Journal of Chromatography, 194 (1980) 343–352
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,748

COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY FOR THE ANALYSIS OF ECDYSTEROIDS

I. D. WILSON*
Laboratory of Toxicology, University College Hospital Medical School, University Street, London WC1 6JJ (Great Britain)
C. R. BIELBY and E. D. MORGAN*
Department of Chemistry, University of Keele, Keele, Staffordshire ST5 5BG (Great Britain)
and
A. E. M. McLEAN
Laboratory of Toxicology, University College Hospital Medical School, University Street, London WC1 6JJ (Great Britain)
(Received February 7th, 1980)

SUMMARY

High-performance liquid chromatography (HPLC) with ultraviolet absorption detection, and gas chromatography (GC) with an electron-capture detector have been compared for their convenience in the analysis of ecdysteroids in invertebrate tissues. Analysis by HPLC on reversed-phase materials, including C_{18}, C_{22} and CN bonded phases, was explored for the separation of both polar ecdysteroids and some of their possible biosynthetic intermediates of lower polarity. The HPLC method was found to be rapid and easy to use, but less sensitive than GC with electron-capture detection. The greater sensitivity and selectivity of GC was found to be important for the more difficult analyses.

INTRODUCTION

High-performance liquid chromatography (HPLC) was applied to the separation and quantification of ecdysteroids soon after the introduction of the technique; some of the earlier work has been reviewed[1].

Early attempts with absorption columns were not very successful, but recently several useful separations on bonded reversed-phase materials have been reported. Permaphase ETU has been used for the separation of phytoecdysones from *Achyranthus radix*[2], eluting with hexane–ethanol (9:1). Isocratic elution from μBondapak C_{18} with acetonitrile–water was used in the analysis of ecdysteroids in *Heliothis zea*[3], and,

* Present address, Hoechst Pharmaceutical Research Laboratory, Walton Manor, Milton Keynes MK7 7DJ, Great Britain.

0021-9673/80/0000–0000/$02.25

more recently, Lafont *et al.*[4] have demonstrated that excellent separations of pure ecdysteroids are possible on silica C_8 and C_{18} phases with solvent programming.

These rapid developments in HPLC encouraged us to re-examine its use with bonded phases and solvent programming, which had not been possible in earlier investigations[5]. Major benefits of HPLC in analysis for ecdysteroids would be shorter analysis time, and the avoidance of heat and contamination from reagents necessary for the formation of derivatives required for the gas chromatography (GC) method presently in use in our laboratory[6].

We describe here some separations obtained with pure ecdysteroids and model compounds on C_{18}, C_{22} and CN bonded phases, and compare results obtained from HPLC and GC with electron-capture detection for biological samples.

EXPERIMENTAL

Ecdysone, 20-hydroxyecdysone and poststerone were purchased from Simes (Milan, Italy); inokosterone was obtained from Rhoto Pharmaceuticals (Osaka, Japan), and the model sterols 2β,3β-dihydroxy-5β-cholest-7-en-6-one, and 3β-hydroxy-5α-cholest-7-en-6-one were synthesised in our laboratory. Dns-Hydrazine was purchased from Pierce and Warriner (Chester, Great Britain). The Hypersil C_{18} and CN bonded packing materials and the Magnusil C_{22} supergrade H and Magnusil C_{22}X were obtained from Magnus Scientific (Sandbach, Great Britain).

Columns were packed as slurries in methanol (for C_{18} and CN) or in isopropanol (C_{22}) using a Magnus P6000 slurry-packing unit. Two Waters Assoc. 6000A pumps (Waters Assoc., Milford, MA, U.S.A.) were used to pump solvents, controlled by a Waters 660 solvent-programming unit. Samples were introduced via a Waters U6K loop injector, and compounds were detected with a Varian Varichrom variable-wavelength UV–visible spectrophotometer set at 240 nm. GC was carried out as described elsewhere[6], on a 1.5-m column of 1.5% OV-101 silicone gum on Chromosorb Q, with use of a Pye 104 gas chromatograph and ^{63}Ni electron-capture detector (ECD) with a Pye GCV variable-pulse amplifier.

