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SENSITIVE AND SPECIFIC POST-COLUMN FLUORIMETRIC DETERMINATION OF DIETHYLSTILBOESTROL RESIDUES IN EXTRACTS OF URINE AND ANIMAL TISSUES AT THE 1-ppb* LEVEL

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SUMMARY

A rapid method for the selective detection and quantitation of diethylstilboestrol (DES) residues at the 1 ppb level in extracts of urine and animal tissues is described. After selective extraction of the oestrogens, DES is analysed by high-performance liquid chromatography using an in-line specific photochemical reactor followed by oxidation to highly fluorescent products. The reaction products of DES were investigated by thin-layer chromatography (TLC). The specificity of the proposed method was compared with that of a reference TLC method on different extracts of animal origin.

INTRODUCTION

In spite of the EEC ban on the use of the stilbenes as growth promoters in cattle, recent surveys still point to the persistent use of diethylstilboestrol (DES), 4,4′-(1,2-diethyl-1,2-ethenediyl)bisphenol, in different EEC countries[1–4]. Regulatory control, based on rapid and reliable analytical techniques, will stop the illegal use of this potential carcinogen.

Efficient analytical techniques should be able to monitor residue levels encountered in the tissues and excreta of farm animals after the application of this anabolic agent. After treatment of cattle, the residue levels of DES in the animal tissues, urine and faeces are dependent on many factors (*e.g.*, formulation, dose, site of application and time of application before slaughtering[5,6]), but generally lie in the range 0.1–10 ppb[6–8]. Regulatory control thus needs methods with adequate sensitivity to detect illegal DES treatment. In view of the eventual juridical consequences of positive DES findings in the carcasses of slaughtered animals, the techniques used should be very reliable. Moreover, the analytical methods should have sufficient capacity to allow the routine detection of DES residues and should be simple and cheap. At the moment, none of the existing methods fulfill these requirements.

* Throughout this article, the American billion (10^9) is meant.

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Direct radioimmunoassay (RIA) of DES in urine is sufficiently sensitive, rapid, cheap but lacks the desired specificity[1,9]. RIA methods, after pre-purification of the extracts[9], may be utilized to screen a large number of urine samples for the presence of DES at the 1 ppb level. In view of the occurrence of false-positive DES values during RIA assays of cow urine, this method should be complemented by independent chemical methods with a very high degree of specificity[1,2] and which allow detection at a level at least equal to the decision limit proposed for RIA (1 ppb).

At present, DES can be determined in urine, tissues or faeces by a range of chemical methods after suitable clean-up procedures. Procedures have been reported using gas chromatography (GC) with an electron-capture detector[10,11] or mass spectrometric (MS) detection[12–14], high-performance liquid chromatography (HPLC) with electrochemical[15,16] or fluorescence[17] detection and by high-performance thin-layer chromatography (HPTLC) using sulphuric acid-induced fluorescence[18], fluorescence introduced by group-selective reagents[19,20] or fluorescence induced by photochemical reactions[21]. However, many of these methods lack the required sensitivity or selectivity[1,2,6] for the successful detection of DES residues in cattle tissues or excreta. GC–MS (multiple ion monitoring) meets the desired specificity but requires expensive apparatus and expert technical assistance and may lead to bottlenecks during confirmatory analysis in regulatory control. Two-dimensional TLC, using sulphuric acid-induced fluorescence, offers a simple means for the sensitive (0.2–0.5 ppb), selective detection of various anabolics, especially DES[18]. This method has been proposed to the EEC and adopted as one of the recommended methods[22] for the detection of anabolics in meat. Comparative studies in The Netherlands on the detection of DES in urine of calves[1] have shown that, of the methods tested, this method yielded results corresponding closely with those obtained by GC–MS.

However, all chemical methods require a lengthy and tedious clean-up procedure before the successful detection of DES at the 1 ppb level can be attempted. Apparently, in contrast to analyses of steroids in clinical chemistry[23,24], little attention has been paid to the selective extraction of hormonal residues from animal tissues or excreta, so that purification and pre-concentration of the extracts are the rate limiting steps in analysis. Recently, a rapid clean-up procedure was developed in our laboratory, offering a simple, rapid and selective extraction of oestrogens from cattle urine[25]. This extraction procedure, coupled with an automated analytical procedure with a high detection specificity, would increase significantly the efficiency of regulatory control against the illegal use of DES.

In this paper we describe a sensitive and rapid automatic procedure for the detection of DES residues at the 1 ppb level in biological extracts from cattle. The method is based on the transformation of DES (I) into a photochemical reaction product (II) and specific oxidation of the latter to highly fluorescent products [*e.g.*, phenanthrenediol (III)] (see Fig. 1). The mechanism of these reactions has been investigated previously[26,27] and some of the reaction products have been utilized for the detection of DES in urine[17,21] and animal tissues[28,29].

In the procedure described, the extracts are injected on to an RP-18 HPLC column and the effluent is directed into an in-line post-column derivatization system. The resulting reaction mixture is passed through the LC flow cell of a fluorescence detector. For optimization of the reaction, the main reaction products were isolated and studied by TLC and fluorescence spectroscopy. The specificity of the procedure

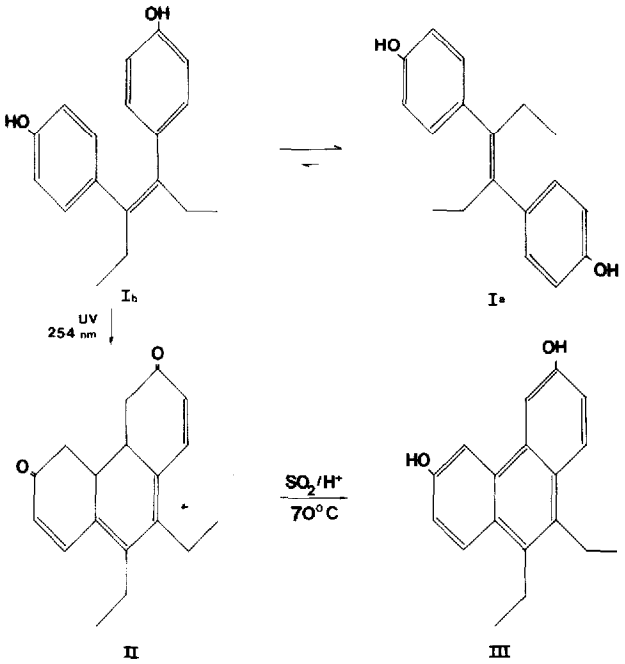

Fig. 1. Reaction scheme for the conversion of DES (I) to phenanthrenediol (III).

has been checked by analysing more than 60 extracts of urine or animal tissues by both the proposed and the TLC[7,18] methods.

