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Miniaturized HPLC and ionspray mass spectrometry applied to the analysis of Paclitaxel and taxanes

Jinping Liu [a,*], Kevin J. Volk [a], Michelle J. Mata [a], Edward H. Kerns [a], Mike S. Lee [b]

[a] *Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT 064921, USA*
[b] *Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 085432, USA*

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Abstract

Analysis of the antitumor agent Paclitaxel, related taxane analogues and yew tree bark extracts has been carried out using an HPLC system capable of performing chromatographic separations with conventional, small-bore, and micro-bore columns. Both diode array detector and mass spectrometry were incorporated into this system, providing additional spectral and structural information for identification of unknown samples. In conjunction with some basic theoretical studies dealing with miniaturized HPLC systems, experiments were designed to minimize the contribution of extra-column variances. Three chromatographic columns, 4.6, 2, and 1 mm i.d., were evaluated using a standard mixture consisting of Paclitaxel and three analogues. The experimental results obtained in these columns demonstrated good correlation with theoretical calculations with respect to the sensitivity enhancement. Studies on the combination of miniaturized HPLC with ionspray mass spectrometry for Paclitaxel samples showed dramatic improvement of MS performance as compared to conventional LC/MS. The advantages of this miniaturized LC/MS system are evidenced by enhanced mass sensitivity, which was more than two order of magnitude higher when changed from a 4.6 mm i.d. column to a 2.0 mm i.d. column, greatly improved peak shape, and the potential gain of efficiency. These studies demonstrate great potential of miniaturized HPLC/MS systems for structural characterization and confirmation of various pharmaceutical compounds. © 1997 Elsevier Science B.V.

Keywords: Ionspray mass spectrometry; Miniaturized HPLC; Paclitaxel; Taxanes; Yew tree bark extracts

1. Introduction

The natural product Paclitaxel (known in the literature as taxol) has been widely known as a novel treatment for refractory ovarian tumors. It was also demonstrated to be effective in treating a variety of human cancers, such as leukemia, breast, skin, and lung tumors [1–4]. Pacific yew (*Taxus brevifolia*) has long been considered as the main natural resource to provide limited Paclitaxel human treatment. Efforts to find alternative methods of extraction and synthesis of Paclitaxel have been explored in the past few years. Partial

* Corresponding author. Tel.: +1 203 2846139; fax: +1 203 2846137.

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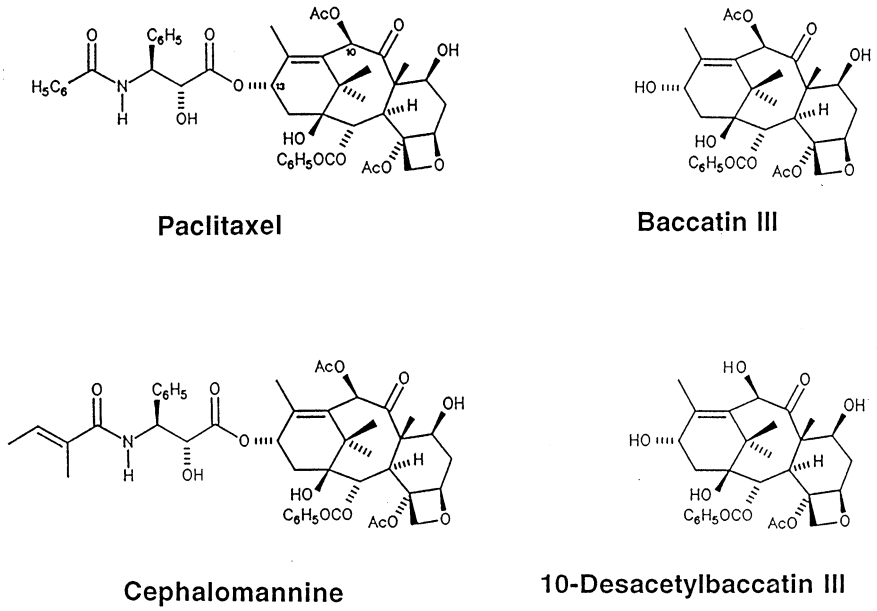

Fig. 1. Structures of Paclitaxel and related taxanes.

synthesis of Paclitaxel and related diterpenoids from corresponding precursors extracted from needle foliage has already been reported [5,6]. Total synthesis has also been successfully achieved although it may still not be practical for pharmaceutical use [7,8]. Since many analogues among taxanes have been shown to have potent antitumor activity [5,6,9], numerous research efforts have been focused on the synthesis of Paclitaxel analogues. These approaches will ultimately provide reasonable resources to offer a possible long-term solution to the availability of Paclitaxel and related active compounds. Continued development of analytical methodologies capable of providing efficient and sensitive separations of Paclitaxel and other taxane analogues is an essential part of this evolving trend.

