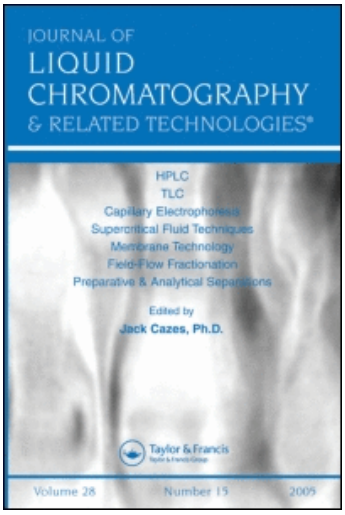

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SEPARATION OF DIETHYLSTILBESTROL AND DERIVATIVES IN BIOLOGICAL FLUIDS AND TISSUES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method was developed to identify radiolabeled DES and DES metabolites in biological fluids and tissues. After a rapid initial clean-up step, the biological samples were analyzed with a single-column, reversed-phase, high-pressure liquid chromatography system. The parent compound and metabolites were simultaneously resolved and tentatively identified by comparing their retention times to those of known standards. Positive identification of some metabolites was achieved by field-desorption and capillary-column mass-spectrometric analysis. The method was found to be rapid and reproducible, and sample recovery was >80%.

INTRODUCTION

Clinical investigations have indicated that diethylstilbestrol (DES) is a teratogen and transplacental carcinogen (1-3). The teratogenic effects of DES have been reported in nonhuman primates (4) and rodents as well (5,6). Despite the well-defined spectrum of genital tract abnormalities following *in utero* DES exposure, the mechanism whereby exposure to DES produces adverse effects is unknown. Possible mechanisms responsible for the *in utero* toxicity are 1) hormonal impact on developing endocrine and reproductive systems, 2) activity as a promoter, or 3) proposed metabolic activation and subsequent covalent binding to critical cellular proteins or genetic material (7-9).

In order to investigate these possible mechanisms, methods are needed to qualitatively and quantitatively define the metabolic profile of DES. Several approaches have been reported in the literature, including gas chromatography, mass fragmentography, and mass spectrometry (10-12). These techniques, however, are often unsuitable for polar conjugates and labile metabolic intermediates of DES. Recently, nondestructive methods of high pressure liquid chromatography (HPLC) have been developed, but these procedures require multiple chromatographic systems (13) or have yet to be validated for biological samples (14).

The present report describes a reversed-phase, HPLC system, capable of separating several DES derivatives and conjugates. The accuracy and reproducibility of the method was investigated by applying it to urine, plasma, and tissue samples, collected from monkeys treated with radiolabeled DES.

MATERIALS

Chemicals

(UL-^{14}C)-Diethylstilbestrol (51.55 mCi/mM) was purchased from Pathfinder Labs (St. Louis, Missouri) and purified by a published HPLC method (15). The radiolabeled E(trans) and Z(cis) isomers are considered to be >98% pure.

(Monoethyl-1-^{3}H)-diethylstilbestrol (53 Ci/mM) was received from Amersham Corporation (Arlington Heights, IL). Purification by HPLC (15) produced a >95% pure sample.

The DES-monoglucuronide (DES-G) was obtained from Aldrich Chemical (Milwaukee, WI) and was found to be 99% pure when analyzed by a HPLC reversed-phase system. (16)

Unlabeled DES (No. 706-2) was purchased from Research Plus Steroids (Denville, NJ) and used as received. An initial assay by HPLC revealed no

UV-absorbing contaminants. Diglucuronide-DES was synthesized by us; the other DES derivatives: 1-hydroxy-DES (omega-hydroxy-DES), Z,Z-dienestrol, and tetramethoxy-DES were synthesized by Southwest Foundation for Research and Education, San Antonio, TX. The purity and conformation were verified by HPLC, nuclear magnetic resonance (NMR), and mass spectrometry. The DES related compounds and derivatives are shown in Figure 1. (17)

Instrumentation

Samples were analyzed on a Waters Associates (Milford, MA) HPLC system. The instrumentation involved was a Model U6K septumless injector, coupled to Model 6000-A high pressure pumps, programmed by a Model 660 gradient/flow programmer. Detection of individual UV-absorbing materials was performed with a Model 440 UV spectrophotometer with an 8 µl flowcell at a fixed wavelength of 254 nm. In some cases, detection of radiolabeled (^{14}C) material was accomplished with a Scaler-Ratemeter SR5 (Nuclear Enterprises LTD-Reading, England) with a flow-thru detector cell, coupled to the outlet of the UV detector. Graphic response of both the UV detector and the isotope monitor was made by an Omniscribe dual pen recorder (Houston Instruments, Austin, TX). Fractions were collected with a Model FC-80H MicroFractionator (Gilson Med. Elect., Middleton, WI). In all cases, aliquots of the fractions were counted for radioactivity with a Packard Tri-Carb Model 3382 Liquid Scintillation Spectrometer (Packard Inst. Co., Downers Grove, IL) in order to calculate percent recovery.

