A GASCHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF SMALL AMOUNTS OF ESTROGENS IN URINE

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ABSTRACT

A sensitive gaschromatographic method for the measurement of estrone,estradiol and estriol in urine is described.After acid hydrolysis, extraction with ether and preliminary purification of the extracts, the three estrogens are separated and purified prior to gaschromatography.Adequate purification was achieved for estrone and estradiol by two thin-layer chromatograms,one with the free steroid and one with the acetate-derivative;for estriol with alumina chromatography of the 3-methylether derivative.The reliability of the method is discussed.Preliminary results obtained from urines of normal postmenopausal women are given.

The determination of estrogens in urine is most commonly done by the method of Brown (4,5).The minimal amount of estrogens that can be estimated reliably with this method is about 2 - 3 µg per 24 hrs urine.This sensitivity is not sufficient for the study of some problems as for instance the estrogen excretion in postmenopausal women.

In recent years it has been shown that a very sensitive detection of estrogens can be obtained by gasliquidchromatography (GLC)(1,7, 8,11,13).This method however is easily disturbed by contaminating substances and therefore requires highly purified urinary extracts.

In this paper two different methods for purification of extracts from urine of postmenopausal women are evaluated.The first method is an extension of the technique described by Wotiz and Chattoraj (13).The se-

cond one is based on the method of Brown (4,5).

Only the three "classical" estrogens : estrone,estradiol and estriol were determined (14).

EXPERIMENTAL PROCEDURES

1 : MATERIAL

Steroids

Radioactive steroids - 4-^{14}C-estrone and -estradiol - were obtained from the Radiochemical Centre,Amersham (U.K.).After purification with thin-layer chromatography they were stored in benzene-methanol 9:1 solution;their purity was checked periodically.

^{14}C-estriol was isolated from the urine of an adrenalectomized,castrated woman after oral administration of radioactive estrone.Recently 16-^{14}C-estriol was obtained as a gift from Dr.M.Levitz,New York.

Non-radioactive steroids were generously supplied by N.V.Organon, Oss (The Netherlands).

Reagents

All chemicals used were of analytical grade (B.D.H.,Analar). Some solvents were purified prior to use in the following way: diethylether was redistilled just before use; petroleumether (40-60°C) was redistilled once and benzene twice; pyridine was distilled once from bariumoxyde and stored over bariumoxyde;acetic anhydride was stored over calciumcarbide after distillation.

2 : METHODS

Thin-layer chromatography (TLC)

Glass plates (20 x 20 cm) were coated with a 0.25 mm layer of silicagel G (Merck A.G.,Darmstadt,Germany),using Desaga equipment.After preliminary drying at room temperature,the plates were activated by heating at 120°C for 1 hour.After cooling they were stored in a dessicator.

For the final thin-layer chromatogram in the procedure to be described,purified silicagel G was used.This purification was achieved by refluxing the silicagel powder first with distilled water during 2 hrs, and after filtration a second time with methanol.Following filtration, the resulting powder was dried at 80°C.

Chromatography was done with complete saturation;the following systems were used :

benzene : methanol 9 : 1
ethylacetate : methanol 9 : 1
petroleumether (40-60^{0}C) : methanol 9 : 1 (13)
cyclohexane : ethylacetate : ethanol 45 : 45 : 10 (12)

For the first chromatography of the crude urinary extracts,only one to two extracts were run on each plate,together with standards on both sides.After chromatography these standards were detected by spraying with iodine in ethanol.During this procedure the extracts to be analysed were protected by covering them with a glass plate.The area between the standards was scraped off with a razor blade,the silicagel was put into a centrifugetube and steroids were eluted with 3 x 5 ml of ethanol.

Prior to gaschromatography elution was carried out by adding 1 ml of benzene to the silicagel in a small centrifuge tube,followed by 0.2 ml of water.After mixing and centrifugation the benzene layer was separated.Again 1 ml of benzene was added to the tube,mixed and separated. This procedure was repeated once more and the three benzene fractions were combined and evaporated.

Gasliquidchromatography (GLC)

An F & M biomedical gaschromatograph (model 400) with flame ionisation detector was used.U-shaped glass columns , 3 - 6 feet long with an internal diameter of 4 mm,were filled with either 2% XE-60 or 3% SE-30 on Diatoport S or GasChrom Q.Coating was done either by the filtration method (9) or by the evaporation technique.
Peak height measurements were used for quantitation.

Chromatography on Aluminiumoxyde

Neutral aluminiumoxyde (Woelm,Germany),activity grade I,was inactivated by adding 10% w/v distilled water under vigorous shaking.At least 18 hrs later the activity was tested with standards,and if necessary re-adjusted with water or aluminiumoxyde until the results were satisfactory.

Urinary extracts were chromatographed after methylation on columns with 6 mm internal diameter,containing 2 g of aluminiumoxyde.

Measurement of radioactivity

A liquid scintillation counter (Nuclear Chicago,model 725) was used.For counting the dry substance was dissolved in 0.1 ml of 99% ethanol and 10 ml of scintillation fluid were added.This fluid consisted of 4 g of PPO (2,5-diphenyl-oxazole) and 40 mg of POPOP (p-bis(2-(5-phenyloxazolyl))-benzene) in 1 liter of toluene.
Correction for quenching was made by the use of an internal standard or with the channel ration method (2).Sufficient counts were collected to reduce counting errors to less than 3%.

Preparation of derivatives

Acetylation was performed by adding 5 drops of pyridine and 10 drops of acetic anhydride to the dried substance (6).After thorough mixing, the mixture was kept overnight at room temperature or heated at 70°C during 1 hour in a closed test tube.When acetylation was complete,5 ml of water were added and the acetates were extracted with 1 x 10 ml and 2 x 5 ml of petroleumether.The combined extracts were washed with 5 ml of 8% $NaHCO_3$-solution and twice with 3 ml of water.

