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CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF ECDYSTEROIDS

RICHARD P. EVERSHED*, JULIAN G. MERCER and HUW. H. REES
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX (U.K.)
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SUMMARY

Fully trimethylsilylated ecdysteroids have been analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) using flexible fused-silica capillary columns. Best results were obtained using columns coated with cross-bonded apolar (OV-1 type) stationary phases. By employing capillary GC–MS with selected-ion monitoring (SIM), very sensitive and selective analyses for ecdysteroids are possible. Detection limits of 10 and 100 pg have been determined for 20-hydroxyecdysone and ecdysone, respectively. At this level of sensitivity, capillary GC–SIM-MS constitutes a complementary means of ecdysteroid analysis to high-performance liquid chromatography (HPLC) with monitoring of the eluent fractions by radioimmunoassay (RIA), but with the advantage of enhanced selectivity. Examples are given of the application of capillary GC–SIM-MS and HPLC–RIA in combination to investigate the nature and concentration of ecdysteroids at the picogram level in three helminth species.

INTRODUCTION

Ecdysteroids structurally related to ecdysone (1), have been detected in invertebrate species belonging to several different phylla[1] and in some plant species[2]. In arthropods they serve as hormones controlling moulting, whereas in non-arthropod invertebrates their exact functions are yet to be established[1].

Ecdysteroids are polyhydroxylated steroids which are relatively polar and, hence, involatile. Methods of liquid chromatographic analysis, such as high-performance liquid chromatography (HPLC)[3–5] and thin-layer chromatography (TLC)[6], are well developed and used extensively in the isolation and purification of ecdysteroids. However, the relatively poor sensitivity and selectivity of commonly used detection and visualisation methods (*e.g.* UV–VIS spectrophotometry) generally preclude their use in trace analyses. Fluorometric screening of HPLC fractions has been described[7]. Although detection limits were in the nanogram to microgram range there were problems of fluorescence quenching which may affect precision in quantitative analyses. Radioimmunoassay (RIA) is the most sensitive method of ecdysteroid detection currently available[8]. It can be used either to detect ecdysteroids in relatively impure

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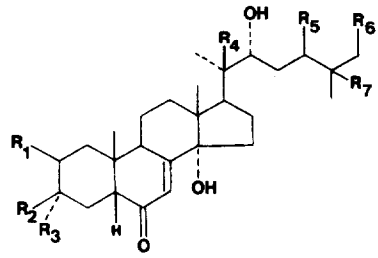

		R_1	R_2	R_3	R_4	R_5	R_6	R_7
1	Ecdysone	OH	OH	H	H	H	H	OH
2	20-Hydroxyecdysone	OH	OH	H	OH	H	H	OH
3	3-Epi-20-hydroxyecdysone	OH	H	OH	OH	H	H	OH
4	Makisterone A	OH	OH	H	OH	CH_3	H	OH
5	20,26-Dihydroxyecdysone	OH	OH	H	OH	H	OH	OH
6	Ponasterone A	OH	OH	H	OH	H	H	H
7	Inokosterone	OH	OH	H	OH	H	OH	H
8	26-Hydroxyecdysone	OH	OH	H	H	H	OH	OH
9	Podecdysone A	OH	OH	H	OH	C_2H_5	H	OH

extracts or highly purified fractions eluting from HPLC columns (HPLC–RIA)[9]. Detection limits of the low picogram level are readily achievable by RIA. The major disadvantage of RIA lies in its relatively poor selectivity and, furthermore, structurally different ecdysteroids cross-react to differing degrees with antisera raised against individual ecdysteroids conjugated to protein.

Another approach to ecdysteroid trace analysis involves converting the ecdysteroids into apolar trimethylsilyl (TMS) ether derivatives and submitting them to gas chromatography (GC)[10]. Procedures for formation of the TMS derivatives, generally using trimethylsilylimidazole (TMSI) as the silyl donor, are extensively documented[3,10,11]. Hitherto, GC analysis of ecdysteroids have tended to be performed on short, packed columns with low stationary phase loadings, there being only one reported example of a capillary GC analysis of such compounds[12]. Owing to the presence of the 6-oxo-7-ene moiety, ecdysteroids can be detected at high sensitivity in an electron-capture detector[8,13]. Absolute detection limits for ecdysteroids by electron-capture detection (ECD) reportedly are in the lower picogram range, comparable to those observed by RIA[8]. However, considerable problems arise through lack of selectivity, leading to interferences from ECD responsive impurities, particularly during the analysis of biological extracts. Procedures for the TLC purification of derivatised ecdysteroids prior to GC–ECD have been described. However, TLC can lead to extensive hydrolytic decomposition of ecdysteroid TMS ethers[10].

