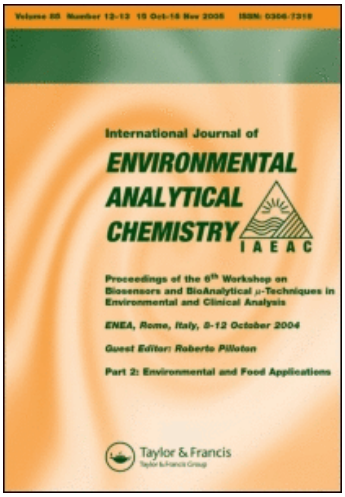

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Derivatives of Diethylstilbestrol (DES), Mass Spectral Properties and Their Use in Biological Analysis

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A series of perfluoroester and chloroacetate derivatives of diethylstilbestrol (DES) were prepared from the corresponding anhydride using trimethylamine as a catalyst. In all cases, accurate mass measurements by high resolution electron impact (EI) mass spectrometry of μg or less quantities showed a strong base peak which corresponded to the disubstituted product. Gas chromatography–mass spectrometric (GC–MS) analysis of the DES derivatives showed no change in any of the patterns due to thermal instability for either the *cis* or *trans* isomers. Beef liver samples containing 2 ng/g of DES were analysed by both the trifluoroacetate (TFA) and heptafluorobutyrate (HFB) derivatives using GC–EC and GC–MS with single ion monitoring. For HFB, no interference was observed by either detection mode but, for the TFA, interferences which showed up by GC–EC were absent on GC–MS. Due to the stability and good mass spectral patterns of the derivatives, GC–MS lends itself well to the confirmation of these DES derivatives in biological samples.

KEY WORDS: Diethylstilbestrol, mass spectrometry, confirmation, derivatives, determination.

INTRODUCTION

Due to its high oral potency and inexpensive production from common precursors, diethylstilbestrol (DES) has become the most important synthetic estrogen. There exists a plethora of methods for analysis of this hormone in biological samples of which the most common determinative step is derivative formation followed by gas chromatography (GC) with electron capture detection (EC). Although GC methods give reproducible results for the presence of DES, due to its instability (being subject to oxidation,

isomerization and photochemical rearrangement), supportative evidence is necessary to confirm its occurrence in biological samples.

Mass spectrometry has been used in the analysis of DES in biological samples including the trimethylsilyl (TMS),[2-7] methyl,[2,8] dichloroacetate (DCA),[9] trifluoroacetate (TFA),[10,11] and heptafluorobutyrate (HFB)[11,12] derivatives. All of these reports have been carried out incidental to other studies on analysis and metabolism. No systematic study has been made on the mass spectral properties of derivatives of DES nor have the derivatives themselves been fully characterized. Our recent studies[13,14] in the electron capture response (EC) of derivatives of DES used in environmental samples have shown that the HFB was superior to the TMS and DCA products on account of its strong EC response and good hydrolytic and thermal stability. It was natural to extend this study to the mass spectral properties of these compounds in order to choose the best conditions for determination of DES in environmental samples.

The basis of the present work is: (i) to identify and characterize a series of derivatives of DES using high resolution electron impact (EI) ionization mass spectrometry on the probe; (ii) compare the results of (i) with GC–MS at low resolution as concerns patterns and relative intensity; and (iii) illustrate mass spectrometry with single ion monitoring with two of the derivatives to confirm the presence of DES in biological samples.

EXPERIMENTAL

DES was the commercially available 100% *trans* isomer from either USP Merck or K & K Lab. Rapid equilibration of this isomer to a 1:3 mixture of *cis* and *trans* takes place[15,16] in benzene, chloroform, or solvents with a little acid or base. Hence, after dissolution of DES in benzene for a short time, two well resolved GC peaks were always obtained from each derivatizing reagent. The TMS and acetate forms were made by standard procedures. The perfluoroesters of DES were synthesized as reported earlier.[11] The chlorinated esters of DES were prepared from trimethylamine and either the corresponding anhydride or chloride. In the case of the chloroacetates and pentadecafluorooctanoate, it was necessary to wash the organic phase several times with the aqueous phase in order to ensure complete hydrolysis and removal of the interfering halogenated acids.