HPLC Column

The column was a commercially available prepacked Altex LiChrosorb RP_{18}, 5 micron, 10 x 250 mm Reversed-Phase Column (Altex Scientific, Inc., Berkeley, CA). The LiChrosorb column packing is a fully porous microparticlulate of irregularly shaped silica gel with a bonded hydrocarbon phase (C_{18}). This reversed-phase material allowed rapid solvent

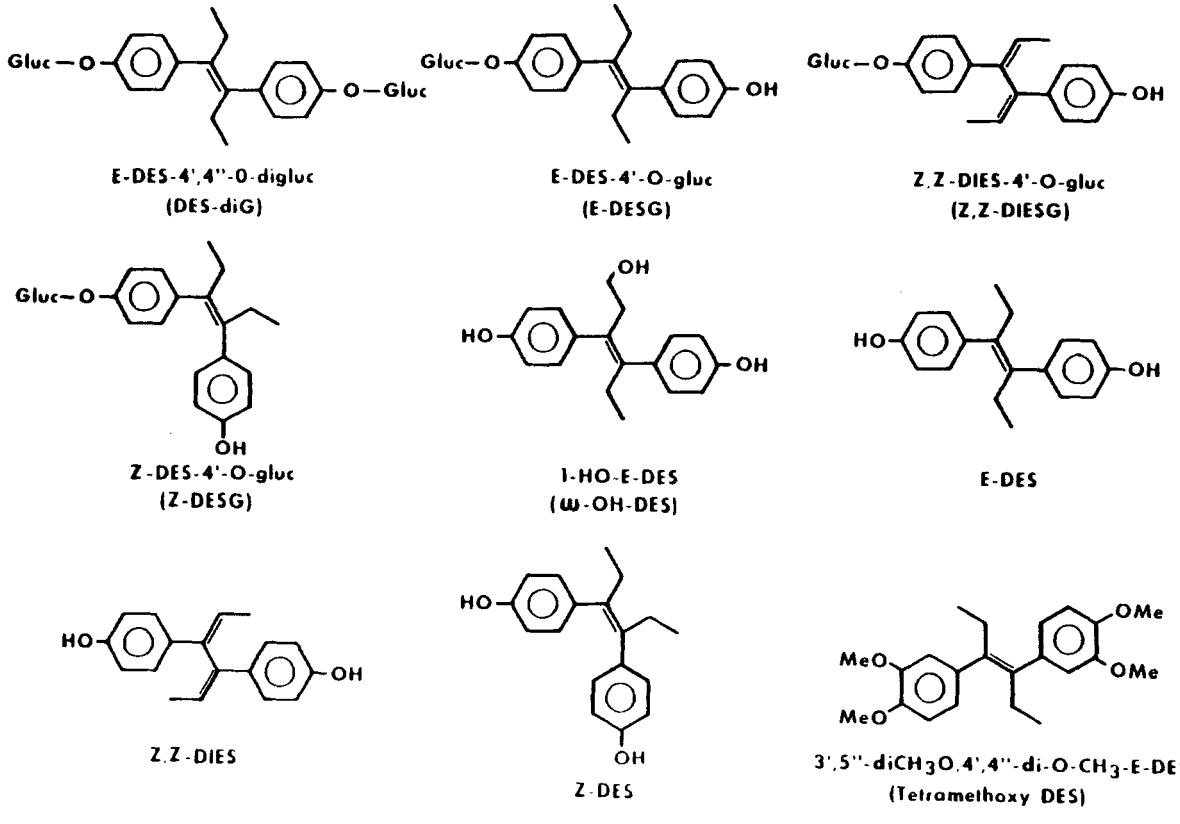

Figure 1: Structures of DES and Derivatives Separated by HPLC.

equilibration, reversible adsorption of solutes, high flow rate with minimal backpressure, and high efficiency and selectivity for the wide polarity range of samples investigated. The large diameter of the column allowed analysis of greater sample volumes without overloading the column. A guard or pre-column was placed in line between the injector and LiChrosorb column. This guard column (3.2 x 35 mm) was dry-packed with 37-50 micron Bondapak C_{18}/ Corasil (Waters). The guard column was designed to protect the LiChrosorb column from the uneluted materials often found in biological samples. It did not alter the retention times of the standards or diminish the resolution or efficiency of the column.

