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Determination of Ethinyl Estradiol in Human Urine by Radiochemical GLC

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Abstract □ A radiochemical GLC analysis was developed for ^{3}H-labeled ethinyl estradiol in human urine. The technique was applied to the unconjugated and aglycone fractions of urine collected from women who were dosed orally with: (*a*) single capsules containing 2.0 mg of ^{3}H-quinestrol (900 μCi) and 2.5 mg of unlabeled quingestanol acetate dissolved in sesame oil and (*b*) single tablets containing 100 μg of ^{3}H-quinestrol (86 μCi). Unconjugated ethinyl estradiol in Day 1 urine collections represented means of 0.02% of the high quinestrol dose and 0.12% of the low dose. Ethinyl estradiol glucuronide in the same collections represented means of 0.55% of the high drug dose and 1.35% of the low dose. The method could detect 1-ng quantities of ^{3}H-ethinyl estradiol and ^{3}H-quinestrol.

Keyphrases □ Ethinyl estradiol—radiochemical GLC analysis in human urine □ Radiochemical GLC—analysis, ethinyl estradiol in human urine □ GLC, radiochemical—analysis, ethinyl estradiol in human urine □ Estrogens—ethinyl estradiol, radiochemical GLC analysis in human urine

Radiochemical GLC (1–14), a system of monitoring the eluates from GLC columns for radioactive compounds, has the capability of detecting only the labeled compounds in complex mixtures of predominantly unlabeled components. Moreover, this system offers flexibility, convenience, specificity, and high sensitivity.

This paper reports a drug metabolism study using radiochemical GLC. Single low doses of ^{3}H-quinestrol (17α-ethinyl estradiol 3-cyclopentyl ether) were administered to women, and their urine was assayed for quinestrol and its metabolite ethinyl estradiol. This biotransformation was described previously (15, 16), but information on the extent of the conversion is lacking.

EXPERIMENTAL

Radioactive Ethinyl Estradiol—Merrill and Vernice (17) described the synthesis of 6,7-^{3}H-ethinyl estradiol with a specific activity of 275 mCi/mmole. Its chemical purity and radiochemical purity were approximately 99%.

Radioactive Quinestrol—The synthesis of this compound (mol. wt. 364.5) from ^{3}H-ethinyl estradiol also was reported previously (17). The oral contraceptive dose consisted of a capsule containing 2.0 mg of ^{3}H-quinestrol (900 μCi) and 2.5 mg of unlabeled quingestanol acetate in 0.2 ml of sesame oil stabilized with 0.05% piperidine. The estrogen replacement dose was a tablet containing 100 μg of ^{3}H-quinestrol (86 μCi).

Radioactivity Measurements—The scintillation counting solution was prepared by dissolving 6 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of ethanol–toluene (17:100). By using internal standardization, 200-μl samples of urine were counted in 20 ml of this solution with a liquid scintillation spectrometer[1] at 4°.

Radiochemical GLC—An oven and proportioning temperature controller[2] were connected to a gas proportional counter[3] by a heated glass-lined metal tube. The operating conditions for the gas chromatograph were: column, 1.8 m coiled glass, 2 mm i.d., 20% SE-30 on 80–100-mesh Gas Chrom Q; carrier gas (helium) flow rate, 50 ml/min; oven temperature, 215°; and injector temperature, 245°. The operating conditions for the gas proportional counter were: quench gas (propane) flow rate, 2.5 ml/min; hydrogen flow rate, 10 ml/min; transfer line temperature, 237°; and oxidizer and reduction furnace temperature, 750°.

