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Analysis of taxol, 10-deacetylbaccatin III and related compounds in *Taxus baccata*

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Abstract

A method is described for the analysis of taxol and related taxanes in the foliage of *Taxus* spp. such as *T. baccata*. The method has been optimised for recovery and analysis of a range of taxanes of differing polarity, namely: taxol, cephalomannine, baccatin III and 10-deacetylbaccatin III (10-DAB-III). Measured recoveries for all these compounds generally exceeded 84%. The reversed-phase high-performance liquid chromatography system developed uses a "sterically protected" C_8 column, and allowed analysis of the components of interest with minimum interference from co-extracted compounds. Typical detection limits for routine operation were in the range of 1–10 mg/kg depending on the component and the particular sample. Analysis of test samples of *T. baccata* have shown variations in taxol concentrations in the range 7–510 mg/kg and of 10-deacetylbaccatin III in the range 52–1185 mg/kg.

1. Introduction

Taxol is a diterpenoid natural product showing distinct promise as an anti-cancer agent [1]. It is derived from many *Taxus* spp., including *T. brevifolia*, *T. canadensis*, *T. cuspidata*, *T. media* and *T. baccata* [2,3]. The compound has been isolated from bark, needles and small twigs, and more recently, from tissue cultures [4]. Typical concentrations of taxol vary between species, and major differences can be found between samples of the same species and depending on locality and environmental factors [3,5]. The high demand for taxol for clinical use has led to intense study of other forms of production, including the sustainable harvesting of *Taxus* spp. and partial synthesis from related compounds. Numerous related compounds have been identified, including those more commonly cited such as cephalomannine, 10-deacetylcephalomannine, baccatin III and 10-deacetylbaccatin III (10-DAB-III). Structures for taxol and 10-DAB-III are shown in Fig. 1.

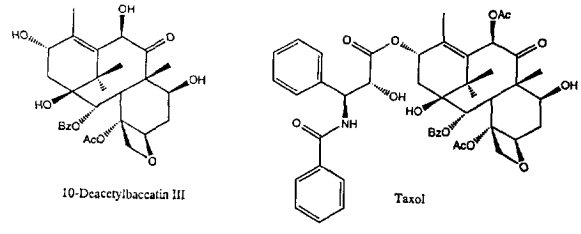

Fig. 1. Structures of two major taxanes found in *T. baccata*.

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As a part of studies of production of taxanes by *T. baccata* from throughout New Zealand, we desired to analyse for taxol, cephalomannine, baccatin III and 10-DAB-III. The main compounds of interest were taxol itself and 10-DAB-III, a useful precursor of the taxol-analogue taxotere and which is reported to be present in *T. baccata* at higher concentrations than taxol [2].

The most commonly used method for analysis of taxanes in plant extracts is high-performance liquid chromatography (HPLC). Methanol [4–8] or methanol–dichloromethane [2,3,9] have been the common solvents of choice for extracting taxanes from plant samples, and earlier published methods [2,5,6] relied on direct analysis of relatively crude plant extracts, generally after simple partition of dried extract between dichloromethane and water. While useful comparative data was presented, problems were reported with high backpressure, short column life and interference from co-extractives. More recent methods have used preliminary hexane washes of the plant material [3,9], or silica [7] or C_{18} solid-phase systems [4,8] for additional clean-up. Our experience was that the more simple procedures, including those with hexane washes, yielded chromatograms with high background baseline absorbance. Accurate quantitation was difficult, especially for samples with low taxane content. Also, the published silica and C_{18} clean-up methods had been optimised for taxol and the closely related cephalomannine, and accordingly were not suitable for other, more polar compounds of interest, such as 10-DAB-III.

The principal purpose of this work was to develop a reliable method for the clean-up of *T. baccata* extracts prior to analysis for taxanes of different polarity. Methanol was chosen as the extraction solvent, and we have adapted a partition clean-up procedure commonly used in our laboratories for the analysis of a number of trace pesticide residues in plant extracts, and combined that with a modification of the silica column clean-up method for taxol described by Castor and Tyler [7]. The analytical system using gradient HPLC developed employs a "sterically protected" C_8 reversed-phase column. This is the first report to describe the use of this type of column for analysis of taxanes.

