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RAPID, SPECIFIC METHOD FOR DIETHYLSTILBESTROL ANALYSIS USING AN IN-LINE PHOTOCHEMICAL REACTOR WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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SUMMARY

Low ppb* levels of diethylstilbestrol in biological samples of human and animal origin can be determined using a unique in-line separation–photoreaction–fluorescence detection analytical system. After a very simple extraction procedure, the diethylstilbestrol is separated from interfering substances by high-performance liquid chromatography, photoconverted to a fluorescent product, and detected by fluorescence spectroscopy at optimised excitation–emission wavelengths. A sample can be extracted and analysed in less than 1 h.

INTRODUCTION

Despite the fact that it is a known carcinogen, diethylstilbestrol (DES, I), 4,4′-(1,2-diethyl-1,2-ethenediyl)-bis-(*E*)-phenol, is used for both human and animal applications because of its estrogenic activity[1]. It is one of the cheapest of the synthetic estrogens to produce and is probably the most controversial. It has been banned from use in animal feeds in the U.S.A. and in many European countries[2]. However, its efficiency in promoting weight-gain is so high that illegal uses persist and the detection of DES continues to be a major concern[2].

Residues of DES expected in animal tissues and fluids from its use as a growth promoting agent can be as low as the ng/g (ppb) range[3]. Two analytical methods that have been found to be sufficiently sensitive to determine such amounts are radioimmunoassay[4] and gas chromatography (GC) with an electron-capture detector (ECD)[5,6] or with mass spectrometric (MS) detection[7]. Nanogram levels of DES have also been detected by thin-layer chromatography (TLC) using sulphuric acid-induced fluorescence[8] or the fluorescence of the dansyl derivative[9–12].

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* Throughout this article, the American billion (10⁹) is meant.

The BP[13] and USP[14] methods for determining large quantities of DES, such as in DES tablets, rely on the well known transformation of DES, I, into a fluorescent tricyclic compound, 3,4,4a,4b,5,6-hexahydro-3,6-dioxo-9,10-diethylphenanthrene, II (eqn. 1)[15,16]:

$$\text{(1)}$$
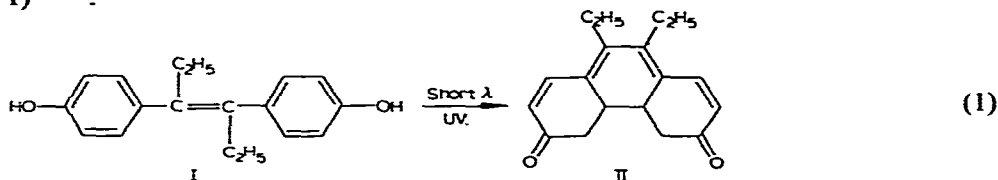
Using this specific photochemical transformation, improved analytical methods have been published for the determination of large[17] as well as very small[18,19] (ppb) levels of DES. The ppb level determinations involve, in one case[18], a very lengthy procedure for biological samples, and, in the other case[19], two-dimensional TLC with visual detection.

The currently available, sensitive methods for determining lower levels of DES suffer from a number of disadvantages. Radioimmunoassay is a complicated and time consuming procedure. The GC–EC and GC–MS methods[6] involve the preparation of derivatives and lengthy extraction and clean-up procedures. Similar disadvantages apply to the TLC procedures[8,12].

In this paper is presented a method for DES analysis which represents a great improvement in speed and simplicity while still being able to detect ppb levels. In our system, the output from an high-performance liquid chromatographic (HPLC) column is fed directly into an in-line photochemical reactor where the reaction in eqn. 1 takes place. The continuously flowing stream is then allowed to pass through the LC flow cell of a fluorescence spectrometer whose excitation and emission monochromators have been set to give a maximum response for the DES photoproduct, II. Extensive sample preparation is not necessary and the entire chromatography is complete, including a column flush cycle, in less than 40 min.