Conventional high performance liquid chromatography (HPLC) has been widely used for the separation of plant materials and the determination of taxane concentrations [10–13]. Mass spectrometry (MS) with various ionization techniques has also been combined with HPLC for taxane determination and identification [14–17]. In particular, electrospray ionization based LC/MS approaches have been demonstrated to be an effective and sensitive method for Paclitaxel analysis and rapid screening of crude materials [16,17]. A recent study utilizing the LC/MS/MS approach for the trace analysis of crude extracts provides a method for rapid and systematic structure elucidation of taxanes in *Taxus* and process intermediates, which was especially shown to be useful for the rapid identification of taxane analogues in mixtures and samples of limited quantity [18,19].

Although LC/MS coupling using conventional 4.6 mm columns has achieved some success, this set up generally suffers from poor sensitivity and inadequate resolution. Alternative analytical approaches are desirable especially while dealing with trace mixture analysis and limited sample conditions. During recent years, there has been a general trend towards the miniaturization of separation techniques especially with configurations involving electrospray MS. The significant advantages of using miniaturized HPLC, such as enhanced sensitivity with the smaller column diameters, increased chromatographic resolution and greatly reduced solvent consumption, has long been recognized [20– Perhaps more importantly the reduced flow rates are beneficial to concentration sensitive detectors such as UV,

fluorescence, and mass spectrometery for on-line detection, which makes the miniaturized HPLC/MS a more attractive technique. Based on previous profiling strategies for trace mixture analysis of taxanes using conventional LC/MS methods [18], this study demonstrates the benefits of a miniaturized HPLC/MS system. A comparison of chromatography columns with different diameters with respect to sensitivity and over all performance has been conducted with an electrospray interface. The rapid and sensitive method has been applied to the analysis of Paclitaxel, taxane analogues and biomass extracts.

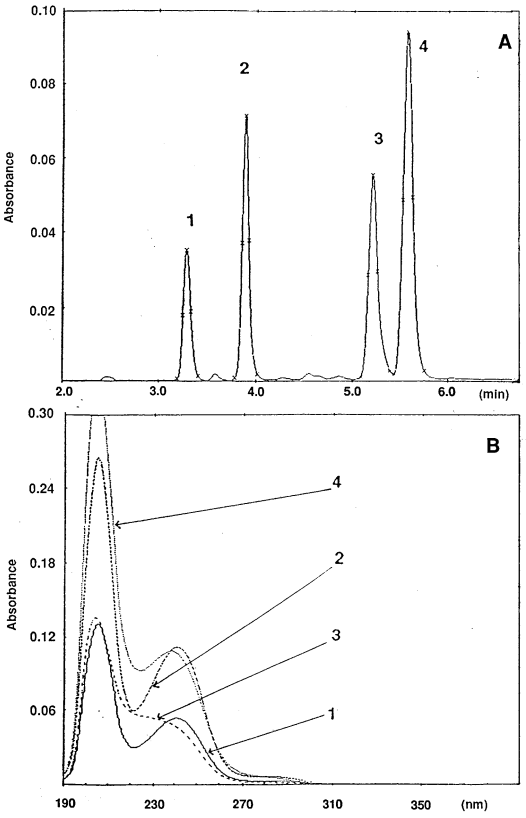

Fig. 2. (A) HPLC chromatogram of taxane standards and (B) corresponding UV spectra using a 2.0 mm i.d. column with isocratic elution (56:44 methanol–water). The UV spectra was acquired from a diode array scanned from 190 nm to 350 nm. Peak correspond to: 1-10-Desacetylbaccatin III, 2-Baccatin III, 3-cephalomannine, 4-Paclitaxel.

2. Experimental

2.1. Apparatus

2.1.1. Liquid chromatography

The HPLC system consists of an ABI 140B solvent delivery system and an ABI 1000S diode array detector, both from Applied Biosystems Division (Foster City, CA), and a PE Nelson 1020 Integrator. The system is capable of performing both conventional and miniaturized chromatographic separations. For comparison, three chromatographic systems were studied with column i.d. ranging from 4.6 mm to 1.0 mm. The injection loop volume was 5 μl and the detector flow cell volume was 3.3 μl. UV spectra were collected from wavelengths 190–350 nm for initial compound identification. For on-line MS detection the detector wavelength was set at 227 nm for monitoring effluents.

2.1.2. Ionspray mass spectrometry

A PE-SCIEX (Thornhill, Ontario, Canada) API III triple-quadrupole mass spectrometer equipped with an ionspray interface was used on-line with a miniaturized LC system and a diode array detector. The LC effluent was introduced directly into the ionspray source for a 1.0 mm i.d. column. A post-column splitter was used with the 4.6 mm and 2.0 mm i.d. columns to produce a flow rate of 50 μl min^{-1} to the ionspray interface. LC/MS experiments were performed while scanning from m/z 150 to m/z 1200 at a scan rate of 2 s per scan. For the LC/MS/MS operation, the parent ions were selected in the first quadrupole mass analyzer and transmitted into the second quadrupole (collision cell) with collision energy of 50 eV and argon collision gas at 450×10^{12} atoms cm^{-2}.

