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Note

Normal-phase high-performance liquid chromatographic analysis of polyhydroxysteroids using non-polar bonded silica columns

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Ecdysteroids represent a class of polyhydroxylated steroids widely represented among invertebrates and plants[1–3]. Their high-performance liquid chromatographic (HPLC) analysis can be performed using various techniques[4,5]. However, the use of normal-phase systems has so far been restricted to medium-polarity ecdysteroids, and polar compounds are analysed by either reversed-phase or ion-exchange HPLC. It would nevertheless be of interest to use normal-phase systems in the latter instance, either as a means of ascertaining compound identity by co-migration with reference compounds or for the final step in the purification of polar ecdysteroids prior to spectrometric analyses.

Up to now, normal-phase HPLC separations of ecdysteroids have been performed by using either silica or polar-bonded silica columns, *e.g.*, aminopropylsilane (APS)[4,6] or diol-bonded[4,7] silicas. Polar bonded phases can provide interesting results, *e.g.*, for the separation of $3\alpha/3\beta$ pairs[6] or the separation of compounds over a wide range of polarity[7].

This paper results from an experimental mistake in which an ODS-bonded column was used instead of a silica column and unexpectedly gave a fairly efficient separation. This induced us to undertake a more extensive analysis of the possible use of polar bonded columns for normal-phase HPLC. The results appear promising and are reported here.

EXPERIMENTAL

Chemicals

Reference ecdysteroids (Fig. 1) were obtained from various sources. Ecdysone and makisterone A were purchased from Simes (Milan, Italy). Ponasterone A, 2-deoxyecdysone and 2-deoxy-20-hydroxyecdysone were gifts from Dr. D. H. S. Horn (Acheron, Australia). 20-Hydroxyecdysone, integristerone A, 20,26-dihydroxyecdysone and 20-hydroxyecdysone glucosides were purified from various plant sources[8]. 25-Deoxyecdysone was synthesized from ecdysone according to Heinrich[9].

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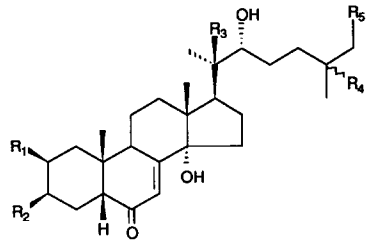

COMPOUND	R_1	R_2	R_3	R_4	R_5
25-Deoxyecdysone	OH	OH	H	H	H
Ponasterone	OH	OH	OH	H	H
2-Deoxyecdysone	H	OH	H	OH	H
2-Deoxy-20-hydroxyecdysone	H	OH	OH	OH	H
Ecdysone	OH	OH	H	OH	H
20-Hydroxyecdysone	OH	OH	OH	OH	H
20,26-Dihydroxyecdysone	OH	OH	OH	OH	OH
20-Hydroxyecdysone 25-glucoside	OH	OH	OH	OGlu	H
20-Hydroxyecdysone 3-glucoside	OH	OGlu	OH	OH	H

Fig. 1. Structures of the ecdysteroids.

Solvents (HPLC grade) were obtained from Prolabo (dichloromethane) or Carlo Erba (methanol, isopropanol). High-purity water was obtained with a Milli-Q system (Millipore).

HPLC equipment

All experiments were performed with a DuPont Model 8800 instrument equipped with a two-solvent gradient system, a fixed-wavelength UV detector (Model 850) and a Rheodyne 7125 injector. Several types of analytical columns were used: Zorbax-Sil (250 × 4.6 mm I.D.), Zorbax-TMS (150 × 4.6 mm I.D.), Zorbax-ODS (250 × 4.6 mm I.D.), Spherisorb 5-ODS-2 (250 × 4.6 mm I.D.), LiChrosorb Diol (250 x 4.0 mm I.D.) ad Nucleosil-NH_2 (150 × 4.6 mm I.D.). All the columns were run isocratically with dichloromethane–isopropanol–water mixtures[10]. Some experiments were carried out using the gradient mode, with methanol as secondary solvent.

