Journal of Chromatography, 156 (1978) 267–273

CHROM. 11,040

STABLE DERIVATIVES FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF SYNTHETIC ANABOLIC STILBENE RESIDUES (DIETHYLSTILBESTROL, DIENESTROL AND HEXESTROL) IN MEAT AND ORGANS OF TREATED CATTLE AT THE SUB-PARTS PER BILLION (10^9) LEVEL

L. LAITEM, P. GASPAR and I. BELLO

Laboratory of Food Analysis, Department of Prof. L. Fiévez, Veterinarian Medicine Faculty, University of Liège, Rue des Vétérinaires 45, B-1070 Brussels (Belgium)

(Received March 6th, 1978)

SUMMARY

A method for the determination of diethylstilbestrol and the related compounds dienestrol and hexestrol residues in meat and organs of treated cattle is described. After extraction and clean-up, these synthetic estrogens are subjected to reaction with pentafluorobenzoyl chloride, which gives very stable perfluoro esters that are suitable for gas chromatographic determination using an electron-capture detector. With the careful clean-up and the very sensitive response of these derivatives, it is possible to reach a limit of detection in the sub-parts per billion (10^9) range starting with only 5 g of sample.

INTRODUCTION

Since the discovery of the anabolic properties of diethylstilbestrol more than 20 years ago, attempts have been made to determine the residues of these compounds in the organs of treated cattle. Two procedures have been found to be sufficiently sensitive: radioimmunoassay and gas–liquid chromatography with an electron-capture detector or single-ion detection by mass spectrometry (MS). Ryan[1] reviewed the chromatographic analysis of hormones in food. Usually dichloroacetyl chloride[2] or perfluoroacid anhydrides such as trifluoroacetic anhydride[3], heptafluorobutyric anhydride[4,5] or pentafluoropropionic anhydride[6] react with hormone residues to enhance the electron-capture detector response. These derivatives were found to give a very sensitive detection of hormonal compounds. However, the perfluoroanhydrides of aliphatic acids and their resulting esters are difficult to handle because of their rapid hydrolysis. It was therefore necessary to develop a determination procedure based on the use of stable perfluoro derivatives. The esterification of residues of diethylstilbestrol (DES) and related compounds with an aromatic compound, pentafluorobenzoyl chloride (PFBC), was selected because this reaction was found to give very stable perfluoro esters. These aromatic esters can be isolated in a crystalline form and stored at room temperature for several days without decomposition.

EXPERIMENTAL

Solvents and reagents

All of the solvents we used were of pesticide grade (Merck, Darmstadt, G.F.R.) and were redistilled twice in an all-glass apparatus. Diethyl ether (Aristar; BDH, Poole, Great Britain) was washed with a saturated aqueous solution of iron(II) sulphate, with 1 *N* sulphuric acid and finally with 1 *N* sodium hydroxide solution before distillation. Sephadex columns were glass tubes (I.D. 5 mm) filled with a suspension of Sephadex LH-20–100 (Sigma, St Louis, Mo., U.S.A.) in benzene–methanol (85:15) to a height of 10 cm and stored in this solvent until use. Pentafluorobenzoyl chloride was purchased from Aldrich-Europe (Beerse, Belgium). Labelled DES (specific-activity 70 Ci/mmole; monoethyl-3-[^{3}H]) was kindly donated by Procida (Romainville, France).

Apparatus

A Tracor Model 560 gas–liquid chromatograph was used with a ^{63}Ni electron-capture detector and a glass column (1.20 m × 2 mm I.D.) packed with 3% OV-17 on Varaport 30 (100–120 mesh). The operating conditions were as follows: carrier gas (nitrogen) flow-rate, 20 ml/min; purge, 20 ml/min; inlet temperature, 300°; oven temperature, 275°; detector temperature, 325°.

