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SELECTIVE EFFECTS OF MOBILE AND STATIONARY PHASES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ECDYSTEROIDS

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SUMMARY

Observation of a number of different solvent systems and reversed-phase packing materials used for the chromatography of ecdysteroids has shown that order of elution and degree of separation between compounds of the series can be varied with useful limits by choice of the appropriate stationary and mobile phase. The effect on retention properties of substituent hydroxyl groups varies considerably with their position in the molecule.

INTRODUCTION

The ecdysteroids are a group of steroidal compounds interesting in themselves, as the moulting hormones of insects and crustaceans. Attention has been focused on their separation by chromatography recently, by the discovery of several minor ecdysteroids among the metabolites of ecdysone in the ovaries and embryos of a number of insects[1,2]. They are interesting for chromatographic studies in another sense, because they all have the same rigid 5β-cholestane skeleton, with a 7-ene-6-one chromophore and a variety of substituent hydroxyl groups, with occasionally an alkyl or lactone substituent. Over fifty such compounds are known[3]. A number of compounds are therefore available to study the effect on chromatographic properties of small alterations in structure.

In the course of our studies with these compounds we have (in common with others, *e.g.* Lafont's group in France) observed that order of elution and degree of separation of some members of the class varied unexpectedly with both packing material (stationary phase) and organic modifier in the solvent (mobile phase), in reversed-phase high-performance liquid chromatography (RP-HPLC). We have examined these effects more closely, because they may be used to advantage to achieve difficult separations and here describe the chief points of interest. We have also observed the effect of extra substituent hydroxyl groups on retention properties within the series. The twelve ecdysteroids used in this study are illustrated in Fig. 1.

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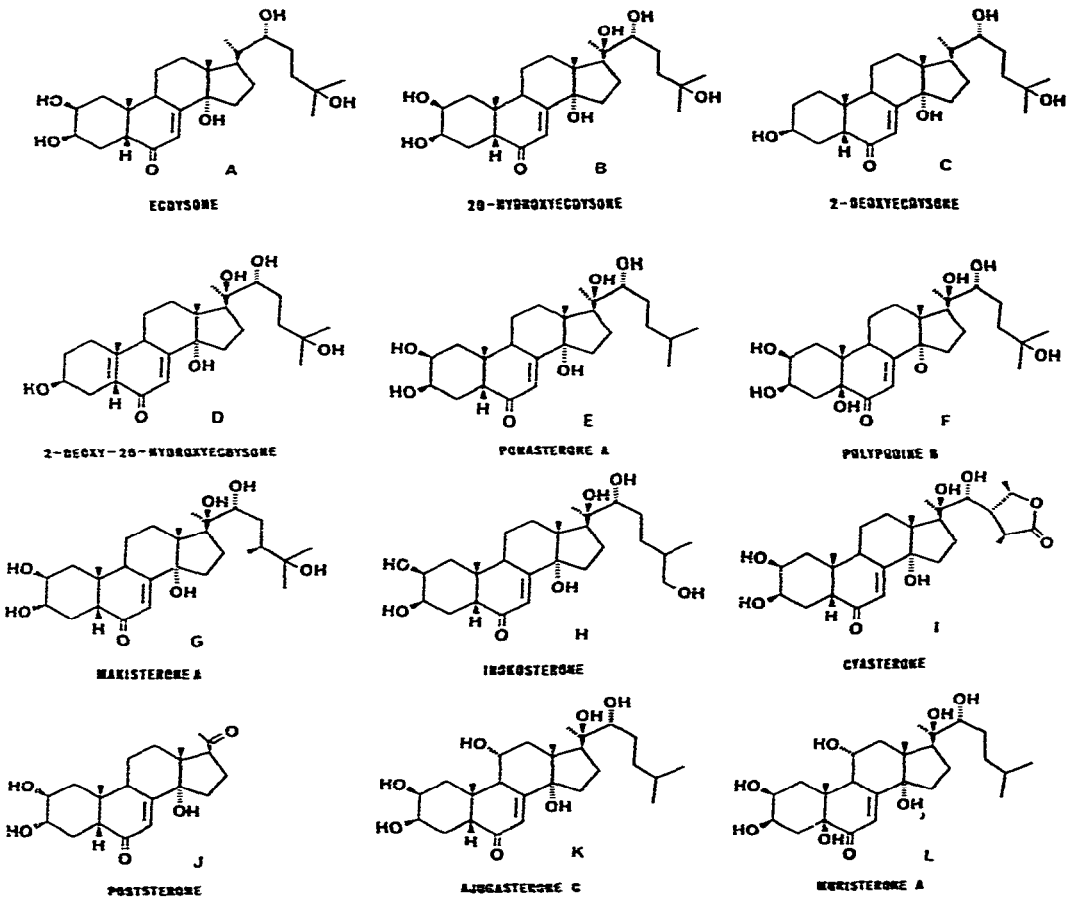

Fig. 1. Structures of the ecdysteroids used in this study.

