ELSEVIER

Journal of Chromatography A, 722 (1996) 267–271

JOURNAL OF CHROMATOGRAPHY A

Analysis of aryltetrahydronaphthalene lignans and their glucoside conjugates in podophyllin resin by high-performance liquid chromatography

C.K. Lim

MRC Toxicology Unit, Hodgkin Building, University of Leicester, P.O. Box 138, Lancaster Road, Leicester, LE1 9HN, UK

Abstract

A reversed-phase high-performance liquid chromatography (HPLC) system has been developed and optimised for the separation of aryltetrahydronaphthalene lignans of the podophyllotoxin series and their glucoside conjugates. The separations were carried out on an ODS-Hypersil column with CH_3OH–water, CH_3CN–water, CH_3OH–ammonium acetate or CH_3CN–ammonium acetate as the mobile phase.

The optimised HPLC system has been applied to the analysis of lignans in podophyllin resins. The separations showed the presence of podophyllotoxin, 4′-demethylpodophyllotoxin, podophyllotoxin β-D-glucoside and 4′-demethylpodophyllotoxin-β-D-glucoside in *Podophyllum emodi* resin, while in *Podophyllum peltatum* resin, α- and β-peltatin and their glucoside conjugates were also detected, in addition to podophyllotoxin.

1. 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). Aryltetrahydronaphthalene lignans of the podophyllotoxin series have been found to have antimitotic properties. These compounds can be extracted in high yield from the roots and rhizomes of plants of the genus Podophyllum, e.g. *Podophyllum emodi* and *Podophyllum peltatum* [1]. The anti-cancer drugs Etoposide (VP 16-213) and Teniposide (VM26) are both well-known semi-synthetic glucoside derivatives of podophyllotoxin [2]. Since the aqueous extracts of these plants (termed podophyllin) may be important sources for potential new drugs, a

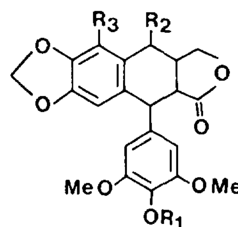

Fig. 1. Structures of aryltetrahydronaphthalene lignans. Podophyllotoxin: R_1 = Me, R_2 = OH, R_3 = H; 4′-demethylpodophyllotoxin: R_1 = H, R_2 = OH, R_3 = H; α-peltatin: R_1 = H, R_2 = H, R_3 = OH; β-peltatin: R_1 = Me, R_2 = H, R_3 = OH; podophyllotoxin-β-D-glucoside: R_1 = Me, R_2 = β-D-glucoside, R_3 = H; 4′-demethylpodophyllotoxin-β-D-glucoside: R_1 = H, R_2 = β-D-glucoside, R_3 = H; α-peltatin-β-D-glucoside: R_1 = H, R_2 = H, R_3 = β-D-glucoside; β-peltatin-β-D-glucoside: R_1 = Me, R_2 = H, R_3 = β-D-glucoside.

0021-9673/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)00433-5

method capable of separating the complex components in podophyllin is needed.

High-performance liquid chromatography (HPLC) has been used to separate lignans from various sources [3–6]. HPLC methods for the determination of VM26 and VP16-213 in body fluid have also been reported [7–9]. The present paper describes the development and optimisation of a reversed-phase HPLC system for the separation of podophyllotoxin, α- and β-peltatin and their glucoside conjugates. The retention behaviour of the lignans has been studied and the optimised system has been applied to the analysis of lignans in podophyllin resins.

2. Experimental

2.1. Materials and reagents

P. emodi resin, *P. peltatum* resin and the lignan standards podophyllotoxin, α-peltatin, β-peltatin, 4′-demethylpodophyllotoxin, podophyllotoxin-β-D-glucoside, 4′-demethylpodophyllotoxin-β-D-glucoside, α-peltatin-β-D-glucoside and β-peltatin-β-D-glucoside were gifts from Dr. D.C. Ayres, Queen Mary and Westfield College, University of London. Acetonitrile and methanol were HPLC grade from Rathburn Chem. (Walkerburn, Peeblesshire, UK). Ammonium acetate was AnalaR grade from BDH (Poole, Dorset, UK).