2. Experimental

2.1. Chemicals and reagents

Standards of taxol, cephalomannine and baccatin III were donated by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA). 10-DAB-III was purchased from Sigma (St. Louis, MO, USA). All solvents were either Nanograde (Mallinckrodt) or HPLC grade. Davisil silica (pore size 60 Å, particle size 149–250 μm) was supplied by Alltech (Deerfield, IL, USA).

2.2. Equipment

HPLC was performed on a Shimadzu LC-6A gradient system fitted with a 6AG mixing chamber, SCL-6A autoinjector, CTO-6A column oven, SPD-6AV variable-wavelength UV–Vis absorbance detector and a C-R4AX data system. Multi-wavelength and spectral acquisitions were also obtained for some samples, using a Linear 206 PHD multiple-wavelength detector. The analytical column was a 5-μm Zorbax SB-C_8 (150 × 4.6 mm I.D.) (Rockland Technologies, Newport, DE, USA), and was preceded by a 2-μm in-line filter (Rheodyne, Cotati, CA, USA).

2.3. Sample extraction and clean-up

Foliage samples of *T. baccata* were composed of leaves and small twigs collected around the circumference of individual trees at a height of 1.4 m. The samples were separated into leaf and stem fractions, dried in a forced-air oven at 35°C, finely ground and then stored at −20°C until analysis.

Ground plant material (2 g) was placed in a stoppered 130-ml tube with methanol (100 ml)

and the mixture shaken on a flat-bed orbital shaker for 12–16 h. The mixture was allowed to settle out and clarify, then an aliquot (10 ml) was removed and diluted with 5% saline solution (10 ml). This solution was washed with hexane (2 × 10 ml) and the hexane washes discarded. The solution was then partitioned with dichloromethane (4 × 10 ml). These partition fractions were collected together by filtering each sequentially through anhydrous sodium sulphate (3 g). The combined dichloromethane fraction was evaporated to dryness, redissolved in dichloromethane (2 ml), and then added to a glass column (250 × 10 mm I.D.) dry-packed with silica (1 g). The column was washed with dichloromethane (2 × 2 ml), then with acetone–dichloromethane (4:96) (2 × 5 ml), and each of these washes discarded. Taxanes were eluted from the column with acetone–dichloromethane (50:50) (2 × 5 ml). These eluates were combined and evaporated to dryness (N_2, 35°C), and then dissolved in methanol (1 ml) for analysis by HPLC.

2.4. HPLC conditions

The analytical column was held at 35°C and the mobile phase flow-rate was 1 ml/min. Separation was achieved with a two-pump gradient programme for pump A (acetonitrile) and pump B (methanol–water, 20:80) as follows: 80% B for 5 min, then jump to 75% B at 5.1 min; hold at 75% B until 14 min then change to 65% B over 10 min and hold at 65% B until 41 min. At this time a column flush-out sequence was commenced by changing to 35% B over 3 min, holding at 35% B from 44 to 55 min, then resetting to 80% B over 5 min, and allowing 20 min for equilibration of the column and stabilisation of the baseline. Thus the total run-time per sample was 80 min. The programme delay through the system was 5–6 min from event to appearance at the detector.

The detector was set at 227 nm. Injection size for standards and samples was 10 μl. Quantitation was by peak height relative to a mixed external standard of 25 μg/ml for each component in methanol. The standard components gave responses of 60–120% of full scale deflection at 0.02 AUFS depending on the compound. The analysis solution concentration was multiplied by a factor of five to convert to mg/kg dry weight. A further multiplication factor of 0.0001 could be used to convert mg/kg to the commonly quoted units of percent of dry weight.

Spectral scanning acquisitions used 31 wavelengths at 4 nm intervals from 201 to 321 nm inclusive. Wavelength response ratios were calculated using 229 and 273 nm.

