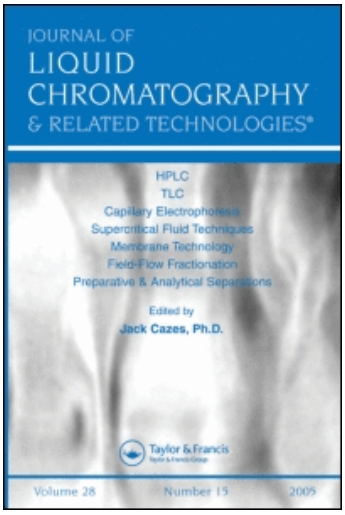

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INVESTIGATIONS ON PREPARATIVE THIN-LAYER CHROMATOGRAPHIC SEPARATION OF TAXOIDS FROM *TAXUS BACCATA L.*

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ABSTRACT

Many mobile phases, including solvent systems with one and two polar modifiers as well as some multiple development techniques and gradient elutions, were examined to select the best thin-layer chromatographic conditions on silica gel F_{254} plates for micropreparative isolation of 10-deacetylbaccatin III (10-DAB III), baccatin III, paclitaxel, and cephalomannine from methanolic extracts obtained from fresh and dried needles and stems of *Taxus baccata* L. In case of each solvent system and development technique, R_F, k, and α values of 10-DAB III and paclitaxel were determined as well as the separation of compounds coextracted with taxoids, and the whole yew extract. The best mobile phase, benzene-chloroform-acetone-methanol (20:92.5:15:7.5) used twice over a distance of 15 cm was applied for isolation of analyzed taxoids by preparative TLC in connection with RP-HPLC, (C-18) quantitative determination using two mobile phases (30% acetonitrile in water to measure of 10-DAB III levels and 50% acetonitrile in water for baccatin III, paclitaxel, and cephalomannine).

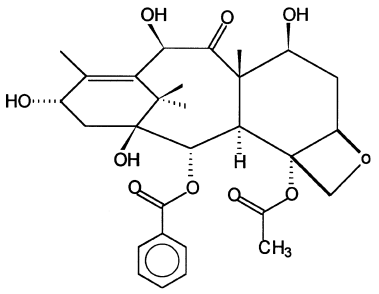
In this way, concentrations of 10-DAB III, baccatin III, paclitaxel, and cephalomannine were determined in the fresh needles and stems of *Taxus baccata* L. collected during the late autumn-spring period (November to April) as well as in dried plant material. Observed retention behavior of baccatin III on silica gel in comparison with its precursor (10-DAB III) is discussed. Some of the investigated solvent systems can be applied for improved column chromatographic separation of the analyzed taxoids.

INTRODUCTION

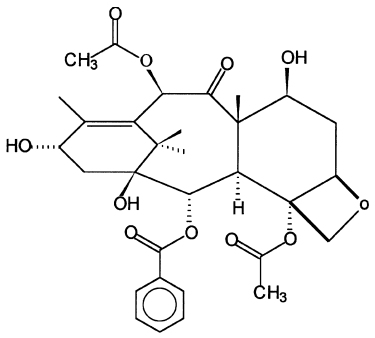
Several species of yew (*Taxus* spp.) contain diterpenoid compounds possessing strong anticancer activity.[1] They were called taxoids. These compounds appeared after screening of medicinal plants with possible antimitotic properties carried out by the National Cancer Institute in the 1960s.[2] Paclitaxel (Taxol), the first taxoid, was isolated by Wani and Wall from the extract of the bark of western yew, *Taxus brevifolia* Nutt.[3] It possessed strong activity against various leukaemias and other tumors.[4] Taxol, which is now a registered trade name for paclitaxel of Bristol-Myers Squibb, is among the most active anticancer drugs. It is mainly applied in the treatment of ovarian cancers resistant to other anticancer compounds[5-6] but it has also been tried to apply in the treatment of other cancers.[6-9]

Among the most important taxoids is a group derived from 10-deacetylbaccatin III (10-DAB III), a diterpenoid compound occurring in high concentration in European yew, *Taxus baccata* L. (more than 0.1% in the needles in some periods).[10] It possesses scheme of structure of four skeletons (6/8/6/4-membered) which was named taxan and 10-DAB III is a derivative of hexahydroxy-11-taxen-9-one (Scheme 1). Baccatin III which is another compound from this group has additionally an acetyl group estrificated with β-OH group at position C-10. Paclitaxel and cephalomannine are less polar taxoids because they possess amide-acid side chains at position C-13. In case of paclitaxel this is (2R,3S)-N-benzoyl-3-phenylsoserine and cephalomannine (2R,3S)-N-tigloyl-3-phenylsoserine side chains. There are also compounds in this group which have an epimer OH group at position C-7 and other substituents are also met.[11-12] So there are many compounds usually with similar polarity and small amounts thus difficult to separate.

