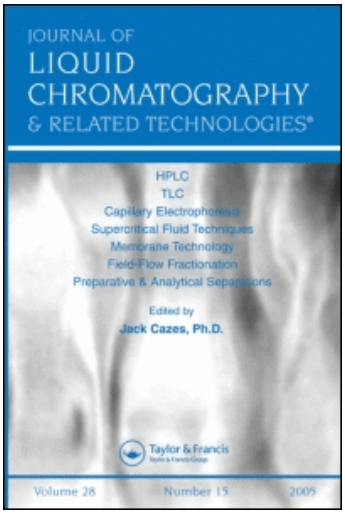

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EVALUATION OF TAXOIDS FROM *TAXUS SP.* CRUDE EXTRACTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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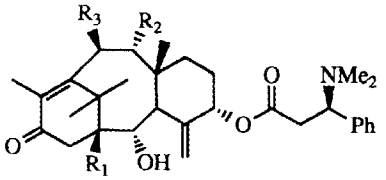
ABSTRACT

The extraction procedures and analysis methods by high-performance liquid chromatography (HPLC) for taxine B (**1**), isotaxine B (**2**) and 10-deacetylbaccatin III (**3**), expected or already known "precursors" of paclitaxel or active analogues, were developed for in series analyses of *Taxus* needles.

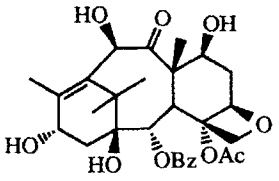
INTRODUCTION

Paclitaxel (Taxol®) and docetaxel (Taxotere®) are two new chemotherapeutic agents which are used for the treatment of cancer. These two drugs are currently prepared from 10-deacetylbaccatin III (10-DAB),[1] a neutral taxoid extracted from the leaves of the yew tree (*Taxus sp.*).[2,3] Taxine B and isotaxine B are basic taxoids, also isolated from the leaves of *Taxus sp.*[4-6] Taxine B, isotaxine B (further noted taxines B) and 10-DAB can be used to synthesize other taxane analogues of biological interest.

In order to select the highest taxoid plants, convenient methods for in series analyses were needed : a number of methods have already been described for paclitaxel content determination, some for 10-DAB evaluation,[7-12] very few for taxines B.[6] As part of our studies on the analysis of taxoids, we wish to report herein the analytical methods that we developed, involving extraction procedures and final quantitation by HPLC of taxines B and 10-DAB.



1 $R_1 = R_2 = OH$, $R_3 = OAc$
2 $R_1 = R_3 = OH$, $R_2 = OAc$
4 $R_1 = H$, $R_2 = OH$, $R_3 = OAc$
5 $R_1 = H$, $R_2 = OAc$, $R_3 = OH$



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MATERIALS

Plant Material

The yew stems were air-dried at room temperature. Dry needles were then removed from the stems and ground. Taxines B and 10-DAB standards were prepared from this plant material (*vide infra*).

Apparatus

The HPLC system consisted of a Waters 616 pump, Waters 717 plus autoinjector, and Waters 996 photodiode array detector (PDA) with a NEC Image 466es computer (Millennium software system) for controlling the analytical system and data processing.

HPLC columns

All the colums were Waters : Analytical columns : Nova-Pak® Silica, 4 µm, 3.9 x 150 mm ; Nova-Pak® C_{18}, 4 µm, 3.9 x 150 mm ; Symmetry™ C_{18}, 5 µm, 4.6 x 250 mm. Semi-preparative columns: Prep. Nova-Pak® HR Silica, 6 µm, 25 x 100 mm.

