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Determination of taxol by high-performance liquid chromatography–thermospray mass spectrometry

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ABSTRACT

A high-performance liquid chromatography–thermospray mass spectrometry method has been developed for the determination of taxol, found in the *Taxus* species. The compound is chromatographed by isocratic elution in 14 min and is quantitated by selected-ion recording of the protonated molecule. The method is linear over the range 1–1000 ng (1.2 pmol–1.2 nmol) of taxol per injection. The standard deviation of replicate bark samples ($n = 6$) was 12.8%.

INTRODUCTION

Taxol is a diterpene (Fig. 1), which has been shown to have important anti-neoplastic activity in preclinical and clinical studies [1]. The most common source of the chemical is the bark of the western yew, *Taxus brevifolia* Nutt. (Taxaceae), but it is also found in other *Taxus* spp., including *T. baccata* L., *T. media* Rehder and *T. cuspidata* Sieb. et Zucc. The taxol content of the plant materials is low, varying from 0.00003 to 0.069% dry weight [2–4]. Taxol has also been produced in a cell suspension culture of *T. brevifolia*, with a yield of 1–3 mg/l [5].

High-performance liquid chromatography (HPLC) with UV detection has been used for the determination of taxol in plant materials [3–5] and in pharmacokinetic studies [6]. Optimal separation of taxol and related compounds has been achieved using cyano- or phenyl-phase columns with gradient elution [4], and the strong absorbance at 235 nm allows their detection at concentrations of 50 pmol [7].

The identity and purity of the peaks detected is always a problem when HPLC samples are derived from complex biological matrices. This is a particular problem when new production methods and the metabolism of a drug are investigated, as new and unpredictable compounds are often present. As HPLC–thermospray mass spectrometry (TSP-MS) has been effectively used to overcome these problems [8], its suitability for the determination of taxol was studied. A method using a reversed-phase cyano-phase column and selected-ion recording (SIR) of the MH$^+$ ion was developed for the verification of the HPLC peak and for the quantitation of taxol in bark and needle samples of *T. cuspidata*.

EXPERIMENTAL

The *T. cuspidata* samples were obtained from the Botanical Garden of the University of Kuopio. The bark and needle samples were freeze-dried and ground in a mortar. The samples (240 mg) were extracted with 3 ml of methanol for 24 h at 4°C. The samples were sonicated for 30 min and centrifuged
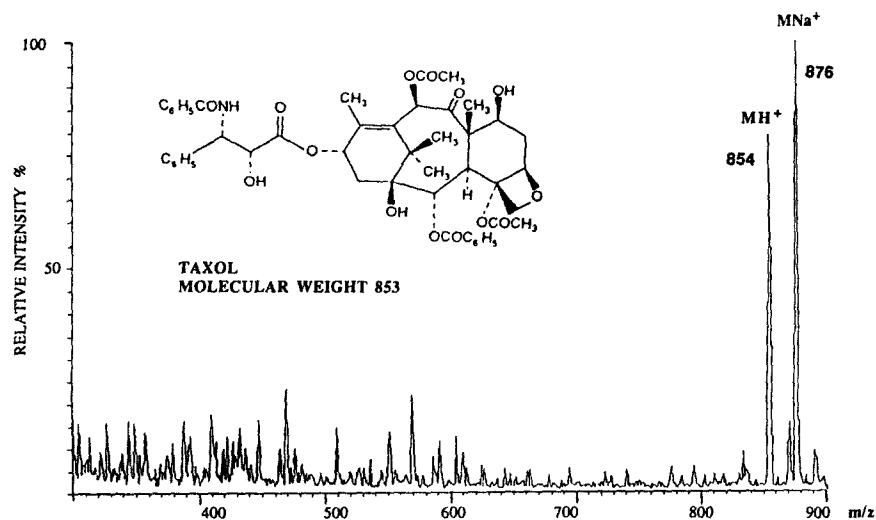
Fig. 1. Molecular structure and thermospray mass spectrum of taxol. Conditions: 50% methanol–water, flow-rate 1 ml/min, capillary temperature 200°C, ion source temperature 200°C. A 100-ng amount of taxol was injected via a loop.

for 10 min (500 *g*) with an FP-510 centrifuge (Labsystems, Finland). The supernatant was collected. The sonification and centrifugation were repeated three times with 1 ml of methanol and the supernatants were combined and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1.5 ml of methanol–water (30:70).

