CHROMATOGRAPHIC PATTERNS OF URINARY ETHYNYL ESTROGEN METABOLITES IN VARIOUS POPULATIONS

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ABSTRACT

Radioactive mestranol (ME) and/or ethynylestradiol (EE) were administered to women in Nigeria, Sri Lanka, and the USA, and the types and patterns of radioactive urinary conjugates examined by Sephadex LH-20 chromatography. There are no differences in the total excretion of urinary radioactivity over 3 days. Consistent geographic differences appear to be present in the proportion of 3-, 17-, and 3,17-glucuronides. If confirmed on larger population samples, these observations may indicate significant geographic differences in the hepatic metabolism of ethynyl estrogens.

High performance liquid chromatographic patterns of the urinary aglycone metabolites of ME and EE were examined in a number of women. The separation was accomplished on a Chromegaprep Diol column with a gradient of isopropanol in heptane. Ethynyl estrogen metabolism shows considerable individual variation. EE is usually the principal compound excreted following ME or EE administration. Unmetabolized ME is present in the ME profiles. The profiles of EE and ME are similar, with EE demonstrating a more complex pattern. Oxidative metabolism occurs chiefly at positions 2, 6, and 16 and is fairly extensive in the USA subjects. The Sri Lankan women generally show less of the oxidative products and the Nigerian group display a notable lack of oxidative metabolism. There is no difference in the metabolic patterns of long-term oral contraceptive users vs. non-users. Using silver sulfoethylcellulose column chromatography, from 14.1 to 34.7% of the excreted radiolabeled aglycones are non-ethynyl (i.e., either D-homo or de-ethynylated estrogens).

INTRODUCTION

Approximately one third to one half of an orally administered dose of ethynylestradiol or mestranol is excreted in the urine (1) in the form of conjugates which can be separated into five major groups by chromatography on Sephadex LH-20 (2,3). Their relative polarities, reactions with glucuronidases and sulfatases, as well as the availability of authentic 17β-glucosiduronate and 3,17-diglucosiduronate (4,5), make it likely that they are eluted in the order: 3-glucuronide, 17-glucuronide, diglucuronide, mixed conjugates (i.e.,

sulfo-glucuronides), and sulfates. It was noted (3) that there was variation from individual to individual in the relative proportions of these conjugate groups; however the number of subjects was too small to permit firm conclusions. Subsequently, we (6) examined the conjugate profiles from *in vitro* incubations of radioactively labeled ethynylestradiol with human liver tissue obtained at biopsy in five individuals, and again noted considerable individual variation, suggesting that this profile delineated one particular stage of ethynyl estrogen metabolism by the liver. Furthermore, the variations in the conjugate profile in urine might provide a convenient reflection of this aspect of hepatic ethynyl estrogen metabolism. We therefore investigated the urinary conjugate profiles after oral administration of mestranol and/or ethynylestradiol in women who had or had not been previously exposed to these agents, and carried out these studies in the USA as well as in Nigeria and Sri Lanka, where extensive studies of the peripheral effects of these compounds were in progress (7-11).

In previous publications (12,13) we have shown that the plasma pharmacokinetics of ethynyl estrogens, administered orally as a single dose, appear to differ in various localities (Nigeria, Singapore, Sri Lanka, Thailand, and the USA). In urinary conjugate studies (14), we found that the proportions of the various types of conjugated metabolites excreted in the urine also appear to differ from one locality to another. These observations suggest the possibility of differences in the hepatic metabolism of ethynyl estrogens. In order to investigate this question further, we have developed a method for the separation of deconjugated urinary ethynyl estrogen metabolites by high performance liquid chromatography. Together with chromatography on silver-impregnated sulfoethylcellulose columns (16) to study de-ethynylation, we examined the variety and proportions of steroid metabolites in women from Nigeria, Sri Lanka, and the USA.

MATERIALS AND METHODS

Steroids

Tritium-labeled mestranol (ME) and ethynylestradiol (EE), with specific activity of 296 mCi/mmol prepared as described by Rao (17), 4-^{14}C-ethynylestradiol (58 mCi/mmol) prepared from estrone-4^{14}C (New England Nuclear) (2) and 4-^{14}C-mestranol (45 mCi/mmol) (New England Nuclear) were repurified by chromatography on Sephadex LH-20 (Pharmacia Fine Chemicals) with 85:15 benzene:methanol for ethynylestradiol and 95:5 benzene:methanol for mestranol. The ethynylestradiol labeled in the 9α,11ξ position with ^{3}H (29 mCi/mmol*) and in the 20,21 position with ^{14}C (119 mCi/mmol*) was purified by high pressure liquid chromatography on a Chromegaprep Diol column (ES Industries) using a gradient of isopropanol in heptane (15). Nonradioactive material in the form of 50-μg tablets of EE or ME was provided by Wyeth Laboratories; these were the same galenical

formulations used in our other clinical investigations (7-11). Authentic 17β-D-glucosiduronate (4) and 3,17-diglucosiduronate (5) of ethynylestradiol were used as received.* Ethynylestradiol monosulfates and disulfates were prepared by the method of Joseph *et al.* (18).

Subjects

In Nigeria and Sri Lanka, women of reproductive age interested in using oral steroid contraceptives were enrolled. In each locality, five women currently using oral contraceptives and five never-users participated in the study. The ethanolic solution of radioactive ME and/or EE was diluted with cherry wine and given in the morning in the postabsorptive state. In Sri Lanka the ethanolic solution contained 7.5 μCi of EE and 5 μCi of ME; in Nigeria 25 μCi of EE alone was given. In addition, 25 μg (1/2 tablet) each of EE and ME was given in Sri Lanka immediately following the labeled dose, and again on each of the 2 following days; in Nigeria, 50 μg of EE only was given in the same fashion.

Three of the USA women were normals of reproductive age (26-31); the other six were castrate-hysterectomized subjects who were currently on estrogen replacement therapy with oral estradiol or estrone sulfate. Two subjects received 8 μCi 4-^{14}C-EE and 16 μCi ^{3}H-ME; two received 25 μCi ME only, and four received 22.3 μCi 9α,11ξ-^{3}H-EE plus 2.5 μCi 20,21-^{14}C-EE. In all instances a carrier dose totaling 50 μg of the appropriate nonradioactive estrogen(s) was given at the time of radioactive dosage and for each of 2 subsequent days.

Twenty-four hour urine collections were obtained for 3 days following the administration of the radioactive material; the urine was collected into bottles containing boric and ascorbic acids (19,20) with refrigeration whenever possible. Aliquots of each urine were stored frozen until processed.

Preliminary Concentration and Purification of Urine

Small aliquots of urine were extracted with chloroform to determine "unconjugated steroid radioactivity"; this also served to indicate whether adequate care had been exercised in collection and preservation of the urine. One ml aliquots from each 24-hr collection were counted for the determination of total radioactivity excreted per day.

For each individual, a 20% aliquot from each of the three 24-hr urine collections was pooled, and this was processed through an Amberlite XAD-2 column (21) using the modifications recently suggested by Bradlow (22), i.e., 0.1M triethylamine and pH 7.2. By this means, the poor recoveries occasionally observed with current batches of XAD-2 were improved, and recovery of radioactivity ranged between 70 and 95%.

Sephadex LH-20 Chromatography of Radioactive Conjugates

The methanolic eluate was evaporated to dryness under nitrogen at 40°C and applied to 19 x 530 mm columns of Sephadex LH-20 using the eluting solvent, which consists of chloroform:methanol 1:1 containing 0.01M NaCl. One hundred 5-ml fractions were collected into scintillation vials and the solvent evaporated. Water (0.2 ml) was added to each vial, followed by scintillation solvent and counting by liquid scintillation spectrometry

*Kindly provided by Drs. E. D. Helton and H. E. Hadd of the National Center for Toxicological Research.

(23). Residual radioactivity on the column was examined by elution of the column with methanol and counting this material. Almost invariably it constituted less than 1% of the applied radioactivity.

Hydrolysis of Urinary Conjugates

(1) Sequential. A 5-ml sample of urine from each individual was first hydrolyzed with beef liver β-glucuronidase (Sigma), 100 U per ml, in pH 5.0, 0.2M acetate buffer for 48 hr at 37°C; then with solvolysis at pH 1 in ethyl acetate overnight, and finally with acid hydrolysis (hot HCl) for 15 min. The steroids liberated by hydrolysis were extracted 3 times with equal volumes of ethyl ether:chloroform (2:1). The percent of radioactivity in each hydrolysis extract was determined by scintillation counting.