2.5. Recovery tests

Preliminary recovery tests were conducted using a mixed standard solution in methanol. Aliquots equal to 5 μg of each component were processed separately through both the partition and silica clean-up sections of the method. Each of the four partition fractions was collected and analysed separately for these preliminary tests, and silica column recoveries were tested using washes of 4:96, 20:80 and 50:50 acetone–dichloromethane, and 100% acetone. Plant extracts known to contain high levels of taxanes were also tested in these preliminary tests, and the identity of peaks of interest was confirmed by spectral scans and spiking tests.

The final recovery tests were conducted in two identical batches, processed on different days using mixed standard solutions and an extract of a very low taxane content sample of *T. baccata* both with and without spiked taxanes. These five samples plus two samples of high taxane content material were also used to determine the reproducibility of the method. In both batch tests duplicate analyses were performed on the following samples: mixed standard in methanol (5 μg each component per 10 ml), mixed standard in methanol (25 μg each component per 10 ml), low-taxane extract, low-taxane extract plus 5 μg of each component per 10-ml aliquot, low-taxane extract plus 25 μg of each component per 10-ml aliquot, extracts of two different high-taxane materials. Results for the two batches were used as one set for estimation of recoveries and repeatability of the method.

3. Results and discussion

Analysis of individual fractions from the partition clean-up step showed that the hexane wash, incorporated to remove potential interfering waxy non-polar components [3], removed less than 1.5% of the compounds of interest. The four dichloromethane extractions were necessary for high recovery of 10-DAB-III. While taxol, cephalomannine and baccatin III were typically 95–100% recovered by two dichloromethane extractions, 10-DAB-III was only 70% recovered, and required an extra two extractions to achieve about 90% recovery. Attempts were made to improve the extraction efficiency for 10-DAB-III by modifying the quantity, concentration and pH of the saline solution, by alternate extraction solvents, or by centrifuging the partition mixture. None of the options tested improved the described method.

Preliminary testing of fractions from the silica clean-up column showed that none of the peaks of interest were removed by the 4:96 acetone–dichloromethane wash, while 20:80 acetone–dichloromethane removed most of the taxol, as reported by Castor and Tyler [7]. This fraction also contained most of the cephalomannine and baccatin III, but only 10–20% of the 10-DAB-III. Washing the silica column with 50:50 acetone–dichloromethane as described in the method, removed all the compounds of interest, and none were found when a subsequent wash with 100% acetone was used. We have found that fractions off silica have a lower background baseline absorbance and are noticeably cleaner than samples through the partition clean-up only, especially in the first 10 min of the chromatogram. This is illustrated by comparing Figs. 2A and 2B.

The HPLC conditions were selected after investigation of a number of gradient options and also the alternative of isocratic analysis in two polarity groupings. Conditions were optimised to give good separation of the two major components, taxol and 10-DAB-III. In some samples interferences occurred for the minor components cephalomannine and baccatin III. For the purpose of our studies, which was to