GC with EC detection was carried out on a Hewlett Packard 5713 equipped with a linear Ni-63 detector and an all glass system. The glass column was 180 cm long × 4 mm i.d., contained 3% OV-17 on Chromosorb W 80–100 mesh, was maintained at 200°C with injection port at 200°C and a flow rate of 60 ml/min helium.

Electron impact ionisation spectra were recorded by a Varian Mat 311A instrument coupled with a Varian 1440 GC and Watson-Biemann separator. The ionization voltage was 70 eV, interface temperature 225°C, source 250°C, and probe 25°C. Acceleration voltage was 3000 V and, for GC-MS, scan speed was 2.5 sec for 0-500 m/z units. Effluent through column was monitored by either a total ion current detector or by single ion monitoring (SIM) with a recorder of 10 mV for full scale deflection. Columns were, for standards, 90 cm long × 2 mm i.d. containing 3% OV-101 on Chromosorb W, 80-100 mesh, and, for samples, 180 cm long × 4 mm i.d. containing 2% OV-17 + 1% QF-1. The choice of the different column stationary phases and lengths is not critical since DES derivatives usually elute with well defined peak shapes. Flow rate of helium was 30 ml/min and column temperature was adjusted to give retention times between 2 and 6 min. Quantities of DES injected after derivatization were about 1 μg for high resolution work on probe, 100 ng for GC-MS of standards at low resolution and entire spectrum scanning, and 1 ng for GC-MS and single ion monitoring (SIM) in biological samples.

RESULTS AND DISCUSSION

All derivatives of DES were readily formed in less than one hour at room temperature to give reproducible GC-EC and GC-MS patterns. The technique of trimethylamine catalysis gave high conversion of phenolic hydroxyl to the corresponding ester derivative with the fluoro-[13] and chloroanhydrides.[14] Donoho *et al.*[17] observed that acid chlorides were superior to the anhydrides using sodium hydroxide and a heterogeneous reaction medium to convert DES to its chloroacetates. However, we could find no consistent difference between the anhydride or acid chloride with our conditions and preferred the former due to its easier handling properties. As the main interest of this work was the use of GC-MS for detection and confirmation of residue levels in biological samples, the total quantity of DES used was not more than 10 μg. Hence, crystalline derivatives at the milligram level were not prepared and analysed by other physical methods. However, preparation of some of the DES derivatives in milligram amounts and serial dilution for GC-EC analysis at the μg and ng level gave the same quantitative results as preparation at the ng level. The only exception was with the TCA compound where its poor GC-EC detection below 50 ng injection was believed to be due to thermal instability as noted previously[17] and discussed recently.[14]

The mass spectral characteristics of the series of derivatives using EI ionisation at the highest m/z value is shown in Table I at a resolution of 10,000. These accurate mass measurements were all carried out on the probe at 25°C with the peak matching technique. In every case analysed, each derivatizing

reagent reacted with DES to produce a disubstituted product as evidenced by the agreement between the theoretical and found elemental composition of the principal molecular ion. Most probably, this acylation occurs on the para-hydroxy groups of both phenyl groups. The consistency of the disubstituted derivative shows the reliability of mass spectrometry and the derivatizing technique for analysing the elemental composition of DES.

