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SEPARATION OF ECDYSTEROIDS BY USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY ON MICROPARTICULATE SUPPORTS

RENÉ LAFONT and GHISLAINE MARTIN-SOMMÉ

École Normale Supérieure, Laboratoire de Zoologie, 46 rue d'Ulm, F-75230 Paris Cedex 05 (France)

and

JEAN-CLAUDE CHAMBET

DuPont de Nemours, Division Instruments, Z.A. de Courtaboeuf, F-91403 Orsay (France)

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SUMMARY

Baseline resolution of many ecdysteroids can be achieved by high-pressure liquid chromatography on columns of microparticulate material. Both silica and reversed-phase (C_{18} or C_8) supports can be efficiently used, provided that the solvent systems are optimised. C_8 bonded phases appear to be the most convenient and, when used with linear gradients, can resolve most ecdysone metabolites in a single pass.

INTRODUCTION

Ecdysteroids are an important family of steroids mainly found in invertebrates (*e.g.*, insects, crustacea, arachnids and nematodes) and some plants. Their identification or quantitative measurement requires several steps including at least one fractionation by thin-layer (TLC) or column chromatography[1]. The most widely used technique is TLC on silica gel containing a fluorescent dye, with a mixture of chloroform with methanol (or ethanol) as mobile phase. More recently, column-chromatographic procedures have been developed, using Amberlite XAD-2[2–6], silica[7–11] or reversed-phase columns[9,12–14]. However, the resolution achieved was not significantly improved by these procedures (except with Amberlite, but in this instance one run took over 10 h, and the technique was thus not suitable for routine analyses).

High-pressure liquid chromatography (HPLC) is a recent and very promising technique, mainly because of the rapid diversification and improvement in column design. HPLC offers the important advantages over TLC that recovery of injected compounds is quantitative and direct determination of ecdysteroids by UV monitoring of the column effluent is possible provided that interfering substances have been separated.

We describe here the results of our experiments with three different systems: a normal silica column and two reversed-phase packings.

MATERIALS AND METHODS

Chemicals

The ecdysone standards used in the present study were obtained from various sources: ecdysone (Simes Labs, Milan, Italy and Fluka, Buchs, Switzerland), ecdysterone (Simes and Rohto Pharmaceutical Co., Osaka, Japan), makisterone (Simes), poststerone (Simes), cyasterone (Rohto) and inokosterone (Schwartz/Mann Research Labs., Orangeburg, N.Y., U.S.A.). Solvents, purchased from Mallinckrodt were of Nanograde quality; those from Merck and from Solvants, Documentation et Synthèse (Marseille, France) were of Spectrosol grade.

Chromatographic procedures

We used a DuPont Model 848 module pump equipped with a universal septumless injector (50-μl sample loop) and a Model 830305 pre-programed gradient former. UV detection was carried out either at 254 nm (fixed-wavelength detector) or at the peak value (243 nm) with a spectrophotometer; in fact, the difference was not significant and the former procedure gave satisfactory results.

Three different columns were used; each was the same size (25 cm $\times$ 4.6 mm I.D.) and was packed with 6-μm particles of Zorbax SIL, Zorbax ODS or Zorbax C_8 (DuPont).

RESULTS

Silica: normal phase

We tried a number of different solvents consisting of a mixture of chloroform (or dichloromethane), an alcohol (methanol, ethanol or isopropanol) and water. It appeared that the quality of the separations increased with the molecular weight of the alcohol, but, because of their smaller eluting power, heavier alcohols led to a considerable increase in analysis time. We also observed that the presence of 1–1.5% of water (*i.e.*, just below saturation) in the mixture noticeably increased the resolution by reducing peak tailing. The most promising results were obtained with two mobile phases, *viz.*, dichloromethane–ethanol–water (840:145:15) and dichloromethane–isopropanol–water (125:25:2). In both these solvent systems (see Fig. 1), ecdysone and makisterone are base-line resolved (this is not the case with a 10-μm silica column), but in the first system, ecdysterone and inokosterone are not resolved. However, the second system can resolve these compounds and even (see the presence of a shoulder) partially resolve two compounds in the inokosterone standard (see also Fig. 7). The main problem with such a column is the need for the injected compounds to differ little in polarity in order to avoid column contamination. This problem does not arise with reversed-phase columns, which can easily be cleaned by the use of a solvent gradient and which re-equilibrate quickly.