(2) Enzymatic. An aliquot of the methanolic extract containing approximately 10^5 dpm was dissolved in 0.2M acetate buffer, pH 5.0, and incubated at 37°C for 72 hr with *Helix pomatia* glucuronidase-phenolsulfatase (Sigma), 100 U/ml urine; bovine glucuronidase (Sigma), 100 U/ml urine, was added after the first 48 hr of incubation. The liberated aglycones were extracted with 3 x 50 ml diethyl ether, and the ether extracts combined and evaporated to dryness. This residue was dissolved in 0.1M citrate buffer, ph 5.0, and β-glucosidase (almond emulsion, Sigma) at 0.1 ml/ml urine was added and incubation at 37°C carried out for 24 hr. This aqueous material was extracted with ether in the manner described. The spent, ether-extracted urine was incubated for a further 24 hr with β-glucuronidase and then extracted with ether. All ether extracts were then combined, evaporated to dryness, and stored temporarily in chloroform:methanol (1:1).

Chromatography of Enzymatically Released Aglycones

(1) Sephadex LH-20 chromatography. Chromatography of the individual consecutive daily urines of some USA subjects was performed as described in a previous publication (2).

(2) High performance liquid chromatography. The free steroid extract was evaporated to dryness and dissolved in 0.2-0.3 ml isopropanol. This was applied to a Chromegaprep Diol column (ES Industires), 9.6 mm x 50 cm in a Waters Model 204 high performance liquid chromatograph (equipped with an additional Model 6000 solvent delivery system, a Model 660 solvent programmer, a Model 440 dual channel UV detector, and a Model U6K injector) and processed at a flow rate of 7.5 ml/min at a pressure of 1000 psi. Linear gradient programming achieved an effective change from 2.25% to 15.00% isopropanol in heptane during a 158-min interval (Waters Solvent Programmer, using heptane for Pump A and 15% isopropanol in heptane for Pump B, gradient 6; 15 to 100% B in 158 min). Upon completion of the program, Pump B was used to rinse the column with 100% MeOH to remove traces of pigment and residual radioactivity. During the program, 1 min fractions were collected (Gilson Mini-escargot scintillation vial fraction collector), dried, and counted by liquid scintillation spectrometry (23). Graphs of radioactivity in each fraction vs. time provided a profile of the radiolabeled metabolites. Using this same program for authentic samples of the D-homo and natural estrogens as well as for metabolites of ethynyl estrogens (2,24-30), it was possible to determine retention times (or volumes) relative to ethynylestradiol for each compound.

De-ethynylation Studies

To obtain a profile of ethynyl and non-ethynyl metabolites, a silver sulfoethylcellulose column as described by Pellizzari *et al.* (16) was prepared but with an increased capacity, 5

x 100 mm. Duplicate aliquots of a free steroid extract were applied to a freshly prepared column in 1 ml of redistilled HPLC-grade MeOH. After a reaction time of 10 min, the non-ethynyl metabolites were eluted with 10 ml MeOH. Then the ethynyl metabolites were released with 15 ml of MeOH saturated with NaCl. The non-ethynyl and ethynyl eluates were evaporated to dryness, dissolved in chloroform and washed with water to remove the salts. The two fractions were chromatographed separately on LH-20 columns (2).

The use of silver columns as an analytical procedure to study de-ethynylation was validated by processing various radiolabeled authentic estrogens, both ethynyl and non-ethynyl, through the procedure. The non-ethynyl (MeOH) and ethynyl (NaCl-MeOH) fractions were counted and the percent recovery in each fraction calculated.

Counting of Radioactivity

Counting was performed in a Packard Model 3320 Tri-Carb liquid scintillation spectrometer using a scintillation fluid consisting of 42 ml Liquifluor (New England Nuclear) and 66 ml Biosolve BBS_3 (Beckman) per liter of toluene. An on-line computer interfaced with the spectrometer provided all computations and insured a precision of ± 2%. Efficiencies were obtained from a curve generated by counting a series of quenched standards with and without irradiation by the automatic external radiation source.

RESULTS

Excretion of Urinary Radioactivity

Table 1 shows the percent of orally administered ethynyl estrogen radioactivity excreted in the urine during the first 3 days. The daily rate of excretion and the total amount eliminated in 3 days (ranging from 13.6 to 26.9%) is consistent with previous findings (31). The similarity of the excretion rates from one country to another is noteworthy, and is observed with both ethynyl estrogens.

Chloroform-extractable Radioactivity

The percent of total urinary radioactivity extractable with chloroform is shown in Table 2. In general, this represents unconjugated steroid, and is a useful measure of stabilization of the urine against bacterial hydrolysis during collection. Thus it provides assurance that the steroid conjugate patterns have not undergone significant deterioration. The amount of chloroform-extractable radioactivity was consistently higher with ME than with EE. This observation requires further study, since it has been shown that certain estrogen *conjugates* are extractable into organic solvents from an aqueous phase (32).

Consistency of the Day-to-day Excretion Pattern of Conjugates

It is probable that the various conjugates of ethynyl estrogens have different biological half lives, and this might influence the number of days of urine collection required after a single oral dose in order to obtain a representative pattern of the urinary conjugates. In two

TABLE 1

PERCENT (Mean ± S.D.) OF ADMINISTERED RADIOACTIVITY EXCRETED IN URINE, BY DAY

	Day 1	Day 2	Day 3	Total in 3 Days
		Ethynylestradiol		
Nigeria (N = 10)	12.9 ± 8.5	6.5 ± 4.2	4.0 ± 2.6	23.2 ± 14.0
Sri Lanka (N = 10)	13.5 ± 3.0	5.1 ± 2.2	3.1 ± 1.4	21.6 ± 5.9
USA (N = 6)	13.4 ± 6.2	7.7 ± 3.3	4.9 ± 1.8	26.9 ± 10.8
		Mestranol		
Sri Lanka (N = 10)	7.9 ± 2.6	3.4 ± 1.6	2.3 ± 1.1	13.6 ± 4.9
USA (N = 4)	10.5 ± 5.3	5.8 ± 2.6	3.4 ± 1.4	19.6 ± 8.9

TABLE 2

PERCENT (Mean ± S.D.) OF TOTAL URINARY RADIOACTIVITY EXTRACTABLE BY CHLOROFORM, BY DAY

	Day 1	Day 2	Day 3
		Ethynylestradiol	
Nigeria (N = 10)	2.7 ± 1.6	3.2 ± 2.6	3.0 ± 2.1
Sri Lanka (N = 10)	3.9 ± 1.2	2.6 ± 1.5	3.1 ± 1.7
USA (N = 6)	4.2 ± 5.5	2.7 ± 2.3	1.4 ± 1.0
		Mestranol	
Sri Lanka (N = 10)	7.1 ± 1.5	8.0 ± 4.4	7.4 ± 3.2
USA (N = 4)	11.8 ± 14.3	9.5 ± 8.3	3.5 ± 1.3

USA subjects (G, SCH) in whom simultaneous administration of EE and ME was carried out, the individual daily urinary conjugate extracts were chromatographed. The results are shown in Figure 1. The pattern of urinary conjugates does not appear to change qualitatively from day to day in either subject or for either estrogen. It was therefore considered justifiable in subsequent studies to pool proportionate aliquots of the 3 collection days of each individual and to analyze the averaged 3-day urinary conjugate pattern.

Excretion of Various Types of Conjugates

The nature of the urinary conjugates was first examined by classical hydrolytic procedures (Table 3). The relative quantities of radioactivity released by glucuronidase hydrolysis, by solvolysis, and, finally, by mineral acid hydrolysis were remarkably similar in all three localities, and for both types of ethynyl estrogen.

TABLE 3

PERCENT (Mean ± S.D.) OF TOTAL CONJUGATED URINARY RADIOACTIVITY RELEASED BY SEQUENTIAL HYDROLYSIS PROCEDURES

	Glucuronidase	Solvolysis	Acid Hydrolysis
		Ethynylestradiol	
Nigeria (N = 10)	67.5 ± 16.8	18.1 ± 8.1	5.8 ± 5.0
Sri Lanka (N = 10)	57.2 ± 9.0	17.7 ± 4.3	3.6 ± 1.3
USA (N = 7)	70.4 ± 15.6	16.6 ± 10.8	5.8 ± 6.1
		Mestranol	
Sri Lanka (N = 10)	68.2 ± 6.9	18.6 ± 7.0	3.4 ± 1.1
USA (N = 4)	71.3 ± 10.5	11.1 ± 3.8	3.3 ± 2.0

Elution Pattern of Radioactive Conjugates

In previous studies of human and baboon urinary conjugates of ethynyl estrogens, the radioactivity tended to elute in distinct peaks (3,31), each probably representing a similar type of conjugate of several steroid aglycones. The availability of authentic glucuronides and