EXPERIMENTAL

Solvents and reagents

Water was distilled, deionized, and pH-adjusted to 3.5 with phosphoric acid. Diethyl ether (Aristar, 99.7%) was distilled through a 30-cm Vigreux column. Acetonitrile (BDH, "for liquid chromatography") was used without further purification. Phosphate buffer solution was 0.1 N, pH 7.4 and made from BDH phosphate buffer, 0.1 M. Ethanol was BDH (AnalaR, 99.7–100%) and was used without further purification. The acetonitrile–water mixture and the acetonitrile itself were thoroughly degassed under vacuum.

A DES (donated by S. Dixon, Agricultural Research Council, Great Britain) solution was made by dissolving 1.0 mg in 20 ml of ethanol. This solution was diluted 1/100 to give a solution containing 0.5 ng/μl of DES. This diluted solution was used for the experiments described in this paper.

A hexestrol (HES, II) (donated by S. Dixon, Agricultural Research Council, Great Britain) solution was made up in a similar manner with 1.1 mg in 20 ml of ethanol and diluted accordingly.

Apparatus

The liquid chromatograph was a Perkin-Elmer Series 3B model which contained an acetonitrile–water (50:50) mixture in one pump module and pure acetonitrile in the second pump module. Sample injection was made via a 20-μl loop injector (Rheodyne). The column used was a 25 × 0.45 cm RP-8 (reversed phase octylsilyl) column (Perkin-Elmer Serial Number P 798). The design of the in-line photoreactor has been described by Twitchet et al.[20].

The stream, including the DES photoproduct, was passed into an LC flow cell incorporated into a Perkin-Elmer Model 3000 fluorescence spectrometer. Excitation and emission monochromators were set at 280 and 390 nm, respectively, with both slits set at 10 nm. Chromatograms were recorded on a Parker-Elmer Model 56 recorder (Fig. 1).
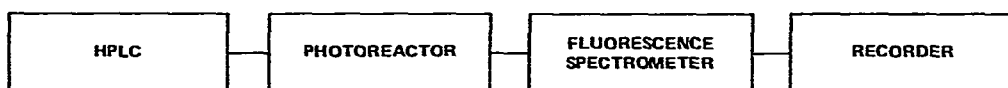

Fig. 1. Diagram of analytical system.

Sample preparation

DES extraction from human urine. To 10 ml of human urine (several sources used) in a separating funnel was added the appropriate amount of DES in ethanol. This mixture was extracted with 10 ml of diethylether. (Note: the initial addition of 6 N HCl to the urine, as specified in ref. 21 upon which this procedure is based, gave difficult emulsions.) The ether layer was then washed with 10 ml of the phosphate buffer solution. The ether solution was filtered through a cone (spatula tip amount) of anhydrous sodium sulphate into a 5-ml test-tube. The separating funnel was washed with 3 ml of diethyl ether and this was added, through filtration, to the original ether extract. The ether extract was evaporated to dryness with a warm water-bath. A 100-μl volume of ethanol was added to the tightly stoppered test-tube and this solution was used for the analyses described later.

DES addition to cow urine, plasma, and sera extracts. Ethanol and DES in ethanol were added to test-tubes containing extracts from cow urine, plasma, or sera to give a total volume of 100 μl. The extracts were supplied to us by S. Dixon, Agricultural Research Council, Great Britain and were prepared in the following manner. To 0.5 ml of urine, plasma, or sera (pretreated with Sigma β-glucuronidase) are added 2.5 ml of double distilled water. This solution is extracted with 5 ml of diethylether. After homogenization, the layers are separated and the ether layer is frozen in a dry ice–acetone bath. The ether extract is decanted from the frozen layer and evaporated to dryness.

Chromatography procedure

A sample is injected into the liquid chromatograph via a 20-μl loop injector. Isocratic elution is then performed with an acetonitrile–water (50:50) (pH 3.5) mixture (pump A) at a flow-rate of 1.5 ml/min. When the DES photoproduct II has completely eluted (about 10 min), the chromatograph is switched to pump B which is set to flush pure acetonitrile at a flow-rate of 2.0 ml/min through the column. After 20 min of flushing, pump A is re-activated and the 50:50 mixture is allowed to flow

through the column for 10 min. After this equilibration period, the recorder baseline has become re-established at its original setting and a subsequent analysis can be performed. Total chromatography time is about 40 min.

