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DETERMINATION OF TAXANES IN *TAXUS BREVIFOLIA* EXTRACTS BY TANDEM MASS SPECTROMETRY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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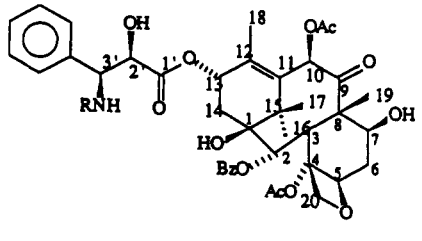
ABSTRACT.—A tandem mass spectrometric (ms/ms) method using desorption chemical ionization is described for the quantitation of taxol [**1**], cephalomannine [**2**], and baccatin III [**3**] found in *Taxus brevifolia* bark and needle extracts. A parent ion scan was used to simultaneously determine the weight percentages of **1–3** in bark and needle samples by the method of standard addition. In an alternative experiment, the concentration of **1** in the same samples was determined by ms/ms using trideuterated 10-acetyltaxol [**7a**] as an internal standard. High-performance liquid chromatography (hplc) was also used to determine the weight percentages of **1–3** in the same *T. brevifolia* bark and needle extracts with an external standard. The ms/ms method of quantitation by internal standard is the best overall method of analysis examined. With this method, **1** was quantitated in the *T. brevifolia* extracts at the low picomole level with a relative standard deviation of 17% or better for all samples analyzed with an analysis time of less than five min per sample. The precision, level of quantitation, and speed of analysis of the three methods of taxane quantitation are compared.

The ability to promote microtubule assembly which is exhibited by the natural product, taxol [**1**] (1,2), is associated with valuable cytotoxic properties. Taxol has recently been approved by the U.S. Food and Drug Administration for the treatment of



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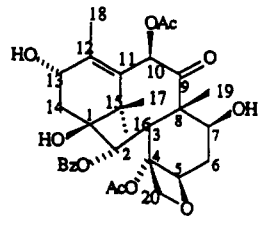

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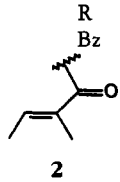

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ovarian cancer and it has also shown activity in screens of many other cancers (3–6). These developments have led to an enormous demand for taxol. Large quantities of this natural product are currently isolated from the bark of the Pacific Yew tree, *Taxus brevifolia* Nutt. (Taxaceae). However, there is concern over the potential destruction of a large fraction of the yew tree population for the purpose of taxol procurement (7). One estimate is that this natural source of taxol will be exhausted in the next 5–10 years (8).

Several alternative sources of taxol have been considered. One such alternative is the total synthesis of taxol. Significant progress has been made in this area (9–11); however, the total synthesis of taxol has not been accomplished because of the complexity of the diterpene skeleton and there may be significant challenges to scaling up a total synthesis. A second alternative is the culturing of plant tissues that produce taxol (12). This procedure has yielded small quantities of taxol; however, the method is still in the exploratory stage. Yet a third method of taxol procurement uses natural sources of taxol and related taxanes as alternatives to *T. brevifolia.* Optimally, a high yielding, plentiful, renewable source of taxol is being sought. Another objective is the discovery of a high yielding, renewable source of a taxol analogue. This would be advantageous because of a synthetic strategy in which the taxol diterpene skeleton is bonded to the C-13 carboxylic acid side-chain (13). In this way, the effective yield of taxol from a given source might be increased by chemically synthesizing taxol from other taxanes. All of these considerations establish the value of screening *Taxus* species for compounds related to taxol such as cephalomannine [**2**] and baccatin III [**3**], both of which contain the same diterpene skeleton as taxol. Cephalomannine has a slightly modified side-chain at the C-13 position, while baccatin III is the 13-hydroxy compound.

Currently, several analytical methods exist to determine the content of taxol and related compounds in plant extracts. High-performance liquid chromatography (hplc) is widely used for this purpose (14–17). Enzyme-linked immunosorbant assays (ELISA) have been used to screen plant extracts for taxanes, but with current technology, cephalomannine cross-reacts with taxol (18). Other methods of taxane analysis include fast-atom bombardment (fab) mass spectrometry (19), matrix-assisted laser desorption (mald) mass spectrometry (20), and spray-ionization techniques such as electrospray (21–22) and thermospray mass spectrometry (23). Both electrospray and thermospray have been used to couple hplc with mass spectrometry and have shown promising results in the on-line analysis of taxanes in plant extracts.