HPLC Solvents and Chemicals

The organic solvents used were Distilled-in-Glass quality from Burdick and Jackson, Muskegon, Mich. Water was purified through a Millipore Milli-Q System (Bedford, MA), fed by a deionized water source, and ultra-pure ammonium acetate (NH_4OAc) (Mallinckrodt), which was used as a solvent buffer. All solvents were filtered through a Millipore BDWP 0.6 μm filter and degassed prior to use.

METHODS

Urine Collection and Analysis

A 2.0-mg/kg dose of (UL-^{14}C) DES, 20 μCi, plus 12.0 mg of unlabeled crystalline DES, was administered intravenously to a pregnant rhesus monkey, #4036. Another pregnant monkey, #1162, received 68 μCi of [monoethyl-1-^{3}H] DES plus 18.0 mg (3 mg/kg) DES. Urine was collected via a Foley catheter for 4 hours after administration.

The urine was processed by the addition of two volumes of methanol-ethanol (MeOH-EtOH) (1:1), whereby proteins and salts were precipitated. After centrifugation, the precipitate was washed twice with an equal volume of MeOH-EtOH (1:1). The supernatants were pooled and evaporated to near

dryness under a nitrogen stream at 37°C. The sample was then brought up to 0.20 ml with addition of MeOH-H_2O (3:1) for HPLC analysis.

Blood Collection and Analysis

Blood samples were collected through a femoral artery catheter and centrifuged to separate the plasma from the red blood cells. The plasma samples (0.3 - 0.5 ml) were diluted to 1.0 ml by addition of reagent-grade H_2O. They were then centrifuged at 10,000 rpm in a Beckman (Palo Alto, CA) Micro-fuge to sediment the "buffy coat". The buffy coat of lipid material was not found to contain significant radioactivity and was discarded. Two volumes of MeOH-EtOH 1:1 was added to the plasma to precipitate proteins and salts. After centrifugation, the precipitate was washed two times with equal volumes of MeOH-EtOH. The washes were added to the initial plasma supernatant. The total sample was evaporated under N_2 at 37°C and resuspended in 0.2 ml of MeOH-H_2O (3:1). The plasma samples were chromatographed in the same reversed-phase system as the urine.

Tissue Extraction and HPLC Analysis

Fetal tissue samples were collected and frozen 21 hours after administration of the dose to the maternal monkey. Tissue samples were weighed and placed in glass-stoppered Erlenmeyer flasks, which were packed in ice. After adding 5 ml of cold distilled H_2O per gram of tissue, each sample was homogenized for approximately one minute, using a Polytron (Brinkman, Westbury, NY). Five volumes of a 1:1 mixture of methanol (MeOH) and dimethoxymethane (DMM) was added to extract the unbound radioactivity and to precipitate the macromolecular components. This mixture was shaken for 18 hours at ambient temperature. Following centrifugation at 12,000 rpm, the supernatant was transferred to a graduated cylinder. The pellet was washed three times with MeOH-DMM (1:1) and these washes were combined with the original supernatant. The volume of the H_2O-MeOH-DMM supernatant

was recorded and a 1.0-ml aliquot was removed for determination of radioactivity. The pellet was solubilized by addition of 1 to 10 ml of 1% sodium dodecyl sulfate (SDS), and a 0.5-ml aliquot from a known volume was counted. The pellet contained only a small percentage of the total radioactivity (<4%) and was not further analyzed. The combined supernatant was reduced to a small volume (1-2 ml) on a rotary evaporator at 40°C. After the addition of 25 to 50 ml of MeOH, the sample was filtered through a 0.6 um BDWP Millipore filter, the filter was rinsed with additional MeOH, and the filtrate was saved. Before discarding the precipitate (if any), it was dissolved and counted for radioactivity. The filtrate was concentrated to less than 10 ml and passed through a C_{18} Sep-Pak (Waters Associates, Inc., Milford, MA), followed by about 5 ml of MeOH. The C_{18} Sep-Pak retained lipid material which would otherwise contaminate the column. The sample was evaporated to near-dryness in a 15-ml conical tube under a stream of N_2 at 37°C. To the residue was added 150 µl of MeOH and 100 µl of H_2O, containing 0.5% SDS. The sample was well mixed immediately before transfer, and a 25-µl aliquot was dispensed into a vial for counting. The remaining 225-µl sample was chromatographed in the reversed-phase HPLC system. An aliquot was counted from each of the 1.0-ml (0.5-min) fractions that were collected.