Insect haemolymph (1.0 ml) was obtained from adult male *Schistocerca gregaria* and divided into two equal portions. To one was added 20-hydroxecdysone (250 ng). Each sample was then diluted with methanol (2 ml) to precipitate proteins, left overnight, then passed through a C_{18} reversed-phase "Sep-pak" (Waters Assoc.) and washed with a further 2 ml of methanol, evaporated to dryness, then taken up in 100 μl of methanol; 20 μl of this solution were injected on to the HPLC column.

Extraction of ecdysteroids from biological samples has been described elsewhere[1,6]. For the barnacle sample, a sample of *Balanus balanoides* (100 g) was ground in methanol, and the methanol-soluble portion was partitioned between light petroleum (b.p. 40–60°C) and aqueous methanol. The aqueous extract was partitioned between butanol and water, and the butanol extract was finally partitioned between ethyl acetate and water. The residue from the aqueous portion was taken up in methanol (2 ml), ecdysone (10 μg) was added, and the sample was silylated, cleaned-up by thin-layer chromatography and subjected to GC. To the residue from a similar sample in methanol (2 ml) was added ecdysone (13.2 μg), and 10 μl of this mixture were injected on to a C_{18} HPLC column.

RESULTS

Comparison of four reversed-phase packing materials for their ability to resolve a test mixture of ecdysteroids and model compounds

The ability of a C_{18}, a CN, and two C_{22} bonded reversed-phase materials to resolve a mixture of two ecdysteroids (ecdysone and 20-hydroxyecdysone) and two model compounds (2β,3β-dihydroxycholest-7-en-6-one and 3β-hydroxycholest-7-en-6-one) was tested. To resolve this mixture of compounds, a gradient elution system was used from 15 to 95% of methanol with water as the counter-phase. This mixture gave two distinct groups of compounds differing greatly in polarity, the ecdysteroids being polar, and the model compounds relatively non-polar. Within these two groups, the polarity of the two compounds differed by only one hydroxyl group. This mixture therefore allowed an assessment of the ability of a particular column to separate compounds with both large and small differences in polarity over the whole range of ecdysteroids and their biosynthetic intermediates likely to be found in extracts of biological material. Representative chromatograms for each phase tested are shown in Fig. 1. From these, it can be seen that the C_{18} and C_{22} supergrade H phases give excellent results, all four compounds being resolved; with the CN and $C_{22}X$ phases, although there is adequate resolution of ecdysone and 20-hydroxyecdysone, the two non-polar model compounds are not resolved. This inability to resolve the non-polar components of the test mixture seems to be an intrinsic property of the phase rather than any function of column length, as even a short C_{18} column (4 cm) gives similar resolution to that seen on the 25-cm C_{18} column under these conditions. The C_{22} supergrade H phase appears to have slight advantages over the C_{18} phase that we tested: slightly improved resolution for ecdysone and 20-hydroxyecdysone, and much improved separation of the non-polar components of the mixture.

Analysis time for these samples under these conditions was approximately 20 min (with the exception of the short, 4 cm, C_{18} column, for which it was *ca.* 12 min).

