The high bed density means that compounds are collected in narrow bands, and results in higher efficiency separation. A sample having similar purity for **I** obtained by using Biotage FLASH150i system in one run must be purified by several self-packed glass columns and recrystallizing step. Because of fewer steps, the yield is higher than those reported earlier. Moreover, the isocratic elution with ethyl acetate: hexane (4:3) was easily reused by distillation under vacuum ($\leq 1 \times 10^3$ Pa, 40℃). The solvents used in gradient elution in the earlier reports could not be recycled by such a valid simple means.

We also found that the mixture containing main **I** (≥98%) and a little **II** (≤2%) recrystallized from ethyl acetate and hexane (3:5) to give white needles, the high purity **I** (≥99.5%). However, the recrystallizing condition must be carefully controlled and it needed a long period and multisteps of recrystallization to get adequate high purity **I**. Because of its low efficiency, it was not suitable as a routine process for high purity **I**. Nevertheless the step of recrystallization can be used as an aided process to purify the little fraction (90% ≤ **I**) to the 95 + % level which can be used in the next step of preparative HPLC.

Step 4: Purification in the preparative reverse-phase high performance liquid chromatography system

The last step of the purification was the most important one in which we selected Waters PrepLC™ 4000 system as a key instrument for isolation of the highest purity **I** in a production-scale. High flow rate and back pressure capabilities allow us to use the more higher resolution, smaller-sized particle sized column packing, so a six micron column, Nova-pak™ C_{18} column (50 mm × 300 mm, 6 μm) may be used for preparative scale isolations of up to grams per sample loading. In a run within 60 min, 1.9 grams 99.5 + % level of purity for **I** was gained from 2.0 grams 95 + % **I** in a yield more than 98%.

The solvent and column regeneration play an important role in preparative LC which was not mentioned in analytical HPLC. In the whole process we used isocratic elution which can be regenerated through the samplc step of redistillation under vacuum below 50℃ in 98% yield using a second cold trap with the common rotated evaporator. The column also can be easily purged by flushing them with 5 column volumes methanol and then be reconditioned by mobile phase.

Selectivity of the mobile phase

Most published papers[8-11] discussed the effect of stationary phase selectivity on separation, but no studies on the selectivity of the mobile phase was reported. However, we found that the selection of mobile phase had obvious impacts on the peak sequence and resolu-

tion of **I**, **II** and **III** on a common C_{18} column. In a way we can say the effects of the selectivity of mobile phase are more remarkable than that of stationary phase.

Two mobile phase systems were intensively studied on a C_{18} bonded phase column in isocratic reverse phase mode. When System I, an acetonitrile/water mobile phase was used, the peak sequence was **III**, **II** and **I** in turn. The percentage of acetonitrile did not alter the elution order and only changed the resolution. According to the relation between retention time and the percentage of acetonitrile in water shown in Fig. 4A, System I gave a reasonable separation of **III** and **I**, but a poor resolution of **II** and **I**. When a 40:60 acetonitrile/water mobile phase was used, the C_{18} column gave the best separation of the three closely taxanes in System I within 40 min ($Rs_{I\text{-}II} = 1.42$, $Rs_{I\text{-}III} = 3.13$)(as shown in Fig. 3A).

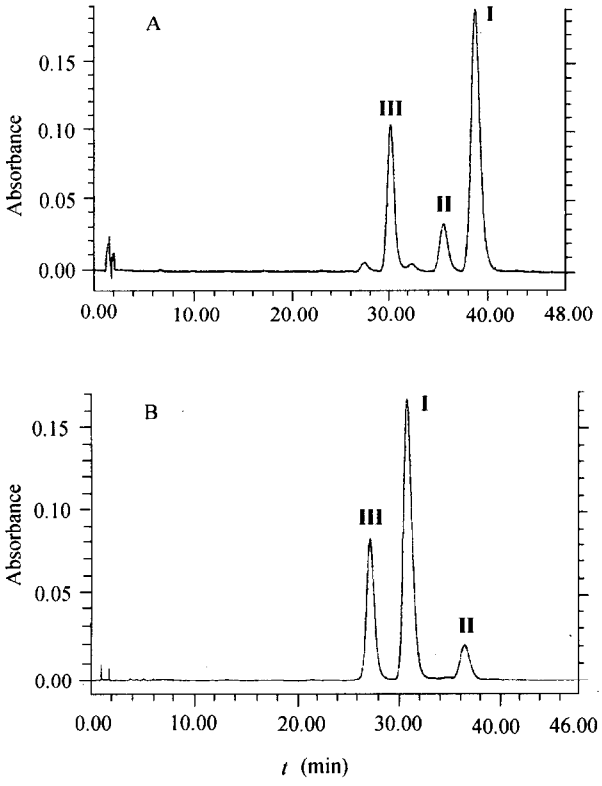


Fig. 3 Chromatograms of separation of **I**, **II** and **III** with (A) 40:60 acetonitrile:water mobile phase and (B) 60:40 methanol:water mobile phase. Column: LUNA™ (2), 4.6 mm × 150 mm, 5 μm, C_{18}; flow rate: 1 mL/min.