Tissue samples from an undosed animal were spiked with ^{14}C-DESG and ^{3}H-DES to compare their HPLC profiles and recovery percentages to those from tissues of a dosed animal. The ^{14}C-DESG and ^{3}H-DES were added to the homogenized tissue at a level of 100,000 dpm of each in 0.5 ml of MeOH per gram of tissue.

Mass Spectrometry

Samples for mass spectrometry were prepared in the following manner. Adjacent fractions separated by HPLC and containing radiolabeled DES or derivatives were pooled and evaporated to dryness under a stream of nitrogen.

In the case of urinary metabolites, the samples were either dissolved in MeOH and their mass spectra determined by field desorption (F.D.) mass spectrometry (Varian CH5 DF) (Florham Park, N.J.) or dissolved in aqueous acetic acid buffer (pH, 5) and hydrolyzed with bovine β-glucuronidase, as previously described (15). The aglycones were then treated with acetic anhydride-pyridine (1:1) overnight. Separation of the acetylated aglycones by capillary column GC and subsequent mass spectrometric analysis were accomplished by using a Finnigan Model 9500 gas chromatograph (Finnigan Corp., Sunnyvale, CA) that was modified by installing a direct capillary-mass spectrometer interface and a split-less capillary injector. This allowed the use of a 50-meter SP2250 Support-Coated Open Tubular (SCOT) column. The helium carrier gas was maintained at a flow of 3 ml/min by a Brooks 5841 flow controller. This flow rate allowed operation of the ionization source at or below 0.1 torr. The manifold temperature was maintained at 70°C. Mass spectral analysis was performed with a Finnigan Gas Chromatograph-Mass Spectrometer (Model 1015D). Electron-impact (70 eV) ionization mode was used.

In the case of the plasma samples, adjacent HPLC fractions containing significant amounts of radioactivity were pooled, concentrated and derivatized in preparation for mass spectrometry. More specifically, DES standards (DES and DESG), method blanks (HPLC purified plasma from untreated monkeys) and experimental samples were evaporated to dryness and allowed to react with diazomethane in order to methylate any carboxylic acids. The reaction was allowed to continue for 2 hr at room temperature. Then, the samples were again brought to dryness and treated with equal volumes of pyridine and acetic anhydride in order to acetylate hydroxyl groups. After 30 min at 70°C, the samples were taken to dryness and then dissolved in MeOH.

Mass-spectrometric identification of derivatized components separated by HPLC was performed on a Finnigan 4023 mass spectrometer combined with an Incos data system. Samples were deposited into glass sample cups (5 μl volume), evaporated to dryness with a stream of dry helium, and introduced into the mass spectrometer via the solid probe. The probe was heated by the ballistic heater to 400°C. Data were collected throughout the heating cycle. For greater sensitivity the mass spectrometer was operated in the multiple specific ion mode. Ionization was accomplished in conventional electron-impact (E.I.) mode.

RESULTS

DES Standards

Of the many systems tried, the reversed-phase chromatography system with gradient elution was found to be best for separating the more polar water-soluble conjugates from the non-polar metabolites and the DES parent compound. A 50-min convex gradient elution (program-#5:Waters 660 programmer) with initial conditions of 10% MeOH-90% H_2O (0.01M NH_4OAc), pH 6.9, and ending with 100% MeOH was used. A 50-min gradient program allowed polar pigmented material to be eluted before the compounds of interest. The column was operated at 1600 psi with a solvent delivery of 2.0 ml/minute and at ambient temperature (25° ± 2°C). The ammonium acetate buffer was used to control ionization of the solutes. This prevented the solutes from tailing. Figure 2 shows the HPLC separation of DES and its derivatives. The reversed-phase system is capable of resolving at least seven DES derivatives and isomers. Separation of compounds with widely different polarities (e.g., DES-diglucuronide and tetramethoxy-DES) is accomplished by a convex gradient elution program without loss of resolution between closely related compounds (e.g., E- and Z-DES and Z,Z-dienestrol).