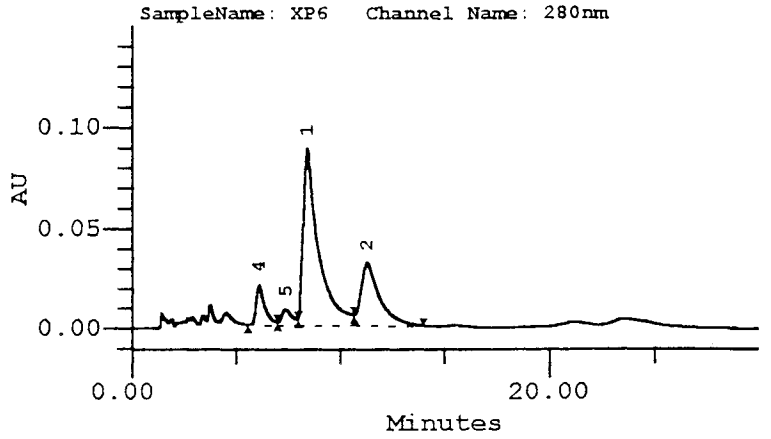

Figure 1. Crude alkaloidal mixture : isocratic, normal phase, separation of "Taxines B" on Nova-Pak Si 4 mm, 3.9 mm x 150 mm column. Mobile phase : $CHCl_3$/MeOH/Et_3N (99.4/0.5/0.1, v/v), 1 mL/min. flow rate **1** = taxine B ; **2** = isotaxine B ; **4** = 1-deoxytaxine B ; **5** = 1-deoxyisotaxine B.

METHODS

Extraction

Procedure for basic taxoids

The ground dry needles (50 g) were moistened with 25% NH_4OH and extracted with CH_2Cl_2 (5 x 200 mL) at room temperature for 7 days. The concentrated CH_2Cl_2 solution was extracted with 2% aqueous HCl until Mayer's test was negative. The combined aqueous layers were basified to pH 9 with 25% NH_4OH and extracted with CH_2Cl_2 (until negative Mayer's test). The CH_2Cl_2 solution was successively washed with H_2O, dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* to provide a crude alkaloidal mixture (TA = total alkaloids).

Procedure for neutral taxoids[2,3]

The ground dry needles (10 g) were extracted with ethanol 95% (60 mL) at room temperature for 1 H. This was repeated 6 times: the resulting organic extracts were evaporated *in vacuo* to give the "ethanolic extract". This

"ethanolic extract" was partitioned between CH_2Cl_2 and H_2O, and the aqueous fraction extracted with CH_2Cl_2. The combined organic layers were finally dried over anhydrous Na_2SO_4, filtered and evaporated to dryness (35°, under reduced pressure) and provided the "CH_2Cl_2 extract".

Standards

Taxine B and isotaxine B were separated and purified by preparative HPLC from the crude alkaloidal mixture of *Taxus baccata*. 10-DAB was also isolated from the leaves of *Taxus baccata*, by classical chromatography, from the crude extract of neutral taxoids.[2,3] Taxines B and 10-DAB were identified in our laboratory from their spectral characteristics.

HPLC Analysis

Taxines B determination

Two methods were developed. Samples for injection were prepared in $CHCl_3$ for method 1, in DMF for method 2 (concentration : 5 mg/mL). The injection volumes were 10 µL at once for standards and samples.

The data were collected over 200-400 nm range of the absorption spectrum and all the chromatograms were plotted at 280 nm.

Method 1

The HPLC column was a Nova-Pak® silica (4 µm, 3.9 x 150 mm). The eluent was isocratic, consisting of $CHCl_3$/MeOH/Et_3N (99.4/0.5/0.1, v/v) at a flow rate of 1 mL/min., in 35 min. The t_R value (retention time) for taxine B and isotaxine B were 8.9 min and 11.2 min (after 35 min all the polar compounds were eluted) (Figure 1). Standards were purified by using a semi-preparative column : Prep Nova-Pak® HR Silica (6µm, 25 x 100 mm). The eluent was isocratic, consisting of heptane/isopropanol/Et_3N (90/10/0.1, v/v) at a flow rate of 8 mL/min, in 50 min. The t_R value for taxine B and isotaxine B were 20.0 min and 25.5 min.

