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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ESTRADIOL-17β AND METABOLITES IN BIOLOGICAL MEDIA

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SUMMARY

A method was developed to resolve radiolabeled estradiol-17β and its various metabolites in biological fluids and tissues. After a rapid initial clean-up step, samples were analyzed with the sequential use of reversed-phase and normal-phase high-performance liquid chromatographic systems. Approximately 25 conjugated and non-conjugated standards could be resolved by the combined use of six systems. Radiolabeled parent compound and metabolites from biological samples were separated and tentatively identified by comparing their retention times to those of known standards. The method was found to be reproducible and quantitative for the majority of the estrogens and their conjugates, and semiquantitative for some of the more polar and di-conjugated estrogens.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the analysis of estrogens has become widespread [1—9]. The reasons for the popularity of HPLC over classical open-column and paper chromatographic techniques include excellent resolving power, short analysis time, good reproducibility and small sample size requirements [3, 5, 7, 9]. Gas chromatographic (GC) methods with resolving power equal to HPLC have been reported [10—16], but are generally inadequate for preparative work. In addition, since many of the estrogen conjugates have high molecular weights, low vapor pressure, and are relatively polar and in some instances, chemically unstable, the general utility of GC techniques would appear to be compromised.

The majority of the reported HPLC methodologies has been developed to resolve non-conjugated estrogens [1—7]. Analysis of estrogen conjugates

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generally required that the steroid nucleus first be liberated by enzyme hydrolysis or solvolysis [1, 3, 5]. Although selective cleavage of estrogen conjugates is considered possible [17—20], this approach precludes the simultaneous gathering of information concerning the relative abundance of individual conjugates and non-conjugates. In view of the growing evidence that specific estrogen conjugates are associated with particular physiological states (e.g., pregnancy) or toxicities (e.g., cholestasis) [21], methods providing a complete estrogen metabolic profile are needed.

Several reports have appeared in the literature describing HPLC methodologies for the resolution of estrogen conjugates but generally have centered around only a few of the many possible metabolites without regard for the accompanying non-conjugated estrogens [22] or have not demonstrated the utility of the methodology for biological media [23].

The present paper describes a series of HPLC systems designed to qualitatively and quantitatively resolve radiolabeled conjugated and non-conjugated estrogens. In addition, the effectiveness of the technique was assessed by its application to biological samples collected from a subhuman primate administered radiolabeled estradiol-17β.

EXPERIMENTAL

Instrumentation

The HPLC systems were composed of Waters Assoc. (Milford, MA, U.S.A.) equipment: Model 6000A pumps, Model U6K injectors, Model 660 solvent programmers and Model 440 dual-channel UV detectors equipped with 280-nm UV filters. Commercial pre-packed reversed-phase columns were used: LiChrosorb RP-18 (5 μm), 250 × 10 mm, and LiChrosorb C_2 (10 μm), 250 × 3.2 mm from Altex Scientific (Berkeley, CA, U.S.A.); LiChrosorb RP-18 (5 μm), 250 mm × 9 mm from Chrompack U.S.A. (Whittier, CA, U.S.A.); Chromegabond Diol (10 μm), 300 × 4.6 mm from ES Industries (Marlton, NJ, U.S.A.) and two (in series) μBondapak C_{18} (10 μm), 300 × 3.9 mm columns from Waters Assoc. Chromatograms were recorded on Fisher Recordall Series 5000 dual-pen recorders. Radioactive fractions were collected on a Micro-Fractionator Model FC-80K (Gilson Medical Electronics, Middleton, WI, U.S.A.) or an Isco Model 328 Golden Retriever (Instrumentation Specialties Company, Lincoln, NE, U.S.A.). The scintillation fluid was Scintisol from Isolab (Akron, OH, U.S.A.). Scintillation counting was performed on a Tracor Analytic Mark III 6881 Liquid Scintillation System (Tracor Analytical, Atlanta, GA, U.S.A.).

Estrogen standards

The estrogens and estrogen conjugates investigated in this study are listed in Table I. The compounds were purchased as sodium or potassium salts or as free acids and were used without further purification. Stock solutions of these estrogens and estrogen conjugates were maintained in 100% methanol. Prior to use, the desired amount of standard was pipetted into a 5.0-ml centrifuge tube and reduced to near dryness in a stream of nitrogen on an N-Evap (Organomation Assoc., Worcester, MA, U.S.A.). The residue was then dissolved in 100—200 μl of the required solvent system.

TABLE I

ESTROGENS AND ESTROGEN CONJUGATES

Trivial name	Abbreviation	Source*
Estrone	E_1	RP
Estradiol-17β	E_2	RP
Estriol	E_3	S
Estrone 3-methyl ether	E_1-3-Met	RP
Estradiol 3-methyl ether	E_2-3-Met	RP
Estriol 3-methyl ether	E_3-3-Met	RP
2-Methoxyestrone	2-MeO-E_1	RP
2-Methoxyestradiol	2-MeO-E_2	RP
2-Hydroxyestrone	2-OH-E_1	SWFRE
2-Hydroxyestradiol	2-OH-E_2	SWFRE
2-Hydroxyestriol	2-OH-E_3	RP
6α-Hydroxyestradiol	6α-OH-E_2	RP
16-Epiestriol	16-Epi-E_3	S
15α-Hydroxyestriol	15α-OH-E_3	Ster
Estrone 3-glucuronide	E_1-3G	S
Estrone 3-sulfate	E_1-3SO_4	RP
Estradiol 3-glucuronide	E_2-3G	S
Estradiol 17β-D-glucuronide	E_2-17G	S
Estradiol 3-sulfate	E_2-3SO_4	RP
Estradiol 17β-sulfate	E_2-17SO_4	RP
Estradiol 3,17-disulfate	E_2-3,17SO_4	RP
Estradiol 3-sulfate,17β-D-glucuronide	E_2-3SO_4.17G	S
Estriol 3-glucuronide	E_3-3G	S
Estriol 16α-glucuronide	E_3-16G	S
Estriol 17β-glucuronide	E_3-17G	S
Estriol 3-sulfate	E_3-3SO_4	RP
Estriol 17-sulfate	E_3-17SO_4	RP
17α-Estradiol 17β-D-glycoside	αE_2-17Glyc	SWFRE

*S = Sigma, St. Louis, MO, U.S.A.; RP = Research Plus Steroid Laboratories, Denville, NJ, U.S.A.; Ster = Steraloids, Wilton, NH, U.S.A.; SWFRE = Southwest Foundation for Research and Education, Custom Synthesis, San Antonio, TX, U.S.A.