EXPERIMENTAL

Reagents and reference compounds

Silica gel 60 thin-layer micro-plates without fluorescence indicator were obtained from E. Merck (Darmstadt, G.F.R.) (Cat. No. 5631) and Amberlite XAD-2 (300–1000 μm) and Polyclar AT (polyvinylpyrrolidone) from Serva (Heidelberg, G.F.R.).

Glucuronidase–sulphatase enzyme suspension (suc d'*Hélix pomatia*: 100,000 Fishman units of glucuronidase + 1,000,000 Roy units of sulphatase) was obtained from IBF (Clichy, France).

Reference diethylstilboestrol and dienestrol were obtained from Bios-products (Brussels, Belgium). Radioactive DES ([monoethyl-^{3}H]diethylstilboestrol) was purchased from Amersham International (Amersham, Great Britain).

Methyl Cellosolve (ethylene glycol monoethyl ether) of Sequanal grade, was purchased from Pierce (Rockford, IL, U.S.A.) and PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene] from Packard (La Grange, IL, U.S.A.).

High-purity HPLC water was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents were of analytical-reagent grade and used without further purification.

Solutions

Solvent systems for HPTLC were (1) *n*-hexane–diethyl ether–dichloromethane (4:3:2) and (2) chloroform–ethanol–benzene (36:1:4). Dipping solutions for the HPTLC plates were 5% sulphuric acid in ethanol–diethyl ether (1:1) and 30% liquid paraffin in *n*-hexane.

Stock solutions of DES or dienestrol were prepared in ethanol at a concentration of 200 μg/ml. These solutions were stored in the dark.

Phosphate buffer stock solution (0.1 *M*) was prepared by dissolving 4.6 g of $K_2HPO_4 \cdot 3H_2O$ in 200 ml of distilled water. The bisulphite stock solution (0.35 *M*) contained 6.7 g of $Na_2S_2O_5$ in 100 ml of distilled water. For the automated analysis, each day fresh solutions in HPLC water were prepared of 0.022 *M* K_2HPO_4, 0.052 *M* $Na_2S_2O_5$, 3.7 *M* hydrochloric acid and 4.75 *M* sodium hydroxide.

The methanol–water mixtures for chromatography were degassed under vacuum during ultrasonification.

Apparatus

The following apparatus was used: homogenizer (Ultra-Turrax), centrifuge, rotary vacuum evaporator with vacuum source, water-bath, chromatographic tanks, heating cabinet, UV (366 nm) transilluminator (C-62; UV Products, San Gabriel, CA, U.S.A.), sample applicator[18] and Zeiss KM 3 TLC scanner (Zeiss, Oberkochen, G.F.R.) operated with a mercury lamp.

The UV–visible spectrophotometer used was a Varian Techtron Model 635. The fluorescence of the different intermediates was measured either in a Mark I

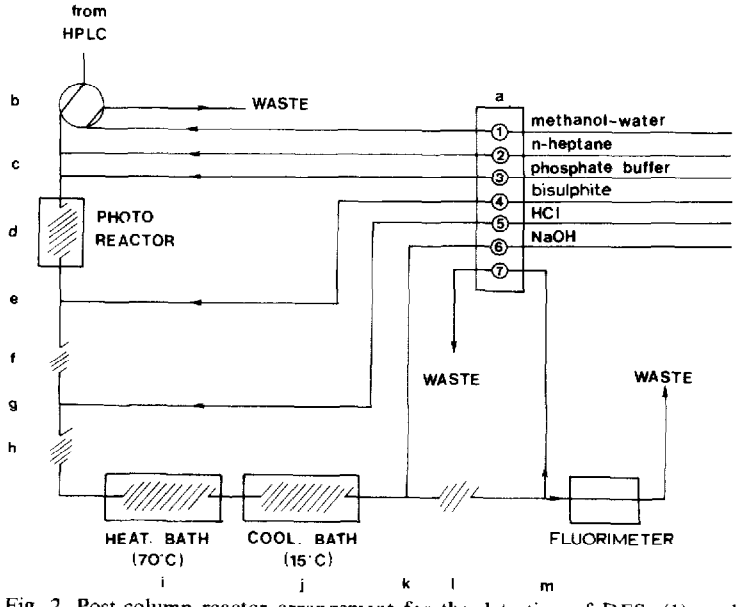

Fig. 2. Post-column reactor arrangement for the detection of DES: (1) methanol–water (65:35) (0.70 ml/min); (2) *n*-heptane (0.4 ml/min); (3) phosphate buffer, 0.02 *M* (0.1 ml/min); (4) hydrogen sulphite solution, 0.05 *M* (0.1 ml/min); (5) 3.7 *M* HCl (0.2 ml/min); (6) 4.8 *M* NaOH (0.2 ml/min); (7) organic solvent from phase separator (0.9 ml/min).

spectrofluorimeter (Farrant Optical, New York, NY, U.S.A.) or in a Model LS5 (Perkin-Elmer, Norwalk, CT, U.S.A.) apparatus.

The liquid chromatograph was a Varian (Palto Alto, CA, U.S.A.) 8500 gradient system. Pump A contained a thoroughly degassed water–methanol mixture (9:1) and pump B contained the same mixture in a 1:9 ratio. Sample injection was made via a Valco six-port injection valve, with a 10-μl loop injector.