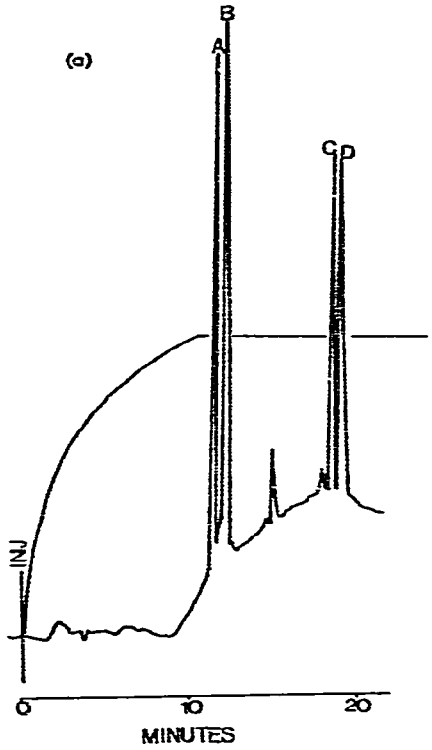
COMPARISON OF GC AND HPLC METHODS

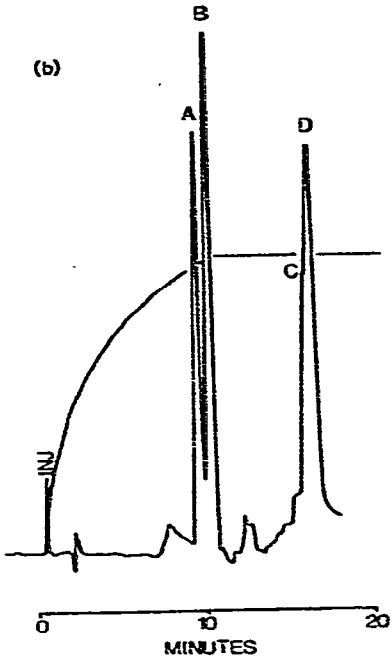
Sensitivity

The least detectable quantities of pure 20-hydroxyecdysone and ecdysone that can be determined by GC–ECD are in the sub-nanogram range ($\approx 10^{-11}$ g); a standard curve is shown in Fig. 2. For HPLC with UV detection at 240 nm, measured under conditions where the 20-hydroxyecdysone was eluted from the column only slightly retained (*i.e.*, conditions designed to give the sharpest possible peaks and minimum band spreading), 5 ng represented the limit of detection. The use of gradients resulted in large baseline disturbances due to the change in solvent composition during chromatography. This, together with somewhat broader peaks, acted to reduce the sensitivity of the method. The use of gradients increased the quantity of ecdysteroid required for detection by about one order of magnitude (to 50 ng).

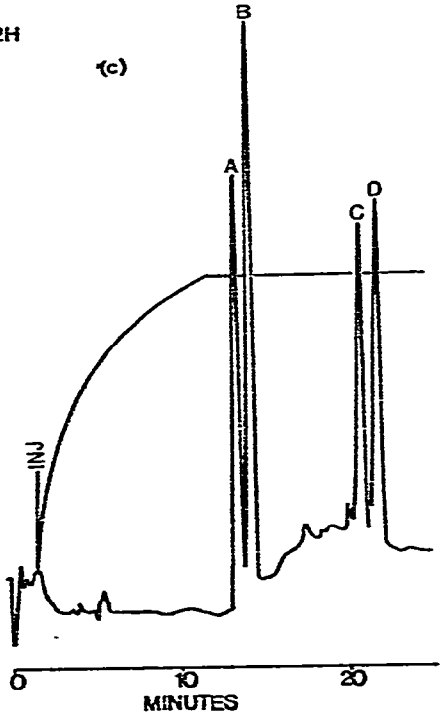
Selectivity

The partition properties, and hence the separation, of the silyl ethers of an ecdysteroid on GC are affected by a combination of at least three factors; molecular weight, polarity and conformation. Thus, the silyl ethers of ecdysone and 20-hydroxy-