2.2. High-performance liquid chromatography

A Varian (Walton-on-Thames, Surrey, UK) Model 9012 liquid chromatograph was used, with a Varian 9050 UV–Vis detector set at 280 nm. Injection of sample was via a Rheodyne 7125 injector fitted with a 100-μl loop.

The lignans were separated on a 250×5 mm I.D. ODS-Hypersil column (5-μm silica chemically bonded with octadecylsilyl groups) from Shandon HPLC, Cheshire, UK. The mobile phases were methanol–water (40:60), methanol–0.5 *M* ammonium acetate (40:60), acetonitrile–water (25:75) and acetonitrile–ammonium acetate (25:75). All HPLC eluents were made up by volume percent. The flow-rate for all separations was 1.5 ml/min. The samples were dissolved in methanol–water (90:10) for injection.

2.3. Liquid secondary-ion mass spectrometry (LSIMS)

A VG Analytical (Manchester, UK) ZAB2-E mass spectrometer, operated at 8 keV accelerating voltage and fitted with a caesium gun (35 keV, 0.5 μA emission) was used for LSIMS. Lignans were dissolved in a small volume (10 μl) of methanol and 1 μl was added to the stainless-steel LSIMS target probe previously prepared with thioglycerol (1 μl) as liquid matrix. Mass spectra of lignans and their glucosides were acquired in the positive-ion mode using a VG Analytical 11-250J data system in continuous multichannel analysis mode at a resolution of 1500 RP.

3. Results and discussion

It has been shown in a previous study on the retention behaviour of aryltetrahydronaphthalene lignans in reversed-phase HPLC that retention is organic-modifier selective and specific [6]. It is therefore reasonable to assume that the solvent-selectivity and -specificity effects will also be important factors to consider in developing an optimised system for the separation of the lignan glucosides. With acetonitrile–water (25:75, v/v) as the eluent, on an ODS-Hypersil column, "normal" reversed-phase behaviour was observed with the polar lignan glucoside conjugates eluted before the unconjugated parent compounds. The elution order was as follows: α-peltatin-β-D-glucoside, 4′-demethylpodophyllotoxin-β-D-glucoside, β-peltatin-β-D-glucoside, podophyllotoxin-β-D-glucoside, 4′-demethylpodophyllotoxin, α-peltatin, podophyllotoxin and β-peltatin. It can be seen from the capacity ratios (k') of the compounds shown in Table 1, that there was insufficient resolution between podophyllotoxin-β-D-glucoside, 4′-demethylpodophyllotoxin and α-peltatin.

Replacing acetonitrile with methanol as the

Table 1
Capacity factors (k') of aryltetrahydronaphthalene lignans on ODS-Hypersil with 25% CH_3CN in water (k'_1), 40% CH_3OH in water (k'_2), 25% CH_3CN in 0.5 *M* ammonium acetate (k'_3) and 40% CH_3OH in 0.5 *M* ammonium acetate (k'_4) as mobile phases

	k'_1	k'_2	k'_3	k'_4
α-Peltatin-β-D-glucoside	1.03	2.60	0.71	2.00
4′-Demethylpodophyllotoxin-β-D-glucoside	1.77	4.50	1.07	3.50
β-Peltatin-β-D-glucoside	3.40	7.40	2.21	5.60
Podophyllotoxin-β-D-glucoside	5.13	12.20	3.00	8.50
4′-Demethylpodophyllotoxin	5.27	8.60	3.93	6.70
α-Peltatin	5.53	6.40	4.36	5.00
Podophyllotoxin	13.80	20.50	10.36	14.80
β-Peltatin	17.00	17.30	12.36	11.50

The results are the means of three determinations.

organic modifier had a dramatic effect on the elution order and resolution of the lignans and their glucoside conjugates. Fig. 2. shows the separation of the lignans with methanol–water (40:60, v/v) as the mobile phase. The k'-values are listed in Table 1. Complete separation of all eight compounds was achieved, demonstrating the superiority of methanol as an organic modifier for the reversed-phase HPLC of lignans. The general increase in retention time and the improvement in resolution are attributed to the ability of methanol, an H-bonding organic modifier, to form a different degree of H-bond with different lignans, depending on their structures and stereo-configurations. The H-bonding effect also led to the reversal of elution order for some compounds when compared with the acetonitrile mobile phase system (Table 1). Lignans with the OH or glucoside group attached to the reduced ring (Fig. 1), seem to form a stronger H-bond with the layer of methanol 'sorbed' onto the hydrophobic C_{18} stationary-phase surface than those with the OH or glucoside group located on the aromatic rings. This explains why podophyllotoxin and its glucoside were more strongly retained than their β-peltatin analogues.