The RP-18 analytical column system had a total length of 68 cm; a 5 × 0.46 cm (10 μm) guard column (Alltech Europe, Eke, Belgium) and two Hibar RT 250-4 (25 × 0.46 cm LiChrosorb, 5 μm) columns (E. Merck, Darmstadt, G.F.R.) were used in series. The columns were mounted in a water jacket and held at a constant temperature of 55°C. The column effluent was led via a Hamilton (Bonaduz, Switzerland) miniature inert valve with a four-port housing and a four-port plug to an in-line post-column reactor system. The arrangement is shown in Fig. 2.

The system consists of a Technicon (Tarrytown, NY, U.S.A.) AutoAnalyzer II proportioning pump (a), with flow-rated pump tubes. The injector (c) (116-0489-01)*, T-pieces (e, k) (115-B034-01) and (g) (116–0200-01S), mixing coils (f) (170-0103-01), (h) (157-0251-01) and (l) (157-0248-01), heating bath (70°C) with heating coil (i) (7.7 ml) (157-0225-01) and phase separator (m) (116-0200-02) were Technicon parts. The cooling bath consisted of a five-turn coil (170-0103-01) immersed in a jar with a water-jacket (j). All solutions or solvents were sucked through a glass filter. To minimize band broadening[30], *n*-heptane was used for solvent segmentation.

The photochemical reactor (d) consisted of a twin-lamp module (Model 3090) containing two low-pressure mercury (254 nm) lamps (Model 3020; Applied Photophysics, London, Great Britain) placed in a reflector. The solvent stream was passed through a PTFE capillary[31] of 1 mm I.D., 1.59 mm O.D. and length of 5.4 m (purchased from Alltech Europe) coiled around the two UV lamps.

A Schoeffel FS-970 LC fluorescence detector (Schoeffel, Westwood, NY, U.S.A.) with a 5-μl flow-through cell was employed to detect the fluorescence reaction products (excitation wavelength 260 nm; emission cut-off filter 370**). The signal was recorded on a Varian A-25 strip-chart recorder.

Sample preparation

Hormones were extracted from cow urine by using a specific adsorption column of oestrogens from a urine hydrolysate[25]. Urine (50 ml) was allowed to percolate through an Amberlite XAD-2 column (80 × 10 mm) at 2 ml/min. The column was washed with 50 ml of methanol–water (30:70) and the eluates and washings were discarded. The conjugated and free steroids were eluted with 15 ml of methanol. The eluates were reduced to a volume of 1 ml under a jet of nitrogen in a Reacti-term heating Module (Pierce) at 60°C. The residue was taken up in 10 ml of acetate buffer (0.2 *M*, pH 5.2) and 0.20 ml of glucuronidase–sulphatase was added. The mixture was incubated for 2 h at 62°C, then the enzyme digest was cooled and percolated through a polyclar column (50 × 10 mm) at a rate of 2 ml/min. After washing the column with 50 ml of distilled water, the estrogens were eluted with 16 ml of methanol in reaction tubes. The methanol was concentrated to *ca.* 1 ml under a jet

* Technicon part number.

** According to the FS-970 LC manual, the filter number denotes the wavelength at 50% normalized transmission.

of nitrogen at 60°C. The contents of the tube were quantitatively transferred into a conical graduated tube (fibrinogen tube) and concentrated to 100 μl under a stream of nitrogen.

Hormones were extracted from 50 g of animal tissues as described by Verbeke[18,22]. After adsorption of the estrogen fraction by a basic Celite column, the column was eluted with diethyl ether. The eluate was evaporated to dryness and dissolved in 100 μl of methanol.

Determination of ^{3}H-labelled DES

A Packard Tri-Carb 3003 liquid scintillation spectrometer was used, with toluene–methyl Cellosolve (4:1) containing 1.2% of PPO and 0.004% of dimethyl-POPOP as scintillation liquid. Samples were spiked with 250,000 cpm of tritiated DES. Samples taken at different steps in the procedure were assayed as described earlier[18].

Quantitative determination of DES after thin-layer chromatography

The amount of DES in the extracts was determined through an adaptation of the antidiagonal technique described by Beljaars *et al.*[32]. Appropriate amounts of the sample extract (1–5 μl) and three standard amounts of DES (1, 2 and 4 ng) were applied in the left-hand corner of a pre-coated silica gel 60 HPTLC plates along an antidiagonal[7]. Chromatographic development was carried out in a non-saturated tank, using solvent 1, over a distance of 6 cm. The plate was air-dried and then run in the second direction, using solvent 2, over a distance of 6 cm. After drying, the plate was immersed in 5% sulphuric acid in ethanol–diethyl ether for 30 sec. The plate was air-dried, incubated at 95°C for 10 min and then observed under transillumination at 366 nm. The fluorescent spots of *cis*- and *trans*-DES in the sample and standards are situated along a line. The fluorescence intensity of *trans*-DES was scanned using a Zeiss TLC scanner with excitation at 366 nm and a cut-off filter at 620 nm. A linear relationship was observed between the fluorescence intensity and the amount of DES on the HPTLC plate. Recoveries of 0.5–5 ppb of DES in urine, corrected for extraction yield, were 100 ± 10%[25].

HPLC procedure

An injected sample (10 μl) was eluted isocratically with methanol–water (65:35) at a flow-rate of 0.7 ml/min. After complete elution of DES (retention time 25 min) the column was flushed for 12 min with methanol–water (9:1). The column was then re-equilibrated with methanol–water (65:35). The second analysis can be performed 55 min after the first injection.