An alternative to GC–ECD is to employ GC–mass spectrometry (MS). This has been applied to ecdysteroid analysis[3,12]. When of the order of 10–100 ng of material is available full mass spectra can be acquired[3]. Where smaller quantities of ecdysteroids are available, as is often the case from non-arthropod species, GC–MS with selected-ion monitoring (SIM) has been employed[3,9,14], leading to a large increase in sensitivity. GC–SIM-MS has the notable advantage of being highly selective.

Our current interest in the ecdysteroids of nematodes and cestodes requires

greater sensitivity and resolution than are currently offered by packed column GC or other techniques. Owing to the potential offered by modern capillary GC technologies, we have developed capillary GC–MS as a routine technique for the trace analysis of ecdysteroids. This paper reports the results of these investigations. The applicability of capillary GC–SIM-MS is demonstrated, in combination with HPLC–RIA, to the determination of the nature and concentration of picogram amounts of ecdysteroids in three helminth species.

EXPERIMENTAL

Reagents

N-Trimethylsilylimidazole (TMSI) was obtained from Pierce and Warriner (U.K.) and distilled before use. Solvents were generally of laboratory grade and distilled before use.

Preparation of trimethylsilylethers of ecdysteroids

Biological extracts containing ecdysteroids, or authentic compounds (pg–μg amounts of ecdysteroid as a solution in methanol) were transferred into disposable screw-topped vials [$\frac{1}{2}$ dram (*ca.* 1.70 ml); FBG Trident] and evaporated to dryness under a stream of dry nitrogen (*ca.* 40°C). The samples were then vacuum desiccated (10^{-2} Torr; 30 min) to remove solvent or moisture which interferes with the subsequent derivatisation. TMSI (80 μl) was then added to the vials under a constant stream of dry nitrogen. The vial caps were then secured and the mixture heated (100°C, 15 h).

The silylated ecdysteroids were purified from the excess TMSI prior to GC or GC–MS by adsorption chromatography. Hexane (200 μl) was added to the reaction mixture and the solution transferred to a column of alumina (1.5 g Woelm Brockmann grade 1, neutral) prepared in a Pasteur pipette plugged with glass wool, the column being pre-washed successively with ethyl acetate–hexane (3:7, v/v; 2 ml) and hexane (2 ml). The vial was washed with further aliquots of hexane (2 × 200 μl) and the washings transferred to the same column. The silylated ecdysteroids were eluted in ethyl acetate–hexane (3:7; 4 ml) and collected in a disposable vial [2 dram (*ca.* 7.10 ml), FBG Trident] prewashed with ethyl acetate–hexane. The eluent was then reduced to *ca.* 100 μl under a stream of dry nitrogen (*ca.* 40°C) and transferred to a pointed glass vial ($\frac{1}{2}$ dram). After evaporation to dryness, the silylated ecdysteroids were taken up in a minimum volume of hexane (5–10 μl) and 1 μl injected into the gas chromatograph or gas chromatograph–mass spectrometer.

Gas chromatography–mass spectrometry

GC–MS of ecdysteroids was carried out on a Pye-Unicam 204 gas chromatograph coupled to a VG micromass 7070H mass spectrometer. Data acquisition and processing were under the control of a Finnigan 2300 INCOS data system. A variety of capillary columns was evaluated. Best results were obtained on a 25 m × 0.22 mm I.D. BP-1 coated (0.1 μm film thickness, OV-1 equivalent) flexible fused-silica capillary column (S.G.E., Milton Keynes, U.K.). Samples were introduced in solution in hexane via an SGE OCI-III on-column injector, the gas chromatograph oven temperature was raised ballistically to 200°C, then temperature programmed to 320°C

at 12°C per min and then held isothermally. Helium was the carrier gas at a column head pressure of 0.5 kg cm^{-2} producing a linear flow velocity (u) of *ca.* 100 cm s^{-1}. The GC–MS interface incorporated a wide bore re-entrant jet allowing the flexible fused-silica capillary column to feed directly into the ion source. The interface oven was modified[15] to facilitate heating to > 300°C. The mass spectrometer was operated in both full scan and SIM modes depending upon analytical requirements. In the full scan mode, an accelerating voltage of 2 kV was employed in scanning the range m/z 90–1000. Both electron impact (EI) and chemical ionisation (CI, using ammonia or isobutane reagent gases) were employed.

For high sensitivity analyses, SIM was performed to detect ions at m/z 567 and 561. The former ion is characteristic of fully silylated ecdysone (1) and other ecdysteroids possessing an ecdysone-type nucleus but no C-20 hydroxyl group. The latter ion is observed for fully silylated 20-hydroxyecdysone (2) and other related C-20 hydroxylated ecdysteroids having an otherwise normal ecdysone ring structure.

