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Short communication

Analysis of taxol and major taxoids in Himalayan yew, *Taxus wallichiana*

Shashi Bala[a], G.C. Uniyal[a,*], S.K. Chattopadhyay[b], V. Tripathi[b], K.V. Sashidhara[b], M. Kulshrestha[b], R.P. Sharma[b], S.P. Jain[c], A.K. Kukreja[d], S. Kumar[d]

[a]*Analytical Instrumentation, Central Institute of Medicinal and Aromatic Plants (CIMAP), P.O. CIMAP Lucknow 226015, India*
[b]*Phytochemical Technology Division, Central Institute of Medicinal and Aromatic Plants (CIMAP), P.O. CIMAP Lucknow 226015, India*
[c]*Botany Division, Central Institute of Medicinal and Aromatic Plants (CIMAP), P.O. CIMAP Lucknow 226015, India*
[d]*Genetic Resources and Biotechnology Division, Central Institute of Medicinal and Aromatic Plants (CIMAP), P.O. CIMAP Lucknow 226015, India*

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Abstract

A reversed-phase column liquid chromatography method for the analysis of taxol, 10-deacetylbaccatin III, baccatin IV, 1-hydroxybaccatin I, 2-acetoxybrevifoliol, brevifoliol, 2′-deacetoxydecinnamoyltaxinine J and 2′-deacetoxytaxinine J in yew needles has been developed using a Nova-Pak Phenyl column and a binary gradient profile. The various aspects of analysis such as extraction efficiency, detection limits, reproducibility and peak purity were validated using UV–Vis as well as photodiode array detection.

Keywords: *Taxus wallichiana*; Plant materials; Taxol; Taxoids

1. Introduction

Taxol, a promising anti-cancer drug, is in great demand for clinical use [1,2]. This has led to the discovery of alternative sources of taxol. A number of *Taxus* spp. as well as their cell cultures have been subjected to intensive research in the search for this drug [2–18]. Besides, efforts are made to find alternative analogues from more common *Taxus* species which may have improved activity, less toxicity and better solubility in water. As part of these studies, a number of compounds were isolated and characterised from *Taxus wallichiana* at this institute [15–17].

A search of the literature shows that for quick screening of taxol and related taxanes in plant extracts as well as in cell cultures, reversed-phase column liquid chromatography (RPLC) has been employed using several stationary and mobile phases, detection modes, extraction procedures and techniques. A number of high-performance liquid chromatography (HPLC) analyses are reported for the analysis of plant material or cell cultures of *T. brevilofolia*, *T. baccata*, *T. canadensis*, *T. cuspidata*, *T.X media*, *T. X media Nigra*, *T. X media Hicksii*, *T. X media Densiformis*, *T. cuspidata capitata*, *T. chinensis*, *T. floridana*, *T. yunnanensis* [3,4,18–27],

*Corresponding author.

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but none of these deal with the chemical analysis of *T. wallichiana*. Most of the HPLC analyses reported are related to the analysis of taxol and its precursors, cephalomannine and its precursors, 10-deacetylbaccatin III and baccatin III, but none of them deal with the identification of other taxoids present in the needles of these plants (Fig. 1). The possible co-elution of cinnamoyl taxanes with taxol was taken into account by different workers [11,20,23,24]. In the present study, a reliable, simple and reproducible method for the extraction of taxol and other taxoids and their quantitative estimation in the needles of *T. wallichiana* has been developed. The analyses were performed on Symmetry C_{18}, Curosil-B and Nova-Pak Phenyl columns, using different mobile phases and gradient profiles. It was found that the best resolution was achieved on the Nova-Pak Phenyl column for these compounds. Photodiode array (PDA) detection was also used for the peak identification, peak homogeneity and peak purity of each individual compound by comparing the PDA-generated UV spectra with those of reference compounds using library matching as the retention times and spiking technique may lead to ambiguous assignments.