RESULTS AND DISCUSSION

Study of the reaction products of DES

The reaction is based on the conversion of DES by irradiation to a yellow photoproduct followed by conversion at 75°C by sulphur dioxide in alcoholic solution to a highly fluorescent DES derivative (Fig. 1). In order to effect on-line detection in HPLC the reaction conditions should be optimized with respect to a continuous flow system and the reaction products should be characterized for optimal measurement of

TABLE I

CHARACTERISTICS OF REACTION PRODUCTS FORMED FROM DES

Substance	*Reaction yield (%)*	*R_F value in solvent 1*	*Colour in UV (366 nm) on HPTLC plate*	*Detection limit of DES*		
				On HPTLC plate (ng)	*In flow cell (ng/ml)*	*In HPLC–PCD§ analysis (ng on-column)*
trans-DES		0.60	Red*	0.5		
cis-DES		0.34				
Phenanthrene-dione	65	0.26	Yellow	<0.5	6	60
Reaction mixture					0.2***	1.5***
Product c	37	0.49	Light brown	<3		
Product b	22	0.22	Light brown**	<0.5		
Product d	6	0.76	Blue**	<0.5		

* After dipping in ethanolic sulphuric acid and heating.
** After prolonged irradiation at 366 nm (30 min).
*** Values expressed as ng DES.
§ PCD = Post-column derivatization.

the fluorescence. In preliminary studies the reaction sequence was studied and the fluorescent products were extracted and characterized by TLC and fluorescence spectroscopy (Table I).

The optimum irradiation time was studied by pumping standard DES solution (20 μg/ml), diluted with an equal volume of 0.1 *M* phosphate buffer, through the reactor and measuring the absorbance of the yellow derivative at 418 nm. Maximal conversion was obtained after irradiation for 0.5 min. Prolonged irradiation of the solution decreased the absorption slightly: after 2 and 6 min, 80 and 70% of the maximal absorption were measured, respectively.

The irradiation product was isolated after passing a solution containing 300 ml of stock DES (200 μg/ml, spiked with $3 \cdot 10^6$ cpm of [^{3}H]DES) and 200 ml of 0.1 *M* phosphate buffer through the photochemical reactor at a rate of 2 ml/min. The irradiated, yellow solution was extracted three times with 100 ml of dichloromethane. The dichloromethane phase was washed successively three times with 50 ml of 1 *M* sodium-hydroxide solution followed by 2 × 100 ml of water to a neutral reaction. After drying over sodium sulphate, the solvent was evaporated and the orange-yellow syrup was taken up in 10 ml of benzene; 76% of the added label was recovered in this extract.

A stock solution of the irradiation product was prepared by evaporating 0.1 ml of the benzene solution in a 100-ml flask and dissolving the residue in 100 ml of ethanol–0.1 *M* phosphate buffer (1:1). The absorption spectrum of this solution was similar to that described by Banes[26] for phenanthrenedione with maximum absorption at 418 and 290 nm. The uncorrected emission spectrum ($\lambda_{em.}$ = 388 nm) was similar to that reported by Rhys-Williams *et al.*[17] with an excitation wavelength of 280 nm. The recovery of DES as the phenanthrenedione in the original solution was calculated from the known molar absorptivity (18.9 l mmol^{-1} cm^{-1} at 418 nm[26]) to 65%.

The irradiation product was examined by TLC: 5 μl of the benzene solution was diluted to 10 ml with toluene (1.9 ng/μl of diketone) and increasing amounts of this solution (0.25–10 μl) were applied on the plate. After one-dimensional development, the plates were dipped in liquid paraffin solutions and viewed under UV light. The product yielded a single bright fluorescent spot (spot a; Table I); the detection limit was less than 0.5 ng/spot. The phenanthrenedione has the same relative mobility and fluorescence characteristics as when a DES spot on the plate, prior to development, is exposed to UV irradiation.

The conversion of the diketone by acidic hydrogen sulphite was performed as described by Ponder[29]: 10 μl of the diketone solution in benzene (39 μg) was evaporated, the residue was dissolved in 3 ml of ethanol and after addition of 3 ml of $Na_2S_2O_5$ (0.35 *M*) and 2 ml of 12 *M* hydrochloric acid the mixture was heated at 70°C for 15 min. The mixture was extracted three times with 2 ml of toluene (extract A). A 1-ml volume of the toluene solution was evaporated and the residue dissolved in 0.25 ml of ethanol. Different amounts (1–5 μl) of this solution were applied to HPTLC plates. Phenanthrenedione (10 ng) and DES (10 ng) were applied in the side lanes. Immediately after development and drying, the plate was examined under UV light: a brown, weakly fluorescent spot (spot c) with a higher mobility of the diketone (spot a) was observed (see Table I). During prolonged irradiation at 366 nm this spot disappeared gradually while two other spots became visible. After 30 min of irradiation an intense blue fluorescent spot (spot d, $R_F = 0.76$) and a light brown spot (spot b) with an R_F value slightly lower than that of the diketone was observed.

In an attempt to study the conversion products of the phenanthrenedione, the toluene extract (A) was extracted with three portions of 2 ml 1 *M* of sodium hydroxide solution (Fig. 3). The toluene phase was washed with water to a neutral reaction, evaporated and the residue dissolved in 1 ml of ethanol (extract C). The alkaline aqueous phase was acidified with 1 *M* hydrochloric acid (6 ml; pH = 1) and re-extracted with three portions of 2 ml toluene. The toluene was evaporated and the residue dissolved in 1 ml of ethanol (extract B). Increasing amounts (0.5–5 μl) of extracts B and C were spotted, developed and viewed under UV light. After 30 min of irradiation at 366 nm it was found that extract B contained substance b while the blue fluorescent product d was present in extract C. Extracts B and C were diluted with methanol and their excitation and emission spectra were recorded. Spectra of the blue fluorescent product ($\lambda_{exc.} = 320$ nm and $\lambda_{em.} = 385$ nm) and substance b ($\lambda_{exc.} = 315$ nm and $\lambda_{em.} = 390$ nm) were obtained. The spectrum of substance b was similar to that of the phenanthrenediol[26]. From the recovery of the label in the different extracts the yield of the products in each fraction was calculated (see Table I).

From these experiments it is evident that conversion of the phenanthrenedione in acidic methanolic solution results in the formation of at least three substances detected by TLC. Two substances were isolated: the phenanthrenediol (substance b) and an intensely blue fluorescent product (d).

On-line fluorimetric detection of the tricyclic diketone

In preliminary experiments alternatively ethanol or an ethanolic solution of tricyclic diketone were pumped (1 ml/min) through the flow cell of the fluorimeter ($\lambda_{exc.} = 280$ nm and cut-off filter 370) at 5-min intervals. A linear relationship was obtained between the height of the response and the concentration of the solution; the detection limit was about 6 ng/ml of diketone.