EXPERIMENTAL

Chromatography was performed on 150 × 3 mm I.D. stainless-steel columns slurry packed with either 5 μm ODS Spherisorb (Phase Separations, Great Britain), or 5 μm ODS Nucleosil (HPLC Technology, Great Britain). Mobile phase was pumped using an LDC Constametric III pump (LDC, Stone, Great Britain), at 1 ml min^{-1}. Eluent was monitored at 254 nm using an LC3-UV detector (Pye Unicam, Cambridge, Great Britain). Samples were introduced onto the column via a Rheodyne Model 7120 loop injector (Magnus Scientific, Great Britain) using a syringe. The mobile phase was degassed before use and chromatography performed at ambient temperature.

Ecdysteroids were used as solutions in methanol, and were gifts from various sources.

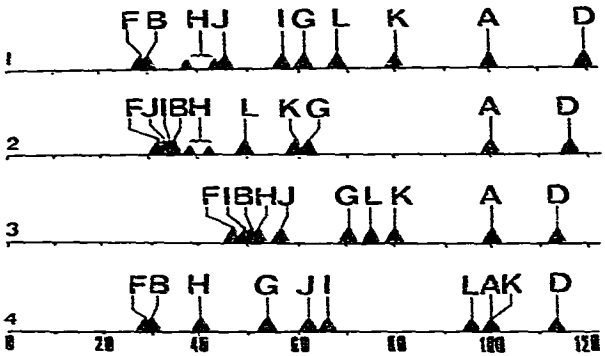

Fig. 2. Diagramatic representation of differences in selectivity observed between ODS Nucleosil and ODS Spherisorb, using methanol–water and acetonitrile–water solvent systems. 1 = ODS Spherisorb acetonitrile water (15:85); 2 = ODS Spherisorb methanol–water (35:65); 3 = ODS Nucleosil methanol–water (50:50); 4 = ODS Nucleosil acetonitrile–water (20:80). Retention is measured relative to ecdysone (A) which is given a value of 100 units. Compounds A–L as in Fig. 1.

RESULTS AND DISCUSSION

Selective effects of the stationary phase

These observations have been made using water–methanol as the mobile phase for simplicity of comparison. However, as seen in the text section, changing the organic component can alter the elution order on a given stationary phase.

Among the group of twelve familiar ecdysteroids (see Fig. 1), for six of them, 2-deoxy-20-hydroxyecdysone, 20-hydroxyecdysone, inokosterone, makisterone A, polypodine B, and ponasterone A (all containing the C-20, C-22 diol), elution order was unchanged in chromatography on ODS Nucleosil or ODS Spherisorb.

Small differences in the degree of separation of particular pairs are observed (Fig. 2). For example, 20-hydroxyecdysone and inokosterone are better resolved on ODS Spherisorb than on ODS Nucleosil, while for 20-hydroxyecdysone and polypodine B the reverse is true. Inokosterone consists of a mixture of 25-*R* and 25-*S* isomers which are resolved on ODS Spherisorb but not by ODS Nucleosil.

Much greater differences between ODS Nucleosil and ODS Spherisorb were noted when the two 11α-hydroxyecdysteroids were chromatographed. Ajugasterone C and muristerone A eluted before makisterone A on ODS Spherisorb, but after it on ODS Nucleosil. Although this pair is better retained on ODS Nucleosil, the separation is only half as efficient. The separation of posterone and cyasterone is poor on ODS Spherisorb, and close to 20-hydroxyecdysone. By contrast, on ODS Nucleosil, separation is much better, cyasterone eluting before 20-hydroxyecdysone. ODS Spherisorb and ODS Nucleosil require different quantities of methanol in the mobile phase for similar retention. The ODS Nucleosil requires more methanol, because it has a higher carbon loading (16% as compared with 8% for Spherisorb). Unfortunately pore volume and specific surface area as well as carbon loading, differ between Spherisorb and Nucleosil, preventing any conclusion being drawn on what factors govern differences of retention.