To determine the recovery of each estrogen standard after chromatography, standards were chromatographed and the eluted peak was collected, reduced in volume, and rechromatographed. Peak height or peak area as determined by UV absorbancy at 280 nm was compared between the first and second chromatograms.

HPLC systems

The estrogen compounds of interest varied widely as to their polarity, therefore, several HPLC systems were necessary to resolve these agents. Two gradient elution systems and three isocratic systems were developed (Fig. 1).

System A consisted of a LiChrosorb RP-18 (5 μm) 250 × 10 mm (A_1) or a 250 × 9 mm (A_2) reversed-phase column protected by an in-line guard column (25 × 6 mm) packed with Bondapak C_{18}-Corasil. The 50-min convex gradient program (No. 5 on the Model 660 solvent programmer) began with 10% methanol in 0.01 *M* ammonium acetate buffer (pH 6.9) and concluded

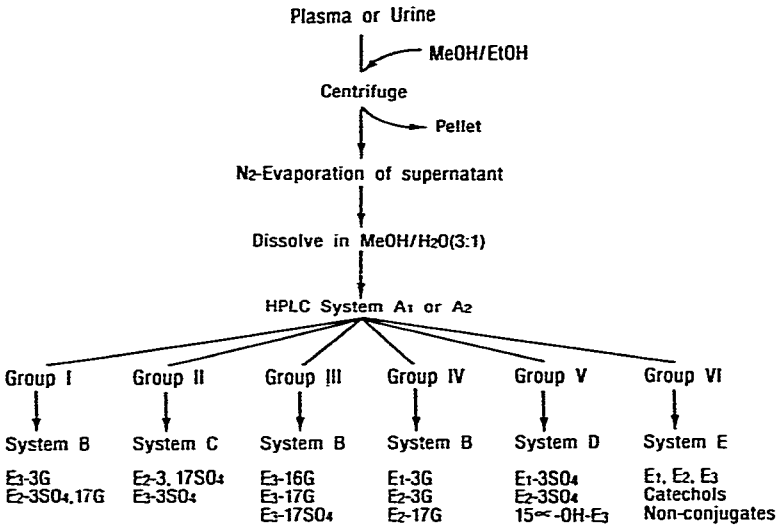

Fig. 1. Flow diagram of the urine and plasma preparation for separation of estrogens and estrogen metabolites into groups by various HPLC systems.

with 100% methanol. The flow-rate was 2.0 ml/min and 1.0-ml fractions were collected.

System B consisted of two μBondapak C_{18} (10 μm) 300 × 3.9 mm reversed-phase columns in series. The solvent contained 45% methanol in 0.01 *M* ammonium acetate buffer adjusted to pH 3.97 with glacial acetic acid. The isocratic system had a flow-rate of 1.0 ml/min and 0.5-ml fractions were collected.

System C was similar to system B except 35% methanol was used and the pH was adjusted to 7.74 with ammonium hydroxide.

System D consisted of a LiChrosorb C_2 (10 μm) 250 × 3.2 mm reversed-phase column. The solvent contained 25% methanol in 0.01 *M* ammonium acetate buffer adjusted to pH 7.56 with ammonium hydroxide. The flow-rate was 1.0 ml/min and 0.5-ml fractions were collected.

System E consisted of a Chromegabond Diol (10 μm) 300 × 4.6 mm normal-phase column. The 100-min linear gradient program (No. 6 on the Model 660 solvent programmer) began with 100% hexane and concluded with hexane—isopropanol (80:20). The flow-rate was 1.5 ml/min and 0.75-ml fractions were collected.

All of the solvents and chemicals used to prepare the solvent systems were reagent grade. The methanol and hexane were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); the isopropanol was from Fisher (Pittsburgh, PA, U.S.A.) and the ammonium acetate from Mallinckrodt (St. Louis, MO, U.S.A.). Deionized water was filtered through a Millipore system and all solvents were degassed prior to use.

Biological sample preparation

In order to assess the utility of the developed HPLC methodology for the analysis of biological samples, an in vivo experiment was performed. Under ketamine (Parke-Davis, Detroit, MI, U.S.A.) anesthesia (10 mg/kg, intra-

muscular) a pregnant 130-day gestational age rhesus monkey (*Macaca mulatta*) was prepared with a femoral artery catheter (No. 5 French) and with a foley catheter (No. 10 French). A HPLC-purified dose of [6,7-^{3}H]estradiol-17β (42.0 Ci/mmole, Amersham, Arlington Heights, IL, U.S.A.) was administered in a 20% ethanol—saline solution via the radial vein. Maternal blood and urine, and fetal blood and tissue samples were collected. The blood samples were immediately centrifuged. The resulting plasma samples and the urine and tissue samples were then stored at —60°C until analysis.

Proteins and salts were removed from urine samples by precipitation with two volumes of methanol—ethanol (1:1). After centrifugation, the precipitate was washed twice with equal volumes of methanol—ethanol (1:1) and the supernatants combined. The supernatant pool was then reduced to near dryness on the N-Evap and subsequently dissolved in 100 μl of methanol—water (3:1) in final preparation for HPLC analysis (Fig. 1).