Preparation of Standard Curves—Quantitation of ^{3}H-ethinyl estradiol peaks was accomplished by the following absolute calibration method. A stock solution of ^{3}H-ethinyl estradiol in methanol was prepared; its concentration was 140,380 dpm/4.0 μl. From this stock solution, serial twofold dilutions were made to give a set of standards corresponding to 70,190, 35,100, 17,550, and 8775 dpm/4.0 μl. Aliquots (4.0 μl) of the standards were injected into the gas chromatograph so that a standard curve could be constructed by integration (planimetry) of the resultant peaks. Data plotted in this manner yielded acceptable straight lines. However, it was necessary to generate a new curve each day because the slope varied from day to day.

The standard curves from one column are shown in Fig. 1; curve A was obtained on Day 1, curve B on Day 2, curve C on Day 3, and curve D on

[1] Tri-Carb model 3320, Packard Instrument Co., Downers Grove, Ill.
[2] Warner-Chilcott, Morris Plains, N.J.
[3] Model 894, Packard Instrument Co., Downers Grove, Ill.

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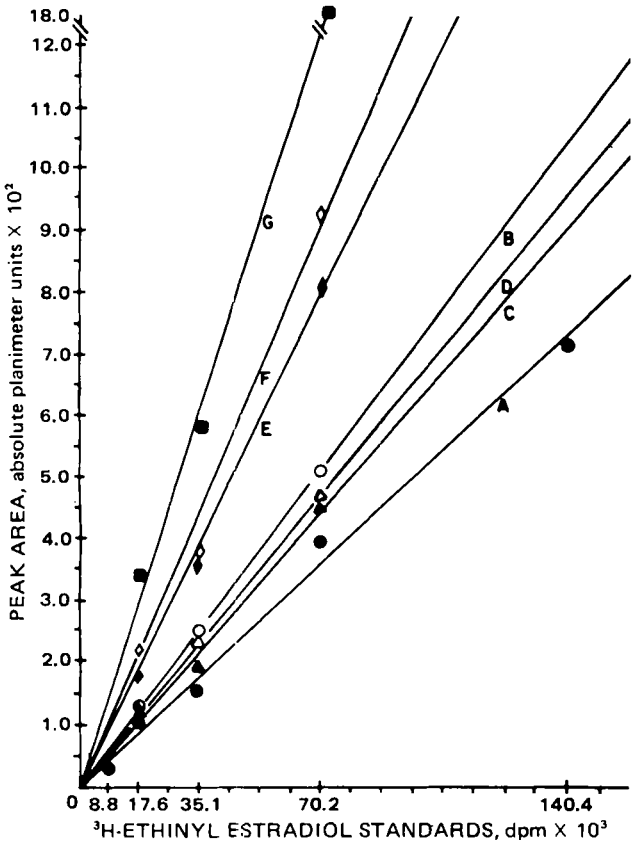

Figure 1—*Representative standard curves used for quantitation of ^3H-ethinyl estradiol in the unconjugated and aglycone fractions.*

Day 6. The second family of curves in Fig. 1 (E, F, and G) was obtained from a second column prepared identically. Their slopes increased in a chronological fashion in contrast to the first family of curves (A, B, C, and D). Sample peaks corresponding to ^3H-ethinyl estradiol were integrated by planimetry, and the disintegrations per minute of tritium present were determined by reference to the appropriate standard curve.

For quantitation of ^3H-quinestrol, a set of five standards was prepared by serial twofold dilutions of a ^3H-quinestrol stock solution with a concentration of 69,600 dpm/2.0 μl. Aliquots (2.0 μl) of the standards (34,800, 17,400, 8700, 4350, and 2175 dpm/2.0 μl) were injected into the gas chromatograph for standard curve preparation. The two standard curves are shown in Fig. 2. Curve A was obtained on Day 1 and curve B on Day 2.

Subjects, Doses, and Urine Collection—Eight healthy women participated in the study after being informed of all potential hazards. Four women (Group I), 21–26 years of age, each were given one capsule of the oral contraceptive formulation. The second four women (Group II), 42–47 years of age, each were given one estrogen replacement tablet. All subjects collected all urine for 24 hr after drug administration. These collections were stored frozen.