2.2. Reagents and materials

All taxane standards were obtained in-house: Paclitaxel (BMY-45622, lot # 36700891A), cephalomannine (BMS-180518-01), Baccatin III (BMS-32108-130), and 10-Desacetylbaccatin III (BMS-182252-01). The Paclitaxel impurity sample was BMY-45622 (lot 28935291A). Methanol, ace-

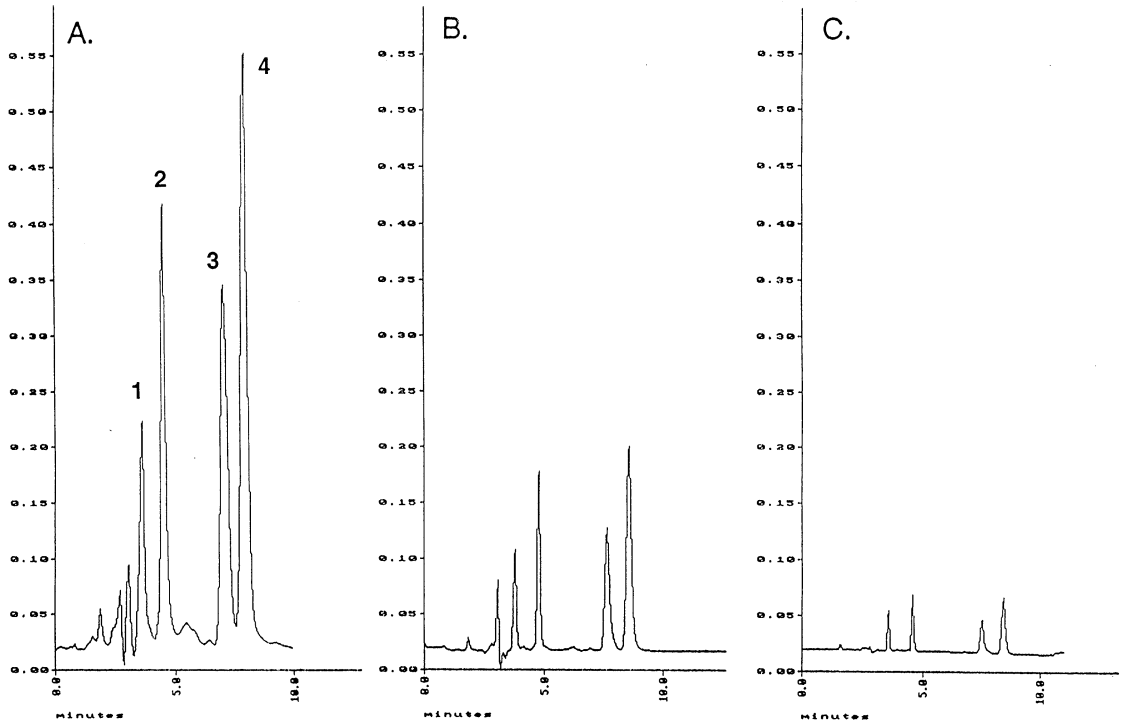

Fig. 3. Taxane profiles obtained from (A) 1.0 mm i.d. micro-bore, (B) 2.0 mm i.d. small-bore and (C) 4.6 mm i.d. standard analytical columns using a Isocratic separation (53:47 acetonitrile–water). A 1 μl injection of standard mixture with 250 μg ml^{-1} of each taxane for all the three columns was used. Peak assignments are the same as those in Fig. 2.

tonitrile, and water were obtained from Fisher Scientific and were of HPLC grade or higher. A standard mixture of Paclitaxel and three related taxanes was prepared such that the concentration of each component was 250 μg ml^{-1} in methanol. A diluted sample of 25 μg ml^{-1} of each component in methanol was also prepared. The impurity sample was prepared in two concentrations, 10 mg ml^{-1} and 5 mg ml^{-1}, both in methanol with 0.1% acetic acid.

All chromatographic columns were from Keystone Scientific (Bellefonte, PA) with a length of 25 cm. Internal diameters of 4.6, 2.0, and 1.0 mm were used with flow rates of 1000, 200, and 50 μl min^{-1}, respectively. Both Hypersil Phenyl-2 and Phenyl/B stationary phase were used for the LC/UV and the on-line LC/MS studies. The LC/UV analysis of this study utilized a mobile phase system consisted of A, acetonitrile–water (35:65) and B, ACN–H_2O (80:20). For on-line LC/MS analysis, mobile phases A and B were slightly modified by adding 2.0 mM ammonium acetate to the solvents.