Ammonium acetate has been shown to be an excellent mobile-phase additive for reversed-

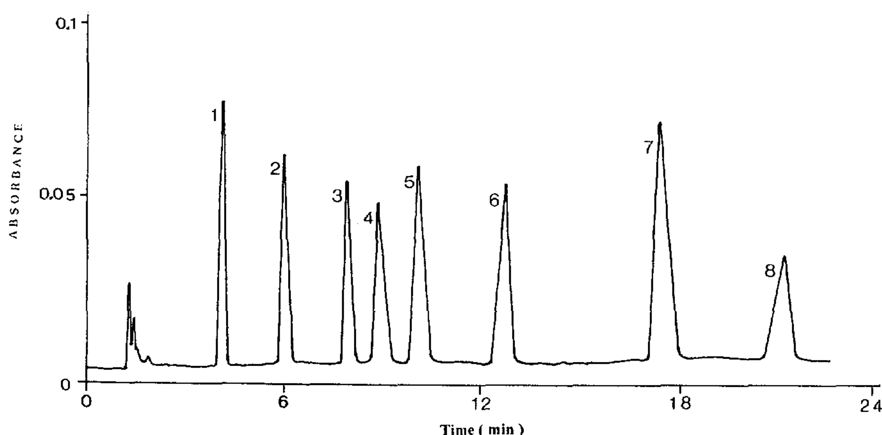

Fig. 2. Separation of aryltetrahydronaphthalene lignans. Column, ODS-Hypersil (25 cm × 5 mm I.D.); eluent, methanol–water (40:60, v/v); flow-rate, 1.5 ml/min; detector, UV, 280 nm. Peaks: 1 = α-peltatin-β-D-glucoside, 2 = 4′-demethylpodophyllotoxin-β-D-glucoside, 3 = α-peltatin, 4 = β-peltatin-β-D-glucoside, 5 = 4′-demethylpodophyllotoxin, 6 = podophyllotoxin-β-D-glucoside, 7 = β-peltatin, 8 = podophyllotoxin.

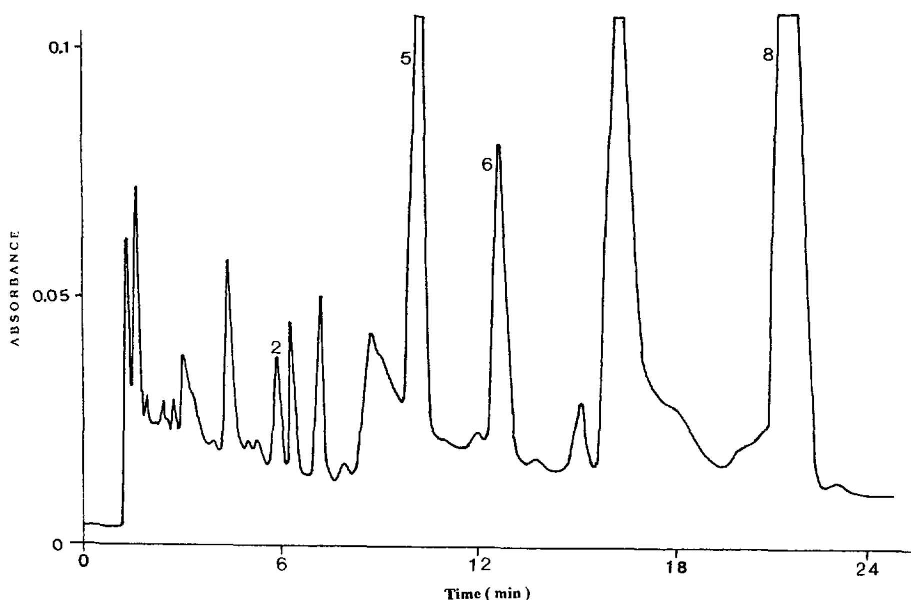

Fig. 3. Separation of aryltetrahydronaphthalene lignans in *P. emodi* resin. HPLC conditions and peak identification as in Fig. 2.

phase HPLC [10]. Addition of ammonium acetate to the mobile phase decreased the k'-values of all lignans (Table 1), while maintaining the resolution. However, since the simple methanol–water system was equally efficient, addition of ammonium acetate was not necessary in this case.