Fractionation of Urine—Thirty percent of the 0–24-hr urine collected by each subject was removed for individual fractionation. First each aliquot was concentrated to 30–50 ml by flash evaporation at 40–50° and poured onto an ion-exchange resin[4] column (2.5 × 50 cm). After the column was rinsed with 1 liter of deionized water, the adsorbed materials were eluted with methanol. Thirty fractions (10 ml each) were collected.

In general, a major peak of radioactivity was eluted in tubes 13–26. The contents of these tubes were pooled and evaporated to dryness under nitrogen. After 50 ml of deionized water was added to the residue, the pH was adjusted to 7; then the solution was extracted four times with 30-ml portions of ether to collect unconjugated steroids. The ether extracts were combined and concentrated to 30 ml under nitrogen. A 0.5-ml aliquot was removed and assayed for tritium. After the remaining ether solution was evaporated to dryness under nitrogen, the residue was dissolved in 50 μl of methanol for injection into the system.

[4] Amberlite XAD-2.

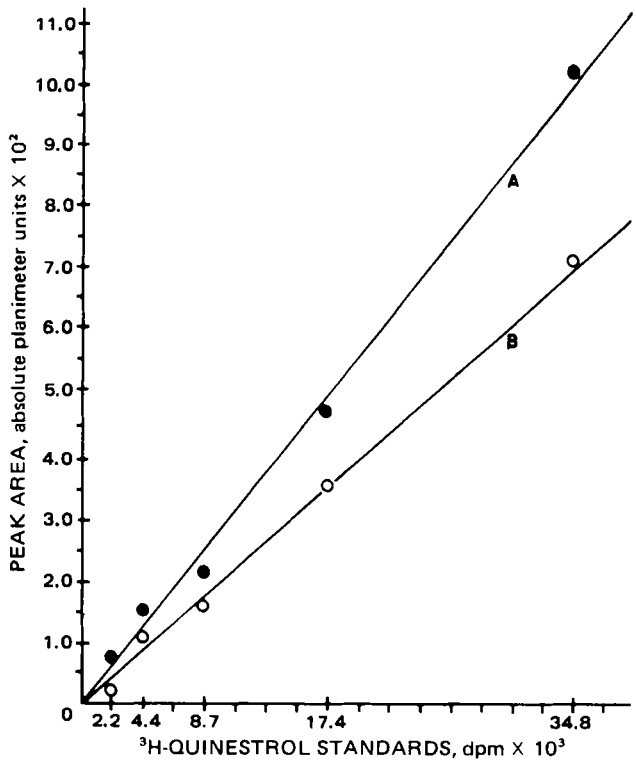

Figure 2—*Representative standard curves used for quantitation of ^3H-quinestrol in the unconjugated and aglycone fractions.*

The aqueous solution remaining after ether extraction was adjusted to pH 5.5 and incubated for 24 hr at 37° with 50,000 units of β-glucuronidase. The solution was then extracted with four 20-ml volumes of ether, and an additional quantity of β-glucuronidase was added to the aqueous phase. Following reincubation, the solution was extracted with ether as already described. The ether extracts were combined, evaporated under nitrogen to 2.0 ml, and chromatographed on a 1 × 36-cm column[5] with methanol as the eluant.

Twenty-five fractions (3.0 ml/9.0 min/fraction) were collected, and a 0.3-ml aliquot of each was assayed for tritium to find the center of the radioactive peak. In general, the peak was located between tubes 6 and 13. The contents of these tubes were pooled and evaporated to 2.0 ml; aliquots were counted to determine ^3H-recovery and injected into the system for analysis.