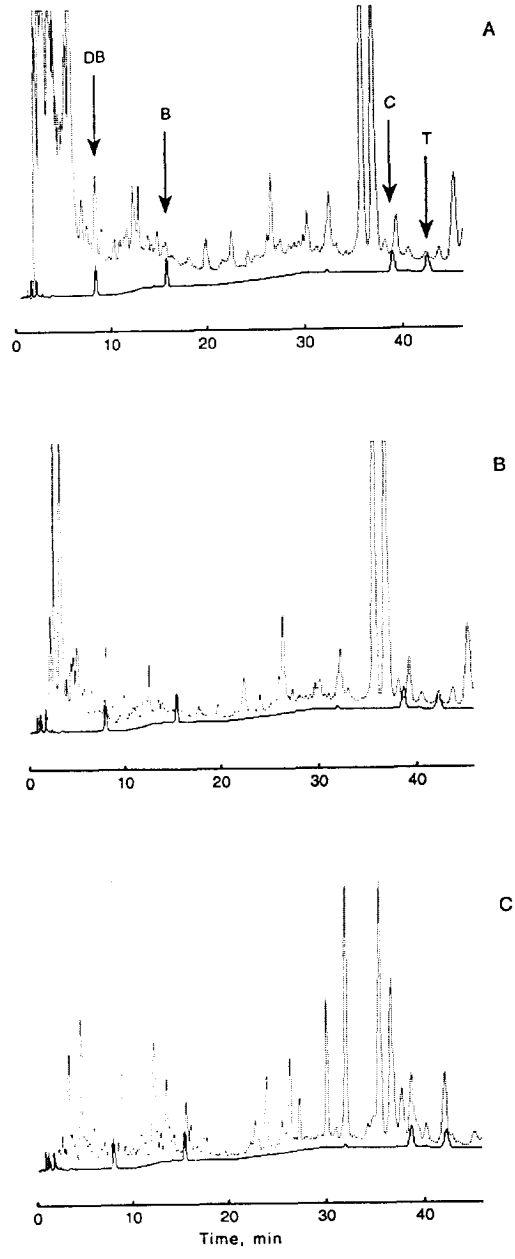

Fig. 2. HPLC chromatograms of (A) low taxane content sample after partition clean-up only and with 5 μg/ml mixed standard as overlay, showing 10-DAB-III (DB), baccatin III (B), cephalomannine (C) and taxol (T). Note that the overlay chromatogram shows the true baseline absorbance of the system. (B) as (A) but after full clean-up of sample. (C) High taxane content extract H1 after full clean-up and with 5 μg/ml mixed standard as overlay. Chromatographic conditions as in Experimental. Figure shows detection at 227 nm with 0.04 AUFS.

identify high-productive plants, it was more important to have accurate values for the major components. Retention times for the peaks of interest were: 8.0 min (10-DAB-III), 15.8 min (baccatin III), 39.0 min (cephalomannine) and 42.5 min (taxol). Sample chromatograms are shown in Fig. 2.

Previous published data suggests that there is no one HPLC column type that will separate or resolve all the taxanes completely. Most recent reports have used specialised columns with phenyl-type phases [2–4,7,8,10]. The present results show that good separations can be achieved using the "sterically protected" C_8 column. This column therefore offers another selectivity option to researchers investigating new and known taxanes from *Taxus* species. We have found the SB-C_8 column and the separation conditions to be robust. To date over 400 samples, including about 100 partly cleaned up fractions, have been run through the column without any apparent deterioration in the column characteristics or increase in backpressure. The use of an elevated column temperature and a flush-out with each sample may help in this respect.

The integrity of the peaks of interest during method development was confirmed by spiking tests and spectral scans. After development, the assignments were similarly confirmed in selected samples put through the method. These scans showed the two main peaks of interest (taxol and 10-DAB-III) to have spectra very similar to authentic standard materials, with UV maxima at about 230 nm, and little absorbance above 260 nm (see Fig. 3). Similar results were obtained for baccatin III and cephalomannine in some samples, but often these two compounds were present in too low quantities to obtain definite spectra. An alternative approach was to compare chromatograms acquired at 229 and 273 nm. Those acquired at 273 nm showed much lower response for all four peaks of interest, and therefore supported the assignments. For example, measured response ratios (A_{229}/A_{273}) in a sample were 14.5, 12.5, 18.8 and 17.0, respectively, for 10-DAB-III, baccatin III, cephalomannine and taxol. These compare with equivalent values for standards of 14.6, 13.9, 20.4 and 15.5. Wavelength response ratios have been used elsewhere [7,10] to estimate taxane peak purity in the presence of potential interfering co-extractive compounds that have strong absorbance near 280 nm.

