THE ISOLATION OF URINARY ESTROGENS AND DETERMINATION OF THEIR SPECIFIC ACTIVITIES FOLLOWING THE ADMINISTRATION OF RADIOACTIVE PRECURSORS TO HUMANS

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ABSTRACT

In vivo studies of estrogen production and metabolism in humans by isotope dilution methods frequently necessitate the isolation of small quantities of estrogen metabolites from large amounts of urine. In addition to large capacity, the isolation methods used in such studies must have a high degree of specificity since a large number of radiometabolites are excreted following the intravenous administration of a radioactive precursor. Under certain conditions such as pregnancy, the specific activities of all these urinary metabolites may not be identical. Therefore, accurate methods for determining the specific activities of these metabolites are also essential. A gradient elution, liquid-liquid partition chromatographic system has been devised which is capable of isolating in purified form most of the estrogens present in crude phenolic extracts from 10 or more liters of human urine. Other partition and thin layer chromatographic systems have been developed for the further purification of estrone, estradiol and estriol. A rapid method for determining the approximate specific activities of these metabolites by formation of their nitroso derivatives on thin layer chromatographic plates is presented. Finally, procedures used for the precise determination of estrogen specific activities by acetylation with radioactive acetic anhydride are described.

INTRODUCTION

The isotope dilution principle has been applied to the measurement of the rates of secretion of many steroid hormones in humans.[1-3] Of the several approaches to this problem, the 'urinary' method has most commonly been used. Endogenous hormone production is estimated from the extent of dilution of an intravenously administered radioactive tracer dose of the hormone as indicated by the specific activity of a particular urinary metabolite. Although under certain conditions the results obtained may not represent the secretory rate of a single substance,[4] this approach has yielded useful informa-

tion concerning steroid hormone production and metabolism.

Utilizing this method for the evaluation of hormone production, it is imperative that urine collection is continued until excretion of the specific radiometabolite(s) is completed. This may require several days, depending on the hormone in question. Since the amounts of radioactivity which may be administered, particularly to pregnant subjects, is limited, it is frequently necessary to utilize the entire urine collection in order to obtain sufficient amounts of radioactive metabolites to insure accurate determination of their specific activities. Thus, the problem of isolating small amounts of steroids from 5 to 15 or more liters of urine must be solved. The study of estrogen production during pregnancy by this technique is further complicated by the excretion of a large number of radioactive estrogen metabolites some of which may have different specific activities following the administration of a radioactive precursor.[5,6] Therefore, efficient methods possessing a high degree of specificity and accuracy must be utilized in order to obtain meaningful results.

Reports of improved chromatographic procedures for purification and separation of urinary estrogens have recently appeared,[7,8] but these methods are not applicable to the problem at hand because of their limited capacity. The present report describes a gradient elution, liquid-liquid partition chromatographic system with which separation into individual components of most of the estrogens present in crude phenolic extracts from large amounts of normal or pregnancy urine is accomplished.[9] Other chromatographic systems are described for the further purification of estrone [estra-1,3,5(10)-trien-17-one], estradiol [estra-1,3,5(10)-trien-3,17β-diol], and estriol [estra-1,3,5(10)-trien-3,16α,17β-triol]. Finally, procedures used in these laboratories for the determination of the specific

activities of radioactive urinary estrogen metabolites by the double isotope derivative method are described.

EXPERIMENTAL

The procedures used for isolating and determining the specific activities of radioactive urinary estrogens are outlined in Figure 1. The basic steps involve differential hydrolysis of estrogen conjugates by treatment of urine with β-glucuronidase and by solvolysis. Following extraction with ethyl acetate, the liberated steroids are separated into neutral and phenolic fractions by partition between diethyl ether and 2N NaOH solution. Individual estrogens are separated and purified by gradient elution chromatography on celite. Estrone, estradiol and estriol are purified further by column partition chromatography in different systems containing isooctane, t-butanol, methanol and water. The identity and purity of these compounds are confirmed by thin layer chromatography of small aliquots together with standards. The free estrogens are detected by the formation of nitroso derivatives from which approximate specific activities are obtained by spectrophotometric and radioassay. Acetylation of the purified estrogens with either tritium or carbon-14-labelled acetic anhydride is followed by purification of the acetates by thin layer chromatography. The purified acetates are recrystallized together with non-radioactive acetate carriers until identical isotope ratios are observed for crystals and mother liquors. Final specific activities of the urinary estrogens are calculated from these final ratios.

Materials. Commercially obtained tritium and carbon-14-labelled steroids were purified by column chromatography on celite using the appropriate solvent systems shown in Table I. Carbon-14 and tritium-labelled acetic anhydride were diluted with solutions of non-radioactive acetic anhydride in benzene (1:10, v:v) to yield materials with a variety of suitable specific activities. All solvents with the exception of ethylene glycol were