These derivatives were then analysed by GC–MS at low resolution after passage through a hot (225°C) separator and measuring the entire mass

TABLE I

High resolution[a] mass spectrum of derivatives of DES

Derivative	Chemical Formula[b]	m/z Theory	m/z Found
Trimethylsilyl (TMS)	$C_{24}H_{36}O_2Si_2$	412.2254	412.2261 ± 9
Trifluoroacetate (TFA)	$C_{22}H_{18}O_4F_6$	460.1109	460.1118 ± 10
Pentafluoroproprionate (PFB)	$C_{24}H_{18}O_4F_{10}$	560.1046	560.1041 ± 11
Heptafluorobutyrate (HFB)	$C_{26}H_{18}O_4F_{14}$	660.0982	660.0987 ± 13
Pentadecafluorooctanoate (PDFO)	$C_{34}H_{18}O_4F_{30}$	1060.073	1060.074 ± 2
Acetate (AC)	$C_{22}H_{22}O_4$	352.1675	352.1684 ± 10
Monochloroacetate (MCA)	$C_{22}H_{22}O_4\ ^{35}Cl_2$	420.0895	420.0894 ± 10
Dichloroacetate (DCA)	$C_{22}H_{20}O_4\ ^{35}Cl_3\ ^{37}Cl$	490.0086	490.0084 ± 10
Trichloroacetate (TCA)	$C_{22}H_{18}O_4\ ^{35}Cl_5\ ^{37}Cl$	557.9307	557.9314 ± 11

[a] $M/\Delta M = 10{,}000$, measured at 5% peak height.

[b] For addition of two derivatizing groups to DES.

spectrum. Since all standard solutions of DES were equilibrated in benzene solution, two measurements were made at the retention times of the *cis* and *trans* isomers. The results, presented in Table II, demonstrate the thermal stability of the derivatives up to at least 225°C as the most intense mass peaks did not change under the specific conditions. All molecular ion peaks after GC were in most cases the base peak. In a few cases, the number and/or intensity of some of the fragmented peaks decreased with respect to the probe values but this was only a minor variation.

A common fragmentation pattern is loss of ethyl (M-29) and, less so, loss of methyl (M-15). The fluorinated esters showed a homologous series of molecular ion peaks (460, 560, 660, 1060) which were constant and

TABLE II

GC–MS[a] peaks of derivatives of DES at low resolution

Derivative[b]	Column temperature eluted, °C	Detectable[c] peaks of both *cis* and *trans* isomers
None[d]	—	268, 253, 239
TMS	200	412 (M^+), 397, 383, 231, 217
TFA	200	460 (M^+), 431, 363, 335, 317, 241
PFP	200	560 (M^+), 531, 510, 481, 460
HFB	200	660 (M^+), 631, 464, 435, 417, 341
PDFO	210	1060 (M^+), 1031, 542, 503, 518
AC	210	352 (M^+), 310, 268, 239
MCA[e]	240	424 (13), 423 (16), 422 (62), 421 (25), 420 (M^+, 100), 344, 239
DCA[e]	240	496 (3), 495 (4), 494 (11), 493 (8), 492 (53), 491 (29), 490 (M^+, 100), 489 (21), 488 (79), 461, 378, 361, 349, 255
TCA[e]	240	566 (7), 565 (5), 564 (14), 563 (17), 562 (42), 561 (23), 560 (67), 559 (21), 558 (M^+, 100), 557 (14), 556 (49), 530, 397, 289

[a]Conditions as given in experimental.
[b]Abbreviated as in Table I.
[c]All peaks >5% relative to strongest.
[d]Recorded only on probe.
[e]Numbers in brackets are relative intensities of molecular ion peaks only due to chlorine isotopes compared to most intense as 100 (underlined).

reproducible. The chlorinated esters exhibited multiple molecular clusters due to the presence of either 2, 4 or 6 chlorine atoms. The abundance of the principal molecular ions of these chloro esters was close to the theoretical values of (10-6-1), (3-4-2) and (5-10-8-3), respectively. No systematic difference was found between the *cis* and *trans* GC peaks except one of total ion abundance due to the smaller amount of the *cis* isomer (ratio 1:3) derived from an equilibrium mixture of the two.