Quantitative analysis of ecdysteroids

Quantitative analyses of ecdysteroids were performed as described previously by use of an internal standard[9]. Standard curves were prepared by TMSI derivatisation of a series of mixtures containing ecdysone (10^5–10^2 pg) and 20-hydroxyecdysone (10^4–10 pg) and a constant amount (usually 1 ng) of makisterone A (4) as internal standard. Peak areas resulting from silylated ecdysone, 20-hydroxyecdysone and makisterone A were determined by GC–SIM-MS (see above). The standard curves were obtained by plotting the logarithm (× 100) of the ratio of the peak areas for ecdysone or 20-hydroxyecdysone to that of the makisterone A internal standard against the mass of ecdysteroid originally added. In the case of biological extracts (see below), a known amount of makisterone A (1 ng) was added prior to silylation. The levels of extracted ecdysteroids were determined by comparing their peak areas in the m/z 561 or 567 chromatograms with that obtained for the internal standard in the m/z 561 chromatogram.

Biological material

Anisakis simplex. Third stage larvae (750 worms) of *A. simplex* were removed from the body cavity of herring and maintained *in vitro* for 24 h[16]. Free ecdysteroids were extracted from the lyophilized nematodes as described previously[9] and aliquots quantified by RIA with ICT-1 antiserum[17]. Aliquots of the free ecdysteroid fraction were either analysed by HPLC, with fractions being collected every minute for RIA[9], or, following derivatisation, by GC–SIM-MS.

Caenorhabditis elegans. Egg producing hermaphroditic adults of the N2 strain of the nematode, *C. elegans*, were grown in liquid cultures. Wild type *Escherichia coli* were grown to saturation in "3XD" medium[18] and resuspended in "S" medium[19]. Cultures were inoculated with *C. elegans* dauer larvae and shaken at 130 rpm for 48 h at 23°C. Gravid adult nematodes were recovered from the cultures and washed to remove bacteria. The free ecdysteroids extracted from the lyophilized nematodes (25.6 g dry weight) were quantified by RIA, HPLC–RIA and (after partial purification by HPLC and derivatisation) by GC–SIM-MS.

Hymenolepis diminuta. Mature *H. diminuta* were removed from the intestines of rats 24 days after infection. Polar conjugated ecdysteroids were extracted from the

lyophilized tapeworms (107 g dry weight) as described previously[9] and were hydrolysed with a crude preparation of *Helix pomatia* hydrolases to release free ecdysteroids. The fraction containing ecdysteroids released from polar conjugation was analysed by RIA, HPLC–RIA and GC–SIM-MS.

RESULTS AND DISCUSSION

Silylation

The polar nature of ecdysteroids requires that they be derivatised before GC or GC–MS analysis can be undertaken. Procedures for the partial and full silylation of ecdysteroids using TMSI have been described in detail elsewhere[3,10,11]. It has been firmly established that silylation of the hydroxyl groups of 20-hydroxyecdysone occurs with varying ease, in the positional order 2, 3, 22, 25 > 20 ⩾ 14[11]. Problems arising from incomplete silylation result in a mixture of derivatisation products which may lead to confusion when interpreting results from analyses of biological extracts. The 14α-hydroxyl group can only be silylated under relatively forcing conditions, but the rate of silylation at this position may be increased by the presence of catalysts[11].

The aim in this work was to achieve full silylation to avoid confusion which might result from the formation of mixed derivatives. Experiments with authentic compounds, conducted under a variety of conditions, showed that fully silylated ecdysone and 20-hydroxyecdysone were conveniently formed by heating (100°C, 15 h) with neat TMSI of high purity (freshly distilled), GC and GC–MS showed a single product peak. Full silylation was confirmed in the case of ecdysone by the presence of a molecular ion ($M^{\ddagger}$, *m/z* 824) in the EI mass spectrum. The presence of the high mass ion at *m/z* 567 and absence of one at *m/z* 564 provided further confirmation that full silylation had occurred[3]. A molecular ion was undetectable in the EI mass spectrum of 20-hydroxyecdysone. Although an intense (relative abundance 40%, base peak *m/z* 73) ion was observed at *m/z* 561, this is not diagnostic of full silylation as it can arise in partially silylated ecdysteroids[3]. By performing CI, using ammonia as reagent gas, full silylation was confirmed. The protonated pseudo-molecular ion [$(M + H)^+$, *m/z* 913, *ca.* 35%; base peak *m/z* 171] is that expected from hexa-TMS-20-hydroxyecdysone.

TMSI is a powerful silyl donor, particularly sensitive to the effects of moisture, decomposing rapidly to give imidazole and hexamethyldisiloxane. It was found that in cases where excessive reagent decomposition occurred during additions prior to heating, or where reagent of poor quality was used, silylation consistently produced a second product peak in the GC or GC–MS analysis. In the case of 20-hydroxyecdysone, this peak eluted at a slightly longer retention time than the hexa-TMS derivative. The EI mass spectrum of this peak was similar to that observed for 20-hydroxyecdysone. However, in the CI mode, using ammonia or isobutane as reagent gases, a strong ion (*ca.* 20%; base peak *m/z* 171) was observed at *m/z* 985. This corresponds to the protonated pseudo-molecular ion of hepta-TMS-20-hydroxyecdysone, *i.e.* that of the enol-silyl ether which has been reported as being formed previously[11]. This latter product was rarely observed in >20% yield. Formation of this component was avoided provided freshly distilled reagent was used and inert conditions maintained during reagent manipulation to avoid decomposition. Comparable results were also obtained using TMSI stored in sealed glass ampoules (1 ml)

for up to 3 months provided sealing of the ampoules is performed immediately subsequent to distillation. This latter observation agrees with that of Bielby *et al.*[10]. Formation of the enol-TMS ether is catalysed by the addition of imidazole, but as this reaction did not proceed to completion, this was not adopted as a derivative for routine ecdysteroid analysis.