Tandem mass spectrometry is particularly well suited for analysis of target compounds in complex matrices (24–28). It can be applied in conjunction with virtually any method of ionization and hence it should facilitate the identification and quantitation of taxanes at low levels in crude plant matrices with little sample preparation. In this study, desorption chemical ionization was performed by resistively heating a rhenium wire filament to thermally desorb sample molecules in an ammonia chemical ionization source. Negatively charged ions were formed by electron attachment (29–31) and fragmentation of the taxane molecular anions to give *m/z* 526 was used as a diagnostic reaction for the presence of the taxol ring system.

The objective of the present study was to evaluate the precision, the level at which taxanes can be quantitated, and the speed of the ms/ms and hplc procedures for the determination of taxanes in *T. brevifolia* bark and needle extracts. The method of standard addition and the use of a d_3 internal standard for quantitation by ms/ms will be examined, as will the use of an external standard for hplc. A preliminary study of the quantitation of taxanes by ms/ms using standard addition has been applied to extracts from a variety of *Taxus* species (32).

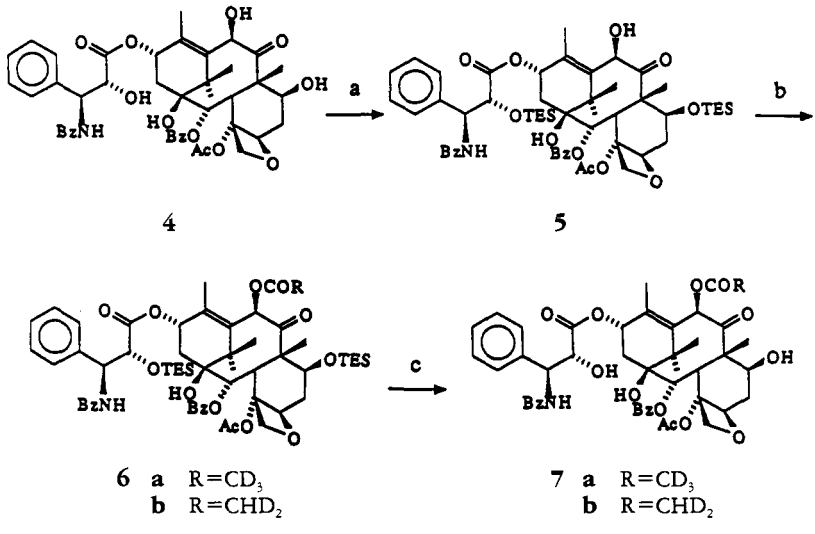
EXPERIMENTAL

SAMPLE COLLECTION.—The samples were collected from the Siuslaw National Forest as part of a survey conducted by the USDA Forestry Service (33). For the purpose of this study, bark and needle samples from each of four trees were utilized. Tree identification numbers were 061200402 (T0730 and T0731), 061200403 (T0732 and T0733), 061200101 (T0734 and T0735), and 061200102 (T0736 and T0737). The samples were shipped overnight on ice to the Frederick Cancer Research and Development Center where they were stored at −20° until grinding.

SAMPLE EXTRACTION.—The samples were vacuum-dried and ground in a Wiley Mill to pass through 4-mm pore sieves. The ground biomass was stored at −20° until extracted. The extraction was performed as described by Wheeler *et al.* (34).