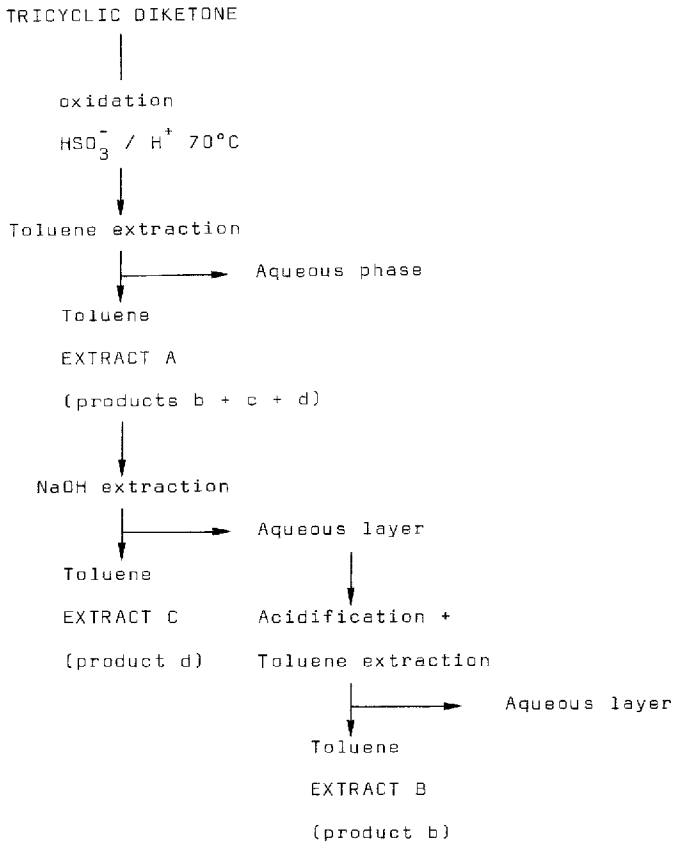

Fig. 3. Extraction scheme for the isolation of the oxidation products of phenanthrenedione.

On-line detection of DES as the tricyclic diketone was studied by injecting 10 μl of the DES stock solution on to an HPLC column (see Experimental) and passing the effluent stream, after mixing with a phosphate solution, through the photochemical reactor prior to fluorescence detection. The use of PTFE coils in a UV reactor has been reported by Scholten *et al.*[31]. The length and diameter of the capillaries are a function of the optimized irradiation time and the solvent flow. The influence of different phosphate concentrations was tested; the optimal reaction yield was observed between 0.25 and 0.02 *M* phosphate. If the reaction was performed in the presence of water or 1.10 *M* phosphate, 25% or 60% of the DES, respectively, was converted into the tricyclic diketone.

The influence of different solvents on the yield of the photochemical conversion reaction was studied by measurement of the absorption of the cyclization product. The highest conversion was obtained in acetonitrile–water (1:1). In the presence of ethanol–water (1:1) or methanol–water (1:1) the yield (95% or 80%, respectively) was only slightly decreased. Tetrahydrofuran–water (3:2) was unsuitable for the photochemical conversion (reaction yield about 15%).

Conversion of trycyclic diketine during automatic analysis

An alcoholic solution of the diketone was converted in the presence of acidic hydrogen sulphite at 70°C for 15 min as described previously. The solution was neutralized with sodium hydroxide and different dilutions in methanol–water (1:1) were prepared. The solutions were pumped through the fluorimeter ($\lambda_{exc.}$ = 260 nm; emission cut-off filter 370) at a rate of 1 ml/min. The detection limit was about 0.2 ng/ml of the reaction product of DES, *i.e.*, a sensitivity approximately 50 times higher than that found for the tricyclic diketone. The conversion reaction performed in acetonitrile–water gave only a very low conversion (about 20% in comparison with the reaction in ethanol–water) of the tricyclic diketone to the fluorescent reaction products.

To minimize band broadening during post-column derivatization in long reaction coils with tubing of about 2 mm I.D., segmentation of the solvent stream is necessary[30]. A two-phase system, with solvent segmentation, entails the risk of some extraction of the reaction products in the separation fluid. Alternatively, owing to its compressibility, air segmentation may cause resonance phenomena in the reaction coil, disturbing the bubble train. After trying different solvents, *n*-heptane was selected for solvent segmentation.

In subsequent experiments, the reaction train was studied under normal reaction conditions. The parameters investigated were the residence time, the temperature of the heating bath and the concentrations of the reagents. The residence time of the solvent stream in the heating bath was studied by substituting different reaction coils. A reaction time of at least 3 min at 70°C gave an optimal fluorescence intensity. Lower temperatures gave a lower relative fluorescence intensity, precluding the use of lower boiling organic solvents for segmentation.

Detection in acidic solution may affect the stainless-steel tubing of the fluorimeter and yielded a very low response (about 33%) compared with neutralized solutions. Exact neutralization of the acidic solvent could not be realized owing to minor fluctuations in the rate of reagent addition by the different pump tubes to a reagent stream of low buffer capacity. The pH variations in the solvent stream distinctly increased the noise levels, which were four times as high as with an alkaline solvent. In alkaline medium (pH > 10) the relative fluorescence intensity was about 75% of that of a neutral solution but a flat baseline was obtained. Addition of buffers to the solvent stream before fluorimetric detection was also attempted; however, the high salt concentration in the solvent stream occasionally clogged the fluorimeter cell.

Fig. 4 shows a chromatogram of 10 ng of DES under the selected chromatographic conditions (retention time 38 min); the limit of detection was about 1.5 ng on the column (signal to noise ratio = 3). A linear relationship was obtained between the amount of DES injected and peak height or peak area (r = 0.99; data not shown). As is expected from the similarity in structure with DES, dienestrol (DIEN) showed the same conversion to phenanthrenediol; the efficiency of conversion of DIEN was about 10% of that obtained for DES. Injection of high concentrations of DIEN (> 100 ng per 10 µl) resulted in a non-linear response between the amount injected and peak height. Lower concentrations gave a linear response.