GC column selection

A number of capillary columns were employed in this work. These ranged from 5 m to 25 m in length and were all flexible fused-silica columns coated with cross-bonded stationary phases. Stationary phases ranged from apolar polydimethyl siloxane (OV-1 type) to medium polarity cyanopropyl–phenylmethyl siloxane (OV-1701 equivalent). While silylated ecdysteroids could be chromatographed on the medium polarity phases, the relatively low maximum operating temperature limit (*ca.* 270°C) of such phases means that elution even from short columns (5–10 m) occurs in the isothermal period at the end of the GC run. Consequently, considerable peak broadening occurs. Optimum results were obtained using apolar polydimethylsiloxane phases. On such columns, fully silylated ecdysteroids display excellent GC characteristics chromatographing with peak shapes comparable to n-alkanes of similar retention times. The relatively low volatility of silylated ecdysteroids (Kováts' retention indices *ca.* 4000) requires that relatively thin film (*ca.* 0.1 μm) coatings of stationary phase are necessary to produce acceptable retention volumes. Short (5–10 m) columns coated with thicker films (*ca.* 0.25 μm) can also be used very effectively. For routine analyses, a 25 m × 0.22 mm I.D. column coated with BP-1 (0.1 μm, OV-1 equivalent) is used.

Fig. 1 shows a partial total ion current (TIC) chromatogram obtained from the capillary GC–SIM-MS of a mixture of biologically important ecdysteroids. Baseline resolution is attainable for all compounds except for the 3-epimers of 20-hydroxyecdysone which were not resolved. The high efficiencies of capillary columns is reflected in the superior resolution obtained compared to packed column GC analysis (Table I). This is particularly important in the case of ecdysteroids where biological extracts often contain mixtures of structurally very similar compounds. The elution order of the various silylated ecdysteroids, as reflected in their relative retention times (compared to silylated makisterone A; Table I), is analogous to that observed previously using packed GC columns employing a similar stationary phase (compare our Table I to Table 1 in ref. 9). GC resolution of the 3-epimers, which do occur naturally, has yet to be achieved. Co-elution also occurs on packed GC columns, but they may be resolved in the underivatised form by HPLC; 3-epi-20-hydroxyecdysone elutes after 20-hydroxyecdysone on reversed-phase HPLC[4].

Capillary GC–MS analyses

Capillary GC–MS analyses of trimethylsilylated ecdysteroids are readily performed. To ensure optimum sample transfer, the direct inlet mode of GC–MS interfacing, whereby flexible fused-silica capillary columns are fed directly into the ion source, is to be preferred. The integrity of the GC profile is largely retained provided good high-temperature (*ca.* 300°C) continuity is maintained across the interface line, in conjunction with a complementary high ion source temperature (*ca.* 300°C).