Methylation was carried out with dimethylsulfate,as described by Brown (4,5).1 ml of dimethylsulfate was added to the substances dissolved in 50 ml 0.4 N NaOH-solution to which 0.9 g boric acid had been added.The solution was shaken until all dimethylsulfate had been dissolved and then incubated at 37°C during 20 minutes.Thereafter were added : 1 ml of 10 N NaOH and 1 ml of dimethylsulfate;the mixture was shaken and again incubated for 20 minutes at 37°C.After this incubation were added 5 ml of 10 N NaOH and 2.5 ml of 30% H_2O_2.

Preparation of urinary extracts

Hydrolysis,extraction and preliminary purification

400 ml of a 24 hrs urine are placed in a round bottomed flask of 1 L attached to a condenser,and brought to boiling.Through the condenser 60 ml of concentrated hydrochloric acid are carefully added,and refluxing is continued for 45 minutes.After cooling under tap water,radioactive estrogens are added to the urine (about 5000 dpm = 0.02 µg of each estrogen) and extraction is done with freshly distilled ether, once with 400 ml and a second time with 200 ml.

The combined extracts are washed with :

120 ml of a saturated carbonate buffer pH 10.4 (1000 ml of 8% $NaHCO_3$ plus 150 ml 5 N NaOH);the aqueous layer is discarded;

30 ml of 2N NaOH and,after shaking for a few minutes,120 ml of 8% $NaHCO_3$ are added.Shaking is continued for a few minutes.Again the aqueous layer is discarded;

finally the ether extract is washed with 30 ml of 8% $NaHCO_3$-solution and with 15 ml of water.Each time the aqueous layer is discarded after careful separation of the two phases.

The ether extract is evaporated to dryness with a rotary evaporator.

Phenolic separation and saponification (3)

The dry residue is dissolved in 1 ml of 99% ethanol and with the aid of 25 ml of benzene transferred to a separatory funnel already containing 35 ml of petroleumether (40 - 60°C).This mixture is extracted with :

I : 2 x 25 ml of water.This water extract contains the estriol.

II : 2 x 25 ml 0.4N NaOH solution.This alkaline solution contains estrone and estradiol.

The remaining organic solvent layer contains the neutral steroids.It is discarded.

To the water extract 2 g of NaOH,and to the alkaline extract 1.2 g of NaOH are added.Each extract is refluxed for 30 minutes and then cooled under tap water.After cooling each extract is buffered with 6 g of $NaHCO_3$.

For further purification preliminary to gaschromatography two different methods were evaluated.

METHOD I

The two fractions,obtained in the way described,are brought to pH 8.8 with 6 N sulphuric acid and each is extracted with 2 x 100 ml of ether. The ether extracts are washed with 2 x 5 ml of water and then taken to dryness.The residues are subjected to thin-layer chromatography in the systems

benzene : methanol 9 : 1 for fractions containing estrone and estradiol;

ethylacetate : acetone 9 : 1 for estriol fractions.

Estrone,estradiol and estriol are eluted separately and each steroid is acetylated and again subjected to thin-layer chromatography in the system petroleumether : methanol 9 : 1.
Elution is carried out with benzene and water,as described.

For GLC the dried extracts are dissolved in 50 µl of heptane and 5-10 µl samples are injected into the column.Similar aliquots are used for measurement of radioactivity.

Method I is summarized in figure 1.

METHOD II

The extracts containing estrone and estradiol (I) are extracted with 25 ml of benzene.After washing with 2 x 5 ml of water,the benzene is mixed with 25 ml of petroleumether and the mixture is extracted with 2 x 25 ml of 0.4 N NaOH solution.The NaOH layers are combined, 0.9 g of boric acid is added and methylation is performed,as described.

After this modifying step estrone-3-methylether and estradiol-3-methylether are extracted with 25 ml of petroleumether.The extract is washed with 2 x 5 ml of water.Then it is applied to an alumina column and eluted with

12 ml of petroleumether-benzene 75-25 (v/v)
15 ml of petroleumether-benzene 60-40 "
12 ml of petroleumether-benzene 40-60 "
15 ml of benzene

Estrone-3-methylether is eluted with petroleumether-benzene 60-40;estradiol-3-methylether with benzene.

The extracts containing estriol (II) are extracted with 50 ml of ether;the ether is extracted with 2 x 25 ml of 0.4 N NaOH solution.The NaOH layers are combined, 0.9 g of boric acid are added and methylation is performed,as described.