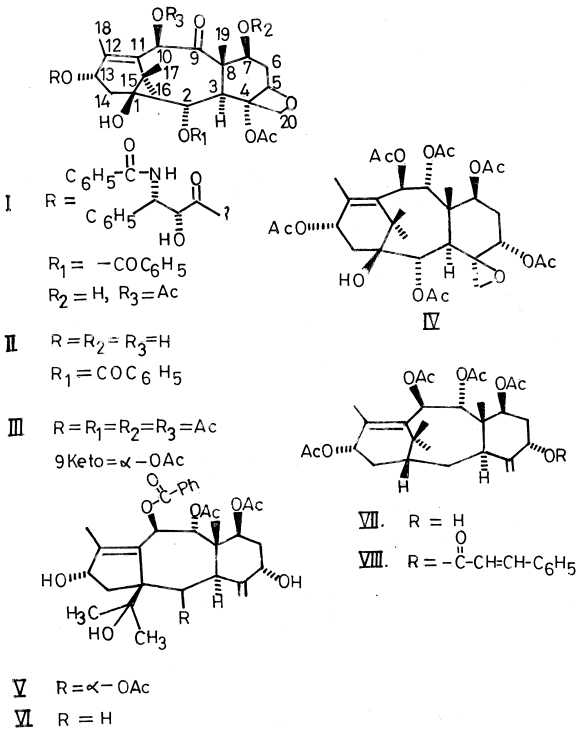

Fig. 1. Structures of taxol (**I**), 10-deacetylbaccatin III (**II**), baccatin IV (**III**), 1-hydroxybaccatin I (**IV**), 2-acetoxybrevifoliol (**V**), brevifoliol (**VI**), 2′-deacetoxydecinnamoyltaxinine J (**VII**) and 2′-deacetoxytaxinine J (**VIII**).

2. Experimental

2.1. Chemicals and reagents

All the standards of taxol, 10-deacetylbaccatin III (10-DAB III), baccatin IV, 1β-hydroxybaccatin I, brevifoliol, 2-acetoxybrevifoliol, 2′-deacetoxytaxinine J and 2′-deacetyldecinnamoyltaxinine J were isolated and characterised at CIMAP [15–17]. The purity of each compound was ascertained by HPLC–PDA. The 10-DAB III and taxol were 98% pure. Methanol and acetonitrile used were of HPLC-grade (Omnisolv, EM Science, USA). The distilled water was prepared from deionised water which was subjected to double distillation through a quartz distillation apparatus at the institute. The water was filtered through a 0.45-μm filter before use.

2.2. Equipment

HPLC analysis was done on a Waters modular system consisting of two 501 pumps, an automated gradient controller, a U6K injector, an in-line degasser, a 484 tunable absorbance detector, a 996 photodiode array detector and a Millennium 2010 chromatography manager. The injector, gradient controller and Millennium 2010 chromatography manager were integrated to give reproducible results. Nova-Pak C_{18} (4 μm, 150×3.9 mm, Waters, USA), Symmetry C_{18} (5 μm, 150×3.9 mm, Waters), Nova-Pak Phenyl (4 μm, 150×3.9 mm, Waters) and Curosil-B (3 μm, 250×4.6 mm, Phenomenex, USA) columns were used for the analysis and the spectral analysis was carried out at 228 nm.

2.3. Plant material

The needles of *T. Wallichiana* were collected from west Kameng District, Arunachal Pradesh and Darjeeling District of India, in November 1997. The plant samples were identified by the botany depart-

ment of our institute and specimens have been deposited at the institute's herbarium (CIMAP Field Nos. 8759, 8754, 8747 and 8760).

2.4. Sample extraction and clean-up

A 20-g amount of green needles of *T. wallichiana* was crushed and extracted with 100 ml (×3) of methanol for 48 h at room temperature (25°C). The aliquots were pooled together and concentrated under reduced pressure and stirred with water (50 ml) and extracted with hexane (3×60 ml). The hexane portion was discarded. It was then extracted with chloroform (3×60 ml) and ethyl acetate (3×60 ml), successively. The chloroform extracts were pooled together and dried over anhydrous sodium sulphate, concentrated and redissolved in methanol–chloroform (1:1, 100 ml). The resulting solution was filtered through a Waters sample clarification kit (organic) and concentrated. The ethyl acetate extract did not contain any taxoid but it contained phenolic compounds. A 10-mg amount of the chloroform extract was dissolved in acetonitrile (1 ml) and subjected to HPLC analysis. Alternatively, the methanol extract was also extracted with dichloromethane instead of chloroform as above and it was found that the recovery yield of taxoids was better by 7% in the case of chloroform, as more taxoids were extracted by chloroform.

