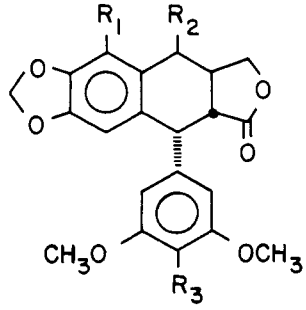
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PODOPHYLLOTOXINS AND RELATED LIGNANS

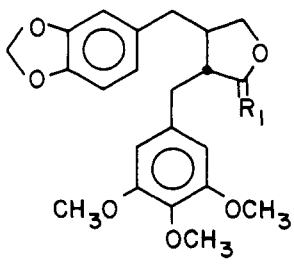
DAVID A. CAIRNES, DAVID G. I. KINGSTON and M. MADHUSUDANA RAO

Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

ABSTRACT.—A reversed-phase liquid chromatographic system has been developed for the separation and quantitation of podophyllotoxin and related lignans from crude plant extracts. This method should prove valuable in screening plant extracts for anticancer agents.

The largest and most successful plant screening program in recent years has been that developed by the U. S. National Cancer Institute to discover new anticancer agents from microbial and plant sources (1, 2). In this program, plants are selected for detailed investigation on the basis of the antileukemic activity and cytotoxicity of their crude extracts (3, 4); thus a plant which happens to contain the same active constituents as other plants which have previously been investigated is likely to be selected for detailed study. Since the fractionation of a plant and the isolation of its active anticancer constituents is a lengthy and expensive process, it would be highly desirable if an assay procedure could be developed to test for the presence of known active agents. In this way a plant suspected to contain a known active compound on the basis of botanical or other evidence could be tested for the presence of this agent prior to the initiation of a full-fledged fractionation study, and the study could be avoided if the test proved positive.





1	R_1=OH,	R_2=H,	R_3=OH	**5**	R_1=O
2	R_1=H,	R_2=OH,	R_3=OCH_3	**7**	R_1=H_2
3	R_1=OH,	R_2=H,	R_3=OCH_3		
4	R_1=H,	R_2=H,	R_3=OCH_3		
6	R_1=OCH_3,	R_2=H	R_3=OCH_3		

The lignan podophyllotoxin (**2**) and related compounds have consistently shown activity in both *in vivo* (PS) and *in vitro* (KB) screens of the National Cancer Institute. Thus podophyllotoxin itself has been known to have anticancer properties for many years (5), and related compounds such as desoxypodophyllotoxin (**4**), α-peltatin (**1**), and β-peltatin (**3**), have also long been known to be active (5). In recent years various related compounds have been isolated and found to be active, such as β-peltatin-A methyl ether (**6**), which shows activity in both the WA16 tumor system (6) and the KB cell culture system (7). In

addition, aryl tetrahydrofuran lignans such as burseran (**7**) have been reported as possessing cytotoxicity (8, 9), although the related compound yatein (**5**) (10–11) does not show activity (12).

The lignan desoxypodophyllotoxin is of particular interest, since it has been reported to occur in a wide variety of plants and because its strong cytotoxicity (ED_{50} 0.0026 μg/ml in the KB cell culture (13)) means that any plant yielding a crude extract containing at least 0.1% of it will give a positive response in the KB pre-screen of the National Cancer Institute. In recent years desoxypodophyllotoxin has been isolated as the active cytotoxic constituent of a number of plants, including *Juniperus phoenicea* (7), *Callitris preissii* (12), *Libocedrus decurrens* (14), *Juniperus bermudiana* (15), *Bursera morelensis* (16), *Bursera microphylla* (17), *Callitris columellaris* (18), and *Hyptis tomentosa* (19). The widespread distribution and high activity of this lignan make it desirable to have a rapid test for it in crude plant extracts. Previous methods for the analysis of the podophyllotoxins by thin-layer chromatography (20) have been useful but not entirely definitive, and an hplc method was thus selected as the method of choice for this determination.

EXPERIMENTAL

ANALYTICAL LIQUID CHROMATOGRAPHY.—Liquid chromatographic separations were performed on the apparatus previously described (21). The column used, a prepacked LiChrosorb RP-8 column 25 cm long and with a 4.6 mm internal diameter, was obtained from MCB, Inc., and was used with a precolumn packed with C_{18}-Corasil obtained from Waters Associates. Sample injection was by a six-port valve, and the injector and column were maintained at ambient temperature. Acetonitrile (Fisher certified A.C.S., catalog A-24) was distilled in glass before use, and distilled water was redistilled in glass before use. A solvent composition CH_3CN, 40 : H_2O, 60 (v/v) was used in most of the work described in this paper, and the flow rate was 2.0 ml/min. Sample injections were typically 10 μl of a 0.1% solution for pure compounds and proportionately more concentrated for complex mixtures. Detection was by uv absorption at 254 nm on a Pharmacia Model 110 uv detector.

