Rapid Qualitative and Quantitative Determination of Seven Valuable Taxanes from Various *Taxus* Species by UFLC-ESI-MS and UFLC-DAD

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Key words
- Taxaceae
- *Taxus*
- yew
- taxane
- paclitaxel
- target analysis

Abstract

The distribution and level of yew constituents vary with species and tissues. In this study, a rapid and valid method incorporating ultra-fast liquid chromatography (UFLC) with MS and UV detection was developed for simultaneous determination of paclitaxel and its six semisynthesis precursors in needles and hair roots from various *Taxus* species. All target analytes could be identified by comparing their retention times as well as UV and MS spectra with authentic standards, while seven valuable taxanes in botanical samples can be rapidly determined by UFLC-DAD with excellent sensitivity. Analysis of more than one hundred yew samples from nine species showed significant variations in distribution and content of seven evaluated taxanes. Thus, different developmental schemes should be used for better utilization of various yew resources.

Abbreviations

UFLC-DAD:	ultra-fast liquid chromatography-diode array detector
ESI-MS:	electrospray ionization-mass spectrometry
DAB:	10-deacetylbaccatin III
B:	baccatin III
DHB:	9-dihydro-13-acetylbaccatin III
DAXP:	10-deacetyl-7-xylosylpaclitaxel
DAP:	10-deacetylpaclitaxel
C:	cephalomannine
P:	paclitaxel

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

received Nov. 4, 2009
revised April 5, 2010
accepted April 20, 2010

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DOI http://dx.doi.org/10.1055/s-0030-1249959
Published online May 26, 2010
Planta Med 2010; 76: 1773–1777 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

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Introduction

Taxane drugs including paclitaxel (**1**) and docetaxel (**2**) are currently standard first-line anticancer agents for the clinical treatment of most solid tumors such as ovarian, breast, and non-small cell lung cancers [1]. The extensive clinical use in cancer chemotherapy and the expanded indications for taxane drugs caused a supply crisis and raised serious environmental concerns, due to the slow growing of yew trees and the low yield of paclitaxel in yew materials [2]. Several solutions have been utilized to produce taxane drugs, including plant cell culture [3,4] and semi-synthesis from common natural precursors (**Fig. 1**) which can be isolated from renewable yew resources such as yew needles [5]. To date, it has been reported that several natural taxanes including 10-deacetylpaclitaxel (**3**), 10-deacetylbaccatin III (**4**), baccatin III (**5**), 9-dihydro-13-acetylbaccatin III (**6**), cephalomannine (**7**), and 10-deacetyl-7-xylosylpaclitaxel (**8**) can be converted to paclitaxel or docetaxel via few steps, and thus recognized as paclitaxel-equivalents or valuable taxanes [6–10]. Paclitaxel and these precursors are found in most yews, but their distribution and level are very variable with species and tissues [11,12]. Therefore, screening of the valuable yew constituents from different species and tissues are crucial to a good agricultural practice in the cultivation of yew trees and cost-effective manufacturing of taxane drugs.

The rapid identification and accurate quantification of target compounds are of great significance for the quality control of medicinal plants. During the past decade, LC-based techniques have been widely used for assignment and quantification of taxanes in biological or botanical samples [13,14]. However, the rapid, sensitive, and simultaneous determination of target taxanes from yew materi-