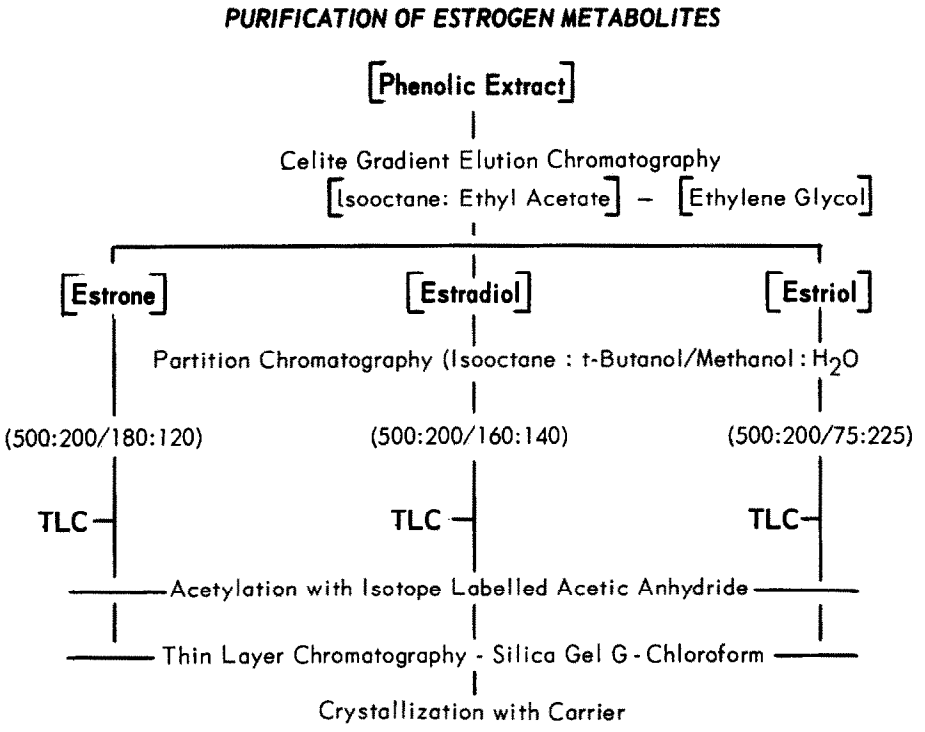

Figure 1. Procedure for isolation of radioactive urinary estrone, estradiol and estriol and determination of their specific activities.

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of reagent grade and were distilled before use.

Johns-Manville celite 545 was prepared for use as stationary phase support for partition columns as follows. A large column (4" x 60") was filled with the dry powder and sufficient 6N HCl was added to completely wet the celite. After standing overnight, the acid was displaced with tap water and washing continued until the pH of the effluent was neutral. The celite was then washed with distilled water, methanol, and finally diethyl ether. Silica gel G obtained from the Brinkmann Company, Great Neck, New York, was used for thin layer chromatography.

Hydrolysis of Conjugates and Extraction of Free Estrogens. The urine, which was kept at 0 to +5° C. until the collection was completed, was acid-

TABLE I

COMPOSITION BY VOLUME OF SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF ESTROGENS

COLUMN CHROMATOGRAPHY

System	Iso-Octane	t-Butanol	Methanol	Water	Peak Position (Hold Back Volume)
E-1	500	200	180	120	Estrone - 2.6
E-2	500	200	160	140	Estradiol - 4.3
E-3	500	200	75	225	Estriol - 4.0

THIN LAYER CHROMATOGRAPHY

		Approximate Rf
TLC-E1-2	isooctane:ethyl acetate 1:1	Estrone - 0.6 Estradiol - 0.4
TLC-E3	ethyl acetate	Estriol - 0.5
TLC-EAc	chloroform	Estrone acetate - 0.43 Estradiol diacetate - 0.72 Estriol triacetate - 0.52

E-1, E-2, and E-3 refer to estrone, estradiol and estriol, respectively.

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ified to pH 5.0 with 50% H_2SO_4. One-tenth normal sodium acetate buffer (pH 5.0) was added (5 ml/100 ml of urine) together with sufficient β-glucuronidase (Ketodase) to give a final concentration of 1000 units per ml of urine. The solution was incubated at 37° for 72 hours and free steroids were extracted with 3 one-half volume portions of ethyl acetate. The organic extracts were combined and solvent removed in a rotary evaporator at 40° C. The dry residue was dissolved in 400 ml of diethyl ether and phenolic steroids were extracted with four 100 ml portions of 2N NaOH. The ether layer was washed with water and evaporated to dryness in vacuo. The combined alkaline extracts were immediately acidified to pH 2-3 with 5N HCL and phenolic steroids were re-extracted with four 200 ml portions of ether.

The combined ether extracts were washed successively with three 100 ml portions of 5% aqueous $NaHCO_3$ solution and three 100 ml portions of distilled water. Removal of the ether yielded a phenolic "glucuronide" extract.

The residual urine was acidified to pH 1 with 50% H_2SO_4 and solid NaCl was added to a final concentration of 20% (w/v). Steroid sulfates were removed by 4 extractions with 1/2 volume of freshly distilled tetrahydrofuran. The combined extracts were neutralized with excess concentrated NH_4OH and the tetrahydrofuran removed in vacuo at 40° C. The volume of the resulting aqueous solution was brought to 400 ml with water and the extraction procedure repeated. The combined extracts were filtered through glass wool and steroid sulfates were cleaved by the addition of perchloric acid as described by Burstein and Lieberman.[10] The liberated steroids were partitioned between diethyl ether and 2N NaOH solution as described above to yield phenolic and neutral "sulfate" fractions.