Plasma samples (0.3—0.5 ml) were made up to 1.0-ml volume by addition of Millipore-filtered water. They were then centrifuged at 8730 *g* in a Beckman micro-fuge to remove the buffy coat. The supernatant was then treated with methanol—ethanol (1:1) and prepared for HPLC analysis in the same manner as the urine.

The procedure employed for tissue extraction and HPLC analysis has been published [9]. Briefly, samples were weighed and placed in ice-packed Erlenmeyer flasks. After adding 5 ml of cold distilled water per g of tissue, each sample was homogenized for 1 min. Five volumes of a mixture of methanol—dimethoxymethane (DMM) (1:1) were used to extract the unbound radioactivity and to precipitate macromolecular components. This mixture was shaken for 18 h and then centrifuged. The supernatant was removed and the pellet washed three times with methanol—DMM (1:1) which was then added to the supernatant. The supernatant was reduced to 1—2 ml volume on a rotary evaporator at 40°C. The sample was washed from the evaporator flask with methanol and filtered through a 0.6-μm BDWP Millipore filter. The filtrate was concentrated to less than 10 ml and passed through a C_{18} Sep-Pak (Waters Assoc.) followed by 5 ml methanol. An aliquot was taken for radioactivity determination and the volume was reduced under a stream of nitrogen to near dryness. The sample was then brought to 225 μl in methanol—water (3:2) in preparation for HPLC.

RESULTS

Estrogen standards

Due to the wide range of polarities, several chromatographic systems were developed to adequately resolve the compounds of interest. The LiChrosorb RP-18 Gradient System A_2 separated the compounds into six arbitrarily defined groups, I—VI, as shown in Figs. 1 and 2. Group I includes the most polar compounds E_3-3G and E_2-3SO_4,17G. Group II includes E_2-3,17SO_4 and E_3-3SO_4. Group III consists of E_3-16G and E_3-17G, which have proven inseparable in our laboratory, and E_3-17SO_4. E_1-3G, E_2-3G, and E_2-17G constitute Group IV and E_1-3SO_4 and E_2-3SO_4 are in Group V with 15α-OH-E_3. The least polar compounds consisting mainly of non-conjugates make up the large Group VI.

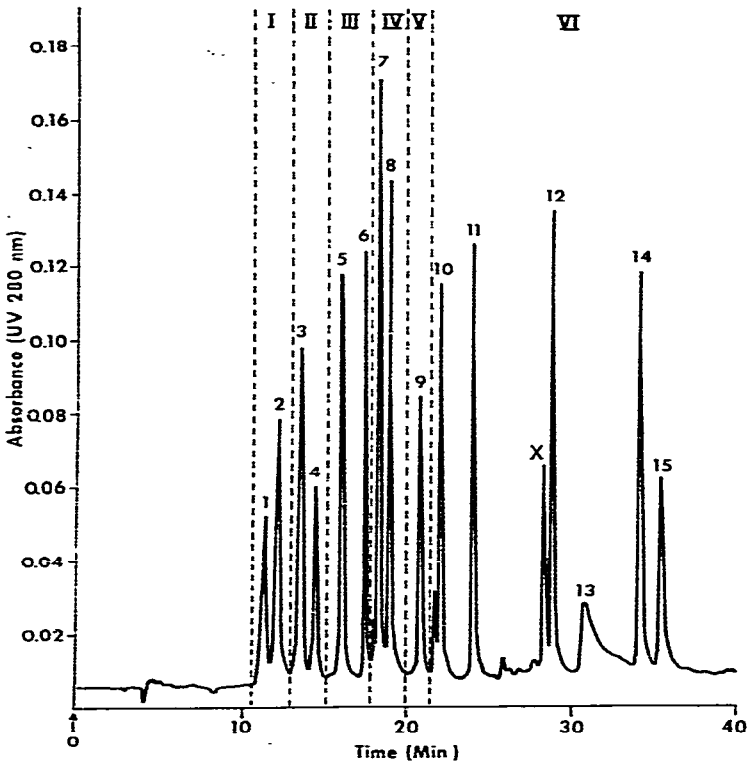

Fig. 2. Chromatogram of estrogen and metabolite standards. System A_2: LiChrosorb RP-18, 5 μm, 250 × 9 mm, reversed-phase column; mobile phase, 10% methanol in 0.01 *M* ammonium acetate, pH 6.9, to 100% methanol, convex gradient program (No. 5) in 50 min; flow-rate, 2.0 ml/min at 2500 p.s.i.; chart speed = 1 cm/2 min; standards: (1) E_3-3G, 20 μg; (2) E_2-3SO_4,17G, 200 μg; (3) E_2-3,17SO_4, 80 μg; (4) E_3-3SO_4, 100 μg; (5) E_3-16G, 20 μg; (6) E_3-17SO_4, 10 μg; (7) E_1-3G, 40 μg; (8) E_2-17G, 20 μg; (9) E_1-3SO_4, 100 μg; (10) 6α-OH-E_2, 10 μg; (11) E_3, 10 μg; (x) unknown degradation product; (12) αE_2-17Glyc, 20 μg; (13) 2-OH-E_2, 15 μg; (14) E_2, 10 μg, E_1, 10 μg; (15) 2-MeO-E_2, 5 μg.

The eluted fractions comprising the groups thus separated were reduced in volume under nitrogen to near dryness and re-dissolved in 100—200 μl of the appropriate solvent to be further chromatographed. The HPLC System B was found to be effective in further separating Group I standards, E_3-3G from E_2-3SO_4,17G (Fig. 3A). Group II standards, E_2-3,17SO_4 and E_3-3SO_4, were resolved by System C as shown in Fig. 3B. System B provided excellent resolution of Group IV standards (Fig. 3C). Group V was resolved by System D (Fig. 3D). This system also separates E_1-3SO_4 and E_2-17SO_4. However, E_2-3SO_4 and E_2-17SO_4 could not be resolved. System E successfully separates the parent compound, E_2, from its primary metabolites E_1 and E_3 and the catechol estrogens (Fig. 4). The conjugate, E_2-17Glyc was also resolved by this normal-phase system. Table II summarizes all the retention times of the estrogen standards in the various HPLC systems. The HPLC system which provides the best resolution of the respective estrogen compounds in biological media is indicated with an asterisk.