Experiments on extracts of urine, meat and fat tissues from cattle

The chromatograms of a urine extract from a blank urine and a urine extract

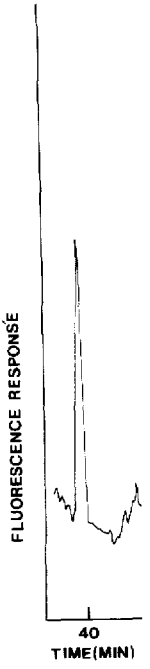

Fig. 4. Chromatogram after post-column derivatisation of 10 ng of DES.

contaminated at a level of 1 ppb of DES are shown in Fig. 5a and b, respectively. Separate experiments have established that 60% of the DES added to urine, in the 0.2–10 ppb DES concentration range, was recovered by using this extraction procedure. Therefore, the 50 ng of DES originally present in 50 ml of urine should have been reduced to approximately 30 ng by the extraction procedure. The extracts were concentrated to 100 μl. As 10 μl were injected, the peak reproduced in Fig. 5a represents an amount of 3 ng of DES injected on to the column.

During preliminary experiments on blank urine extracts, a minor peak with the same retention time as DES was frequently (in about 30% of the blank urine extracts) observed. During "lamp off" experiments[17], these extracts showed a more intensely fluorescent peak eluting at the DES position. These findings suggest that the interfering substance may be destroyed by UV irradiation. PTFE coil in the photochemical reactor was then lengthened (5.4 m; see Experimental); the increased residence time (about 3 min) of the DES in the photoreactor resulted only in a slightly lower conversion to the phenanthrenedione (about 78% yield compared with a residence time of 30 sec) but eliminated the occurrence of a false DES peak during the analysis of blank urine extracts.

In a series of experiments, the recovery of DES during the column chromatographic procedure was determined. Blank urine extracts from 50 ml of urine were spiked with 75, 45, 30 and 22.5 ng of ethanolic DES solutions (equivalent to 3, 1.5, 1 and 0.75 ppb of DES, respectively). The recoveries of DES varied between 80% and 104% (mean 87%; coefficient of variation = 11.4%).

Fig. 6 shows the chromatogram of extracts of 50 g of fat tissue (a) containing

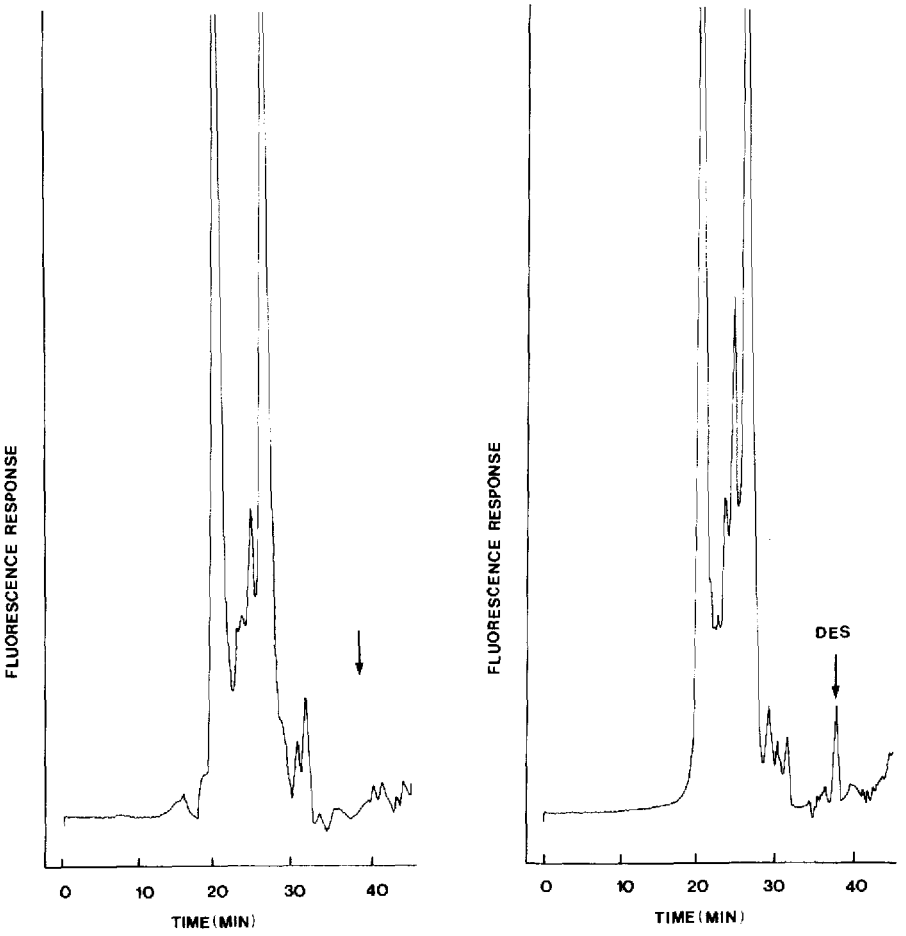

Fig. 5. (a) Chromatogram of a DES-negative urine extract. (b) Chromatogram of an urine extract containing 1 ppb of DES.

no measurable DES concentration (blank) and (b) contaminated at a level of 0.2 ppb of DES (representing less then 1 ng of DES on the column). Comparison of Fig. 5a with Fig. 6a demonstrates that fat or meat extracts contain appreciably less interfering substances than observed in urine extracts. Therefore, the detection limit in the fat or meat extracts is appreciably lower than observed in urine extracts.

Approximately 20 urine samples can be extracted in 1 day by using the described clean-up procedure. The number of sample extracts that can be analysed by this method is primarily limited by the time required for the chromatographic run (55 min). However, the post-column reaction train can be fed by a second HPLC column through the Hamilton valve during the flushing of the first column. In this way, about 15 sample extracts can be analysed by one analyst in 1 day, including the daily calibration of the system. Thus one analyst can handle at least 40 analyses a week.