Chromatography. Celite columns for partition chromatography were prepared by thoroughly mixing stationary phase with dry celite (1:2, v:w) in either a glass beaker or polyethylene bag. The damp powder was added in small portions to a plain glass tube fitted with a constriction containing a glass wool plug at its lower end. Each portion was settled by tapping the lower end of the column on a rubber pad and was then tightly packed with a close-fitting cork tamper. The final height of each segment was 1.0 to 1.5 times the column diameter. The ratio of the total packed column height to its diameter was usually 25:1. Samples were dissolved in a minimum volume of a mixture of stationary phase together with an equal or lesser amount of mobile phase. The solution was mixed with the appropriate amount of celite and the mixture was packed onto the column with a foil-covered tamper as described above. Mobile phase was carefully introduced and allowed

to flow by gravity until solvent emerged from the column. The volume of solvent required to fill the column (hold back volume, HBV) was measured directly or by adding a measured amount of solvent and determining the length of column that it occupied. Under these conditions, the ratio of the mobile phase to stationary phase volume ranged from 1.5 for columns containing 10 to 40 g of celite to approximately 3 for larger columns. Flow rates of 10-15 ml/cm^2/hour were used for partition columns and were achieved by application of air pressure to the solvent reservoir. Gradient elution chromatography was carried out with columns packed in a similar manner with the exception that a stationary phase "trap" was packed in the lower portion of the column. This consisted of 10 g of celite mixed with 5 ml of water and served to minimize elution of ethylene glycol from the column.

Gradients of increasing solvent polarity were produced by a system of two cylinders[11] as shown in Figure 2. Cylinder I constitutes the mixing chamber and is fitted with two outlets having size 18/9 standard ball joints. Cylinder II is of identical size but with only one outlet at the bottom. Both cylinders are provided with 24/40 standard taper joints at the top for application of pressure to the system. A tube connects the two cylinders and is fitted with 18/9 socket joints, a teflon stopcock and a coarse sintered glass plug which minimizes spurious flow of solvent between the two cylinders. A delivery tube is also fitted with a socket joint and passes into the column through a rubber stopper. The column was packed as follows for chromatography of urinary estrogen metabolites. Section C contained 10 g of celite mixed with 5 ml of water. Section B contained 30 g of celite mixed with 15 ml of pure ethylene glycol. Section A contained 4 g of celite plus the sample dissolved in 2 ml of ethylene glycol. Isooctane

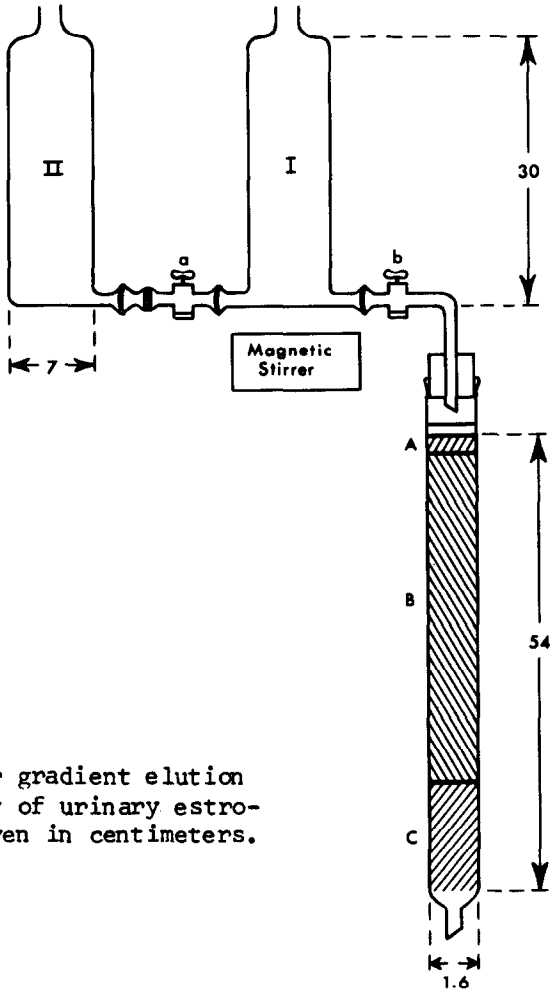

Figure 2. Apparatus for gradient elution partition chromatography of urinary estrogens. Dimensions are given in centimeters.

(2,2,4-trimethyl pentane) was passed into the column until solvent emerged from the bottom. Gradient cylinder I was filled with 900 ml of isooctane and cylinder II with 700 ml of ethyl acetate. The magnetic stirrer was started and stopcock "a" was cautiously opened. If a flow of solvent between cylinders I and II was observed, hydrostatic equilibrium was adjusted by either adding or removing ethyl acetate from cylinder II. Stopcock "b" was then opened and solvent allowed to flow through the column at a

rate of 60 to 100 ml per hour until both cylinders were empty. Finally, 200 ml of ethyl acetate was passed through the column. One-tenth hold back volume fractions were collected and an aliquot of each fraction was removed for radioassay. Those fractions near the end of the gradient contained small amounts of ethylene glycol which formed two phases. One ml of ethanol was added to these fractions prior to removal of aliquots for counting. The desired fractions were pooled and solvents removed in the flash evaporator. Ethylene glycol remaining in estriol and more polar fractions was removed by partitioning the sample between 10 volumes of diethyl ether and one volume of water.

The solvent systems used for further purification of estrone, estradiol and estriol are shown in Table I. These were prepared by thoroughly mixing the appropriate volumes of solvent in a separatory funnel and allowing the two phases to remain in contact until the mobile phase was clear. The columns used for this purpose contained 20 g of celite except those used for the purification of estriol from pregnancy urine which contained 60 g.