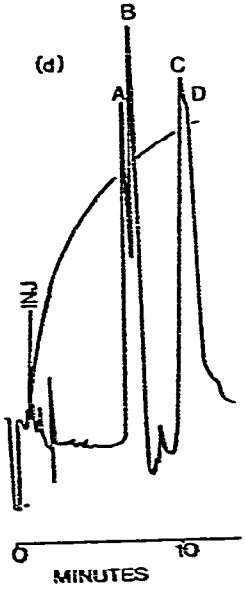

(a)
B
A
C
D
INJ
0
10
20
MINUTES


(b)
B
A
D
C
INJ
0
10
20
MINUTES

C22H


(c)
B
A
D
C
INJ
0
10
20
MINUTES

CN


(d)
B
C
A
D
INJ
0
10
MINUTES

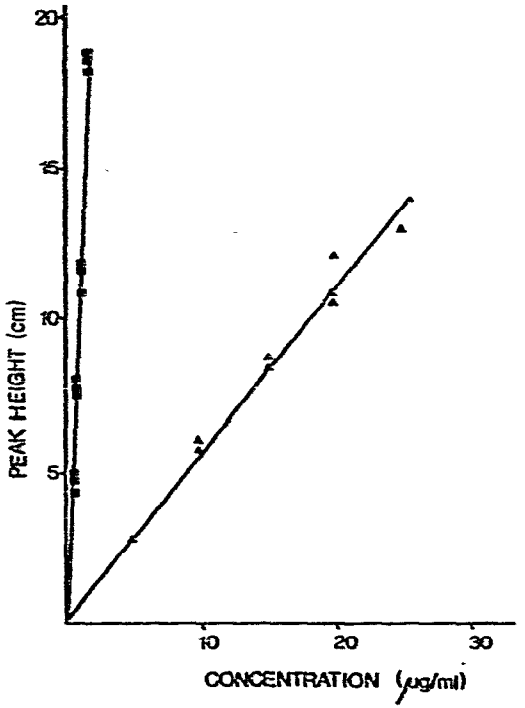


Fig. 2. Calibration curves for 20-hydroxyecdysone determined by HPLC (▲) and GC–ECD (■).

ecdysone, while of similar polarity, differ in molecular weight, and are easily separated by GC. Similarly, inokosterone and 20-hydroxyecdysone, while of similar polarity and molecular weight, are well resolved in GC because of differences in the positions of hydroxyl groups on the side-chain. For HPLC, the most important factor in chromatography of the ecdysteroids on reversed phases seems to be polarity (Lafont *et al.*[4]). As a consequence, we were unable under the conditions we have used to resolve inokosterone and poststerone from 20-hydroxyecdysone. This separation has been achieved by Lafont *et al.*[4] on a C_8 bonded phase and required a very shallow solvent gradient. In HPLC, separation of a wide range of ecdysteroids, metabolites and precursors is possible by solvent programming. In GC, the same is achieved by temperature programming.

Fig. 1. Separation of ecdysteroids and model steroids by HPLC with methanol–water gradients (a), C_{18}; (b), $C_{22}X$; (c) $C_{22}H$; (d) CN. The compounds are: A, 20-hydroxyecdysone; B, ecdysone; C, 2β,3β-dihydroxy-5β-cholest-7-en-6-one; and D, 3β-hydroxy-5α-cholest-7-en-6-one. Detection was by UV at 240 nm; 20 μl of test solution was injected in methanol. The profile of the solvent gradient is shown. With C_{18}, $C_{22}X$ and $C_{22}H$ packings, the flow-rate was 1.5 ml/mm, and the gradient was from 15% methanol to 95% methanol in 10 min. For the CN phase, the gradient was from 0% methanol to 95% methanol (compounds A and B were not retained by 15% methanol). Column lengths were 25 cm for C_{18} and $C_{22}H$ packings, and 10 cm for $C_{22}X$ and CN.

Analysis of biological samples

The biological tissues, fluids or residues that we have examined in previous studies[7–9] have varied widely in their ecdysteroid content, and in the amount of contaminating material present that interferes with ecdysteroid determination. To compare HPLC with GC for routine analyses, we chose two materials, haemolymph from the desert locust *Schistocerca gregaria*, which as we have shown in an earlier study[10] can contain high levels of ecdysone and 20-hydroxyecdysone at certain stages of development (0.5 μg/ml), and the barnacle, *Balanus balanoides*, which typically gives highly contaminated samples, even after extensive purification by solvent partition.

Locust haemolymph was collected at a stage in the life cycle of this insect when ecdysteroids are absent (adult males). The haemolymph was diluted with methanol, with or without the addition of 20-hydroxyecdysone (2.5 μg/ml) as described in the experimental section, and passed through a Sep-pak column. Under these conditions ecdysteroids are not retained by the Sep-pak C_{18} packing material, whereas non-polar lipids are. The solution resulting from this treatment is surprisingly free of UV-absorbing contaminants. These solutions were directly analysed by HPLC on a C_{18} column using the gradient elution method (Fig. 3), although the isocratic elution method is equally acceptable. Fig. 3 also shows the chromatogram obtained with an equivalent solution of pure 20-hydroxyecdysone on this C_{18} column. The determination of ecdysteroids in haemolymph by either HPLC or GC is relatively simple, and determinations by HPLC can be performed on comparatively unpurified material, due to the absence of UV-absorbing contaminants. However, the baseline disturbances created with gradient elution probably make it necessary to analyse samples by using isocratic elution techniques.