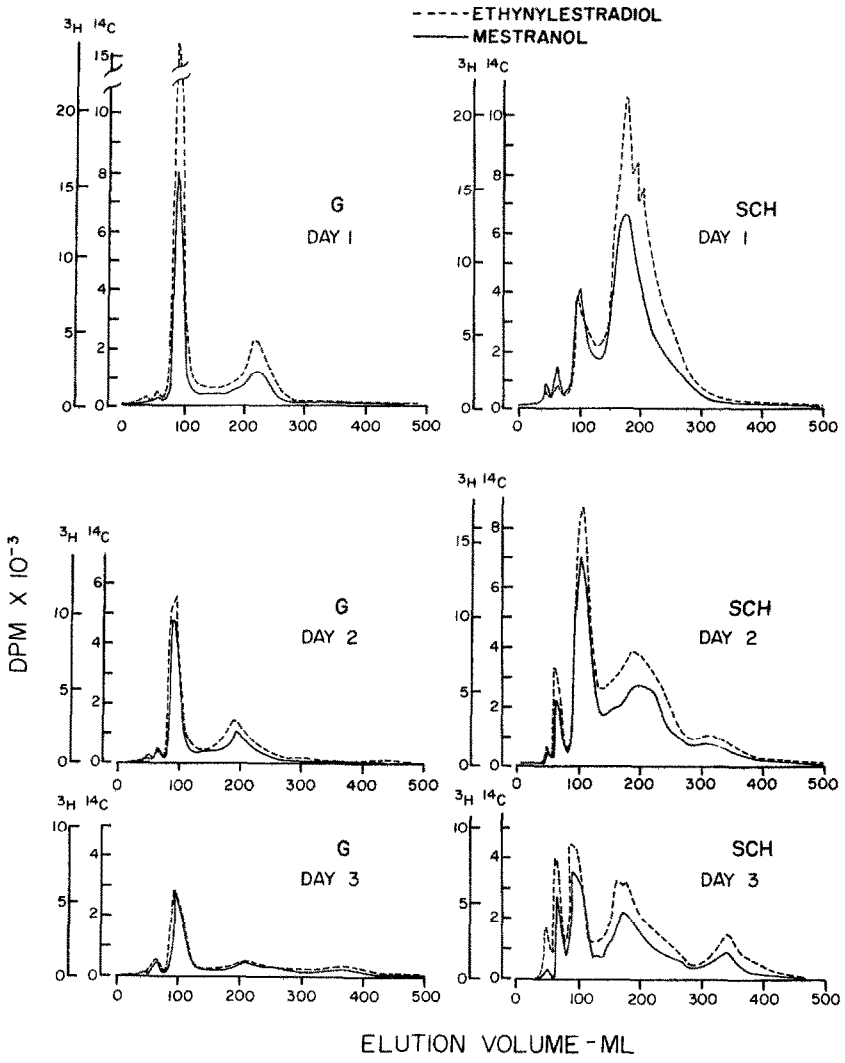

Figure 1. Pattern of urinary conjugate radioactivity, by day, in two USA subjects given radioactively labeled ethynylestradiol and mestranol simultaneously, by mouth.

sulfates has made it possible to suggest structures for these various classes of conjugates (4,5). Figure 2 demonstrates the elution patterns of authentic 17β-D-glucuronide and the 3,17-diglucuronide of EE as well as the elution pattern of the three EE sulfates. The pure conjugates were chromatographed in the LH-20 system without prior triethylamine-XAD treatment. It is to be noted that the disulfate could be eluted only by stripping with methanol. These data, together with information obtained in our previous hydrolysis study (3) and the elution sequence of other steroid conjugates in the same LH-20 system reported by Vihko (33) and Tikkanen and Adlercreutz (34), suggest the following probable order of elution: monoglucuronides (3 before 17), diglucuronides, monosulfates (3 before 17), and disulfates (Figure 2). Mixed glucuronide-sulfate conjugates of EE were not available.

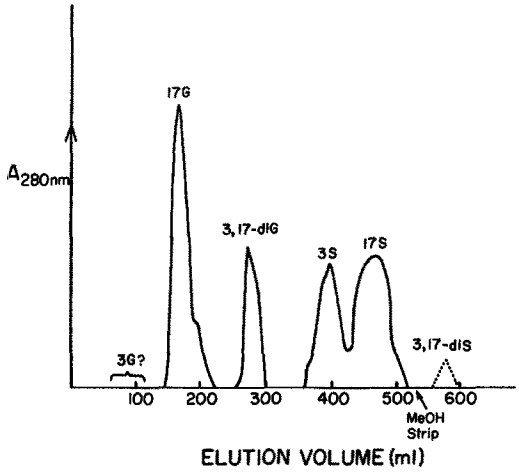

Figure 2. Chromatographic positions of authentic glucuronides and sulfates of ethynylestradiol, chromatographed on LH-20 with chloroform:methanol 1:1 containing 0.01M NaCl. The disulfate could be removed only by stripping with methanol. No prior processing through triethylamine/XAD-2 was used. Authentic 3-glucuronide of EE was not available.

The urinary conjugate profiles from eight USA subjects are shown in Figure 3. The monoglucuronides were usually the major component and in the two subjects (H, S) who received only ME, contained nearly all the radioactivity. The proportion of diglucuronide varied greatly from subject to subject. It was the major constituent in GLD, aged 31, and SDT, aged 40, and was minor to undetectable in the other subjects. Sulfoconjugates were observed with certainty only in subjects A and GLD (with EE), subject SCH (with ME and EE), and subject H with ME.

The patterns in the urines of the Nigerian women (Figure 4) are distinctly different from those of the USA women. With the exception of OLA and FLO a large proportion of the radioactivity falls in the diglucuronide region.

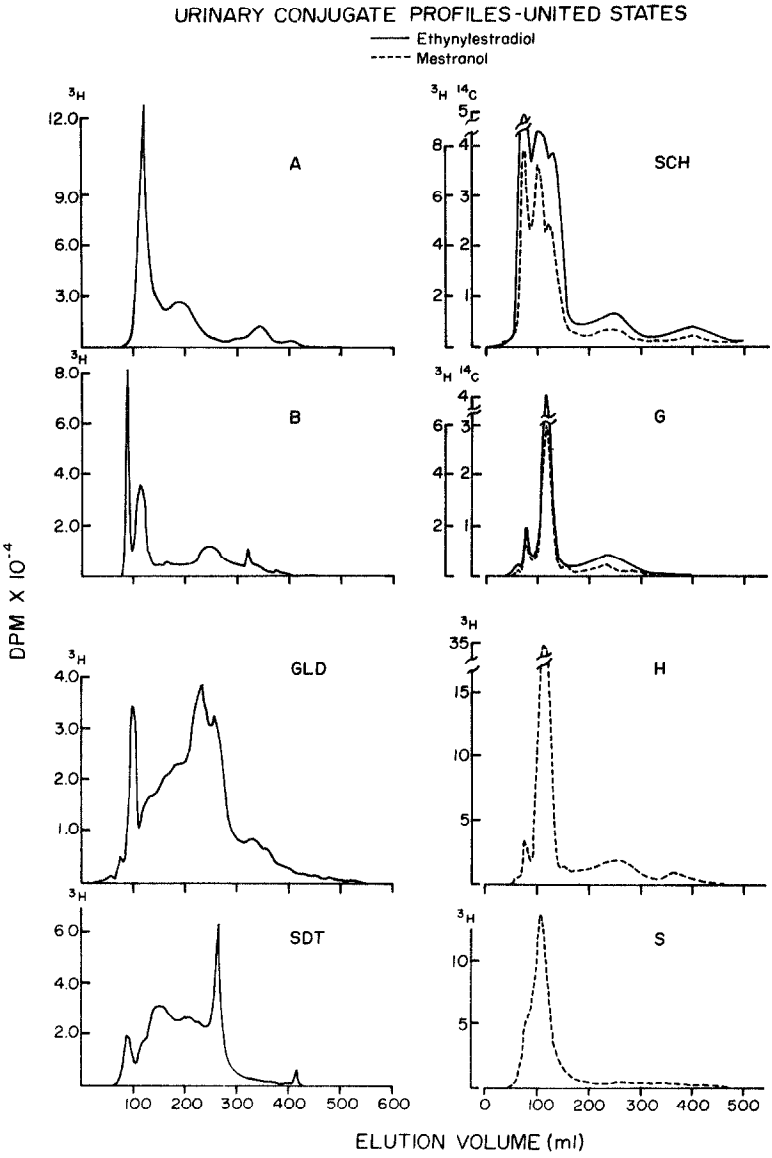

Figure 3. Profiles of urinary ethynyl estrogen conjugates in USA women given mestranol and/or ethynylestradiol.