PREPARATION OF 2′,7-*BIS*-TRIETHYLSILYL-10-DEACETYLTAXOL, [**5**].—10-Deacetyltaxol [**4**] (0.20 g, 0.25 mmol) (Scheme 1) was dissolved in pyridine (2.5 ml) and the solution treated with chlorotriethylsilane (0.42 ml, 2.5 mmol). This was stirred under a nitrogen atmosphere for 7 h at 25°. The reaction mixture was then diluted with EtOAc and extracted with saturated aqueous $CuSO_4$ (25 ml×3). The organic layer was dried (Na_2SO_4) and evaporated under vacuum (20 Torr). The residue was chromatographed over Si gel (20 g) and eluted with EtOAc-hexane (1:4). There was obtained 0.22 g (85%) of product **5** as a colorless, amorphous solid. ^{1}H nmr ($CDCl_3$, 300 MHz) δ 0.35–0.60 (12H, m), 0.82 (9H, t, J=7.9 Hz), 0.94 (9H, t, J=7.9 Hz), 1.10 (s, Me), 1.22 (s, Me), 1.76 (s, Me), 1.85–2.0 (m, H-6), 1.94 (s, Me), 2.05–2.15 (m, H-14), 2.30–2.45 (m, H-14), 2.45–2.55 (m, H-6), 2.56 (s, Me), 3.88 (d, J=7.1 Hz, H-3), 4.21 (d, J=8.3 Hz, H-20), 4.27 (d, J=1.6 Hz, HO-10), 4.32 (d, J=8.3 Hz, H-20), 4.38 (dd, J=6.4 and 10.5 Hz, H-7), 4.68 (d, J=2 Hz, H-2′), 4.9–5.0 (m, H-5), 5.11 (d, J=1.6 Hz, H-10), 5.65–5.75 (m, H-2, H-3′), 6.31 (br t, H-13), 7.12 (d, J=8.7 Hz, NH), 7.25–7.55 (11H, m), 7.76 (2H, d, J=7.0 Hz), 8.13 (2H, d, J=6.9 Hz); ^{13}C nmr ($CDCl_3$, 75 MHz) δ 4.4 (CH_2), 5.1 (CH_2), 6.5 (Me), 6.8 (Me), 10.2 (C-19), 14.2 (C-18), 21.0 (C-16), 23.0 (MeCO, C-4), 26.7 (C-17), 35.9 (C-14), 37.2 (C-6), 43.2 (C-15), 46.4 (C-3), 55.7 (C-3′), 57.8 (C-8), 71.5 (C-7 or C-13), 72.8 (C-7 or C-13), 74.0 (C-2′), 74.8 (C-2), 75.0 (C-20), 79.0 (C-1), 81.0 (C-4), 84.3 (C-5), 126.5, 127.0, 127.9, 128.7, 129.2, 130.2, 131.8, 133.6, 134.0, 136.0, 138.2, 138.5, 166.9, 167.0, 170.2, 171.5, 209.8 (C-9).

PREPARATION OF 2′,7-*BIS*-TRIETHYLSILYL-10-DEACETYL-10-TRIDEUTEROACETYLTAXOL [**6a**].—2′,7-*bis*-Triethylsilyl-10-deacetyltaxol (**5**, 0.211 g, 0.20 mmol) was dissolved in pyridine (2.0 ml), the solution was cooled to 0°, and treated with trideuteroacetylchloride (0.21 ml, 3.0 mmol). The cooling bath was removed and, after the reaction mixture had warmed to 25°, the solution was stirred (1 h). The reaction mixture was diluted with EtOAc and extracted with saturated aqueous $CuSO_4$. The organic layer was dried



SCHEME 1. Reagents and Reaction Conditions: (a) 10 equiv. Et_3SiCl, pyridine, 25°, 7 h, 85%; (b) 4 equiv. CD_3COCl, pyridine, 0°, 3 h → 25°, 57%; (c) 0.1 M HCl/MeOH, 25°, 1 h, 84%.

(Na_2SO_4) and evaporated under vacuum (20 Torr). The residue was chromatographed over Si gel (10 g) and eluted with EtOAc-hexane (1:4 to 1:3). There was obtained 125 mg (57%) of product **6** as a colorless, amorphous solid. ^{1}H nmr ($CDCl_3$, 300 MHz) δ 0.35–0.50 (6H, m), 0.50–0.65 (6H, m), 0.82 (9H, t, J=7.9 Hz), 0.93 (9H, t, J=7.9 Hz), 1.18 (s, Me), 1.22 (s, Me), 1.70 (s, Me), 1.85–1.95 (m, H-6), 2.03 (s, Me), 2.05–2.20 (m, H-14), 2.35–2.50 (m, H-14), 2.50–2.55 (m, H-6), 2.55 (s, Me), 3.83 (d, J=7.0 Hz, H-3), 4.21 (d, J=8.2 Hz, H-20), 4.33 (d, J=8.2 Hz, H-20), 4.45–4.55 (m, H-7), 4.70 (d, J=2 Hz, H-2′), 4.96 (br d, J=7.8 Hz, H-5), 5.70 (br d, H-2, H-3′), 6.25 (br t, H-13), 6.46 (s, H-10), 7.11 (d, J=8.7 Hz, NH), 7.25–7.65 (11H, m), 7.75 (2H, d, J=7.0 Hz), 8.14 (2H, d, J=7.0 Hz); ^{13}C nmr ($CDCl_3$, 75 MHz) δ 4.2 (CH_2), 5.2 (CH_2), 6.4 (Me), 6.6 (Me), 10.0 (C-19), 12.2 (C-18), 21.4 (C-16), 23.0 (MeCO, C-4), 26.4 (C-17), 35.4 (C-14), 37.0 (C-6), 43.2 (C-15), 46.5 (C-3), 55.6 (C-3′), 58.3 (C-8), 71.3 (C-7 or C-13), 72.1 (C-7 or C-13), 74.7 (C-2′), 74.8 (C-2), 76.5 (C-20), 78.7 (C-1), 81.0 (C-4), 84.1 (C-5), 126.4, 126.9, 127.8, 128.6, 129.1, 130.1, 131.6, 133.5 (C-11), 133.9, 138.3, 140.0 (C-12), 166.8, 166.9, 169.2, 170.0, 171.5, 201.6 (C-9). The calculated mass for $C_{59}H_{76}D_3N_1O_{14}Si_2$ is 1084. From the ratio of $[M+H]^+$ peaks at 1085 and 1084, the ratio of trideutero to dideutero material is approximately 2 to 1.

