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Note

Preliminary investigation of the use of high-pressure liquid chromatography for the separation of indole alkaloids

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The separation of complex mixtures of plant constituents is an essential part of much natural products chemistry, and becomes a crucial operation when the objective of the research is to isolate and identify a minor constituent of the mixture. For most practical purposes the separation of mixtures in the range of 100 mg to 1 g is required in this field, so that enough material can be made available for structure elucidation and biological testing.

In our research we are concerned with the isolation of cytotoxic and anti-leukemic compounds from plants of the genus *Tabernaemontana*. These plants are a rich source of indole alkaloids, and we have shown that the biological activity of the crude plant extract is retained by the alkaloid fraction. Preliminary purification by standard open-column techniques led to a number of partially purified active fractions, each showing several spots on thin-layer chromatography (TLC), which could not be satisfactorily resolved by further application of open-column chromatography.

The analytical separation of some indole alkaloids by high-pressure liquid chromatography (HPLC) has already been described in the literature. Thus various ergot alkaloids, including LSD, have been separated on both normal-phase and reversed-phase columns[1–4], and some oxindole alkaloids have been separated on a reversed-phase column[5]. No results have been published, however, on the separation of the common indole alkaloids such as those of the iboga and voacanga classes, which are known to occur in the genus *Tabernaemontana*. In addition, no work has appeared on the preparative separation of any alkaloids. This note describes our evaluation of various support materials and solvent systems for the preparative separation of indole alkaloids.

EXPERIMENTAL

Chemicals

The alkaloid fraction used was obtained from *T. holstii* by a procedure involving acid extraction of the initial plant extract and sequential chromatography on alumina and silica gel columns. The fraction, designated F176, was eluted from the silica gel column with chloroform containing 1% methanol. Solvents used in HPLC

were Burdick and Jackson distilled in glass grade. Anhydrous ammoniacal methanol was prepared by the addition of anhydrous liquid ammonia to anhydrous methanol. Porasil® and Corasil II® packing materials were obtained from Waters Ass. (Framingham, Mass., U.S.A.) and were of 37–75- and 37–50-μm particle-size ranges, respectively. Alumina was Woelm (Eschwege, G.F.R.) alumina N18, and was deactivated with 10% water before use.

Thin-layer chromatography

TLC was carried out on Merck silica gel GF thin-layer plates (layer thickness, 0.25 mm) using chloroform–methanol (19:1) as the solvent system. The spots were visualised on the developed chromatoplates by viewing in ultraviolet light and by spraying with ceric ammonium sulfate reagent[6].

High-pressure liquid chromatography

The experiments were carried out on an instrument equipped with a Waters Ass. M6000 pump and a Pharmacia UV (254-nm) monitor. Two columns (in series) and fittings made of stainless steel were used; columns were each 2 ft. × 3/8 in. The column packings were pre-conditioned according to manufacturer's specifications and then packed by the tap-fill method on an apparatus similar to that previously described[7]. The solvent flow-rate was maintained at 4.0 ml/min.

RESULTS AND DISCUSSION

Since the ultimate objective of the work described was the preparative separation of alkaloid mixtures, the solvents selected for testing were chosen on the basis of their suitability for large-scale work; thus very expensive solvents and also relatively involatile solvents were avoided. For normal-phase chromatography, using Porasil and Corasil II columns, a variety of solvent systems was tested, including methylene chloride–methanol, acetonitrile, chloroform–methanol, and chloroform–acetonitrile. It was found that both chloroform–methanol and methylene chloride–methanol systems gave the best separations on these columns, and the former system was selected for more intensive study.

Initial studies were directed at optimising the solvent system on Porasil C, a moderately high surface-area packing material. It was found that simple methanolic chloroform, for example 5% methanol in chloroform, yielded incomplete recovery and bad tailing of peaks (Fig. 1a). These phenomena are probably due to irreversible adsorption between the acidic silica gel and the basic function(s) of the alkaloids. Salt formation between traces of acid in the solvent and the alkaloids might also be a complicating factor. It was found that tailing could be reduced and sample recovery increased to close to 100% (as verified by preparative-scale runs) by the addition of about 0.2–0.5% anhydrous ammonia to the solution. This was most conveniently done by preparing a stock solution of 20% anhydrous ammonia in methanol and using this in conjunction with pure anhydrous methanol to prepare the methanolic chloroform solvent. Fig. 1b shows the separation obtained with 1% methanolic chloroform containing 0.2% ammonia; tailing has been reduced and sample recovery increased in the run.