As all derivatives exhibited strong molecular ion peaks and thermal stability, all could be used for confirmation. Two of them (TFA and HFB) were selected to illustrate mass spectral confirmation with the GC–MS combination and single ion monitoring. The fluorinated derivatives of DES have been shown[13] to exhibit high EC sensitivity along with thermal and hydrolytic stability. Beef liver was chosen for this analysis since DES is known[7] to accumulate and metabolize in this tissue and, because of the presence of interfering peaks, is the most difficult tissue to analyse for the presence of DES. Two beef liver samples were spiked with DES at the level of 2 ng/g and purified

according to the method of Coffin and Pilon[18] (total recovery of 100 ng in 50g sample was 65%). After cleanup, one sample was derivatized to the TFA ester and the other to the HFB ester. The GC–EC patterns of these food samples are shown in Figures 1A and 2A, respectively, for 0.4 ng injections at two different attenuations. While the HFB derivative shows a well-defined peak due to its high EC response, the TFA tracing is somewhat obscured by EC peaks of similar intensity arising from either reagents or food constituents. The chloro- and TMS derivatives would also be expected to show such interfering background at these low levels.[13,14] Aliquots of these same two samples were then rechromatographed on a GC–MS combination at 4,000 resolution using SIM at m/e 460 and 660 for the TFA and HFB, respectively. Corresponding patterns after injection of 1 ng of DES as derivatives are shown in Figures 1B and 2B. All halo-derivatives of DES except the TCA are known to give a linear EC response from 0.1 ng to 100 ng so that no decomposition is believed to take place at these low levels of injection even though the accurate mass spectral and GC–MS entire scan measurements were carried out with at least 50 ng