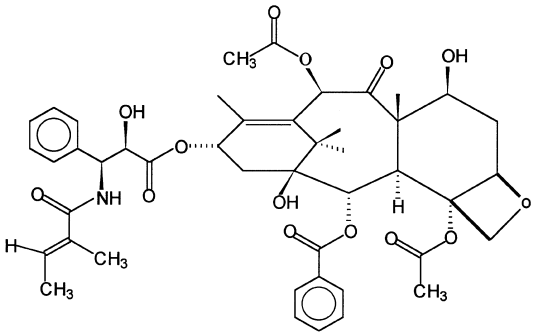
The importance of 10-DAB III and other taxoids lacking a side chain at position C-13 (e.g. baccatin III) has increased since, the 10-DAB III is applied in hemisynthetic procedures involving Taxol[13-14] and another anticancer drug, Taxotere (Docetaxel).[15]



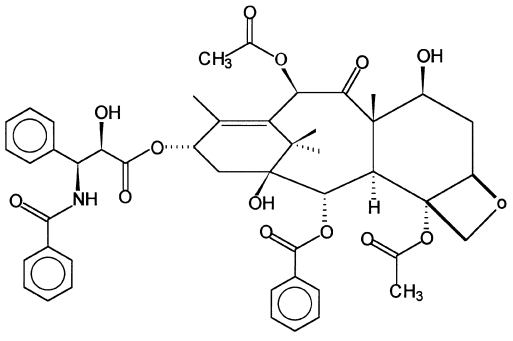
10-deacetylbaccatin III (10-DAB III)



baccatin III



cephalomannine



paclitaxel

Scheme 1. Chemical structures of analyzed taxoids.

This is because there are only a few steps leading to Taxol and Taxotere from 10-DAB III and high amounts of 10-DAB III in the needles of *Taxus baccata* L., which are a renewable source of this compound. Synthetic procedures developed independently by Holton et al. and Nicolaou et al. in 1994 are very complicated [16] and they are not commercially feasible.

In the determination of 10-DAB III and its derivatives (paclitaxel, cephalomannine, baccatin III, and others) in plant material and tissue cultures various RP-HPLC methods with UV detection have been applied.[17-23] Hyphenated techniques: HPLC/MS and HPLC/MS/MS were also used.[24-25] Immunological detection using ELISA method has been applied in the analysis of 10-DAB III and paclitaxel.[26-27]

Micropreparative thin layer chromatography was seldom used in direct separation of yew extracts in connection with HPLC determination of paclitaxel and cephalomannine,[28-29] as well as 10-DAB III and baccatin III[17], or with ELISA analysis of 10-DAB III in tissue cultures of *Taxus baccata* L.[26] There are only a few mobile phases commonly applied in TLC analysis of yew extracts on silica gel plates.[30-31] Two-dimensional TLC (2D TLC) of extracts from the bark of *Taxus brevifolia* Nutt. was carried out by Stasko et al.[32] There are no systematic studies on TLC separation of yew extracts for the purpose of obtaining, on a micro-preparative scale, 10-DAB III and other less polar taxoids. These investigations are important, as TLC can be applied in a pilot test for an improved column chromatographic method for obtaining this compound.

In the present work, the investigations on selection of the best mobile phases to assure the best separation of analysed taxoids, especially of 10-DAB III, as well as its less polar derivatives, baccatin III, paclitaxel, and cephalomannine obtained from the extracts of fresh and dried needles and stems of *Taxus baccata* L., have been undertaken. Simple RP-HPLC isocratic procedures could be further applied for quantification.

EXPERIMENTAL

Plant Material

First and second year twigs of *Taxus baccata* L., grown in the Saski Garden in Lublin, were collected in the following days: 5 XI ’96, 18 XII ’96, 28 I ’97, 3 III ’97 and 22 IV ’97. Then, these twigs were divided into the needles and the remaining stems. The needles and the stems were cut into small parts (2 mm). The remaining twigs collected in November were stored at room temperature for 2 days and then redried at 50°C in a desiccator. After drying they were separated into the needles and stems and cut into small parts.

Figure 1. Scheme of isolation of 10-DAB III, baccatin III, paclitaxel, and cephalomannine from *Taxus baccata L.*

Extraction and Purification (see Figure 1)

The plant material (5g of needles and stems separately) was extracted with hot 100% methanol (3 x 100 cm^3) on a boiling water bath, under reflux, for 2 hr in each case. Then, combined methanolic extracts were evaporated to dryness under reduced pressure, the residue was dissolved in methanol, transferred to a 25 cm^3 volumetric flask, and diluted to the mark with the solvent. These solutions were further used for thin layer chromatographic investigations on silica gel 60 F_{254} plates.