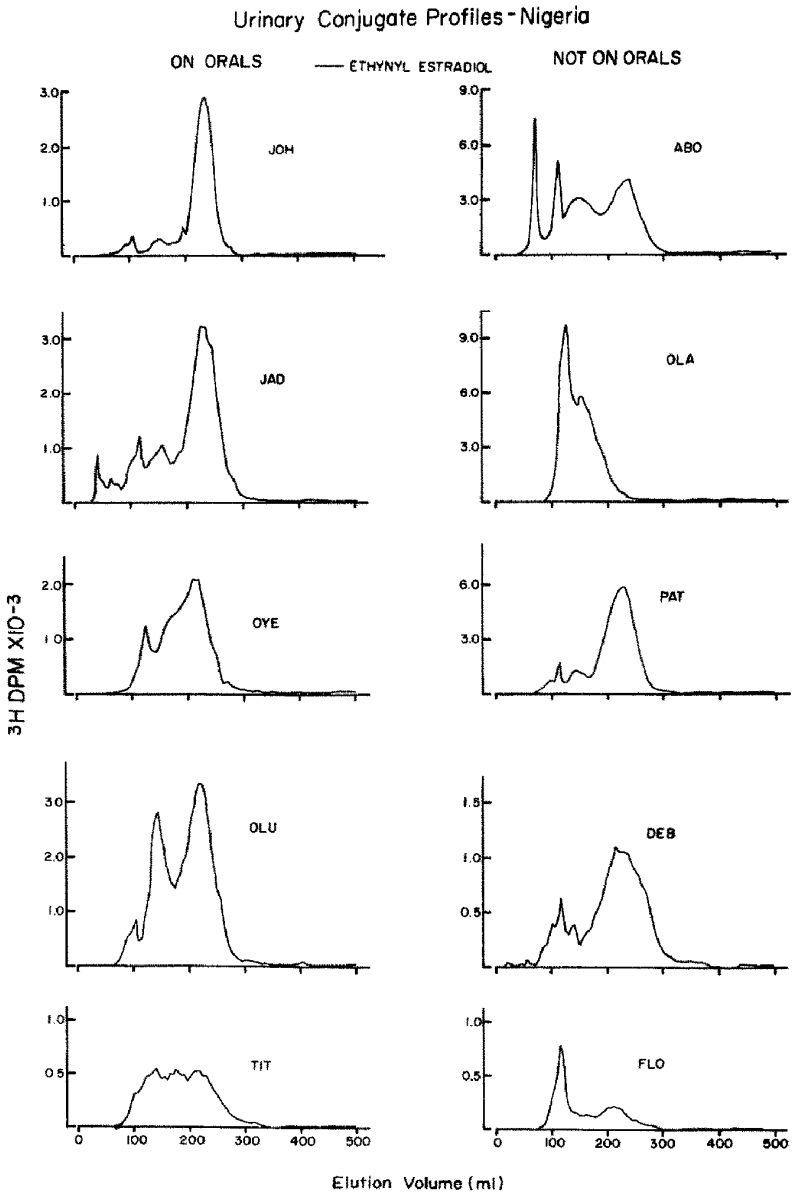

Figure 4. Profiles of urinary ethynylestradiol conjugates in Nigerian women.

The conjugate patterns in the Sri Lanka urines (Figure 5) differ from those of the USA and Nigeria chiefly in the "smearing" of the radioactivity into broad bands instead of relatively sharp peaks, as observed in the other two localities in most instances. If anything, the patterns seem to be intermediate between USA and Nigeria, in that substantial amounts of radioactivity are seen in both the monoglucuronide and diglucuronide regions. The sulfoconjugated radioactivity did not appear as a distinct peak.

In the Sri Lankan women and in the two USA subjects who received EE and ME simultaneously, the nature and proportions of the conjugate types for the two estrogens are remarkably similar. This is to be expected, as the metabolism of ME proceeds chiefly by demethylation to EE or by metabolism similar to that of EE, followed by demethylation (35,36). Subsequent conjugation should then produce similar urinary profiles.

Recovery of Radiolabeled Aglycones Released by Enzymatic Hydrolysis

Initial purification of the conjugates was performed by the use of Amberlite XAD-2 columns according to the method of Bradlow (21). The recoveries shown in Table 4 were substantially improved in subsequent experiments after introduction of modifications (conversion to the triethylamine salts) suggested by Bradlow (22).

Exhaustive hydrolysis with various enzymes was essentially quantitative (Table 4). A hydrolysis step using almond emulsion β-glucosidase was incorporated into the original protocol, since we and others (32,37) had observed the presence of glycosidic estrogen conjugates in tissues or urine from other species. However, in these urines this enzyme did not release significant amounts of radioactivity, and it has been omitted in subsequent work.

TABLE 4

Percent Recovery of Urinary Radioactivity

Locality	No. Subjects	Pre-extraction (before XAD) (mean ± S.D.)	After XAD Purification (mean ± S.D.)	After Enzyme Hydrolysis (mean ± S.D.)
Nigeria	10	3.0 ± 2.1	76.9 ± 15.7	74.9 ± 12.6
Sri Lanka	10	3.2 ± 1.5	86.6 ± 7.8	91.2 ± 9.1
U.S.A.	8	2.8 ± 2.9	85.0 ± 18.8	81.5 ± 10.8

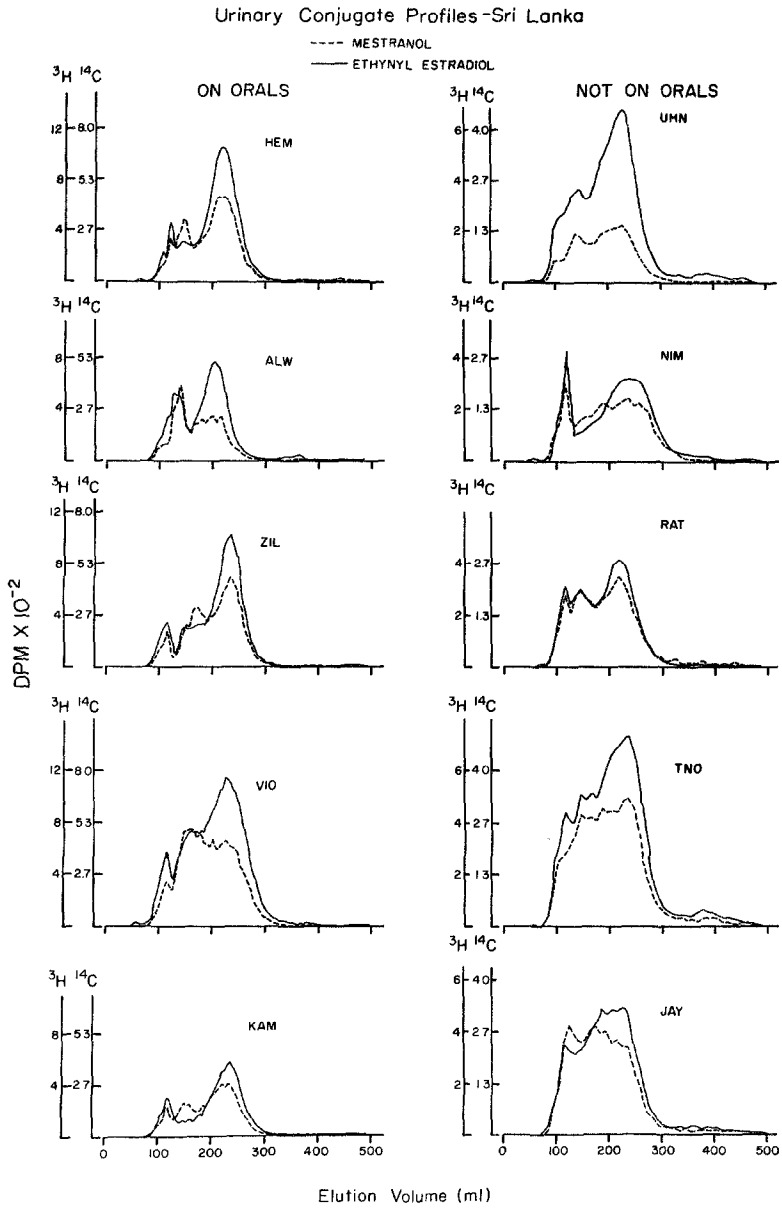

Figure 5. Profiles of urinary ethynylestradiol and mestranol conjugates in Sri Lankan women.

Chromatography of Free Steroids

In our previous investigation of urinary metabolites of ethynyl estrogens (2), chromatography with Sephadex LH-20 was employed. The development of a system for fractionating steroids by high performance liquid chromatography (15) has permitted a far greater degree of resolution. This is shown in Figure 6, which compares chromatograms of an extract of ethynylestradiol metabolites processed through LH-20 and HPLC. In addition, we have repeatedly observed that reference steroids which appeared homogeneous after chromatography on LH-20 showed significant separation of previously undetected contaminants upon rechromatography by HPLC (15).

The resolution of a series of ethynyl and non-ethynyl estrogen reference steroids is shown in Figure 7. Detection was by UV absorption at 280 nm.