Octadecylsilane (ODS) phase

Packings with C_{18} bonded phases are the most common supports for HPLC. They allow the use of solvent gradients and we used them especially with biological extracts. Zorbax ODS has a high content (14%) of stationary phase that provides a plate number of 8000 for a 25-cm column. Ecdysone analyses were first carried out

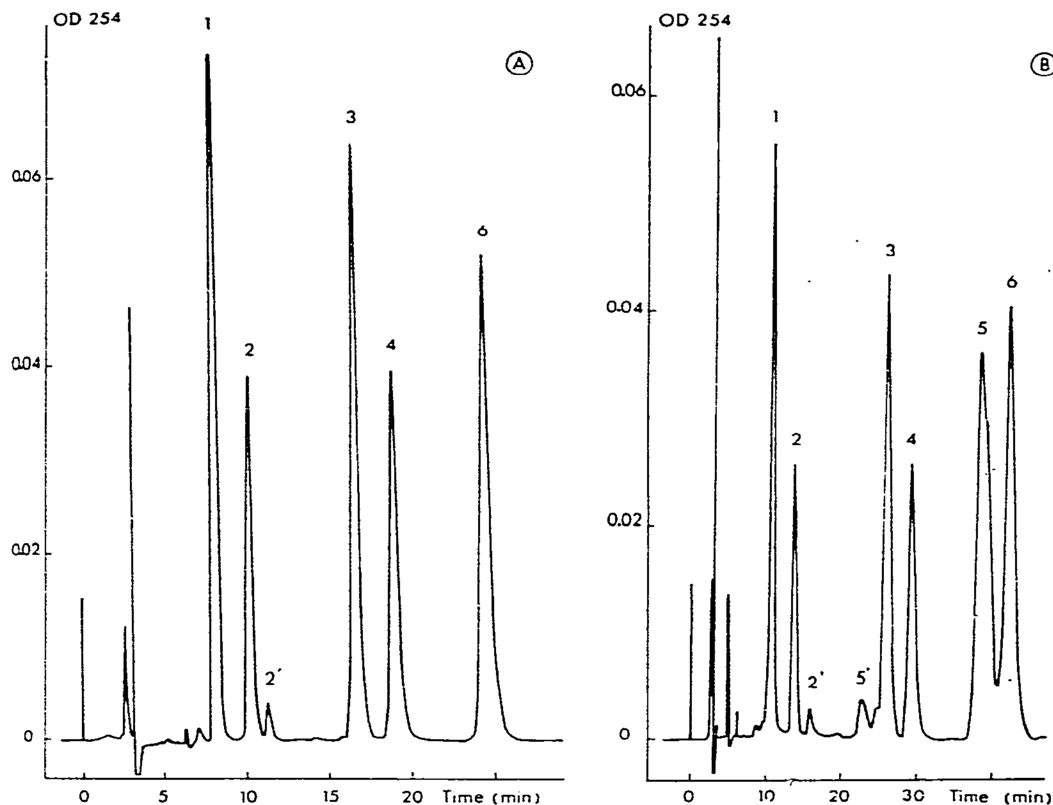


Fig. 1. Separations of ecdysteroid mixtures (*ca.* 1 μg of each component) on Zorbax SIL, with detection at 254 nm (0.1 a.u.f.s.); mobile-phase flow-rate 1 ml/min. Peaks: 1 = poststerone; 2 = cyasterone; 3 = ecdysone; 4 = makisterone; 5 = inokosterone; 6 = ecdysterone. A: dichloromethane–ethanol–water (840:145:15) as mobile phase; in this system, ecdysterone and inokosterone are not separated. B: dichloromethane–isopropanol–water (125:25:2) as mobile phase; inokosterone and ecdysterone are partly resolved, and the inokosterone peak has a shoulder (see Fig. 7). Peaks 2′ and 5′ represent impurities of cyasterone and inokosterone standards, respectively.