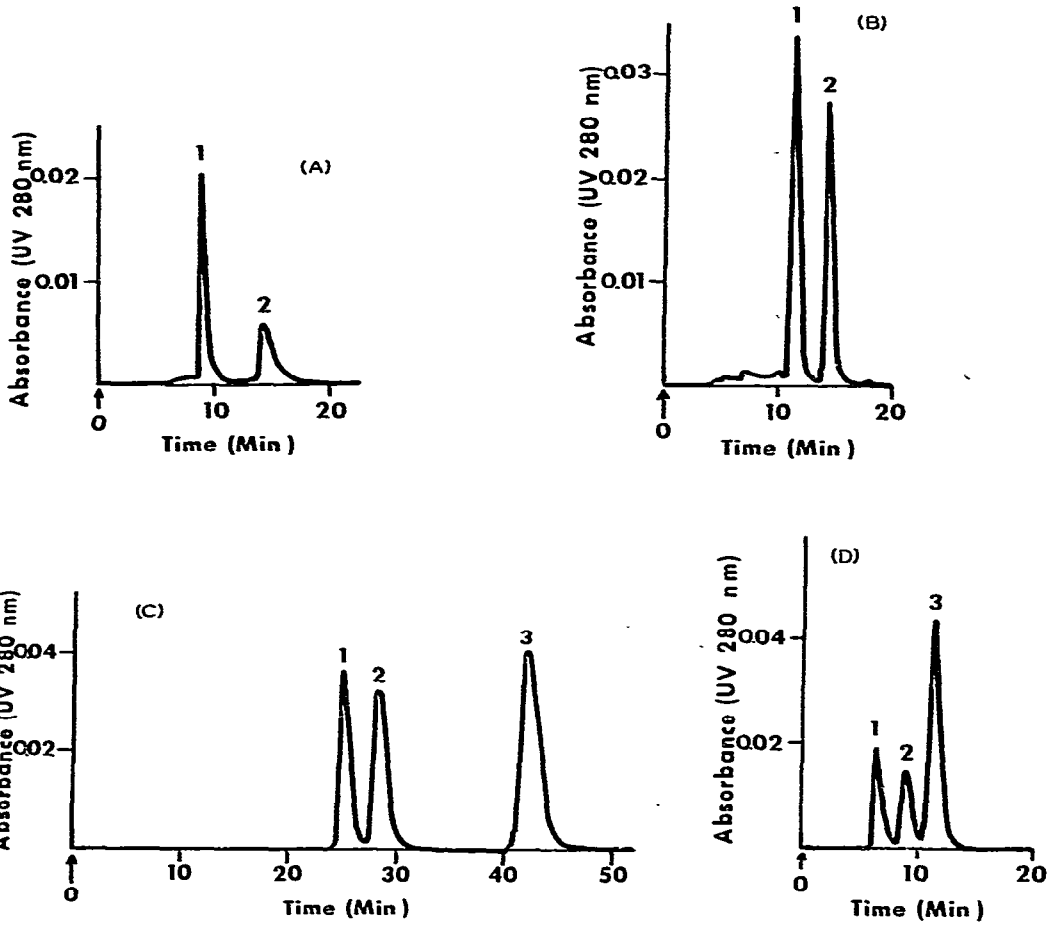

Fig. 3. Separation of Group I, II, IV and V metabolites. A, Group I. System B: two μBondapak C_{18}, 10 μm, 300 × 3.9 mm reversed-phase columns; mobile phase, 45% methanol in 0.01 *M* ammonium acetate buffer, pH 3.97; flow-rate, 1.0 ml/min at 2750 p.s.i.; chart speed = 1 in./10 min; standards: (1) E_3-3G, 15 μg; (2) E_2-3SO_4, 17G, 60 μg. B, Group II. System C: same as Fig. 3A but methanol at 35% concentration and pH 7.74; standards: (1) E_2-17SO_4, 40 μg; (2) E_3-3SO_4, 60 μg. C, Group IV. System B: same as Fig. 3A; standards: (1) E_1-3G, 30 μg; (2) E_2-3G, 30 μg; (3) E_2-17G, 30 μg. D, Group V. System D: LiChrosorb C_2, 10 μm, 250 × 3.2 mm reversed-phase column; mobile phase 25% methanol in 0.01 *M* ammonium acetate, pH 7.56; flow-rate, 1.0 ml/min at 2000 p.s.i.; chart speed = 1 in./10 min; standards: (1) E_1-3SO_4, 60 μg; (2) E_2-3SO_4, 60 μg; (3) 15α-OH-E_3, 10 μg.

To determine the recovery of each estrogen standard after chromatography, standards were chromatographed and the eluted peak was collected, reduced in volume, and rechromatographed. Peak height or peak area as determined by UV absorbancy at 280 nm was compared between the first and second chromatograms. The percent recovery of each standard for HPLC System A_2 and subsequent system of choice is listed in Table II. The recovery of standards on the reversed-phase System A_2 ranged between 87 and 111% for all compounds with the exception of the catechol estrogens 2-OH-E_1 (74%), 2-OH-E_2 (65%)