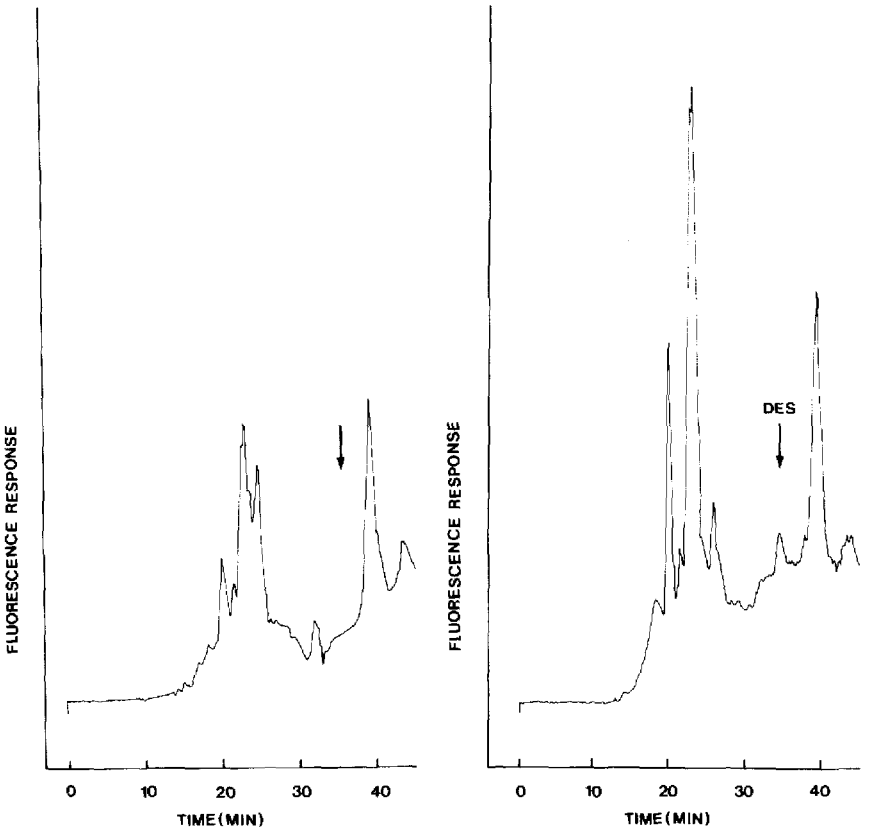

Fig. 6. (a) Chromatogram of a DES-negative fat extract. (b) Chromatogram of a fat extract containing 0.2 ppb of DES.

Determination of DES in biological extracts by HPLC and HPTLC methods

In total 64 samples (37 cow urine, 21 fat and 6 meat samples), selected during regulatory control, were extracted and analysed for the presence of DES residues by both the described HPLC method and a quantitative HPTLC technique[7,18]. HPTLC

TABLE II

CONTINGENCY TABLE OF OBSERVED FREQUENCIES OF DES CONCENTRATIONS IN BIOLOGICAL SAMPLES USING HPLC WITH POST-COLUMN DERIVATIZATION (HPLC–PCD) AND HPTLC METHODS

HPTLC (ppb)	*HPLC–PCD (ppb)*			
	<0.2	*0.2–0.9*	*1–6*	*>6*
<0.2	19	4	–	2
0.2–0.9	–	10	3	–
1–6	–	1	19	–
>6	–	–	–	6

analysis involves two-dimensional TLC of the sample in the presence of appropriate standard DES concentrations and detection by sulphuric acid-induced fluorescence as described under Experimental. Scanning of the characteristic red fluorescence spots produced by *trans*- and *cis*-DES permits a specific and dependable determination of 0.5–5 ng of DES on the plate (coefficient of variation = 10%). The technique is very sensitive; concentrations of 0.2 ppb of DES in different tissues or urine can be determined.

The results obtained during the analysis of the same sample extracts by the two independent detection techniques are summarized in Table II. The presence of DES was detected in 39 samples by both methods. As shown in Fig. 7, in samples contaminated with DES at levels of 0.2–6 ppb, a good correlation ($r = 0.91$) was found between the amounts of DES determined by the two methods. No DES was detected by TLC in 25 samples (Table II). According to HPLC analysis, 19 of these samples