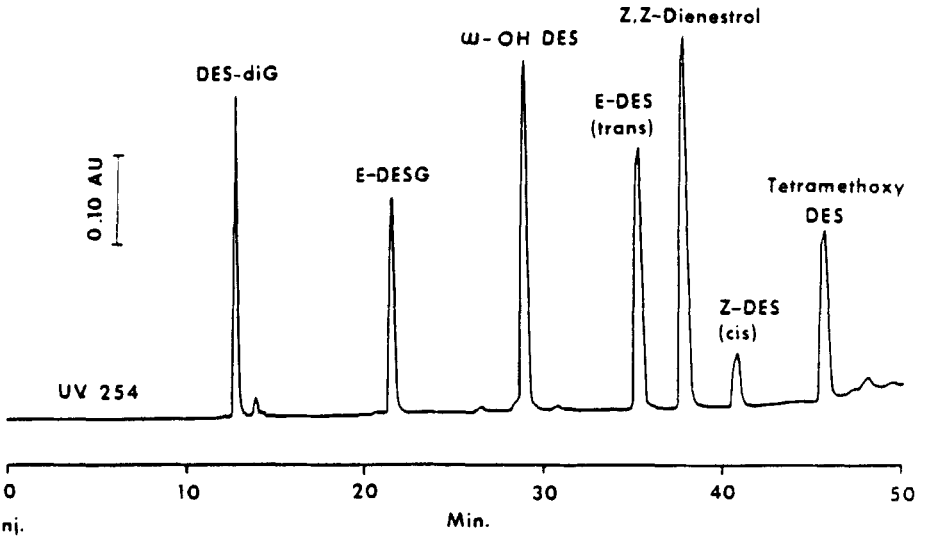

Figure 2: HPLC Profile of DES Standards. Chromatographic conditions: Column - LiChrosorb RP-18, 5 μm, 10 x 250 mm; mobile phase - 10% MeOH-90% H_2O (0.01 M NH_4OAc), pH 6.9, to 100% MeOH, convex gradient elution in 50 min; flow rate - 2.0 ml/min at 1600 psi; sample size - 10 μg each, except DES-diglucuronide = 20 μg and tetramethoxy DES = 5 μg.

Urinary Metabolites

The 0-4-hr urine sample collected from Monkey #4036 was processed as described under Methods. A typical chromatogram (Fig 3) defines the five major DES metabolites (radiolabeled peaks I-V). It can be observed that the more polar endogenous material and urinary pigments are eluted early in the gradient program and precede the DES metabolites.

Five aliquots of this 4-hr urine sample were chromatographed, and fractions were collected and subsequently counted by liquid scintillation. The retention times and the percent radioactivity of each major metabolite are shown in Table 1. The standard error of the mean of five determinations is small, indicating high reproducibility. As shown in the right column of Table 1, three of the five metabolites were subsequently identified by