By employing the full scan mode of GC–MS operation with either EI or CI,

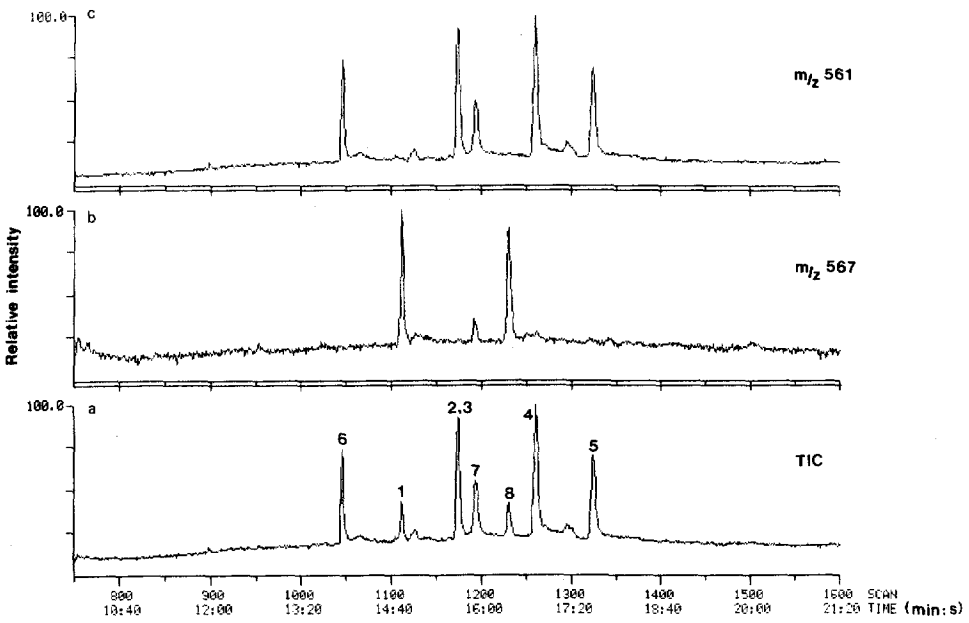

Fig. 1. Partial TIC chromatogram (a) and *m/z* 567 (b) and *m/z* 561 (c) mass chromatograms for the capillary GC–SIM-MS analysis of a mixture of fully silylated biologically important ecdysteroids. The peak numbers refer to structure numbers. Analyses were performed on a 25 m × 0.22 mm I.D. BP-1 coated (OV-1 equivalent, 0.1 μm film thickness) flexible fused-silica capillary column. Following on-column injection the oven temperature was raised ballistically to 200°C, then programmed at 8°C/min to 320°C and held isothermally.

TABLE I

GC CHARACTERISTICS OF FULLY SILYLATED TMS ETHERS OF ECDYSTEROIDS

Compound silylated	*Retention times relative to silylated makisterone A (RRT)*	*Resolution (R_s)*	
		*Capillary column**	*Packed column***
Ponasterone A	0.83		
Ecdysone	0.88	5.3	1.0
20-Hydroxyecdysone	0.93		
3-Epi-20-hydroxyecdysone			
Inokosterone	0.95	6.3	1.2
26-Hydroxyecdysone	0.98		
Makisterone A	1.00		
20,26-Dihydroxyecdysone	1.05		
Podecdysone A	1.10		

* Experimental conditions identical to caption to Fig. 3.

** From ref. 9.

structure information is readily obtained provided 10–100 ng of each component are present. Such amounts of ecdysteroids can be readily obtained from comparatively small quantities of tissues in the case of arthropods[1]. However, in recent investigations of non-arthropod species, amounts of ecdysteroids several orders of magnitude lower have been regularly encountered[9,14]. In such circumstances, GC–SIM-MS is employed. As can be seen from the standard curves shown in Fig. 2, detection limits of 10 and 100 pg are achieved for 20-hydroxyecdysone and ecdysone, respectively. These detection limits constitute a 5–10 fold improvement on those attained previously using packed columns[9]. The improved sensitivity arises from a combination of optimised (on-column injection), chromatographic performance (narrower capillary GC peaks increasing signal-to-noise ratio) and GC–MS interfacing (direct capillary inlet). The slight deviation from linearity in the standard curve for 20-hydroxyecdysone has been observed consistently. There is no obvious explanation for this. The deviation implies that an impurity is present which co-elutes with 20-hydroxyecdysone. As all reagents have been rigorously purified, contamination by 20-hydroxyecdysone itself is improbable. Whatever the explanation, the deviation is reproducible and the curve is used as such in quantitative analyses.

A series of experiments were performed to test whether or not the aforementioned levels of detection were achievable in the presence of biological impurities. Amounts of ecdysone (100 or 500 pg) and 20-hydroxyecdysone (10 or 50 pg) together