The extraction procedures given above do not completely clean-up the biological samples. Therefore, with continuous heavy use, it is recommended that the column be flushed overnight with a small flow (0.1 ml/min) of acetonitrile and the column frits be cleaned periodically to prevent excessive pressure build-up in the system.

Determination of excitation–emission wavelengths for DES

A solution of DES was placed in a fluorescence spectrometer (Perkin-Elmer Model 3000) and irradiated at 280 nm for 5 min (15-nm slit). Using 280 nm (2.5-nm slit) as the excitation wavelength, the emission spectrum (5.0-nm slit) of the irradiated DES solution was recorded between 300–500 nm. The emission spectrum (300–500 nm), excitation 280 nm, of the same DES solution before irradiation was also recorded.

RESULTS AND DISCUSSION

Excitation–emission wavelengths

The selection of optimum excitation and emission wavelengths will give the greatest sensitivity for the fluorescence detection of the desired photoproduct II, while minimizing or eliminating altogether interfering peaks. Therefore, the emission spectrum from 300–500 nm, using excitation at 280 nm, was recorded for DES solutions before and after irradiation at 280 nm. Fig. 2a and 2b clearly show that DES is converted to a fluorescence photoproduct, presumably II, which has a maximum emission at 390 nm. DES itself has only a small absorption under these conditions. The fluorescence detection in the experiments to be described were, therefore, all done at excitation and emission wavelengths of 280 and 390 nm, respectively.

Calibration curve for pure DES solutions

The standard DES solution (0.5 ng/μl) was diluted to 0.75, 0.50 and 0.25 of its original concentration. Each diluted sample and the original were subjected to liquid chromatography, photoreaction and fluorescence detection. The peak heights were graphed versus the dilution factor to give the graph in Fig. 3. These 20-μl injections place from 10 to 2.5 ng of DES onto the column and the analytical system responds in a linear fashion to this successful attempt to construct a calibration curve for low ng amounts of pure DES in ethanol solution. The single peak (other than a short retention peak ethanol) for the DES photoproduct appears in 9.5 min but is completely absent if the experiment is repeated with the lamp in the photoreactor turned off. This "lamp on–lamp off" effect[20] is demonstrated in later figures in this paper.

Human urine experiments

Fig. 4 shows the chromatogram (a) resulting from the extraction (according to the scheme described in the Experimental section) of 12.5 ng of DES added to 10 ml of human urine and (b), a blank, resulting from the extraction of 10 ml of the same
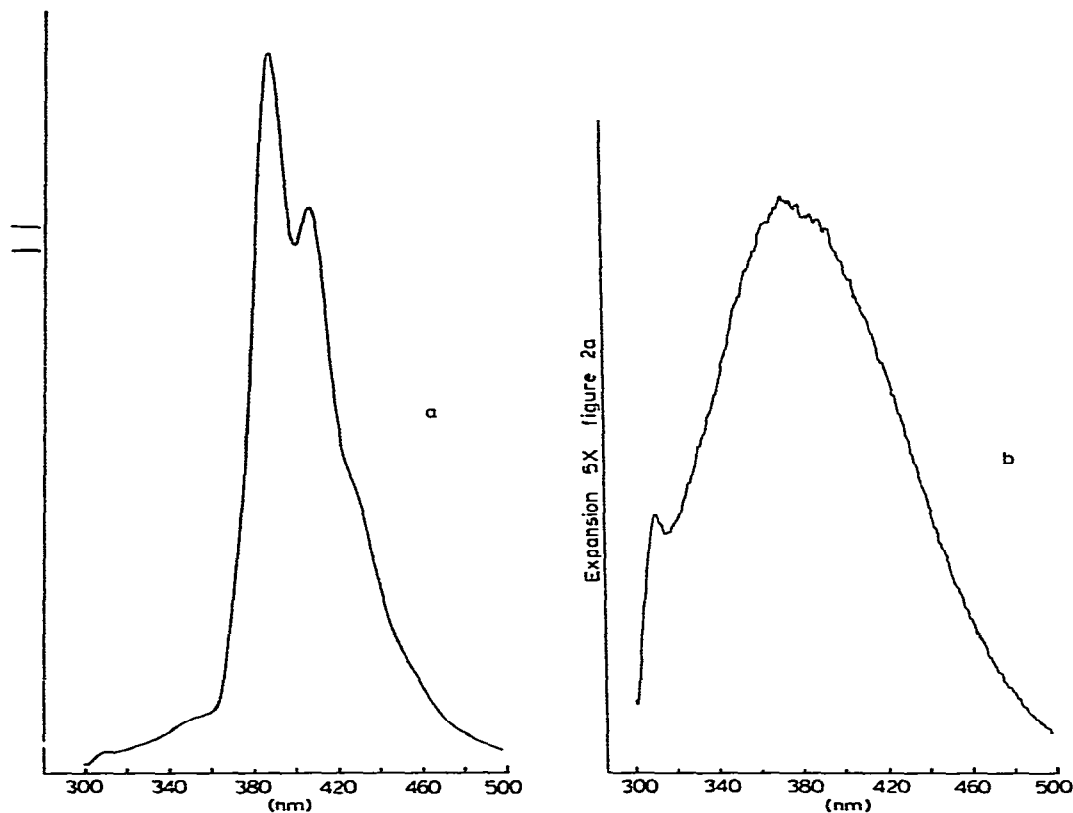
Fig. 2. a, Emission spectrum (excitation 280 nm) of DES photoproduct (II) after irradiation of DES solution at 280 nm for 5 min. b, Emission spectrum of DES before irradiation (expansion 5 × that of a).