2.5. HPLC conditions

2.5.1. Solvent system I

Solvents: (A) Methanol–acetonitrile–water (20:5:75, v/v), (B) methanol–acetonitrile–water (20:45:35, v/v). Initial conditions: 80% A at an initial flow-rate of 0.8 ml/min for the first 15 min, then changing to 60% A at 20 min at the same flow-rate and then changing to 40% A at 30 min at a flow-rate of 1 ml/min. At 40 min solvent B was 100% at a flow-rate of 1 ml/min, held until 50 min, and then changing to 80% A over 5 min and allowing 10 min for the equilibration of the column. Thus the total run time per sample was 65 min A linear gradient profile was used throughout the analysis at a temperature of 27°C.

2.5.2. Solvent system II

The composition of solvents A and B was as in system I but a concave gradient (3) was used throughout the analysis. The other parameters were as above.

2.5.3. Solvent system III

Solvents: (A) methanol–acetonitrile–water (20:5:75, v/v), (B) methanol–acetonitrile–water (30:35:35, v/v). Initial conditions: 80% A at a flow-rate of 0.8 ml/min changing to 60% A at 20 min at a flow-rate of 1.0 ml/min, changing to 47% A at the end of 26.0 min, 20% A at 31.0 min and 100% B at 37.0 min at a flow-rate of 1.2 ml/min until 45.0 min. A concave curve (profile 3) was used throughout the analysis.

The spectral acquisitions were done at 228 nm. Injection size for standard and sample was 20 μl each. Calibration curves for each standard in different concentration ranges were plotted by using computer software and were found to be linear in the range of 5 μg/ml to 50 μg/ml for all compounds.

3. Results and discussion

For quick screening of *Taxus* spp. for taxol content a number of methods are reported. Most of the extraction methods published so far are for dried or vacuum dried needles, grounded and extracted with chloroform or dichloromethane–methanol, followed by evaporation of solvents and partitioning in CH_2Cl_2–water and subjecting the dichloromethane-soluble compounds to HPLC analysis; this resulted in co-extraction of chlorophyll, high back pressure and short column life [3,4,21]. Recent methods, however, reported the use of ethanol–water–acetic acid (80:19:1) as extraction solvent followed by further purification on a polypropylene solid-phase extraction column. In yet another method the extracted methanol aliquot was diluted with 5% saline solution followed by washing with hexane, partitioning with dichloromethane and clean-up of the CH_2Cl_2 fraction on a dry-packed silica column [21,24]. All these reported methods are time consuming. Therefore the main objective of this work was to devise a more simple, economically feasible, fast and reproducible method which can be used by a less-

experienced analyst and with an extraction procedure that yields better recoveries of the more polar taxanes without high back pressure during analysis.

The analytical parameters were selected after screening a number of solvent systems, gradient profiles and reversed-phase adsorbents such as C_8, C_{18}, phenyl, diphenyl, cyano and other special phases like Curosil which is used for the analysis of taxanes present in bark, leaves, roots, needles, callus extracts and cell suspensions of different plant species as reported earlier [22,27]. The initial analysis was done on a Curosil-B (Phenomenex, 3 μm), taxol column as reported earlier [26] but the desired resolution was not achieved for compounds **V** and **VI** while compound **VIII** did not elute at all from the column. This led us to consider other mobile phases and gradient profiles to achieve the required resolution for all the eight compounds.