Extraction and clean-up

A 5-g sample of tissue was placed in an extraction tube. After homogenization in 10 ml of acetone, the mixture was shaken for 15 min, the extraction tube was centrifuged at 1600 *g* for 5 min and the supernatant was decanted. This extraction step was repeated with the same volume of solvent. The combined acetone extracts were dried under a stream of nitrogen on a water-bath at 50° and the residue was dissolved in 5 ml of cyclohexane–diethyl ether (1:1) and extracted twice with 5 ml of 1 *N* sodium hydroxide solution. The organic layer was discarded and the combined aqueous extracts were cooled in an ice-bath and acidified with 1.5 ml of concentrated orthophosphoric acid. This solution was extracted three times with 3 ml of cyclohexane–diethyl ether (1:1). The combined organic layers were finally washed with 5 ml of 5% sodium hydrogen carbonate solution. The organic solvent was then dried and the residue was redissolved in 0.5 ml of benzene–methanol (85:15). This solution was transferred to the top of a Sephadex column and eluted with the same solvent. The first 3 ml of the eluate were discarded and the following 3 ml, containing the DES and the other synthetic estrogens hexestrol (HE) and dienestrol (DE), were collected in a reaction vial.

Reaction

After evaporation of the eluate under a stream of nitrogen, the residue was dissolved in 0.4 ml of benzene and to the solution were added 0.1 ml of a 0.1 *M* solution of pyridine in benzene and 10 μl of a 1% solution of PFBC in benzene. After homogenization, the reaction mixture was heated in a water-bath at 60° for 10 min. The solvent was evaporated and the residue was transferred to the top of a second Sephadex column in 0.5 ml of benzene–methanol (85:15) and eluted with this solvent. The first 1.5 ml of the eluate were collected, the solvent was evaporated, the residue was

dissolved in 0.5 ml of benzine and 0.5–2 μl of this final solution were injected into the chromatograph.

RESULTS AND DISCUSSION

Extraction and clean-up

The procedure used was based on the phenolic properties of DES and related compounds. As final clean-up, the use of gel chromatography in an organic medium was found very effective. Fig. 1 shows the elution profile of *cis*- and *trans*-DES from a Sephadex column. These two isomers were eluted practically into the same fraction. DE and HE were found to behave in a similar manner to the *trans*-isomer of DES. The Sephadex column provided an excellent clean-up in spite of its short length, and most coextratives could be eliminated in this step. This column could be re-used after washing with 20 ml of the eluent. We measured the recovery of DES by adding 0.1 ml of ^{3}H-labelled DES in methanol to 34 meat samples (added activity, 1000 cpm) of different origins. The mean recovery was 40.2% with extreme values of 22% and 58% and a standard deviation of 8.3%.

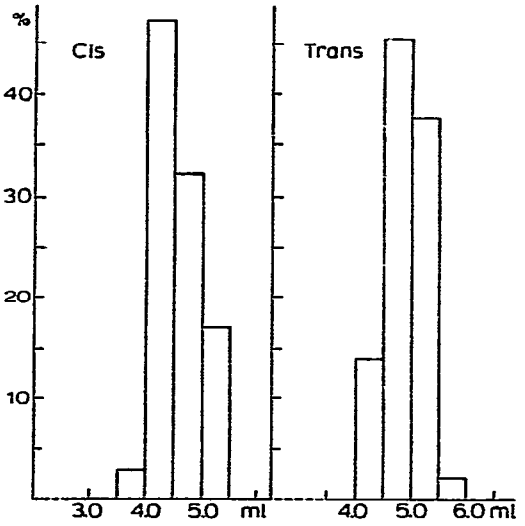


Fig. 1. Elution profile of *cis*- and *trans*-DES from a Sephadex column.

Reaction of PFBC with synthetic estrogens

This work was performed in order to select a derivatization reagent that could lead to perfluoro esters that are more stable than the compounds obtained by reaction with trifluoroacetic (TFA) anhydride or heptafluorobutyric (HFB) anhydride. PFBC was found to give very stable perfluoro derivatives on reaction with DES or related compounds. These aromatic perfluoro esters were isolated in crystalline form and remained unchanged after storage at room temperature for several days in stoppered glass tubes. Benzene solutions of these compounds are also very stable and no decomposition occurred after storage in stoppered glass tubes in a refrigerator for a week.