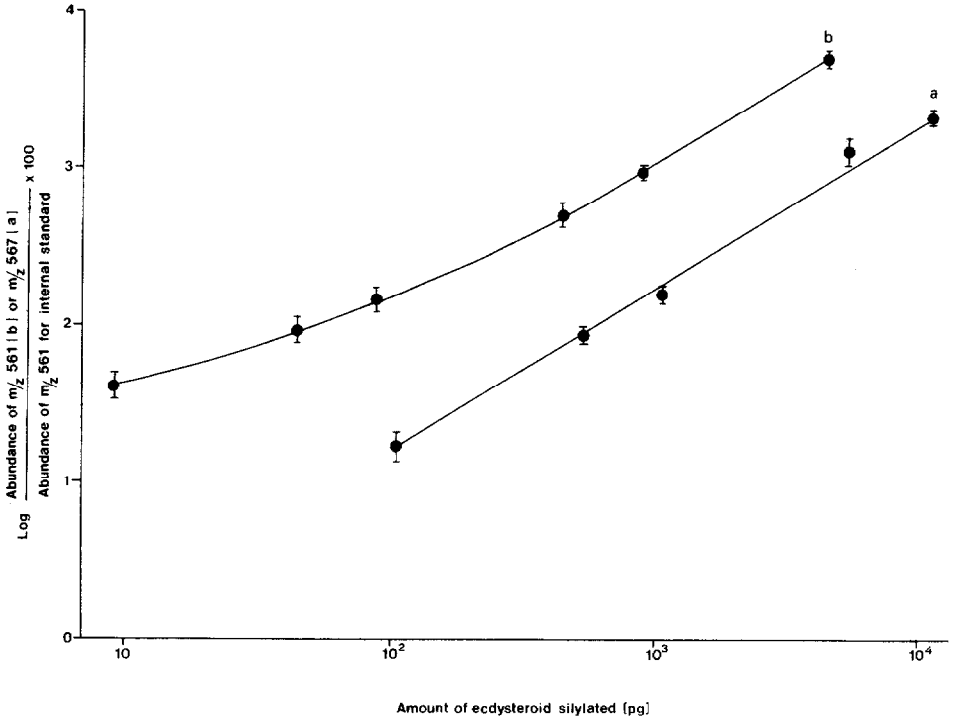

Fig. 2. Standard curves of the amount of (a) ecdysone and (b) 20-hydroxyecdysone silylated *versus* the abundance of selected ions observed during GC–SIM-MS relative to that of makisterone A (1 ng) internal standard. All data points are from replicate analyses. The error bars correspond to ± S.D.

with makisterone A (1 ng; internal standard) were added to aliquots (5 mg) of a biological extract known to be free from ecdysteroids. The biological extract chosen was a chromatographic fraction of slightly higher polarity than that containing free ecdysteroids, and was obtained from a methanolic extract of the nematode, *C. elegans*. Triplicate analyses were performed at each ecdysteroid concentration. GC–SIM-MS analysis showed that at the higher levels added, recoveries (mean ± S.D.) were 407 ± 77 pg and 55 ± 21 pg for ecdysone and 20-hydroxyecdysone, respectively. Hence, as the reasonable recoveries are achievable in the presence of biological impurities, quantitative analyses of ecdysteroids can be carried out at these levels. At the lower levels of added ecdysteroids, quantitative precision is poorer, and at these levels the technique is best regarded as a semi-quantitative means of analysis.

Analysis of biological extracts

RIA is the most sensitive method of ecdysteroid analysis with detection limits at the low picogram range[8]. The major disadvantage of RIA, *viz.* its poor selectivity, is improved when it is employed as a means of monitoring HPLC eluent (HPLC–RIA)[9]. GC–MS offers markedly enhanced selectivity compared to RIA. The demonstrably higher sensitivities now attainable by GC–SIM-MS with capillary columns are comparable to those achieved by RIA. This is especially significant, as it means that capillary GC–SIM-MS and HPLC–RIA can be used as complementary means of ecdysteroid analysis. This is illustrated here by analyses of three helminth species the results of which are summarised in Table II.

In our work, we aim to use both HPLC–RIA and GC–SIM-MS qualitatively and quantitatively. As can be seen from Table II in all but one case, the GC–SIM-MS data corroborate those obtained by HPLC–RIA and so provide strong evidence

TABLE II

GC–SIM-MS AND HPLC–RIA ANALYSES OF ECDYSTEROIDS IN THREE HELMINTH SPECIES

Helminth species	*Ecdysteroid**	*Structure*	*GC–SIM-MS (pg)*	*HPLC–RIA (pg)***
*Caenorhabditis elegans****	Ecdysone	1	635§	660
	20-Hydroxyecdysone	2	360§	500
Anisakis simplex§§	Ecdysone	1	70	74
	20-Hydroxyecdysone	2	50	62
	20,26-Dihydroxyecdysone	5	30	120
Hymenolepis diminuta§§§	Ecdysone	1	870	740
	20-Hydroxyecdysone	2	300	120
	20,26-Dihydroxyecdysone	5	160	Not detected

* Assignments based on RRTs of peaks in *m/z* 561 or 567 mass chromatograms compared to internal standard (Table I).

** After correction for cross-reactivities.

*** Aliquot equivalent to extract of 2.5 g of worms dry weight.

§ Quantification based on duplicate determinations.

§§ Aliquot equivalent to extract of *ca.* 110 worms.

§§§ Aliquot equivalent to extract of 10.7 g of worms dry weight.