While System II, a methanol/water mobile phase was used, the peak sequence was **III**, **I** and **II** in turn. According to the relation between retention time and the percentage of methanol in water shown in Fig. 4B, it gave an efficient separation of **I** and **II**, but a poor resolution of **III** and **I**. When a 60:40 methanol/water mobile phase was used, the C_{18} column gave the best separation of the three compounds in System II within 40 min ($Rs_{I\text{-}II} = 3.14$, $Rs_{I\text{-}III} = 1.62$)(as shown in Fig. 3B).

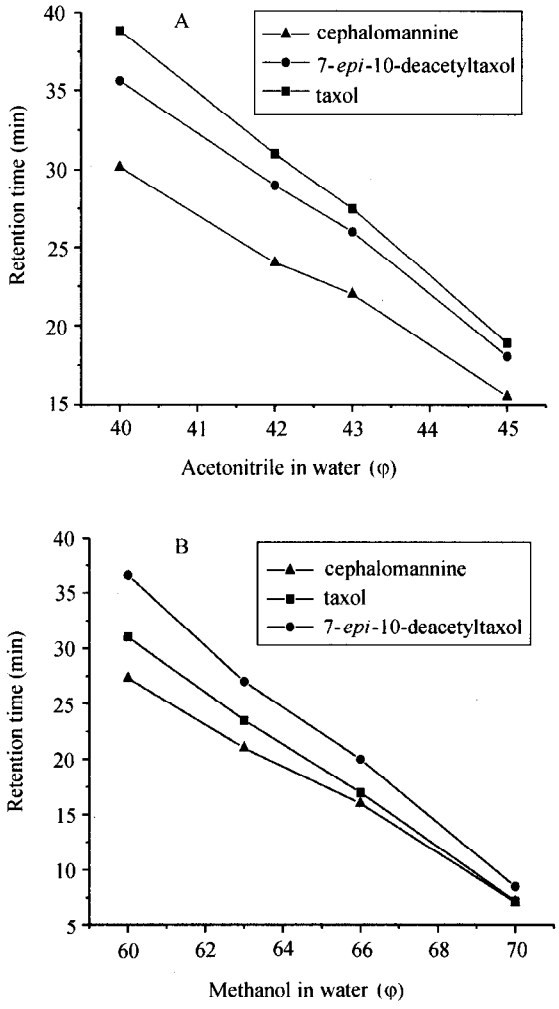


Fig. 4 Relations between retention time and mobile phases. (A) acetonitrile in water; (B) methanol in water. Column: LUNA™ (2), 4.6 mm × 150 mm, 5 μm, C_{18}; flow rate: 1 mL/min.

Obviously, judging from the selectivity of the acetonitrile and methanol in mobile phase, we can draw a conclusion that acetonitrile tends to elute compound **II** in front of **I**, while methanol tends to elute **II** behind **I**.

The resolution of **III** and **I** can be improved by adding more acetonitrile in the mobile phase, while that of **II** and **I** by adding more methanol. When using acetonitrile and methanol together as an elution, it could improve the resolutions of each pair peaks of **I**, **II** and **III** in a fast run. However, it should be avoided merging the peaks of **I** and **II** into one band with an unsuitable proportion of methanol and acetonitrile in water. After many times of testing, a 30:30:40 methanol/acetonitrile/water mobile phase was used on the C_{18} column in an analytical method, because it provided a base-resolutions of **I**, **II** and **III** in a shorter running time within 20 min (as shown in Fig. 2B).