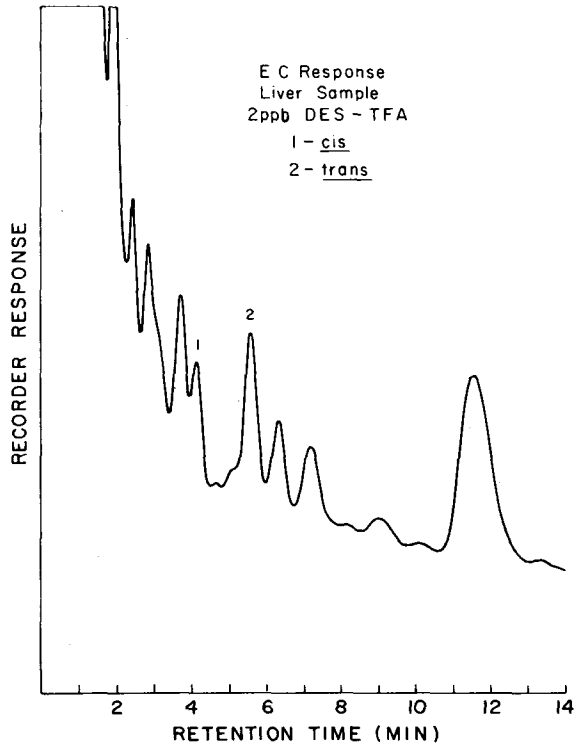

FIGURE 1A

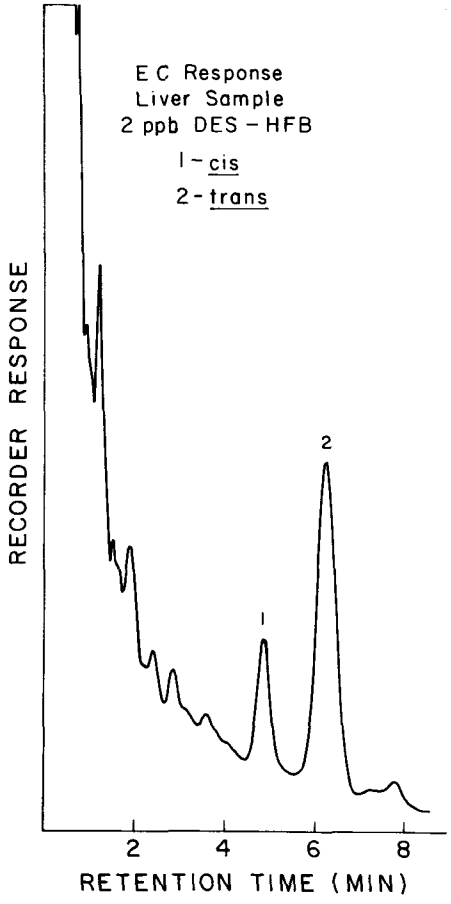

FIGURE 2A

FIGURE 1A, 2A GC–EC tracing of beef liver sample containing 2 ng/g DES after derivatization to the TFA and HFB ester, respectively. Injected 0.4 ng of each at attenuation 128 for 1A and 512 for 2A.

DES. The mass spectral patterns are much cleaner particularly for the DES-TFA and show distinctly the two *cis* and *trans* isomeric peaks at the same retention time as by EC and in the same relative ratio.

CONCLUSION

Accurate mass measurements of nine derivatives of DES have shown that, in every case, a disubstituted product is formed with the molecular ion as the

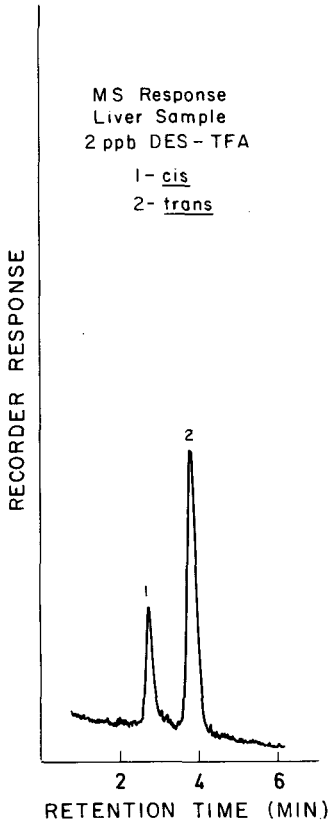

FIGURE 1B

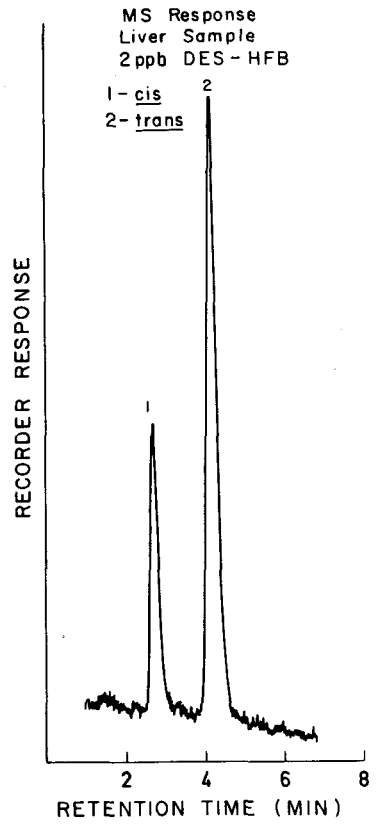

FIGURE 2B

FIGURE 1B, 2B GC-MS tracing of same sample as in Fig. 1A, 2A with SIM at 460 for 1B and 660 for 2B. $M/\Delta M = 4{,}000$, 70 eV. Injected 1 ng of parent DES as its derivative.

most abundant ion. On GC-MS analysis, no loss of intensity occurs with the base peak due to thermal instability of any of these derivatives. The use of fluorinated derivatives of DES in combination with GC-MS and SIM at the molecular ion value has demonstrated the specificity and simplicity for confirming the presence of DES in biological samples. The choice of the most useful derivative for analysis or confirmation of DES must be made on their electron capturing response and thermal and hydrolytic stability since all showed equally good mass spectral characteristics.

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