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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ARYLTETRAHYDRONAPHTHALENE LIGNANS

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SUMMARY

The retention behaviour of some aryltetrahydronaphthalene lignans is studied. Adsorption chromatography on silica eluted with *n*-heptane–dichloromethane–methanol (90:10:4) and reversed-phase chromatography on a C_{18} column with methanol–water (45:55) as mobile phase are effective for the resolution of the seven known diastereoisomers of podophyllotoxin. Methanol is essential for the separation of the diastereoisomers by reversed-phase chromatography, but is less useful for the separation of the functional-group derivatives of podophyllotoxin. Acetonitrile and/or dimethyl sulphoxide are superior for the resolution of functional derivatives, but are unable to separate the diastereoisomers. A ternary mobile phase of acetonitrile–methanol–water (22.5:22.5:55), dimethyl sulphoxide–methanol–water (10:25:35) or a quaternary solvent mixture of acetonitrile–dimethyl sulphoxide–methanol–water (20:20:10:50) is required for the separation of a mixture containing diastereoisomers and functional-group derivatives. The relative retention of the lignans can be explained by the relative ability of a lignan to interact with the adsorption site or by its relative hydrophobicity in reversed-phase chromatography.

INTRODUCTION

Lignan[1] is the name given to a large family of natural products that contain the 2,3-dibenzylbutane skeleton. Lignans with a fully reduced B-ring are aryltetrahydronaphthalene lignans (Fig. 1). Lignans, especially those closely related to podophyllotoxin, are of interest to chemists and medical scientists because of their strong antimitotic activity and hence are a possible source for anticancer agents. The podophyllotoxin derivatives VP16-213 and VM26 have been widely and successfully used in the chemotherapy of cancers[2].

The biological activity and toxicity of podophyllotoxin and its derivatives are stereochemically specific. The base-catalysed isomerization of the rigid, strained podophyllotoxin structure with a 2β-H configuration[3] to the flexible picropodophyllin

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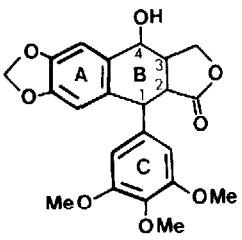

1	Isopodophyllotoxin	1β,2α,3β,4α
2	Podophyllotoxin	1β,2β,3α,4β
3	Epipodophyllotoxin	1β,2β,3α,4α
4	Picropodophyllin	1β,2α,3α,4β
5	Epiisopodophyllotoxin	1β,2α,3β,4β
6	Epipicropodophyllin	1β,2α,3α,4α
7	Epiisopicropodophyllin	1β,2β,3β,4β
8	Isopicropodophyllin (Unknown)	1β,2β,3β,4α

Fig. 1. Structures of the diastereoisomers of podophyllotoxin showing H-configurations at C-1, C-2, C-3 and C-4 positions.

with a 2α-H configuration (Fig. 1) led to the loss of toxicity as well as of antimitotic activity. It is therefore important to develop an effective method for their separation, as contamination of the active compound with inactive isomers is often a problem.

High-performance liquid chromatography (HPLC) has been used to isolate[4] and to check the purity[5] of lignans isolated from natural sources. Several workers have reported the determination of VP16-213 and VM26 in biological fluids[6–9]. This paper describes the separation and retention behaviour of podophyllotoxin diastereoisomers (Fig. 1) and the functional-group derivatives podophyllotixin, deoxypodophyllotoxin and acetylpodophyllotoxin (Fig. 2).

The diastereoisomers can be resolved by both adsorption on silica (Hypersil) and reversed-phase chromatography on octadecylsilica (ODS-Hypersil) with *n*-heptane–dichloromethane–methanol (90:10:4) and methanol–water (45:55) as mobile phases, respectively.

The podophyllotoxin derivatives are easily and conveniently separated by reversed-phase chromatography with acetonitrile–water (40:60) as the eluent. The simultaneous resolution of diastereoisomers and functional derivatives, however, re-