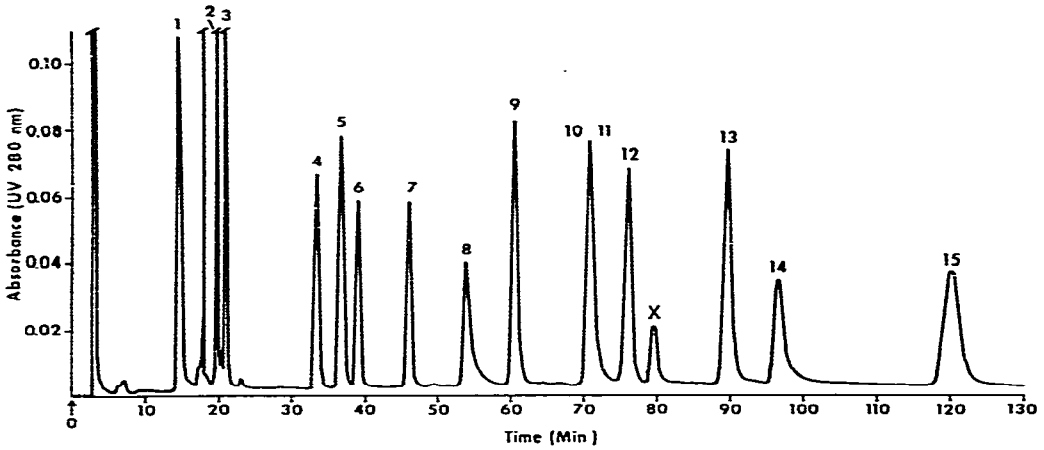

Fig. 4. HPLC profile of estrogen and metabolite standards. System E: Chromegabond Diol, 10 μm, 300 × 4.6 mm normal-phase column; mobile phase, 100% hexane to hexane—isopropanol (80:20), linear gradient program (No. 6) set for 100 min; flow-rate, 1.5 ml/min at 300 p.s.i.; chart speed = 1 in./10 min; standards: (1) E_1-3-Met, 30 μg; (2) E_2-3-Met, 10 μg; (3) 2-MeO-E_1, 10 μg; (4) E_1, 10 μg; (5) 2-MeO-E_2, 10 μg; (6) E_3-3-Met, 10 μg; (7) E_2, 10 μg; (8) 2-OH-E_1, 20 μg; (9) 16-Epi-E_3, 30 μg; (10) 2-OH-E_2, 15 μg; (11) E_3, 10 μg; (12) 6α-OH-E_2, 10 μg; (x) unknown degradation product; (13) 15α-OH-E_3, 25 μg; (14) 2-OH-E_3, 30 μg; (15) αE_2-17Glyc, 30 μg. The unlabeled UV spike between standard (1) and (2) is the isopropanol solvent front.

and 2-OH-E_3 (71%). These 2-hydroxylated estrogens also exhibited lower recovery on the normal-phase Chromegabond Diol column (System E). It would appear that the juxtaposition of the two OH-groups in the A-ring is responsible for the poor recovery since the structurally related metabolic precursors E_1, E_2 and E_3 or metabolic products (2-methoxy compounds) showed good recovery (87—96%) in HPLC System A_2 (Table II). In general, the sulfoconjugates exhibited slightly less recovery than other conjugates in the reversed-phase System D (59—87%) but not in the initial system, A_2. In all cases, the retention times of the estrogen standards were the same for the first and second chromatograms.

Biological samples

In order to demonstrate the utility of the HPLC methodology, radiolabeled E_2 was administered to a rhesus monkey and samples of urine, blood and tissue were collected. These samples were prepared for analysis as described in Biological Sample Preparation and then co-chromatographed with appropriate estrogen standards.

Fig. 5 is a typical chromatogram of a sample of monkey urine from System A_1. The majority of the polar endogenous material and pigments absorbing at 280 nm are eluted before the radioactivity. Then followed a large amount of endogenous UV-absorbing material co-migrating with the radioactivity of the polar Groups I and II and the intermediate Group III. The UV profiles of the crystalline standards eluting in these three groups were partially screened by the high UV-background. The percent recovery of seven conjugates based on three replicate analyses of a 1-h urine sample is seen in Table III. Approximate-

TABLE II

RETENTION TIME IN MINUTES OF THE ESTROGEN STANDARDS IN THE VARIOUS HPLC SYSTEMS

Values in parentheses are the percent recovery of the standards after rechromatography in the designated HPLC system.

Estrogen	HPLC system					
	A_1	A_2	B	C	D	E
E_3-3G	14	11(88)	8(100)*	9	2	
E_2-3SO_4,17G	15	12(105)	14(91)*	7	2	
E_2-3,17SO_4	16	13(108)	9	11(88)*	2	
E_3-3SO_4	16	14(90)	9	15(84)*	2	
E_3-16G	19	16(96)	16(98)*	17	4	
E_3-17G	19	16(96)	16(100)	17	4	
E_3-17SO_4	19	17(96)	16	20(83)*	5	
E_1-3G	21	18(102)	25(100)*	22	4	
E_2-3G	21	18(98)	28(90)*	26	4	
E_2-17G	22	19(104)	42(95)*	32	6	
15α-OH-E_3	24	20(92)	—		12	90(79)*
2-OH-E_3	24	20(71)*	—		—	97(39)
E_2-17SO_4	—	20(95)	26		9(87)*	—
E_1-3SO_4	24	21(99)*	24		7(70)*	—
E_2-3SO_4	24	21(88)*	26		9(59)	—
6α-OH-E_2	25	22(111)				76(81)*
E_3	27	24(96)*				71
16-Epi-E_3	31	28(95)				61(81)*
αE_2-17Glyc	—	28(97)*				120
2-OH-E_2	34	30(65)				71(89)*
2-OH-E_1	34	30(74)*				54(54)
E_1	36	34(94)				34(85)*
E_2	37	34(88)				46(80)*
2-MeO-E_2	39	38(87)				37(83)*
2-MeO-E_1	—	38(88)				21(90)*
E_1-3-Met						14(85)*
E_2-3-Met						20(83)*
E_3-3-Met						39(84)*

*HPLC system of choice for resolution of estrogens in biological samples.

ly 62% of the radioactivity in the sample co-migrated with known estrogen standards while the other 38% was not identifiable. The largest portion of urinary radioactivity co-chromatographed with E_1-3G, 27.63% ± 1.76 (mean ± S.E.) as seen in Fig. 6. Another 17% migrated with the standards E_2-3G, E_2-17G and E_1-3SO_4 (Table III). The standard error of the mean and coefficient of variation (C.V.) for these four estrogen conjugates is quite small indicating a reproducible assay. Three more polar conjugates were also found to co-migrate with significant amounts of radioactivity (17.3%). As indicated by the larger C.V., these polar conjugates were more difficult to reproducibly quantify. The total recovery of radioactivity after complete analysis was >92%.