with isocratic elution, but excessive peak tailing and rapid dilution of retained compounds rendered this procedure impractical. Of the various solvent mixtures tested acetonitrile–water gave the most satisfactory results. The use of linear gradients (from 5–10% to 30 or 40% of acetonitrile in water) gave quite good separations, although still with some tailing of peaks (see A in Fig. 2). This phenomenon was particularly noticeable with new columns and disappeared progressively with use (Fig. 2B shows the same separation with a 6-month-old column that had been used repeatedly with biological extracts). It seems that the very active sites responsible for tailing have been progressively blocked. The limit of detection is below 10 ng when this procedure is used. Fig. 3 shows the separation of several ecdysteroids on an "old" column: with a "new" one, ecdysone and poststerone are not resolved when using the same elution gradient, and give a single peak with no visible shoulder.

C_8 bonded columns

Zorbax C_8 is a new non-polar bonded phase designed for reversed-phase

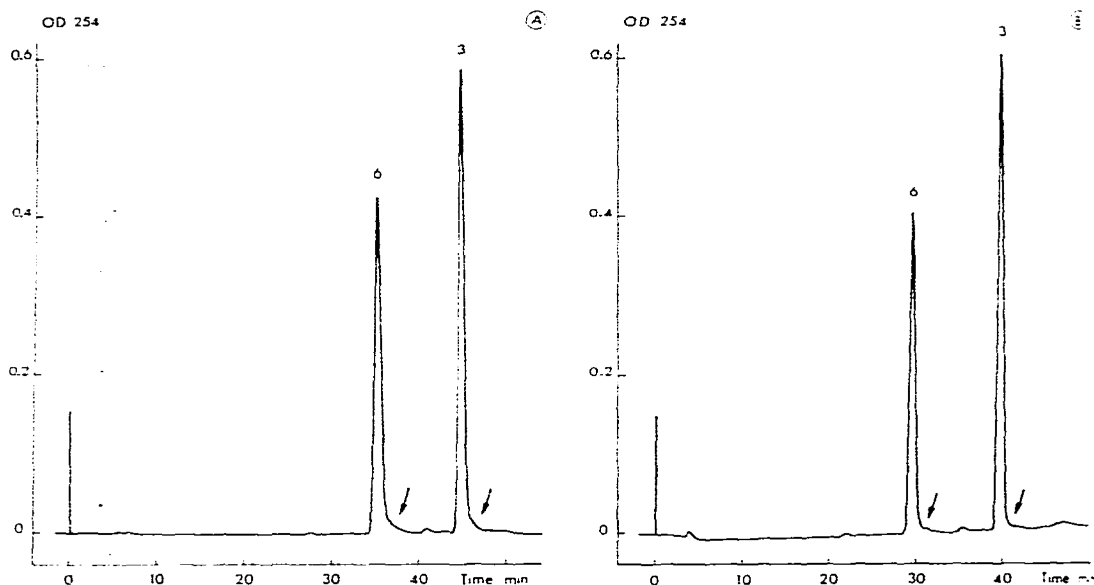


Fig. 2. Separation of ecdysone (peak 3) and ecdysterone (peak 6) (*ca.* 10 μg of each) on Zorbax ODS, with detection at 254 nm (1 a.u.f.s.) and mobile-phase flow-rate *ca.* 1 ml/min (1000 p.s.i.). Linear gradient increasing (at 2% per min) from 0 to 100% of secondary solvent (30% acetonitrile in water) in primary solvent (10% acetonitrile in water). A: 1-month-old column. B: 6-month-old column. Arrows show how peak tailing is reduced with the older column.