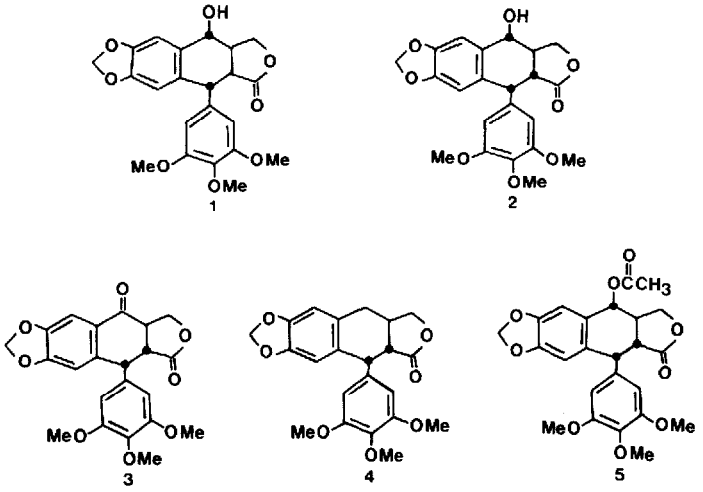

Fig. 2. Structures of some aryltetrahydronaphthalene lignans. 1, Picropodophyllin; 2, podophyllotoxin; 3, podophyllotoxone; 4, deoxypodophyllotoxin; 5, acetylpodophyllotoxin; ● denotes β-H.

quires the use of ternary or quaternary mobile phases such as acetonitrile–methanol–water (22.5:22.5:55), dimethyl sulphoxide–methanol–water (10:25:35) or methanol–acetonitrile–dimethyl sulphoxide–water (10:20:20:50).

The retention behaviour of the diastereoisomers on silica is determined by the relative ease with which the hydroxyl group at C-4 (Fig. 1) interacts with the adsorption site. In reversed-phase chromatography, hydrophobicity is of prime importance, but solvent specificity and selectivity are also involved.

EXPERIMENTAL

Materials and reagents

The lignans used in the present study were isolated and synthesized at Westfield College, London, except for isopodophyllotoxin, epiisopodophyllotoxin and epiisopicropodophyllin, which were gifts from Prof. W. J. Gensler, University of Boston. Acetonitrile and methanol were HPLC grade from Rathburn Chem. (Walkerburn, Great Britain). Dichloromethane, *n*-heptane and dimethyl sulphoxide were reagent grade from BDH (Poole, Great Britain).

High-performance liquid chromatography

A Pye Unicam (Cambridge, Great Britain) PU4010 liquid chromatograph was used, with a Pye Unicam variable-wavelength UV detector set at 280 nm. Injection of samples was via a Rheodyne 7125 injector fitted with a 20-μl loop. All HPLC solvents were made up by volume per cent.

The podophyllotoxin diastereoisomers were separated on a 25 cm × 5 mm I.D. Hypersil column (5 μm spherical silica; Shandon Southern, Runcorn, Great Britain) and on a 25 cm × 5 mm I.D. ODS-Hypersil column (5-μm silica chemically bonded with octadecylsilyl groups). The mobile phases were *n*-heptane–dichloromethane–methanol (90:10:4) and methanol–water (45:55), respectively.

A mixture of picropodophyllin, podophyllotoxin, podophyllotoxone, deoxypodophyllotoxin and acetylpodophyllotoxin was chromatographed on a 10 cm × 5 mm ODS-Hypersil column. The following mobile phases were used: methanol–water (50:50), acetonitrile–water (40:60), acetonitrile–methanol–water (25:25:50), acetonitrile–methanol–water (22.5:22.5:55), dimethyl sulphoxide–methanol–water (10:25:35) and acetonitrile–dimethyl sulphoxide–methanol–water (20:20:10:50). The mobile phase flow-rate for all separations was 1 ml/min.

RESULTS AND DISCUSSION

Separation of the diastereoisomers of podophyllotoxin

The separation of the seven diastereoisomers of podophyllotoxin by adsorption chromatography on Hypersil with *n*-heptane–dichloromethane–methanol (90:10:4) as mobile phase is shown in Fig. 3. The peaks are numbered to correspond with the numbering of the compounds listed in Fig. 1 for easy identification. The capacity-ratio values (K') of the diastereoisomers are shown in Table I.

The retention behaviour of the diastereoisomers on silica is mainly determined by the configuration and hence the ability of the hydroxyl group at C-4 to interact with the adsorption site. The lactone function has less influence on retention. Com-

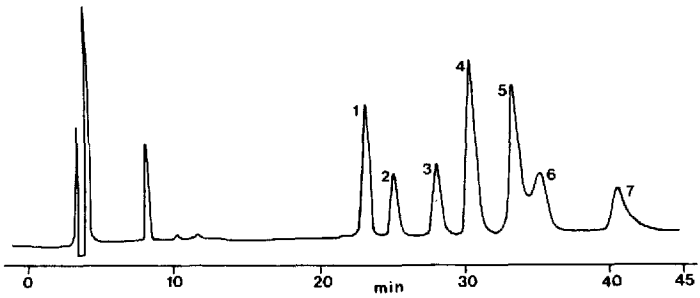

Fig. 3. Separation of podophyllotoxin diastereoisomers. Column, Hypersil (25 cm × 5 mm); eluent, *n*-heptane–dichloromethane–methanol (90:10:4); flow-rate, 1 ml/min. Peak numbers correspond to compound numbers in Fig. 1.