Method 2

The HPLC column was either a Nova-Pak® C_{18} (4 µm, 3.9 x 150 mm) or a Symmetry™ C_{18} (5 µm, 4.6 x 250 mm).

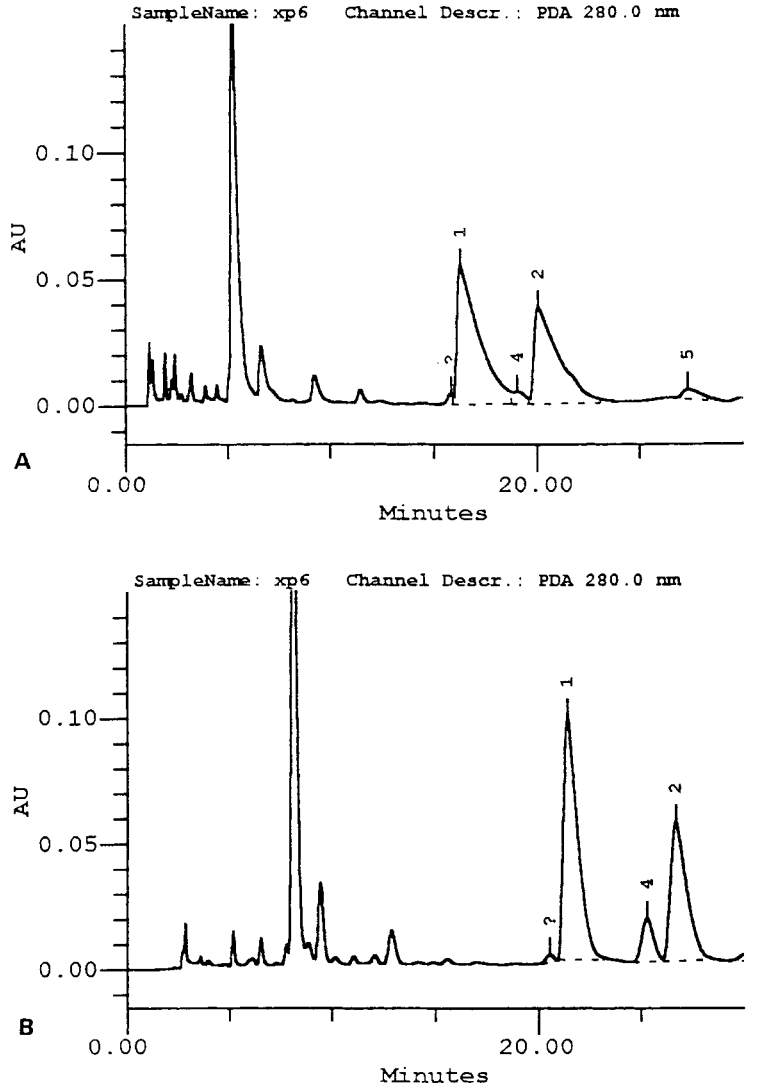

Figure 2. Crude alkaloidal mixture : isocratic, reversed phase, separation of "Taxines B". **2A**. Separation on Nova-Pak C18, 4µm, 3.9 x 150 mm column. **2B**. Separation on Symmetry, 5 µm, 4.6 x 250 mm column. Mobile phase for both columns was CH_3CN/H_2O/TFA (23/77/0.05, v/v),1 mL/min flow. **1** = taxine B ; **2** = isotaxine B ; **4** = 1-deoxytaxine B ; **5** = 1-deoxyisotaxine B.

The eluent was isocratic consisting of CH_3CN/H_2O/TFA (23/77/0.05, v/v) at a flow rate of 1 mL/min in 35 min; then, a linear gradient to CH_3CN/H_2O/TFA (50/50/0.05, v/v) over 10 min. The taxine B and isotaxine B were clearly resolved respectively at 21.4 and 26.7 min (Figure 2).