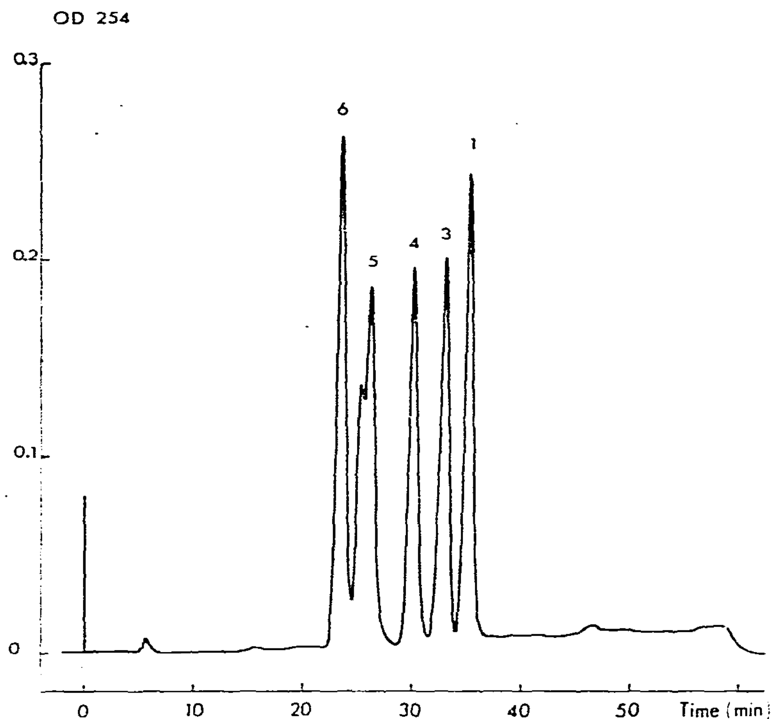


Fig. 3. Separation of an ecdysteroid mixture on a 6-month-old column of Zorbax ODS. Conditions as in Fig. 2; peaks as in Fig. 1.

chromatography. This material, which shows weaker retention, was introduced in the hope of reducing the peak tailing seen with C_{18} columns. Fig. 4 shows the separation of ecdysone from ecdysterone on a C_8 column; there is almost no tailing and the resolution is far better than with C_{18}. The analysis of the same mixture as in Fig. 3 is shown in Fig. 5 (the inokosterone had previously been purified). All five compounds are base-line resolved in this system. Use of the column for 2 months did not produce any significant change in its properties. Many gradient systems have been tested; depending on the nature of the compounds being analysed, gradients can be used with, *e.g.*, 1, 5, 10 or 15% of acetonitrile in water as primary solvent and 30% or 40% of acetonitrile as secondary solvent, with linear programming at 1 or 2% per min and a solvent flow-rate of 0.8–1.5 ml/min.

Some applications of HPLC to ecdysteroid analysis

Standard purification. Ecdysteroid standards usually contain 1–2% of various impurities, especially other ecdysteroids. These may be undesirable for physiological studies and can be readily removed by using HPLC. Fig. 6 shows the chromatographic pattern of two ecdysterone standards (from Rohto and Simes) containing different impurities. Impurities (traces of ecdysterone or other ecdysteroids) are also present in some ecdysone standards and could account for certain *in vitro* effects of this hormone.

The results are more impressive with the inokosterone standard (see Fig. 7).