Variation in Daily Metabolite Patterns

Since ethynyl estrogens are excreted relatively slowly, it is important to know whether the pattern of metabolites changes from day to day, and consequently if the duration of urine collection is sufficient to provide a representative picture of the metabolic conversions. The extracts from three consecutive 24-hr collections in two subjects given both EE and ME were processed and the LH-20 chromatograms are shown in Figure 8. It is evident that, during this phase of urinary excretion at least, there is no significant alteration in the pattern of metabolites.

Metabolite Patterns Derived from EE

It is clear from Figure 9 that the metabolism of EE differs qualitatively, to a marked degree, from individual to individual. Subject A, for example, excreted EE chiefly in unaltered form, with only three metabolites of any significance; the same pattern is observed in the metabolism of ME by subject H. On the other hand, subject GLD showed extensive metabolism of EE, to the extent that at least two metabolites were present in greater quantities was than the parent steroid. Except for this case, however, the predominant urinary metabolite, both from the administration of EE and ME, is EE itself. In Sri Lanka (Figure 10), EE itself is the predominant metabolite of EE and ME in every instance, but the numerous peaks, although of low degree, bespeak a fairly diverse attack on the EE molecule. There appears to be no difference in the patterns of users and never-users. In the Nigerian group (Figure 11), the consistent absence of metabolites other than unaltered EE is noteworthy. Subject TIT is a distinct exception, while the patterns of OLU and FLO resemble those seen commonly in the Sri Lankan subjects.

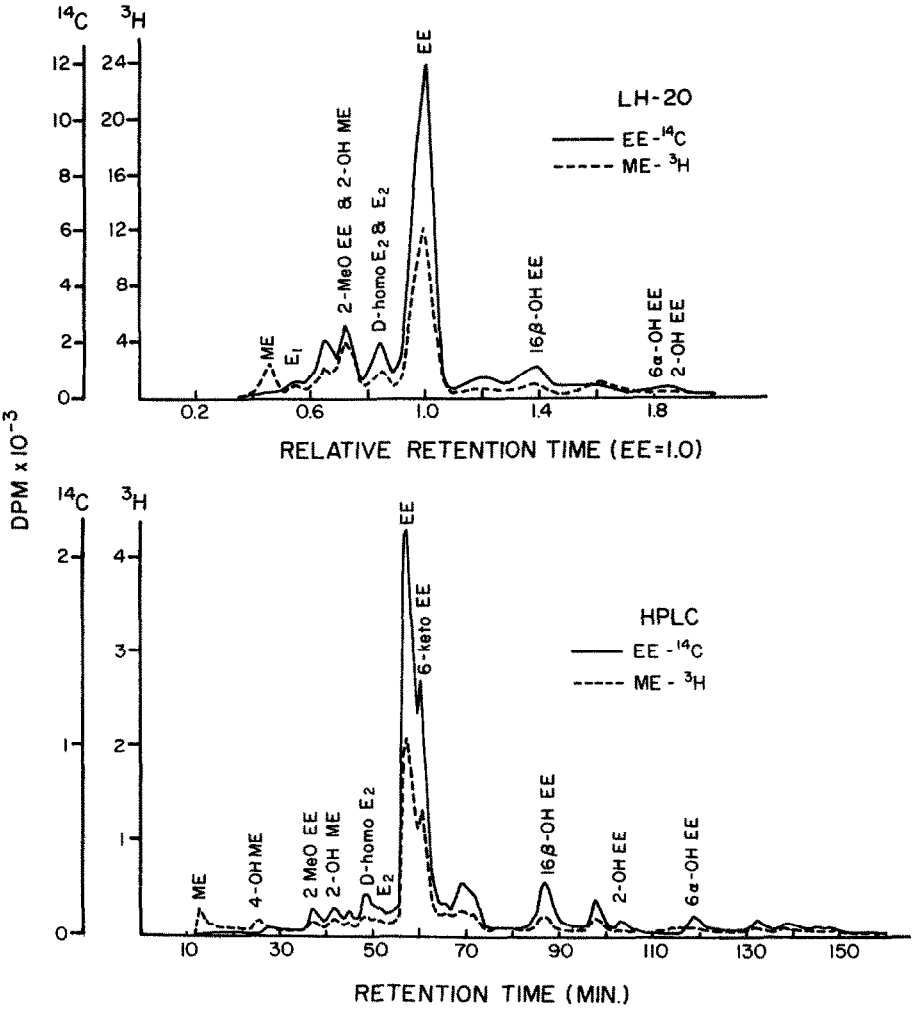

Figure 6. Pattern of radiolabeled ethynylestradiol urinary metabolites run on Sephadex LH-20 vs. HPLC preparative Diol column. Sephadex LH-20 column, 1.9 cm x 50 cm; benzene:methanol (85:15 v/v); scintillation counting of 5-min fractions. Chromegaprep Diol column, 9.6 mm x 50 cm, 10 μ; flow 7.5 ml/min; pressure 1000 psi; linear gradient of 2.25 to 15% isopropanol in heptane in 158 min; scintillation counting of 1-min fractions.

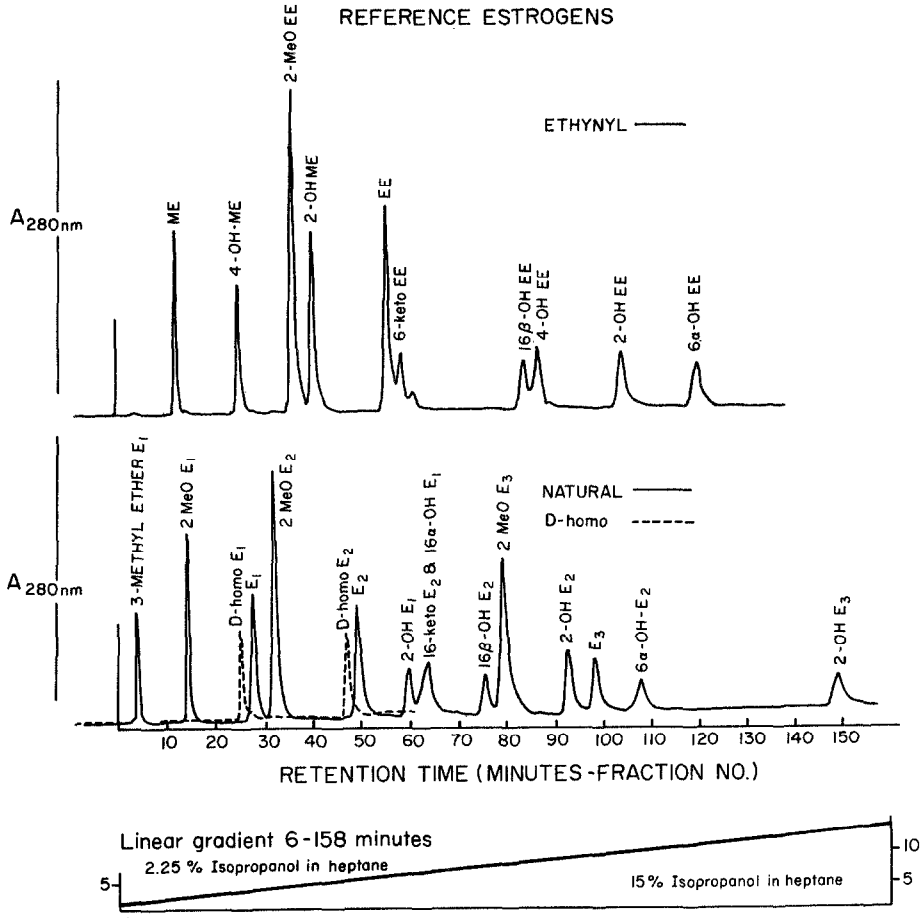

Figure 7. Pattern of reference ethynyl and non-ethynyl estrogens on a HPLC Chromegaprep Diol column, 9.6 mm x 50 cm, 10 μ; flow 7.5 ml/min; pressure 1000 psi; linear gradient 2.25 to 15% isopropanol in heptane in 158 min; detection by UV absorption at 280 nm.