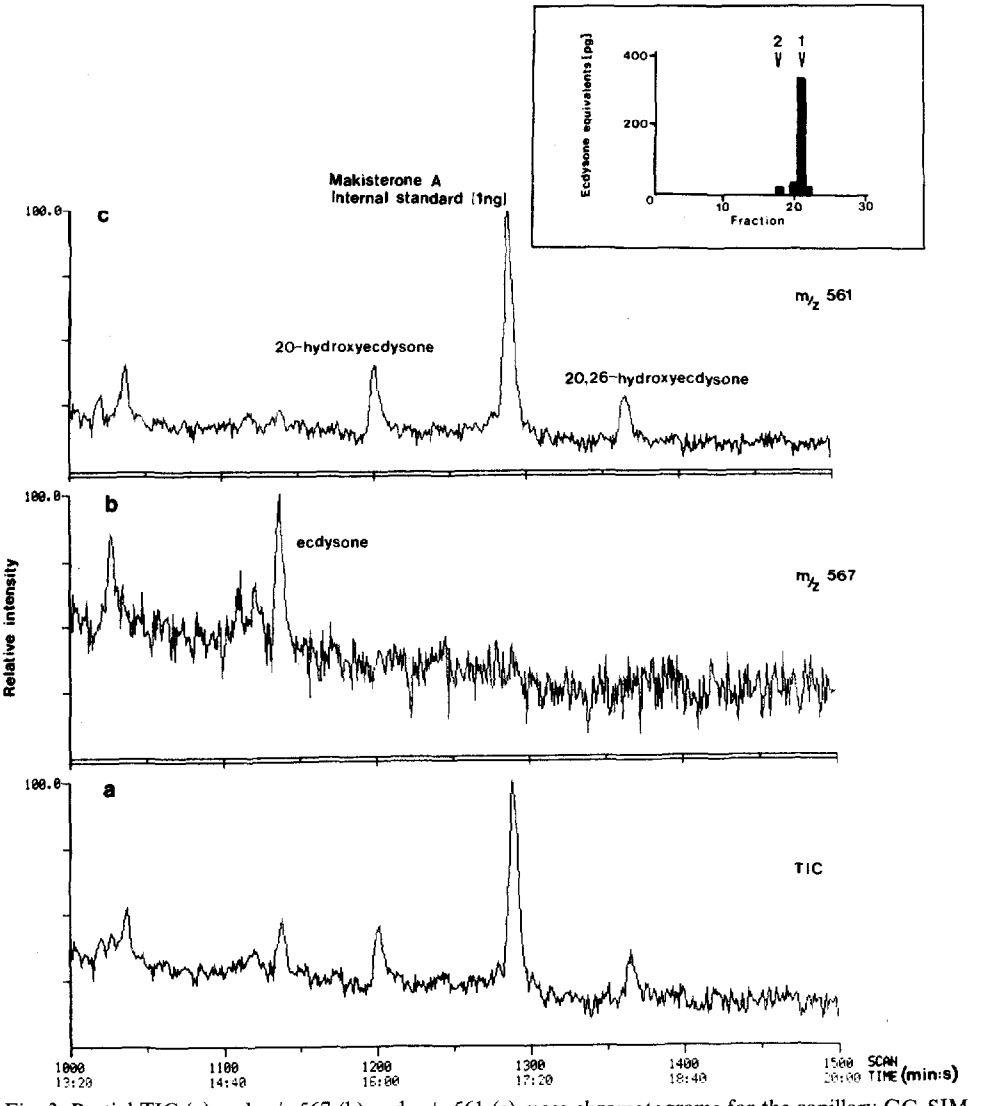

Fig. 3. Partial TIC (a) and *m/z* 567 (b) and *m/z* 561 (c) mass chromatograms for the capillary GC–SIM-MS analysis of the silylated ecdysteroids released by hydrolysis of the polar conjugate fraction from the cestode, *Hymenolepis diminuta*. All experimental details are the same as for the caption to Fig. 1. The inset shows the HPLC–RIA chromatogram obtained using ICT antiserum from a smaller aliquot (80%) than that derivatised for GC–SIM-MS (data not corrected for different cross-reactivities). Reversed-phase HPLC (C_{18} Rad-Pak; Waters Assoc., Harrow, U.K.) eluted with a linear gradient of methanol–water (35:65) to 100% methanol over 30 min at 1 ml/min with fractions being collected every minute for RIA. Elution volumes of ecdysteroids are indicated by structure numbers.

for the occurrence of the various ecdysteroids in the three helminth species. This clearly emphasises the power of GC–SIM-MS and HPLC–RIA for trace analysis when used in combination. The only ambiguous result is the indication by GC–SIM-MS (Table II and Fig. 3) of the presence of 20,26-dihydroxyecdysone in *H. dimi-*

nuta which is not corroborated by the HPLC–RIA data. This discrepancy could be explained by the occurrence of a biological impurity, which could be ecdysteroidal or non-ecdysteroidal, having a similar GC relative retention time to 20,26-dihydroxyecdysone. Thus, the occurrence of this latter ecdysteroid in *H. diminuta* awaits substantiation.