Before preparative TLC, crude methanolic extracts were preliminarily purified by liquid-liquid extraction using the following procedure: bidistilled water was added to suitable volume of methanolic extract (1+1) (5 cm^3 in the case of extracts from fresh stems, 4 cm^3 in the case of extracts from fresh needles and dried stems, and 2 cm^3 in the case of extracts from dried needles). The obtained solutions were first extracted with n-hexane (2 x, the volumes of n-hexane were equal to the volumes of methanolic extracts used) to remove waxes and some chlorophylls, and the hexane fractions were discarded; then, they were extracted with dichloromethane (5 x, the volumes of dichloromethane were equal to the volumes of methanolic extracts used). A 5 min delay was maintained after each process of extraction to reach equilibrium. The dichloromethane soluble fractions (MC fractions) containing taxoids were combined together by filtering over an anhydrous sodium sulphate and then evaporated to dryness under reduced pressure.

Thin-Layer Chromatography (TLC)

The purified methanolic extracts of *Taxus baccata* L. as well as, methanolic solutions of taxoid standards (10-deacetylbaccatin III, baccatin III, paclitaxel, and cephalomannine, purchased from Sigma, St. Louis, MO), were spotted or applied as bands with a Teflon applicator on the start line of silica gel 60 F_{254} pre-coated plates (E Merck, Darmstadt, Germany), 0.25 mm thickness (10 x 10 cm, 10 x 20 cm, 20 x 20 cm), as well as, with 2 cm wide pre-concentration zone of silica gel, and were run in horizontal DS Chambers (Chromdes, Lublin, Poland).

In the case of micropreparative TLC separation of 10-DAB III, baccatin III, paclitaxel, and cephalomannine, the dry residues of MC fractions were dissolved in 1-1.2 cm^3 of methanol and subjected into silica gel 60 F_{254} pre-coated plates, 20 x 20 cm, 0.25 mm thickness (E Merck, Darmstadt, Germany), and developed with a quaternary mobile phase: benzene-chloroform-acetone-methanol (20:92.5:15:7.5), (2 x 15 cm) in horizontal DS Chambers (Chromdes, Lublin, Poland). After development the plates were analyzed under UV light (254 nm). The located common bands of baccatin III, paclitaxel, and cephalomannine (R_F = 0.55-0.66), as well as, 10-DAB III bands (R_F = 0.30-0.37) were scrapped off the plates. These bands were eluted separately from silica

using acetone-methanol (1:1) mixture (3 x 20 cm^3), followed by centrifuging (2800 r.p.m., for 3 min.). Supernatants were combined, evaporated to dryness under reduced pressure, and dissolved in 3 cm^3 of methanol (Baker, HPLC grade) to final RP-HPLC analysis.

HPLC Analysis

A Hewlett-Packard (Palo Alto, CA) Model 1050 liquid chromatograph, equipped with a stainless-steel 200 x 4.6 mm i.d., Hypersil ODS 5 μm column (Shandon, UK), was used for quantification of 10-DAB III, baccatin III, paclitaxel, and cephalomannine in fresh and dried needles and stems of *Taxus baccata* L. The mobile phases consisted of mixtures of acetonitrile in water 50:50 v/v (for baccatin III, paclitaxel, and cephalomannine), and 30:70 v/v for 10-DAB III. In each case, 10 μL aliquots after TLC were injected into the chromatograph. The analyses were made at 200 nm. An external standard HPLC method was used in the quantitative determination of the analysed taxoids.

RESULTS AND DISCUSSION

The chemical structures of 10-deacetylbaccatin III, baccatin III, paclitaxel, and cephalomannine are shown in Scheme 1. Some of the solvent systems used as the mobile phases in TLC investigations of these compounds on silica gel 60 F_{254} plates are presented in Table 1.

The following criteria in choosing the best mobile phases were included:

a) Suitable range of retardation factor R_F (3-7)

b) Good selectivity which was described by separation factor (relative retention) α

$$\alpha = \frac{k_A}{k_B} = \frac{(1 - R_{F(A)}) * R_{F(B)}}{(1 - R_{F(B)}) * R_{F(A)}} > 1$$

where: k_A = retention factor (capacity factor, k) of 10-DAB III, k_B = retention factor (capacity factor, k) of paclitaxel, or by ΔR_M values, where

$$\Delta R_M = \log\alpha = R_{M(A)} - R_{M(B)}; R_M = \log k = \log\frac{1 - R_F}{R_F}$$

Table 1

Some Solvent Systems (in V Ratios) Used as the Mobile Phases and Means of Development