It is nevertheless possible, by the judicious selection of ODS Spherisorb or ODS Nucleosil, to obtain most required separations of pairs of ecdysteroids. Further examples of the use of different stationary phases for ecdysteroid separations are given by Lafont *et al.*[4] and Dinan *et al.*[5].

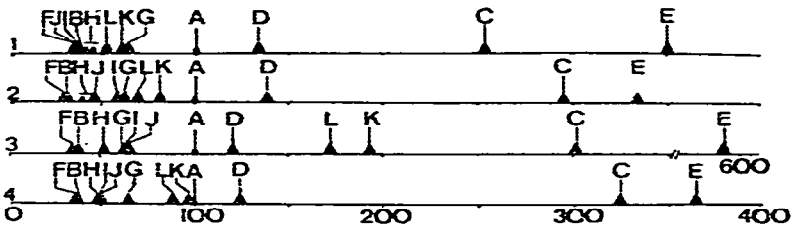

Fig. 3. Diagramatic representation of changes in selectivity seen with four different organic modifiers with ODS Spherisorb. 1 = Methanol–water (35:65); 2 = acetonitrile–water (15:85); 3 = tetrahydrofuran–water (10:90); 4 = dioxan–water (20:80). Retention is measured relative to ecdysone (A) which is given a value of 100 units.

Selective effects of mobile phase composition

It is well known from other studies that changes in the mobile phase can produce unexpected differences in relative retention in RP-HPLC. For example, changing the organic modifier in the chromatography of a large number of organic compounds, even on a silica surface highly covered with *n*-octyl groups, greatly influenced separation[6]. In another study, small amounts of organic ethers, added as a third component to the mobile phase significantly altered the resolution of some steroidal hormones[7].

Replacing methanol by acetonitrile causes changes in retention on both ODS Nucleosil and ODS Spherisorb, so that on ODS Spherisorb cyasterone and poststerone are better resolved and eluted after 20-hydroxyecdysone. Ajugasterone C and muristerone A which elute before makisterone A with methanol, now elute after it, but the resolution is unchanged (Fig. 3). On ODS Nucleosil, the positions of poststerone and cyasterone relative to makisterone A are reversed on changing between acetonitrile–water and methanol–water. Other improved separations on changing to acetonitrile can be seen in Fig. 3, including a better separation of inokosterone and 20-hydroxyecdysone.

Using tetrahydrofuran as organic modifier, the most notable change is in the retention of the two 11-hydroxysterols, ajugasterone C and muristerone A, which are retained considerably longer, relative to ecdysone, and though having more hydroxyl groups than ecdysone, elute after it. The relative retention of ponasterone A is also increased to six times that of ecdysone. This emphasized the importance of the C-25 hydroxyl group in ecdysteroids in conferring polar character and affecting chromatographic behaviour. Cyasterone and poststerone, though at the extremes of variation of chemical structure, have similar chromatographic properties, but perversely, the separation of this pair is not much affected by change of solvent.

An example of the marked difference of behaviour of ecdysteroids between tetrahydrofuran–water and acetonitrile–water is illustrated in Fig. 4.

Dioxan also has its effect on separation or relative retention compared with methanol, expecially for the 11-hydroxyecdysteroids which are strongly affected by each change of organic modifier.

Effect of substituent groups

The retention volumes of ecdysteroids relative to ecdysone on 5 μm ODS Spherisorb using methanol–water shows clearly that the number of hydroxyl groups per molecule is less important than their position. The three pentahydroxy-ecdysteroids, ecdysone, 2-deoxy-20-hydroxyecdysone and ponasterone A, display large

