Journal of Chromatography, 236 (1982) 224–229
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,379

Note

Evaluation of some phytoecdysteroids as internal standards for the chromatographic analysis of ecdysone and 20-hydroxyecdysone from arthropods

I. D. WILSON*,*, C. R. BIELBY and E. D. MORGAN
Department of Chemistry, University of Keele, Keele, Staffordshire ST5 5BG (Great Britain)
(Received September 14th, 1981)

In the analysis of trace constituents such as ecdysteroids some losses during sample preparation are likely. In different tissues, or where levels are fluctuating considerably such loss may vary from sample to sample. Use of an internal standard can remove uncertainty resulting from these factors. In addition the percentage recovery of the internal standard may be used to determine the efficiency of the sample preparation.

We have examined the chromatographic properties of a number of common phytoecdysteroids to assess their suitability for use as internal standards in the determination of insect and crustacean ecdysteroid titres.

EXPERIMENTAL

HPLC

High-performance liquid chromatography (HPLC) was performed on a 15 cm × 3 mm I.D. stainless-steel column packed with either 5 μm ODS Spherisorb or 5 μm ODS Nucleosil (HPLC Technology, Wilmslow, Great Britain). Solvents were mixtures of water and methanol or water and acetonitrile, and were degassed before use. Solvents were delivered using an LDC Constametric III pump (Laboratory Data Control, Stone, Great Britain), at 1 ml min^{-1}. Ecdysteroids were detected in the column effluent at 254 nm using a Pye Unicam LC3 UV detector (Pye Unicam, Cambridge, Great Britain). Ecdysteroids were made up in methanol, and injected onto the column via a Rheodyne 7125 loop injector (Magnus Scientific, Stone, Great Britain).

GLC

Samples of ecdysteroids were prepared for gas–liquid chromatography (GLC) by silylation with trimethylsilylimidazole in pyridine (35 and 65 μl, respectively), at 120°C for 5 h contained in a 1-ml Reacti-vial (Pierce and Warriner, Chester, Great Britain). The sample was diluted with ECD-grade toluene prior to injection[1]. Samples

---

* Present address: Analytical Department, Hoechst Pharmaceutical Research Labs., Walton Manor, Milton Keynes, Bucks., Great Britain.

0021-9673/82/0000–0000/$02.75 © 1982 Elsevier Scientific Publishing Company

(1 μl) were then injected onto a 0.9 m × 4 mm I.D. column of OV 101 (1.5%), on Chromosorb W. Chromatography was performed on a Pye series 104 gas chromatograph at 285°C with nitrogen as carrier gas at a flow-rate of 50–60 ml min^{-1}. Derivatives were detected using an ^{63}Ni electron-capture detector (ECD) at 300°C.

Ecdysteroids were gifts.

RESULTS AND DISCUSSION

Miyazaki *et al.*[2] have used cyasterone as a GLC internal standard, as have Lafont and co-workers[3,4]. More recently, Lafont *et al.*[4] have also used makisterone A. At about the same time we investigated a number of alternative compounds in our laboratory to see what advantages, if any, these had over makisterone A and cyasterone. Both HPLC and GLC are used by us for ecdysteroid analysis, depending on levels of ecdysteroid in the sample. Each candidate internal standard has been examined using both techniques. Ideally the internal standard should be compatible with both methods, as it is often useful to analyse the same sample by both techniques. In this case it is important that the internal standard should not interfere with any of the compounds of interest.

Five compounds were examined: cyasterone, inokosterone, makisterone A, polypodine B and ponasterone A. Inokosterone and makisterone A, and more recently ponasterone A, have been detected in some arthropod extracts[5–7], which may complicate their use as internal standards.

The chromatographic and other relevant properties of each compound are discussed below.

Cyasterone

The poor GLC characteristics of this compound (long retention times, multiple product formation on silylation, and poor peak shape) preclude its use as an internal standard for the GLC analysis of ecdysone and 20-hydroxyecdysone (see also ref. 4). Cyasterone may be used for HPLC analysis if care is taken in the choice of organic modifier used in the mobile phase. We have found that water–methanol based solvents give poor resolution of cyasterone and 20-hydroxyecdysone[8]. However, adequate resolution may be obtained with acetonitrile–water systems (Fig. 1 and Table I).

Inokosterone

Differing from 20-hydroxyecdysone only in possessing a C-26 rather than a C-25 hydroxyl, inokosterone has very similar chromatographic properties. On GLC the presumed penta-trimethylsilyl (TMS) ether of inokosterone has a longer retention time (9.7 min) than those of ecdysone and 20-hydroxyecdysone (6.15 and 8.5 min, respectively). Separation from 20-hydroxyecdysone is not large in this system, but could be improved at the expense of an increase in analysis time. The ECD response of this compound is similar to that of ecdysone. On HPLC (as was the case with cyasterone) acetonitrile–water mobile phases, but not methanol–water systems, resolve inokosterone from 20-hydroxyecdysone. Inokosterone exists as a mixture of C-25 *R* and *S* isomers, and under some circumstances (*e.g.* chromatography on ODS Spherisorb) these are resolved, resulting in two peaks for the internal standard.