Thin layer chromatography (TLC) was performed on silica gel G. Plates were prepared by applying a strip of plastic tape to two edges of one or more clean 20 x 20 cm glass plates and spreading a slurry of silica gel G in distilled water (1:2, w:v) with a clean glass rod. The tape served not only to immobilize the plates on the work area but also determined the thickness of the resulting layer (approximately 0.2 mm). The plates were allowed to air dry for 10 to 15 minutes and were then heated in an oven at 110° C for one hour. The plates were stored prior to use in an oven whose temperature was maintained at 45° C. Estrogens and estrogen acetates were applied to the plates dissolved either in methylene chloride or ethyl acetate. Solvents used for TLC are also shown in Table I. Free estrogens

were detected following development of the plates by formation of yellow nitroso derivatives[12,13] (see below) or by very brief exposure to iodine vapor.[14] Estrogen acetates were also detected by exposure to iodine vapor or by 30-60 second exposure to ultraviolet light which resulted in the formation of a light yellow fluoresence. Free estrogens were eluted from silica gel G with ethanol and estrogen acetates with diethyl ether.

Determination of Specific Activities. Radioactivity was measured with a Packard Tricarb Model 500 D scintillation spectrometer. Samples were counted in 5 ml of toluene containing 0.3% of 2,5-triphenyloxazole and 0.01% of 1,4-bis-2-(5-phenyloxazolyl)-benzene. Radioactivity associated with estriol and other polar metabolites was assayed by the addition of 1 ml of methanol to the above medium. Quenching of these samples was corrected by the internal standard technique. Radioactivity was determined in samples which contained both tritium and carbon-14 by the methods of Okita, et al.[15]

The specific activities of purified radioactive estrogens were determined by formation of acetates with radioactive acetic anhydride and application of the double isotope derivative principles described by Kliman and Peterson.[16] However, colorimetric procedures were used to obtain preliminary values which guided the selection of radioactive acetic anhydride whose specific activity was such that estrogen acetates having a $^{3}H/^{14}C$ cpm ratio in the range of 3 to 20 were formed upon acetylation. This was accomplished in early experiments by utilization of the Ittrich modification of the Kober reaction.[17] More recently, free estrogens have been measured quantitatively by the formation of yellow nitroso derivatives directly on TLC plates as described below.

Following chromatography of an aliquot of purified estrogen in parallel with the appropriate standard (10, 20 and 30 µg), the plate was exposed

to an atmosphere of nitrogen dioxide for exactly one minute. This was accomplished by placing the plate in a dry chromatography jar in which the atmosphere had previously been saturated with nitrogen dioxide. The gas was formed by the action of concentrated nitric acid on metallic copper in a small beaker placed in the jar. Following complete removal of excess nitrogen dioxide by placing the plate in a stream of clean air for 30 minutes, the plate was placed in a second chromatography jar containing concentrated NH_4OH for 2 minutes. All of the estrogens tested immediately developed a bright yellow color. The colored zones of silica gel G were transferred to clean centrifuge tubes and the nitroso derivatives were eluted by the addition of 1.0 ml of concentrated NH_4OH. The tubes were briefly shaken and centrifuged at 2000 RPM for 5 minutes. Aliquots of the NH_4OH eluates were removed for optical density measurements using 1 cm light path micro-cuvettes and the Beckman Model DU spectrophotometer. Readings were made against an NH_4OH blank which was prepared from an uncolored area of the TLC plate. Under these conditions the nitroso derivatives of estrone, estradiol and estriol exhibited an absorption maximum at 420 mμ with a molecular extinction coefficient of approximately 3,000. Measurements were also made at 380 and 460 mμ and the Allen correction[18] applied to the 420 mμ readings. Larger aliquots were removed and transferred to counting vials. These samples were dried and counted using the methanol-toluene system described above.

The above procedure allowed both the final characterization and approximation of the specific activity of the isolated urinary estrogen in the same operation. In instances when the amount of an estrogen metabolite isolated was small, the entire sample was placed on the TLC plate by applying a series of adjacent spots at the origin. Following development with

solvent, the estrogens were detected with iodine vapor and their positions marked. After removal of the iodine, most of the silica gel G containing the urinary estrogen was removed from the plate and the sample was recovered by elution with ethanol. The specific activity of the metabolite was approximated from the portion of the sample remaining on the TLC plate by formation of its nitroso derivative as described above. This additional purification step materially reduced the amount of radioactive impurities formed during subsequent acetylation of estrogen samples with tritium-labelled acetic anhydride of high specific activity.

Aliquots of the purified estrogens were placed in the tip of glass-stoppered, conical centrifuge tubes by repeated solution in ethanol followed by evaporation with a stream of dry nitrogen. The tubes were kept in an evacuated dessicator over P_2O_5 overnight. The residue was dissolved in 0.05-0.1 ml of anhydrous pyridine and 0.1 ml of the appropriate solution of acetic anhydride was added.[19] The tubes were tightly stoppered and allowed to stand at room temperature for 18 hours. Solvents and excess acetic anhydride were removed with a stream of dry nitrogen and the residue was transferred to a TLC plate. If the amount of radioactive estrogen which was acetylated was less than 100 µg, this amount of the appropriate non-radioactive estrogen acetate was added to the dried reaction mixture prior to chromatography. Similar amounts of standard acetates were applied adjacent to the urinary acetates and the plates were developed with chloroform. Following detection, the zones of silica gel containing radioactive acetates were transferred to small test tubes and the estrogen acetates were eluted with 2 ml of diethyl ether. Silica gel was removed by filtration through small medium porosity sintered glass filters and the ether was evaporated to dryness. Approximately 10 mg of non-radioactive estrogen