The solid-phase extraction procedure described by Vidensek *et al.* [3] was modified to purify the crude plant extracts. The extraction cartridges (Bond-Elut C_{18}, 1 ml, Analytichem, Harbor City, CA, USA) were conditioned with 1 ml of methanol and 1 ml of methanol–water (30:70). The sample was added and the columns were washed once with 1 ml of water and twice with 1 ml of acetonitrile–water (30:70). Taxol was eluted from the column with 0.5 ml of methanol. Pure standard taxol (NSC-12973), supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA) was used to optimize the extraction and HPLC procedures.

HPLC conditions

The HPLC pump used with the mass spectrometer was a Model 2900-0374 solvent delivery system (Applied Biosystems, USA). The injector was a Rheodyne Model 7125 instrument with a 20-μl loop (Rheodyne, Cotati, CA, USA). The cyano-phase

HPLC column was Zorbax CN (25 cm × 4.6 mm, 5 μm) (DuPont, Wilmington, DE, USA). The isocratic eluent system was acetonitrile–methanol–0.1 *M* ammonium acetate buffer (pH 5) (26.5:26.5:47).

Mass spectrometry

The HPLC–MS system used was a VG thermospray–plasmaspray probe coupled to a VG Trio-2 quadrupole mass spectrometer (VG Masslab, Manchester, UK). The measurements were carried out using the TSP mode. Tuning of the mass spectrometer and the eluent composition experiments were performed with triplicate injections of 100 ng of taxol via a loop.

The vaporizer orifice was adjusted by crimping with wire cutters to obtain a 25–30 bar back-pressure at a 1 ml/min flow-rate without heating, as described by Robins and Crow [9]. The original back-pressure was 11 bar. The capillary temperature of the TSP probe was optimized by increasing the temperature from 180 to 230°C in 10°C steps using a constant ion source temperature of 200°C. The capillary temperature was then set at 220°C and the source temperature was tested at 150, 200 and 227°C. The ion repeller potential of the TSP probe was optimized by increasing the voltage from 180 to 340 V in 30-V steps.

The effect of the eluent pH on ionization was

studied with methanol–water (50:50) using an ion source temperature of 200°C and a capillary temperature of 220°C. The pH was adjusted with trifluoroacetic acid, acetic acid or ammonium hydroxide to 2.1, 4.1, 6.0, 8.0 or 9.9.

The effect of the ammonium acetate concentration on the spectrum was studied with methanol mixed with buffer solution at 0.001, 0.01 and 0.1 M concentrations (50%). The pH was adjusted to 5.1.

The intensities and signal-to-noise ratios of the protonated molecular ion at m/z 854 and the sodium adduct ion at m/z 876 were observed by SIR.

For the determination of taxol in the *T. cuspidata* samples the ion source temperature was set to 200°C, the capillary temperature to 190°C, the solvent flow-rate to 1 ml/min, and the ion repeller voltage to 290 V. The mass spectrum of taxol was recorded by injecting 100 ng of taxol via a loop. Taxol was quantitated by SIR of the MH^+ and MNa^+ ions. The external standard method was used. The calibration graphs were created by using 1, 10, 100 and 1000 ng of taxol per injection (duplicate injections). Linear regression analysis was used to calculate the curve parameters. The precision of the TSP-MS system was determined by analysing one needle sample six times and the inter-day precision was tested by analysing one bark and one needle sample on six different days. The precision of the whole analytical method was tested by extracting and analysing six replicate bark samples.

RESULTS AND DISCUSSION

The primary process for the production of ions in a TSP ion source in the absence of external ionization is considered to be gas-phase ionization by the ammonium ion originated from the volatile ammonium acetate buffer [10]. The direct-ion evaporation process in the TSP source is dependent on the type and relative amount of ionic species and the pH in solution. Typical ions for the ion evaporation spectra are the adduct ions of organic compounds and alkali metal cations originated from the residual salts in most solvents [11]. The efficiency of ionization by the TSP source is dependent on the solvent composition, the proton affinity of the analyte and the conditions in the vaporizer capillary and ion source [12–14]. As the mass spectrum of taxol

(Fig. 1) showed an abundant protonated molecule at m/z 854 and a sodium adduct ion at m/z 876 suitable for the identification and quantitation of the chromatographic peak, SIR of these ions was used to tune the instrument and optimize the solvent compositions.