pounds with a 4α-OH configuration are generally eluted before those with a 4β-OH configuration. This is because the 4α-OH configuration is slightly more hindered than the 4β-OH configuration and is therefore less able to interact with the adsorption sites of the silica. Thus, podophyllotoxin (4α-OH) is eluted before epipodophyllotoxin (4β-OH), and epipicropodophyllin (4β-OH) is retained longer than picropodophyllin (4α-OH).

Flexible molecules, which are able to arrange themselves in conformations allowing for maximum interaction of the polar groups with the adsorbent, are more strongly retained than rigid, inflexible molecules where molecular movement is restricted. The k' values (Table I) of the flexible picropodophyllin, epipicropodophyllin and epiisopicropodophyllin are therefore larger than those of the corresponding rigid C2-isomers podophyllotoxin, epipodophyllotoxin and epiisopodophyllotoxin.

Aiyar and Chang[10] have reported the unusual behaviour of epiisopicropodophyllin during hydrogenolysis as a result of its ability to adopt a conformation that brings the hydroxyl group at C-4 closest to the oxido-oxygen of the lactone. This conformation will also explain why epiisopicropodophyllin was the most strongly

TABLE I

CAPACITY-RATIO (k') VALUES OF PODOPHYLLOTOXIN DIASTEREOISOMERS ON HYPERSIL AND ODS-HYPERSIL

Solutes 1, 2, 3 and 5 are rigid molecules; solutes 4, 6 and 7 are flexible.

*Compound No.**	*Compound*	*k'*	
		Hypersil	*ODS-Hypersil*
1	Isopodophyllotoxin (4β-OH)	5.82	8.66
2	Podophyllotoxin (4α-OH)	6.41	11.73
3	Epipodophyllotoxin (4β-OH)	7.29	7.66
4	Picropodophyllin (4α-OH)	7.94	8.20
5	Epiisopodophyllotoxin (4α-OH)	8.88	9.06
6	Epipicropodophyllin (4β-OH)	9.35	6.53
7	Epiisopicropodophyllin (4α-OH)	10.94	7.20

* See Fig. 1.

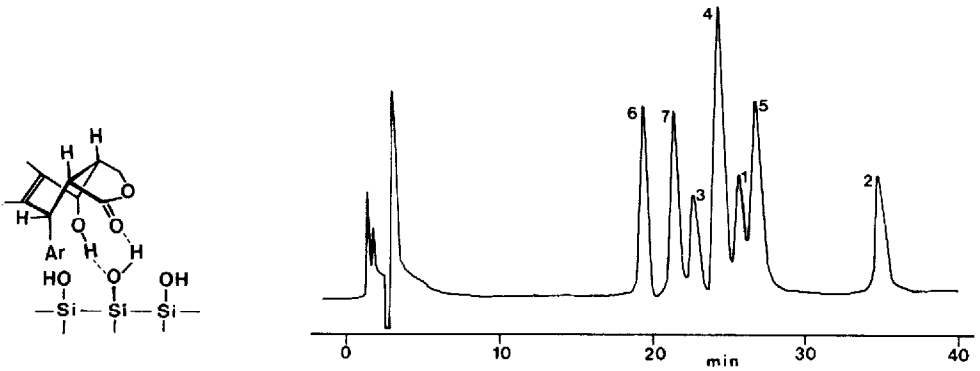

Fig. 4. Diagram showing H-bonding between the C4-OH and oxido-oxygen of the lactone with the hydroxyl group of silica. The partial structure of epiisopicropodophyllin is shown. Ar = ring C (see Fig. 1).

Fig. 5. Separation of podophyllotoxin diastereoisomers. Column, ODS-Hypersil (25 cm × 5 mm); eluent, methanol–water (45:55); flow-rate, 1 ml/min. For peak identification see Fig. 3.

retained compound. The proximity of the hydroxyl group at C-4 and the oxido-oxygen of the lactone allows maximum hydrogen bonding with the hydroxyl groups of the silica adsorbent (Fig. 4).

The reason why isopodophyllotoxin is the first compound to be eluted is not so clear, but it is interesting to note that, while epiisopicropodophyllin has the all-*cis* configuration at the C-1, C-2, C-3 and C-4 positions, isopodophyllotoxin has the all-*trans* configuration (Fig. 1).