We next investigated the separations obtainable with three different varieties

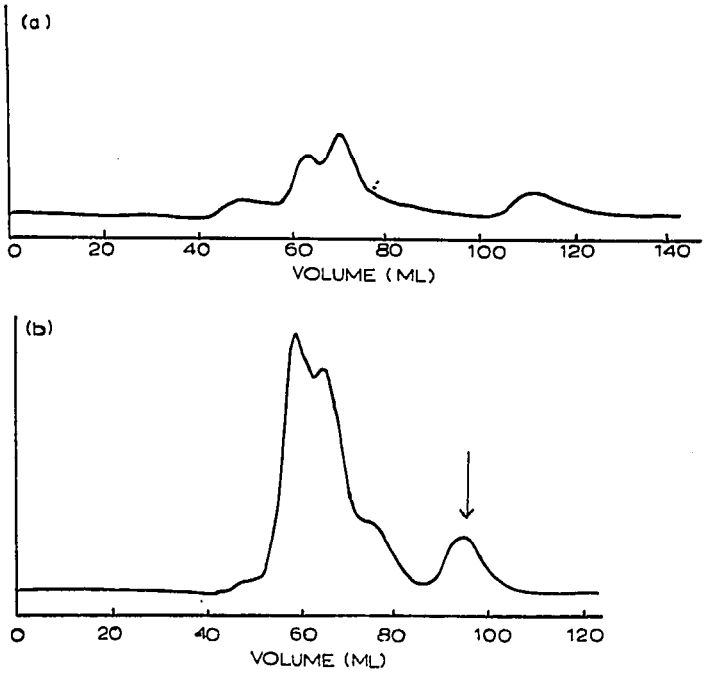

Fig. 1. Comparison between the separation of F176 on Porasil C with and without added ammonia in the solvent. (a) Solvent: chloroform–methanol (95:5), no added ammonia. (b) Solvent: chloroform–methanol–ammonia (99:0.8:0.2).

of Porasil, the Porasils A, C and E, differing in surface area. Porasil A has a surface area of 350–500 m^2/g, Porasil C has an area of 50–100 m^2/g, and Porasil E one of 10–20 m^2/g. The methanol content of the solvent was adjusted to give comparable retention times on each packing material; the results obtained are indicated in Fig. 2. The separation achieved by Porasil E is clearly inferior to that obtained by the Porasils C and A, and since the sample capacity of this material is limited by its low surface area, its use for preparative separations is contraindicated. Porasil C, on the other hand, appears to separate our standard mixture about as well as Porasil A, and this, coupled with the fact that we have had some trouble obtaining reproducible results on Porasil A (perhaps due to the highly active nature of this packing material and hydration effects, or possibly to sample decomposition) makes Porasil C the packing material of choice for normal-phase separations.

We also investigated the use of the pellicular silica packing material Corasil II as the adsorbent. Although pellicular packings are not normally thought of in a preparative context, Corasil II has a surface area comparable to Porasil E and would be expected to have a capacity similar to this material. On the other hand, the pellicular nature of Corasil II should result in a greatly improved resolution of the sample components. In the event, it transpired that the separation obtained on Corasil II (Fig. 3) is indeed superior to that obtained on the Porasil E column of similar surface area, but is only slightly better than that obtained on Porasil C. In view of the high cost and low capacity of Corasil II, its use would also seem to be contraindicated for preparative work in this area in all but the most demanding separations.