3. Results and discussion

Paclitaxel and taxane analogues are a class of compounds with a unique core structure and different chain substitutes as the four taxanes, Paclitaxel, cephalomannine, Baccatin III, and 10-Desacetylbaccatin III show in Fig. 1. These compounds generally produce UV spectra with absorption maxima at approximately 227 and 250 nm. LC/UV was initially utilized to establish chromatographic conditions aimed at rapid profile analysis. Various chromatographic conditions were evaluated using the of the four taxanes. Fig. 2A shows a representative chromatogram of four standard taxanes which was

Table 1
Sensitivity enhancement: comparison of theoretical calculation and experimental results for taxanes

Column diameter (mm)	Theoretical calculation	Experimental results			
		Peak area ratios			
		Peak 1	Peak 2	Peak 3	Peak4
Isocratic separations					
4.6	1	1	1	1	1
2.0	5	6.4	6.0	6.1	5.9
1.0	20	18.1	16.3	18.2	18.2
Gradient separations					
4.6	1	1	1	1	1
2.0	5	4.6	5.4	4.5	4.4
1.0	20	17.2	15.0	17.5	15.3

obtained using a 2.0 mm i.d. narrow-bore column. Baseline separation was achieved for the four taxanes within 6 min. The corresponding UV spectra shown in Fig. 2B were acquired from the diode array detector and can be used as a preliminary means for compound identification and confirmation of peak identity. As expected, the spectra are similar with minor differences in the range 210–270 nm. In a more complex mixture, such as the Paclitaxel impurity sample, identification of specific taxanes by this method would be more difficult as the spectra overlap. Alternative approaches such as LC/MS techniques are desirable.

There has been tremendous demands in recent years to search for more efficient and economic HPLC methods while maintaining experimental integrity. Advances in LC/MS interface technologies has been one of the major driving forces towards the miniaturization of analytical HPLC. The advantages of using a miniaturized LC system such as micro-bore and capillary LC has long been recognized. These techniques may, once refined, provide a viable and efficient economical alternative to analytical HPLC. Fig. 3 illustrates the analysis of the four taxanes using several HPLC columns with different diameters ranging from conventional (4.6 mm i.d.) to micro-bore (1.0 mm i.d.) columns. A mobile phase composition consisting of 40% B (53:47 ACN–H_2O) was determined optimum for the isocratic separation of the taxane standards on each of the three column diameters. The flow rates were set at 1000, 200, and 50 μl min^{-1} for the 4.6 mm, 2.0 mm, and 1.0 mm columns, respectively. As seen from these chromatograms (Fig. 3A to C), sensitivity increases as the column i.d. decreases when 1 μl of mixture with 250 μg ml^{-1} concentration was injected into column. In order to make reasonable comparisons of three different column diameters for the enhancement of sensitivity, the experimental configuration was carefully optimized to minimize the extra-column contributions to peak variance and distortion. All chromatographic components such as injection sample loop and detector flowcell, except the flow rate which was restricted by column diameters, were the same for each column.

Recent literature indicates that sensitivity enhancement, or the ratio of the maximum solute concentration within the detector cell, favors miniaturized columns over analytical columns because of their smaller i.d. [20]. The mass sensitivity in a chromatographic system is inversely proportional to the square of column diameters. A decrease in column diameter from 4.6 to 1.0 mm can result in a substantial enhancement of mass sensitivity which is beneficial to concentration-sensitive detectors (UV, fluorescence, etc.). Under the current optimized chromatographic conditions, some experimental results together with theoretical calculations are

summarized in Table 1. The integrated peak areas for all four taxanes from experimental data vary approximately 20:5:1 for column diameters, 1.0, 2.0 and 4.6 mm i.d., respectively, as predicted by theory. Similar results were also obtained for gradient separations. The detection sensitivity with the 1.0 mm i.d column was enhanced about 20 times more than that for the 4.6 mm i.d column, which was about 5 times more than for the 2.0 mm i.d column. The observed deviations from theory are most due to differences in column packing and extra-column variances. The differences in sensitivity is quite impressive. Gen-

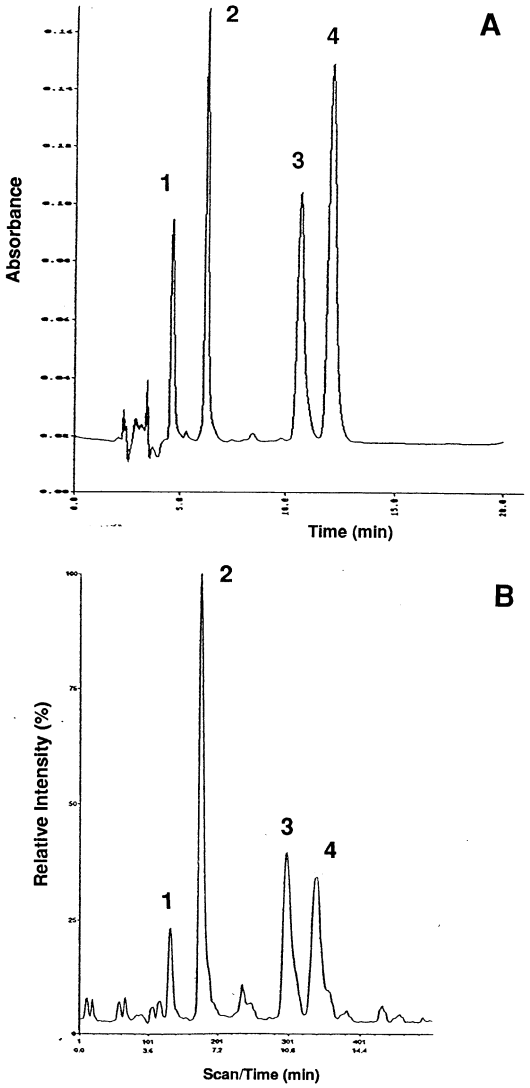

Fig. 4. On-line LC/ionspray MS of Paclitaxel and analogues using 2.0 mm i.d column. (A), UV chromatogram; (B), total ion chromatogram. A 1 μl injection of mixture with 250 μg ml^{-1} of each taxane was used. Peaks were confirmed as those assignments indicated in Fig. 2.