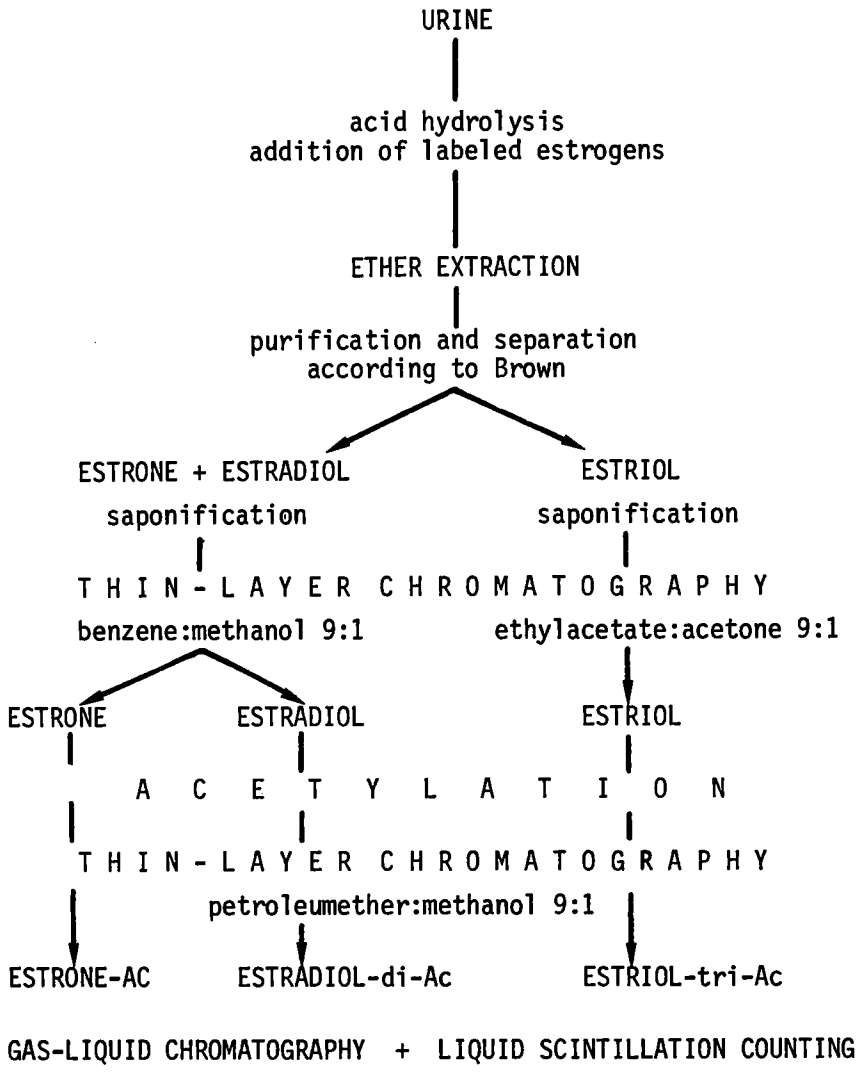

Figure 1 : Flow-sheet,summarizing the method in which the acetate-derivatives of the three estrogens are prepared (Method I).

Estriol-3-methylether is extracted with 25 ml of benzene,the benzene is washed with 2 x 5 ml of water and then applied to an alumina column.The elution is done with

12 ml of benzene-ethanol 99-1 (V/V)
15 ml of benzene-ethanol 97.5-2.5 (V/V)

This last fraction contains estriol-3-methylether.

The fractions collected are evaporated in vacuo.The residues are dissolved in 50 µl of heptane and 5-10 µl aliquots are injected into the co-

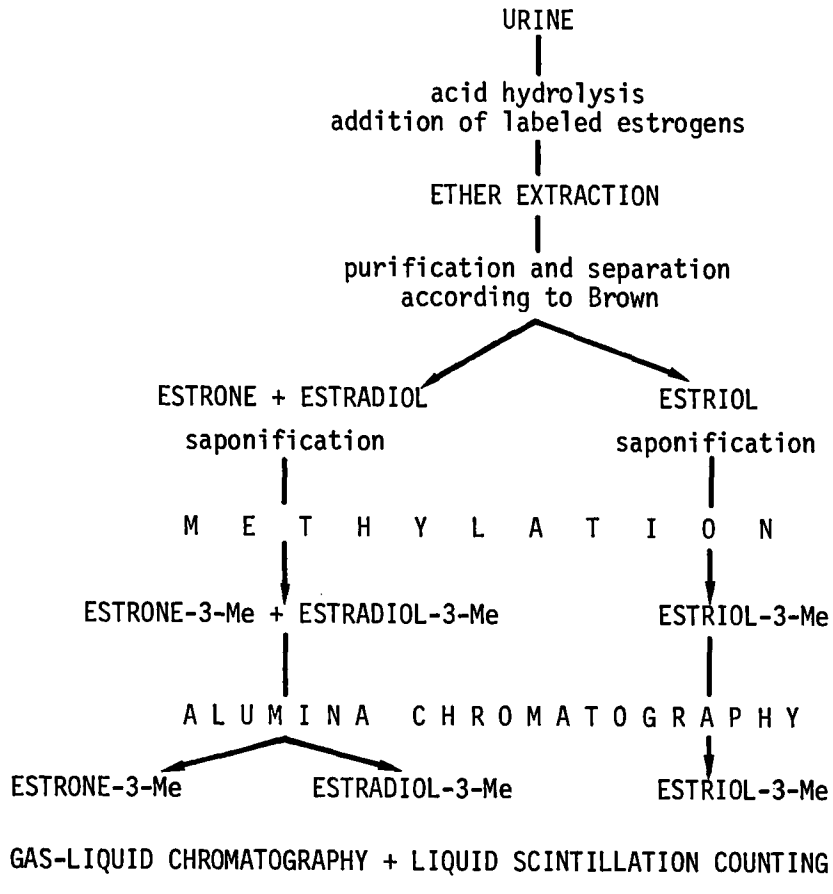

Figure 2 : Flow-sheet,summarizing the method in which the methylether-derivatives of the three estrogens are prepared (Method II).

lumn for GLC.Similar aliquots are prepared for the measurement of radioactivity.

A summary of method II is given in figure 2.

RESULTS

The two methods described were applied to the urine of postmenopausal women.The results will be discussed separately for each method.

RESULTS WITH METHOD I :

Figures 3,4 and 5 show gaschromatographic tracings obtained with extracts prepared by method I.The amount injected into the column was equi-