The restriction of the capillary increased the peak area of the MH^+ and MNa^+ ions and the signal-to-noise ratio by more than five times. This is presumably due to the smaller droplets produced, which resulted in a greater charge-to-surface area ratio and an enhanced ion evaporation process [9,15]. However, the restriction prevented the use of capillary temperatures above 230°C, which caused too high a back-pressure (over 350 bar) for the HPLC pump.

The best response in ionization by a TSP source is achieved with capillary temperatures at which nearly complete vaporization occurs. Strong heating of the TSP ion source further enhances the vaporization of the droplets in the jet and improves the sensitivity for less volatile compounds [16]. As shown in Fig. 2, the intensities of the MH^+ and MNa^+ ions of taxol remained stable at capillary temperatures between 180 and 200°C and then increased with temperature from 200 to 230°C. When the source temperature was increased from the initial 200 to 227°C to enhance the desolvatation of the droplets, the response of these two ions could be further increased
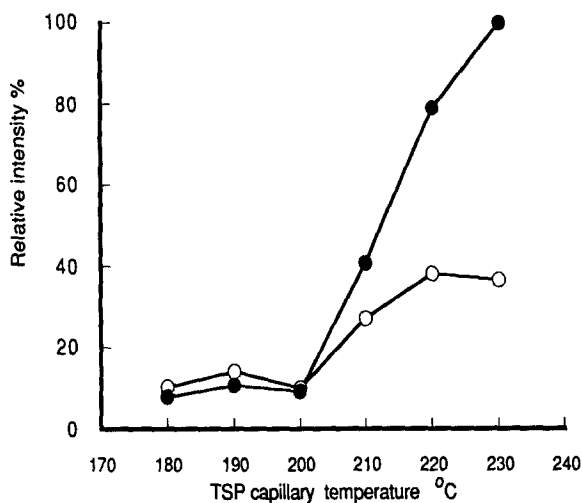

Fig. 2. Relative amounts of MH^+ (○) and MNa^+ (●) ions produced at different thermospray capillary temperatures. Other conditions as in Fig. 1.

two-fold. The temperature of 227°C was the maximum for the ion source of this instrument. For the analytical method, a capillary temperature of 190°C was chosen to prevent small changes from affecting the response and the source temperature was set to 200°C to conserve the heater wire.

The use of a high repeller electrode voltage has been observed to improve the sensitivity of ionization by a TSP source, especially at high masses (near m/z 1000). The presence of the electrostatic field may increase the evaporation of ions from equilibrated droplets [9,17] and it may increase the extraction efficiency of high mass ions with a higher kinetic energy [15]. The increase in the repeller potential from 160 to 340 V increased the response of the MNa^+ ion more than the response of the MH^+ ion (Fig. 3). This indicates that in addition to increasing the sampling efficiency of the ions, by increasing the ion mobility in the source, the evaporation ionization process is also affected, possibly by inducing expulsion of the ions from the droplets.