ISOLATION OF DESOXYPODOPHYLLOTOXIN FROM *Austrocedrus chilensis*.—A partially purified fraction obtained from *Austrocedrus chilensis* (D. Don) Florin and Boutelje (Cupressaceae) (22) was subjected to preparative liquid chromatography on a 0.7 x 50 cm Partisil 10 ODS column with CH_3CN, 50 : H_2O, 50 (v/v) as solvent. The peak corresponding to desoxypodophyllotoxin as determined by the analytical runs described below was collected, and the solvent was removed by evaporation *in vacuo*. The isolated material had spectral data (pmr and ir) identical with those of an authentic reference sample of desoxypodophyllotoxin.

RESULTS AND DISCUSSION

Of several solvent system—column packing combinations investigated, the simple system acetonitrile—water, 50:50 or 40:60, on a Lichrosorb RP-8 column proved convenient and satisfactory, and the separation of a standard mixture of eight lignans with the 40:60 system is shown in fig. 1. With the exception of the burseran-sesamin mixture, all the test compounds were well separated with this system. An alternate system consisting of chloroform-hexane, 1:1, on a silica gel column gave poorer separations of the test compounds. Retention volumes and capacity factors for the lignans are given in table 1.

Quantitative estimation of the amount of lignan present could also be obtained with this system. Thus with a 3 mm pathlength uv detector operating at 254 nm, a water-acetonitrile, 50:50 solvent system, a sensitivity of 0.08 AUFS, and a flow rate of 2.0 ml/min, the peak height per microgram of sample injected was 14 mm with podophyllotoxin, 8.2 mm with desoxypodophyllotoxin, and 4.6 mm with sesamin, with correlation coefficients of 0.996, 0.997, and 0.992, respectively. The technique can thus easily detect and quantitate quantities of lignan in the

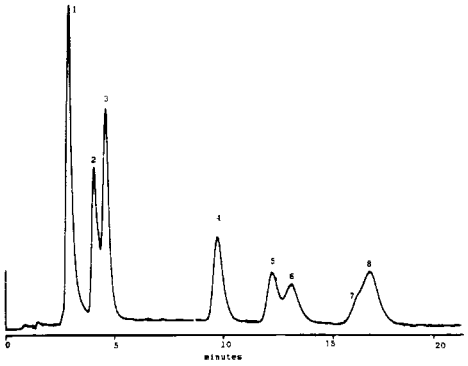


FIG. 1. Separation of a mixture of 10 μg each of various lignans under the conditions described in the text; sensitivity 0.08 AUFS. Compound identification: 1, α-peltatin; 2, podophyllotoxin; 3, β-peltatin; 4, desoxypodophyllotoxin; 5, yatein; 6, β-peltatin-A methyl ether; 7, burseran; 8, sesamin.

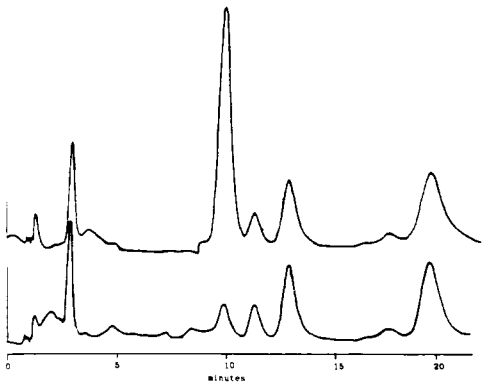


FIG. 2. Chromatogram of a fraction derived from *Austrocedrus chilensis* as described in the text. Overlay: Same fraction spiked with authentic desoxypododphyllotoxin.

nanogram range, particularly if longer pathlength detectors and more sensitive settings are used.

The ultimate usefulness of the method, however, is in detecting the presence of cytotoxic lignans in new plant samples. Since desoxypodophyllotoxin is one of the more common lignans, it was selected for the initial study.

The plant *Austrocedrus chilensis* was selected for investigation since it was known to contain desoxypodophyllotoxin from independent studies (22). A 10 g sample of *A. chilensis* was extracted with ethanol, the ethanol extract was evaporated, and the residue (1.6 g) was dissolved in chloroform. Addition to a short column of silica gel with elution by chloroform gave a crude fraction (0.107 g)) which was dissolved in 10 ml acetonitrile. Injection of a 50 μl aliquot of the solution yielded the chromatogram shown in figure 2. The presence of desoxypodophyllotoxin in the crude fraction is clearly indicated, and it can be calculated

TABLE 1. Retention volumes and capacity factors of lignans.[a]