Fig. 5 shows the separation of the diastereoisomers by reversed-phase chromatography on an ODS-Hypersil column with methanol–water (45:55) as the eluent; the k' values are listed in Table I.

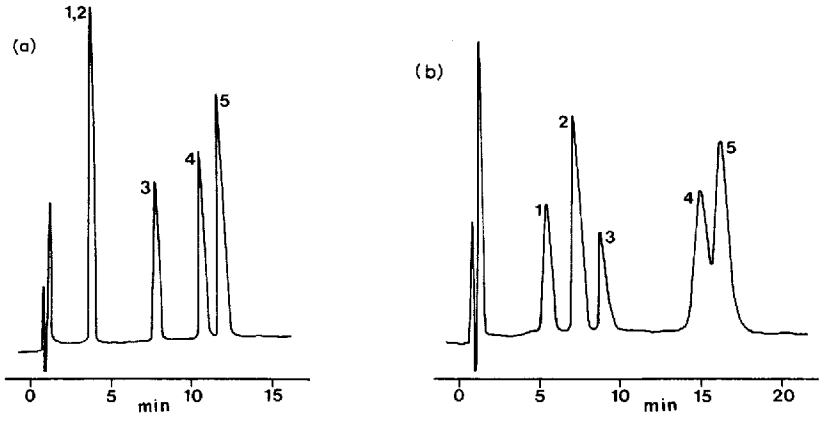

Fig. 6. Separation of picropodophyllin (1), podophyllotoxin (2), podophyllotoxone (3), deoxypodophyllotoxin (4) and acetylpodophyllotoxin (5). Column, ODS-Hypersil (10 cm × 5 mm); eluent, (a) acetonitrile–water (40:60) and (b) methanol–water (50:50); flow-rate, 1 ml/min.

Hydrophobic interaction is probably the major factor in determining the relative retention of the diastereoisomers by reversed-phase chromatography. The flexible compounds, which are able to interact freely with the polar hydroxyl groups of the adsorbent, are also more hydrophilic for the same reason. They were therefore eluted before their corresponding rigid, inflexible C2-isomers (Fig. 5 and Table I). This is also consistent with the fact that compounds with a less hindered 4α-OH configuration have longer retention times than the 4β-OH epimers (Table I). However, solvent selectivity and specificity effects may also influence the relative retention. Considering the seven diastereoisomers as a whole, the elution order is thus not strictly the reverse of that of adsorption (normal-phase) chromatography.

The solvent-selectivity and -specificity effects are best demonstrated by studying the separation of a mixture containing picropodophyllin, podophyllotoxin, podophyllotoxone, deoxypodophyllotoxin and acetylpodophyllotoxin (Fig. 2).

With acetonitrile–water (40:60) as the mobile phase, no separation of picropodophyllin and podophyllotoxin was possible, whereas resolution of the functional-group derivatives was satisfactory (Fig. 6a). Methanol–water (50:50), on the other hand, caused the loss of resolution of deoxypodophyllotoxin and acetylpodophyllotoxin, but completely separated picropodophyllin from podophyllotoxin (Fig. 6b). An attempt to improve the separation of deoxypodophyllotoxin and acetylpodophyllotoxin by decreasing the methanol content of the mobile phase was not successful. The specificity of methanol in effecting the separation of diastereoisomers is clearly demonstrated in Fig. 5. The same separation could not be achieved with solvent systems containing acetonitrile and/or dimethyl sulphoxide as organic modifier.

The effective separation of picropodophyllin and podophyllotoxin and its derivatives is achieved by use of ternary mobile phases. The addition of methanol to the acetonitrile–water mixture had an immediate effect on the separation of picropodophyllin and podophyllotoxin while maintaining the resolution of the functional derivatives (Fig. 7).