urine with no DES added. The extra peak at 9.5 min has the same retention time as in the pure DES experiments and exhibits the same "lamp on–lamp off" behaviour.

Separate experiments established that about 60–70% of the DES was recovered by the very simple extraction procedure. Furthermore, these extractions of low ng levels of DES could be duplicated to within 10% or less. Therefore, the 12.5 ng of DES originally added, as described above, should have been reduced to less than 10 ng by the extraction procedure. This DES and the urine residues are dissolved in 100 µl of ethanol and 20 µl of this solution is injected. Therefore, the peak for the DES photoproduct in Fig. 4a represents less than 2 ng of DES on the column. Also, because there was originally 12.5 ng in 10 ml of urine, Fig. 4a demonstrates that our system can detect levels of DES of 1 ng/g (1 ppb) or less in human urine.

The calibration curve of Fig. 5 was the result of separate extractions in which 37.5, 25, and 12.5 ng, respectively, of DES were added to urine samples before extraction. Fig. 5 shows that very low ppb levels of DES can be quantitatively extracted and determined by this method. Furthermore, once the reagents are prepared and the instrumental system is established, the entire extraction and chromatography of DES in human urine can be accomplished in less than 1 h. Daily calibration of the system is desirable.
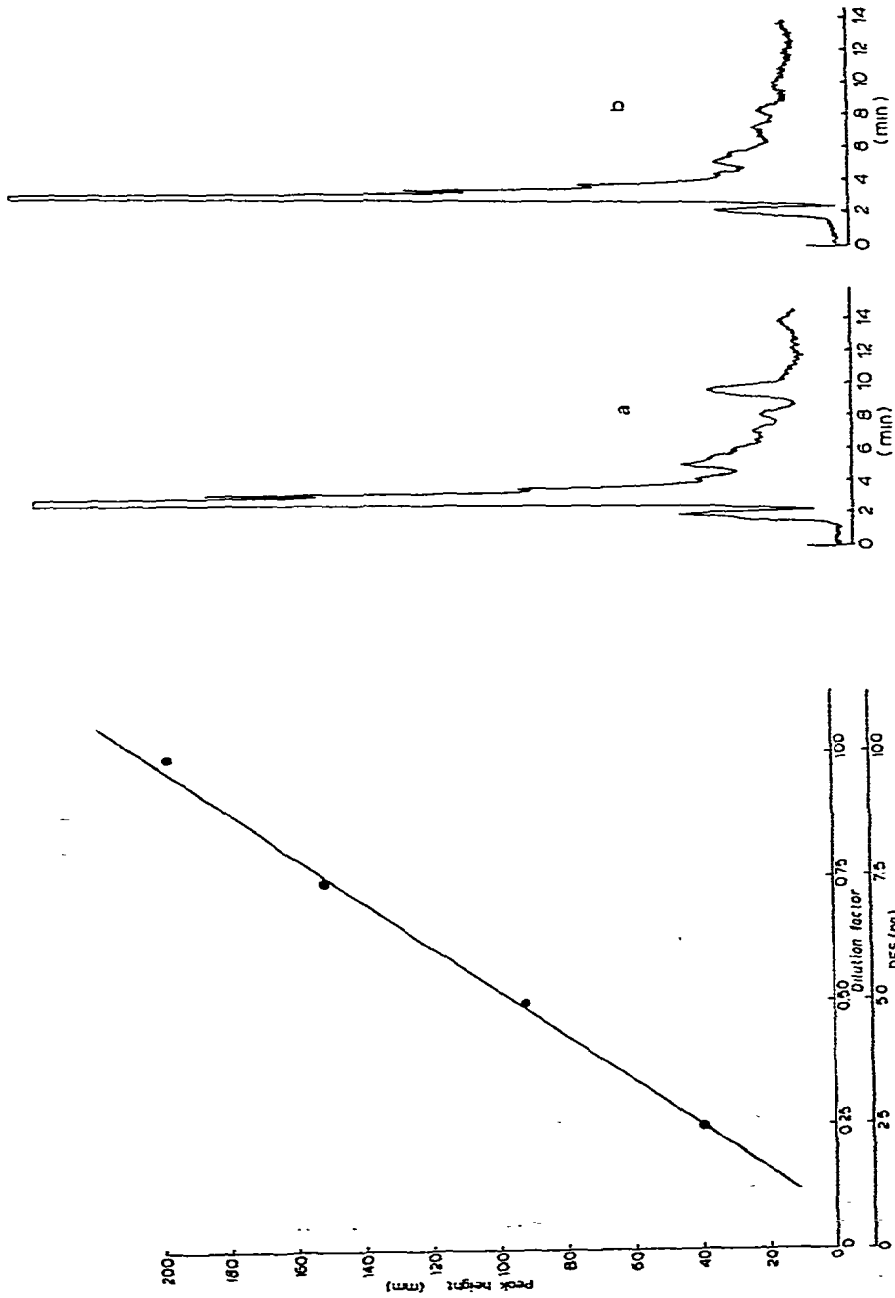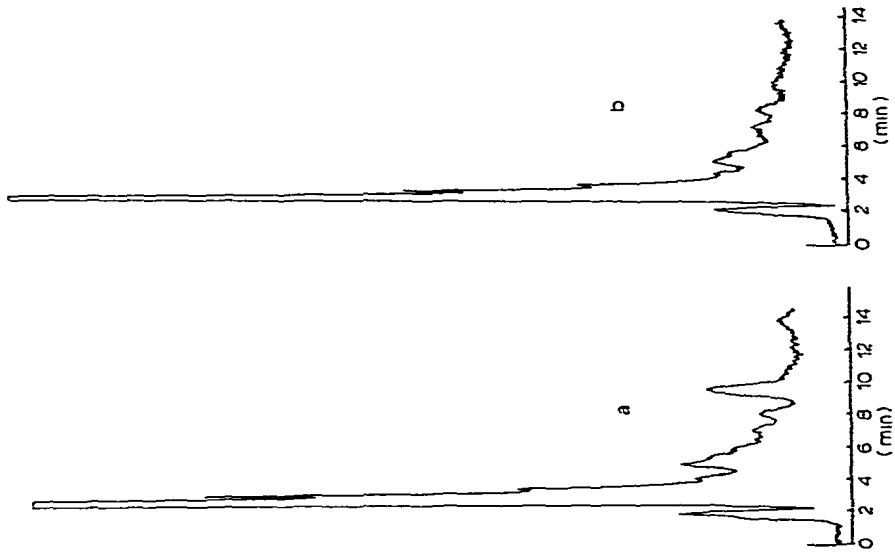
Fig. 3. Calibration curve for low ng levels of pure DES in ethanol solution.