The effect of pH on the response was studied with a methanol–water eluent between pH 2 and 10. As shown in Fig. 4, the responses of the MH^+ and MNa^+ ions were good between pH 4 and 8, but decreased sharply beyond these. At pH 2.1 the adduct ion MNa^+ could no longer be seen. This indicates that the solvent pH affects the spectra, and that the ion evaporation process is responsible for
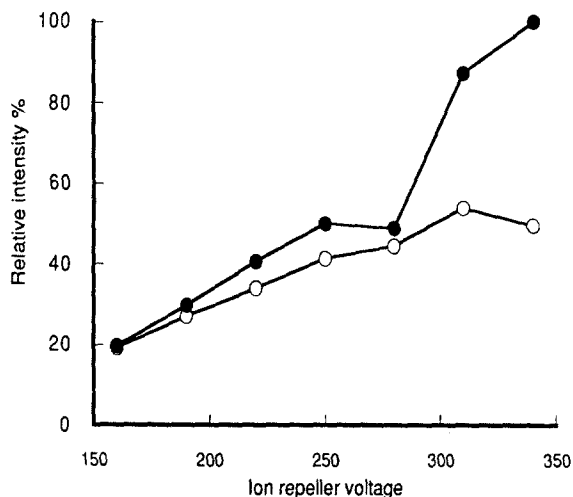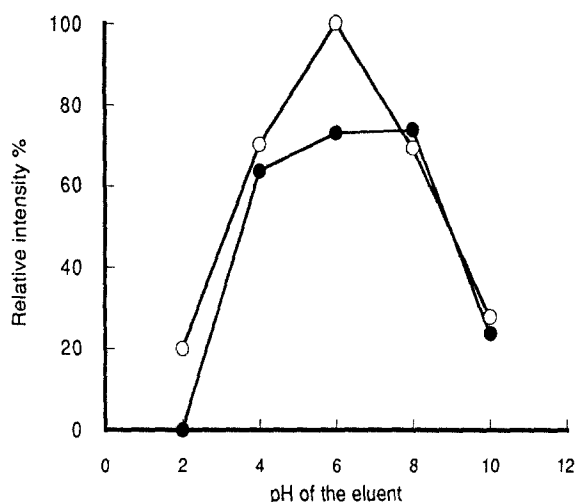
Fig. 4. Effect of the eluent pH on the amount of MH^+ (○) and MNa^+ (●) ions. Conditions as in Fig. 1, but the capillary temperature was set to 220°C.

the ionization. The use of 0.001, 0.01 or 0.1 M ammonium acetate buffer only slightly affected the MH^+/MNa^+ ratio (Fig. 5). This is much less than the decrease of one or two orders of magnitude in the MNa^+ ion intensity observed for nucleosides by Voyksner [11]. With buffer concentrations of 0.01 and 0.1 M the background was higher, but the
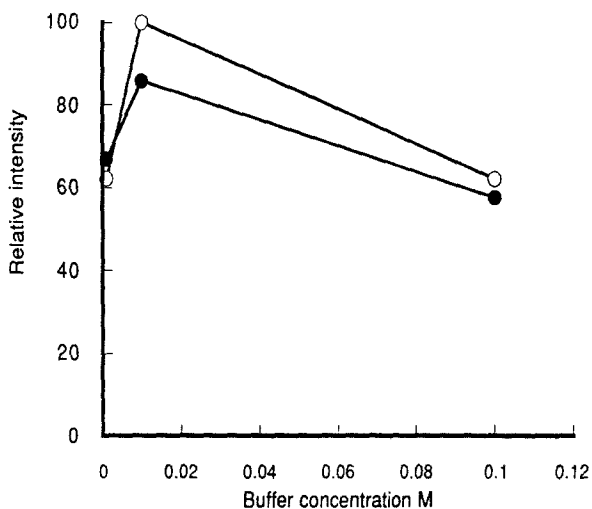
Fig. 3. Effect of the thermospray ion repeller potential on the amount of MH^+ (○) and MNa^+ (●) ions.

Fig. 5. Effect of the ammonium acetate buffer concentration on the amount of MH^+ (○) and MNa^+ (●) ions. The pH was set to 5.1; other conditions as in Fig. 4.

signal-to-noise ratio was as good as without the buffer.