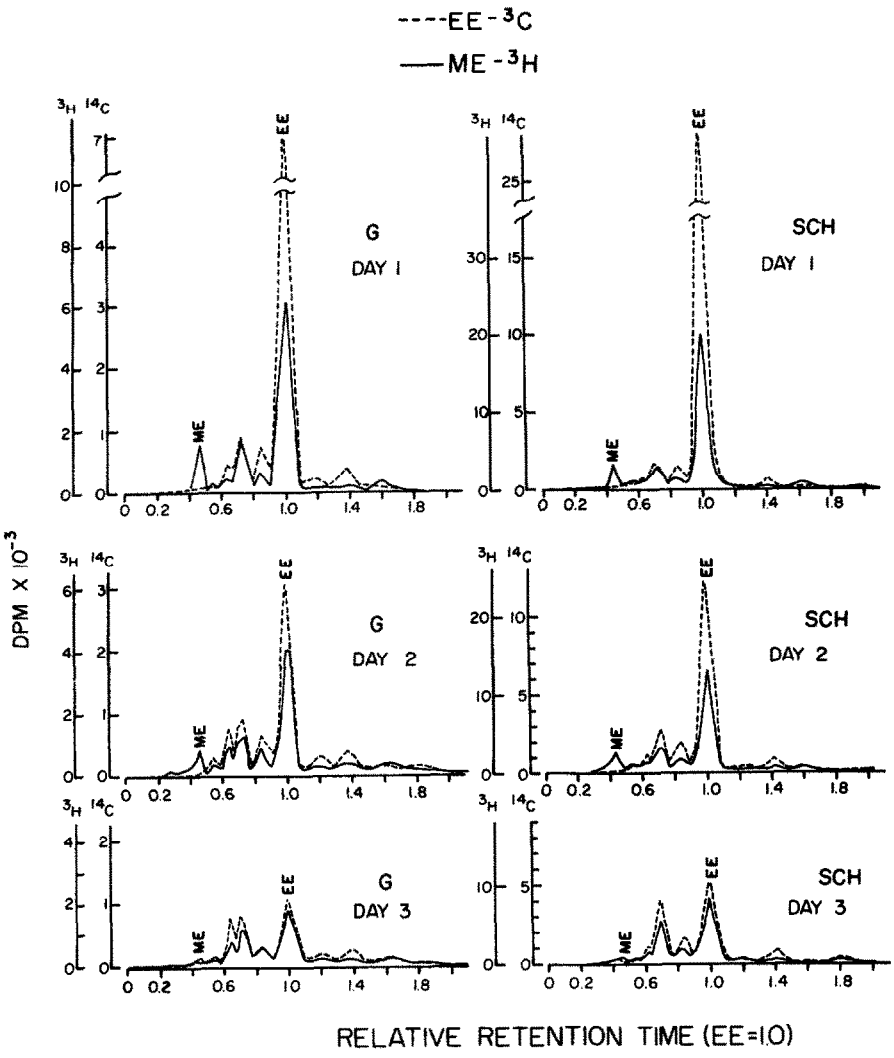

Figure 8. Variation in daily urinary metabolite pattern following the ingestion of radiolabeled mestranol and ethynylestradiol. Sephadex LH-20 column, 1.9 cm x 50 cm; benzene:methanol (85:15 v/v); scintillation counting of 5-ml fractions.

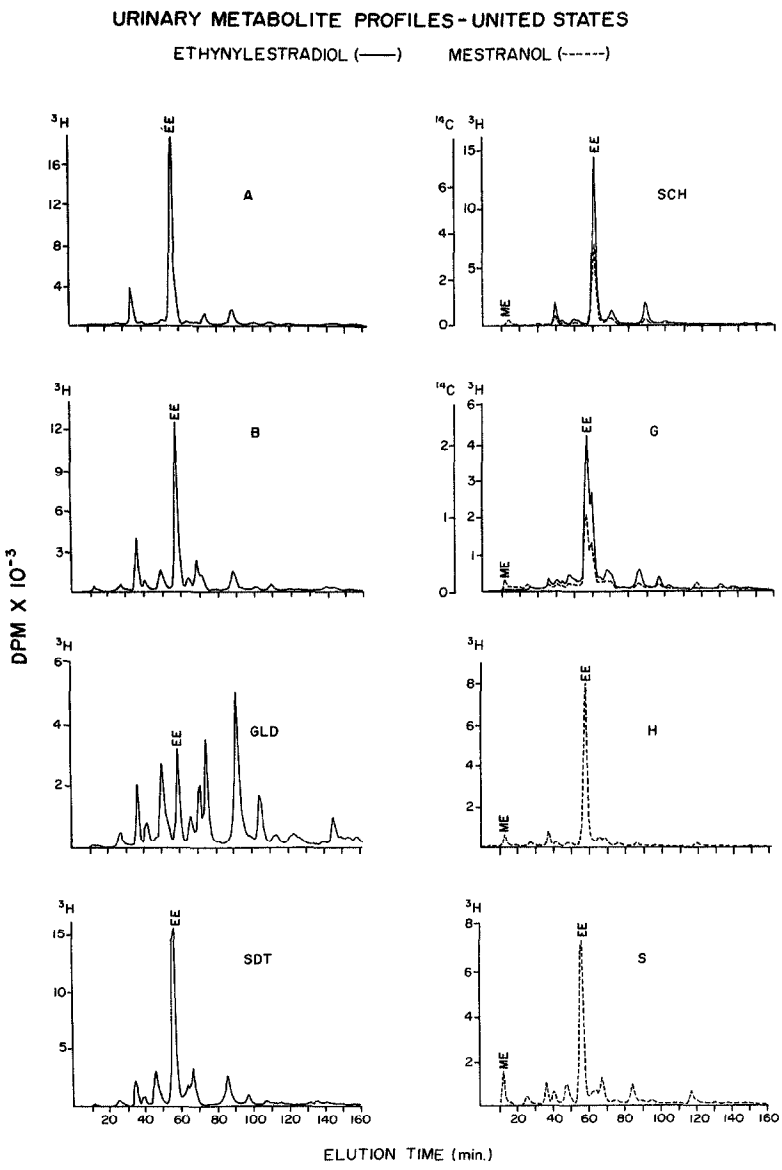

Figure 9. Patterns of urinary mestranol and ethynylestradiol metabolites in United States women. HPLC Chromegaprep Diol column, 9.6 mm x 50 cm; 10 μ; flow 7.5 ml/min; pressure 1000 psi; linear gradient 2.25 to 15% isopropanol in heptane in 158 min; scintillation counting of 1-min fractions.

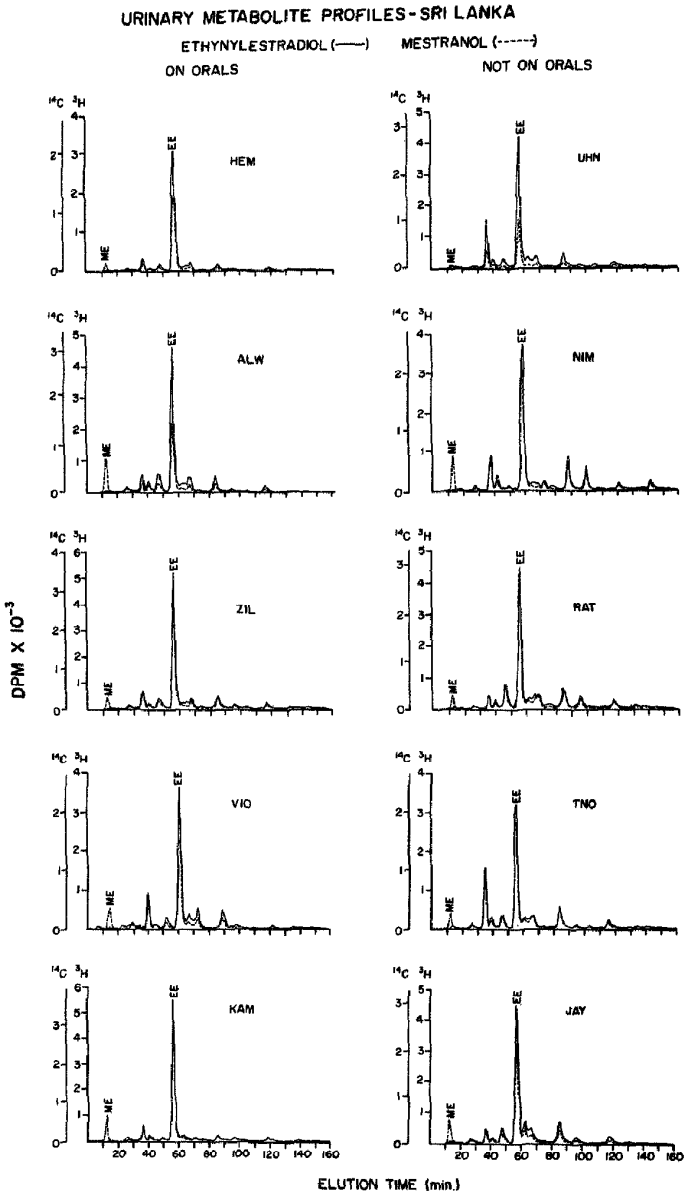

Figure 10. Patterns of urinary mestranol and ethynylestradiol metabolites in Sri Lankan women. HPLC Chromegaprep Diol column, 9.6 mm x 50 cm; 10 μ; flow 7.5 ml/min; pressure 1000 psi; linear gradient 2.25 to 15% isopropanol in heptane in 158 min; scintillation counting of 1-min fractions.