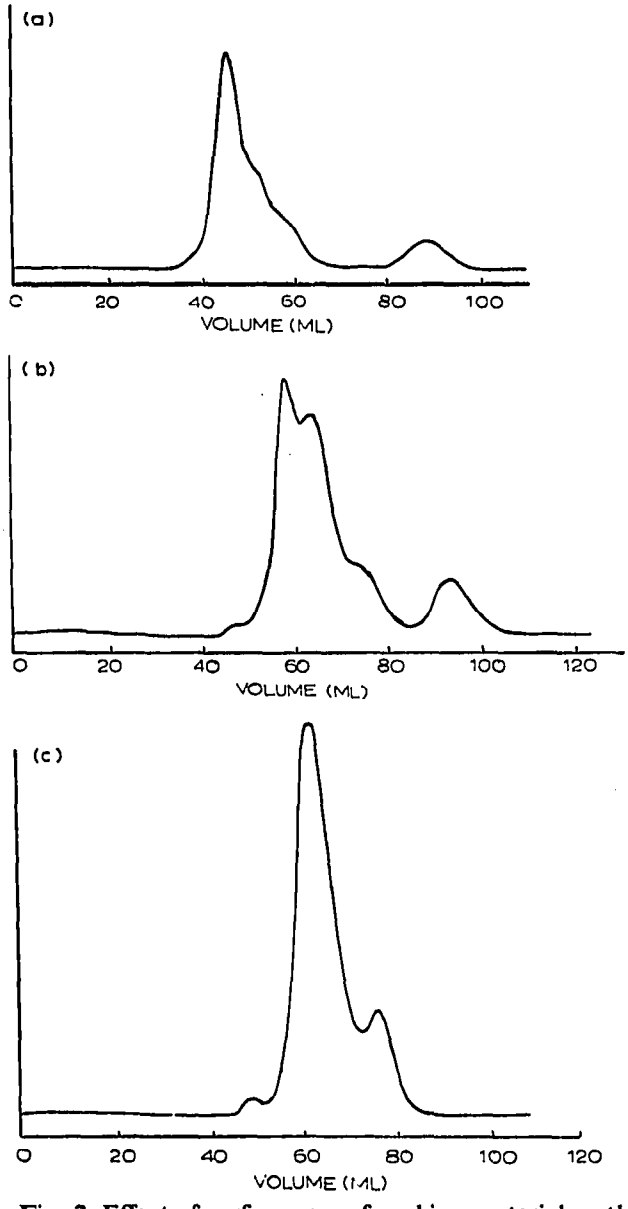

Fig. 2. Effect of surface area of packing material on the separation of F176. (a) Porasil A with chloroform–methanol–ammonia (97:2.4:0.6). (b) Porasil C with chloroform–methanol–ammonia (99:0.8:0.2). (c) Porasil E with chloroform–methanol–ammonia (99.5:0.4:0.1).

We next studied the use of a reversed-phase C18–Porasil B column for the separation of our standard mixture. Two solvent systems were used, *viz.* 100% methanol and methanol–water–ammonia (90:5:5). Chromatograms for these two mixtures are shown in Fig. 4, and it can be seen that both systems provide an adequate separation of the standard mixture.

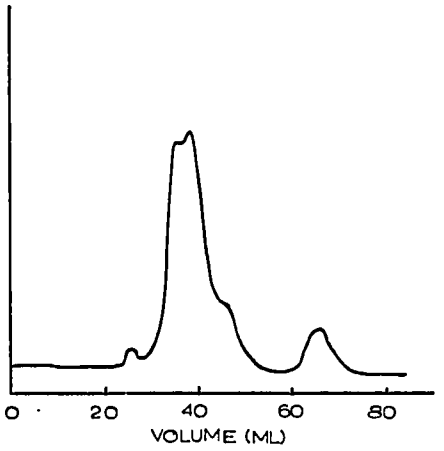

Fig. 3. Separation of F176 on a pellicular packing material, Corasil II. Solvent: chloroform–methanol–ammonia (99:0.8:0.2).

Finally, we investigated the use of alumina as an adsorbent for this type of separation. The alumina used was first treated with 10% water to deactivate it, and was then packed in the usual way. The separation obtained with this material and a mobile phase of chloroform–hexane (9:1) is illustrated in Fig. 5. Here again, an adequate but not complete separation of the components of our standard mixture was obtained on the alumina column.