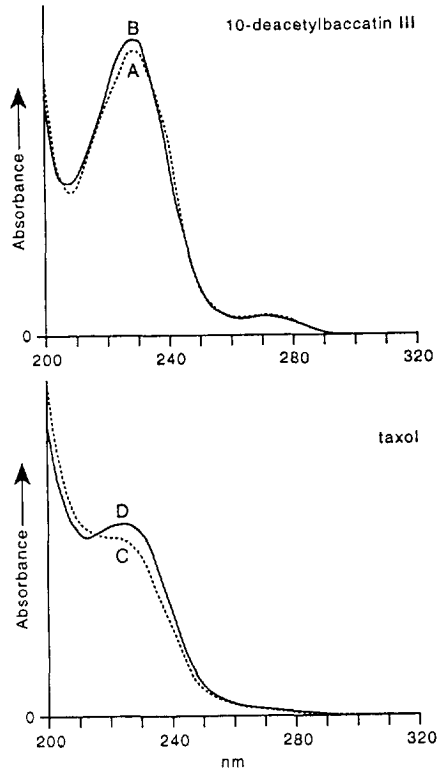

Fig. 3. Spectra for 10-DAB-III and taxol acquired during chromatography of a standard mixture (A and C) and a cleaned up sample of *T. baccata* (B and D).

The effectiveness and reproducibility of the chosen method was confirmed by replicate recovery tests on standards, on a low taxane content *T. baccata* extract with and without spikes, and on two *T. baccata* extracts containing moderately high levels of taxol and 10-DAB-III. The results are given in Table 1, and show good repeatability of results. Standard deviations are typically less than 10% of the mean value obtained. Also, the results represent recoveries better than 76% and generally better than 84%.

Detection limits for standards under routine operating conditions were below 0.13 μg/ml

Table 1
Reproducibility ($n = 4$) and recovery data for taxanes through the method[a]

Sample	Mean concentration (μg/ml in final analysis solution) for taxanes			
	10-DAB-III	Baccatin-III	Cephalomannine	Taxol
Tests for recovery and reproducibility				
5 μg/10 ml standard in methanol	4.2 (0.2)	4.8 (0.1)	4.5 (0.2)	4.6 (0.3)
25 μg/10 ml standard in methanol	19.2 (1.9)	21.5 (2.0)	22.4 (0.5)	22.3 (0.6)
Extract L (low taxane content)	10.4 (1.0)	1.1 (0.2)	n.d.	1.4 (0.2)
5 μg/10 ml spike in extract L	15.0 (0.9)	5.1 (0.1)	br.	6.2 (0.2)
25 μg/10 ml spike in extract L	32.2 (3.0)	24.6 (1.5)	24.5 (1.2)	25.1 (0.9)
Tests for reproducibility only				
Extract H1 (high taxane content)	126.1 (6.3)	8.9 (0.4)	14.7 (1.3)	21.3 (2.1)
Extract H2 (high taxane content)	70.6 (2.5)	9.0 (0.25)	22.0 (1.1)	48.0 (5.4)

[a] Samples of 10 ml of *T. baccata* extracts or standard solutions in methanol were processed through the method and then made up in 1 ml of methanol for analysis by HPLC. Expected concentrations for the two levels of standards are 5 and 25 μg/ml. The four replicates represent results from two batches of duplicate samples processed on different days. Values in parentheses represent the standard deviation.
n.d. = not detected at detection limit.
br. = broad peak. Component not resolved from adjacent co-extractive. Accurate analysis not possible.

(equivalent to 0.66 mg/kg) (signal to noise ratio at least 5), while detection limits in low-taxane samples of *T. baccata* were more likely to be in the range 1–10 mg/kg depending on the component and the particular sample. In samples analysed to date, taxol concentrations have varied from 7 to 510 mg/kg, while 10-DAB-III levels have been in the range 52–1185 mg/kg.

4. Conclusion

The method developed gives clean analytical samples and allows reliable analysis of four taxanes with very different polarity in extracts of *T. baccata*. Recently, another method was reported for the analysis of taxanes with a range of polarity in extracts cleaned up by a combination of partition and selective solubility off a solid-phase (celite) column [10]. We had earlier investigated using celite in a similar manner, and although promising results were obtained, considered that silica clean-up was potentially more adaptable. Clean-up efficiency by the two methods was similar but there were selectivity differences. Therefore, it is probable that investigation of combinations of the two phases would be useful for samples found to be intractable by either method alone.

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