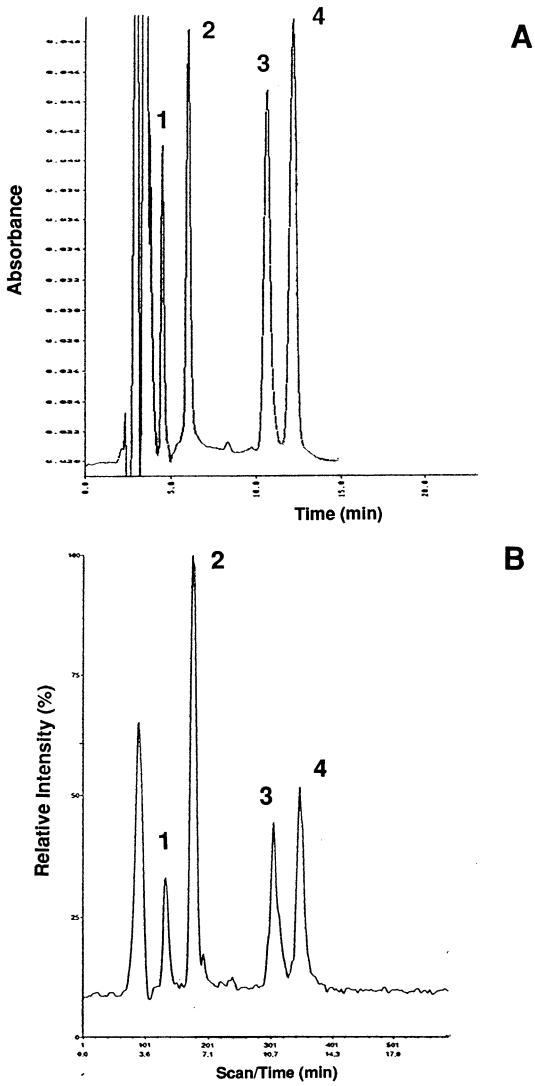

Fig. 5. On-line LC/ionspray MS of Paclitaxel and analogues using 1.0 mm i.d column. (A), UV chromatogram; (B), total ion chromatogram. A 1 μl injection of mixture with 25 μg ml^{-1} of each taxane was used. Peaks were confirmed as those assignments indicated in Fig. 2.