SIR of the MH^+ ion at m/z 854 was used for the quantitation of taxol in plant extracts and the sodium adduct ion at m/z 876 was also monitored to confirm the identity of the peak (Fig. 6). Taxol was chromatographed in 14 min using an isocratic solvent system, and baseline separation was achieved for taxol on both channels. The abundant peak eluting at 11 min is probably a related compound, cephalomannin (molecular weight 831 dalton). In the cyano-phase columns, cephalomannin elutes before taxol when acetonitrile–methanol–water is used as the eluent [4]. The calibration graphs were determined by plotting the chromatographic peak areas (y) against the amount of taxol per injection
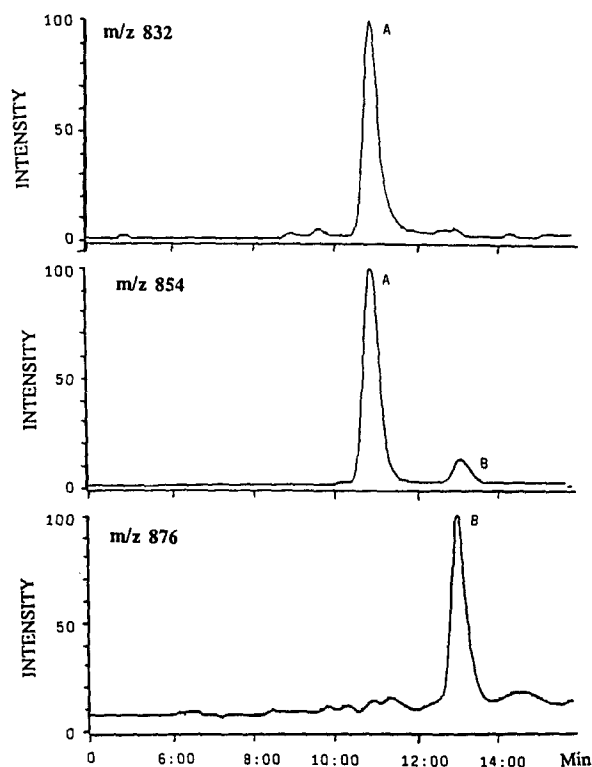

Fig. 6. Selected ion chromatograms of a *Taxus cuspidata* needle sample. Compounds monitored: peak (B) is taxol, MH^+ at m/z 854 and MNa^+ at m/z 876; peak (A) is probably cephalomannin, MH^+ at m/z 832 and MNa^+ at m/z 854. Conditions: acetonitrile–methanol–0.1 M ammonium acetate (26.5:26.5:47), flow-rate 1 ml/min, capillary temperature 190°C, source temperature 200°C, repeller voltage 290 V.

(x) (1, 10, 100 and 1000 ng). When the MNa^+ ion was recorded, the regression equation of the linear relationships was $y = 0.9953x + 5.51$ ($r = 1.00$); the confidence limits of the slope and intercept were 0.9953 ± 0.069 and 5.51 ± 34.7 ($p = 0.05$), respectively. When MH^+ was recorded the regression equation of the linear relationships was $y = x + 2.17$ ($r = 1.00$) for the range 10–1000 ng; the confidence limits of the slope and intercept were 1 ± 0.044 and 2.16 ± 25.4 ($p = 0.05$), respectively. Although the response of the method is almost linear for a wide concentration range, calibration graphs for narrower concentration ranges should be determined to obtain the optimum results, especially at low analyte concentrations.

The precision of the TSP system was tested by analysing six needle samples in parallel (approximately 715 ng per injection). When the MH^+ ion was recorded, the relative standard deviation (R.S.D.) was 6.8%; it was 11.2% when the sodium adduct ion was used for quantitation. However, as changes in the capillary or ion source parameters may considerably change the response, the performance must be evaluated daily.

The precision of the whole assay method was determined by analysing six replicate bark samples (280 ng). The R.S.D. was 12.8%. The inter-day precision was tested by analysing one needle sample (607 ng) and one bark sample (260 ng) on six days. The R.S.D. values were 10.8 and 7.4%, respectively.

The amount of taxol in the bark samples of *T. cuspidata* grown in Finland varied from 0.0027 to 0.0049% and in the needle samples from 0.0048 to 0.0061%. These values are comparable with the results of Vidensek *et al.* [3].

CONCLUSIONS

These results show that ion evaporation is at least partly responsible for the ionization of taxol by a TSP source, as the mass spectrum of the compound showed abundant MH^+ and MNa^+ ions in the absence of ammonium acetate buffer. The presence of the MNa^+ ion and the dependence of the ionization on the eluent pH indicate that the ions are formed in solution. These processes made it possible to use HPLC–TSP-MS and SIR of the MH^+ ion for the determination of taxol. The selectivity and sensitivity of the method is needed as taxol is only a

minor component in the *Taxus* species. The precision of the method is adequate for screening the taxol content of plant extracts.

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