Three main goals of any chromatographic separation are resolution, separation speed and capacity. In analytical chromatography, speed and resolution are a requirement, whereas in preparative chromatography, capacity is the most important. To obtain maximal throughput, the column must be overloaded to the point where the peaks still separate (Rs, mini), namely a higher resolution of **I** and **II** meant a possibility to get a higher throughput of **I** within the limit of the instrument. The preparative C_{18} column gave a high resolution of **I** and **II** ($Rs = 3.17$) with a 60:40 methanol/water mobile phase, when loaded with a sample of 15 mg binary mixture of **I** and **II** (as shown in Fig. 5A). Fig. 5B showed that a sample of 2 g mixture of **I** ($\geqslant 95\%$) and **II** ($\leqslant 5\%$) was overloaded on the preparative C_{18} column to the point where the peaks of **I** and **II** still separate ($Rs = 1.25$). Although the Rs decreased from 3.17 to 1.25, the throughput of **I** on the same column was increased more than 100 folds. However, the chromatogram (Fig. 5C) demonstrated a superposition of two nearly rectangular peaks of **II** and **I** on the same column with a 40:60 acetonitrile/water mobile phase, when the column was overloaded with the same sample used in Fig. 5B. Concerning such chromatographic experiments, we can state: in the large-scale preparative chromatographic separation of the **I** and **II** mixture, an optimized strategy, especially a 60:40 methanol/water mobile phase would be chosen to give the highest resolution of **I** and **II** on a C_{18} column within a reasonable running time. If this is done, as large a sample as possible should be placed on the column so as to increase the throughput per chromatogram.

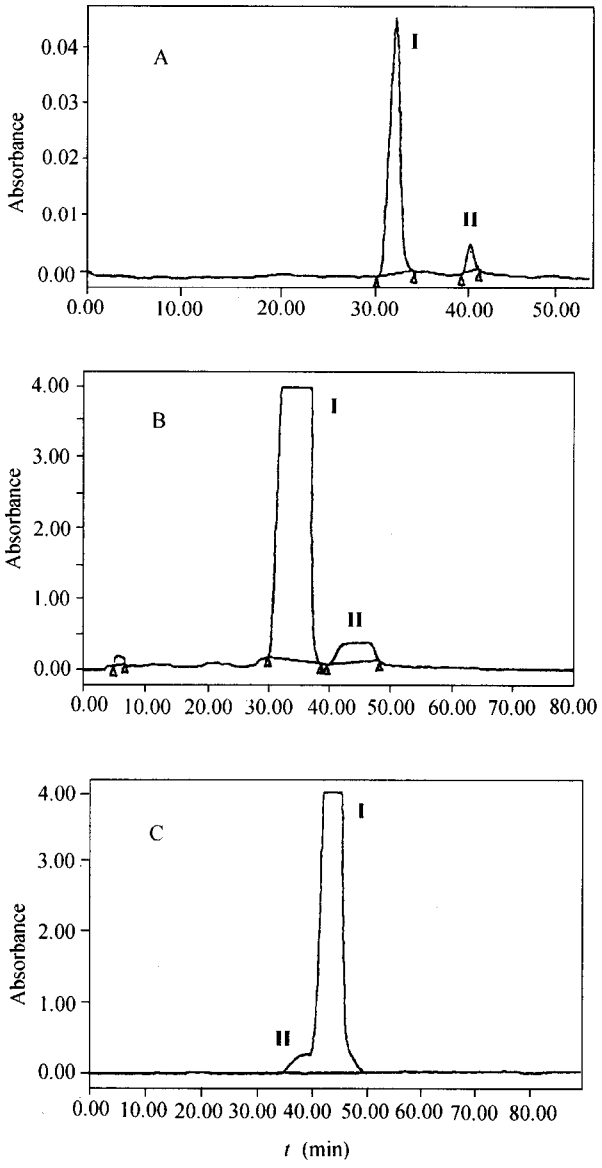


Fig. 5 Chromatograms of separation of (A) a sample (5 mg) of **I** and **II** with a 60:40 methanol:water mobile phase, (B) an overloading sample (2 g) of **I** and **II** with a 60:40 methanol/water mobile phase and (C) an overloading sample (2 g) of **I** and **II** with a 40:60 acetonitrile:water mobile phase in preparative RP-HPLC. Column: Nova-pak™ C_{18} column (50 mm × 300 mm, 6 μm); flow rate: 50 mL/min.

Identification of **I**, **II** *and* **III**

I, **II** and **III** were respectively isolated for structure elucidation. Based on the results of the selectivity of the

mobile phase, a 40:60 acetonitrile/water mobile phase was selected to separate **III** from the fractions of the column effluent corresponding to the binary mixture of **III** and **I** without ozonolysis. A 60:40 methanol: water mobile phase was optimized to separate **II** from the effluent containing **II** and **I** collected in Step 3. NMR, MS, UV and FT-IR(KBr) of **I** and **II** matched values given in the literature.[12-19]