acetate was added to each tube and the mixture was recrystallized from diethyl ether-petroleum ether (boiling range 30-50° C) until the $^{3}H/^{14}C$ cpm ratios of successive crystals and mother liquors were identical. The specific activity (sa) of the estrogen metabolites was then calculated by using one of the following formulas:

$$saE_m\ (cpm^{3}H/\mu gE2) = (T/C) \cdot {}^{14}C/E2Ac \cdot n, \text{ or}$$

$$saE_m\ (cpm^{14}C/\mu gE2) = \frac{{}^{3}H/E2Ac \cdot n}{(T/C)}$$

where (T/C) is the tritium:carbon-14 cpm ratio of an estrogen acetate formed by acetylation of an estrogen metabolite (E_m) containing tritium (or carbon-14) with carbon-14- (or tritium-) labelled acetic anhydride whose specific activity was such that $^{14}C/E2Ac$ (or $^{3}H/E2Ac$) cpm per acetyl group were introduced per microgram of estradiol. The number of acetyl groups in the estrogen acetate is represented by n. It should be noted that the above formulas yield values for estrone and estriol which are expressed as cpm per microgram of estradiol. This manner of expressing specific activities allows direct comparison of values of the three metabolites, as is the case when molar quantities are used, and also permits rapid calculation of production rates, etc. in commonly used terms, such as mg/day. True values for estrone and estriol may be obtained from the above by use of the appropriate molecular weight corrections.

In certain experiments to be described here and elsewhere,[20] the isolated urinary estrogens contained both tritium and carbon-14. The specific activity with respect to one of the isotopes of such metabolites was determined as follows. Separate aliquots of the purified metabolite were acetylated with both non-radioactive and either tritium or carbon-14-labelled acetic anhydride. The choice of isotope-labelled acetic anhydride and

its specific activity depended upon the $^{3}H/^{14}C$ cpm ratio and approximate specific activity of the isolated urinary estrogen. Following purification and crystallization of each acetate with carrier as described above, a $^{3}H/^{14}C$ cpm ratio, corrected for the isotope content of the free estrogen, was calculated from one of the following expressions. When carbon-14-labelled acetic anhydride was used, $(T/C)_{corr.} = \frac{(T/C)_I}{1 - \frac{(T/C)_I}{(T/C)_0}}$, and when tritium-labelled acetic anhydride was used, $(T/C)_{corr.} = (T/C)_I - (T/C)_0$, where $(T/C)_I$ and $(T/C)_0$ represent $^{3}H/^{14}C$ cpm ratios of acetates prepared with isotope-labelled and non-radioactive acetic anhydride, respectively. These corrected ratios were then substituted in the appropriate formula given above.

RESULTS AND DISCUSSION

The use of the above procedures can be illustrated by the following experiment. After the administration of a tracer dose of pure estradiol-6, 7-^{3}H (3.0×10^{6} cpm) and dehydroisoandrosterone-4-^{14}C-sulfate (5.0×10^{6} cpm) to a pregnant woman in her 26th week of gestation, urine was collected for 5 days. Eighty per cent of the total volume (13.8 liters) was processed as described above. A phenolic "glucuronide" extract weighing 0.48 g and containing 5.4×10^{5} cpm of tritium and 1.8×10^{5} cpm of carbon-14 was obtained. The results of gradient elution chromatography of this extract are shown in Figure 3 where fraction numbers are plotted against their tritium content. Preliminary experiments[9] had shown that the peaks labelled 2-methoxy-E1, E1, E2, epi-E3 and E3 contained 2-methoxy-estrone [2-methoxy-3-hydroxy-estra-1,3,5(10)-trien-17-one], estrone, estradiol, 16-epi estriol [estra-1,3,5(10)-trien-3,16β,17β-triol] and estriol, respectively. Removal of solvent from the fractions comprising the estrone and estradiol peaks yielded