Analysis of the lignan composition in the resin of *P. emodi* showed the presence of podophyllotoxin, 4′-demethylpodophyllotoxin and

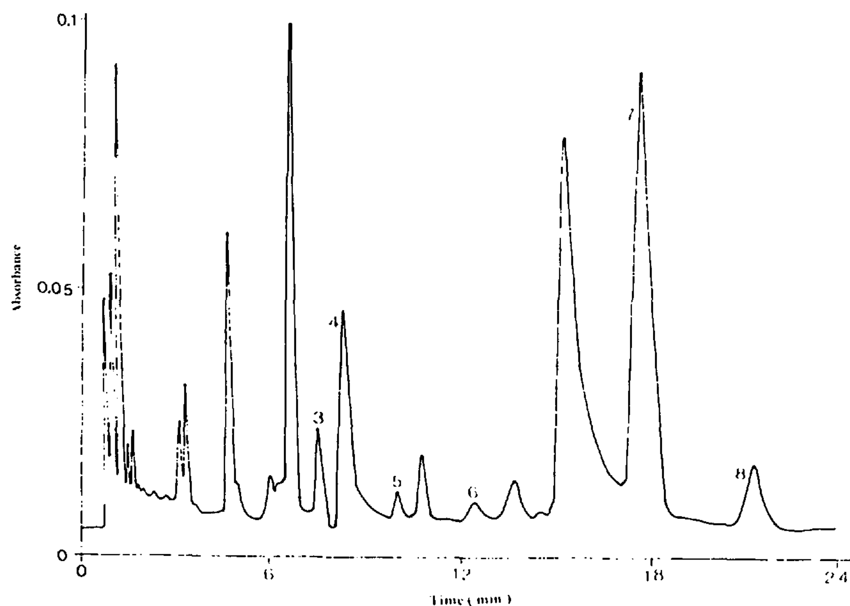

Fig. 4. Separation of aryltetrahydronaphthalene lignans in *P. peltatum* resin. HPLC conditions and peak identification as in Fig. 2.

their glucoside conjugates, respectively (Fig. 3). The lignan composition in the resin of *P. peltatum* was more complex (Fig. 4) than in the resin of *P. emodi*. The main components were β-peltatin and its glucoside conjugate. Small amounts of podophyllotoxin, 4′-demethylpodophyllotoxin, podophyllotoxin-β-D-glucoside and α-peltatin were also detected. The peaks were identified by retention time in comparison to authentic standards and were confirmed by liquid secondary ion mass spectrometric analyses.

4. Conclusions

The simple reversed-phase system with methanol–water (40:60, v/v) as mobile phase is applicable to the analysis of lignans present in podophyllin resins. There are still many unidentified components present in the resins. The characterisation of these will probably require on-line HPLC–MS, in which the methanol–0.5 *M* ammonium acetate (40:60, v/v) system will be more suitable, since ammonium acetate is commonly added to solvents to enhance ionisation in mass spectrometric analysis.

References

[1] R.D. Haworth, Ann. Rep. Progr. Chem., 33 (1936) 266.
[2] B.F. Issell, Cancer Chemother. Pharmacol., 7 (1982) 72.
[3] S. Treppendahl and P. Jakobsen, J. Chromatogr., 189 (1980) 276.
[4] D.A. Cairnes, D.G.I. Kingston and M.M. Rat, J. Nat. Prod., 44 (1981) 34.
[5] R.J. Strife, I. Jardine and M. Colvin, J. Chromatogr., 182 (1980) 211.
[6] C.K. Lim and D.C. Ayres, J. Chromatogr., 255 (1983) 247.
[7] L.M. Allen, J. Pharm. Sci., 69 (1980) 1440.
[8] J.J.M. Holthuis, W.J. van Oort and H.M. Pinedo, Anal. Chim. Acta, 130 (1981) 23.
[9] P. Farina, G. Marzillo and M. D'Incalci, J. Chromatogr., 222 (1981) 41.
[10] C.K. Lim and T.J. Peters, J. Chromatogr., 316 (1984) 397.