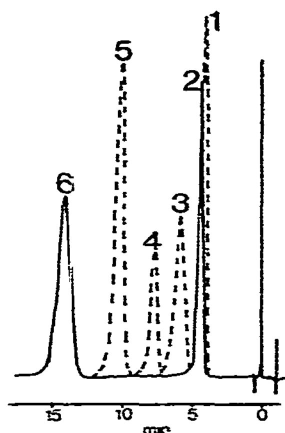

Fig. 1. A composite chromatogram showing the positions of polypodine B (1), 20-hydroxyecdysone (2), inokosterone (3), makisterone A (4), cyasterone (5), and ecdysone (6) on reversed-phase chromatography on ODS Nucleosil with acetonitrile–water (20:80) as mobile phase.

Makisterone A

On GLC this 24-methyl homologue of 20-hydroxyecdysone is well resolved from 20-hydroxyecdysone and ecdysone (Figs. 2 and 3). In addition its response to the ECD is similar to that of ecdysone. On reversed-phase HPLC, makisterone A elutes between 20-hydroxyecdysone and ecdysone, and there appears to be no limitation on the organic modifier used in the mobile phase.

TABLE I

HPLC RETENTION DATA FOR ECDYSTEROIDS WITH ACETONITRILE-BASED MOBILE PHASES

Under these conditions ponasterone A is not eluted from the column. On ODS spherisorb with 20% acetonitrile, ecdysone has retention time (t_R) 5.2 min, 20-hydroxyecdysone has retention time (t_R) 1.6 min, and ponasterone A retention time (t_R) 17.4 min.

Compound	*ODS Spherisorb: acetonitrile–water (15:85)*		*ODS Nucleosil acetonitrile–water (20:80)*	
	*t_R (min)**	*t_R relative to ecdysone*	*t_R (min)**	*t_R relative to ecdysone*
Ecdysone	22.4	1	14.2	1
20-Hydroxyecdysone	6.8	0.3	4.4	0.31
Makisterone A	14	0.63	7.8	0.55
Inokosterone	8.6, 10**	0.38, 0.45	5.8	0.41
Polypodine B	6.4	0.29	4.2	0.30
Cyasterone	13	0.58	9.5	0.67

* Measured from the solvent front.
** C-25 *R* and *S* isomers.

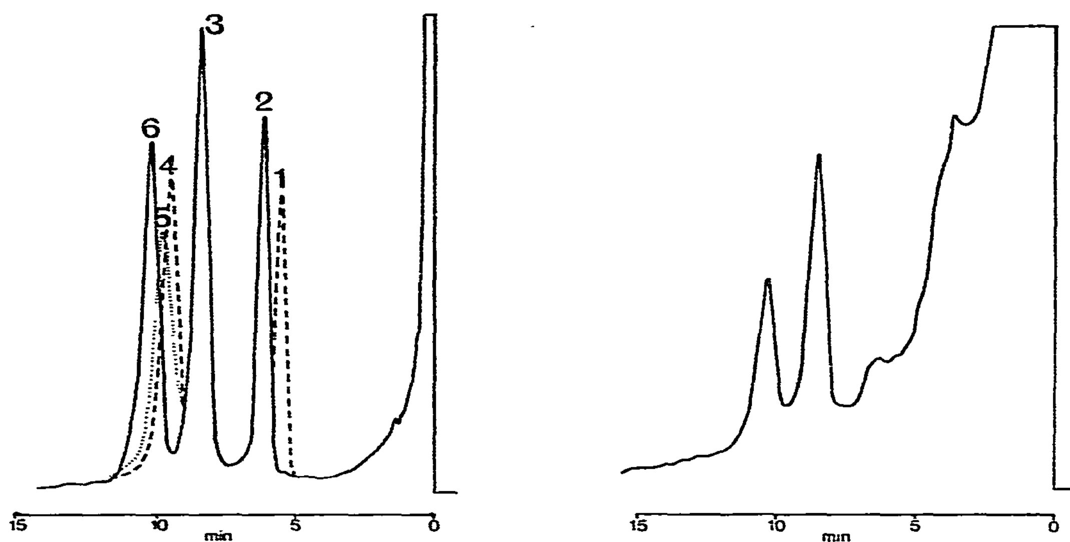

Fig. 2. A composite chromatogram showing the positions of ponasterone A (1), ecdysone (2), 20-hydroxyecdysone (3), polypodine B (4), inokosterone (5), and makisterone A (6), on GLC on 1.5% OV 101 at 285°C.

Fig. 3. A 0.7 μl^{-1} sample from the barnacle *Balanus balanoides* with makisterone A as internal standard. 20-Hydroxyecdysone is present (1.8 ng μl^{-1}). Chromatographic conditions as described in the Experimental section. The area of the makisterone A peak indicates an overall recovery of 86%.