RESULTS AND DISCUSSION

A typical separation of ecdysone and 20-hydroxyecdysone obtained on three different columns from the same manufacturer is shown in Fig. 2. The retention times of ecdysone and 20-hydroxyecdysone on the different columns are given in Table I (values are the means of three separate assays and the fluctuations did not exceed ±3%, provided that the ambient temperature was kept constant). It is obvious from these data that TMS- and amino-bonded columns give results close to those with non-bonded silica, and that ODS-bonded columns give much reduced although significant retention times. The latter result was surprising, as it was expected that ecdysteroids would elute with the solvent front, as they do when using pure methanol. The result seems understandable, however, when it is considered that a significant percentage of silanol groups remain free in such columns, which would be responsible for the chromatographic process. This idea is supported by the fact that when using two ODS columns from the same manufacturer, the new column gave lower retention times than the older column (data not shown). The TMS-bonded column appeared of

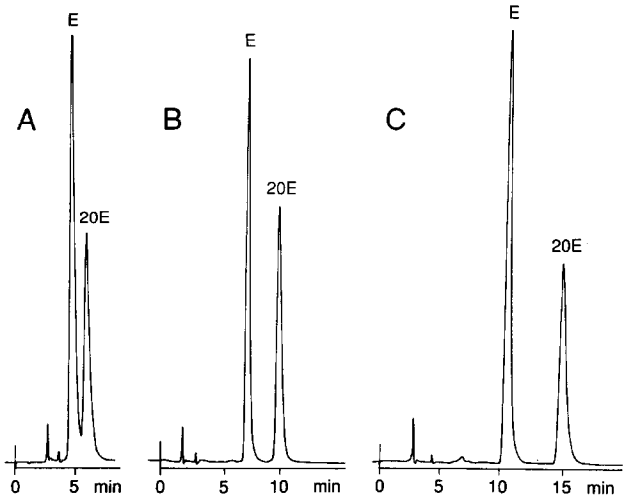

Fig. 2. Separation of an ecdysone (E)–20-hydroxyecdysone (20E) mixture using three different HPLC columns. Solvent system: dichloromethane–isopropanol–water (125:40:3); flow-rate, 1 ml min^{-1}. A, Zorbax-ODS (25 cm); B, Zorbax-TMS (15 cm); C, Zorbax-SIL (25 cm).

interest because the eluted peaks were perfectly symmetrical, whereas in the other instances there was always some tailing. For that reason, we carried out a more extensive study of this particular type of column.

TMS packings have good efficiency and are particularly interesting for polar compounds, which elute within reasonable times. Such columns can be used not only in the isocratic mode, but also in the gradient mode, in order to optimize separations of compounds over a wide range of polarity. This was checked by using a slow linear gradient of methanol (0–20% in 45 min) in the primary solvent (dichloromethane–

TABLE I

COMPARISON OF THE RETENTION TIMES OF ECDYSONE (E) AND 20-HYDROXYECDYSONE (20E) USING DIFFERENT COLUMNS AND TWO DIFFERENT SOLVENT SYSTEMS

Column	*Solvent system 1*[a]			*Solvent system 2*[a]		
	$t_R(E)$[b]	$t_R(20E)$[b]	N/m[c]	$t_R(E)$[b]	$t_R(20E)$[b]	N/m[c]
Zorbax-SIL	44.8	78.9	34 400	13.0	18.8	15 600
Zorbax-TMS	46.0	76.3	25 200	14.3	20.3	17 200
Zorbax-ODS	13.7	22.3	8 000	5.6	6.6	–
Spherisorb ODS-2	4.6	6	–	2.8	3.1	–
Lichrosorb DIOL	17.7	27.1	–	6.4	7.9	–
Nucleosil-NH_2	32.5	62.3	–	13.2	17.7	–

[a] Solvents: dichloromethane–isopropanol–water: system 1 125:20:1.5 (v/v/v); system 2, 100:30:2 (v/v/v).

[b] t_R = Retention time in minutes; values standardized for a 25 cm × 4.6 mm I.D. column; flow-rate, 1 ml min^{-1}

[c] N/m = Number of plates per metre column length.