A representative HPLC chromatogram of fetal liver tissue as resolved by

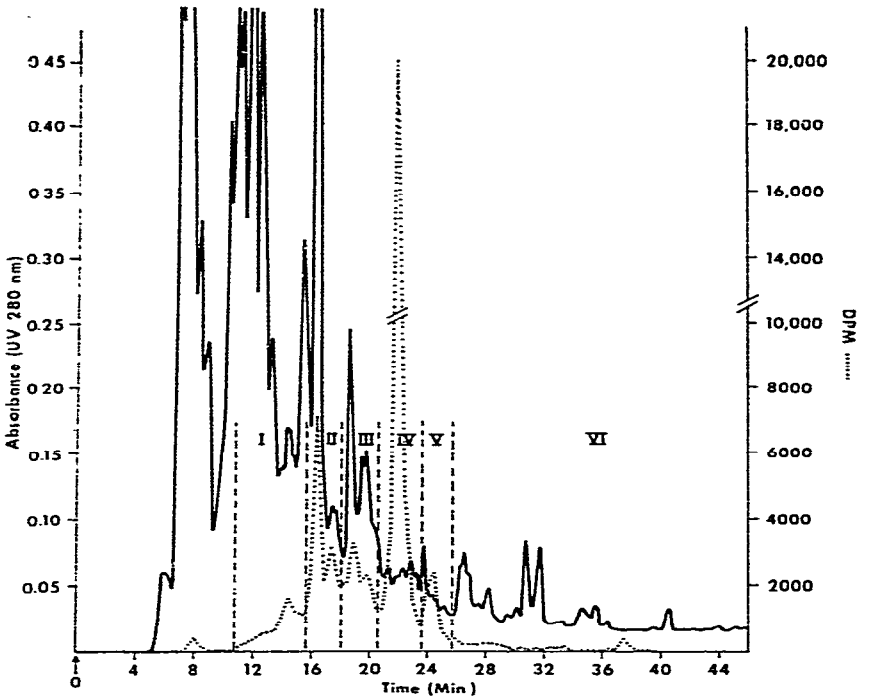

Fig. 5. A typical chromatogram of a sample of monkey urine containing tritium-labeled metabolites of [^{3}H]estradiol-17β. System A_1: same conditions as Fig. 2. Standard, E_3-16G, 25 μg; retention time, 19 min. The solid line is the UV absorbancy at 280 nm. The dotted line represents the distribution of tritium radioactivity in dpm.

TABLE III

PERCENT RECOVERY OF ESTROGEN CONJUGATES FROM URINE

Conjugate	Trial no.			Mean	S.E.	C.V.*
	1	2	3			
E_3-3G	3.2	5.6	5.1	4.63	0.73	27
E_2-3,17SO_4	4.1	5.0	8.5	5.87	1.34	39
E_3-3SO_4	4.1	10.3	6.0	6.80	1.84	47
E_1-3G	26.4	31.3	25.4	27.63	1.76	11
E_2-3G	6.2	7.5	6.1	6.60	0.45	12
E_2-17G	6.1	7.1	5.5	6.23	0.47	13
E_1-3SO_4	4.4	4.5	3.3	4.07	0.39	17
Unknown	29.1	24.5	37.2	30.27	3.72	21
Total	83.6	95.6	97.1	92.10	—	—

*C.V. = $\frac{\text{standard deviation}}{\text{mean}} \times 100$.

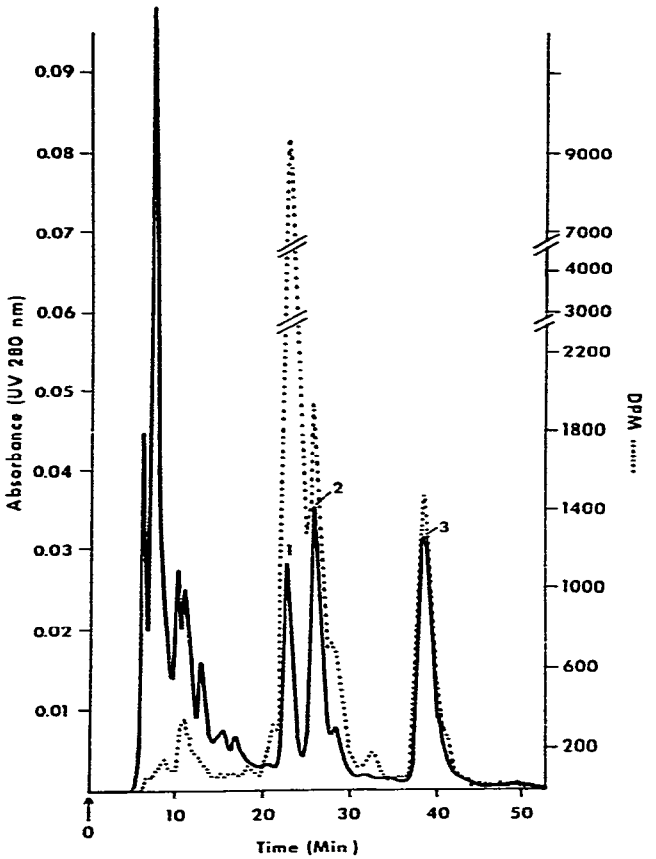

Fig. 6. Separation of metabolites upon rechromatography of Group IV fraction from monkey urine (HPLC System B). Chromatographic conditions same as in Fig. 3C; standards: (1) E_1-3G, 10 μg; (2) E_2-3G, 20 μg; (3) E_2-17G, 20 μg. The solid line is UV absorbancy at 280 nm. The dotted line represents the distribution of tritum radioactivity in dpm.

system A_1 is shown in Fig. 7. The majority of the radioactivity administered to the maternal monkey as [^{3}H] estradiol-17β was converted to a metabolite which co-migrated with E_1-3SO_4. Further, chromatography of this radioactivity in system D demonstrated that over 95% co-migrated again with E_1-3SO_4 and not E_2-3SO_4 or 15α-OH-E_3 (data not shown). Fetal plasma samples showed similar results following HPLC analysis (Fig. 8). In either the tissue or plasma, the endogenous UV-absorbing material near the front of the chromatogram did not interfere with the identification of estrogen standards. Total recovery of radioactivity from the plasma and tissue samples was always greater than 80%.