I_1: Dichloromethane-methanol (95:5); 2 x 8 cm
I_2: Dichloromethane-methanol (92.5:7.5); 8 cm
I_3: Dichloromethane-acetone (70:30); 8 cm
I_4: Dichloromethane-acetone (75:25); 8 cm
I_5: Benzene-acetone(70:30); 8 cm
I_6: Benzene-methanol (90:10); 8 cm
I_7: Chloroform-dioxane (75:25); 8 cm
I_8: n-Heptane-dichloromethane-methanol (20:70:10); 2 x 8 cm
II:$_1$ Dichloromethane-acetone-methanol (75:20:5); 2 x 8 cm
II_2: Dichloromethane-acetone-methanol (80:15:5); 2 x 8 cm
II_3: Chloroform-acetone-methanol (75:20:5); 3 x 8 cm
II_4 Chloroform-acetone-methanol (80:15:5); 3 x 8 cm
III_1: a) Dichloromethane-acetone (75:25); 4 cm
b) Dichloromethane-methanol (92.5:7.5); 4-8 cm (3x)
III_2: a) Dichloromethane-acetone-methanol (80:15:5); 8 cm
b) Dichloromethane-methanol (92.5:7.5); 8 cm
III_3: a) Dichloromethane-methanol (92.5:7.5); 8 cm
b) Dichloromethane-acetone-methanol (80:15:5); 2 x 8 cm
III_4: a) Dichloromethane-acetone-methanol (80:15:5); 9 cm
b) Chloroform-acetone-methanol (80:15:5) 9 cm
III_5: a) Dichloromethane-acetone-methanol (80:15:5), 2 x 10 cm
b) Chloroform-acetone-methanol (85:10:5); 10-15 cm
III_6: a) Chloroform-methanol (92.5:7.5); 8 cm
b) Chloroform-ethylmethylketone-methanol (75:22.5:2.5); 8-15 cm
III_7: a) Chloroform-methanol (92.5:7.5); 8 cm
b) Benzene-acetone-methanol (70:20:5); 8-16 cm
IV: Benzene-chloroform-acetone-methanol (20:92.5:15:7.5); 2 x 15 cm

Not only was good selectivity (high values of α or log α) respective to 10-DAB III and paclitaxel (the former was the most polar and the latter was the less polar among the investigated taxoids) taken into consideration, but it has been also aspired to obtain the best separation of closely eluted coextracted compounds such as polar compounds (especially closely the bands of 10-DAB III) and some chlorophylls (especially in the extracts from the needles). The separation of the whole yew extracts was also considered. All of these parameters are put together in Table 2.

Using 5 or 7.5% of methanol in dichloromethane (or in chloroform) caused the separation of 10-DAB III and paclitaxel with α values of 2.1-2.3, but in the first case second development was necessary. The separation of coextracted

Table 2

The Values of R_F x 100; k, α of 10-DAB III and Paclitaxel*

Solvent Systems	R_F x 100		k		α	Sep'n. of Coextracted Compounds	Sep'n. of the Whole Yew Extracts
	10-DAB III	Paclitaxel	10-DAB III	Paclitaxel			
I_1	15	27	5.7	2.7	2.1	X	X
I_2	35	55	1.9	0.8	2.3	X	X
I_3	32	73	2.1	0.4	5.7	XX	X
I_4	26	70	2.8	0.4	6.6	XX	X
I_5	26	55	2.8	0.8	3.5	XX	XX
I_6	13	18	6.7	4.5	1.5	X	O
I_7	21	56	3.8	0.8	4.8	XX	XX
I_8	33	47	2.0	1.1	1.8	X	X
II_1	59	86	0.7	0.2	4.3	X	X
II_2	38	71	1.6	0.4	4.1	XX	XX
II_3	63	85	0.6	0.2	3.3	XX	XX
II_4	50	79	1.0	0.3	3.7	XX	XX
III_1	41	51	1.4	1.0	1.5	XX	XXX
III_2	59	67	0.7	0.5	1.4	X	X
III_3	61	85	0.6	0.2	3.5	XX	X
III_4	41	57	1.4	0.8	1.9	XX	XX
III_5	54	71	0.9	0.4	2.1	XX	XX
III_6	39	66	1.6	0.5	3.0	XXX	XX
III_7	31	54	2.2	0.9	2.6	XX	XX
IV	36	63	1.8	0.6	3.0	XXX	XXX

* As well as sep'n of coextracted compounds with anal. taxoids and the whole yew extracts in some investigated solvent systems. X = medium, XX - good, XXX = very good, O poor (unsatisfactory).

compounds with 10-DAB III was slightly better using more polar solvent system (I_2) (10% of methanol has already been too much), but it got worse in the separation of less polar taxoids. On account of low R_F values the separation of the whole yew extracts was only partial (Table 2).

We did not obtain better separation of less polar taxoids and the separation factor α was low (1.8) with the mixture of two solvents (n-heptane + dichloromethane 20+70 v/v) with 10% of methanol (I_8).