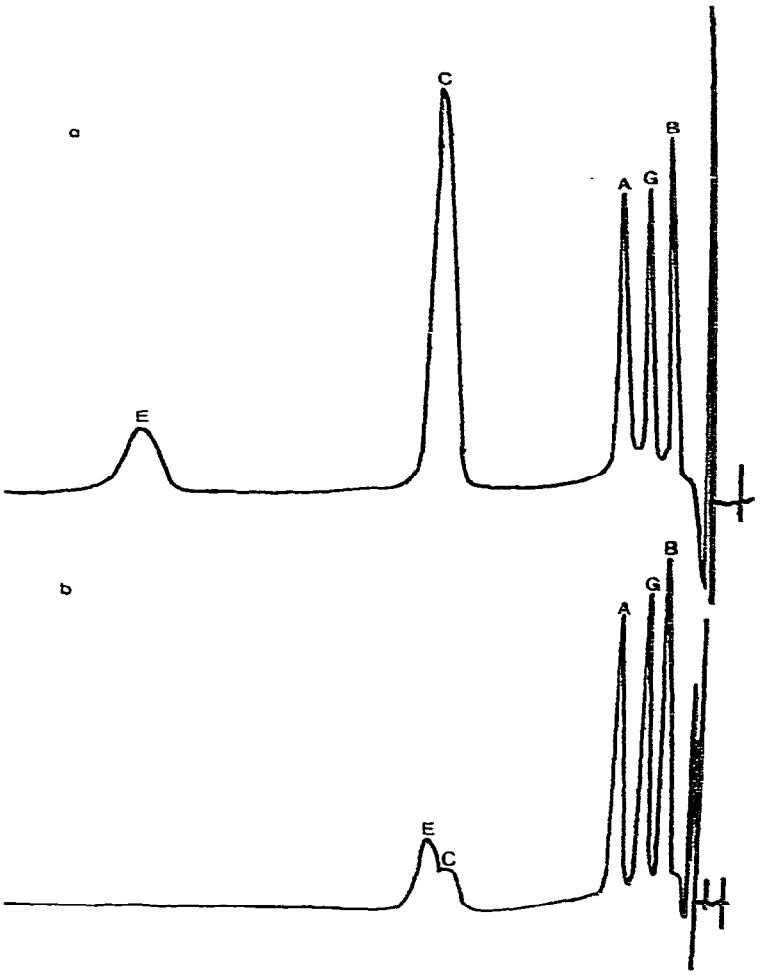

Fig. 4. Chromatogram showing changes in resolution between ponasterone A (E) and 2-deoxyecdysone (C) with acetonitrile (b) and tetrahydrofuran (a) based solvent systems.

differences in retention. The effect of an hydroxy at C-25 is much greater than at C-2 and this in turn is greater than that at C-20. The combined effect of hydroxyls at C-2 and C-20 in methanol–water is about the same as one at C-25. In the same way, addition of an α-OH at C-11 to ponasterone A to give ajugasterone C produces a large decrease in retention. Comparing ajugasterone C and 20-hydroxyecdysone (each with six OH groups), the hydroxyl at C-11α has a much smaller effect on retention than that at C-25. An hydroxyl at C-5β (polypodine B) has a negligible effect on retention, indeed the separation of polypodine B and 20-hydroxyecdysone is one of the most difficult to achieve in the group. The polarity of the 5β-OH may be reduced through hydrogen bonding to the adjacent carbonyl group. The effect of the C-5β-OH is much greater when comparing ajugasterone C and muristerone A.

Evidently, the effect of hydroxyl groups on ecdysteroid retention is not simply one of polarity, but steric effects are important. On ODS Spherisorb, in methanol–water the eluotropic effect of some of the hydroxyls decreases in the order: 25 > 26 > 2β > 20 > 11α > 5β. But it must be added that the effect of an added hydroxyl

groups depends also upon those already present. As Bush[8] has pointed out, it is not enough for a polar group in an organic compound to be able to associate with solvent molecules, there must be room around the group to fit in several molecules of solvent if it is to interact strongly with the lattice of the solvent.

The effect of an extra methyl group attached to the side chain is surprisingly large. Makisterone A has a retention volume about 50% greater than 20-hydroxyecdysone. Shortening the side chain to only two carbon atoms (poststerone) seems to produce a balance of polar and non-polar effects so that its retention is not very different from 20-hydroxyecdysone. Conversion of the end of the chain to a lactone (as in cyasterone) also produces little change.

CONCLUSIONS

The selective effects of different stationary phases may lie in the efficiency of covering of the silica surface, the proportion of residual silanol groups and in the pore size. We cannot, in the present state of knowledge, study these factors separately. Moreover, the selectivity of a column may change slightly with age and batch from the manufacturer. Stationary phases have to be chosen by trial and error. The collection of data here is helpful in making a choice for the ecdysteroids. As yet, not many studies have been made on effects of mobile phase and since it is evident that other effects than partition influence retention on reversed phases, no predictive powers are available in this respect either. The data displayed here can be used to aid the choice of suitable stationary and mobile phases for the RP-HPLC of ecdysteroids. In general, acetonitrile–water seems to be the best mobile phase, since it gives the best spread of retention values. Lafont *et al.*[4] recommended acetonitrile–water systems as in their hands these mixtures gave least peak tailing.

Two practical examples of the use of this data are illustrative. We have been able to choose a system that altered the retention of muristerone A to remove impurities, and also to separate mixtures of 2-deoxyecdysone and ponasterone A, which are very difficult to separate by thin layer chromatography, or by HPLC with some common mobile phases.

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