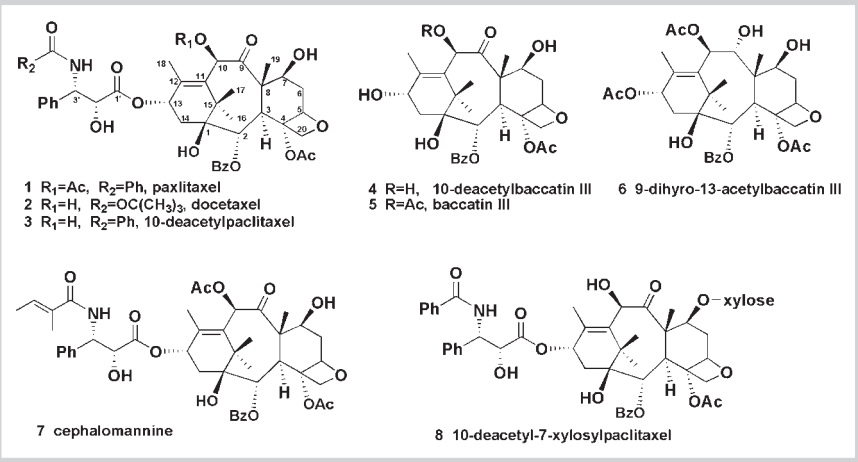

Fig. 1 Structures of taxane drugs and major valuable taxanes.

als is still difficult for phytochemists because of the chemical complexity of crude yew extract and the fact that it contains just trace amounts of taxanes [15,16]. Recently, ultra-high performance liquid chromatography (UHPLC) and ultra-fast liquid chromatography (UFLC), which employ fine stationary phase particles to achieve extreme high resolution with short analytical time as well as good sensitivity, have attracted wide attention of pharmaceutical analysts for its rapid determination of trace constituents from complex samples [17,18]. These advantages facilitate the large scale screening of trace constituents from crude yew samples which is difficult to perform by conventional means.

The objectives of this study were to develop a rapid and sensitive screening approach to complex yew samples and to determine the distribution and content of paclitaxel and six other valuable taxanes in yew samples from various species. The UFLC-ESI-MS was used to identify target taxanes in crude yew extracts based on their retention times and mass spectra by comparison with standards. Meanwhile, a practical and sensitive UFLC-DAD method was used to quantify the content of seven valuable taxanes from complex yew samples. Additionally, in light of the distribution and contents of these valuable taxanes from various *Taxus* species, the potential developing strategies of various yew resources were also discussed.

Materials and Methods

Reagents and materials

Millipore water, HPLC grade acetonitrile, and methanol were used throughout; other reagents used were of analytical grade. The authentic standards (purity > 98%) including 10-deacetylbaccatin III (DAB), baccatin III, and paclitaxel were purchased from Sigma. Other taxane standards (purity > 96%) used in this study including 9-dihydro-13-acetylbaccatin III (DHB), 10-deacetyl-7-xylosylpaclitaxel (DAXP), 10-deacetylpaclitaxel (DAP), and cephalomannine were purchased from Shanghai Jinhe Bio-technology Co., Ltd. *T. canadensis* and *T. baccata* needles were provided by Shanghai Jinhe Biotechnology Co., Ltd. and *T.* × *hunnewelliana* needles were collected from the University of Toronto, Canada. *T. wallichiana* samples were collected from wild trees in Tibet and other plant samples were collected from several yew plantations in China (**Table 1S**). All plant samples were collected from trees whose age was known. Representative samples of each species from different locations were identified by Dr. Dacheng Hao (Dalian Jiaotong University) based on morphological characters and molecular markers including 18S rDNA and internal transcribed spacer [19].

Sample preparation

Yew needles were separated from stem and yew hair roots (i.d. ≤ 2 mm) and washed with water to remove soil before drying. Yew materials were air-dried (≤ 50 °C), ground to fine particles and sieved through a 40-mesh screen. The extraction and sample preparation were reported previously [14,20]. In brief, each sample (2.5 g) of powdered yew material was extracted with methanol (12.5 mL) by ultrasonication at room temperature for 30 min after 4 h immersion. The extraction was repeated three times, and the methanolic extracts were combined and concentrated *in vacuo*. The extract was then dissolved in methanol: water (2 : 1, 7.5 mL) and twice washed with hexane (2 × 7.5 mL). The aqueous layer was then thrice extracted with dichloromethane (DCM, 3 × 10 mL) after adjusting the ratio of methanol: water to 1 : 1. The DCM phase was combined and concentrated *in vacuo*. The residue was dissolved in methanol, diluted to a 25-mL volumetric flask as sample solution and filtered through a 0.22 µm filter before UFLC analysis.