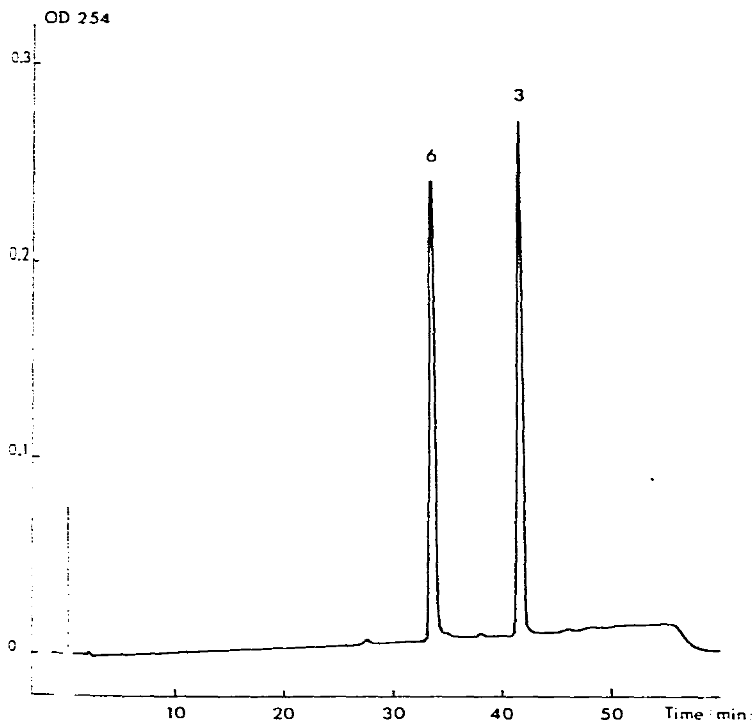


Fig. 4. Separation of ecdysone (peak 3) and ecdysterone (peak 6) (*ca.* 5 μg of each) on Zorbax C-8, with detection at 254 nm (0.5 a.u.f.s.) and mobile-phase flow-rate *ca.* 0.8 ml/min (800 p.s.i.). Linear gradient increasing (at 2% per min) from 0 to 100% of secondary solvent (30% acetonitrile in water) in primary solvent (5% acetonitrile in water).

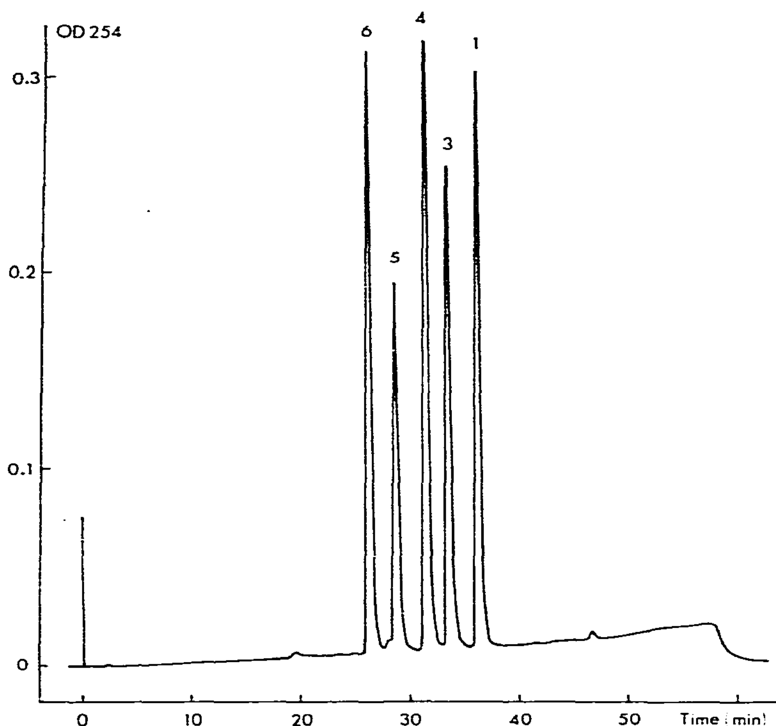


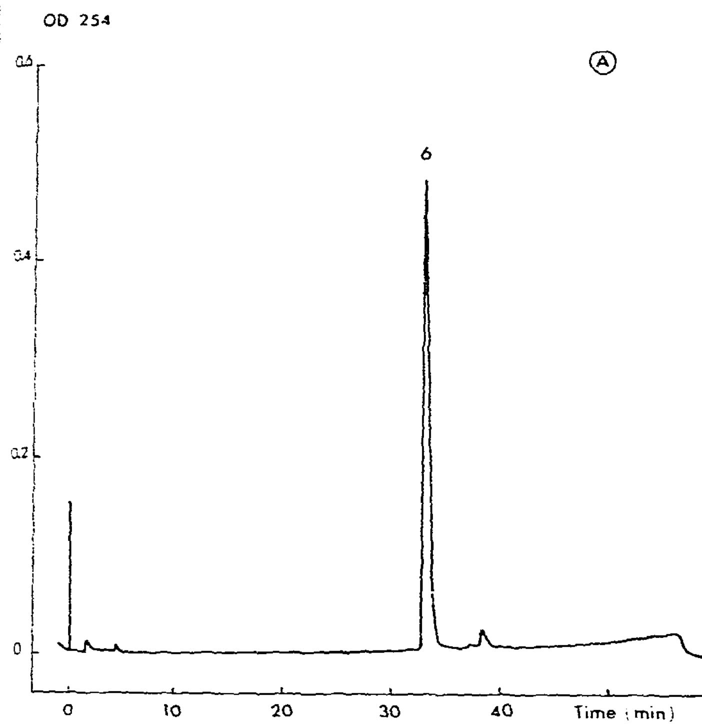
Fig. 5. Separation of an ecdysteroid mixture (*ca.* 3–5 μg of each component) on Zorbax C-8. Conditions as in Fig. 4 except for pressure (1000 p.s.i.) and primary solvent composition (10% acetonitrile in water). Inokosterone purified before injection. Peaks as in Fig. 1