PREPARATION OF 10-DEACETYL-10-TRIDEUTEROACETYLTAXOL (10-D_3-ACETYLTAXOL) [**7a**].—2′,7-*bis*-Triethylsilyl-10-deacetyl-10-trideuteroacetyltaxol (**6a**, 50 mg, 0.046 mmol) was dissolved in methanolic HCl (0.1 N, 0.5 ml) and the solution stirred 1 h at 25°. The reaction mixture was then partitioned between CH_2Cl_2 and 5% aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4) and concentrated under vacuum (20 Torr). The residue was chromatographed over Si gel (5 g) and eluted with EtOAc-hexane (1:1 to 2:1). There was obtained 33 mg (84%) of product **7** as a colorless, amorphous solid. ^{1}H nmr ($CDCl_3$, 300 MHz) δ 1.14 (s, Me), 1.23 (s, Me), 1.68 (s, Me), 1.79 (s, Me), 1.85–1.95 (m, H-6), 2.20–2.30 (m, H-14), 2.30–2.40 (m, H-14), 2.38 (s, Me), 2.50–2.60 (m, H-6), 3.65 (br s, OH), 3.79 (d, J=6.9 Hz, H-3), 4.19 (d, J=8.3 Hz, H-20), 4.30 (d, J=8.3 Hz, H-20), 4.35–4.45 (m, H-7), 4.79 (dd, J=2.7, 5.3 Hz, H-2′), 4.94 (br d, J=7.8 Hz, H-5), 5.67 (d, J=7.0 Hz, H-2), 5.78 (br d, J=8.7 Hz, H-3′), 6.22 (br t, H-13), 6.27 (s, H-10), 7.02 (d, J=8.7 Hz, NH), 7.30–7.65 (11H, m), 7.74 (2H, d, J=7.2 Hz), 8.13 (2H, d, J=7.1 Hz); ^{13}C nmr ($CDCl_3$, 75 MHz) δ 9.3 (C-19), 14.5 (C-18), 21.5 (C-16), 22.3 (MeCO, C-4), 26.5 (C-17), 35.3 (C-14), 35.3 (C-6), 42.8 (C-15), 45.3 (C-3), 54.8 (C-3′), 58.3 (C-8), 71.9 (C-7), 72.0 (C-13), 72.9 (C-2′), 74.6 (C-2), 75.2 (C-10), 76.2 (C-20), 78.7 (C-1), 80.8 (C-4), 84.0 (C-5), 126.8, 128.0, 128.4, 128.7, 128.8, 129.9, 131.7, 132.8 (C-11), 133.3, 133.4, 137.7, 141.7 (C-12), 166.7, 166.8, 170.1, 171.0, 172.4, 203.3 (C-9). The calculated mass for $C_{47}H_{48}D_3N_1O_{14}$ is 856. Ms analysis of the 10-d_3-acetyltaxol internal standard showed that this compound was approximately 72% pure. The majority of the contamination was from 10-d_2-acetyltaxol.

MS/MS ANALYSIS OF *T. BREVIFOLIA* EXTRACTS.—Taxane-containing solutions were examined by placing 1-μl aliquots on a rhenium wire filament of a direct evaporation probe. This probe was then introduced into the source of a Finnigan Triple Stage Quadrupole (TSQ) 700 (Finnigan MAT, San Jose, CA) mass spectrometer. Radical anions of **1–3** were produced by desorption chemical ionization using NH_3 as the reagent gas at a pressure of 7000 mTorr (uncorrected). Under these ionization conditions, some $[M-H]^-$ was also produced but this form of the molecular ion was not utilized in the ms/ms screening procedure. Collisional activation was achieved by accelerating ions to a nominal 30 eV and colliding them with an argon target at 0.5 mTorr.