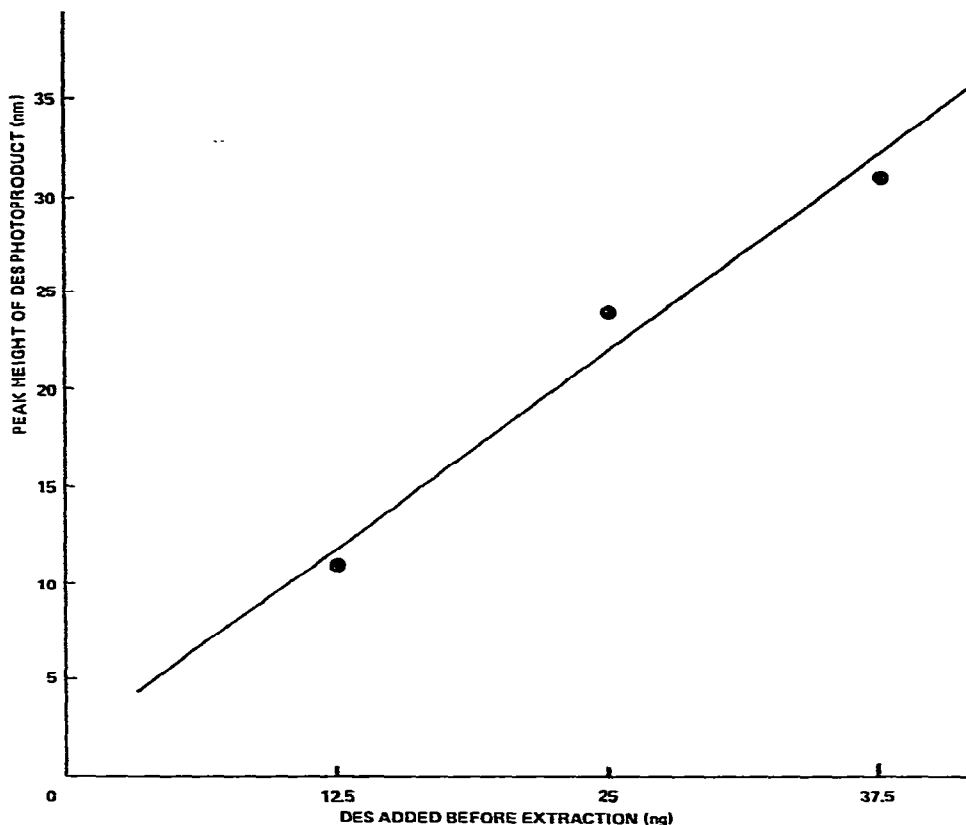Fig. 4. Chromatograms of human urine extracts: a, 12.5 ng DES added before extraction; b, blank.

Fig. 5. Calibration curve for DES extracted from human urine.

Cow urine, plasma, and sera experiments

A limited number of blanks made up by the ether extraction of cow urine, plasma, and sera as described in the Experimental section were available to us[21]. To each of these were added 50 μl of the 0.5 ng/μl DES solution, i.e., 25 ng of DES, and 50 μl of ethanol. These samples were chromatographed according to the conditions previously described with the following results.

. · Fig. 6a shows the expected peak for the DES photoproduct at about 9.5 min in the cow urine samples. Fig. 6b is a repeat injection of the same sample as that in 6a except that the lamp is turned off off and no photoconversion of DES into II takes place. Fig. 6c represents a different cow urine sample with no DES added, i.e., simply dissolved in 100 μl of ethanol, with the lamp on.