The bands of analyzed taxoids were very sharp using 10% methanol in benzene (I_6) but R_F values both of 10-DAB III and paclitaxel were very low and the value of α was only about 1.5, also the separation of the whole yew extract was poor.

The mobile phases containing 25-30% of acetone (electron donor polar modifier) in dichloromethane (I_4 and I_3 respectively), caused considerable increase of separation factor values α (6.6 and 5.7 respectively), and high R_F values of paclitaxel were observed (0.70 and 0.73 respectively). The polar

coextracted compounds were adsorbed on the start line. The increase of α value, in the case of a decrease of acetone concentration in dichloromethane (from 30 to 25% v/v), was followed by only a slight increase in separation of less polar taxoids. When dioxane (another electron donor solvent) in the same concentration (25%) in chloroform was used (I_7), the separation factor α decreased to 4.8 but the separation of the whole yew extract was good.

Benzene (or toluene) used as solvent with 30% of acetone (I_5) caused good separation of chlorophylls neighbouring to less polar taxoids (paclitaxel and cephalomannine), and the polar coextracted compounds were poorly eluted. In this case α value was 3.5.

Double development using mobile phase containing dichloromethane-acetone-methanol (75:20:5) (II_1) obtained high R_F values for 10-DAB III and paclitaxel (0.59 and 0.86 respectively) and α value was 4.3 but the separation of less polar taxoids was not enough good. The decrease of acetone concentration from 20 to 15% in the same solvent system, again using double development (II_2), caused considerably better separation of the bands closely eluted with paclitaxel and cephalomannine, and the separation of the whole yew extracts was also good. Separation factor α amounted to 4.1. When chloroform, instead of dichloromethane, in such solvent systems was used (II_3 and II_4), triple development was necessary to obtain the separation with α values similar (or slightly lower) to those obtained with dichloromethane (3.3 and 3.7 respectively), but the bands of taxoids were sharp and the separation of the whole yew extract was also good. The majority of polar compounds were concentrated on the start line.

Using a development technique composed of two solvent systems with one polar modifier (the first was 25% of acetone in dichloromethane over a half of development distance (4 cm), and the second was 7.5% of methanol in dichloromethane (4-8 cm, 3 x) (III_1)), we obtained very good separations of the whole yew extracts with α value equal to 1.5 and R_F values: 0.41 and 0.51 for 10-DAB III and paclitaxel respectively. When we changed the first mobile phase, adding 5% of methanol together with 15% of acetone in dichloromethane, and also applied development over the whole distance, and then over the same distance the mobile phase containing 7.5% of methanol in dichloromethane (III_2) was used, the separation of yew extracts became considerably worse and the separation of coextracted compounds was realized with the analyzed taxoids. Reversing the mobile phases order applied in this development technique (III_3) and additionally using second development with dichloromethane-acetone-methanol (80:15:5) mixture, the values of separation factor α increased to 3.5, and the R_F values for 10-DAB III and paclitaxel to 0.61 and 0.85 respectively. The separation of coextracted compounds with 10-DAB III was better than those ones with paclitaxel and cephalomannine.

The values of α and R_F decreased when two mobile phases with two polar modifiers were used: dichloromethane-acetone-methanol (80:15:5) was first applied over 2/3 of the whole development distance (10 cm) (2 x), and then chloroform-acetone-methanol (85:10:5) (III_5); but in this case the separations of the whole yew extracts considerably got better, as well as, the separation of coextracted compounds especially with 10-DAB III.

When we first applied chloroform-methanol (92.5:7.5) over half of the whole distance, and then the mobile phase containing 22.5% of ethylmethylketone (2-butanon) together with 2.5% of methanol in chloroform (III_6) we obtained very good separations of the whole yew extracts as well as the separation of coextracted compounds, both with 10-DAB III and paclitaxel. Separation factor α amounted to 3.0 and R_F values of 10-DAB III and paclitaxel were 0.39 and 0.66 respectively; but on account of above 20% addition of ethylmethylketone the time of development was too long.

Benzene used instead of chloroform and acetone for ethylmethylketon in the second mobile phase (III_7) caused better separation in the region of less polar taxoids thus cleaning of the band of paclitaxel and cephalomannine got better. The selectivity using this development technique was similar to ethylmethylketon mode (III_6) (α values were 2.6 and 3.0, respectively).