RESULTS

Standard Curves—The ability of the detector to respond in a proportional manner to tritium became evident from radiochromatograms of serially diluted solutions of ^3H-ethinyl estradiol. Twofold dilutions yielded proportional results with respect to both peak height and peak area (Fig. 3). Under operating conditions that achieved satisfactory resolution, standard curves were developed for ^3H-quinestrol (retention time of 53 min) and ^3H-ethinyl estradiol (retention time of 15 min). The ranges covered were 2175–34,800 dpm for quinestrol and 8775–140,380 dpm for ethinyl estradiol. The curves generated by plotting disintegrations per minute *versus* absolute planimeter units are presented in Figs. 1 and 2.

Reference to Fig. 1 shows clearly that a family of standard curves was obtained from each chromatographic column. Curves A, B, C, and D were developed in that sequence after injecting the indicated quantities of ^3H-ethinyl estradiol onto the same column on different days; curves E, F, and G were developed similarly with another column, presumably identical to the first. The slopes of the curves and, therefore, the sensitivity of the assay varied considerably and indicated the necessity of preparing a standard curve on the same day that the assays were performed to maintain reproducibility within replicates at approximately 5%.

Figure 2 presents two standard curves generated for ^3H-quinestrol on

[5] Sephadex LH-20.

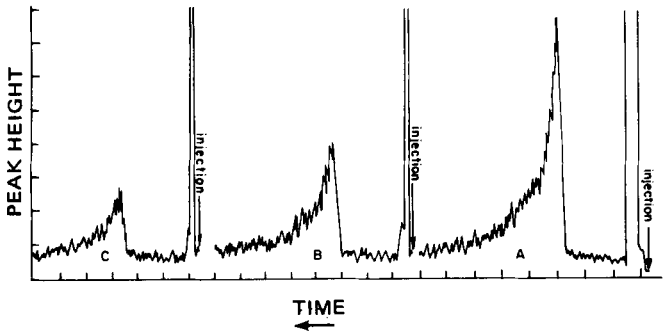

Figure 3—*Radiochromatograms of known ^{3}H-ethinyl estradiol representing serial twofold dilutions for standard curve preparation. The height and area of peak B are one-half those of peak A; peak C has a height and an area that are one-half those of B.*

different days. Like the ^{3}H-ethinyl estradiol curves, they differ markedly, confirming the conclusion that standard curves must be run on the same days as assays.

Urinary Excretion of Radioactivity—Table I shows the quantities of tritium excreted within 24 hr by each subject. The data are expressed in terms of quinestrol equivalents. The mean excretion of the dose was 4.6 ± 0.7% by Group I and 7.7 ± 1.0% by Group II.

Radioactivity in Unconjugated and Aglycone Fractions of Urine—The distribution of tritium between the unconjugated and aglycone urine fractions of each subject is listed in Table II. Each urine specimen contained small quantities (1.7–3.9%) of labeled unconjugated steroids and significant amounts (15.3–54.8%) of labeled steroid glucuronides, including ethinyl estradiol glucuronide (mol. wt. 472.5).

Radiochemical GLC of Urine Fractions—Typical responses following the injection of unconjugated and aglycone fractions appear in Figs. 4 and 5. All fractions showed the presence of only one peak, and the retention time of this peak corresponded to ^{3}H-ethinyl estradiol (Fig. 3). When a stream splitter was used to direct one part of the column effluent into a flame-ionization detector and 10 parts into the gas proportional counter, the mass response showed the presence of several components in addition to ethinyl estradiol. The splitter was not employed for quantitative purposes because steroids adsorb to stainless steel.

Table I—Description of Women and Their Urinary Excretion of Tritium from 0 to 24 hr after Treatment with ^{3}H-Quinestrol

Group	Subject	Age, years	Weight, kg (lb)	Equivalents of Quinestrol in 0–24-hr Urine, μg
I[a]	1	26	50 (111)	115.80
	2	24	89 (196)	68.00
	3	24	63 (138)	70.40
	4	21	49 (107)	113.20
			Mean ± *SE*	91.85 ± 13.10
II[b]	5	45	61 (135)	10.36
	6	45	57 (126)	5.27
	7	47	68 (151)	7.37
	8	42	49 (108)	7.67
			Mean ± *SE*	7.67 ± 1.04

[a] Given capsule containing 2.0 mg of ^{3}H-quinestrol (900 μCi) and 2.5 mg of unlabeled quingestanol acetate. [b] Given tablet containing 100 μg of ^{3}H-quinestrol (86 μCi).