After derivatization, it was necessary to separate the esters from the excess of reagent. Owing to the different molecular sizes of the compounds to be separated, Sephadex columns were effective for this purpose. As shown in Fig. 2, the large molecules of esters were eluted in the first 1 ml, whereas the smaller molecules of PFBC in excess were retained on the Sephadex and required much more solvent for elution.

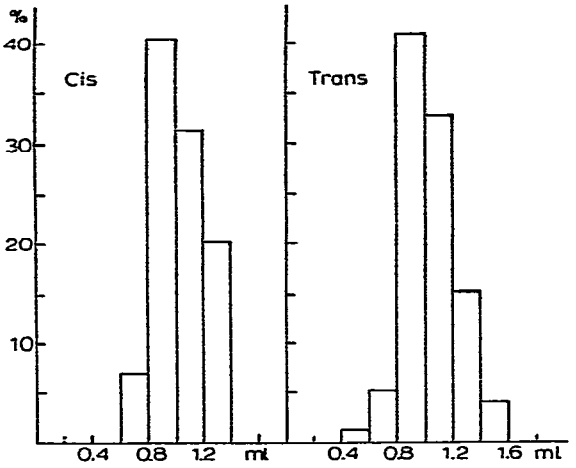


Fig. 2. Elution profile of *cis*- and *trans*-DES pentafluorobenzoates from a Sephadex column.

Sensitivity of the method in electron-capture gas chromatographic (GC) assays

When measured on standards, the sensitivity obtained with pentafluorobenzoyl derivatives was of the same order as the response given by the corresponding heptafluorobutyrates. The latter compounds were prepared according to Ryan and co-workers[4,5]. When the analysis was performed on meat samples to which DES had been added in the parts per billion (10^9) range or less, we found that the procedure described here gave clearer final chromatograms than those obtained by using heptafluorobutyric anhydride. This result can be explained in terms of the use of a more effective clean-up procedure and especially by the fact that perfluorobenzoyl esters have a lower volatility and were thus well separated from the coextracted compounds. The limit of detection was determined by analysis of samples of meat to which had been added decreasing amounts of standards. Under the described conditions, the procedure allowed the detection of 0.5 ppb of DES and 0.1 ppb of DE and HE.

Possibility of single-ion scanning in GC–MS assays

The mass spectra of *cis*- and *trans*-DES trifluoroacetates and heptafluorobutyrates were recorded by GC–MS and the data are reported in Table I. Table II gives the mass spectral data for *trans*-DES pentafluorobenzoate. The latter mass spectrum was recorded by direct introduction of the solid compound into the mass spectrometer. With the exception of these different modes of introduction, these three spectra were obtained under the same instrumental conditions. It should be noted that *trans*-DES pentafluorobenzoate was the only isomer that we could isolate in

TABLE I

MASS SPECTRA OF DES TRIFLUOROACETATE AND HEPTAFLUOROBUTYRATE

m/z (*cis* or *trans*)	*Relative abundance (%)*	
	Cis	*Trans*
461	9.5	12
460	40	46
432	4.8	5.9
431	24	25
347	4.8	5.9
317	14	13
242	13	13
241	100	100
217	22	18
203	73	63
661	13	23
660	31	39
632	5.5	8.4
631	11	24
448	3.6	4.2
447	11	14
446	3.6	4.2
419	3.6	6.1
418	5.5	10
417	13	17
342	18	22
341	100	100
318	1.8	3.5
317	22	17
304	5.5	8.4
303	40	39
275	—	8.4

TABLE II

MASS SPECTRUM OF *trans*-DES PENTAFLUOROBENZOATE

m/z	*Relative abundance (%)*	*m/z*	*Relative abundance (%)*
658	0.26	340	1.5
657	7.1	339	7.7
656	20	316	0.45
641	0.32	315	2.7
628	0.71	302	0.25
627	2.0	301	1.7
462	0.52	295	0.77
461	1.0	266	0.32
446	0.65	249	0.26
445	2.3	238	0.71
444	1.4	237	1.9
433	0.65	221	0.77
432	1.1	196	7.7
417	0.81	195	100
416	0.52	168	1.9
415	0.81	167	7.0
353	0.58		

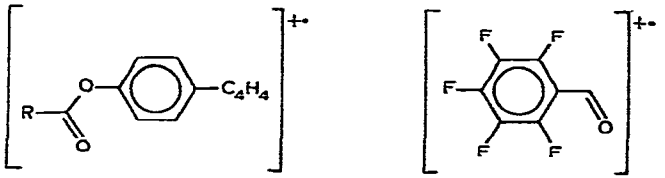
crystalline form from the reaction of DES with pentafluorobenzoyl chloride in pyridine in stoichiometric amounts. This compound was purified by crystallization from toluene.

