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Analysis of diethylstilbestrol, dienestrol and hexestrol in biological samples by immunoaffinity extraction and gas chromatography–negative-ion chemical ionization mass spectrometry

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SUMMARY

A method has been developed for the detection of diethylstilbestrol, together with dienestrol and hexestrol, using extraction with a single immunoaffinity column containing antibodies raised against diethylstilbestrol, followed by gas chromatography-negative-ion chemical ionization mass spectrometry. Immunoaffinity columns were prepared by coupling immunoglobulin G fractions obtained from rabbit antisera with a Sepharose matrix. The immunizing agent was synthesized by introducing a carboxyl group into the diethylstilbestrol molecule and coupling this product to bovine serum albumin. The columns were used for immunoadsorption of diethylstilbestrol and other estrogens, after dilution of samples with phosphate buffer, and were eluted with acetone-water (95:5 v/v). A derivatization method suitable for gas chromatographic-mass spectrometric analysis of diethylstilbestrol and other estrogens was developed using pentafluorobenzyl bromide and ethanolic potassium hydroxide as reagents. The derivatives obtained were detectable at the sub-picogram level using gas chromatography with negative-ion chemical ionization mass spectrometry. Recoveries of *cis*- and *trans*-diethylstilbestrol, dienestrol and hexestrol from the im-

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munoaffinity columns, determined after extraction from urine, plasma and buffer, ranged from 28 to 96%. The sensitivity for diethylstilbestrol in urine samples was ca. 10 ppt. The method was applied to the analysis of urine from calves given a single dose of 10 mg of diethylstilbestrol. Free and glucuronic acid conjugated diethylstilbestrol decreased with time, but their ratio was variable.

INTRODUCTION

Diethylstilbestrol (DES) is a powerful synthetic estrogen that has been used since the 1950s in human medicine and, as a growth promoter, in cattle. For the latter purpose, however, its use is now prohibited in Western Europe and in the U.S.A. because of its carcinogenicity [1]. Nevertheless DES may sometimes by illegally used in some countries, together with other anabolic substances. The possibility of residues of these carcinogenic substances appearing in edible tissues [2] means that simple and reliable methods must be available for their analysis in biological samples. Critical for this kind of analysis is the preparation and purification of samples, which is often laborious and time-consuming. In fact most published methods require complex clean-up steps before instrumental analysis, or else rely on instrumentation (such as tandem mass spectrometry) that is not always available [3–14].

The use of immunoaffinity columns makes the purification of the samples simple, with high efficiency and recovery. The subsequent use of gas chromatography–mass spectrometry (GC–MS) for instrumental analysis, preferably with negative-ion chemical ionization (NICI), achieves high selectivity and sensitivity. This approach has been described for the analysis of several compounds, such as prostaglandins [15], 17β-estradiol [16] and 19-nortestosterone plus methyltestosterone [17], with good results.

This paper describes the development and use of anti-DES immunoaffinity columns for the extraction of DES and similar cross-reacting compounds, such as dienestrol and hexestrol, from urine and plasma. DES and the other compounds were determined by GC–NICI–MS as their pentafluorobenzyl derivatives. This type of derivatization was also used for the analysis of other natural estrogens, such as estrone, 17β-estradiol and estriol.

EXPERIMENTAL

Materials

Diethylstilbestrol (*trans*-diethylstilbestrol), dienestrol (α-dienestrol or *E,E*-dienestrol), hexestrol, estrone, 17β-estradiol, estriol, bovine serum albumin (BSA), complete Freund's adjuvant and sodium ethylmercurythiosalicylate (thimerosal) were from Sigma (St. Louis, MO, U.S.A.). All solvents, of analytical grade, were from Merck (Darmstadt, F.R.G.) or Farmitalia Carlo Erba (Milan, Italy). Ethyl chloroacetate, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC) and pentafluorobenzyl bromide (PFBBr) were

from Aldrich (Milwaukee, WI, U.S.A.). N,O-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was from Fluka (Buchs, Switzerland). Protein A-Sepharose and CNBr-Sepharose 4B were from Pharmacia (Uppsala, Sweden). ELISA microtitration plates were from Flow Labs. (Irvine, U.K.). Anti-rabbit immunoglobulin G (IgG), peroxidase labelled, was from Kirkegaard & Perry Labs. (Gaithesburg, MD, U.S.A.). β-Glucuronidase/arylsulphatase from *Helix pomatia* was from Boehringer (Mannheim, F.R.G.).