To start with, resolution of seven compounds was achieved on the Curosil-B column by using solvent system I (Fig. 2A), but the last compound – much less polar – did not elute. Decreasing the polarity of

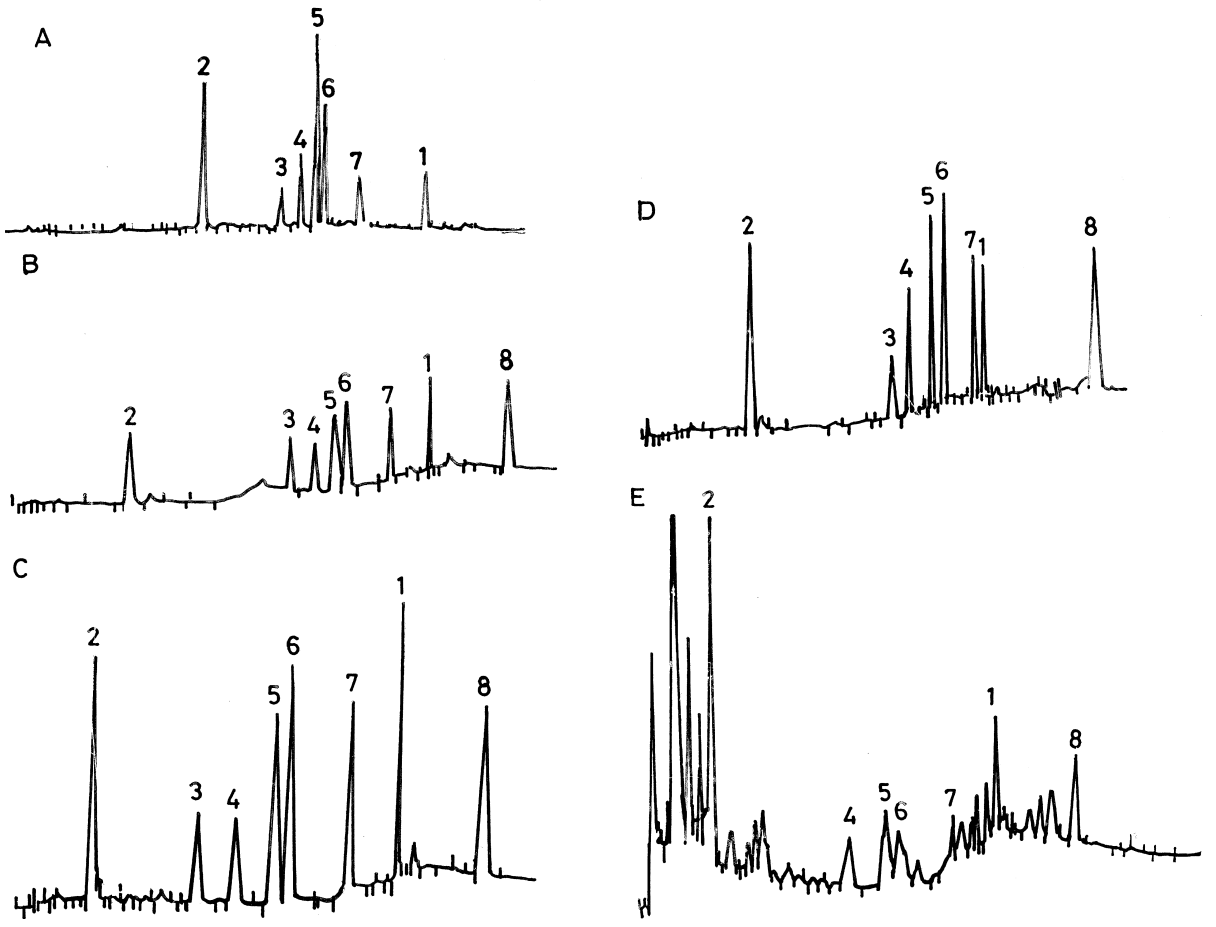

Fig. 2. (A) HPLC chromatogram of standards on Curosil-B column. Analytical conditions as in system I. t_R values for compounds 1–7; 40.52, 18.92, 26.62, 28.51, 29.97, 30.81, 34.21 min, respectively. (B) HPLC profile of standards on Nova-Pak Phenyl column. Analytical conditions as in (A). t_R values for compounds 1–8; 38.75, 10.76, 25.69, 28.01, 29.92, 31.01, 35.04, 45.83 min, respectively. (C) HPLC profile of standards on Nova-Pak Phenyl column using solvent system III. t_R values for compounds 1–8; 33.78, 6.95, 16.89, 20.53, 23.66, 24.89, 29.78, 41.57 min, respectively. (D) HPLC profile of standards on Symmetry C_{18} column. Analytical conditions as in (C). t_R values for compounds 1–8; 37.30, 11.59, 27.08, 29.03, 31.44, 32.74, 35.82, 69.87 min, respectively. (E) HPLC chromatogram of crude extract of *T. wallichiana*. Analytical conditions as in (C). Peaks: 1=taxol, 2=10-DAB III, 3=baccatin IV, 4=1-hydroxybaccatin I, 5=2-acetoxybrevifoliol, 6=brevifoliol, 7=2′-deacetoxydecinnamoyltaxinine J, 8=2′-deacetoxytaxinine J.

mobile phase resulted in the merger of compounds **V** and **VI** though compound **VIII** could be eluted in 60 min. Thus the column was not considered suitable for the analysis of taxoids present in *T. wallichiana*.

Then an attempt was made to resolve all the compounds by using a Nova-Pak Phenyl column (4 μm, 150×3.9 mm, Waters) as reported by Theodoridis et al. [27]. Under identical conditions we could not achieve resolution for compounds **IV** and **V** as they co-eluted though baseline resolution was achieved for all other compounds. The analysis time was also reduced by 12 min as the last compound eluted in 29.35 min but the method could not be considered suitable as the desired resolution was not achieved for all compounds. This led us to consider other mobile phases and baseline resolution was achieved for all the eight compounds within 46 min using solvent system I (Fig. 2B). Attempts were also made to reduce the analysis time by changing the gradient profile (concave curve 3) but sufficient resolution could not be achieved for the last compound though