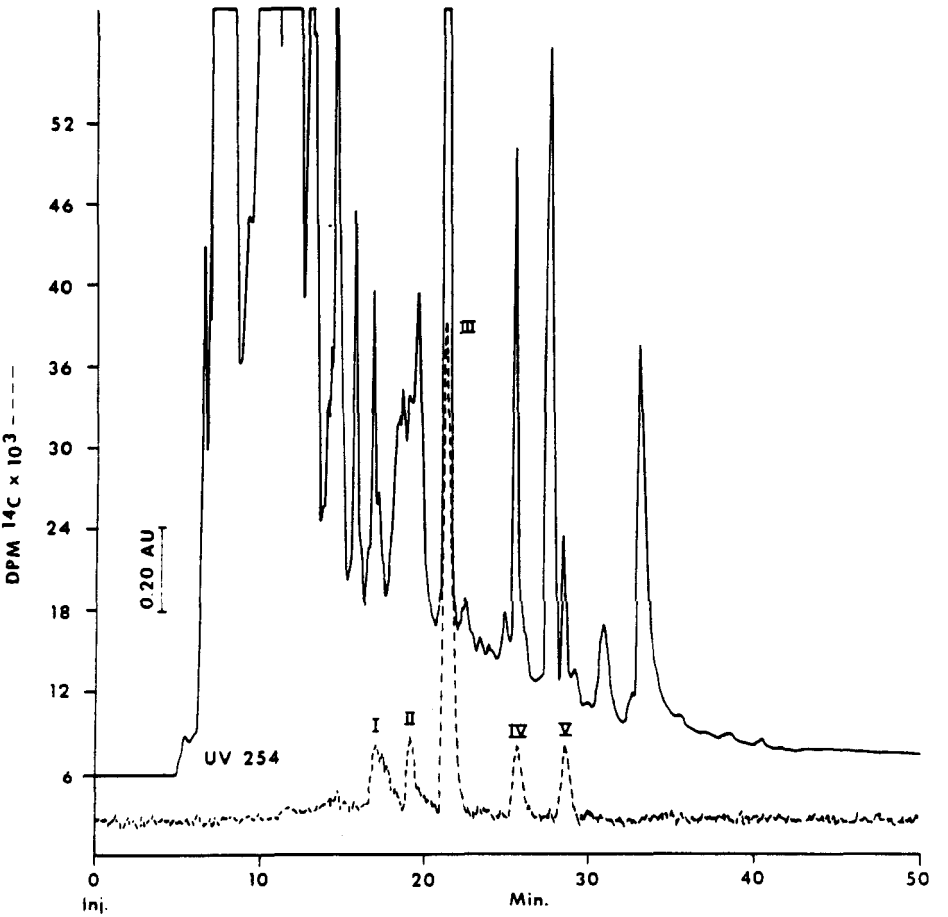

Figure 3: HPLC Profile of Urinary Metabolites. Chromatographic conditions same as Fig 2. Peak III was subsequently identified as E-DESG; Peaks IV and V were identified as Z,Z-DIES and Z-DESG respectively.

field desorption (F.D.) mass spectrometry and GC-MS (E.I.) as E-DES monoglucuronide (III), Z,Z-dienestrol monoglucuronide (IV) and Z-DES monoglucuronide (V).

Plasma Metabolites

A 0.5-ml plasma sample, collected 5 min after ^{14}C-DES administration, was processed and the resulting chromatogram is shown in Fig 4. The

TABLE 1

CHARACTERISTICS OF THE URINARY DES METABOLITES SEPARATED BY HPLC.

HPLC Retention Time (Min.)	% Radioactivity Mean ± S.E.	F.D. M/E	% Hydrolyzed by β-Glucuronidase	E.I. M/E	Compound
13	3.8 ± 0.4	---	64	---	---
15	6.7 ± 0.2	---	63	---	---
17	13.4 ± 0.7	---	90	---	---
19	10.3 ± 0.5	---	96	---	---
21	48.5 ± 1.2	444	98	352	E-DESG
26	9.0 ± 0.5	442	98	350	Z,Z-DIESG
28	8.3 ± 0.5	444	90	352	Z-DES

peak of ^{14}C radioactivity (dotted line) with a retention of 22-23 min was confirmed by mass spectrometry to be E-DES monoglucuronide. Also, the peaks at 36 min and 40 min are E-DES and Z-DES, respectively. The remaining radiolabeled peaks are currently undergoing identification.

Tissue Metabolites

The HPLC profile from fetal liver of an untreated animal, after the tissue was spiked with ^{14}C-DESG and ^{3}H-DES, is shown in Fig 5. These radiolabeled compounds co-chromatographed with the unlabeled standards, which were added to the sample. After extraction and HPLC analysis, 95% of the ^{14}C-DESG and 82% of the ^{3}H-DES, added to the homogenized tissue, was recovered.

A fetal liver tissue sample, collected 21 hr after administration of the ^{14}C-DES dose, was analyzed as described under Methods. Fig 6 depicts the resulting chromatogram which defines four major peaks of radioactivity.