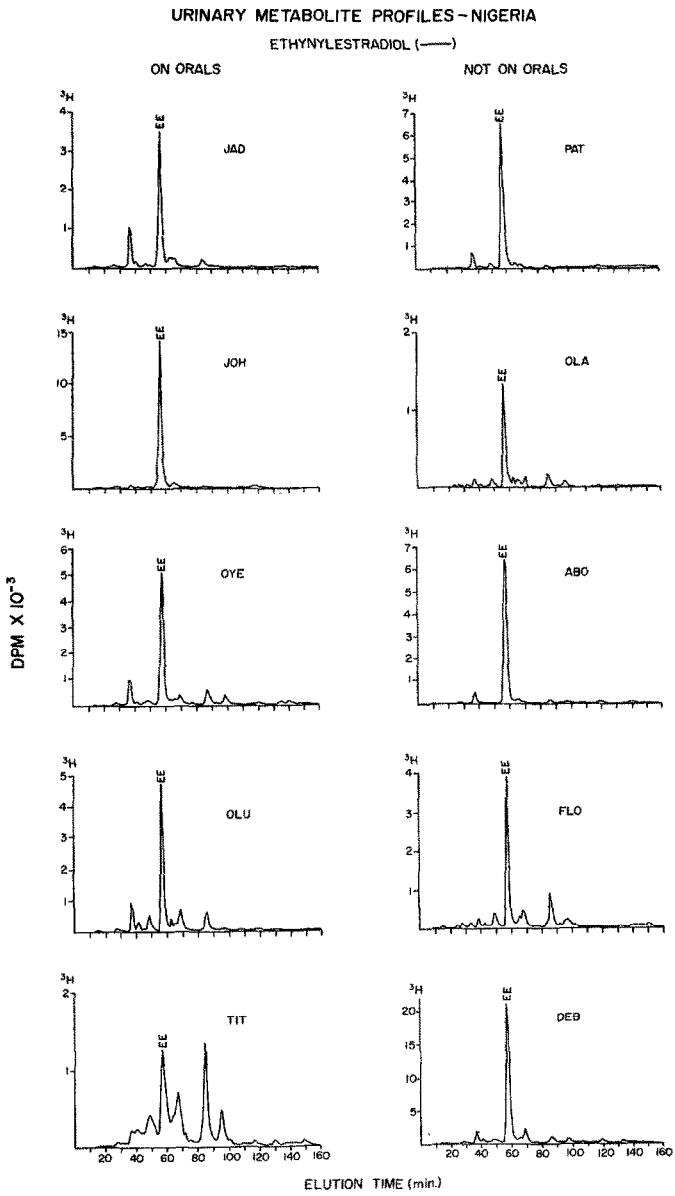

Figure 11. Patterns of urinary ethynylestradiol metabolites in Nigerian women. HPLC Chromegaprep Diol column, 9.6 mm x 50 cm; 10 μ; flow 7.5 ml/min; pressure 1000 psi; linear gradient 2.25 to 15% isopropanol in heptane in 158 min; scintillation counting of 1-min fractions.

Simultaneous Administration of EE and ME

The pattern of urinary metabolites resulting from the simultaneous administration of both ethynyl estrogens may be examined in the two USA subjects (G and SCH) and in all 10 of the subjects from Sri Lanka. In every instance, a peak of 11 min, corresponding to unmetabolized ME, may be observed. The presence of the ME label alone in this peak confirms this interpretation. Figures 9 and 10 also show the close parallelism of the metabolite patterns derived from EE and ME in the same individual. If anything, the pattern of EE metabolites tends to show more complexity and somewhat larger amounts of the minor components. Both these observations are consistent with the view that extensive demethylation is the initial step in the metabolism of ME (26,27).

In two early studies in the USA group ME alone was administered. The patterns of subjects S and H have a major peak coincident with the retention time of EE, and a metabolite pattern similar to that observed with EE; however, the dual-label studies in subjects G and SCH show direct comparisons which are much more informative.

The tritium/carbon-14 ratios of selected metabolites provide a means of studying the simultaneous metabolism of EE and ME (Table 5). Individual differences for the Sri Lankan women in the rate of demethylation are indicated by a ratio which varies from 1.6 to 4.3, compared to the ingestion ratio of 1.5. The two USA women demonstrated a preferential conversion of EE to 16β-OH EE rather than to 2-MeO EE.

De-ethynylation of Ethynyl Estrogens

The results of processing urinary extracts through silver sulfoethylcellulose columns are presented in Table 6. The percent of non-ethynyl (D-homo and/or natural estrogen) metabolites in the four urines studied ranged from 14.1 to 34.7%. When ME and EE were administered simultaneously the percent conversion to non-ethynyl compounds was less for ME.

Three successive recrystallizations of the radiolabeled material from the retention volume of D-homo E_1 with the authentic compound resulted in constant specific activity. Recrystallizations of the D-homo E_2 area yielded evidence for the presence of more than one radiolabeled compound.

The radiolabeled ethynyl and non-ethynyl metabolites isolated by silver sulfoethylcellulose and re-chromatographed on Sephadex LH-20 are presented in Figure 12. Five small non-ethynyl peaks are visible on the ME chromatogram. Only three can be seen in the EE study.

TABLE 5

Tritium/Carbon-14 Ratios of Radiolabeled Ethynyl and Non-ethynyl Urinary Metabolites Isolated by HPLC

Tentative Identification of Compounds*	$^3H/^{14}C$ Ratio in Metabolites of Sri Lankan Women Receiving Oral Dose of ^3H-EE/^{14}C-ME = 1.5										$^{14}C/^3H$ Ratio in Metabolites of United States Women Receiving Oral Dose of ^{14}C-EE/^3H-ME = 0.5	
	Not on Orals					On Orals						
	TNO	RAT	UHN	NIM	JAY	KAM	VIO	ALW	HEM	ZIL	SCH	G
2-MeO EE	2.1	1.7	2.8	0.9	0.8	1.6	4.5	2.8	2.5	3.1	0.4	0.3
2-OH ME	1.6	1.5	3.9	1.9	1.7	1.7	2.3	2.9	2.2	2.0	1.1	0.9
D-Homo E_2	1.5	1.4	4.1	1.6	1.9	1.8	2.8	3.0	1.8	2.0	1.0	0.9
EE	1.8	1.6	4.3	1.8	1.7	1.9	2.3	3.1	2.3	1.9	1.1	0.9
16β-OH EE	1.6	1.6	4.0	1.8	2.1	1.7	2.8	2.6	2.3	2.0	1.6	1.3

*Based on retention volume of reference compounds.

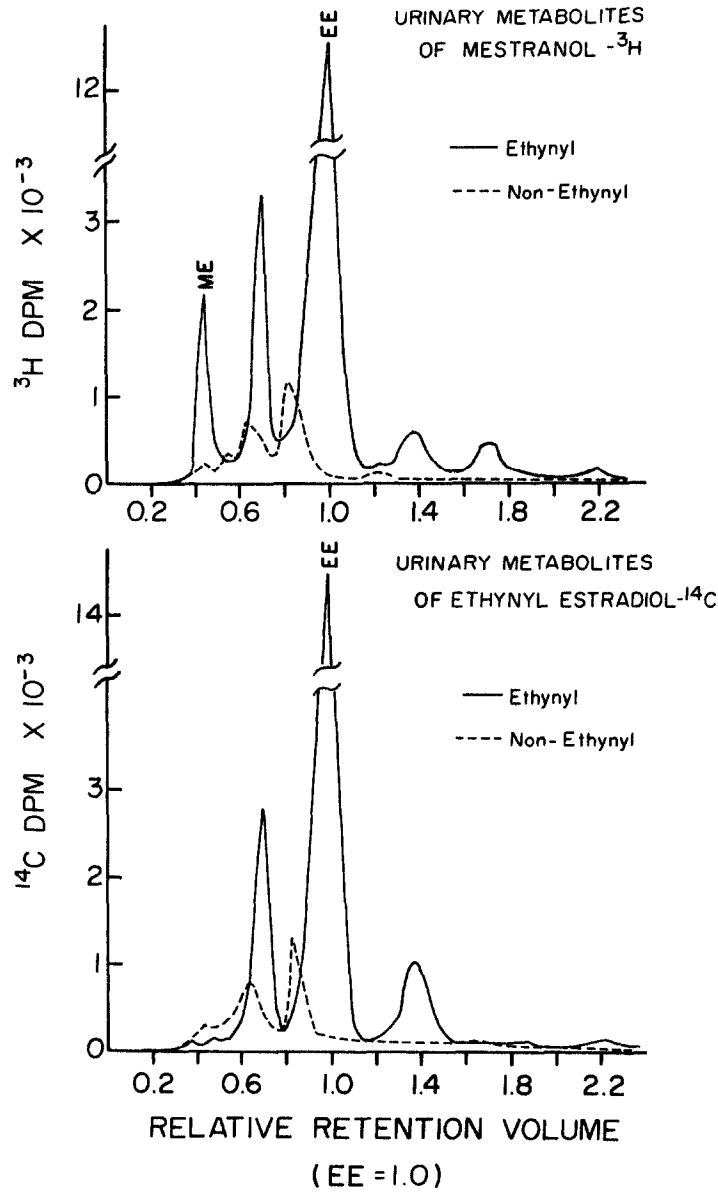

Figure 12. Patterns of urinary ethynyl and non-ethynyl metabolites of mestranol and ethynylestradiol in United States women. Sephadex LH-20 column, 1.9 cm x 50 cm, benzene:methanol (85:15 v/v); scintillation counting of 5-ml fractions.