Preparation of DES–BSA conjugate and anti-DES antiserum

To obtain an immunogenic compound, DES was conjugated with BSA. First, a carboxyl group was introduced into the molecule of DES by a modification of the method reported by Rao and Moore [18]. Briefly, *trans*-DES (1 g) was treated for 30 min, under reflux, with ethyl chloroacetate (0.8 ml added dropwise), in 30 ml of anhydrous ethanol, in which 350 mg of pure sodium had been dissolved. The monocarboxymethyl-DES ethyl ester obtained was hydrolysed with 1% potassium hydroxide in methanol–water (9:1, v/v), with heating under reflux for 1 h. After drying, the reaction mixture was redissolved in water and unchanged DES was removed by extracting twice with diethyl ether and twice with ethyl acetate. The reaction product, monocarboxymethyl-DES, was extracted in the same way after acidification of the aqueous phase to pH 2. Recrystallization from ether–petroleum ether gave 140 mg of 99% pure product (analysed by GC–MS).

The carboxymethyl-DES was then conjugated with BSA according to the method reported by Abraham and Grover [19] for some steroids, with minor modifications. Water-soluble carbodiimide (EDC) was used as condensing agent: 0.3 ml of a solution of 80 mg/ml EDC in pyridine–water (1:1, v/v) were added dropwise, at room temperature and under stirring, to 30 mg of carboxymethyl-DES in 0.5 ml of the same solvent. The mixture was stirred constantly for 30 min, and then 0.5 ml of a solution of 40 mg/ml BSA in distilled water was added dropwise. The reaction was continued for 2 h, after which the DES–BSA conjugate was purified by dialysis. After lyophilization, the conjugate was characterized by UV spectroscopy, as described by Erlanger et al. [20], and gave a DES/BSA molar ratio of 42:1.

Two New Zealand rabbits were then immunized with subcutaneous injections of 1 mg of conjugate in 1 ml of complete Freund's adjuvant. Booster injections were repeated after three weeks and then every two weeks for two months. Immunoglobulin production was checked by immunoenzymic assay (ELISA), using DES–BSA and BSA as coating antigens and peroxidase-labelled anti-rabbit IgG as second antibody. After the fifth immunization the antisera did not show any further increase in the dilutions that gave positive results with ELISA. At this time these dilutions were 1:500 000 and 1:240 000 for the two antisera, using DES–BSA as coating antigen. The antisera were

purified and used for the preparation of affinity columns without further characterization.

Preparation and use of immunoaffinity columns

Immunoglobulins were purified from other serum proteins using a protein A-Sepharose column following the manufacturer's instructions. About 7 mg of IgG were recovered per ml of rabbit serum.

The IgG fractions of the two antisera were pooled and coupled with CNBr-activated Sepharose 4B (5 mg IgG per ml of gel) as recommended by the manufacturer.

Immunoaffinity columns were prepared by introducing 1 ml of coupled gel into glass columns (0.5 cm diameter, 10 cm length, with reservoir on top and Teflon stopcock at the bottom). Before use, the columns were washed with 10 ml of acetone–water (95:5, v/v), 2 ml of distilled water and 2 ml of 0.05 *M* phosphate buffer (pH 7.4). For extraction, the samples (0.5–2 ml of centrifuged plasma or urine) were diluted to 10 ml with phosphate buffer and left in contact with the gel in the columns for 30 min, under gentle mixing, and then allowed to elute slowly from the gel. The columns were washed with 10 ml of phosphate buffer and 2 ml of distilled water, and finally immuno-adsorbed substances were eluted with two 1.5-ml volumes of acetone–water (95:5, v/v). After washing with another 10 ml of elution solvent and regeneration with distilled water, the columns were stored at 4°C in phosphate buffer containing 0.02% of thimerosal.

Synthesis of internal standard

In order to measure the levels of DES and the recovery in each sample, a deuterated analogue of DES, to be used as internal standard, was synthesized. *trans*-2,2,3′,3″,5,5,5′,5″-Octadeuterodiethylstilbestrol (*trans*-$DESD_8$) was obtained by isotope exchange according to the method described by Liehr and Ballatore [21], except that the final reaction mixture, containing also *cis*-2,2,3′,3″,5,5,5′,5″-octadeuterodiethylstilbestrol (*cis*-$DESD_8$) and *cis*-2,3′,3″,4,5,5,5′,5″-octadeutero-3,4-bis(*p*-hydroxyphenyl)-2-hexene (ψ-$DESD_8$) was used without isolation of the single isomers. For quantitative purposes a constant amount of this isomer mixture (containing 0.4 ng of *cis*-$DESD_8$, 0.75 ng of ψ-$DESD_8$ and 1 ng of *trans*-$DESD_8$ in 10 μl of ethanol, determined by GC–MS) was added to samples and to standards.