10-Deacetylbaccatin III determination

Samples for injection were prepared in DMF and kept at 10°C (20 mg/mL). The injection volumes were 10 µL at once for standards and samples. The data were collected over 200-350 nm range of the absorption spectrum and all the chromatograms were plotted at 240 nm. The HPLC column was a Nova-Pak® C_{18} (4 µm, 3.9 x 150 mm).

The eluent was isocratic consisting of 100% solvent A (MeOH/H_2O/AcOH, 39/61/0.1, v/v) in 14 min, then linear gradient to 100% solvent B (0.1% AcOH in MeOH) in 10 min and isocratic with solvent B (100%) in 10 min. The flow rate was 1 mL/min. The t_R value for 10-DAB was 10.7 min (Figure 3).

RESULTS AND DISCUSSION

As part of an ongoing project on the selection of the "best" plants from which a biotechnological production of active taxoids or "precursors" of them will be developed, as well as silviculture in open field conditions and new semisyntheses of paclitaxel (= Taxol®) analogues, we needed to develop convenient methods for in series taxines B and 10-DAB content evaluation.

For Taxines B

From total alkaloids two HPLC methods were developed to separate the taxines B. The first one consisted in using a silica column; two elution systems were tested with analytical and preparative objectives :

A. $CHCl_3$/MeOH/Et_3N (99.4/0.5/0.1, v/v)

B. Heptane/isopropanol/Et_3N (90/10/0.1 v/v)

From the crude basic taxoid extract the elution order of the constituents differs with the elution system. Purified taxine B and isotaxine B were isolated by using HPLC semi-preparative column. When eluent (A) is used with preparative objective a solvatation is suspected between Et_3N and taxines B and