The "lamp on–lamp off" effect is shown again in the chromatograms of Fig. 7. Fig. 7a and b are from the same solution of cow plasma in ethanol to which DES (25 ng) had been added. Some differences in the chromatograms when the lamp is turned off, other than the disappearance of the peak for II, are to be expected. The UV lamp undoubtedly causes other photochemical transformations which destroy or create fluorescent substances in these biological materials.
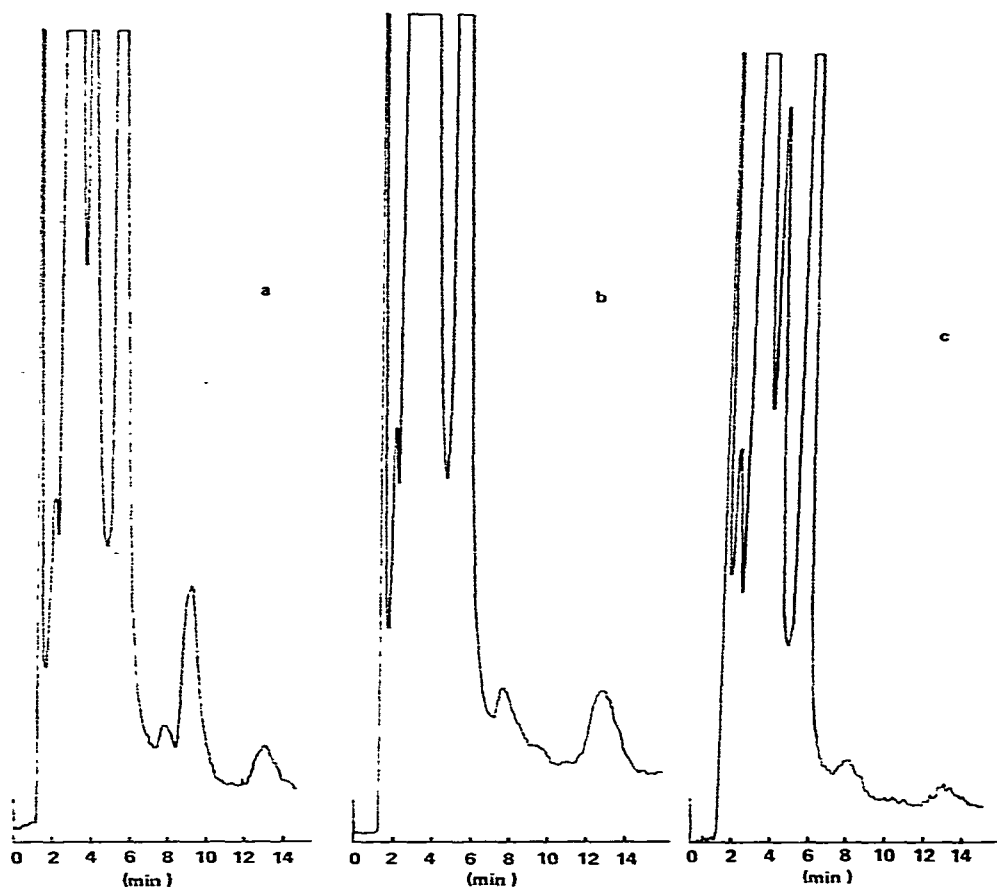
Fig. 6. Chromatograms of cow urine extracts: a, 25 ng DES added, lamp on; b, as a, lamp off; C, no DES added, lamp on.

Fig. 8 shows the chromatograms that result when 25 ng of DES are added to cow sera with the lamp on (a) and the lamp off (b).

Because the amount of cow urine, plasma or sera used to prepare the extracts was originally 0.5 ml, the chromatograms of Fig. 6–8 represent 25 ng in 0.5 ml of sample or levels of 50 ppb. The expansion control of the fluorescence spectrometer used could be increased 5 × over that used to obtain these chromatograms. It is evident that, especially with the plasma and sera samples, very low ppb levels of DES can be determined.