Derivatization and GC–MS

Samples were derivatized for GC–MS after drying the acetone–water eluates in a stream of air at 60°C. Pentafluorobenzyl derivatives of DES, hexestrol, dienestrol, estrone, 17β-estradiol and estriol were obtained by reaction with PFBBr, using the following experimental conditions: 50 μl of a solution of PFBBr in acetonitrile (1:20, v/v) and 50 μl of a solution of potassium hydrox-

ide in anhydrous ethanol (8 mg/ml) were added to the dried samples in a conical tube and heated at 60 °C for 30 min. After evaporation of the solvents the samples were redissolved in 20–50 μl of BSTFA, heated at 60 °C for 30 min, then injected into the gas chromatograph.

Under these conditions PFBBr reacts with phenolic groups and BSTFA with the remaining aliphatic hydroxyl groups. Bispentafluorobenzyl derivatives were thus obtained for DES, hexestrol and dienestrol, and pentafluorobenzyl-trimethylsilyl derivatives for estrone, 17β-estradiol and estriol. BSTFA also seems useful as injection solvent for optimal chromatographic conditions.

Instrumental analysis was performed on a VG TS-250 mass spectrometer, equipped with an HP 5890 gas chromatograph. GC conditions were as follows: oven temperature from 160 °C (1 min) to 300 °C at 15 °C/min; injector temperature 240 °C, in splitless mode. The column was a CP Sil 5 CB (Chrompack Italia, Cernusco sul Naviglio, Italy), 25 m × 0.32 mm I.D., film thickness 0.12 μm, operated with a head-pressure of 30 kPa of helium. The mass spectrometer conditions were: source temperature, 180 °C; electron energy, 40–100 eV (CI negative) and 70 eV (electron impact, EI); emission current, 1000 μA (CI negative); trap current 500 μA (EI). Ammonia or isobutane was used as the reacting gas for the formation of negative ions by electron capture.

Treatment of animals

For validation of the method with biological samples, DES-positive urines were collected from three calves (two females, one male) injected subcutaneously with 10 mg of DES in 1 ml of coconut oil. Samples were collected three, six and ten days after treatment and analysed for DES content. Since glucuronic acid conjugation was previously reported [2,3,6,9], DES was analysed before and after hydrolysis with β-glucuronidase and arylsulphatase (100 μl/ml urine, 16 h at 38 °C in 0.05 *M* acetate buffer, pH 4.5).

RESULTS AND DISCUSSION

GC–MS determination with pentafluorobenzyl derivatives

The NICI and EI mass spectra and the structures of the derivatives obtained with the derivatization method described are shown in Fig. 1. The NICI spectra of all the compounds showed a significant fragment ion corresponding to the loss of a PFB group (M − 181) and the compounds containing two PFB groups also showed a fragment ion corresponding to further loss of a hydrogen fluoride group (M − 201). The EI spectra showed more fragmentation, although the molecular ion was present (except for hexestrol).

NICI was chosen as the ionization mode because it is more sensitive, on account of the low GC–MS background noise and the predominant formation of a single negative ion, which carries most of the total ion current. Selected-ion recording (SIR) was done on the most intense NICI fragments of each