DISCUSSION

The HPLC methodology herein presented provides a means to qualitatively and quantitatively describe the metabolic profile of the naturally occurring estrogen, estradiol-17β. With the aid of radiolabeled isotopes, the described method may be used to determine the pharmacokinetics of estradiol and its

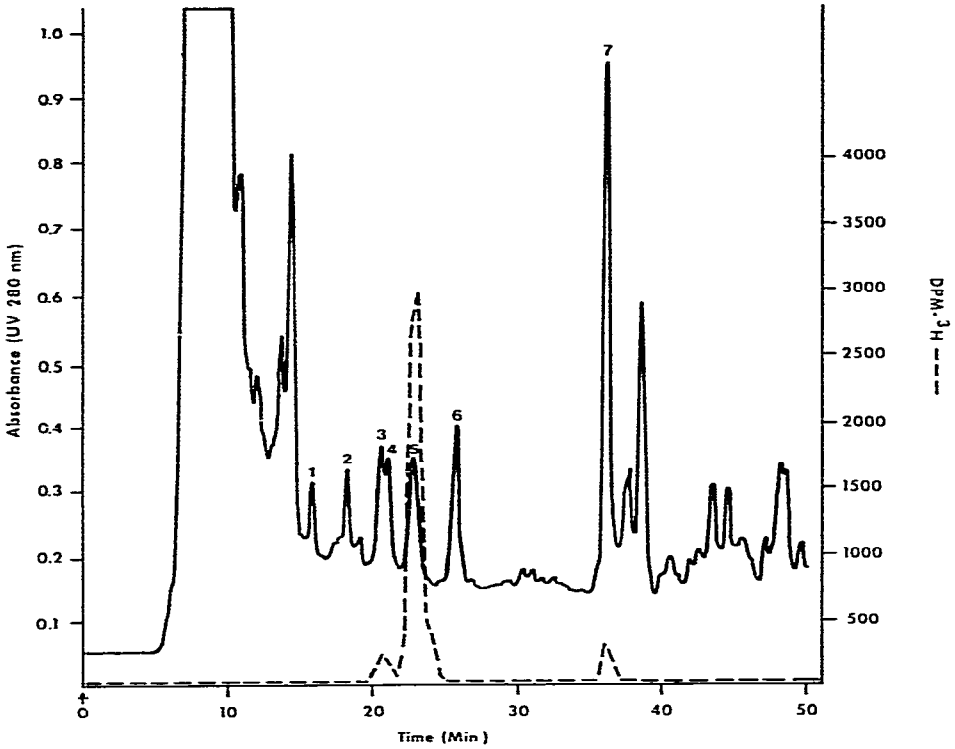

Fig. 7. A typical chromatogram of fetal monkey liver tissue containing tritium-labeled metabolites of [^{3}H]estradiol-17β. System A_1: same conditions as Fig. 2; standards: (1) E_2-3,17SO_4; (2) E_3-16G; (3) E_1-3G; (4) E_2-17G; (5) E_1-3SO_4; (6) E_3; (7) E_2. The solid line is UV absorbancy at 280 nm. The dashed line represents the distribution of tritium radioactivity in dpm.

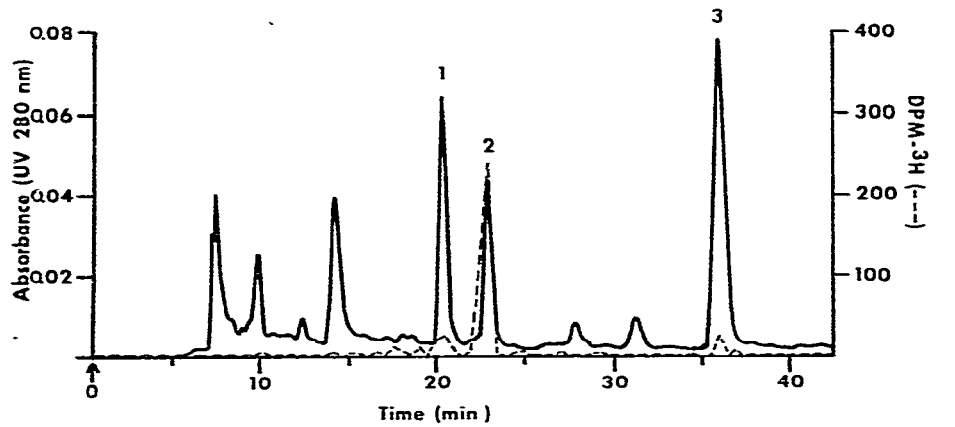

Fig. 8. A typical chromatogram of a fetal plasma sample containing tritium-labeled metabolites of [^{3}H]estradiol-17β. System A_1: same conditions as Fig. 2; standards: (1) E_1-3G; (2) E_1-3SO_4; (3) E_2. The solid line is UV absorbancy at 280 nm. The dashed line represents the distribution of tritium radioactivity in dpm.

metabolites in the plasma, tissue and excretory products of experimental animal models. A variety of methods are available in the literature concerning estrogen analysis [1—3, 5, 22, 24], but to our knowledge, this report is the first to exemplify the utility of a HPLC method for biological samples which provides for the profiling of estrogen metabolites including conjugates and non-conjugates simultaneously. By eliminating the need for solvent extraction and hydrolysis steps, the time required for quantitation of metabolites has been reduced as compared to previous techniques [25].