Quantitative analysis of **1–3** was performed using a parent scan, that is by setting quadrupole 3 to pass the common product ion of mass-to-charge ratio 526 while scanning quadrupole 1. To optimize sensitivity, a modified form of the multiple reaction monitoring (mrm) procedure was utilized in which a ten-dalton window was scanned in 0.05 sec around each of the parent ions. The instrument response was obtained by integrating the reconstructed ion chromatogram for each of the ions corresponding to the radical species of **1–3**. The desorption time typically ranged between 3 and 10 sec.

For quantitation by the method of standard addition, the instrument response to the standard taxanes was evaluated by placing aliquots of solutions of **1–3** onto rhenium wire filaments. Compounds **1** and **2** were analyzed at levels of 0.313, 0.625, 1.25, 2.5, 5.0, 10.0, and 20.0 ng. Concomitantly, **3** was analyzed at levels of 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, and 5.0 ng. The lowest level of each compound was placed onto one filament and all three compounds were analyzed simultaneously in the mrm scan mode. The instrument response to the next level of each compound was then measured by placing an aliquot of each compound onto a single filament. This procedure was followed until all levels had been analyzed. The instrument response at each level was measured 5 times.

Quantitation of the *T. brevifolia* samples by the ms/ms method of standard addition was performed by weighing dried extracts and reconstituting in CH_2Cl_2 to achieve a concentration of approximately 1 mg/ml. Of this solution, 1 μl was analyzed. The sample was spiked at one level of each taxane standard, typically in the 500 pg to 2 ng range. A second spike of the sample was performed at a second level of taxane standard, in the 2 to 10 ng range. Each of these measurements was made in triplicate. The determination of **1–3** in

a single sample required nine separate measurements taking a total time of approximately 35 min. Taxane concentrations were determined by plotting the instrument response vs. the amount of taxane analyzed and extrapolation to the *x*-axis gave the absolute value of the taxane concentration in the sample.

Ms/ms quantitation using the 10-d_3-acetyltaxol internal standard was also performed. The instrument response was calibrated for **1–3** vs. 10-d_3-acetyltaxol but the quantitation results for **2** and **3** were unsatisfactory; therefore, 10-d_3-acetyltaxol was not used as a calibration compound for the analysis of **2** and **3**. The instrument response to 10-d_3-acetyltaxol was established by analyzing samples of 1, 3, 5, 10, and 15 ng of taxol spiked with 5 ng of 10-d_3-acetyltaxol. A plot of the ratio of instrument responses to taxol and 10-d_3-acetyltaxol vs. the mass ratio of taxol and 10-d_3-acetyltaxol was constructed for instrument calibration. Each of these ratios was measured three times. The ms analysis of the 10-d_3-acetyltaxol internal standard showed that this compound was approximately 72% pure. Ms/ms instrument responses used for quantitation were mathematically corrected for the impurity of this standard.

T. brevifolia samples were prepared for analysis by making solutions of the extracts at concentrations of approximately 1 mg/ml. One 1-μl aliquot was then spiked with 5 ng of 10-d_3-acetyltaxol and analyzed. The complete analysis time of one sample is less than five min, requiring only the analysis of the sample containing the 10-d_3-acetyltaxol spike. Each of these determinations was performed in triplicate and the amount of taxol in a given sample was determined from the calibration curve.

HPLC ANALYSIS OF *T. BREVIFOLIA* EXTRACTS.—A Rainin Dynamax, phenyl, 5 μm, 4.6×150 mm column was used for the hplc analysis of *T. brevifolia* extracts. Isocratic elution was achieved with a MeOH/MeCN/aqueous ammonium acetate buffer adjusted to pH 4.4 with HOAc (20:32:48) at a flow rate of 1 ml/min. Samples were injected with the Waters WISP 700 Autosampler and effluent was monitored by uv absorption at 228 nm with the Waters 990+ photodiode array detector. With this system, the R_t of **3** was 4.41 min, **2** was 12.01 min, and **1** was 14.45 min. A further discussion of the hplc procedure can be found in Wheeler *et al.* (34).

For the hplc determination of **1–3**, 10 μl of a 1 mg/ml solution of the standard compounds was injected as the external standard. For the analysis of plant material, 25 μl of a 20 mg/ml solution was injected. This corresponds to a total plant sample of 0.5 mg injected per analysis. Considering the weight percentages of **1–3** that are present in *T. brevifolia* bark and needle extracts, this corresponds to actual taxane amount in the high picomole to low nanomole range for each analysis. The peak ratios corresponding to **1–3** for the standard injection and the sample injections were used to determine the weight percentages of **1–3** in the plant extracts.