Instruments and analytical conditions

All assays were performed on a Shimadzu Prominence UFLC system equipped with a CBM-20A communications bus module, an SIL-20ACHT autosampler, two LC-20AD pumps, a DGU-20A3 vacuum degasser, a CTO-20AC column oven, and an SPD-M 20A diode array detector. A Shim-pack XR-ODS (100 mm × 2.0 mm, 2.2 µm; Shimadzu) analytical column with an ODS guard column (5 mm × 2.0 mm, 2.2 µm; Shimadzu) was used and kept at 50 °C. The mobile phase consisted of water (A) and CH_3CN (B). The following gradient condition was used: 0–11 min, 69–52.5% A; 11–12 min, 52.5–5% A; 12–15 min, 5% A; and 15–18 min, 69% A. The flow rate was set at 0.4 mL/min, and the injection volume was 1 µL. DAD detection was achieved in the range of 190–370 nm, and the wavelength was set at 230 nm for quantitative analysis.

Mass detection was performed on a Shimadzu LCMS-2010EV instrument with an ESI interface both in positive and negative ion modes (ESI-) from *m/z* 100 to 1100. The detector voltage was set at + 1.55 kV and − 1.55 kV for positive and negative ion detection, respectively. In order to obtain potential fragment ions, the curved desolvation line (CDL) temperature and the block heater temperature were both set at 250 °C, while the CDL voltage was set at 40 V. Other MS detection conditions were as follows: inter-

Table 1 Retention times (t_R), UV λ_{max} values, molecular weights (MW), and product ions in negative ion mode for seven major valuable taxanes.

t_R (min)	Identification	λ_{max} (nm)	MW	Product ions in negative ESI, *m/z* (relative intensity, %)
1.94	10-deacetylbaccatin III	230.2	544	579 (100), 543 (22), 391 (8)
3.73	baccatin III	231.2	586	621 (100), 585 (6)
4.92	9-dihydro-13-acetylbaccatin III	228.6	630	665 (100), 629 (6), 587 (61)
6.88	10-deacetyl-7-xylosylpaclitaxel	230.8	943	978 (78), 942 (8), 284 (100)
7.94	10-deacetylpaclitaxel	233.3	811	846 (100), 810 (51), 658 (6), 501 (12), 284 (8)
9.89	cephalomannine	231.5	831	866 (100), 830 (6), 525 (48)
10.61	paclitaxel	233.2	853	888 (100), 852 (6), 525 (52)

face voltage, 4 kV; nebulizing gas (N_2) flow was 1.5 L/min, and the drying gas (N_2) pressure was set at 0.06 MPa. Data processing was performed using the LCMS Solution version 3.41 software (Shimadzu).

Validation of the quantitative analysis

Calibration curves: Every taxane stock solution was prepared by dissolving the standard in methanol with seven different contents for establishing calibration curves. The calibration curves were constructed by plotting the peak area versus the corresponding content, and each calibration curve data point represented the mean of triplicate injections.

Limits of detection and quantification: A mixed solution containing seven taxanes was diluted with methanol to a series of diluted solutions with appropriate concentrations, and these samples were injected for UFLC-DAD analysis. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the lowest amount of analyte that gives a peak with a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Precision, stability, and recovery: The intraday precision was determined by three standard solutions with certain concentrations for six times within one day, and the inter-day precision was evaluated with nine injections of standard solutions in three consecutive days. Stability test was performed with two samples (a needle extract of LJ001 and one root extract of LJ001R) on three consecutive days. The recovery was evaluated by adding three standards (DAB, DAP, and paclitaxel) with known amounts to a plant sample (LJ001R) and calculated with the formula:

$$\text{recovery}\,(\%) = (\text{amount detected} - \text{original amount})/\text{amount spiked} \times 100\%$$

Supporting information

The detailed information on yew samples and the explanations of the major fragment ions of several taxanes are available as Supporting Information.