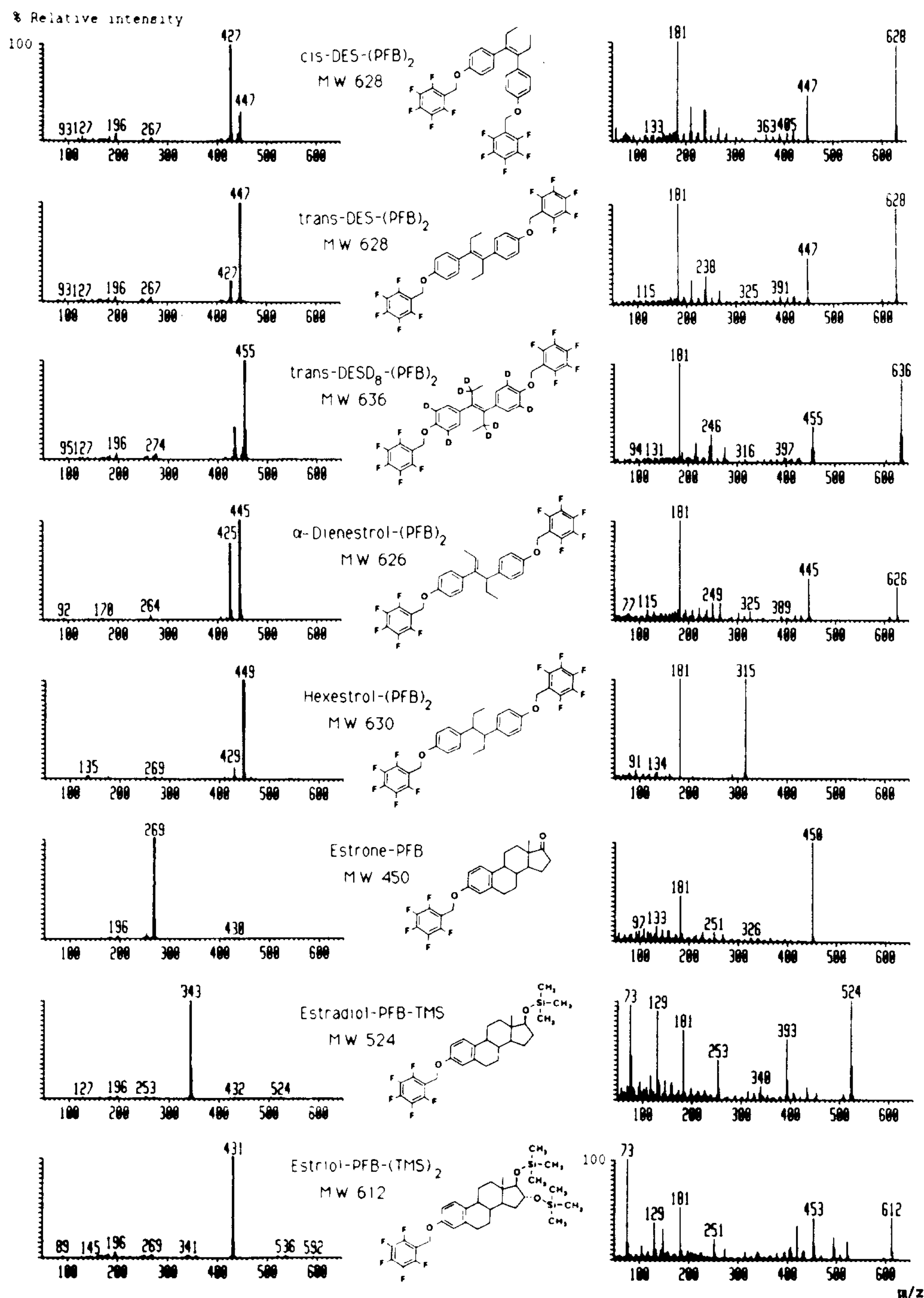

Fig. 1. Negative-ion chemical ionization (left) and electron-impact (right) mass spectra of pentafluorobenzylated derivatives of the estrogens indicated. For a description of the derivatization method and instrumental conditions see Experimental.

compound: m/z 427 for *cis*-DES, m/z 447 for *trans*-DES, m/z 435 for *cis*-$DESD_8$, m/z 455 for *trans*-$DESD_8$, m/z 445 for dienestrol and m/z 449 for hexestrol. The absolute sensitivity for standards of bispentafluorobenzyl-DES, using this type of ionization, was ca. 200 fg injected into the gas chromatograph.

An advantage of the derivatization method used is the simplicity and rapidity of the pentafluorobenzylation reaction, compared with those previously reported, which require phase transfer catalysis [22].

Immunoaffinity extraction

The characteristics of the immunoaffinity columns were determined by capacity tests and recovery tests from reconstituted blank samples of urine, plasma and buffer. Column capacity was determined by extracting 1 μg and 10 μg of *trans*-DES from phosphate buffer, and in both cases gave maximum recovery of 0.7 μg per ml of gel.