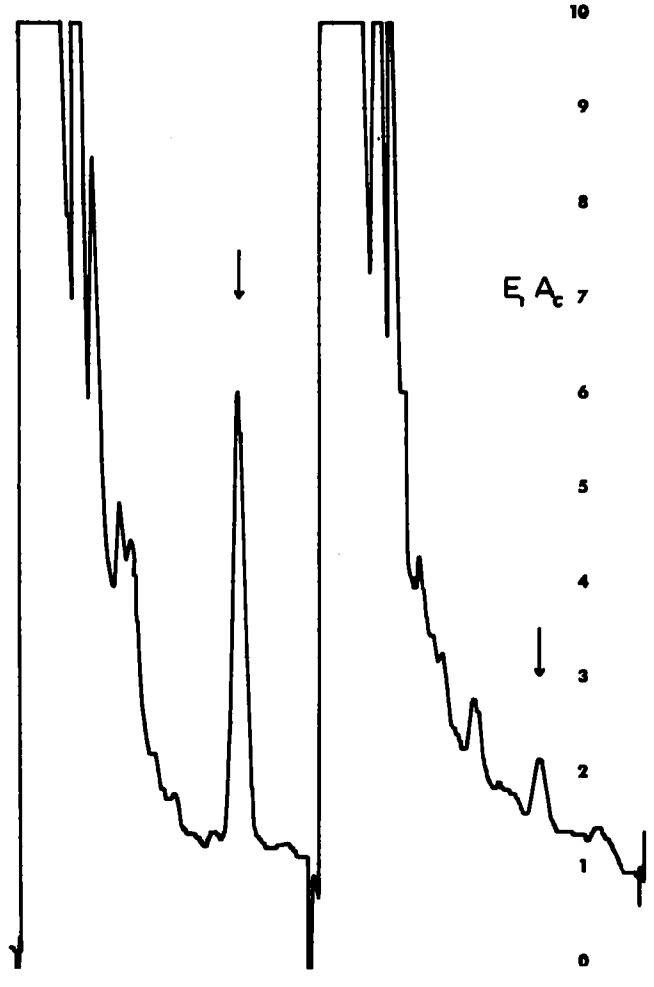

Figure 3 :
Chromatograms obtained with urinary extracts containing estrone-acetate, prepared with method I.

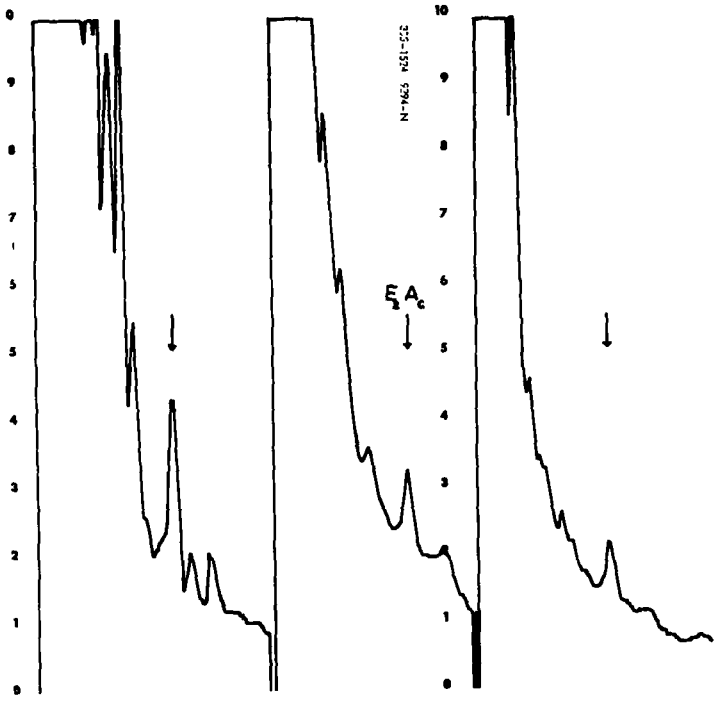

Figure 4 : Chromatograms obtained with urinary extracts containing estradiol-diacetate, prepared with method I.

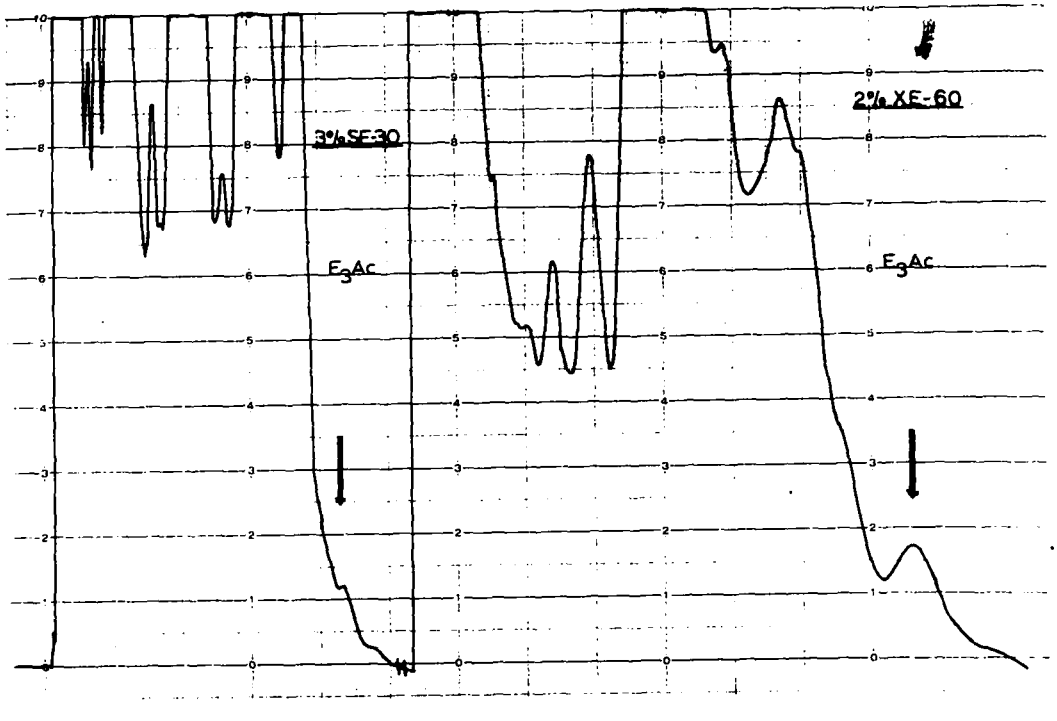

Figure 5 : Chromatograms obtained with urinary extracts containing estriol-triacetate, prepared with method I.