RESULTS AND DISCUSSION

For the quantitation of taxanes in plant matrices by ms/ms, desorption chemical ionization was used in conjunction with a parent ion scan. Desorption chemical ionization was chosen because of its rapid sample throughput, low limits of detection, and ability to handle crude extracts. The parent ion scan was used as the scan mode for the tandem experiment because of the ability to quantitate **1–3** simultaneously. A parent ion scan of *m/z* 526 is very selective because a signal only results from ions that pass through quadrupole 1 as it is scanning and then fragment with loss of a neutral species upon collisional activation in quadrupole 2 to form *m/z* 526. Quadrupole 3 was set to pass *m/z* 526 only, an ion which is characteristic of the taxol diterpene skeleton. While this method is very selective, it may respond to isomers of **1–3** which could be present in the plant extracts.

To decrease the limit of detection, a multiple reaction monitoring parent ion scan was utilized. Rather than scan the entire mass range from **3** (*m/z* 586) to **1** (*m/z* 853), a 10-dalton window around the molecular ion regions of **1**, **2** and **3** was examined. The masses between the molecular anion regions of **1**, **2**, and **3** were not scanned so that more time was spent acquiring data in the mass ranges of interest.

Four *T. brevifolia* bark and needle extracts were quantitatively analyzed by three different methods. Two of these methods employ the ms/ms methodology described above. The first ms/ms procedure uses the method of standard addition and the second ms/ms procedure uses an isotopically labeled taxol derivative, 10-d_3-acetyltaxol, as an internal standard. The third method of quantitation is hplc with the use of an external standard. The relative performance of each of these methods for the analysis of the *T.*

brevifolia extracts was evaluated on the basis of i) precision, ii) level of analysis, and iii) speed of analysis.

For taxane analysis by the ms/ms method of standard addition and by hplc, standard compounds of **1–3** were used as reference materials; however, for ms/ms analysis using an internal standard, **7a** was prepared as the quantitation reference compound. 10-d_3-Acetyltaxol [**7a**] was prepared (Scheme 1) by converting 10-deacetyltaxol [**4**] (35) to its 2′,7-*bis*-triethylsilyl derivative [**5**] which was then reacted at the 10-hydroxyl position with trideuteroacetylchloride in pyridine. The reaction was carried out on **5** which had not previously been exchanged with deuterium. The reaction was found to produce **6a** contaminated to about ⅓ with the dideutero compound **6b**. The TES protecting groups were readily removed by treatment with 0.1 N HCl in MeOH. The resulting 10-d_3-acetyltaxol has ^{1}H-nmr and ^{13}C-nmr spectra which are essentially identical to those reported for taxol (36), except that the ^{1}H-nmr peak for the C-10 acetate at δ 2.23 is missing.

The mass spectrometer's response to the taxanes was evaluated by integrating the reconstructed ion chromatograms. Figure 1 shows a reconstructed ion chromatogram for the ions corresponding to taxol (*m/z* 853), cephalomannine (*m/z* 831) and baccatin III (*m/z* 586). This chromatogram represents the instrument response to 1 ng each of **1–3**. The *x*-axes of these ion chromatograms show the total desorption time. In this case, ion current generated by **1** was observed for about 4 sec. The desorption of compound **3** occurs at a slightly lower temperature than the desorption of compounds **1** and **2**. In separate experiments, it was shown that the limit of detection of **3** is approximately 5 pg, while the limit of detection for **1** and **2** is approximately 30 pg when all three compounds are analyzed simultaneously.

The linearity of the mass spectrometer's response to the standard compounds **1–3** was established to be nearly 2 orders of magnitude with the parent ion scan. A plot of