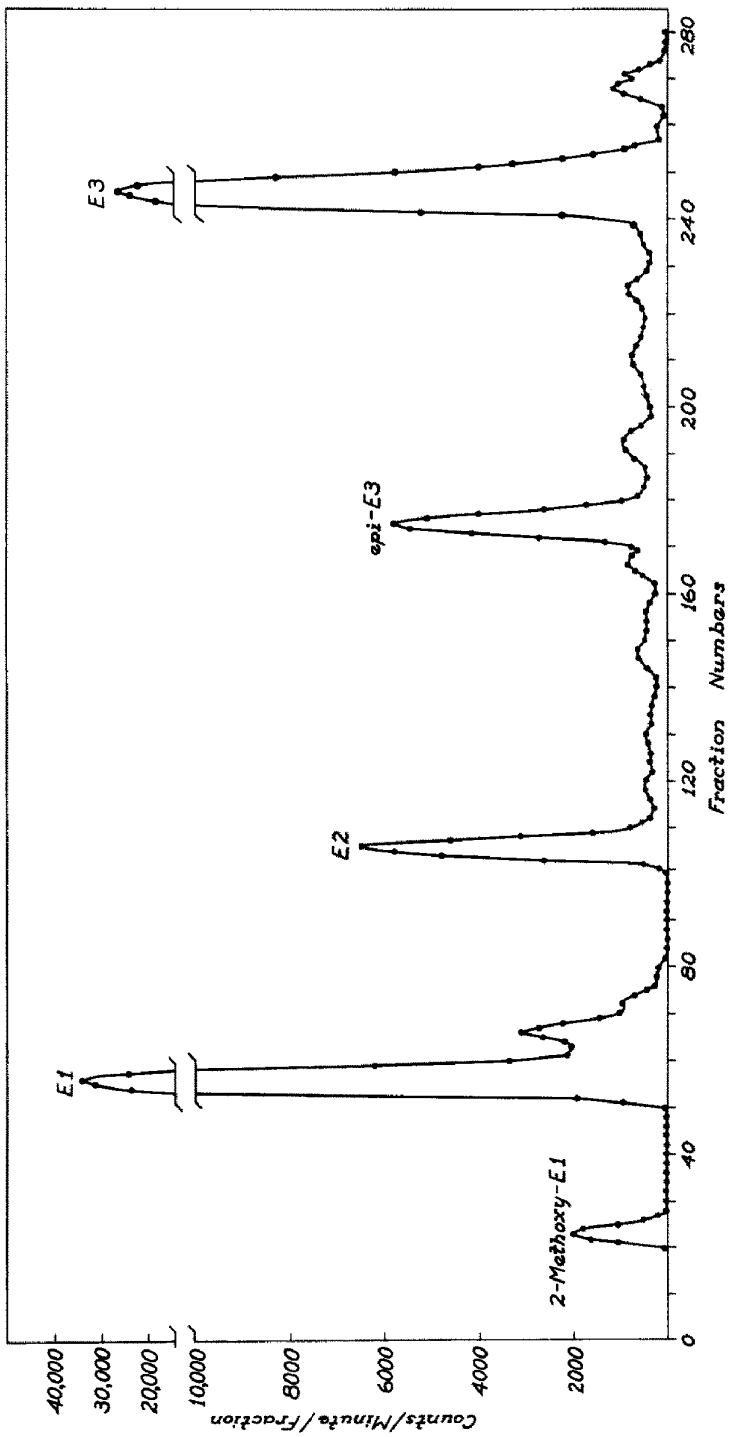

Figure 3. Results of gradient elution chromatography of a phenolic extract from 11.4 liters of human pregnancy urine. The experimental subject, who was in the 26th week of gestation, had received estradiol-6,7-^{3}H (3.0×10^{6} cpm) and dehydroisoandrosterone-4-^{14}C-3β-sulfate (5.0×10^{6} cpm) intravenously and urine was collected for 5 days. Fraction numbers are plotted against their tritium content. The entire contents of fractions 110 to 240 were used for radioassay. The column hold back volume was 56 ml and the fraction volume was 5.6 ml. 2-methoxy-E1 = 2 methoxy estrone, E1 = estrone, E2 = estradiol-17β, epi-E3 = 16-epi estriol, E3 = estriol.

crystalline residues weighing 6.2 and 2.4 mg, respectively. The estriol peak fractions were pooled and following removal of solvents the material remaining was partitioned between ether and water. Evaporation of the ether yielded a crystalline residue weighing 28 mg.

Chromatography of each of these residues was carried out in the appropriate system shown in Table I. A single, symmetrical peak of radioactivity, the position of which agreed with that indicated in the Table, was observed in each case. The peak materials were recovered and one half of the estrone and estradiol samples and a small aliquot of estriol were further purified by TLC. The position of each of the urinary metabolites following solvent development agreed with that observed for authentic standards as detected by exposure to iodine vapor. Following removal of the iodine, most of the areas of silica gel containing the estrone and estradiol samples were removed from the plates and the estrogens were recovered by elution with ethanol. Nitroso derivatives were prepared from the remaining portions and specific activities of estrone, estradiol and estriol were determined as described above.

Separate aliquots of estrone and estradiol, which had been purified by TLC, and of estriol were acetylated with non-radioactive and tritium-labelled acetic anhydride. The resulting estrogen acetates were purified by TLC and their positions on the plates were visualized by exposure to iodine vapor. Iodine was removed and the acetates were eluted with diethyl ether as described above. The $^{3}H/^{14}C$ cpm ratios of the materials in each of these eluates as well as those of successive crystals and mother liquors obtained upon recrystallization of each radioactive acetate with the appropriate non-radioactive estrogen acetate are shown in Table II. Also shown in the Table are the specific activities of the urinary estrogens which

TABLE II

DETERMINATION OF THE SPECIFIC ACTIVITIES OF URINARY ESTROGENS FOLLOWING THE ADMINISTRATION OF RADIOACTIVE PRECURSORS

Acetic Anhydride Used	Tritium/Carbon-14 cpm Ratio							Specific Activity (cpm tritium/microgram)	
	TLC Eluate	M-1	X-1	M-2	X-2	M-3	X-3	Isotope Method	Nitroso Method
ESTRONE									
Non-radioactive	2.63	3.28	2.43	2.54	2.45	-	-	54.8	53
Radioactive*	10.1	12.8	9.80	9.78	9.70	9.75	9.77		
ESTRADIOL									
Non-radioactive	2.50	2.54	2.44	2.52	2.46	-	-	54.4	58
Radioactive	17.7	19.7	16.7	17.5	17.0	17.0	17.1		
ESTRIOL									
Non-radioactive	1.85	1.43	1.96	1.90	2.00	-	-	13.0	18
Radioactive	9.05	7.65	9.30	9.15	9.28	9.21	9.26		

* Tritium-labelled acetic anhydride whose specific activity was such that 162 cpm per acetate group per µg of estradiol was introduced into both estrone and estradiol. The specific activity of that used for estriol was 16.6 cpm ^{3}H per acetate per µg of estradiol. See text for further details.

were calculated from the $^{3}H/^{14}C$ cpm ratios of the final crystals and these are compared with the values obtained by the nitroso method.