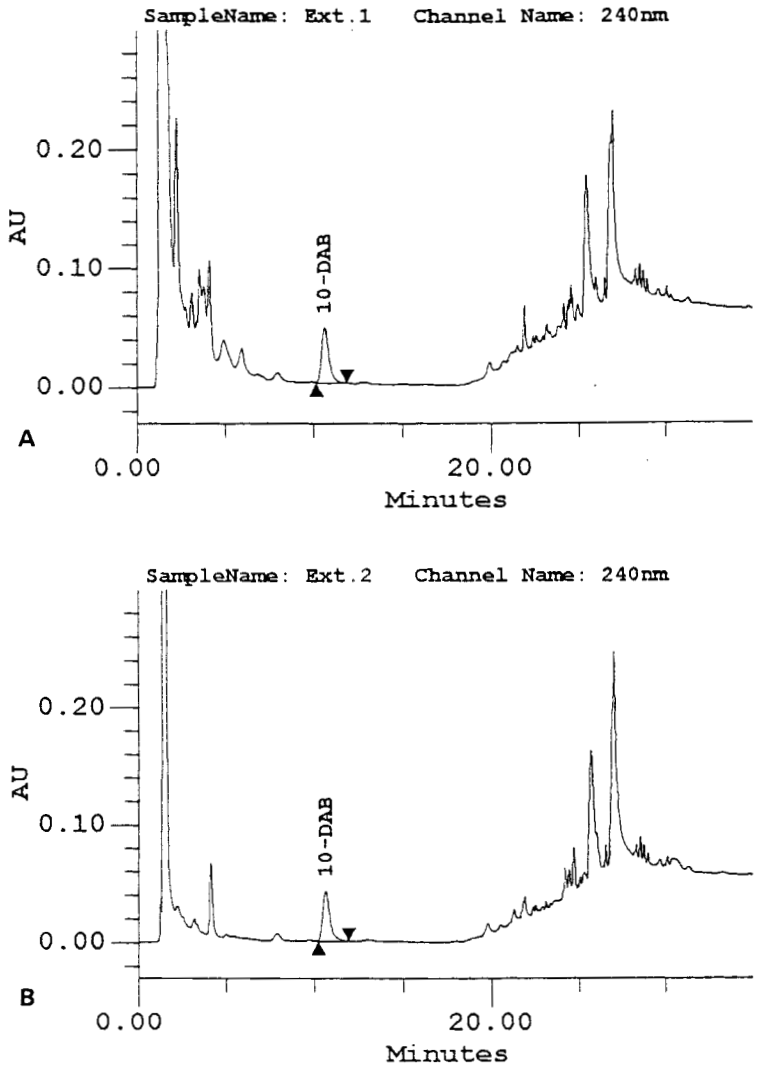

Figure 3. Crude neutral extracts : Separation on Nova-Pak C_{18}, 4 µm, 3.9 x 150 mm column. Mobile phase : MeOH/H_2O/AcOH (39/61/0.1, v/v) during 14 min. then a linear gradient to MeOH/AcOH (100/0.1, v/v) in 10 min., 1 mL/min. flow rate. **3A**. EtOH extract ; **3B** : CH_2Cl_2 extract ; 10 DAB = 10-deacetylbaccatin III.

Table 1

Characteristics of Columns

	Symmetry		Nova-Pak (Silica)		Nova-Pak C_{18}
	Taxine B (1)	**Isotaxine B (2)**	**Taxine B (1)**	**Isotaxine B(2)**	**10DAB (3)**
r^2	0.9999	0.9999	0.9995	0.9997	0.999
lowest RSD	0.01%	0.2%	0.3%	0.1%	0.02
highest RSD	2.6%	2.5%	1.5%	6.2%	2.1

a final partition CH_2Cl_2/H_2O is needed, with only 50% yield; with eluent (B) the higher boiling temperatures of heptane and isopropanol help along the triethylamine evaporation. In both eluents, the expected transesterification is observed, mainly in the way isotaxine B → taxine B; in the conditions of HPLC this transesterification is very fast, as much as it is when isotaxine B is kept in methanolic solution. A ratio 40/60 (isotaxine B/taxine B) is fastly obtained. Chromatograms were interpreted by co-injecting total alkaloids with each standard and recording the UV spectra.

A second method was carried out to try to separate, more efficiently, taxine B and isotaxine B from total alkaloids; it consisted in using reversed phase HPLC.

Two different columns were tested: Nova-Pak® C_{18} and Symmetry C_{18} (the last one was more recently available); the Symmetry column is especially convenient for basic molecules like taxines B: the chromatograms show peaks with very good symmetry.

The best solvent system was CH_3CN/H_2O/TFA (23/77/0.05, v/v). In this mobile phase, transacetylation appeared to be very slight: when taxine B was collected, its solution evaporated and the residue injected again, the measured isomerisation was 0.68 %; in the same conditions, isotaxine B showed 3% isomerisation.

Calibration curves were fixed up for both used methods. The linearity of the detector response between the peak area and the concentration was determined by injecting twice a series of five standard solutions ranging in concentration from 0.2 to 3.2 mg/mL: the relative standard deviation (R.S.D.) was calculated for each concentration (Table 1).