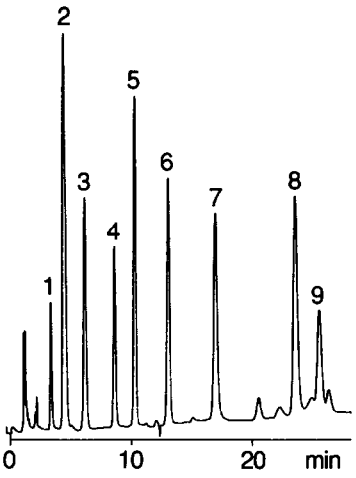

Fig. 3. Separation of an ecdysteroid mixture on a Zorbax-TMS column (15 cm × 0.46 cm I.D.). Operating conditions: flow-rate, 2 ml min^{-1}; primary solvent, dichloromethane–isopropanol–water (125:20:1.5, v/v/v); secondary solvent, methanol, gradient from 0 to 20% methanol in 45 min. Peaks: 1 = 25-deoxyecdysone; 2 = ponasterone A; 3 = 2-deoxyecdysone; 4 = 2-deoxy-20-hydroxyecdysone; 5 = ecdysone; 6 = 20-hydroxyecdysone; 7 = 20,26-dihydroxyecdysone; 8 = 20-hydroxyecdysone 25-glucoside; 9 = 20-hydroxyecdysone 3-glucoside.

isopropanol–water, 125:20:1.5), and this allowed us to separate within 20 min a mixture of compounds bearing between four and nine hydroxyl groups (Fig. 3). Column re-equilibration after such a gradient required no more than 10 min (at 2 ml/min) in order to obtain reproducible analyses. This type of solvent system therefore appeared well suited for metabolic studies.

In conclusion, the use of TMS columns for the normal-phase analysis of polar steroids seems very promising, and such systems might be of more general use. A parallel of the present data can be made with the columns used for supercritical fluid chromatography (SFC), which was recently applied to ecdysteroids[11,12] with either silica[11] or non-polar bonded phases[12]. Supercritical carbon dioxide acts as a non-polar mobile phase that is modified by adding a small percentage of methanol[12]. In SFC, non-polar bonded columns provide reduced retention times in comparison with silica and this seems to have a similar explanation to that proposed here. It is therefore suggested that TMS columns could perhaps also be successfully used for the SFC analysis of polar compounds.

REFERENCES

1 C. Hétru and D. H. S. Horn, in J. A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier, Amsterdam, 1980, p. 13.
2 R. Lafont and D. H. S. Horn, in J. A. Koolman (Editor), *Ecdysone*, Georg Thieme, Stuttgart, 1989, p. 39.
3 H. H. Rees, in J. A. Koolman (Editor), *Ecdysone*, Georg Thieme, Stuttgart, 1989, p. 28.
4 R. Lafont, P. Beydon, B. Mauchamp, G. Sommé-Martin, M. Andrianjafintrimo and P. Krien, in F. Sehnal, A. Zabza and B. Cymborowski (Editors), *Regulation of Insect Development and Behaviour*, Technical University of Wrocław Press, Wrocław, 1981, p. 199.

5 R. Lafont, in H. Kalasz and E. S. Ettre (Editors), *Chromatography '87*, Akadémiai Kiádo, Budapest, 1988, p. 1.
6 L. N. Dinan, P. L. Donnahey, H. H. Rees and T. W. Goodwin, *J. Chromatogr.*, 205 (1981) 139.
7 B. Fournier and D. Radallah *Arch. Insect Biochem. Physiol.*, 7 (1988) 211.
8 J.-P. Girault, M. Bathori, E. Varga, K. Szendrei and R. Lafont, *J. Nat. Prod.*, (1990) in press.
9 G. Heinrich, *Dissertation*, Cologne, 1970.
10 R. Lafont, G.Sommé-Martin and J.C. Chambet, *J. Chromatogr.*, 170 (1979) 185.
11 E. D. Morgan, S. J. Murphy, D. Games and I. C. Mylchreest, *J. Chromatogr.*, 441 (1988) 165.
12 M. W. Raynor, J. P. Kithinji, I. K. Barker, K. D. Bartle and I. D. Wilson, *J. Chromatogr.*, 436 (1988) 497.