Both reversed-phase (Fig. 7A) and suitable normal-phase (Fig. 7B) chromatography show that this compound is a mixture of three major components, with some minor impurities. It appears that ecdysterone accounts for some 10–15% of the whole "compound".

Study of ecdysone metabolites. HPLC on a reversed-phase column can also be useful in metabolic studies: Fig. 8 shows the pattern of ecdysone metabolites 24 h after injection of [^{3}H]ecdysone into *Pieris brassicae* pre-pupae. The main advantage of this technique is that many compounds from polar metabolites (conjugates) to compounds less polar than ecdysone (dehydroecdysterone ?) can be resolved in a single run. Complete resolution of all components, however, would need a second run of some of the peaks on another column, such as normal silica.

DISCUSSION

HPLC was firstly proposed by Hori[2] for use with ecdysteroids. His method gave good resolution, *i.e.*, better than that of TLC on silica gel, but a single run lasted *ca.* 15 h and could only be used in preparative work. In the analytical field, several systems have recently been proposed, but, owing to the rapid evolution of phase design, columns of much higher performance are now available. The use of microparticulate (5–10 μm) material has brought about large increase in the resolving power of columns and has considerably shortened the analysis time.



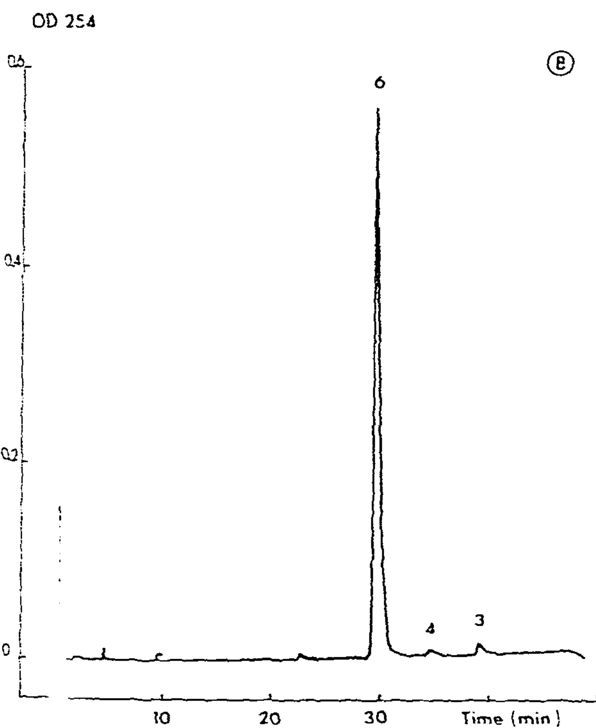


Fig. Comparison of two ecdysterone standards (*ca.* 10 μg of each) using Zorbax C-8, with detection 254 nm (1 a.u.f.s.). Other conditions as in Fig. 4 except for pressure (1000 p.s.i.).A, ecdy rone from Rohto; B, ecdysterone from Simes.