TABLE III

PROTON MAGNETIC RESONANCE SPECTRUM OF *trans*-DES PENTAFLUOROBENZOATE IN $CDCl_3$–HEXAMETHYLDISILOXANE

Chemical shift (ppm)	*Signal*	*Coupling constant (Hz)*
0.73	6 H (*t*)–CH_3	7.35
2.10	4 H (*q*)–CH_2	7.35
7.21	8 H (aromatic)	—

Table III gives the PMR characteristics of this isomer. It was impossible to detect any trace of the *cis*-isomer in chloroform or benzene solutions of this compound after storage of these solutions for several days in a refrigerator (detection by electron-capture GC for benzene solutions and by NMR spectroscopy for chloroform solutions). By comparing the mass spectra of these three derivatives, we concluded that the *cis*- and *trans*-isomers of DES have the same fragmentation pattern. Also, no qualitative difference exists in the fragmentation patterns of TFA and HFB derivatives. Fig. 3 represents the formulae of the base peaks (I and II). These important fragments had m/z values of 241 for the TFA esters and 341 for the HFB esters. These ions are characteristic of the original compounds and can be used for the specific detection of DES residues by the single-ion scanning mode in a GC–MS assay (or single-ion detection mode). The fragmentation pattern of *trans*-DES pentafluorobenzoate was different. In the mass spectrum of this compound, the base peak was a fragment with $m/z = 195$ (Fig. 3, III). Unfortunately, this very important fragment is not characteristic of the original ester, as it can be generated from other molecules (*e.g.*, PFBC itself). This ion therefore cannot be used in a single-scanning mode in GC–MS assays for the detection of DES residues. Only the molecular ion ($m/z = 656$) can be used for this purpose but the intensity of this ion represents only 20% of the base peak.



I: R = CF_3, m/z = 241
II: R = C_4F_7, m/z = 341

III: m/z = 195

Fig. 3. Base peaks observed in the mass spectrum of (I) DES perfluoroacetate, (II) DES perfluorobutyrate and (III) DES perfluorobenzoate.

CONCLUSION

The procedure for the determination of DES and the related anabolic agents DE and HE described here is very sensitive. Starting with only 5 g of meat samples

the method can detect DES residues in the sub-parts per billion range. The perfluorobenzoate derivatives were very stable and very effective for electron-capture GC assays. From a comparative study, we found that the procedure gives chromatograms that can be interpreted more easily than those obtained by using reagents such as TFA or HFB. If specific detection by single-ion monitoring is to be used, heptafluorobutyrate derivatives were more effective owing to their fragmentation pattern.

ACKNOWLEDGEMENTS

The authors thanks Dr. R. Weber, Heterocyclic Organic Chemistry Department, University of Liège, for his assistance with the mass spectrometry, and Dr. C. Vincent, Inspector, Ministry of Public Health, for his constant interest.

REFERENCES

1 J. J. Ryan, *J. Chromatogr.*, 127 (1976) 53.
2 E. W. Day, J. Lynn, E. Vannetta and R. F. Sieck, *J. Ass. Offic. Anal. Chem.*, 58 (1975) 520.
3 D. E. Coffin and J. C. Pilon, *J. Ass. Offic. Anal. Chem.*, 56 (1973) 352.
4 J. J. Ryan and J. C. Pilon, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 817.
5 J. F. Lawrence and J. J. Ryan, *J. Chromatogr.*, 130 (1977) 97.
6 J. R. King, C. R. Nony and M. C. Bowman, *J. Chromatogr. Sci.*, 15 (1977) 14.