Quaternary mobile phases composed of reinvestigated solvents: benzene, chloroform, acetone, and methanol were another approach to the optimizing process. The best of them was the solvent system containing benzene-chloroform-acetone-methanol (20:92.5:15:7.5) developed over a distance of 15 cm (2 x, in some cases third development was necessary). Using this mobile phase we obtained very good separation of co-extracted compounds both with 10-DAB III and paclitaxel, as well as, the separation of the whole yew extracts (see Figure 2). The separation of taxoids was better in the extracts obtained from fresh needles and stems than from the dried plant material. The extracts from the dried needles contained the highest concentrations of ballast compounds interfering with the bands of analyzed taxoids. Localization of 10-DAB III, baccatin III, paclitaxel, and cephalomannine in the isolated bands was confirmed by RP-HPLC assay and by chromatographic separation of fortified yew extracts.

From the table on R_F, k and R_M values of standards of analyzed taxoids (Table 3) in conditions of micropreparative TLC separation, suitable values of retardation factor R_F of the analyzed taxoids (0.4-0.7), with separation factor α (10-DAB III/paclitaxel) amounting to 3.4, have been obtained. Esterification of free the OH group of 10-DAB III at position C-10 by acetic acid, which takes place in baccatin III, caused the considerable slope of baccatin III retention on silica gel in comparison with retention of 10-DAB III. This indicates an increase of hydrophobic properties in the molecule of baccatin III.

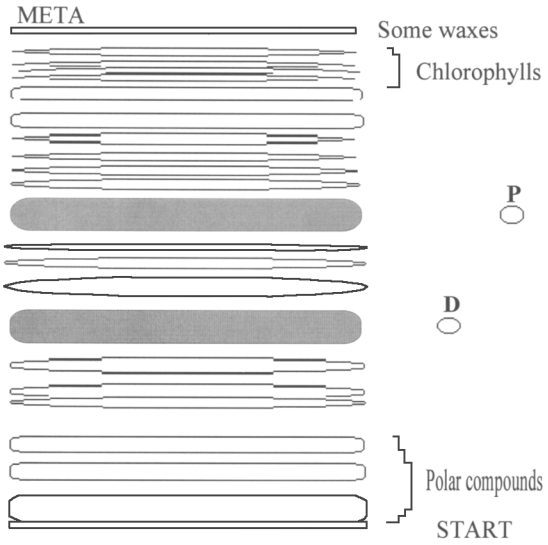


Figure 2. TLC separation of purified methanolic extract from fresh needles of *Taxus baccata* L. on silica gel 60 F_{254} plate. Mobile phase: benzene-chloroform-acetone-methanol (20:92.5:15:7.5). Distance of development: 2 x 15 cm. (**D** – 10-DAB III; **P** – paclitaxel. **Grey band** – band of 10-DAB III; **grated band** – common band of baccatin III, paclitaxel and cephalomannine).

Table 3

R_F, k, and R_M Values of Standards of Analyzed Taxoids in Conditions of Micropreparative TLC Separation*

Analyzed Taxoid	R_F	k	R_M (log k)
10-Deacetylbaccatin III	0.37	1.7	0.2
Baccatin III	0.63	0.6	-0.2
Paclitaxel	0.66	0.5	-0.3
Cephalomannine	0.66	0.5	-0.3

* Mobile phase: benzene-chloroform-acetone-methanol (20:92.5: 15:7.5). Distance of Development: 2 x 15 cm.

Table 4

Average Values of Retention Factor k in RP-HPLC Analysis of 10-DAB III, Baccatin III, Paclitaxel, and Cephalomannine Using ODS Hypersil Column[a,b]

Analyzed Taxoid		k
10-Deacetylbaccatin III	2.1	⊗
Baccatin III	1.0	⊕
Paclitaxel	3.7	⊕
Cephalomannine	3.0	⊕

[a] Column - d_p = 5 μm. [b] ⊗, ⊕ - The conditions of RP-HPLC Analysis. ⊗ - acetonitrile - water (30:70) (v/v), ⊕ - acetonitrile - water (50:50) (v/v).

First, we can explain such chromatographic behavior of baccatin III by lack of the free OH group at position C-10, capable of affecting competitively with free OH groups on the surface of silica gel (hydrogen-bonding interactions), and secondly, the presence of an acetyl (ester) group at position C-10 can impede adsorption of baccatin III on silica gel caused by hydrogen-bonding interactions between carbonyl group at position C-9 and OH groups on the surface of silica gel (kind of spherical hindrance. This is observed in the computer modeling of spatial structure of taxoids by HYPERCHEM) because a role of this carbonyl group in polar properties of taxoids was confirmed by analysis of ^{1}H NMR spectra.[33]

This can be responsible for the decrease of baccatin III retention on silica gel which is similar with retention of paclitaxel and cephalomannine; both possess the acetyl group at position C-10 and low polar side chains at position C-13. Because of very slight differences in the structure of the amide substituent of the acyl side chain at position C-13 (N-benzoyl vs. N-tigloyl) and low polar properties, these two compounds have the same retention on silica gel.