For recovery studies, samples of human and bovine urine and of human plasma (1 ml) were centrifuged and diluted 1:10 with phosphate buffer, and various amounts (20–1000 pg) of *trans*-DES, dienestrol, hexestrol, 17β-estradiol, estrone and estriol were added. The solutions were then extracted by the method described. A known amount of $DESD_8$ mixture was added after extraction and before derivatization, and was taken as reference for calculating the recoveries of the other estrogens. (Recoveries of the deuterated isomers were obtained in the same way using non-deuterated DES as reference.) Recoveries were calculated by comparison of the GC–SIR peak-area ratios (estrogen peak area/*trans*-$DESD_8$ peak area) of extracted samples and of standards containing known amounts of all the derivatized estrogens. Fig. 2 shows the chromatograms of recovery tests for some of these estrogens.

Table I shows the recoveries of DES isomers, dienestrol and hexestrol from buffer, urine and plasma. Estrone, 17β-estradiol and estriol were not recovered from the columns, suggesting that the antibodies did not cross-react with these natural estrogens, which may be present in biological samples (data not shown).

The calculation of the recovery for DES is complicated by *cis–trans* isomerization. In fact, standards made from solutions of pure *trans*-DES plus $DESD_8$ mixture, and recovery studies of *trans*-DES from buffer, showed the presence of *cis*-DES, with a *cis/trans* ratio of 0.33 ± 0.05 S.D., $n=6$, which was independent of the absolute amount of *trans*-DES and $DESD_8$ mixture present. The *cis/trans*-$DESD_8$ ratio in the deuterated standard was, in the same way, constant (0.40 ± 0.08 S.D., $n=12$). It was assumed for the calculations, as reported [23], that the isomerization of added *trans*-DES to *cis*-DES does not take place during or before the extraction procedure but only during derivatization [4–6]. With the deuterated standard, instead, the isomerization was already present before derivatization as a result of the deuteration procedure (see Experimental). Therefore the recovery of added *trans*-DES was calculated from the sum of the peak areas of *cis* and *trans* isomers, and the recoveries of the deu-

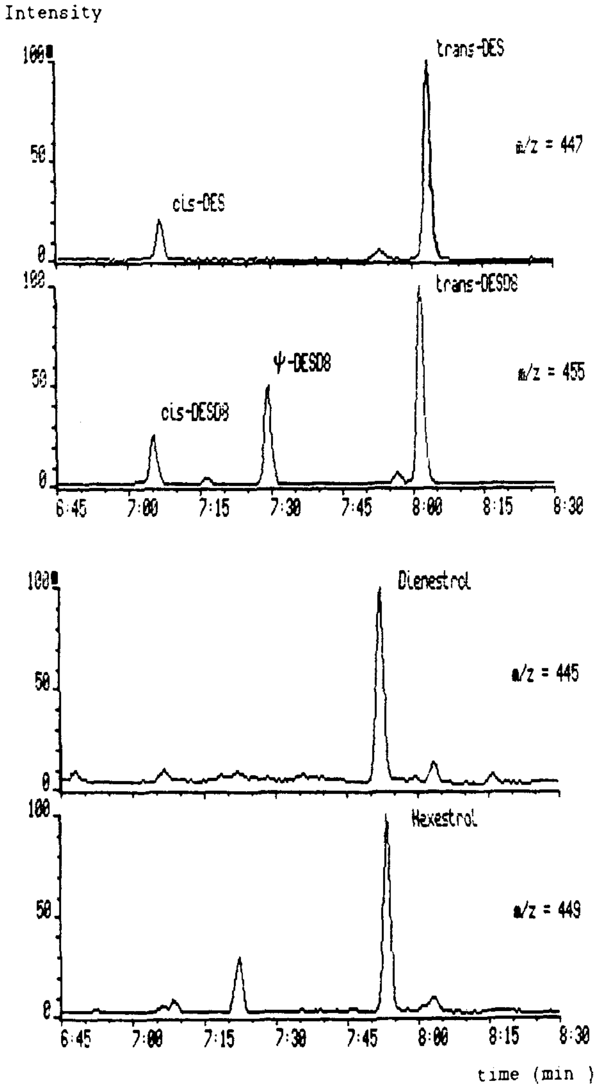

Fig. 2. Selected-ion recording tracings of bovine urine spiked with 200 ppt of diethylstilbestrol (DES), octadeuterated diethylstilbestrol ($DESD_8$, mixture of isomers), dienestrol and hexestrol, and extracted with an anti-DES immunoaffinity column. Conditions as described in Experimental.

terated isomers were calculated separately. On the other hand, the presence of *cis*-DES before extraction has been demonstrated in biological samples of treated animals [3–6], as will be discussed later.