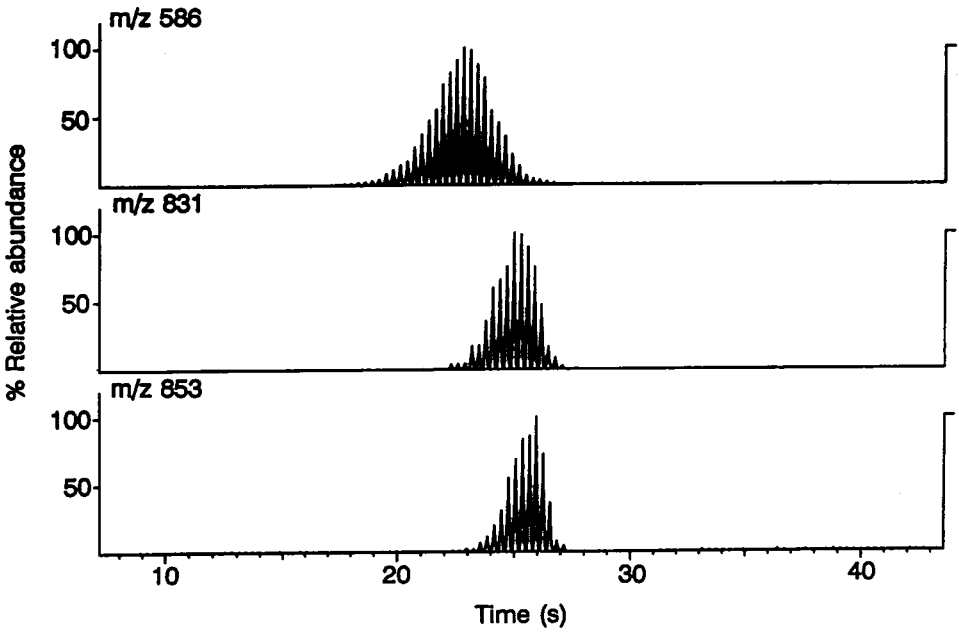


FIGURE 1. Reconstructed ion chromatograms of **1–3** generated by a multiple reaction monitoring parent ion scan of *m/z* 526. A mixture of 1 ng each of **1** (*m/z* 853), **2** (*m/z* 831), and **3** (*m/z* 586) was placed on the direct evaporation probe to generate these traces. Total data acquisition time is shown on the *x*-axis.

instrument response vs. amount of **1** was linear from 0.3 to 10 ng with a slope of 165.9 (relative response), a *y*-intercept of −22.0 pg and a correlation coefficient of 0.9858. The relative standard deviation for this standard compound where $n=5$ ranged from 14 to 44%. The calibration curve for **2** was constructed from 0.3 to 10 ng and showed a slope of 163.9, a *y*-intercept of −27.0 pg and a correlation coefficient of 0.9978. The relative standard deviation for this standard compound, where $n=5$, ranged from 11 to 25%. The mass spectrometer's response to **3** was evaluated from 0.078 to 5 ng. A plot of the instrument's response vs. the amount of **3** produced a line with a slope of 742.5, a *y*-intercept of −22.6 pg and a correlation coefficient of 0.9948. The relative standard deviation for this standard compound, where $n=5$, ranged from 14 to 26%. Data points at one level of **1**, **2**, and **3** for all three calibration curves described above were collected simultaneously.

A calibration curve was constructed for the quantitation of **1** by ms/ms with the d_3 internal standard by spiking 1.0, 2.5, 5.0, 10.0, and 15.0 ng of taxol with 5.0 ng of 10-d_3-acetyltaxol. This corresponds to a taxol to 10-d_3-acetyltaxol ratio ranging from 0.2 to 3.0. The curve has a slope of 1.12 and a *y*-intercept of -0.036 with a correlation coefficient of 0.9998. The range of relative standard deviations for each of these determinations, where $n=3$, is 9 to 16%.

The results of the determination of **1** in the *T. brevifolia* bark samples are shown in Table 1. The percent relative standard deviation for the ms/ms experiment using standard addition as the method of quantitation ranged from 10 to 37%, where $n=3$, for samples T0730, T0734, and T0736. The precision of the ms/ms experiments done using the deuterated internal standard was much better, the relative standard deviation ranges from 4 to 17%, where $n=3$, for all four samples. The precision of the hplc determinations is better than 20% relative standard deviation, where $n=10$.

The results of the quantitation of the *T. brevifolia* needle samples are shown in Table 2. The method of standard addition again displays a large relative standard deviation for the determination of **1**, with a range of 32 to 48% for samples T0731 ($n=3$), T0735 ($n=5$), and T0737 ($n=2$). The relative standard deviation for the ms/ms internal standard quantitation of **1** ranged from 8 to 16%, where $n=3$, for all four samples. Again the precision of the hplc analysis is less than 20% relative standard deviation, $n=10$. The consistency of the determined weight percentages of taxanes among all three of the

TABLE 1. Weight Percentages of **1–3** in *T. brevifolia* Bark Extracts as Determined by Hplc and Ms/ms.[a]