In HPLC taxoids determination more polar a solvent system (30% of acetonitrile in water) was suitable to base-line separation of 10-DAB III (with k value amounting to 2.1, see Table 4) and its closely eluted coextracted compounds (see Figure 3). However an increase of acetonitrile concentration in water to 50% caused overlapping of the 10-DAB III peak and two small neighbouring peaks which usually accompanied the 10-DAB III in yew extracts (especially obtained from the dried needles).

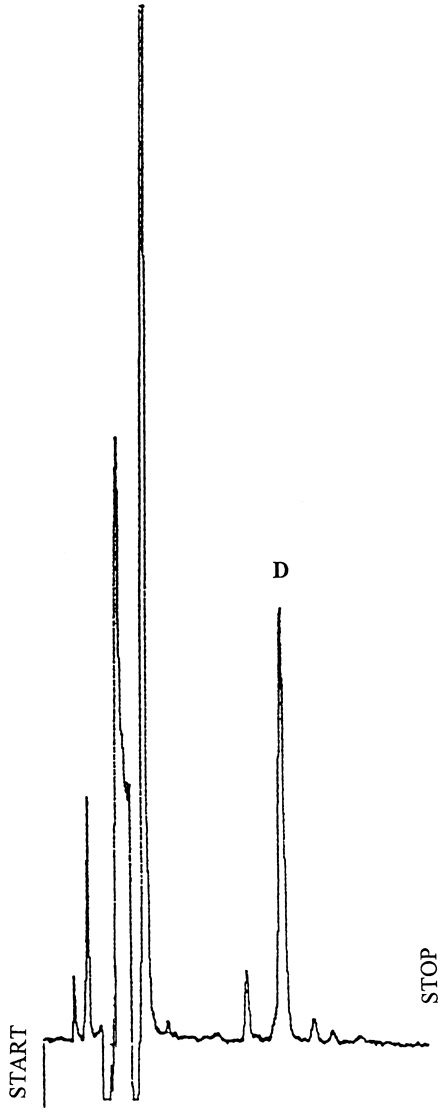


Figure 3. RP-HPLC analysis of 10-DAB III band obtained from the extract of fresh stems of *Taxus baccata* L. (stationary phase: Hypersil ODS 5µm 200 x 4.6 mm i.d.; mobile phase: acetonitrile-water 30:70 v/v) (**D**- 10-deacetylbaccatin III).

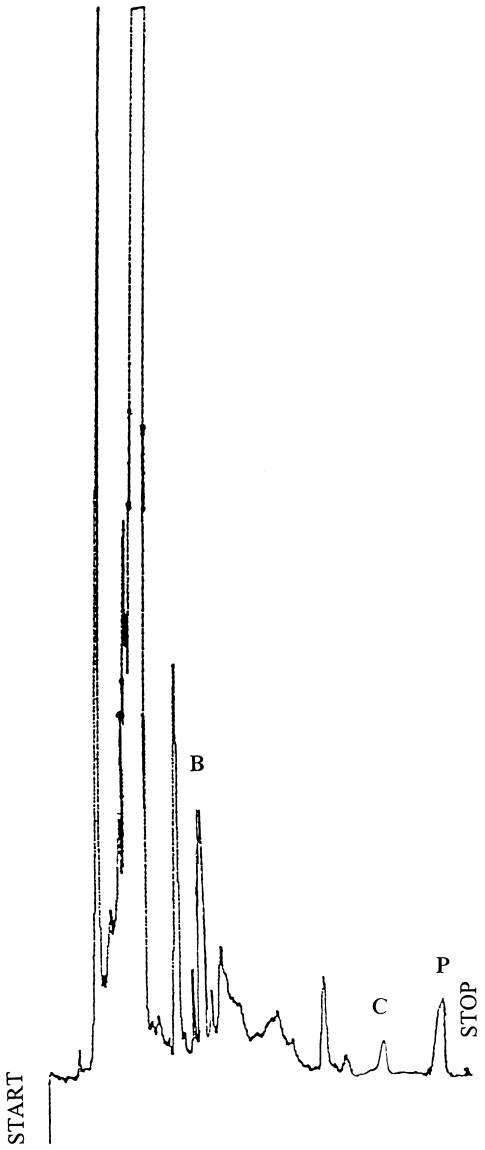


Figure 4. RP-HPLC analysis of the common band of less polar taxoids obtained from the extract of the fresh stems of *Taxus baccata* L. (stationary phase: Hypersil ODS 5µm 200 x 4.6 mm i.d.; mobile phase: acetonitrile-water 50:50 v/v) (**B**- baccatin III, **P**- paclitaxel, **C**- cephalomannine).