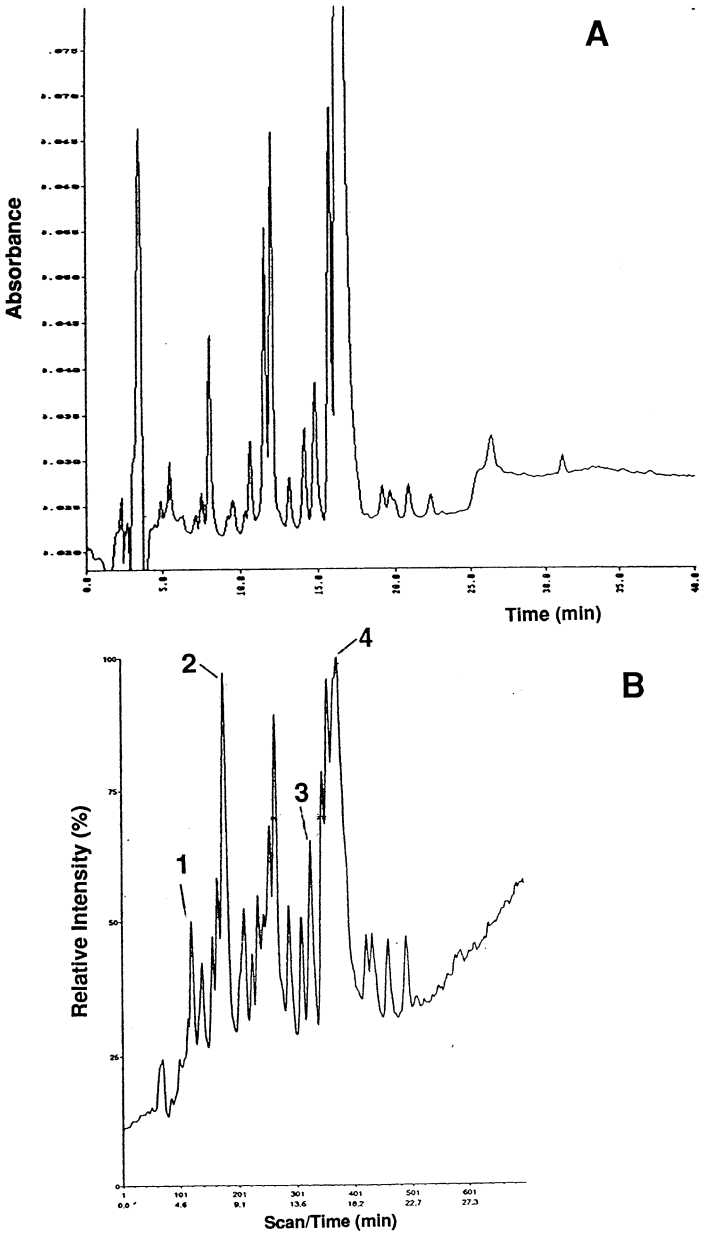

Fig. 6. On-line LC/ionspray MS of Paclitaxel extract from yew tree bark using 2.0 mm i.d column. (A), UV chromatogram; (B), total ion chromatogram. Gradient separation from 20 to 70% mobile phase B in 30 min was applied. A 1 μl injection sample volume with concentration of 0.5 mg ml^{-1} was used. Peaks corresponding to the four standard taxanes were identified as indicated.

erally, in order to achieve the same sensitivity obtained on a 1.0 mm i.d. column on a 4.6 mm i.d. column, 20 times more samples would be needed. Obviously, would be important for trace mixture analyses and for samples that are available in only limited amounts.

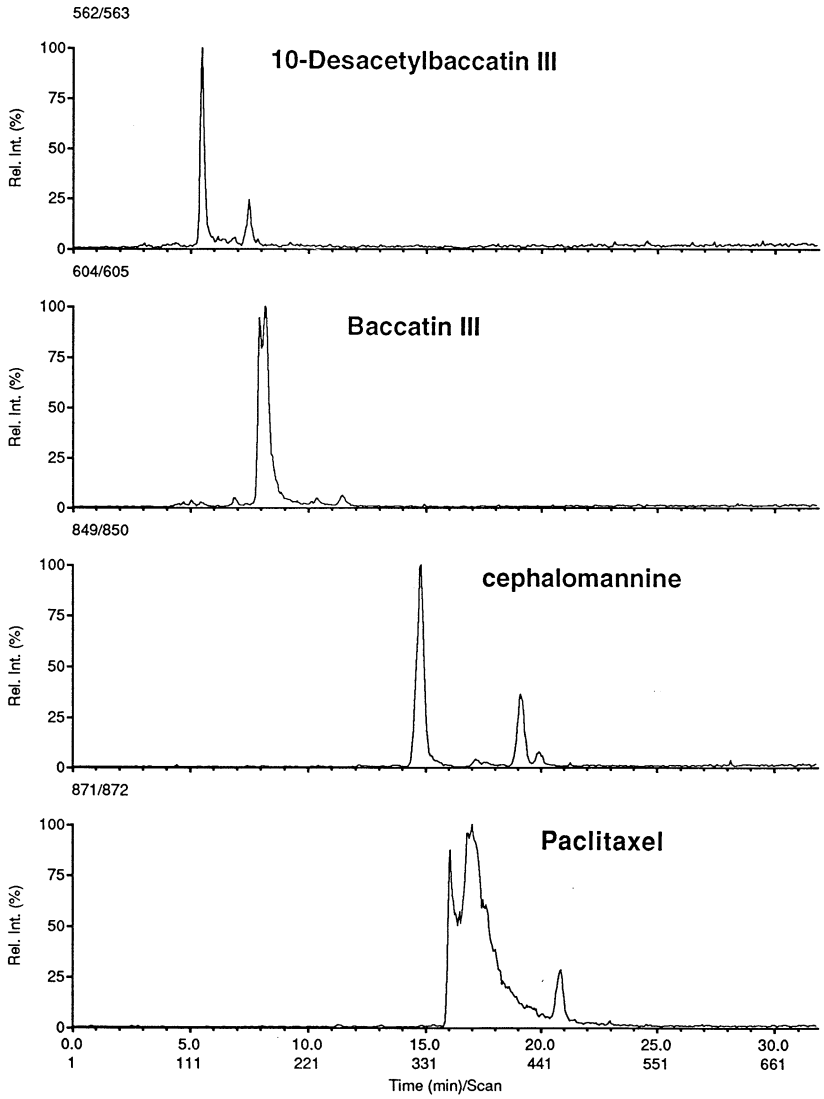

Fig. 7. Identification of Paclitaxel and major taxanes from the Paclitaxel impurity sample by LC/ionspray MS using a 2.0 mm i.d. analytical column. A 1 μl of sample volume with concentration of 0.5 mg ml^{-1} was injected.