Sample	Method	% Taxol	% Cephalomannine	% Baccatin III
T0730	hplc	0.0129	0.0014	0.0010
	ms/ms[b]	0.0114 (0.0035)	0.0018 (0.0004)	0.0011 (0.0004)
	ms/ms[c]	0.0158 (0.0010)		
T0732	hplc	0.0120	0.0051	0.0073
	ms/ms[b]	0.0129	0.0049	0.0057
	ms/ms[c]	0.0165 (0.0029)		
T0734	hplc	0.0324	0.0142	0.0431
	ms/ms[b]	0.0434 (0.0045)	0.0181 (0.0013)	0.0277 (0.0116)
	ms/ms[c]	0.0588 (0.0097)		
T0736	hplc	0.0311	0.0134	0.0486
	ms/ms[b]	0.0262 (0.0097)	0.0218 (0.0088)	0.0344 (0.0161)
	ms/ms[c]	0.0502 (0.0019)		

[a]Hplc relative standard deviations are less than 20%; ms/ms standard deviations are displayed in parentheses.

[b]Ms/ms quantitation by the method of standard addition.

[c]Ms/ms quantitation by internal standard.

TABLE 2. Weight Percentages of **1–3** in *T. brevifolia* Needle Extracts as Determined by Hplc and Ms/ms.[a]

Sample	Method	% Taxol	% Cephalomannine	% Baccatin III
T0731	hplc	0.0011	0.0003	0.0070
	ms/ms[b]	0.0018 (0.0008)	0.0004 (0.0002)	0.0058 (0.0016)
	ms/ms[c]	0.0024 (0.0002)		
T0733	hplc	0.0027	0.0009	0.0100
	ms/ms[b]	0.0042	0.0010	0.0086
	ms/ms[c]	0.0033 (0.0005)		
T0735	hplc	0.0034	0.0010	0.0065
	ms/ms[b]	0.0037 (0.0012)	0.0007 (0.0002)	0.0082 (0.0009)
	ms/ms[c]	0.0048 (0.0008)		
T0737	hplc	0.0056	0.0010	0.0078
	ms/ms[b]	0.0058 (0.0019)	0.0014 (0.0001)	0.0102 (0.0032)
	ms/ms[c]	0.0053 (0.0008)		

[a]Hplc relative standard deviations are less than 20%; ms/ms standard deviations are displayed in parentheses.

[b]Ms/ms quantitation by the method of standard addition.

[c]Ms/ms quantitation by internal standard.

methods is good. The differences can be explained by the uncertainty associated with the reproducibility of the methods.

The relative standard deviations for the ms/ms analysis of **2** and **3** by the method of standard addition was very similar to that recorded for the analysis of **1** (Tables 1 and 2). The analysis of **2** and **3** was attempted using **7a** as the internal standard; however; poor results for the quantitation of **2** and **3** were observed showing that the 10-d_3-acetyltaxol compound could not be used as an internal standard for **2** and **3**. The results for the quantitation of **2** and **3** by hplc also showed relative standard deviations similar to those recorded for the quantitation of **1**.

Compound **1** was determined by the ms/ms method of standard addition at the low picomole level; the determination of **1** with the internal standard was also performed at the low picomole level. The ms/ms method of standard addition was used to perform the quantitation of **2** and **3** at the high femtomole to low picomole level. The hplc method of standard addition was performed at the high picomole to low nanomole level for all compounds.

The analysis time required for the determination of **1**, **2**, and **3** in a single sample by the ms/ms method of standard addition was approximately 35 min. The determination of **1** by ms/ms with the internal standard took less than 5 min. Because only one sample loading is needed, data acquisition time corresponds to the total time of the thermal desorption of the sample, which is only 1.1 min. The time-consuming step with this method is sample preparation. While analyzing yew extracts for **1** with the internal standard, the ion current from **2** and **3** was also monitored. It should be possible to quantitate these compounds in the same run with the appropriate d_3 standards. By contrast, the time required for a single hplc analysis of **1–3** is approximately 40 min.

Taxane determination by the ms/ms method of standard addition provides low levels of quantitation at the high femtomole to low picomole range. However, the time for analysis is long at 35 min per sample and the precision is relatively poor. The method of taxane analysis using an internal standard in conjunction with tandem mass spectrometry provides fast analysis times (less than 5 min per sample), low levels of quantitation at the low picomole range, and relatively good precision. The level of precision achieved with this method is much better than that observed with the ms/ms method of standard addition and is comparable to that observed with hplc. Hplc provides a good means of

taxane analysis; the method was used to quantitate taxanes at the high picomole to low nanomole level with good reproducibility; however, the speed of analysis is relatively slow at 40 min per sample.

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