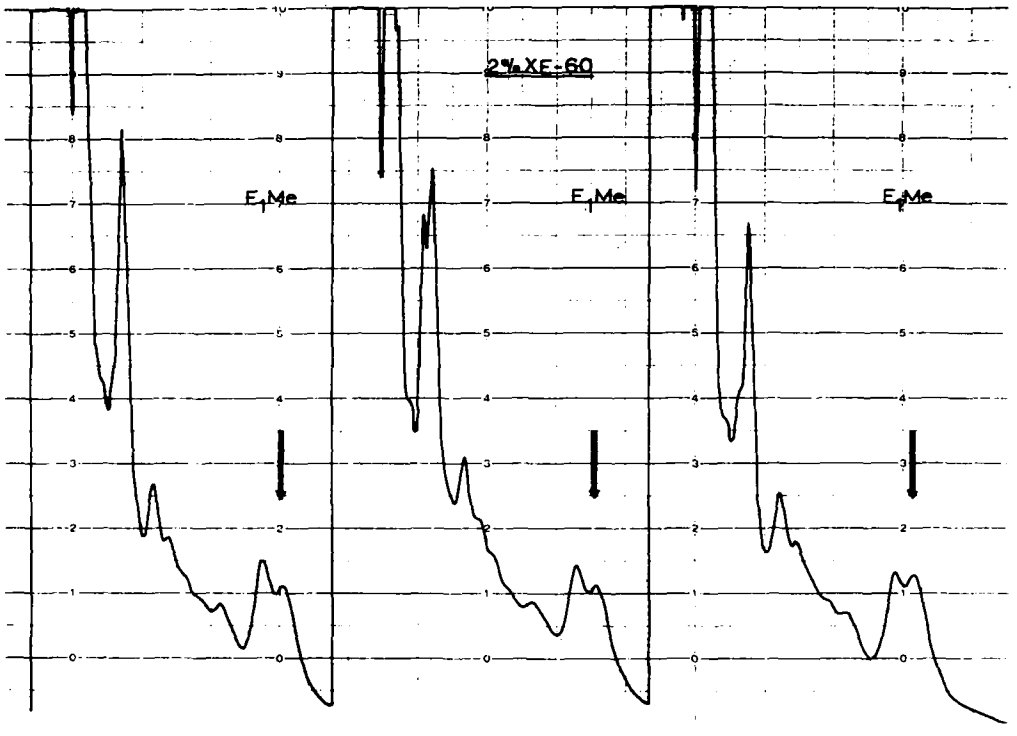

Figure 6 : Chromatograms obtained with urinary extracts containing estrone-3-methylether, prepared with method II.

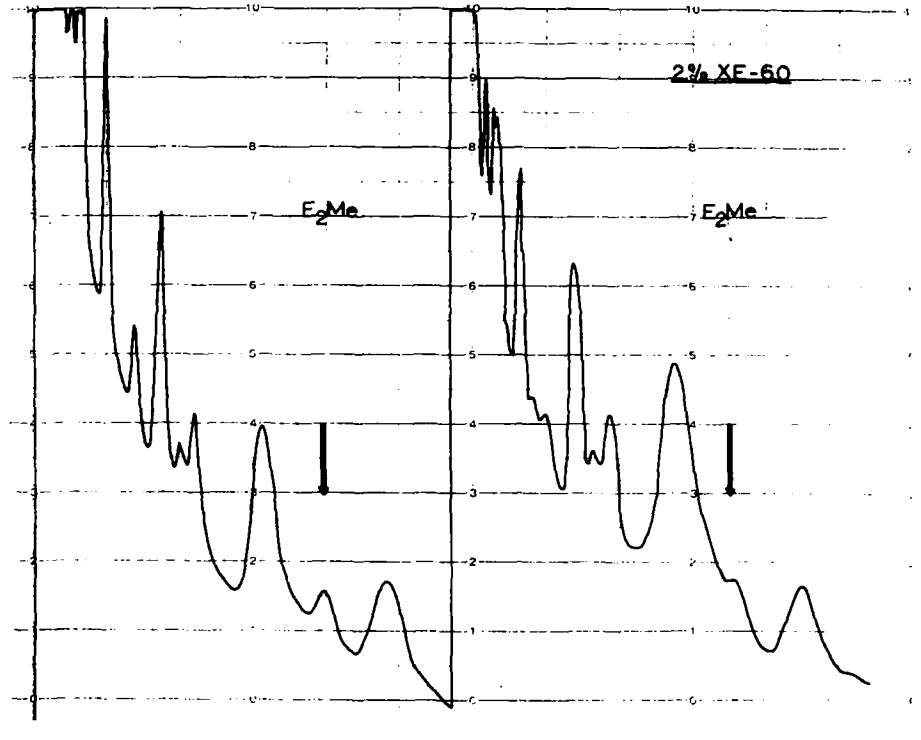

Figure 7 : Chromatograms obtained with urinary extracts containing estradiol-3-methylether, prepared with method II.