In the present report, quantification of the various estrogen metabolites is based on radioactivity. A disadvantage of this technique is that the use of radioisotopes is generally restricted to animal studies and can only rarely be applied to human experiments. On the other hand, an advantage of radioisotope studies is that the sensitivity of the method is determined by the specific activity of the radioisotope in question. In the case of estradiol-17β, an isotope is available commercially which has a specific activity of over 100 Ci/mmole. Therefore, based on the ability to quantify 200 dpm of radiolabeled estrogen, (signal-to-noise ratio of 8:1) the sensitivity of the present radioisotope method is greater than 0.5 pg per 0.5 ml biological fluid (1.0 ppt).

Because of the range of polarities of the many possible estradiol metabolites, a number of HPLC systems and columns were found necessary for the complete resolution of the 25—30 standards. It was essential that the initial HPLC system applied to resolve the various estrogens be capable of separating the entire polarity range of possible metabolites from highly polar diconjugates to low polarity methylated non-conjugated metabolites. A reversed-phase gradient system was found suitable to accomplish this separation. As expected, the relative retention times of standards were the same between the two commercially available columns which contained the same stationary phase (LiChrosorb RP-18) but differed in column diameter (A_1, 250 × 10 mm; A_2, 250 × 9 mm). As was observed in our studies with the HPLC analysis of diethylstilbestrol [9], the ammonium acetate buffer was found to decrease solute tailing.

In order to resolve the several closely related estrogen conjugates in each of Groups I—V, two different reversed-phase packings, three different methanol – ammonium acetate solvents and three different pH adjustments were necessary. As expected, it was observed that the polar conjugates were more readily separated with the use of a more polar solvent mixture (i.e., larger water – methanol ratio).

The single normal-phase system (E) adequately resolved the 14 non-conjugates and one glycoside conjugate studied. Williams and Goldzieher [26] were the first to report the use of a Chromegabond Diol column for the separation of estrogens (i.e., ethynylated estrogens). More recently, this normal-phase column has been used to resolve a variety of estrogens including 17α-ethynylestradiol and diethylstilbestrol [25].

The recovery of the non-catechol estrogen standards following rechromatography ranged between 87 and 111% in the initial HPLC System A_2. This high recovery indicates that the collection, evaporation and chromatography steps of the procedure do not result in alteration or loss of estrogen standard. The catechol estrogens, however, exhibited a lesser recovery (65—74%) perhaps

relecting their susceptibility to oxidation. In an attempt to increase the recovery of the labile catechol estrogens, an inert gas, argon, was bubbled through the HPLC solvents and utilized in all evaporatory steps of the method. The percent recovery of E_1 and 2-hydroxy-E_1 and E_3 was compared with and without the use of argon in HPLC system A_2. The argon gas treatment failed to increase the recovery of the catechol estrogens (unpublished observation). An earlier report in the literature also described reversed-phase and normal-phase HPLC separation of the 2-OH-E_1 and 2-OH-E_2 [6]. However, no estimation of the recoveries of these catechol estrogens was provided for comparison.

As noted in the results, the sulfoconjugates resolved in HPLC System D exhibited lesser recovery than in HPLC System A_2. An explanation of this difference may reside in the fact that the reversed-phase column packing for System D was C-2 and not the more common C-18. The shorter side-chains on the silicon base of the C-2 column may allow for more ionic interactions and thereby reduce the stability and recovery of these relatively unstable conjugates, E_2-17SO_4, E_1-3SO_4 and E_2-3SO_4.

The requirement of more than one chromatographic system to separate the various E_2 metabolites was also observed by Van der Wal and Huber [23]. It is important to note however, that up to 14 metabolites, both free and conjugated, can be resolved with the initial LiChrosorb RP-18 HPLC system (A_1 or A_2). We have found that the significant, plasma-borne conjugates of E_2 are relatively few in number as compared to the urinary products and may be adequately defined by this single chromatographic system (Fig. 8).

A problem area of the described methodology revolves around three of the least polar estrogen standards; E_1-3-Met, E_2-3-Met and E_3-3-Met. These standards are well resolved by HPLC System E and exhibit good recovery (83—90%). However, they are so non-polar that their recovery from the methanol-based reversed-phase HPLC System A_2 is impeded. If these estrogen derivatives are expected to be present in a biological sample, then they may be first extracted with a non-polar solvent such as chloroform or benzene and then chromatographed on HPLC System E.

Based on the relative retention times of the estrogens on the LiChrosorb RP-18 column, it would appear that classical solvent partitioning methods would not discriminate conjugates from polar non-conjugated estrogens. For example, both 2-OH-E_3 and 15α-OH-E_3 exhibited the same retention time as E_1-3SO_4 in System A_1 and A_2, while αE_2-17Glyc had a greater retention time than several non-conjugates including 2-OH-E_3, 15α-OH-E_3, 6α-OH-E_2 and E_3 in these reversed-phase systems.

The reproducibility and recovery of the method was tested by comparing multiple HPLC separations of a single urine sample collected from an animal dosed with radiolabeled E_2. Total recovery of radioactivity from the procedural analysis was over 92% while approximately 62% of this co-migrated with known standards. The coefficient of variation ranged from 11 to 17% for the most abundant and least polar metabolites and up to 47% for the more polar metabolites. This trend, i.e., that the more polar conjugates exhibited greater variability than the others, may in part be produced by the large amount of interfering, endogenous material which co-migrates with these polar compounds.

In conclusion, the described sample preparation and HPLC methodologies are capable of the rapid and efficient resolution of free and conjugated metabolites of radiolabeled estradiol-17β from biological tissues and fluids.

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