Table 5

Concentrations of Analyzed Taxoids in the Needles of *Taxus Baccata L.*

Date of Collection	Concentration, •g/g (dry wt.) 10-DAB III	Baccatin III	Paclitaxel	Cephalomannine
5 XI'96 (f)	50.2 ± 5.7	34.7 ± 2	33.7 ± 4.2	204.8 ± 16.8
5 XI'96 (d)	55.7 ± 8	14.8 ± 1.4	2.8 ± 0.7	6.7 ± 0.8
18 XII'96 (f)	82.4 ± 2	26.0 ± 4.6	12.8 ± 0.1	106.1 ± 8.2
28 I'97 (f)	145.9 ± 1.6	37.2 ± 1.1	12.9 ± 2.2	34.6 ± 4.3
3 III'97 (f)	96.4 ± 0.5	45.3 ± 3.1	18.8 ± 1.3	59.1 ± 4.2
22 IV'97 (f)	57.2 ± 0.8	19.3 ± 1.0	11.7 ± 1.5	38.7 ± 4

f - fresh needles. d - dried needles (stored for 2 days at room temperature and then redried at 50°C.

Table 6

Concentrations of Analyzed Taxoids in the Stems of *Taxus Baccata L.*

Date of Collection	Concentration µg/g (dry wt.) 10-DAB III	Baccatin III	Paclitaxel	Cephalomannine
5 XI'96 (f)	68.0 ± 2	9.8 ± 0.2	3.6 ± 0.4	16.5 ± 4.1
5 XI'96 (d)	44.4 ± 1.9	13.7 ± 0.7	3.9 ± 0.3	2.1 ± 0.4
18 XII'96 (f)	302.2 ± 17.7	45.7 ± 0.8	12.7 ± 2.5	6.2 ± 0.8
28 I'97 (f)	128.8 ± 0.9	47.1 ± 1.9	18.9 ± 2.4	7.9 ± 2
3 III'97 (f)	162.9 ± 1.5	21.6 ± 2.2	9.0 ± 2.2	52.8 ± 3.8
22 IV'97 (f)	91.7 ± 0.7	9.1 ± 0.8	2.3 ± 1	17.8 ± 1.6

f - fresh stems. d - dried stems (stored for 2 days at room temperature and then redried at 50°c.

This mobile phase made possible discrimination of baccatin III, paclitaxel, and cephalomannine peaks in isolated common bands (Figure 4) with k values: 1.0, 3.7 and 3.0 respectively (Table 4). The bands of analyzed taxoids isolated from the extracts from the needles contained more peaks of coextracted compounds with taxoids than those obtained from the stem extracts.

The stems collected on 18th of December contained the highest concentration of 10-DAB III (more than 300 μg/g dry wt.), (see Table 6). In the fresh needles the concentration of this taxoid was the highest in January (about 150 μg/g dry wt.) (see Table 5). The highest concentrations of baccatin III in the fresh stems in December and January were measured, but, in March, the fresh needles amounting to about 50 μg/g dry wt. The concentrations of paclitaxel were usually higher in the fresh needles than in the fresh stems (12-34 μg/g dry wt). High concentration of cephalomannine (about 200 μg/g dry wt.) in the fresh needles, in November, was determined. Dried needles and stems contained usually lower levels of analyzed taxoids, especially of cephalomannine and paclitaxel (dried needles).

CONCLUSIONS

Using binary mobile phases containing 25-30% of one polar electron-donor modifier (acetone, dioxane) in dichloromethane or chloroform, high values of separation factor α (10-DAB III/paclitaxel) are observed, as well as low elution of polar ballast compounds. Such chromatographic systems can be applied in the separation by column chromatography, of polar from less polar taxoids and their polar coextracted compounds in preliminary CC investigations on taxoids before further detailed studies.

Small addition (already about 15%) of π-electron solvents such as benzene gives better separation of the band of less polar taxoids (paclitaxel, cephalomannine) and closely eluted chlorophylls.

The mobile phases with two polar modifiers (acetone-methanol, ethylmethylketone-methanol) assure relatively high values of α factor and the separation in the area of less polar taxoids is better in comparison with the separation obtained by one electron-donor mobile phases.

Because of the complexity of composition of yew extracts, different multiple development techniques and gradient elutions can be considered as further steps of detailed CC or TLC separations of different taxoids on silica gel.

Presence of an acetyl ester group at position C-10 in the structure of baccatin III as probably a kind of sterical hindrance can be responsible for considerable decrease of its retention in comparison with retention of its 10-deacethyl derivative (10-DAB III).

In simple separation of four usually encountered taxoids in yew extracts (the polar one: 10-DAB III and its three less polar derivatives: baccatin III, paclitaxel and cephalomannine) by preparative TLC hyphenated with RP-HPLC

determination (two independent isocratic mobile phase systems) a quaternary mobile phase containing benzene-chloroform-acetone-methanol (20:92.5:15:7.5) can be recommended.

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