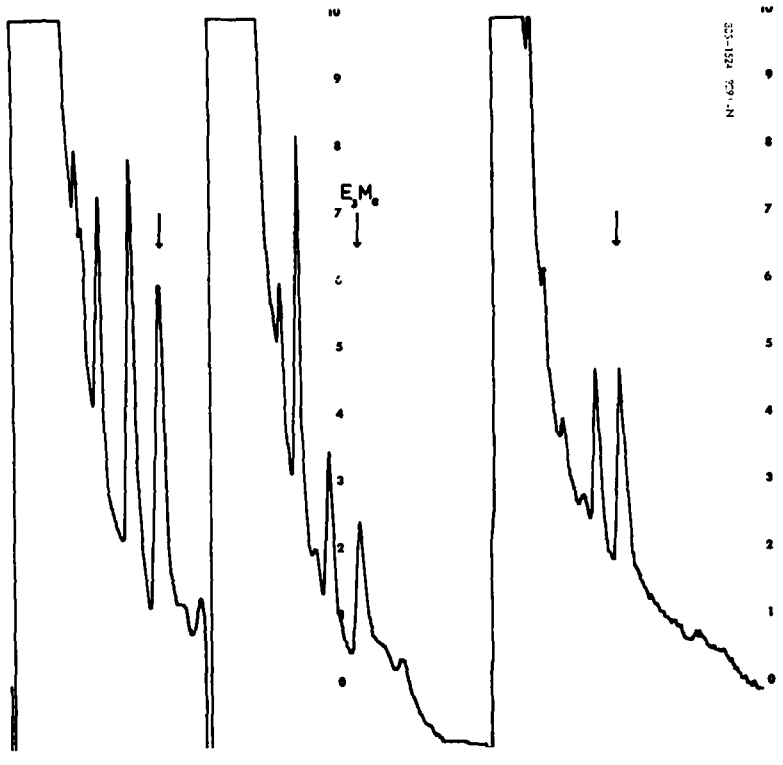

Figure 8 : Chromatograms obtained with urinary extracts containing estriol--3-methylether,prepared with method II.

valent to 3 - 5% of the total 24 hrs urine.
In figure 3 chromatograms from fractions containing estrone-acetate are shown,whereas figure 4 gives fractions containing estradiol-diacetate. The peaks obtained permitted quantitative evaluation of estrone and estradiol.
Figure 5 shows that with fractions containing estriol-triacetate no suitable tracings could be obtained,neither on XE-60 nor on SE-30 columns.

RESULTS WITH METHOD II :

With this method good results were obtained with estriol-3-methylether fractions (figure 8).There is always a sharp peak representing estriol--3-methylether which can be easily distinguished from those caused by contaminating substances.
The results with estrone-3-methylether and estradiol-3-methylether were not satisfactory (figure 6 and 7).

COMBINATION OF METHOD I AND II

It was concluded that good results for all three estrogens could be obtained by combining both methods :

After the first extraction and purification,the urinary extracts are separated into a fraction containing estrone plus estradiol and a fraction containing estriol.

The first fraction is prepared for the final GLC by acetylation (method I),the other fraction by methylation (method II).
The resulting procedure is summarized in figure 9 .

EVALUATION OF THE COMBINED METHOD

The reliability of the combined method (figure 9) was studied by means of the following experiments :

The accuracy was tested by estimating the recovery of non-radioactive estrogens added to 400 ml of hydrolyzed urine.The results are given in table 1.

steroid	amount added (µg)	number of experiments	mean recovery ± s.d. (µg)
estrone	1.0	8	1.07 ± 0.11
estradiol	0.2	3	0.20 ± 0.03
	0.5	3	0.41 ± 0.10
	1.0	4	1.07 ± 0.09
estriol	1.0	8	1.09 ± 0.14
	2.5	3	2.42 ± 0.21

TABLE 1

The precision of a method can be calculated by determining the difference between duplicates in a series of assays.The standard deviation of our method,calculated from the results of duplicate "routine" assays du-

ring a period of several months,are given in table 2,specified for several ranges of estrogen excretion.

Sensitivity : With a flame ionisation detector amounts of 0.005 µg can be detected with certainty.At lower levels the measurement is less reliable.From each urinary extract an aliquot representing about 5% of

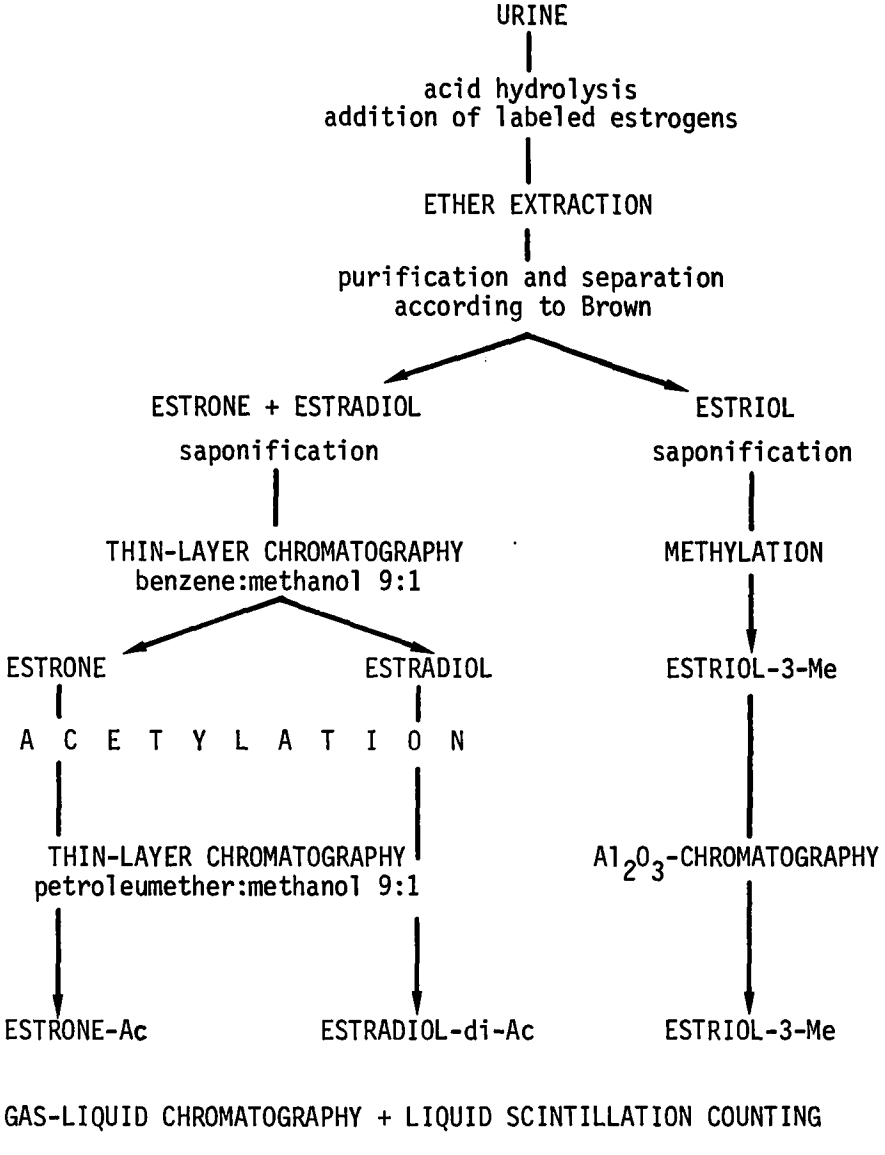

Figure 9 : Flow-sheet,summarizing the combined gaschromatographic method.

the total 24 hrs urine is injected into the column.The sensitivity of detection of our method therefore is about 0.1 µg of each steroid per 24 hrs urine.