TABLE 6

De-ethynylation of Estrogens

Compound		% Recovery of Steroids* As Ethynyl Steroids	% Recovery of Steroids* As Non-ethynyl Steroids
Methodological evaluation of standard: processing of radiolabeled estrogens (+ 25 μg unlabeled carrier) through silver column procedure.			
^{3}H-ethynylestradiol		89.9	0.6
^{14}C-mestranol		94.1	0.5
^{3}H-estradiol-17β		0.8	98.9
^{14}C-estrone		0.6	96.2
Analysis of hydrolyzed urine extracts:			
^{3}H-mestranol	GEM	68.4	28.4
^{14}C-ethynylestradiol		64.9	34.7
^{3}H-mestranol	SCH	78.3	22.6
^{14}C-ethynylestradiol		74.9	24.6
^{3}H-mestranol	S	71.5	26.5
^{3}H-mestranol	H	84.6	14.1

*Average of duplicates

DISCUSSION

There appear to be consistent regional differences in the conjugation of ethynyl estrogens with glucuronic acid: the monoglucuronides predominate in USA subjects, diglucuronides appear to predominate in Nigerian women, and the proportion of the two is more equal in the Sri Lankan women.

There appear to be no differences in the profiles within each country between women exposed to the ethynyl estrogens for the first time and long-term users. Moreover, in women given both estrogens simultaneously, the conjugate profiles for the two estrogens were remarkably similar. However, the number of individuals involved in each of these studies is small, and the observations call for confirmation on a larger population. The significance of differences in glucuronide conjugation, if real, is entirely speculative at this time, but this mechanism does represent one of the major pathways for drug elimination.

Mixed (sulfoglucuronide) conjugates could not be investigated adequately due to a lack of the authentic compounds. The sulfates are relatively minor components in this study and in our previous urinary analyses (2,3). The indistinct separation of the conjugates, seen

uniformly in the Sri Lankan samples and occasionally in urines from the other localities, may represent interference with the resolving power of the Sephadex by contaminants of unknown nature, which were not eliminated by the preliminary purification procedures. Examination of the aglycones in individual peaks of several urines (15) has not shown differences in composition (and therefore polarity) of a magnitude sufficient to account for the broad elution patterns. This problem awaits further clarification. The application of high pressure liquid chromatographic techniques to steroid conjugate mixtures (38-43) promises to be a major advance in the analysis of these substances.

The development of an HPLC method using a preparative Chromegabond Diol column (15) has provided a valuable tool for studies of individual differences in the metabolism of the ethynyl estrogens, ME and EE, by examination of the urinary metabolites following oral administration of tracer doses. The metabolism of EE differs qualitatively, to a marked degree, from individual to individual. Further, the USA women demonstrated a high degree of oxidative metabolism, the Sri Lankans less, and the Nigerians almost none. There were no differences between oral contraceptive users and never-users.

As expected, EE was the principal excreted metabolite, but there were exceptions in the USA and in Nigeria, where the 16-hydroxylated metabolite predominated over the 2-hydroxylated metabolite.

Simultaneous administration of EE and ME showed a close parallelism of the metabolites. De-methylation is the primary step in ME metabolism, but is less complete in some individuals than in others. The presence of mestranol can be seen in the ME profiles. In Figure 1 the HPLC profile clearly demonstrates the presence of a metabolite having the same retention volume as 4-OH ME.

The silver sulfoethylcellulose column was an effective method for determining the relative amounts of ethynyl and non-ethynyl metabolites excreted. In the four subjects studied, the percent of non-ethynyl metabolites ranged from 14.1 to 34.7, again demonstrating considerable individual variation. Recently, a mechanism has been proposed by Ortiz de Montellano *et al.* (44) involving enzymatic oxidation of the triple bond (covalent binding of the ethynyl group to prosthetic heme), explaining the formation of the D-homo compounds. The proportion of D-homo (25,45,46) vs. natural (2,47) estrogens as a product of de-ethynylation could be answered in future studies if the silver column technique were coupled with HPLC analysis. Unfortunately, this portion of our studies was completed prior to the development of the HPLC techniques.

NOMENCLATURE

Trivial Names

EE 17β-glucuronide	17-ethynyl-3-hydroxy-1,3,5(10)-estratrien-17β-yl-β-D-glucopyranosiduronate
EE 3,17β-diglucuronide	17-ethynyl-1,3,5(10)-estratriene-3,17β-diyl-β-D-glucopyranosiduronate
EE 3-sulfate	17-ethynyl-17β-hydroxy-1,3,5(10)-estratrien-3-yl-sulfate
EE 17-sulfate	17-ethynyl-3-hydroxy-1,3,5(10)-estratrien-17β-yl-sulfate
EE 3,17β-disulfate	17-ethynyl-1,3,5(10)-estratriene-3,17β-diyl-disulfate
Ethynylestradiol (EE)	17-ethynyl-1,3,5(10)-estratriene-3,17β-diol
6α-Hydroxy-ethynylestradiol (6α-OH EE)	17-ethynyl-1,3,5(10)-estratriene-3,6α,17β-triol
6-Keto-ethynylestradiol (6-Keto EE)	17-ethynyl-3,17β-dihydroxy-1,3,5(10)-estratrien-6-one
2-Methoxy-17-ethynylestradiol (2-MeO EE)	17-ethynyl-2-methoxy-1,3,5(10)-estratriene-3,17β-diol
2-Hydroxy-ethynylestradiol (2-OH EE)	17-ethynyl-1,3,5(10)-estratriene 2,3.17β-triol
16β-Hydroxy-ethynylestradiol (16β-OH EE)	17-ethynyl-1,3,5(10)-estratriene-3,16β,17β-triol
4-Hydroxy-ethynylestradiol (4-OH EE)	17-ethynyl-1,3,5(10)-estratriene-3,4,17β-triol
Mestranol (ME)	17-ethynyl-3-methoxy-1,3,5(10)-estratriene-17β-ol
2-OH Mestranol (2-OH ME)	17-ethynyl-3-methoxy-1,3,5(10)-estratriene-2,17β-diol
4-OH Mestranol (4-OH-ME)	17-ethynyl-3-methoxy-1,3,5(10)-estratriene-4,17-diol
D-Homoestrone (D-Homo E_1)	3-hydroxy-D-homo-1,3,5(10)-estratrien-17a-one
D-Homoestradiol-17aβ (D-Homo E_2)	D-homo-1,3,5(10)-estratriene-3,17aβ-diol
Estrone 3-Methyl Ether	3-methoxy-1,3,5(10)-estratrien-17-one
Estrone (E_1)	3-hydroxy-1,3,5(10)-estratrien-17-one
2-Methoxyestrone (2-MeO E_1)	2-methoxy-3-hydroxy-1,3,5(10)-estratrien-17-one
Estradiol-17β (E_2)	1,3,5(10)-estratriene-3,17β-diol
16-Keto Estradiol-17β (16-Keto E_2)	3,17β-dihydroxy-1,3,5(10)-estratrien-16-one
16α-Hydroxyestrone (16α-OH E_1)	3,16α-dihydroxy-1,3,5(10)-estratrien-17-one
2-Methoxyestradiol (2-MeO E_2)	2-methoxy-1,3,5(10)-estratriene-3,17β-diol
Estriol (E_3)	1,3,5(10)-estratriene-3,16α,17β-triol

2-Hydroxyestrone (2-OH E_1)	2-hydroxy-1,3,5(10)-estratrien-17-one
2-Hydroxyestradiol-17β (2-OH E_2)	1,3,5(10)-estratriene-2,3,17β-triol
6α-Hydroxyestradiol-17β (6α-OH E_2)	1,3,5(10)-estratriene-2,6α,17β-triol
2-Hydroxyestriol (2-OH E_3)	1,3,5(10)-estratriene-2,3,16α,17β-tetrol

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