It can be seen that the $^{3}H/^{14}C$ cpm ratios of the TLC eluates were only slightly different from those of the first crystals obtained upon crystallization of each of the radioactive acetates with carrier. A high degree of radiochemical purity of each of the urinary estrogens and also of their acetates was indicated by the fact that the observed isotope ratios of these crystals was not significantly altered by recrystallization. Radiochemical homogeneity of purified estrone and estradiol was further evidenced by the identical $^{3}H/^{14}C$ cpm ratios of their acetates prepared with non-radioactive acetic anhydride. The presence of tritium, derived from injected estradiol-6,7-^{3}H, and carbon-14, derived from injected dehydroisoandrosterone-4-^{14}C-sulfate, in the same proportion in both estrone and estradiol makes radioactive contamination of either metabolite highly unlikely. Specific activities of 54.8, 54.4, and 13.0 cpm tritium/µg were calculated for estrone, estradiol and estriol, respectively. It is evident from Table II that the specific activities obtained by the nitroso procedure compared favorably with these values only in the case of estrone and estradiol.

Lisboa and Diczfalusy[13] reported the formation of nitroso derivatives first described by Boute,[12] for the detection of estrogens following TLC. The method described above has proven useful for the stated purpose, that is to obtain rapid approximations of specific activities which aid in the application of the isotope derivative method. However, the method as described cannot be used for exact quantitation of estrogens since discrepant values are occasionally obtained despite vigorous efforts to control variables. The sensitivity of the procedure is approximately 2 µg and the colors formed

with each of the estrogens adheres to Beer's law within the range of 0 to 40 μg. These colors are stable in concentrated NH_4OH solution for at least 24 hours. However, longer exposure of estrogens on TLC plates to nitrogen dioxide causes further chemical transformation as indicated by the disappearance of the absorption maximum at 420 mμ and appearance of a new maximum at about 380 mμ. The intensity of this maximum increases with time of exposure up to 30 minutes and also on standing in concentrated NH_4OH solution. Further efforts are being made to improve the accuracy and reproducibility of the method.

The determination of specific activities by the isotope derivative procedure is more costly and time consuming than procedures which use a separate colorimetric or fluorimetric assay. However, the greater accuracy and sensitivity of the radioactive procedure make it the method of choice particularly when small differences in specific activities are likely to be encountered. Of interest in the above experiment was the finding that the specific activities of estrone and estradiol were in fact identical when expressed on a molar basis. The specific activities of these urinary metabolites have consistently been nearly identical following the administration of isotope-labelled estradiol to both pregnant and non-pregnant women.[20] Similar findings have previously been reported by Fishman, et al.[5] and also by Gurpide, et al.[6] Fishman, et al.[21] have shown that estradiol is rapidly and almost completely converted to estrone following its intravenous administration to nonpregnant women. However, the reverse reaction, estrone to estradiol, proceeds at a much slower rate. Nevertheless, since the amounts of estrone which are secreted are small,[22] it is not surprising that the specific activities of urinary estrone and estradiol are identical following the administration of isotope-labelled estradiol to nonpregnant subjects. Although experiments similar to those of Fishman, et al.[21]

have not been reported for pregnant women, the fact that identical estrone and estradiol specific activities are observed in such subjects suggests that pregnancy does not alter the peripheral metabolism of estradiol.

Recently, column partition chromatography has been widely used for the isolation of steroids because of its inherently good resolving power and higher capacity compared to paper chromatography. Among other advantages of the column method are its predictability and relatively mild conditions. However, the widely different polarity within certain classes of compounds such as the estrogens, makes their separation in a single partition system inconvenient. During the early phases of development of the gradient system described above, two reports appeared which presented methods for overcoming this problem. The first, described by Engel, et al.,[7] utilized a gradient eultion system consisting of a methanol-water stationary phase and a mobile phase of isooctane containing increasing amounts of methylene dichloride. A second approach was reported by Preedy and Aitken[8] who devised an elaborate automatic system for increasing solvent polarity in a stepwise manner in order to elute more polar estrogens. Although both of these methods achieved separation of the classical estrogens, estrone, estradiol and estriol, in a single chromatogram, they were limted to chromatography of extracts obtained from relatively small amounts of urine (100-500 ml) or plasma.

The gradient elution system described in this report evolved from efforts to devise a system which combined high capacity with good resolving power over a broad range of polarity. Ethylene glycol was chosen as stationary phase for the following reasons. This material has good solvent properties and yet is relatively immiscible with non-polar solvents. In addition, ethylene glycol has a high affinity for stationary phase supports

such as celite. Finally and perhaps most important, the polar, more difficult to separate, estrogens exhibit small distribution coefficients (K, concentration of solute in mobile phase/concentration of solute in polar stationary phase) between this solvent and non-polar solvents such as isooctane. This factor was considered to be important in devising a high resolution system since the separation of substances by any partition process is greatest when their distribution coefficients are relatively small. Enhanced separation results from the relatively large number of transfers of solutes between the two phases of the system which are required to complete the process, e.g., elution of the solutes from a partition column. However, a limiting characteristic of partition processes is that solute bands broaden and peak heights decrease as the number of solute transfers between phases increases. It was anticipated that a gradual increase in mobile phase polarity would overcome this latter tendancy. Furthermore it was expected that the use of ethylene glycol as stationary phase would result in efficient separations as a result of the large numbers of transfers required to elute the desired compounds.