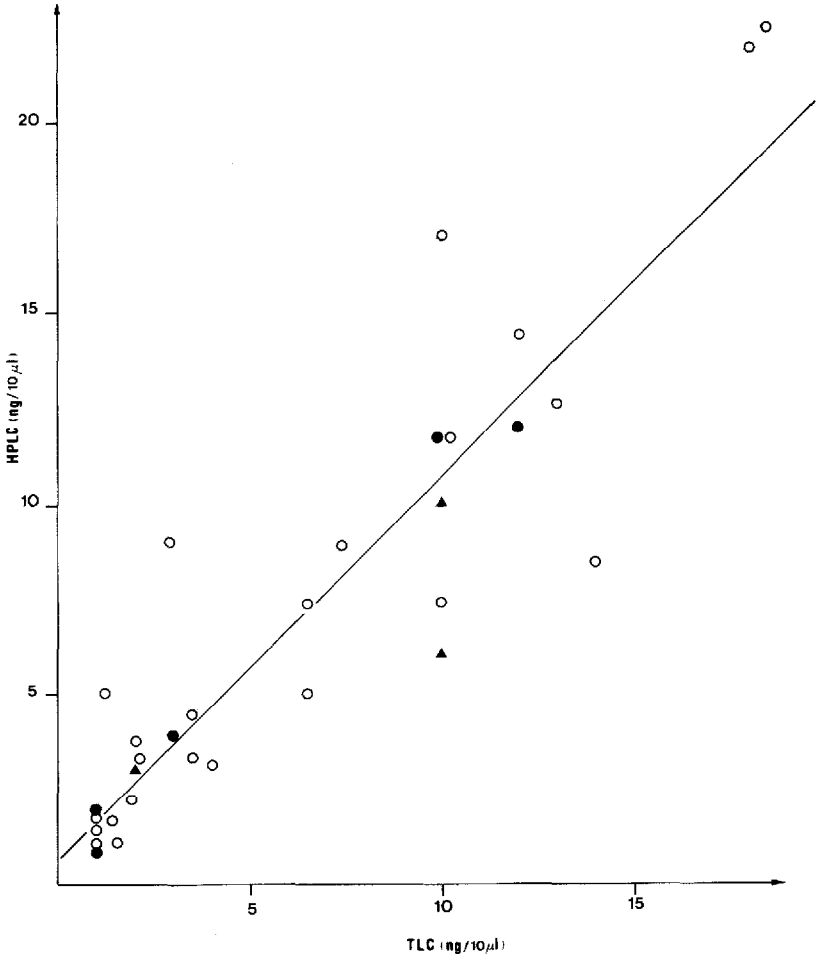

Fig. 7. Comparison of DES determination using HPLC–post-column derivatization and HPTLC methods on urine (○), fat (●) or meat (▲) samples contaminated in the concentration range 0.2–6 ppb. The solid line was determined by regression analysis ($y = 1.01x + 0.69$; $r = 0.91$).

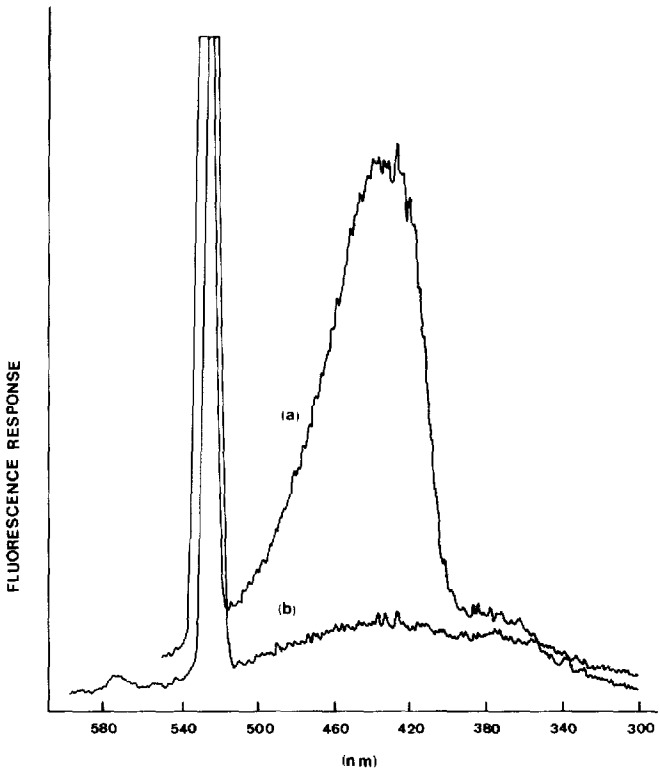

Fig. 8. Emission spectrum ($\lambda_{exc.}$ = 260 nm) of a neutralized reaction mixture after irradiation and subsequent conversion with hydrogen sulphite: (a) 0.180 ng/ml DES; (b) blank.

contained no DES, four samples contained less then 1 ppb of DES but two samples contained a high level of DES residues.

Work in progress indicates that the interfering substances in urine, probably phenolic in nature[33], show different irradiation (*i.e.*, destruction after prolonged irradiation) and emission characteristics compared with the DES reaction products. Recently the opportunity was offered to re-examine some of the samples but using a Perkin-Elmer LS 5 spectrometer ($\lambda_{exc.}$ = 260 nm, $\lambda_{em.}$ = 430 nm). Using this system, the detection limit of DES was similar to that reported on the Schoeffel fluorimeter. However, during analysis of the two false-positive DES samples, no peak could be detected at the retention time of DES. This indicates that the occurrence of false-positive DES samples can be significantly reduced through measurement of the fluorescence at one particular wavelength.

Detection of DES using a fluorescence spectrometer

In the course of this work, Rhys-Williams *et al.*[17] described a method for DES based on in-line fluorescence detection of the tricyclic diketone using a fluorescence spectrometer. A detection limit of 1 ng of DES on-column was reported, *i.e.*, the sensitivity should be at least 50 times that of our fluorescence detection (Table I). However, with both the Schoeffel FS-970 and the LS-5 Perkin-Elmer fluorimeters the

detection limit of the phenanthrenedione in an in-line HPLC–photochemical reactor system amounts only to 60 ng of DES on-column.

The fluorescence spectrum of the reaction mixture, after conversion of the tricyclic diketone, was measured against the blank solution in 1-cm cells. A 3-ml volume of a standard solution, containing 0.5 or 0.1 ng/ml of tricyclic diketone, was mixed with 3 ml of 0.35 *M* $Na_2S_2SO_5$ solution and 2 ml of 12 *M* hydrochloric acid. A blank solution was similarly prepared. After reaction at 70°C for 15 min, the reaction mixture was cooled and neutralized by the addition of 3 ml of 4 *M* sodium hydroxide solution. Fig. 8 shows the fluorescence spectrum ($\lambda_{exc.}$ = 260 nm) of a solution containing the reaction products of 0.180 ng/ml of DES and that of a blank solution. In both solutions the presence of a high fluorescent peak ($\lambda_{em.}$ = 530 nm) is noted after the emission of the DES reaction products. Thus one would expect that, by selection of a particular wavelength, the sensitivity of detection would be increased over that measured in a fluorescence detector equipped with cut-off filters. However, no significant increase in the detection limit of DES was observed when using the fluorescence spectrometer.

CONCLUSIONS

The described procedure allows the specific fluorimetric determination of DES residues in urine extracts and in tissues of slaughtered animals. The method is based on in-line photochemical conversion of DES to its triclyclic phenanthrenedione and specific oxidation to highly fluorescent reaction products. In comparison with existing HPLC methods, the method is more specific and allows the specific determination of DES in biological extracts at the 1 ppb level. The high analytical capacity of this method (40 samples per week per analyst) permits better regulatory control of the abuse of DES in animal production.

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