Table II—Recovery of Tritium in the Unconjugated and Aglycone Fractions of Urine

Group	Subject	Tritium in Urine, %	
		Unconjugated	Aglycone
I	1	3.39	42.29
	2	3.07	29.12
	3	3.89	54.77
	4	1.70	17.59
	Mean ± *SE*	3.01 ± 0.47	35.94 ± 8.05
II	5	2.59	37.91
	6	1.80	15.32
	7	3.83	27.47
	8	2.61	34.25
	Mean ± *SE*	2.71 ± 0.42	28.74 ± 4.97

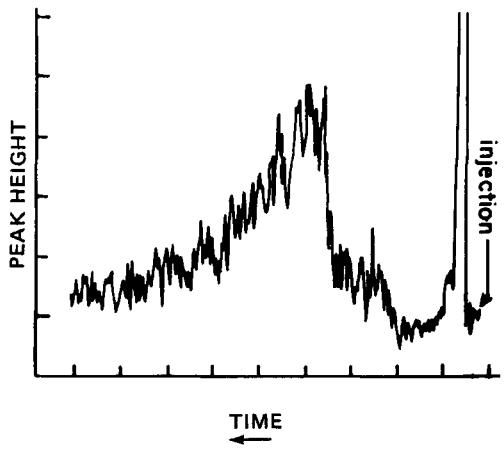

Figure 4—*Typical radiochromatogram of an unconjugated fraction.*

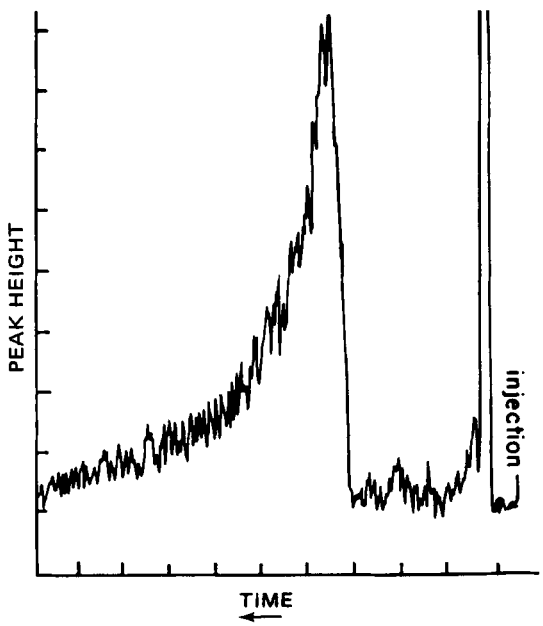

Figure 5—*Typical radiochromatogram of an aglycone fraction.*

Although the ^{3}H-ethinyl estradiol in several unconjugated fractions was represented by small peaks, these peaks were clearly defined and their areas could be measured accurately. With the exception of one fraction, appropriate standard solutions of ^{3}H-ethinyl estradiol were used for calibration so that the area of the metabolite ethinyl estradiol peak was read from a portion of the standard curve that included points on both sides of the metabolite point.