Generally, column diameter will not seriously affect resolution. However, in practice, a change in column diameter may result in resolution gain or loss. In most cases, this will depend on the column packing and instrumental optimization for a specifically chosen column. By using the same injection amount (0.25 μg per injection in this study) for each column, this may cause a capacity overload in the smaller i.d. columns, which may then result in some loss of resolution. Thus, a loading study was performed using a 2 mm i.d. small-bore column. A substantial loss in resolution, especially between cephalomannine and Paclitaxel, was observed at the higher injection amounts. To gain a comparable resolution proper sample reduction for smaller diameters is needed, which is actually one of the advantages of using a miniaturized HPLC system.

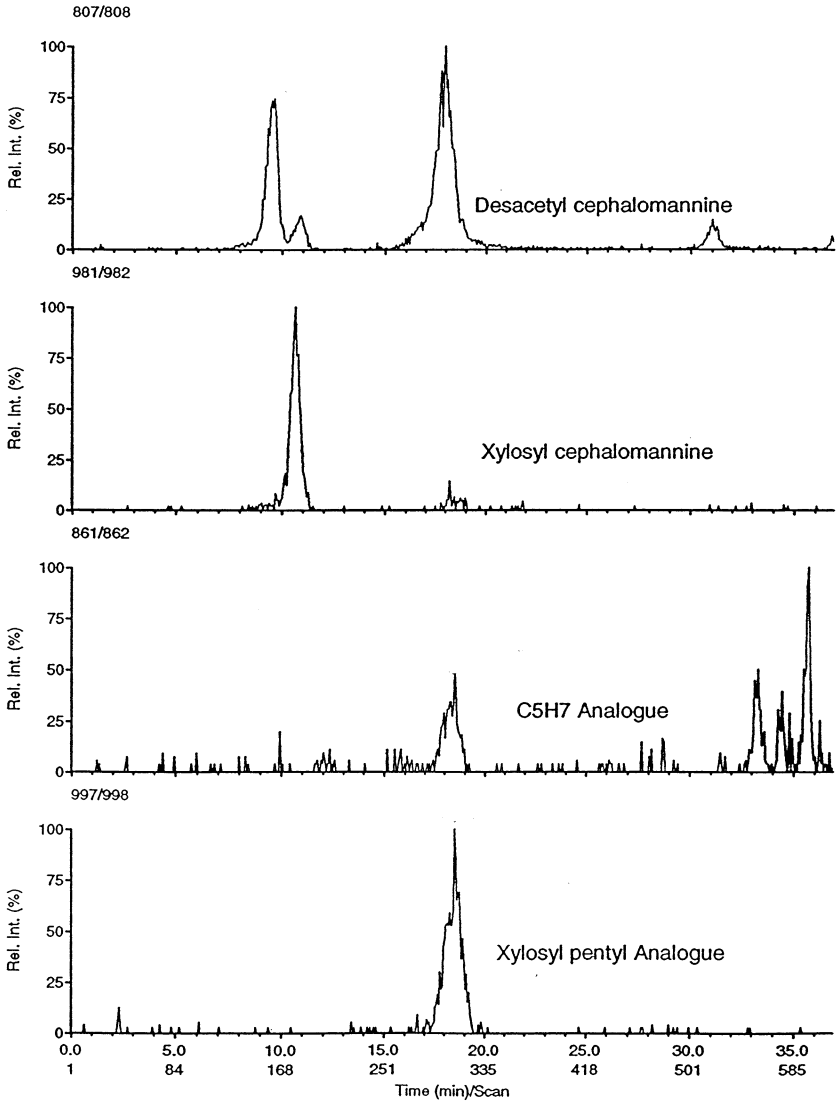

Fig. 8. Extracted ion chromatograms obtained from the Paclitaxel impurity sample by LC/ionspray MS using a 4.6 mm i.d. analytical column. A 20 μl of sample volume with concentration of 10 mg ml^{-1} was injected.

Previous studies established a LC/MS and LC/MS/MS protocol for trace analysis of taxanes and analogues in complex mixtures [18,19]. Coupling of the miniaturized HPLC system with ionspray/MS would be beneficial to further improvement and fine-tuning of the system for obtaining additional complementary information to the data acquired from LC/UV. For LC/MS studies, the chromatographic mobile phase previously optimized for LC/UV was slightly modified by adding 2.0 mM ammonium acetate to the solvents to enhance the detection of taxane ions. Initial on-line LC/MS analysis of the taxanes were performed using the 2.0 mm and 1.0 mm i.d. Phenyl–B columns. Their UV chromatograms and total ion chromatograms (TIC) are shown Fig. 4 and Fig. 5. It was immediately recognized that less concentrated samples would be needed to