Results and Discussion

The identification of paclitaxel and other valuable taxanes is very crucial for the screening and quality evaluation of various yew materials, since numerous taxanes with similar molecular weights and retention behaviors exist in yews [16]. In this study, seven target taxanes can be assigned by comparison of their retention times, UV spectra, and MS spectra with standards (**Table 1**). The maximum absorption wavelengths of these taxanes are near to 230 nm, indicating that they are normal taxanes with a 6/8/6/4 cyclic skeleton, which can be used to differentiate normal taxanes and taxines [13]. More importantly, mass spectra (in particular negative ESI-MS spectra) provide vital evidence for the assignment of taxane analogues. With a negative ESI, most taxanes can produce two molecular ions including $[M + 2H_2O\text{-}H]^-$ and $[M - H]^-$ for molecular mass determination, while some informative fragment ions also can be detected under in-source collision-induced dissociation conditions (**Table 1**). These fragment ions are very helpful for the assignment of taxanes with diverse structures, especially for normal taxanes with ester substitutes [14, 21]. For instance, paclitaxel (10.61 min) and cephalomannine (9.89 min) have a remarkable fragment ion at *m/z* 525, which corresponds to the loss of one mole AcOH combined with the cleavage at the C-1′ ester bond (**Fig. 1S**). DAP (7.94 min) has two remarkable molecular ions (*m/z* 846 and *m/z* 810) and several fragment ions including *m/z* 658, *m/z* 501 and *m/z* 284 (**Fig. 2S**), in which *m/z* 501 can be assigned as the fragment ion after cleavage of the C-13 side chain and the C-4 acetyl group of DAP, and the *m/z* 284 corresponds to the fragment ion of its C-13 side chain [14]. DAXP (6.88 min) can produce a dominant product ion *m/z* 284 corresponding to the fragment ion of its C-13 side chain, which can be used to identify 10-deacetyl-7-xylosyltaxanes combined with their own molecular ions (**Fig. 3S**). For two important paclitaxel precursors without a C-13 side chain, DAB (1.94 min) has a fragment ion of *m/z* 391 (**Fig. 4S**), indicating it undergoes successive neutral losses of one mole BzOH and one mole HCHO (the cleavage of the oxetane ring), while DHB (3.73 min) can produce a fragment ion of *m/z* 587 corresponding to loss of CH_2CO from an acetyl group [22]. With the help of these product ions, as well as retention behaviors of taxanes on reversed-phase column [18], all target taxanes can be rapidly identified by comparison of standards.

Using the UFLC-based method, seven target taxanes can be eluted with satisfactory resolution within 12 minutes (**Fig. 2**), while the time needed for the column regeneration is shortened remarkably compared with conventional HPLC. The detection sensitivity is also improved by the UFLC system; LODs of seven selected taxanes were less than 0.3 ng (**Table 2**). Most target taxanes can be quantified with good linear regression ($R^2 \geq 0.9998$) over the range from 1.0 to 200 ng, indicating that the UFLC-DAD method is precise and sensitive for the simultaneous determination of major valuable taxanes from yew materials. The quantitative method has been validated based on the variations of both retention times and peak areas. The overall intraday variations of retention times and peak areas are below 1.0% and 2.8%, respectively, and the inter-day variations of retention times and peak areas are less than 1.56% and 4.03%, respectively. It is also found that the analytes in the sample solutions are stable within three days with acceptable RSD values (< 5.0%). In addition, the recovery also has been evaluated with satisfying results (> 94%) of three taxanes standards. All of these results indicate that the proposed UFLC-DAD method is valid and applicable for the target analysis of trace constituents from *Taxus* samples.