The results show the usefulness of the immunoaffinity columns for the extraction of DES and similar compounds. These columns' ability to extract all these compounds might be due not only to the natural polymorphism of poly-

TABLE I

RECOVERIES OF DES ISOMERS, DIENESTROL AND HEXESTROL FROM SPIKED BUFFER, URINE AND PLASMA WITH EXTRACTION WITH ANTI-DES IMMUNOAFFINITY COLUMNS

Estrogen	Recovery (mean ± S.D.) (%)		
	From buffer	From urine	From plasma
trans-DES[a]	92 ± 12	89 ± 18	82 ± 21
cis-$DESD_8$[b]	70 ± 14	67 ± 22	54 ± 15
trans-$DESD_8$[b]	88 ± 9	96 ± 16	83 ± 11
ψ-$DESD_8$[b]	42 ± 14	52 ± 20	33 ± 13
Dienestrol[a]	52 ± 8	53 ± 10	28 ± 7
Hexestrol[a]	61 ± 8	67 ± 15	48 ± 9

[a] $n = 4$.
[b] $n = 12$.

clonal antisera, but also to the presence of both *cis*- and *trans*-DES (which have different spatial structures) on the surface of the immunizing agent (DES–BSA conjugate), giving rise to further variability of immunoglobulin populations. The sufficient specificity of the columns, however, is demonstrated by the fact that other natural estrogens were not recovered.

Quantitation methods

Samples were quantitated by an isotope dilution method, adding a constant amount of $DESD_8$ mixture before extraction, as specified in Experimental. Calibration curves were obtained from standards containing different amounts of estrogens (10–20 000 pg) and a constant amount of $DESD_8$ mixture, by plotting peak-area ratios (estrogen peak area/*trans*-$DESD_8$ peak area), obtained with GC–SIR, against absolute amounts of estrogens. These calibration curves gave a standard error of 6.7% (determined with triplicate analyses) for 1-ml urine samples reconstituted with *trans*-DES at the 200 ppt level, and standard errors of 14.5 and 7.2%, respectively, for dienestrol and hexestrol under the same conditions.

In biological samples, containing both *cis*- and *trans*-DES [3–6], these two isomers were quantified separately, by using their peak-area ratios to the respective deuterated isomers. As there was no standard available for *cis*-DES, the standard curve for *trans*-DES was used also for the quantitation of the *cis*-isomer, introducing the correction factor (0.4) calculated from the *cis/trans* ratio in the deuterated standard. The two isomers must be calculated separately because of their different extraction recoveries (Table I).

The lowest level of *trans*-DES detectable with the method, determined with reconstituted blank urine and plasma, was ca. 10 ppt for 1-ml samples, using

GC–NICI-SIR as detection system. For GC–EI-SIR detection on the molecular ion (m/z 628) the sensitivity was ca. 100 ppt.

Analysis of biological samples

Urine samples from calves treated with DES were analysed by the method described. The levels of DES after three, six and ten days were determined, before and after hydrolysis with β-glucuronidase and arylsulphatase.

The results are listed in Table II. Together with the expected decreases in DES levels with time, there were marked differences between individuals. In fact urinary excretion of free *cis*- and *trans*-DES was high in the male calf but very low in the two females. The sum of the total urinary concentrations of DES (free + conjugated), instead, was similar in the three animals.

The *cis/trans*-DES ratio found in urine samples was often abnormally high compared with that observed in standard samples, and quite variable (Table II and Fig. 3), ranging from 0.5 to 2.0. This altered *cis/trans*-DES ratio has been reported in the literature, where enzymic or photo-reactions have been cited as possible explanations [3–6]. We found that incubation with β-glucuronidase and arylsulphatase from *Helix pomatia* strongly affected this ratio and also the relative ratios of *cis*-$DESD_8$, *trans*-$DESD_8$ and ψ-$DESD_8$ in the deuterated internal standard mixture, supporting the hypothesis that enzymic processes are involved [23]. To take account of these changes in the isomer ratios of the internal standard, a known amount of hexestrol was added to all

TABLE II

DES LEVELS IN CALF URINE AFTER A SUBCUTANEOUS INJECTION OF 10 mg OF DES IN OIL

Urine samples of a control calf were always negative (<0.01 ppb).