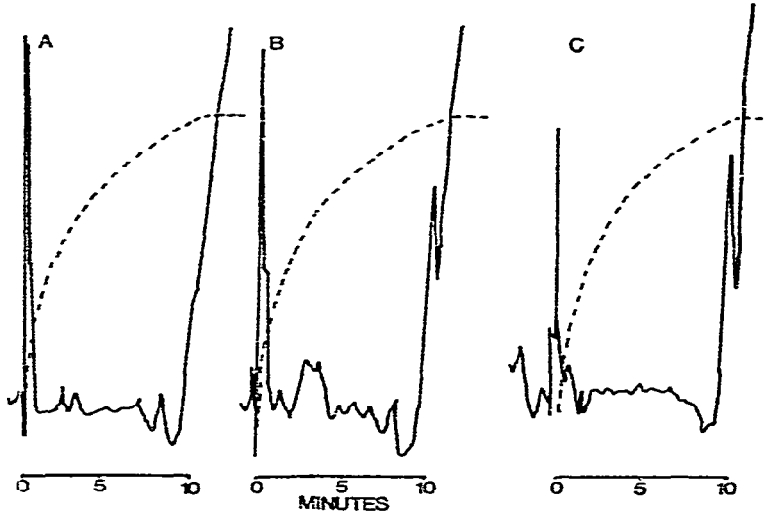


Fig. 3. Insect haemolymph analysed by HPLC on a 25-cm column of Hypersil C_{18}, with a flow-rate of 1.5 ml/min and a gradient of 15 to 95% methanol in 10 min (broken line). A, Haemolymph (free of ecdysteroids) before addition of 20-hydroxyecdysone; B, haemolymph after addition of 50 ng of 20-hydroxyecdysone; and C, 50 ng of pure 20-hydroxyecdysone analysed under the same conditions. The rising baseline is due to change of solvent composition when using high sensitivity.

Samples from barnacles posed a much greater problem as, even after the solvent partition steps outlined in the experimental section, they still contained large quantities of UV-absorbing material, which effectively prevented the detection and quantification of the ecdysone with which they had been spiked (Fig. 4). Indeed, had the sample been concentrated, and the UV detector operated at the sensitivity required to observe the quantity of ecdysone present in the sample, the level of contamination would have rendered the sample completely opaque. The same sample could easily be quantified by GC (Fig. 5).