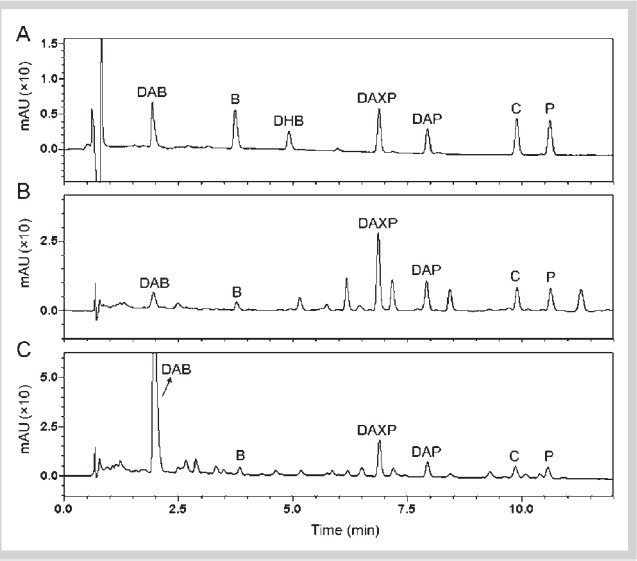

Fig. 2 Representative UFLC-DAD chromatograms of (**A**) mixture of seven standards, (**B**) root extract of *T. yunnanensis* and (**C**) needle extract of *T. yunnanensis*. The detection wavelength was set at 230 nm.

Qualitative analysis reveals that DAXP can be detected from yew roots and needles of four endemic *Taxus* species (*T. yunnanensis, T. mairei, T. chinensis, T. wallichiana*) in South Asia, while DHB only exist in needles of *T. canadensis*. In addition, DAB, DAP, cephalomannine, and paclitaxel appeared both in the needle and root extracts from most *Taxus* species. The average contents of seven taxanes in the needle and root extracts from various *Taxus* species are displayed in ◉ **Table 3**. It is evident that distribution and content of valuable taxanes in yew needles vary significantly, suggesting that different developmental strategies should be utilized for various yew needle resources, based on the distribution of their valuable taxanes.

The most important semisynthetic precursor of taxane drugs, DAB, appeared with high yields (> 1000 µg/g dried needles) in needles of several *Taxus* species including *T. yunnanensis, T.* × *media*, and *T. baccata*, therefore these species were promising resources for supply of DAB. DAXP was found as a major taxane (> 400 µg/g dried needles) in needles of *T. yunnanensis, T. mairei*, and *T. chinensis*; thus, these species have to be regarded as useful resources for the isolation of DAXP. Relatively high contents (300–500 µg/g dried materials) of paclitaxel were detected in needles of *T.* × *media*, as well as roots of most yew species, therefore it makes sense to isolate paclitaxel from these yew resources. In addition, the isolation and utilization of DHB was crucial for the development of *T. canadensis* as a resource, since DHB was the most abundant constitute in needles of this species.

In contrast to yew needles, hair roots from different *Taxus* species have consistent chemical profiles [14]. It also can be seen from ◉ **Table 3** that *Taxus* hair roots have consistent chemical distribution, and all root samples contain paclitaxel and five paclitaxel-equivalent taxanes (compounds **3**–**8** except DHB). The consistent chemical distribution implies that yew root resource can be processed by an identical developmental scheme without consideration of species origins. Notably, DAXP, paclitaxel, and cephalo-

Table 2 Calibration curve, linear range, and precision of seven major valuable taxanes.

Analytes	Linear regression				Precision	
	Calibration curves	Correlation coefficient	Linear range (ng)	LOD (ng)	Intraday R.S.D. (%)	Inter-day R.S.D. (%)
DAB	Y = 3123.8X + 520.74	0.9998	1.0–200	0.25	0.82	0.99
B	Y = 3132.2X − 361.61	0.9999	1.0–200	0.25	2.15	3.72
DHB	Y = 1712X − 335.56	1.0	2.0–200	0.50	1.92	2.56
DAXP	Y = 3770X − 1043.2	1.0	1.0–200	0.20	2.77	3.87
DAP	Y = 4780X − 553.52	0.9999	1.0–200	0.20	2.80	4.03
C	Y = 4282.4X − 955.59	1.0	1.0–200	0.25	0.96	1.74
T	Y = 4009.8X − 1366.6	0.9999	1.0–200	0.29	0.64	0.88

Table 3 The average contents of seven valuable taxanes in needles and roots from various *Taxus* species.