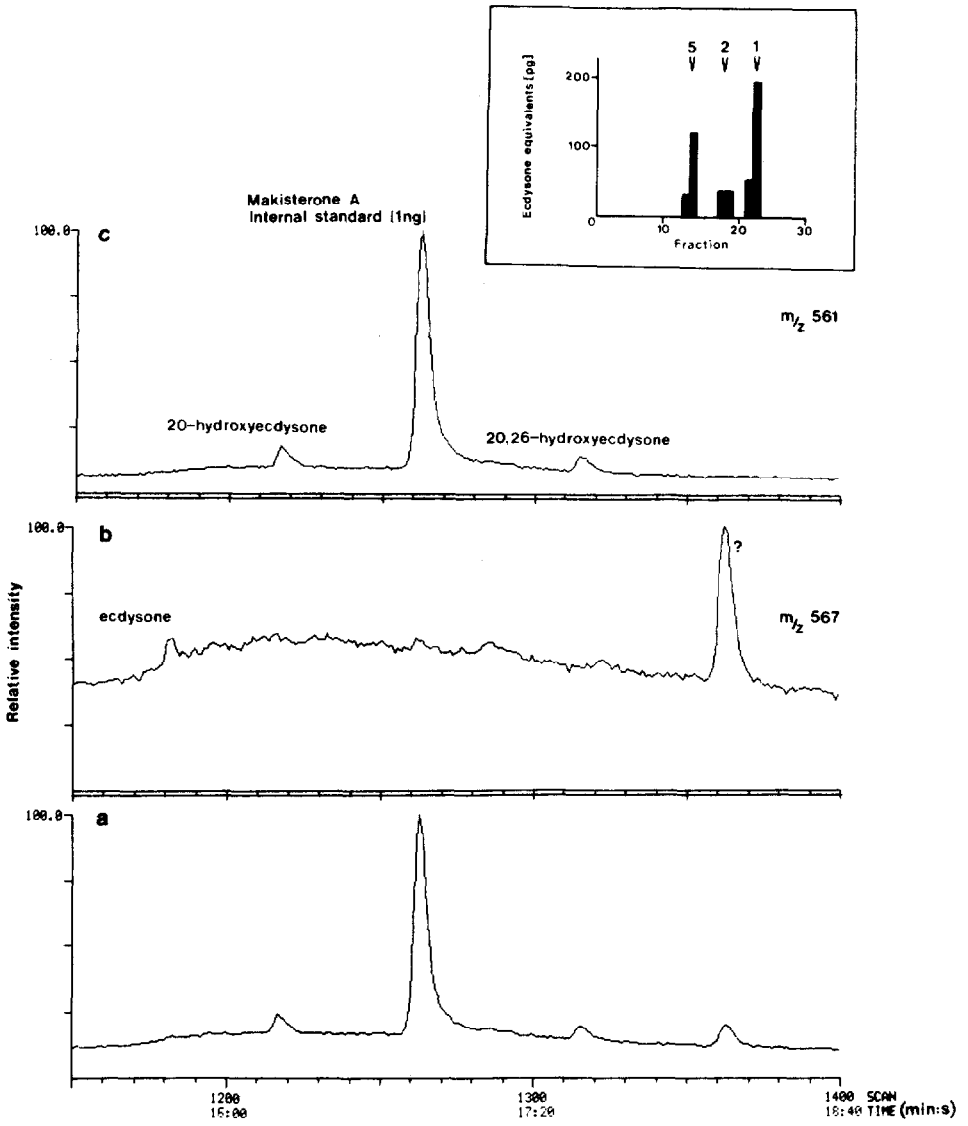

Fig. 4. Partial TIC (a) and *m/z* 567 (b) and *m/z* 561 (c) mass chromatograms for the capillary GC-SIM-MS analysis of the silylated free ecdysteroids from the nematode, *Anisakis simplex*. The inset shows the reversed-phase HPLC–RIA chromatogram obtained using a 30-min linear gradient of methanol–water (35:65) to methanol–water (80:20), from a larger aliquot (330%) than that derivatised for GC–SIM-MS. Other details the same as for caption to Fig. 3.

The quantitative data derived from the GC–SIM-MS and HPLC–RIA are also presented in Table II for the three species. The agreement between the two methods is generally good. The problems of precision as revealed by the spiking experiments (see above) must be borne in mind, particularly where ecdysteroids are present at their limits of detection, *i.e.* of the order of 100 and 10 pg of ecdysone and 20-hydroxyecdysone, respectively. Furthermore, there remains the problem of comparing GC–SIM-MS and HPLC–RIA data for compounds (*e.g.* 20,26-dihydroxyecdysone) for which a GC–SIM-MS standard curve has not been plotted and radioimmunoassay antiserum (ICT-1) cross-reactivity is unknown. For this reason, the quantitative data presented for 20,26-dihydroxyecdysone must be regarded as approximate.

A major disadvantage of GC–SIM-MS arises when a component is encountered whose GC retention characteristics are unlike those of any other available authentic compound for structural confirmation by co-injection or relative retention time comparison. This is a general shortcoming of GC–SIM-MS and one that was encountered in the analysis of the free-ecdysteroids of *A. simplex*. As can be seen from Table II, GC–SIM-MS and HPLC–RIA data confirm the occurrence of ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone. A further intense peak is also present in the *m/z* 567 mass chromatogram (Fig. 4). Its chromatographic behaviour is comparable to that of the other ecdysteroids. If it is an ecdysteroid, its appearance in the *m/z* 567 chromatogram suggests a structure possessing an ecdysteroid nucleus analogous to ecdysone (1) but, lacking a C-20 hydroxyl group. Further structural information on this unidentified component has to await additional physico-chemical analyses.

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