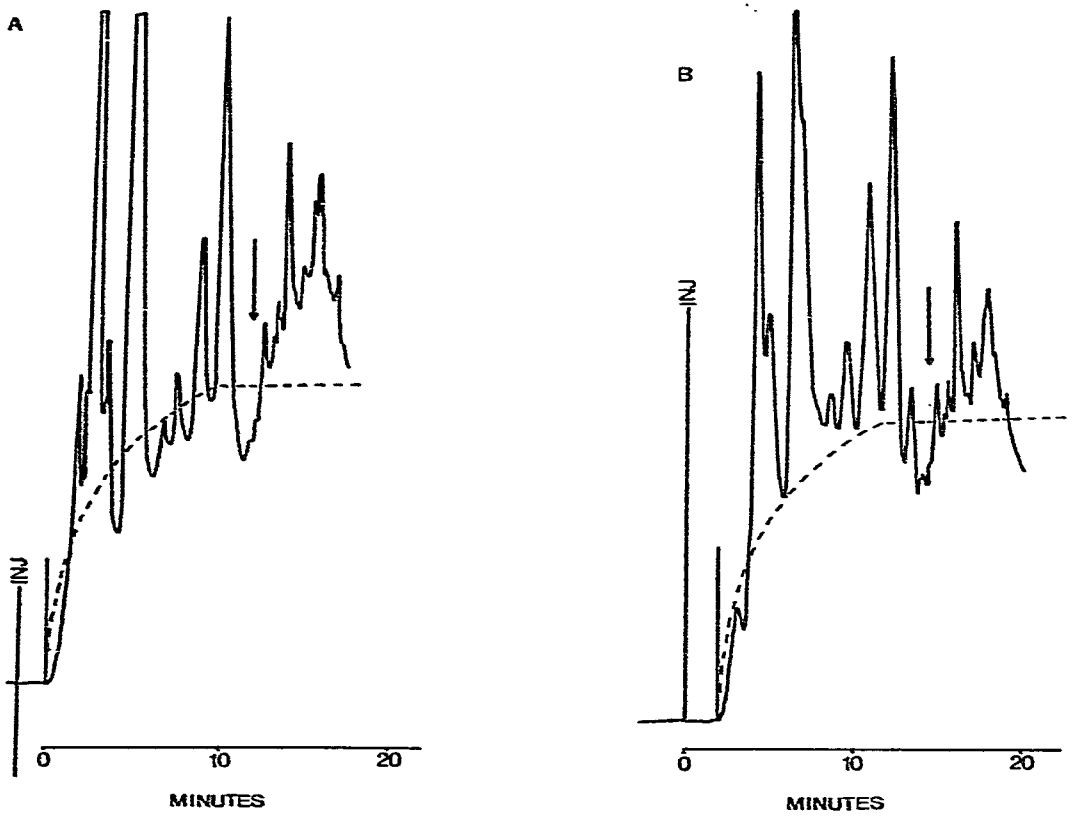


Fig. 4. HPLC trace of barnacle extract on a 25-cm column of Hypersil C_{18}; A, before, and B, after, addition of 66 ng of ecdysone (10 μl of a 6.6 ng/μl solution). Analytical conditions as in Figs. 1 and 3. The arrow indicates the retention time of ecdysone.

Fluorescent derivatives of ecdysteroids for HPLC

We have attempted to find fluorescent derivatives of the ecdysteroids in order to increase both the sensitivity and specificity of their detection after HPLC. Since all ecdysteroids possess a 6-keto group, we have attempted to prepare a fluorescent hydrazone of this group. Although reaction of Dns-hydrazine was rapid and quantitative with the 3-keto group of methyltestosterone, it was slow and incomplete with ecdysone or 20-hydroxyecdysone, where the keto group is rather more hindered (see

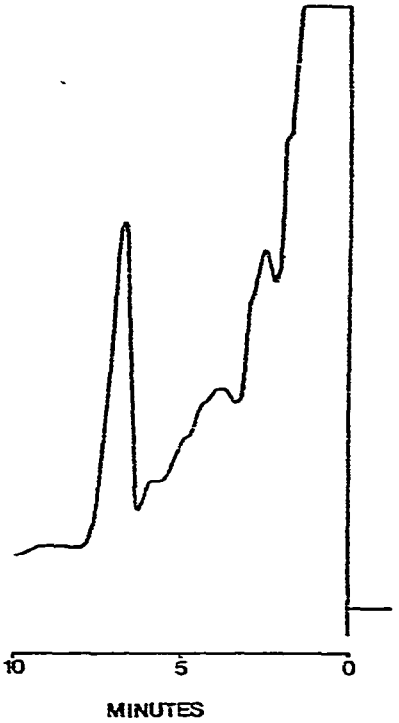


Fig. 5. GC–ECD trace of the same barnacle sample used in Fig. 4 (after addition of ecdysone and conversion to the tetrakis(trimethylsilyl) ether and clean-up on a thin-layer plate. The peak represents 5 ng injected in 1 μl of toluene.

for example, the relative ease of reduction of the 3-keto groups in ecdysteroids compared with the 6-keto group[11]). Although various sets of reaction conditions were tested, none was sufficiently promising to suggest that a Dns-hydrazone of an ecdysteroid could be formed quantitatively.

CONCLUSIONS

For several years, we have routinely used GC–ECD for the determination of ecdysteroids in biological materials. This procedure requires silylation of the ecdysteroids to render them volatile for GC. The process of purification and silylation makes the analysis lengthy and unsuitable for large numbers of samples. The strong UV absorption of the ecdysteroids, combined with their low volatility and sensitivity to heat, would appear to make the ecdysteroids ideal candidates for reversed-phase HPLC. Also, as silylation would not be required, this would lessen the number of operations needed for each sample, thus reducing the analysis time. In addition, if required, ecdysteroids could be collected as they are eluted from the column and further characterised or bioassayed, neither of which is possible when GC methods are used. Indeed, for analysing some samples from biological fluids (*e.g.*, haemolymph) and for testing the efficiency of solvent-partition steps and stability studies, HPLC with UV detection may well be the preferred method of analysis. However, for samples