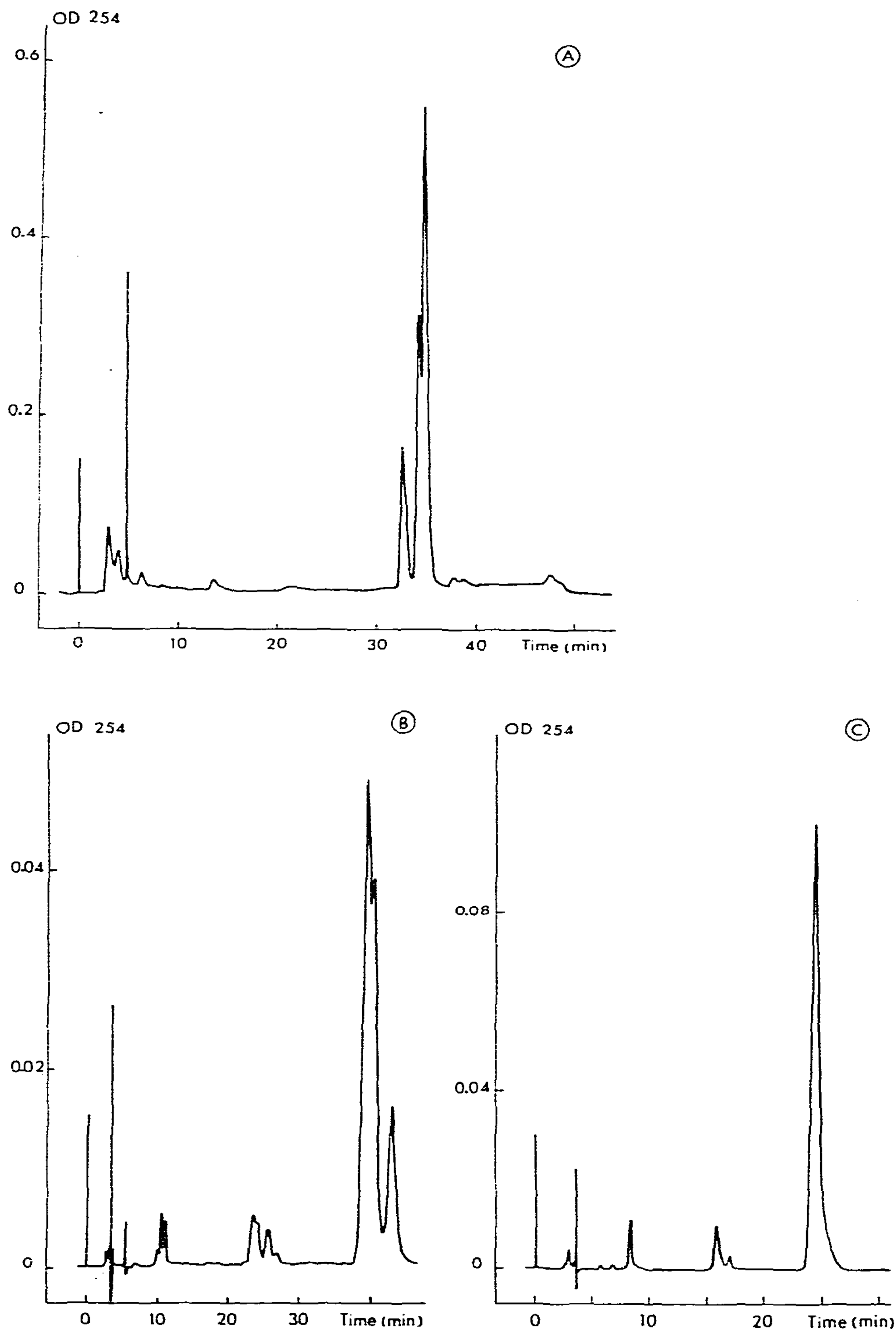


Fig. 7. Analysis of an inokosterone standard in various systems. A: Zorbax C-8; conditions as in Fig. 6. B: Zorbax SIL; conditions as in Fig. 1B. C: Zorbax SIL; conditions as in Fig. 1A.