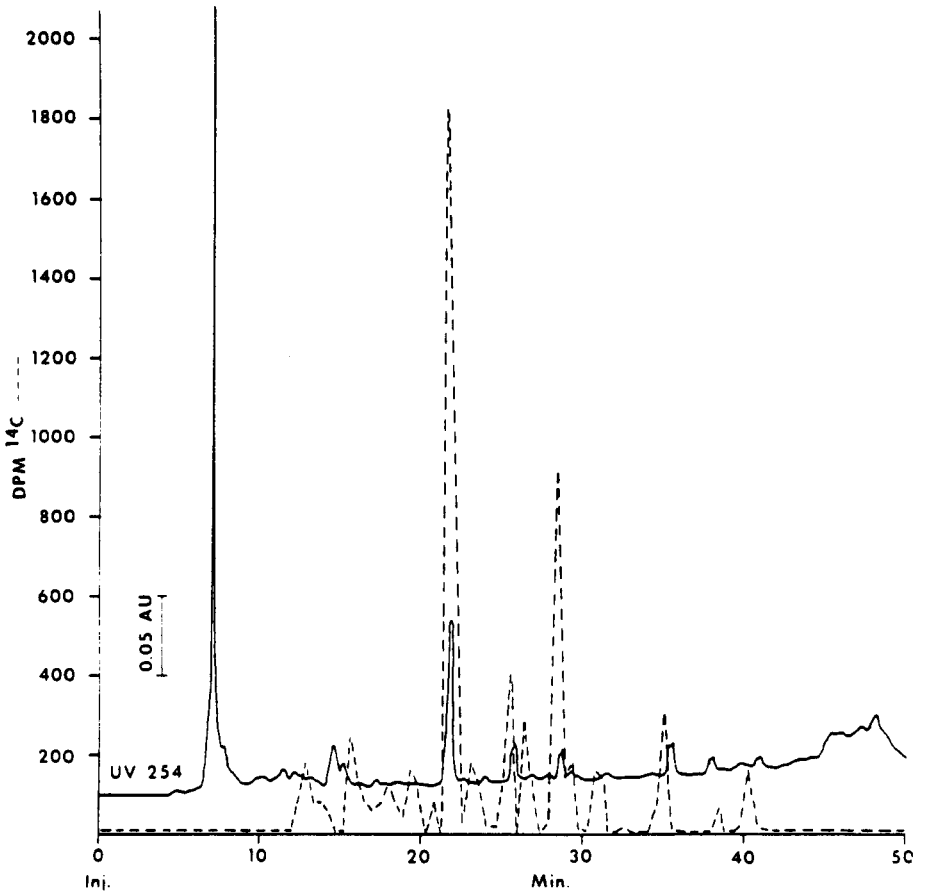

Figure 4: Chromatogram of Plasma Metabolites of DES. Peak (dotted line) at 22-23 min is E-DES-monoglucuronide; peaks at 36 min and 40 min are E-DES and Z-DES, respectively. Chromatographic conditions same as Fig 2.

The retention times indicate the presence of E- and Z-DES-monoglucuronide and E- and Z-DES. The total radioactivity recovered from the column was 84% of that in the initial liver sample.

DISCUSSION

These results complement an earlier report from this laboratory (18) and confirm that the described HPLC method reproducibly separates the major DES

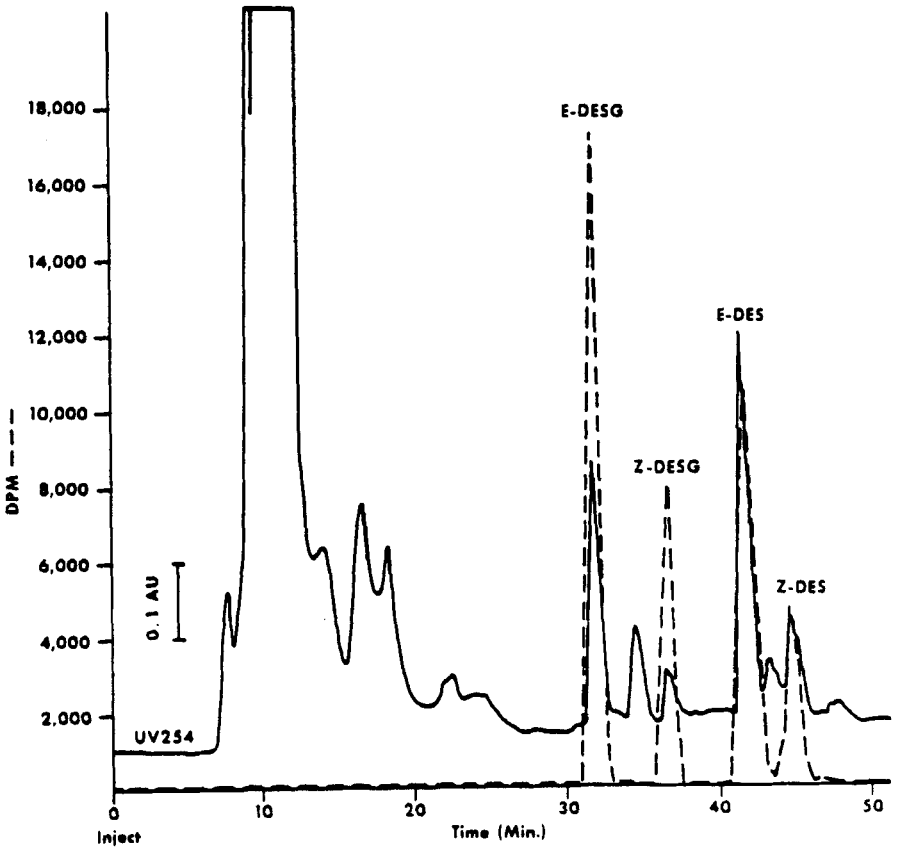

Figure 5: HPLC Profile of fetal liver tissue, spiked with ^{14}C - DESG and ^{3}H-DES. The radiolabeled peaks coincide with the authentic unlabeled DES. Chromatographic conditions: same as Fig 2.

metabolites in urine, plasma, and tissue. Unlike conventional techniques for estrogen analysis (13, 15), the present method provides for the separation of a variety of DES metabolites, including conjugates, in a single chromatographic step. The limited manipulation of samples in preparation for HPLC insures a high recovery (>80%) of DES and metabolites.