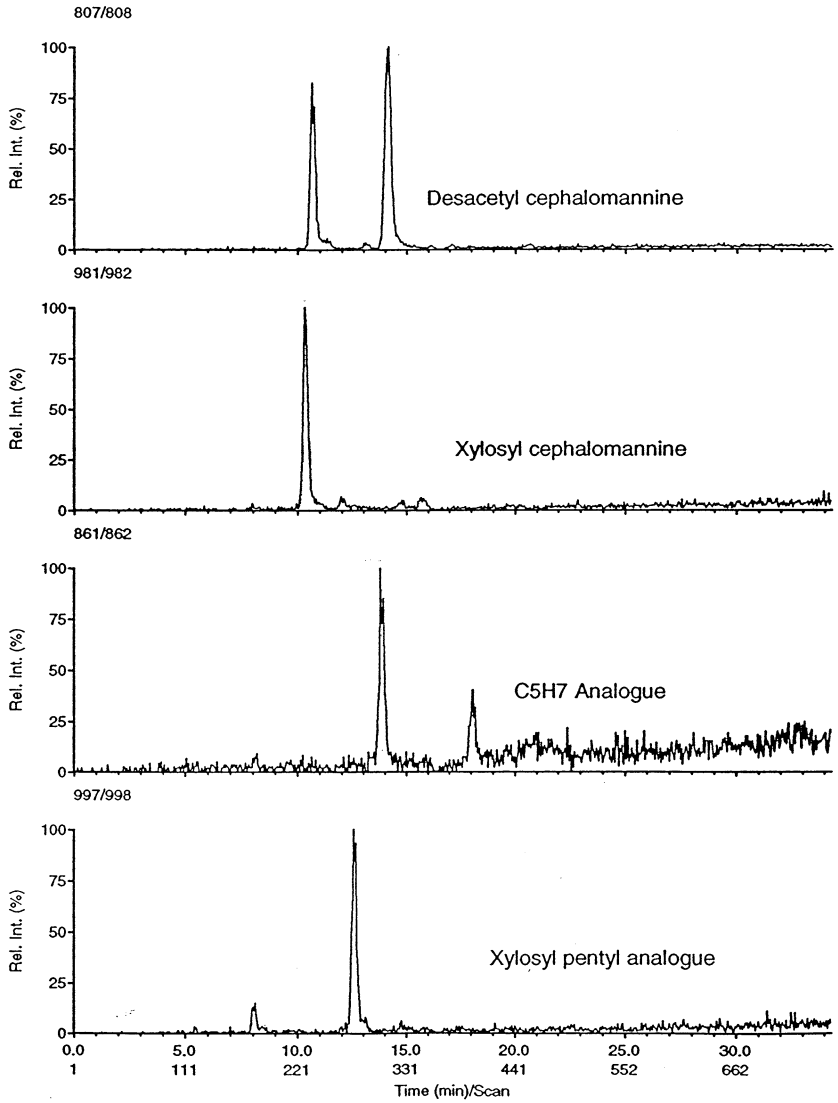

Fig. 9. Extracted ion chromatograms obtained from the Paclitaxel impurity sample by LC/ionspray MS using a 2.0 mm i.d. column. A 1 μl of sample volume with concentration of 0.5 mg ml^{-1} was injected.

produce comparable total ion chromatograms because of the sensitivity enhancement of the MS as in the case of UV detection. With a 1.0 μl of sample volume, 29 pmol of Paclitaxel was injected into the 1.0 mm i.d. column, which represents ten times less the amount of sample needed for the 2.0 mm i.d. column. The typical molecular ions $(M + H)^+$ associated with Paclitaxel, cephalomannine, Baccatin III, and 10-Desacetylbaccatin III, are m/z 854, m/z 832, m/z 587, and m/z 790, respectively. With ammonium ions present in mobile phase, these taxane compounds also characteristically produce abundant adduct ion $(M + NH_4)^+$.

For more complicated mixtures of varying polarity, such as the Paclitaxel impurity samples from yew bark extraction, a gradient separation may be more appropriate. As shown in Fig. 6, the UV chromatogram and TIC were obtained from a

Paclitaxel extraction sample containing various taxane analogues investigated in a previous study. The major components such as Paclitaxel and related taxanes can be easily identified as shown in Fig. 7. With use of a 2.0 mm i.d. column in this analysis the TIC signal is enhanced compared to the earlier studies [18] with much less sample needed for the analysis. The broad Paclitaxel peak is largely due to the overloaded sample amount on the column since this is a predominant component in the mixture. As previously discussed, a significant improvement of peak shape can be achieved using smaller columns resulting in a potential gain of efficiency. Figs. 8 and 9 show the extracted ion chromatograms for selected taxane analogues obtained from the Paclitaxel impurity sample by both conventional analytical LC and miniaturized LC combined with ionspray MS. The peak shape for these taxanes has been dramatically improved with small-bore column as compared to the standard analytical column resulting in increased efficiency as well. It also should be emphasized that the sample injection volume is 20 μl with a crude sample concentration of 10 mg ml^{-1} for a conventional column, while only 1 μl of sample injection volume with a sample concentration of 0.5 mg ml^{-1} is needed for a small-bore column to achieve better MS performance. This represents more than two order of magnitude gain in sensitivity.

The miniaturized HPLC system has been shown to provide fast analysis and sensitive detection of natural products. Enhancement of sensitivity with small-bore and micro-bore columns can be achieved as predicted by theory. The effect of column size on detection sensitivity is significant, and is highly beneficial to systems which incorporate concentration-sensitive detectors. When coupled with other techniques such as ionspray/MS, miniaturized LC can be a powerful tool in analysis of complex substances such as natural products.

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