Using ESI-MS, 1D and 2D-NMR, the structure of **II** was deduced as 7-*epi*-10-deacetyltaxol, whose backbone protons were assigned by Jerry and his coworkers.[20] Our data of ^{1}H NMR (Table 1) were almost the same with theirs. Additionally, we also assigned three aromatic protons and all carbons chemical shifts (Table 2) by TOCSY, DQF-COSY, HMQC. Table 2 also gave all $^3J_{C\text{-}H}$ and $^2J_{C\text{-}H}$ coupling, respectively, with which all carbons were connected. Moreover, the connection C-3′ with NH, and C-6 with C-5, were established by $^2J_{3'H\text{-}NH}$ and $^2J_{5H\text{-}6H}$. The structure of four-membered cycle was supported by the coupling of C-5 and H-20 in HMBC, and the characteristic absorption at 1266 cm^{-1} in IR.

Table 1 ^{1}H NMR data of 7-*epi*-10-deacetyltaxol (**II**) in $CDCl_3$, chemical shifts (δ) and coupling constants (J, Hz)

H	δ_H		δ_H (ref.)	
2	5.726(d)	J = 7.44 Hz	5.73(d)	J = 7.5 Hz
3	3.91(d)	J = 7.44 Hz	3.91(d)	J = 7.5 Hz
5	4.892(dd)	J = 4.09, 9.06 Hz	4.90(t)	J = 6 Hz
6	2.32, 2.287	J = 10.78 Hz	2.3(m)	J = 3,12 Hz
7	3.659(s, broad)		3.66(br, t)	
10	5.415(s)		5.42(s)	
13	6.228(t)	J = 8.55 Hz	6.23(br, t)	J = 9 Hz
14	2.233, 2.36		2.3(m)	
16	1.072(s)		1.20(s)	
17	1.183(s)		1.09(s)	
18	1.738(s)		1.74(br, s)	
19	1.713(s)		1.72(br, s)	
20	4.411, 4.382(AB)	J = 8.55 Hz	4.39(br, s)	
23	2.496(s)		2.51(s)	
2′	4.78(s)		4.79(d)	J = 3 Hz
3′	5.796(d)	J = 5.80, 9.00 Hz	5.80(dd)	J = 3,9 Hz
3′-NH	7.094(d)	J = 7.09, 9.00 Hz	7.02(d)	J = 9 Hz

Table 2 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR for 7-*epi*-10-deacetyltaxol (**II**), including results obtained by heteronuclear 2D shift-correlated HMQC ($^1J_{C,H}$) and HMBC ($^nJ_{C,H}$, n = 2,3), in $CDCl_3$ as solvents and residal $CHCl_3$ used as internal reference (δ_H 7.24 and δ_C 77.0, $CDCl_3$), chemical shifts (δ) and coupling constants (J, Hz)

Carbon No.	^{1}H × ^{13}C-HMQC-$^1J_{(C,H)}$		^{1}H × ^{13}C-HMBC-$^nJ_{(C,H)}$	
	δ_C	δ_H	$^2J_{C,H}$	$^3J_{C,H}$
1	79.275		2H	17H,16H
2	75.513	5.726(d)		
3	40.303	3.91(d)	2H	19H
4	82.121		3H,5H,20H	
5	82.685	4.892(dd)		20H
6	35.358	2.32, 2.287		
7	75.943	3.659(s)		19H,3H
8	57.321		3H,19H	2H
9	215.059		10H	19H
10	77.889	5.415(s)		
11	135.76		10H	18H,17H,16H,13H
12	137.972		18H,13H	10H
13	72.454	6.228(t)		18H

Continued

Carbon No.	$^1H \times ^{13}C$-HMQC-$^1J_{(C,H)}$		$^1H \times ^{13}C$-HMBC-$^nJ_{(C,H)}$	
	δ_C	δ_H	$^2J_{C,H}$	$^3J_{C,H}$
14	36.442	2.233,2.36		2H
15	42.575		16H,17H	10H
16	20.627	1.072(s)		17H
17	26.017	1.183(s)		16H
18	14.419	1.738(s)		
19	16.721	1.713(s)		3H
20	77.814	4.395(AB)		
21	167.108			2H
22	172.499		23H	
23	22.572	2.496(s)		
1′	172.707		2′H	13H
2′	73.256	4.78(s)		
3′	55.034	5.796(d)		
3′-NH		7.094(d)		
4′	167.227		NH	2bH
1a	137.868		3′H	
2a	126.924	7.464		3′H
3a	128.706	7.35		
4a	129.062	7.41		
1b	128.335			
2b	127.102	7.719		
3b	128.706	7.36		
4b	131.98	7.47		
1c	129.419			
2c	130.28	8.17		
3c	128.825	7.51		
4c	133.681	7.61		

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