Other reports have described HPLC methods for analysis of DES and its major derivatives (10, 14), but failed to demonstrate the utility of the

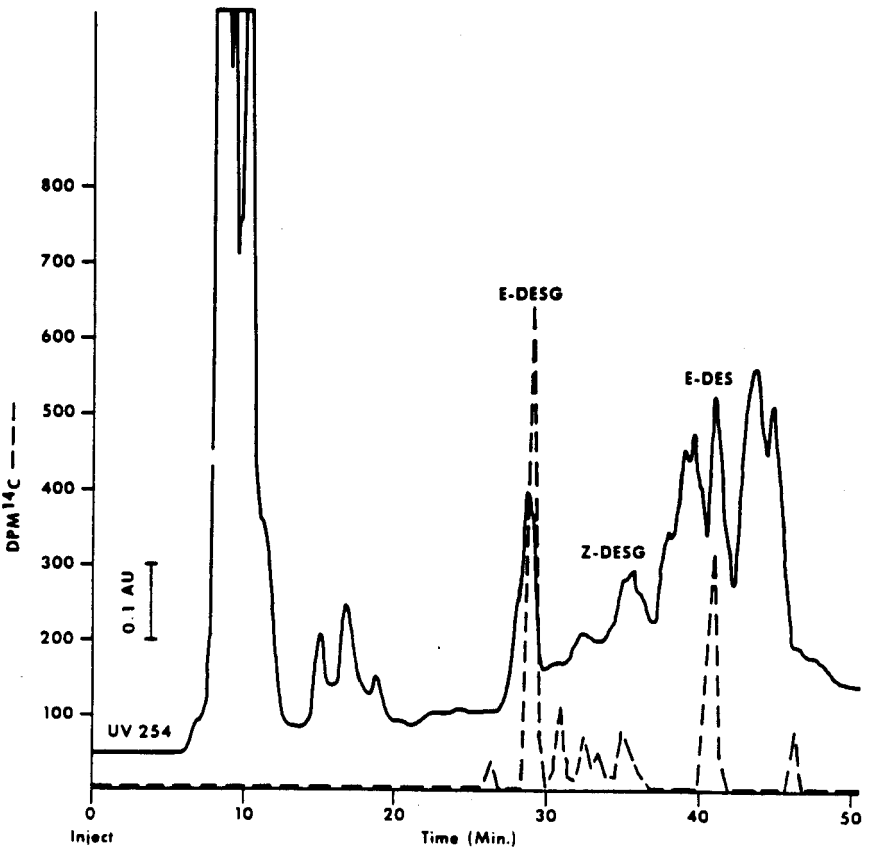

Figure 6: HPLC Chromatogram of fetal liver tissue sample from a treated monkey, showing the four major metabolites separated by HPLC. Chromatographic conditions: same as Fig 2.

techniques for biological samples. The method we have described was used to separate five major DES urinary metabolites. Three of these metabolites were subsequently identified by field desorption and GC-MS as E- and Z-DES monoglucuronide and Z,Z-dienestrol monoglucuronide. Plasma and tissue were likewise analyzed and E-DES monoglucuronide and E- and Z-DES were detected. Thus, the HPLC method has been shown useful for separating DES metabolites from biological fluids and tissue in preparation for subsequent positive identification.

The reversed-phase HPLC method described has several advantages over conventional column chromatography on, e.g., non-ionic polymeric adsorbents (XAD-2), lipophilic gel (Sephadex LH-20) and Florisil. First, the described HPLC method is rapid, requiring only 2 to 3 hours for the analysis of urine or plasma samples, in contrast, some other techniques require 1 to 2 days for similar analyses. Secondly, this single-column separation does not require extraction or hydrolysis steps; simultaneous chromatography of the unaltered conjugated and free metabolites is readily accomplished. Thirdly, authentic reference standards may be co-chromatographed with each sample. Finally, the described method is convenient for small biological samples (0.4 ml plasma and urine or 1 gm tissue). These advantages of this single-column HPLC technique make it suitable for the study of radiolabeled DES and its metabolites from biological fluids and tissues of laboratory animals.

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