That these expectations were realized is evident from the results shown in Figure 3. The number of theoretical "plates" in the column which yielded this chromatogram was calculated to be about 1220. In comparison, a value of 180 was reported for the system described by Preedy and Aitken.[8] In addition to the identified peaks in Figure 3, approximately eleven other minor radioactive metabolites of estradiol-6,7-^{3}H were partially or completely resolved. Although positive identification of all of these compounds has not been made, the total number of components detected approximates the number of estrogen metabolites known to occur in human urine. It should be noted that most of the components were eluted in 10 or less

fractions, that is, less than one hold back volume. This behavior can be compared with the previously described gradient elution system for estrogen chromatography[7] with which elution of a single component required 5 to 10 hold back volumes. In addition to epi-estriol, a number of components intermediate in polarity between estradiol and estriol were resolved. The metabolites appearing between estradiol and epi-estriol consist of other estriol isomers and the corresponding α-ketols. As judged by peak heights, the relative amounts of these metabolites present in both normal and pregnancy urine was found to be highly variable. This may in part be due to the use of strong alkali in the preliminary purification procedure. Certain estrogen metabolites, such as 2-methoxy- and 2-hydroxy- derivatives, and the α-ketols, are known to be unstable under alkaline conditions. Milder procedures, such as the use of ion exchange resins,[7] are indicated if these metabolites are of primary interest.

Although gradient elution principles have been applied to the separation of a wide variety of compounds, most of these applications have involved the use of adsorption or ion exchange chromatography. The use of gradients of increasing solvent polarity in partition columns is obviously limited by the ability of the support to retain the stationary phase. Although ethylene glycol possesses desirable qualities in this regard, small amounts of this material were eluted from these columns beginning near fraction number 200-210 despite the use of a stationary phase "trap". Striping occurred much earlier and in greater amounts if the "trap" was omitted. The non-volatility of ethylene glycol proved to be a disadvantage since its removal from estriol samples required partition between ether and water and a consequent 5-10% loss of this steroid. This was considered to be a minor problem in view of the extremely large capacity which this system offered.

Extracts from late pregnancy urine weighing as much as 3 g have been successfully chromatographed. Such samples contained up to 100 mg of estriol which was usually eluted in approximately the same number of fractions as shown in Figure 2. When dealing with extracts from small amounts of urine (10-1000 ml) the high capacity of this system obviates the necessity of extensive preliminary purification steps and their attendant losses. Although larger columns have not been used, it would appear that the system is well suited for modification to allow the isolation of estrogens from even larger amounts of urine or other biological materials.

Although the gradient partition system has been used only for isolation of radioactive urinary estrogens, the reproducibility of the system suggests that it could easily be adapted to studies using chemical estrogen assays. The fraction numbers of the tubes containing the maximum concentration of estrone, estradiol, epi-estriol and estriol observed in ten consecutive chromatograms of extracts of urine collections from different individuals are shown in Table III. With the exception of estriol it can be seen that the peak position for each estrogen was usually within five fractions of the mean position. The shape of the gradient curve produced by this system (Figure 2) cannot be calculated by the equation derived by Bock and Ling[11] because of the difference in density of isooctane and ethyl acetate. However, it has been experimentally determined that the concentration of ethyl acetate increases in approximately linear fashion. Other types of gradients, using more elaborate devices, have not been investigated. While satisfactory separation of estrone, estradiol and estriol could be achieved using smaller initial volumes of isooctane and ethyl acetate, a loss in resolution of other components was observed.

The flow rates which can be used with this system are remarkably higher than those which can be used with simple partition columns. Chro-

TABLE III

GRADIENT ELUTION CHROMATOGRAPHY OF URINARY ESTROGENS

Column Number	Hold Back Volume (ml)	Peak Fraction Number			
		Estrone	Estradiol	Epi-Estriol	Estriol
1	55	62	105	170	260
2	56	54	98	178	276
3	60	58	103	175	262
4	60	55	97	160	252
5	58	55	104	180	264
6	56	59	105	170	262
7	56	55	101	165	275
8	56	66	108	172	280
9	56	56	106	174	246
10	56	60	109	175	248
Mean	57	58	104	172	262

matography as described above requires 18-24 hours for completion. However, satisfactory separations have been achieved using faster flow rates such that the chromatograms have been completed in about 10 hours. It should be pointed out that the "dry pack" method for preparing celite columns described above is not that which is used by most practitioners of partition chromatography. The majority of published procedures recommend the "slurry" method in which celite, moistened with stationary phase is suspended in mobile phase and packed in the column with the aid of a special packer. Although the latter method may yield more homogeneously packed columns, any small losses in resolution resulting from use of the "dry pack" method are more than compensated for by the time saving which this procedure affords. For example, the preparation of the gradient column described above requires 30-45 minutes, whereas the preparation of a column of similar size, if possible, by the "slurry" method would require many hours.

Gradient elution chromatographic systems similar to that described

above have been applied to the separation of other urinary steroids. These results will be described in future reports.

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ether (boiling range, 30-50° C) until the specific activity of crystals and mother liquors, obtained by counting weighed samples, were identical. High specific activity acetic anhydride was standardized by acetylating estradiol-4-^{14}C or estradiol-6,7-^{3}H of known specific activity. These were prepared by adding the radioactive steroids to weighed samples of pure estradiol. The resulting acetates were purified by TLC and then crystallized together with non-radioactive estradiol diacetate until the isotope ratio in both mother liquor and crystals were identical. Specific activities were then calculated as described in the text.

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