containing either low levels of ecdysteroids or large quantities of UV-absorbing contaminants, HPLC is less useful, and GC–ECD is to be preferred. The major difficulty in the application of HPLC to the analysis of ecdysteroids is that UV detection at 240 nm is neither sufficiently sensitive nor specific enough for all but a limited range of biological samples, and certainly this is the case for some whole-body extracts (*e.g.*, of barnacles). In addition, it is not possible to use any but the shallowest of gradients for eluting samples at high instrumental sensitivities, because of baseline disturbances resulting from the changing solvent composition. This, while not unexpected, is disappointing, since it means that it is not possible to determine the whole range of ecdysteroids and their precursors in a single pass. To measure a range of polarities would be advantageous if, for instance, the stages of a biosynthetic pathway to ecdysone in an organ culture were being monitored.

Attempts to overcome some of the problems associated with sensitivity and specificity by preparing fluorescent derivatives of the ecdysteroids with Dns-hydrazine have proved unsuccessful. The compound 9-phenanthrenylboronic acid[12] may prove to be suitable for forming fluorescent derivatives of the ecdysones for HPLC, but its use is limited to those compounds possessing a vicinal diol grouping.

We examined a number of bonded reversed-phase packing materials for their ability to resolve the ecdysteroids; others have also been examined in a recent study[4], in which it was concluded that a C_8 bonded phase gave the best results. We were particularly interested in comparing C_{18} phases with the relatively recently introduced C_{22} materials. These have been reported to have increased resolving power compared with C_{18} materials[13]. This would indeed seem to be the case for one of the C_{22} phases examined by us (Magnusil C_{22} supergrade H), but not for the other (Magnusil $C_{22}X$), which gave results inferior to those obtained with the C_{18} column. These differences in the resolving power of the C_{22} materials may be attributable to the fact that the "supergrade" material has had all free silanol groups (remaining after the bonding of the C_{22} chain to the silica) capped with trimethylsilyl groups, whereas the other C_{22} material has not been treated in this way.

Recent, and continuing, developments in bonded reversed-phase packing materials have dramatically improved the possibilities of HPLC analysis of the ecdysteroids. Further developments, including the use of new types of HPLC detector, and the combination of HPLC for preliminary purification followed by GC–ECD, or some other method of determination (*e.g.*, radioimmunoassay), are still under investigation.

ACKNOWLEDGEMENTS

We wish to thank the Overseas Development Administration for support for I.D.W. for part of this work, and S.R.C. for an award to C.R.B. We also thank Dr. P. Ellis, Centre for Overseas Pest Research, London, Great Britain, for providing locust haemolymph.

REFERENCES

1 E. D. Morgan and C. F. Poole, *Advan. Insect Physiol.*, 12 (1976) 17.
2 S. Ogawa, A. Yoshida and R. Kato, *Chem. Pharm. Bull.*, 25 (1977) 904.
3 G. M. Holman and R. W. Meola, *Insect Biochem.*, 8 (1978) 275.

4 R. Lafont, G. Martin-Sommé and J.-C. Chambet, *J. Chromatogr.*, 170 (1979) 185.
5 C. F. Poole, *Ph.D. Thesis*, University of Keele, Keele, 1976.
6 C. R. Bielby, A. R. Gande, E. D. Morgan and I. D. Wilson, *J. Chromatogr.*, 194 (1980) 43.
7 I. D. Wilson and E. D. Morgan, *J. Insect Physiol.*, 24 (1978) 751.
8 A. R. Gande and E. D. Morgan, *J. Insect Physiol.*, 25 (1979) 289.
9 P. M. Bebbington and E. D. Morgan, *Comp. Biochem. Physiol.*, 56B (1977) 77.
10 A. R. Gande, E. D. Morgan and I. D. Wilson, *J. Insect Physiol.*, 25 (1979) 669.
11 L. Dinan and H. H. Rees, *Steroids*, 32 (1978) 629.
12 C. F. Poole, S. Singhawangcha, A. Zlatkis and E. D. Morgan, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 1 (1978) 96.
13 C. J. Little, A. D. Dale and M. B. Evans, *J. Chromatogr.*, 153 (1978) 543.