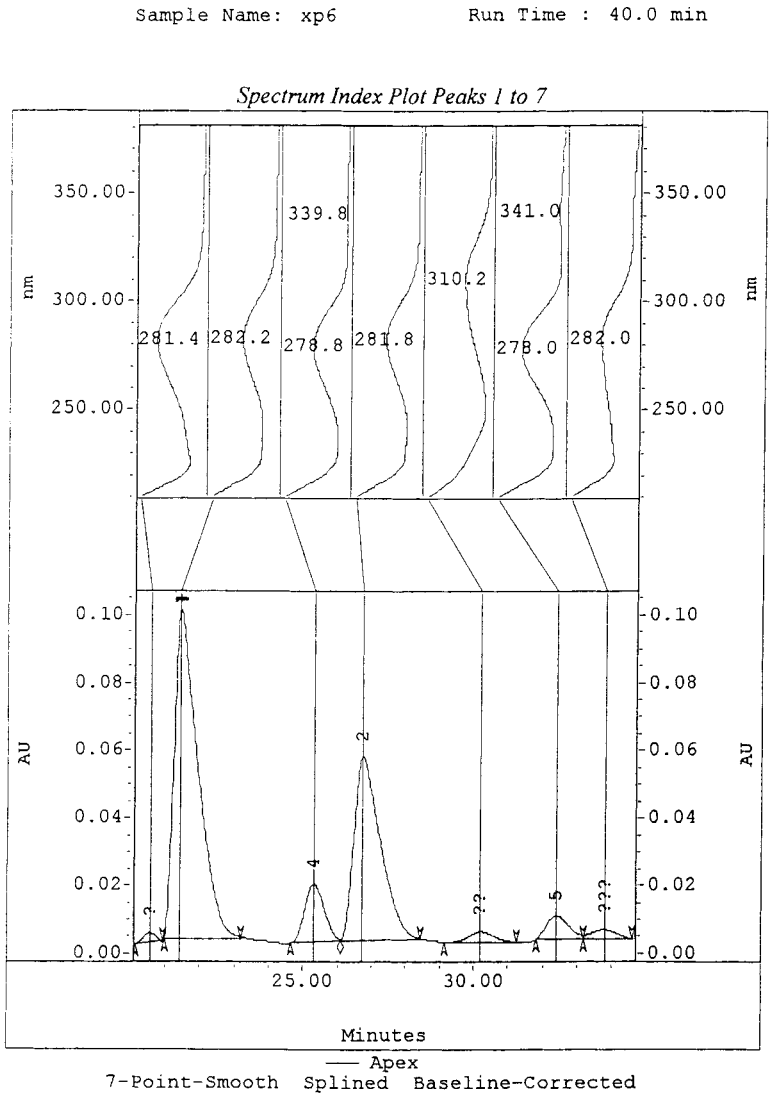

Figure 4. Diode array scans of "Taxines B" over 200 to 400 nm range of the absorption spectrum. **1** = taxine B ; **2** = isotaxine B ; **4** = 1-deoxytaxine B; **5** = 1-deoxyisotaxine B.

So, reversed phase HPLC of total basic taxoid extract provides a good manner to evaluate separately taxine B and isotaxine B contents but HPLC analysis on silica column is sufficient to evaluate globally taxines B. This method allowed to analyse easily more than 100 samples of various *Taxus sp.* needles; compared with the already published method,[6] it presents the advantage of avoiding the use of a buffer (the elimination of which is often tedious and the obtaining of pure compounds difficult).

These analyses showed significant variations in taxines B content : 0.13-7.53 g/kg ; they could represent 8.7-91% of the crude basic taxoid extract. Two other compounds were relatively abundant in some extracts; by semi-preparative HPLC, they have been separated, characterized and identified to 1-deoxytaxine B (**4**) and 1-deoxyisotaxine B (**5**).[6] (Figure 4).

For 10-Deacetylbaccatin III

After evaluation of 10-DAB in both extracts, ethanol and CH_2Cl_2, it was seen that the results were similar. 10-DAB being well "isolated" in both chromatograms; so, for in series evaluation, the EtOH extract, carried out faster, was preferred.

The separation of 10-DAB from the crude "ethanolic extract" by HPLC on a classical C_{18} column in previously described conditions was satisfactory. The 10-DAB peak appears sufficiently free of co-eluting material to allow the content determination.

The linearity of the detector response was determined by injection of standard solutions ranging in concentration from 15 to 200 μg/mL. A linear relationship was observed between the peak areas and the concentration of 10-DAB in the studied range; relative standard deviation (R.S.D.) was calculated for each concentration (Table 1).

To their credit, these new conditions of HPLC analysis provide, from crude ethanolic extracts of *Taxus* needles, clear chromatograms allowing an easy 10-DAB content determination. The analyses also showed important variations for 10-DAB content 3 - 959 mg/kg.

ACKNOWLEDGMENT

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