Calf	Days after treatment	DES concentration (ppb)					
		Free			Glucuronic acid-conjugated		
		cis	*trans*	Total	*cis*	*trans*	Total
1 (female)	3	0.045	0.058	0.103	26.4	22.4	48.8
	6	0.011	0.021	0.032	1.12	1.32	2.44
	10	<0.010	<0.010	<0.010	0.17	0.26	0.43
2 (female)	3	0.047	0.048	0.095	20.0	10.7	30.7
	6	0.053	0.066	0.119	11.8	10.9	22.7
	10	0.014	0.017	0.031	2.07	2.03	4.10
3 (male)	3	7.94	4.34	12.3	15.9	7.86	23.8
	6	0.26	0.22	0.48	1.19	1.06	2.25
	10	0.042	0.041	0.083	0.058	0.079	0.14

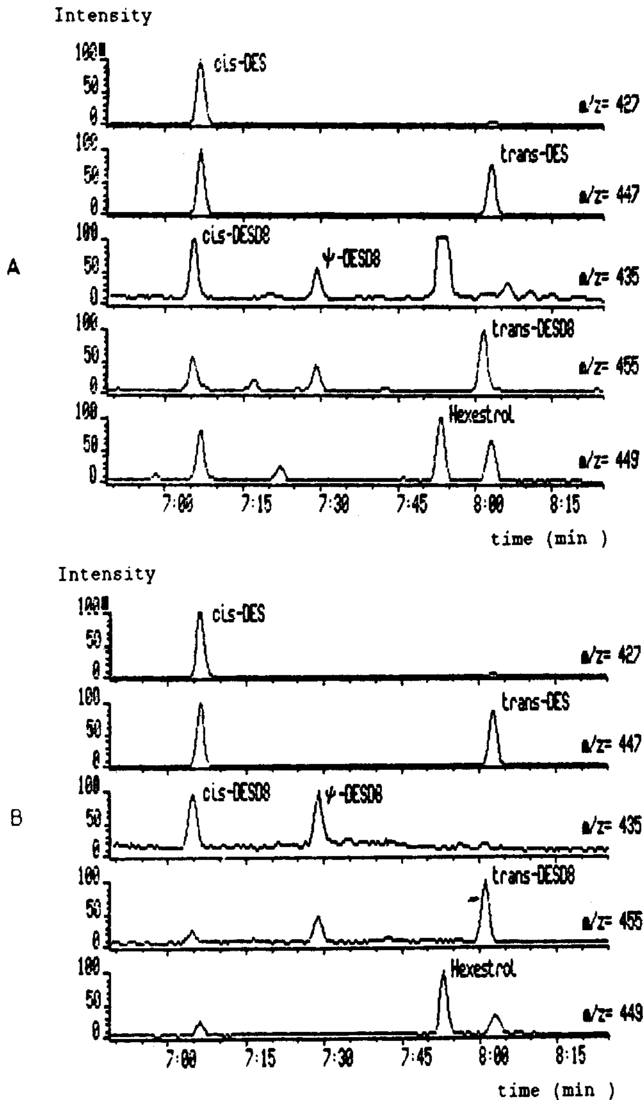

Fig. 3. Selected-ion recording tracings of urine from a calf treated with diethylstilbestrol (DES), showing altered *cis/trans*-DES ratios. Samples were extracted with immunoaffinity columns using octadeuterodiethylstilbestrol ($DESD_8$, mixture of isomers) as internal standard and hexestrol as external standard to check recovery. Conditions as described in Experimental. (A) Without hydrolysis with β-glucuronidase and arylsulphatase; (B) after hydrolysis with β-glucuronidase and arylsulphatase.

samples, after extraction, as external standard. The relative amounts of *cis*-$DESD_8$, *trans*-$DESD_8$ and ψ-$DESD_8$ found after extraction, before and after hydrolysis were, respectively, 0.31:1:0.41 and 0.49:0.8:0.27 (means of twelve

samples). Quantitative determinations after hydrolysis took account of these differences.

CONCLUSION

The experiment with calves shows the applicability of the highly sensitive and specific method described to the analysis of biological samples. The method also allows the simultaneous determination of *cis*- and *trans*-DES, of hexestrol and dienestrol. Another advantage is its rapidity: we found that six to twelve samples can be analysed in one day, making the method suitable for urgent analyses.

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