the elution pattern of compounds **III** to **VI** changed drastically. In the final step the composition of solvent B was decreased and solvent system III was used for the analysis. This resulted in a decrease of the retention time of compound **VIII** by 6 min without affecting the resolution of other compounds (Fig. 2C). Taking into account the recent analytical method reported by Van Rozendaal et al. [24] for the analysis of taxol, the analysis was also carried out on Nova-Pak C_{18} (4 μm, 150×3.9 mm, Waters) and Symmetry C_{18} (5 μm, 150×3.9 mm, Waters) columns but we could not achieve the desired resolution for all the compounds under identical conditions. However, when solvent system III was used with the Symmetry C_{18} column the desired resolution for all eight compounds was achieved but the retention time for compound **VIII** was too large, i.e., 69.87 min (Fig. 2D).

The most interesting finding of this work is that the retention behaviour of taxol remained almost unaffected by changing the mobile phases and the gradient profiles as the t_R values were 40.52, 38.75, 33.83 and 37.30 min on Curosil-B, Nova-Pak Phenyl and Symmetry C_{18} columns, as is evident from Fig. 2A–D. The best baseline resolution was achieved on the Symmetry C_{18} column but the retention time of the last compound, i.e., 2-deacetoxytaxinine J was too large and is not suitable for quick scanning of a large number of samples. The analysis of crude plant extract was also done under identical conditions as reported for standards on these columns. The crude extract was a complex sample matrix and optimum resolution was required to identify each compound. The best resolution for all the peaks, except peak 6 was achieved using the Nova-Pak Phenyl column and solvent system III (Fig. 2E). In case of peak 6 (t_R 24.56 min) an unknown peak (t_R 25.03 min) co-eluted along the downslope of the peak 6 but was integrated separately and did not interfere in quantitative estimation of peak 6. Hence, the analytical method reported here using a phenyl column and solvent system III is best suited for the analysis of a large number of samples for taxol and related compounds present in *T. wallichiana*. The analytical method was applied for the analysis of 90 samples and the results were reproducible.

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