The blank value of the method described is negligible,as was shown by experiments with water and with urine from an adrenalectomized,post-menopausal woman.No substances with retention times identical to those of the three estrogens studied were found on the final gaschromatograms.

The smallest amount of estrogen (x) which can be distinguished from zero has been calculated by applying the formula $t = \frac{x - 0}{\frac{s}{\sqrt{n}}}$

steroid	excretion per 24 hrs (µg)	number of duplicates	standard deviation (µg)
estrone	0 - 3	41	0.25
	3 - 6	18	0.7
	> 6	3	1.0
estradiol	0 - 1	21	0.1
	1 - 3	11	0.5
	> 3	7	1.1
estriol	0 - 6	17	0.8
	6 - 10	13	1.1
	> 10	13	2.4

TABLE 2

where $\frac{s}{\sqrt{n}}$ is the standard deviation of the mean of duplicate determinations (table 2),and t = 1.9 representing the 10% level of significance.

Therefore $x = \frac{t.s}{\sqrt{n}} = \frac{1.9 \cdot s}{\sqrt{n}}$

For duplicate estimations the results for estrone and estradiol are 0.3 and 0.1 µg respectively,for estriol a value of 1.1 µg is found.
The figure for estriol is based on the standard deviation for urines containing between 0 and 6 µg per 24 hrs.With low excretions s becomes very small,as can be seen in table 2.The excretion of estriol was lower than 3 µg per 24 hrs in only 6 urines,and the standard deviation for these urines was 0.3 µg.With this figure the smallest amount of estriol that can be distinguished from zero would be 0.4 µg.Since the number of determinations is so small,this figure can hardly be regarded as fully reliable.

Concluding it seems justified to say that the sensitivity of the method described is between 0.1 and 0.4 µg of each estrogen per 24 hrs urine.

Specificity : There is little doubt about the specificity of this method as the substances quantitated have been compared with standards during several chromatographic steps.Chromatographic comparison has been done for estrone and estradiol as the free steroid on TLC;as the acetate derivative on TLC and during GLC;for estriol by alumina-chromatography after methylation and during GLC.
During GLC both the substances from the urine and the standards yield good-sized peaks with identical retention times.
Additional proof during each estimation is identity of behaviour of the added radioactive steroid with the substances to be determined.

The results obtained remained the same when the stationary phase for GLC was changed from XE-60 into SE-30.When,after TLC of the acetate derivative of estrone and estradiol,several parts of the steroid spot were eluted separately,in all instances the same specific activity was found in them.Collection of the eluted estriol-3-methylether from the alumina column in several fractions,which were estimated separately,gave the same specific activity in all fractions.

For estriol determinations two more procedures were employed for ascertaining the specificity of the method.First the introduction of a further step of purification by TLC in the system cyclohexane:ethylacetate:ethanol 45:45:10;secondly the preparation of another derivative of estriol (the 3-methylether-16,17-diacetate) (8) followed by purification with TLC

in the system petroleumether:methanol 9 : 1.Neither of these procedures changed the results.

A comparison was made between the gaschromatographic method described here and the method of Brown (corrected for losses by the use of radioactive estrogens).It appeared that at lower concentrations of urinary estrogens there were always different results,the gaschromatographic method giving lower values.As at low levels the specificity of Brown's method is dubious,further comparison has been made between the gaschromatographic method and the more sensitive fluorometric method described by Ittrich (10).This was done by dividing the final extract,prepared for GLC as described above,into several parts and subjecting one part to GLC and another part to fluorometry.The amounts of each estrogen in the extracts as measured with both methods are shown in figure 10.

From this figure it can be seen that there exists a good correlation between the results with both methods for estrone and estriol (r = 0.94 and 0.90 resp.),but not for estradiol (r = 0.34).This finding can be explained by the fact that in the series of the compared urinary extracts estradiol values were very low.Especially at extremely low levels the correlation appears to be bad,fluorometry giving higher values,probably because of the presence of estrogenic substances not identical with the steroids studied or because of non-specific fluorescence.In view of the facts that higher readings were only obtained at very low levels and that the tetrabromoethane extracts (as used for fluorescence) of urinary samples were always more coloured than extracts of standard estrogens,we believe that non-specific fluorescence causes the discrepancy at very low levels between the two methods,the gaschromatographic method giving the more reliable figures.

EXCRETION OF ESTROGENS IN NORMAL POSTMENOPAUSAL WOMEN

The urinary excretion of the three estrogens was studied with the method described (figure 9) in 35 normal,healthy,non-hospitalized postmenopausal women,all between 55 and 75 years old.

The results obtained for the three estrogens are shown in figure 11, and are summarized in table 3.