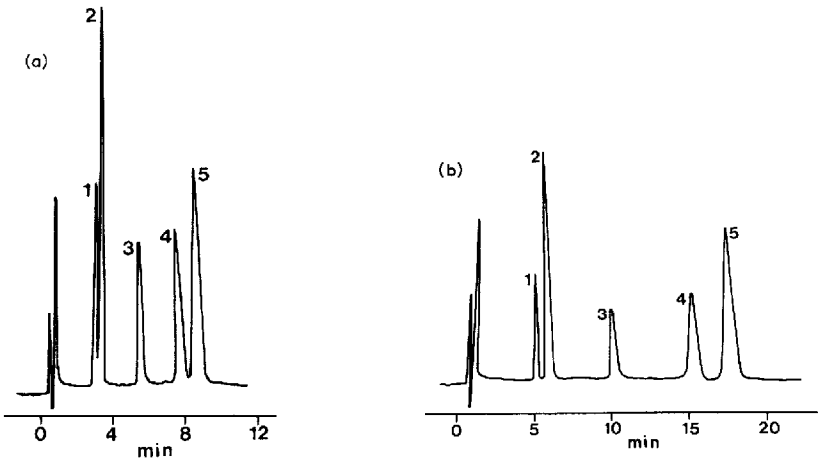

Fig. 7. Chromatograms illustrating solvent-selectivity and -specificity effects. Eluent, (a) acetonitrile–methanol–water (25:25:50) and (b) acetonitrile–methanol–water (22.5:22.5:50). Other HPLC conditions and peak identification as in Fig. 6.

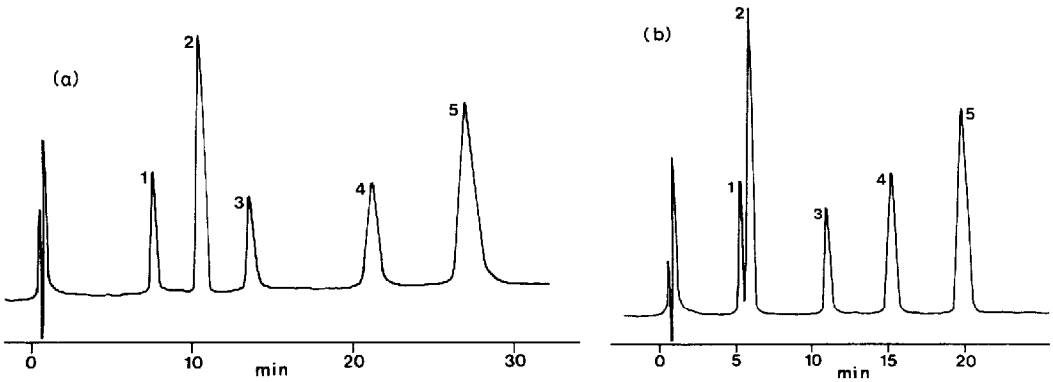

Fig. 8. Separation of podophyllotoxin derivatives by using mobile phases containing dimethyl sulphoxide. (a) Dimethyl sulphoxide–methanol–water (10:25:25) and (b) dimethyl sulphoxide–acetonitrile–methanol–water (20:20:10:50). Other HPLC conditions and peak identification as in Fig. 6.

Dimethyl sulphoxide, like acetonitrile, is effective for the separation of functional derivatives, but less useful for resolving diastereoisomers. Its effect on the separation of deoxypodophyllotoxin and acetylpodophyllotoxin is greater than that of acetonitrile. From Fig. 8, it can be seen that effective separation of the lignans can be attained by using mobile phases containing dimethyl sulphoxide as one of the components.

Dimethyl sulphoxide is a superior solvent for the lignans, especially for picropodophyllin and its derivatives, which are less soluble in alcohols. It is therefore particularly advantageous to use dimethyl sulphoxide in situations where preparative isolation of the lignans is required.

The importance of HPLC in lignan analysis is well illustrated in Fig. 9. A commercially available sample of "pure" podophyllotoxin is shown to contain about 6% of the biologically inactive picropodophyllin.

The purification of ligans by recrystallization is often difficult, because of the tendency of lignans to aggregate or complex with each other; HPLC provides an

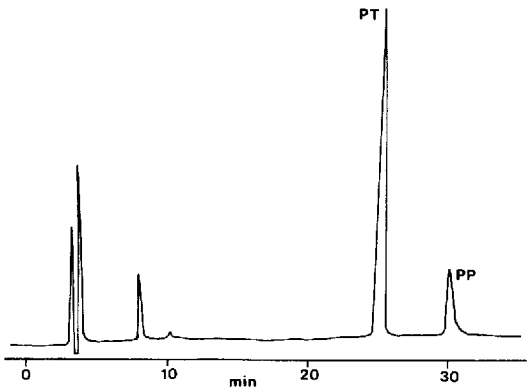

Fig. 9. Separation of podophyllotoxin (PT) from a commercial source showing contamination with picropodophyllin (PP). HPLC conditions as in Fig. 3.

effective and powerful technique for their separation. With the recent isolation and characterization of lignans from animal sources, including human urine[11,12], interest in the field has been greatly increased. It is hoped that the present study will provide the basis for future development in the separation and analysis of lignans by HPLC.

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