It is of interest to compare the separation obtained by HPLC with that

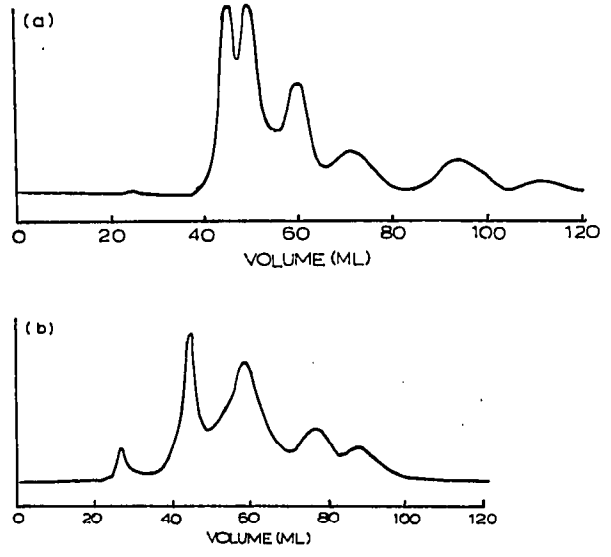

Fig. 4. Separation of F176 on a reversed-phase packing, C18–Porasil B. (a) Solvent: 100% methanol. (b) Solvent: methanol–water–concentrated ammonia (90:5:5).

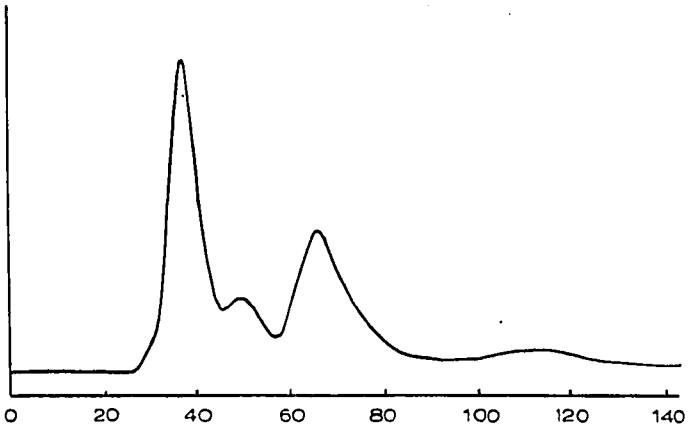

Fig. 5. Separation of F176 on alumina. Solvent: chloroform–hexane (9:1).

obtainable by TLC. The separation of our standard mixture on a silica gel TLC plate using a 5% methanol in chloroform solvent system is shown in Fig. 6, and it can be seen that this separation does not suffer by comparison with the HPLC separations obtained. From a preparative point of view, of course, HPLC has the advantage of ease of operation and, most importantly, the potentiality for recycle operation. Nevertheless, TLC is still an extremely important analytical tool, and we routinely check our fractions obtained by HPLC by TLC.

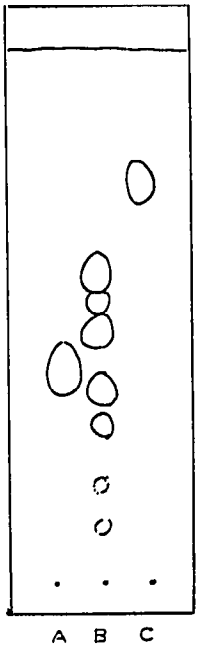

Fig. 6. Separation of F176 by TLC. Silica gel GF_{254} plate. Solvent: chloroform–methanol (95:5). A = Perivine; B = F176; C = Conoduramine.

As a check on the utility of this study from a preparative standpoint, a large sample (100 mg) of F176 was subjected to chromatography on an 8-ft. × 1/2-in. column packed with Porasil C. Elution with chloroform–methanol (99:1) containing 0.2% ammonia yielded a complex first fraction, but the second fraction, indicated by an arrow in Fig. 2b, was found to be pure and crystallised on removal of the solvent. The material (m.p. 231–232°) was identified as pericyclivine, previously isolated from *Catharanthus roseus*[8] and *Gabunia odoratissima*[9].

One possible limitation of the chloroform–methanol systems containing ammonia is that the ethanol present in most commercial samples of chloroform may undergo ester exchange with the methyl ester functions common in many indole alkaloids. This problem may be avoided by the use of alcohol-free chloroform in the preparation of this solvent mixture.

CONCLUSIONS

This work has indicated the usefulness of HPLC in the separation of complex mixtures of indole alkaloids. For preparative purposes the most suitable systems of those tested are ammoniacal chloroform–methanol on Porasil C, methanol (or aqueous methanol containing ammonia) on C18–Porasil B, and chloroform–hexane on alumina.

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