Table III shows the recoveries of tritium as ^{3}H-ethinyl estradiol in the unconjugated and aglycone fractions for both groups. The percentage

Table III—Ethinyl Estradiol Content of Unconjugated and Aglycone Fractions

Group	Subject	Tritium in Fraction as ^{3}H-Ethinyl Estradiol, %	
		Unconjugated	Aglycone
I	1	21.4	74.3
	2	7.8	41.1
	3	12.4	9.4
	4	21.7	30.4
	Mean ± *SE*	15.8 ± 3.4	38.8 ± 13.5
II	5	105.2	59.0
	6	100.1	87.4
	7	23.6	99.6
	8	64.5	61.4
	Mean ± *SE*	73.4 ± 18.9	76.9 ± 9.9

Table IV—Ethinyl Estradiol in Unconjugated and Aglycone Fractions

Group	Subject	Ethinyl Estradiol, μg	
		Unconjugated	Aglycone
I	1	0.68	29.50
	2	0.13	6.59
	3	0.28	2.93
	4	0.34	4.90
	Mean ± *SE*	0.36 ± 0.12	10.98 ± 6.22
II	5	0.23	1.88
	6	0.08	0.57
	7	0.05	1.64
	8	0.10	1.31
	Mean ± *SE*	0.12 ± 0.04	1.35 ± 0.28

recoveries listed are based on the total tritium present in each fraction. For Group I, ^{3}H-ethinyl estradiol constituted approximately 8–22% of the unconjugated fraction; for Group II, the recovery was 24–100%. The aglycone fraction of Group I was composed of 9–74% ethinyl estradiol; again the recovery for Group II was much higher, ranging from 59 to 100%.

Presented in Table IV are the total quantities of ethinyl estradiol found in the unconjugated and aglycone fractions of the urine collected from each subject. The largest amounts of this metabolite were present in the aglycone fraction of every subject. The range for Group I was approximately 3–30 μg; for Group II, it was 0.6–1.9 μg. Unconjugated ethinyl estradiol was found in small amounts: 0.1–0.7 and 0.05–0.2 μg for Groups I and II, respectively. Although more ethinyl estradiol was present in both urine fractions of Group I, the quantities were considerably less than the 20-fold difference in the administered doses. Besides reflecting wide interpersonal differences of the quantities of ethinyl estradiol and its glucuronide excreted by members of a given group, the data show that there were large interpersonal differences in the ratio of these metabolites.

DISCUSSION

The radiochemical GLC unit developed functioned satisfactorily in the present drug metabolism study, making it clear that the method has wide applicability. The instrumentation also can be applied to the assay of body fluids for nonradioactive drugs, drug metabolites, and endogenous compounds after the target compound is derivatized with a radioactive reagent (14).

There are two potential problems with radiochemical GLC. One problem relates to the quantity of radioactivity in the injected aliquot. When this level was too high, radioactivity tended to bleed from the column and to produce a heightened "noisy" baseline, which sometimes persisted for 30 min. This on-column degradation produced a dark, dense plug in the throat of the column. It was impossible to identify the material because it could be dissolved only in concentrated acid. The second problem is the necessity of preparing a standard curve for each day on which assays are performed. The most important operational parameter contributing to the difficulty is the composition of the counting gas. In the described setup, there might have been minor inadvertant differences in mixing the three gases at different times.

Intact quinestrol was not found in the urine of any subject who ingested the compound. In view of the ability of the instrument to detect 1300 dpm of ^{3}H-quinestrol, the specific activities of the administered labeled compound preparations would allow finding 1.3 ng of quinestrol in Group I specimens and 0.68 ng in Group II specimens. Therefore, quinestrol would have submitted to assay if either the Group I fraction had contained the drug to the extent of 0.003% of the dose or the Group II fraction had contained quinestrol to the extent of 0.03% of the dose.

This investigation confirmed the *O*-dealkylation of quinestrol to ethinyl estradiol *in vivo* and quantified this conversion for the first time. This quantification was limited to unconjugated ethinyl estradiol and its glucuronide. The use of reverse isotope dilution in an earlier study (18) indicated that women dosed with ^{3}H-ethinyl estradiol excreted conjugated ethinyl estradiol, 2-methoxy-17α-ethinyl estradiol, 2-hydroxy-17α-ethinyl estradiol 3-methyl ether, and 17αβ-D-homoestradiol.

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