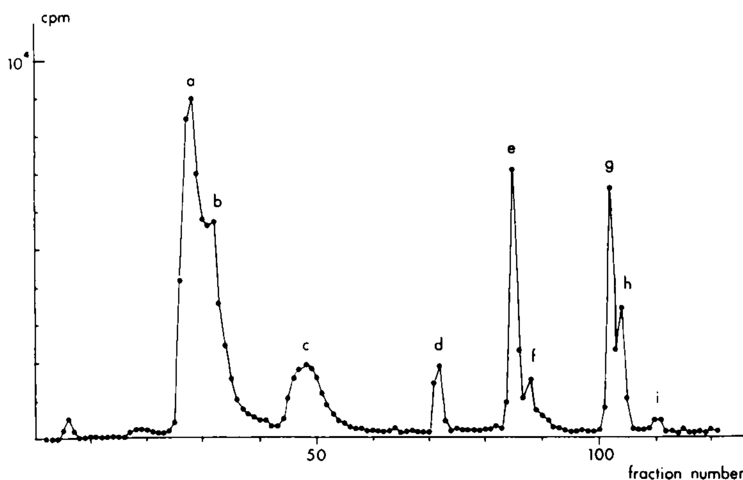


Fig. 8. Use of a C_8 column for studies of ecdysone metabolism. *Pieris brassicae* pharate pupae were injected with *ca.* 10^6 dpm [^{3}H]ecdysone and used 48 h later (*i.e.*, as 30-h-old pupae). Labelled compounds were extracted with methanol and purified on silicic acid, and an aliquot was injected. Fractions corresponding to 0.3 min were collected and counted with a Kontron MR 300 scintillator. Pressure 1800 p.s.i. Linear gradient increasing (at 2% per min) from 0 to 100% of secondary solvent (40% acetonitrile in water) in primary solvent (1% acetonitrile in water). According to the data from various authors, the compounds can be tentatively identified as: a, b, c = polar metabolites (conjugates); d = 20,26-dihydroxyecdysone; e = ecdysterone; f = 3-epiecdysterone (?); g = ecdysone; h = 3-epiecdysone (?); i = 3-dehydroecdysterone (?).

Silica: normal phase

Corasil II (37–50 μm) (Waters Assoc., Milford, Mass., U.S.A.) was used by Nigg *et al.*[7,8] and by Gilgan[9], and Zorbax-SIL by Nigg *et al.*[8], Ohnishi *et al.*[10] and Moribayashi and Ohtaki[11]. The reported procedures were able to resolve ecdysone, makisterone and ecdysterone, but not ecdysterone and inokosterone. This last separation has achieved in the present study because of the smaller size of the particles and the modification to the solvent system. The solvent system is most important, and very good resolution of ecdysteroids has been obtained by paper chromatography[15] with a suitable two-phase solvent system. In HPLC, it appears to be better to use isopropanol rather than ethanol or methanol, because this alcohol seems to provide better wettability of the silica particles and to promote a regular arrangement of water molecules in the stationary phase.

Reversed phases

Previously used column packings include XAD-2[2–6], Bondapak phenyl-Corasil (Waters)[9] and Poragel PN (Waters)[12–14], with isocratic or gradient elution. The reported results indicate poor separation with Poragel PN, and this packing appears more suitable for a preliminary purification step. Bondapak phenyl-Corasil gives better results, especially when gradient elution is used, but appears unable to resolve makisterone and inokosterone[9]. In consequence, Gilgan[9] proposed use of a combination of normal silica and reversed-phase columns for complementary separations. We have reached the same conclusion, but for another purpose. In Fig. 8, there is some indication of duplication for the ecdysone and ecdysterone peaks. This could well be due

to the presence of 3-epimers, which have been found in insects[8,16] and which can be more or less resolved on a normal silica column[8].

CONCLUSIONS

HPLC provides a most suitable tool for ecdysone studies. Its resolution far exceeds that of conventional TLC and it can be used for routine analyses. Its application to the direct measurement of ecdysone levels during insect development is theoretically possible, even with small samples, owing to the large concentration of ecdysteroids in insects as compared with those of steroid hormones in vertebrates. However, it must be noted that UV detection is not specific, and, even with the chromatographic procedures described here, it seems inadvisable to use it without another control system, such as radioimmunoassay, gas–liquid chromatography with mass spectrometry or electron-capture detection. The coupling of HPLC with mass spectrometry is being tried and would provide an ideal detection system. Another possibility for increasing the specificity of detection is the use of fluorescent derivatives of ecdysones, as recently proposed by Poole *et al.*[17]: this improves the sensitivity to less than 1 ng after HPLC purification.

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