Hexestrol (HES) experiment

It has been claimed[12] that the dansyl derivatives of hexestrol (HES, III), DES, and dienestrol are all converted into more intensely fluorescent substances upon exposure to 366-nm UV radiation. The central double bond is an essential feature in the most plausible mechanism for the reaction in eqn. 1. However, because of the close similarity of structure for DES and HES and as a check on the specificity of the
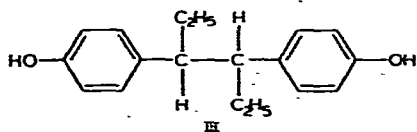
photoconversion of DES into II, a solution of 0.5 ng/μl of HES in ethanol was made. When this solution was subjected to the same chromatographic conditions as DES, no peak appeared after 20 min with either the lamp on or off.
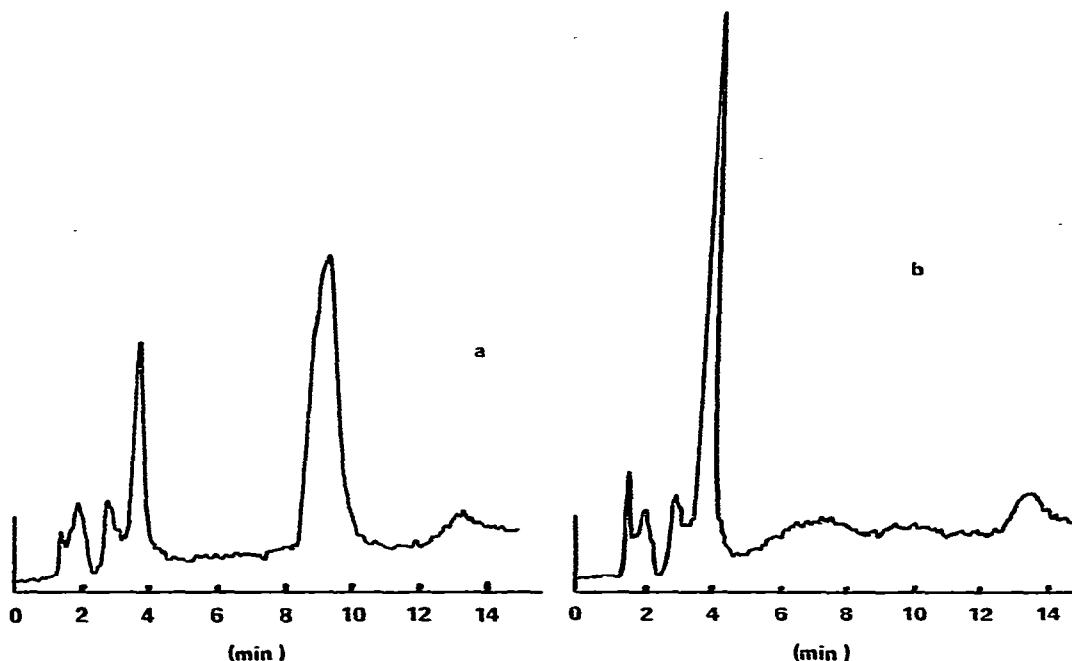
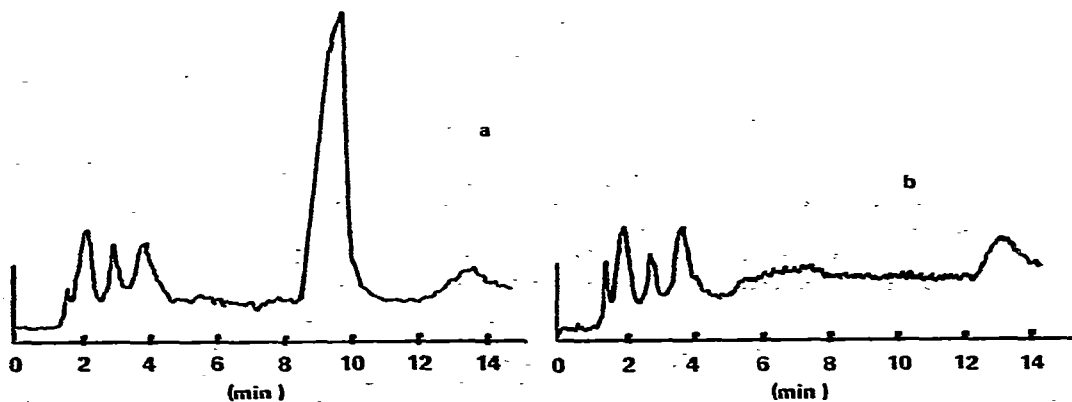Fig. 7. Chromatograms of cow plasma extracts: a, 25 ng DES added, lamp on; b, as a, lamp off.

Fig. 8. Chromatograms of cow sera extract: a, 25 ng DES added, lamp on; b, as a, lamp off.

CONCLUSIONS

The analytical method described in this paper allows for the highly specific, quantitative determination of DES at levels comparable to existing methods.

However, compared to the lengthy extraction, work up, and derivatisation procedures characteristic of other methods, our method is unrivalled for speed and simplicity. It is also free of the subjectivity that is inherent in the visual spot identification of most of the TLC methods.

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