Polypodine B

Polypodine B (5β-20-dihydroxyecdysone) has a similar GLC retention time to inokosterone, eluting after the penta-TMS ether of 20-hydroxyecdysone. However, the ECD response of this compound is only about a fifth of that of ecdysone (Table II), presumably due to the presence of the 5β hydroxyl. On reversed phase HPLC it is very difficult to resolve polypodine B from 20-hydroxyecdysone, even with extended analysis times. This similarity in HPLC properties might be useful in the analysis of

TABLE II

GLC RETENTION DATA FOR TMS ETHERS OF ECDYSTEROIDS

Compound	t_R *(min)*	t_R *relative to*		*ECD response relative to ecdysone*
		Ecdysone	*20-Hydroxyecdysone*	
Ecdysone	6.15	1	0.72	1
20-Hydroxyecdysone	8.5	1.38	1	1
Makisterone A	10.1	1.64	1.2	1
Inokosterone	9.7	1.57	1.14	1
Polypodine B	9.5	1.55	1.12	0.22
Cyasterone*	17.6	2.86	2.07	—
Ponasterone A	5.5	0.89	0.65	1

* Cyasterone also gave peaks at 15.5 and 27 min, amounting to *ca.* 20% of the total.

20-hydroxyecdysone if HPLC was used in sample preparations, and GLC for analysis, as only a single peak would need to be collected.

Ponasterone A

The absence of a C-25 hydroxyl in ponasterone A (25-deoxy-20-hydroxyecdysone), greatly reduces the polarity of the compound and increases the volatility of its silyl ether. This results in a short GLC retention time, when the compound is not well isolated from ecdysone, and a long HPLC analysis time.

CONCLUSIONS

The extensive purification, generally required of samples before chromatographic analysis for ecdysteroids is possible, demands the use of an internal standard, if precise and accurate quantitations are to be assured. However, the internal standard chosen depends to some extent on the type of analysis (GLC or HPLC), and the ecdysteroid present in the sample. For the general analysis of ecdysone and 20-hydroxyecdysone by both GLC and HPLC, makisterone A is suitable. Inkosterone is also suitable provided that acetonitrile–water systems are used for reversed-phase HPLC. A disadvantage of makisterone A and inokosterone is that both have been detected in arthropods[6,7]. This should not restrict their use if a preliminary screen shows them to be absent from the species under investigation at that stage of development. Polypodine B is suitable for GLC, but not HPLC where 20-hydroxyecdysone is present. Cyasterone is unsuitable for GLC, but usable on HPLC with an appropriate mobile phase. Ponasterone A is unsuited for use in HPLC because of its long retention time. On GLC it might be useful for the analysis of 20-hydroxyecdysone in the absence of ecdysone.

The candidate internal standards described here have usually been isolated from mixtures of other ecdysteroids, including ecdysone and 20-hydroxyecdysone. Some contamination with other ecdysteroids may therefore be encountered (see for instance Lafont *et al.*[4] on inokosterone). Standards should therefore always be checked for purity, and purified if necessary.

Provided that such precautions are taken, a suitable internal standard can only improve the quality of results obtained by chromatographic analysis. The internal standard chosen will depend on the type of analysis, and the ecdysteroids present in the sample.

ACKNOWLEDGEMENTS

Gifts of ecdysteroids from Professor C. Wei-shan, Professor G. Ferrari, Dr. D. H. S. Horn, Dr. J. Jizba and Professor K. Nakanishi are gratefully acknowledged. Financial support from the Science Research Council for C.R.B. is also gratefully acknowledged.

REFERENCES

1 C. R. Bielby, A. R. Gande, E. D. Morgan and I. D. Wilson, *J. Chromatogr.*, 194 (1980) 43.
2 H. Miyazaki, M. Ishibashi, C. Mori and N. Ikekawa, *Anal. Chem.*, 45 (1973) 1164.

3 R. Lafont, J. P. Delbeque, L. de Hys, B. Mauchamp and J. L. Pennetier, *C.R. Acad. Sci., Ser. D*, 279 (1974) 1911.
4 R. Lafont, G. Somme-Martin, B. Mauchamp, B. Maume and J. P. Delbeque, in J. A. Hoffman (Editor) *Progress in Ecdysone Research*, Elsevier/North-Holland, Amsterdam, 1980.
5 A. Faux, D. H. S. Horn, E. J. Middleton, N. M. Fales and M. E. Lowe, *Chem. Commun.*, (1969) 175.
6 J. N. Kaplanis, S. R. Dutkey, W. E. Robbins, M. J. Thompson, E. L. Lindquist, D. H. S. Horn and M. N. Galbraith, *Science*, 190 (1975) 681.
7 F. Lachaise, M. Gaudeau, C. Hetru, C. Kappler and J. A. Hoffman, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 521.
8 I. D. Wilson, C. R. Bielby and E. D. Morgan, *J. Chromatogr.*, in press.