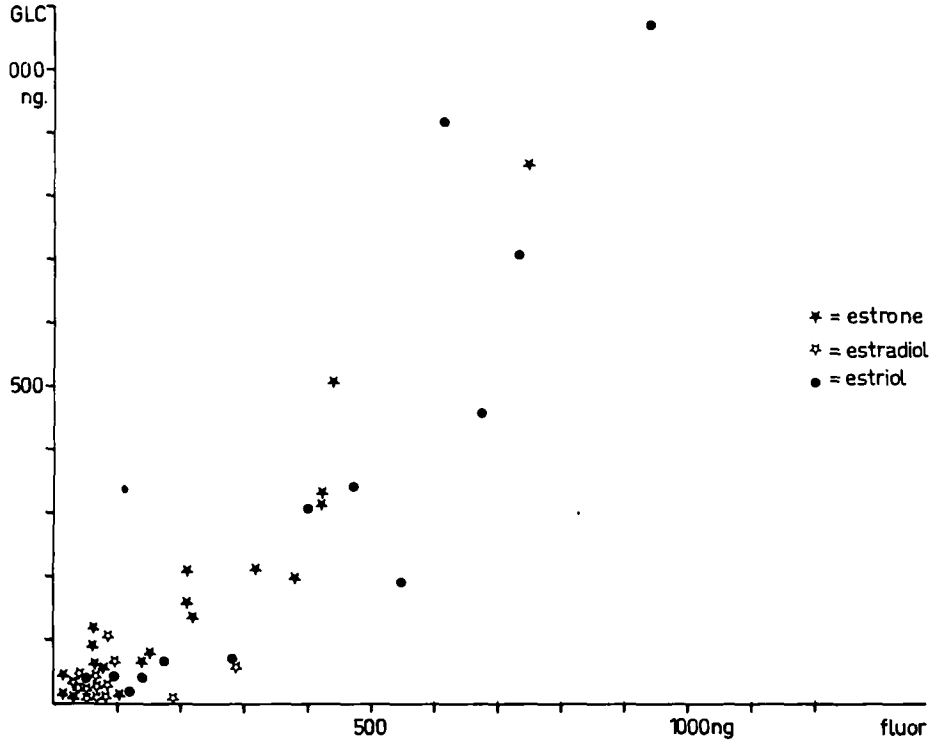

Figure 10 : Correlation between the amount of estrone, estradiol and estriol in urinary extracts, measured with the gaschromatographic method described in this paper and the fluorometric method of Ittrich (10).

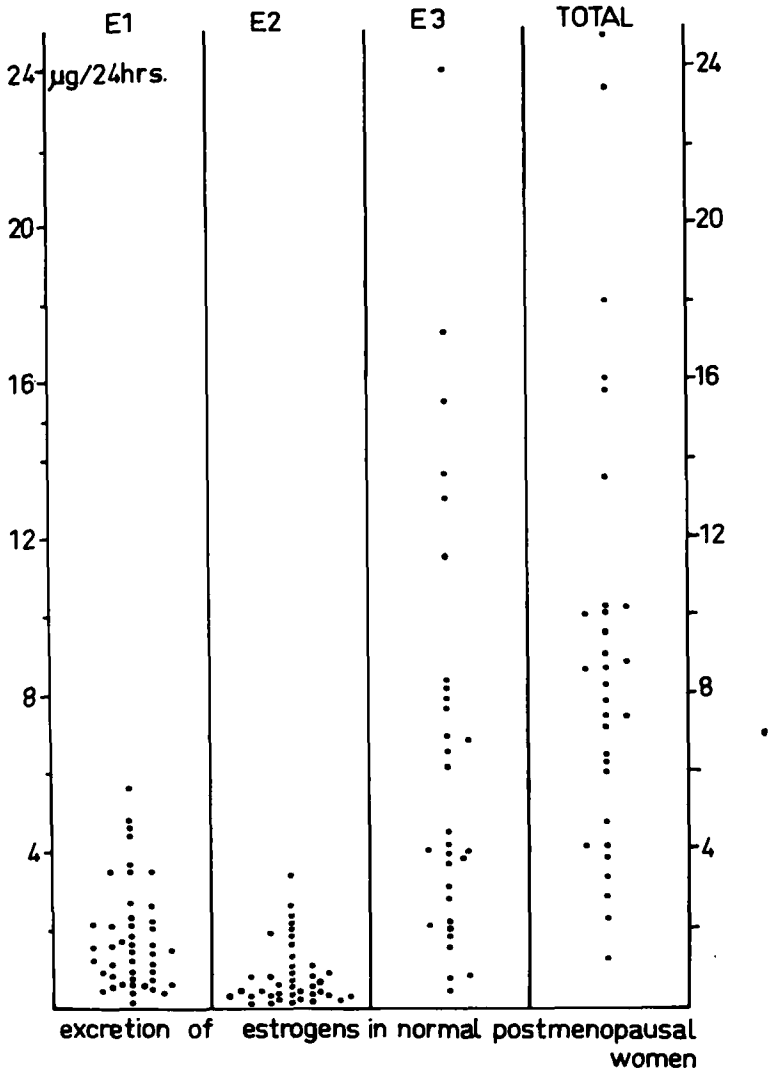

Figure 11

steroid	mean excretion per 24 hrs (µg)	range (µg)
estrone	1.8	0.1 - 5.6
estradiol	0.9	<0.1 - 4.8
estriol	6.6	0.4 - 25.9

TABLE 3

ACKNOWLEDGEMENTS

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14. The following trivial names and abbreviations will be used:

estrone	:3-hydroxyestra-1,3,5(10)-triene-17-one
estradiol	:3,17β-dihydroxyestra-1,3,5(10)-triene
estriol	:3,16α,17β-trihydroxyestra-1,3,5(10)-triene
estrone-acetate = E_1-Ac	:3-acetoxyestra-1,3,5(10)-triene-17-one
estradiol-diacetate = E_2-Ac	:3,17β-diacetoxyestra-1,3,5(10)-triene
estriol-triacetate = E_3-Ac	:3,16α,17β-triacetoxyestra-1,3,5(10)-triene
estrone-3-Methylether	:3-methoxy-estra-1,3,5(10)-triene-17-one
estradiol-3-Methylether	:3-methoxy-estra-1,3,5(10)-triene-17β-ol
estriol-3-Methylether	:3-methoxy-estra-1,3,5(10)-triene-16α,17β-diol