Species	Plant part	Sample number	Average content (µg/g dried material)						
			DAB	B	DHB	DAXP	DAP	C	P
T. yunnanensis	needle	6	1034	121	ND	996	454	69	112
	root	4	217	120	ND	485	780	377	448
T. mairei	needle	51	280	71	ND	470	248	79	163
	root	33	149	269	ND	507	403	247	367
T. chinensis	needle	2	272	42	ND	412	267	147	248
	root	1	104	135	ND	277	311	284	378
T. wallichiana	needle	3	227	59	ND	120	115	68	116
	root	3	110	100	ND	1618	490	74	233
T. × *media*	needle	10	1661	205	ND	ND	421	254	435
	root	8	165	81	ND	587	267	248	463
T. cuspidata	needle	6	47	ND	ND	ND	97	125	249
	root	3	155	75	ND	292	200	109	160
T. canadensis	needle	3	253	ND	479	ND	77	62	194
T. baccata	needle	2	1961	72	ND	ND	225	77	99
T. × *hunnewelliana*	needle	1	100	ND	ND	ND	198	53	67

* ND means not detected or lower than LQD (limit of quantitative detection)

mannine are distributed in the yew hair roots with relative high contents, thus it is very promising to utilize these valuable taxanes for production of taxane drugs. In addition, the interspecific variation in the contents of six target taxanes in the yew hair roots is lower than the corresponding inter-specific variation in the needles in which the average content of DAB ranges from 47 (*T. cuspidata*) to 1961 (*T. baccata*) μg/g dried needles among various species, while the content of paclitaxel ranges from 67 (*T.* × *hunnewelliana*) to 435 (*T.* × *media*) μg/g dried needles.

It should be noted that the large quantitative intraspecific variations also exist among individual samples within the same species, although their chemical profiles are similar. In this study, 51 needle samples and 33 root samples, collected from different *T. mairei* trees at nine locations in different seasons (**Table 1S**), can be used to evaluate the intraspecific variations in contents of paclitaxel and other valuable taxanes. Quantitative analysis of *T. mairei* needle samples reveals significant variations in contents of DAB (32–679 μg/g dried needles) and DAXP (161–672 μg/g dried needles), and relatively large variations of DAP (147–428 μg/g dried needle), cephalomannine (40–171 μg/g dried needle), and paclitaxel (38–264 μg/g dried needle). In hair root samples of *T. mairei*, the large quantitative variations in DAP (131–747 μg/g dried hair root), DAXP (193–736 μg/g dried hair root), and T (87–400 μg/g dried hair root) are also found. The large intraspecific variation in the content of taxanes could be attributed to many factors including the growing conditions, the age, and the sex of the tree, as well as the collecting season of the sample [23, 24]. These findings suggested that it is necessary to quantify the valuable taxanes in each batch of yew materials before manufacturing.

In summary, this paper describes the use of ultra-fast liquid chromatography (UFLC) with MS and UV detection for the simultaneous determination of paclitaxel and its six semisynthesis precursors in needles and hair roots from various *Taxus* species. More than a hundred plant specimens were analyzed with this method, and the results showed significant variations both in distribution and content of valuable taxanes, depending upon the specific *Taxus* species and the investigated plant part.

Acknowledgements

The authors are grateful to Dr. Hai-Tang Wang, University of Waterloo, Canada, Mr. Yong-Hua, Liu Chayu forestry bureau, Tibet, China, Mr. Ming-Lin He, Fang County of the Hubei Province, China, and Mr. Li-Ming Chen, Lishui City of the Zhejiang Province, China, for sample collecting. This project was supported by the 863 program (2009AA02Z205), the National Natural Science Foundation (30973590) of the Ministry of Science and Technology of China, and the Key Direction Program (KSCX2-YW-G-050) of the Chinese Academy of Sciences.

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