Compound	Retention vol. (ml)	Capacity factor
α-Peltatin	5.6	1.1
Podophyllotoxin	8.0	2.0
β-Peltatin	9.2	2.4
Desoxypodophyllotoxin	19.6	6.3
Yatein	24.6	8.1
β-Peltatin-A-methyl ether	26.4	8.8
Burseran	32.8	11.1
Sesamin	33.8	11.5

[a]System: Water-acetonitrile, 60:40, on LiChrosorb RP-8, 4.6 mm x 25 cm.

that the plant investigated contained a total of 0.8 mg desoxypodophyllotoxin in the original 10 g sample. Isolation of desoxypodophyllotoxin, either by classical chromatographic techniques (22) or by preparative liquid chromatography as described in the experimental section, yielded material in amounts corresponding approximately to this figure. Thus classical techniques yielded 9 mg of pure desoxypodophyllotoxin from 1000 g of plant material, but much loss of material occurred during crystallization, and it is estimated that the total amount of podophyllotoxin obtained was closer to 50 mg. The amount isolated directly by preparative liquid chromatography was not measured.

It should be emphasized that the technique described can only suggest the presence of a known lignan in a plant extract. In all cases, direct proof of the presence of a suspected compound should be obtained, either by isolation and comparison with an authentic sample, or by spectroscopic methods before fractionation is discontinued. This procedure will prevent the possibility of misidentifications of inactive compounds having the same chromatographic properties as active compounds.

CONCLUSION

The procedure described represents a rapid and effective method for detecting the presence of desoxypodophyllotoxin and other lignans in crude plant extracts. Final proof of the presence of a particular lignan will, of course, always depend on its isolation and characterization, but the procedure described will serve to accelerate this process significantly in many cases.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research Grant No. CA-12831 from the National Cancer Institute. The authors thank Dr. M. Suffness and the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute, for samples of α- and β-peltatin; Dr. J. R. Cole, University of Arizona, for a sample of β-peltatin-A methyl ether; and Dr. J. Harmatha, Czechoslovak Academy of Science, for a sample of yatein.

Received 9 May 1980

LITERATURE CITED

1. M. Suffness and J. D. Douros in "Methods in Cancer Research, Volume 16", Academic Press, Inc., New York, 1979, p. 73.
2. J. D. Douros, *Cancer Treat. Rep.*, **60**, 1069 (1976).
3. D. Statz and F. B. Coon, *Cancer Treat. Rep.*, **60**, 999 (1976).
4. B. J. Abbott, *Cancer Treat. Rep.*, **60**, 1007 (1976).
5. J. L. Hartwell and A. W. Schrecker, *Fortschritte Chem. Org. Naturstoffe*, **15**, 83 (1958).
6. E. Bianchi, K. Sheth and J. R. Cole, *Tetrahedron Lett.*, 2759 (1969).
7. D. A. Cairnes, O. Ekundayo and D. G. I. Kingston, *J. Nat. Prod.*, **43**, 495 (1980).
8. J. R. Cole, E. Bianchi and E. R. Trumbull, *J. Pharm. Sci.*, **58**, 175 (1969).
9. S. J. Torrance, J. J. Hoffman and J. R. Cole, *J. Pharm. Sci.*, **68**, 664 (1979).
10. P. B. McDoniel and J. R. Cole, *J. Pharm. Sci.*, **61**, 1992 (1972).
11. H. Erdtman and J. Harmatha, *Phytochemistry*, **18**, 1495 (1979).
12. M. M. Rao and D. G. I. Kingston, unpublished work.
13. G. R. Pettit and G. M. Cragg, "Biosynthetic Products for Cancer Chemotherapy, Volume 2," Plenum Press, Inc., New York, 1978.
14. S. M. Kupchan, R. J. Hemingway and J. C. Hemingway, *J. Pharm. Sci.*, **56**, 408 (1967).
15. B. Tammami, S. J. Torrance and J. R. Cole, *Phytochemistry*, **16**, 1100 (1977).
16. S. D. Jolad, R. M. Wiedhopf and J. R. Cole, *J. Pharm. Sci.*, **66**, 892 (1977).
17. E. Bianchi, M. E. Caldwell and J. R. Cole, *J. Pharm. Sci.*, **57**, 696 (1968).
18. Y. Aynehchi, *J. Pharm. Sci.*, **60**, 121 (1971).
19. D. G. I. Kingston, M. M. Rao and W. V. Zucker, *J. Nat. Prod.*, **42**, 496 (1979).
20. E. Stahl, "Thin Layer Chromatography", Springer-Verlag, New York, 1969, p. 87, 710.
21. D. G. I. Kingston and B. T. Li, *J. Chromatogr.*, **104**, 431 (1975).
22. D. A. Cairnes, M.S. Thesis, Virginia Polytechnic Institute and State University, 1979.