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MASS FRAGMENTOGRAPHIC DETERMINATION OF ELEVEN ESTROGENS IN THE BODY FLUIDS OF PREGNANT AND NONPREGNANT SUBJECTS

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SUMMARY

The potentialities of a new mass fragmentographic method for the quantitative determination of 11 estrogens have been explored. These estrogens (estriol, estrone, 2-methoxyestrone, estradiol-17β, estradiol-17α, 16-epiestriol, 17-epiestriol, 16α-hydroxyestrone, 16β-hydroxyestrone, 16-oxoestradiol-17β and 15α-hydroxyestrone) were quantitatively determined in bile from male and female (post-menopausal) subjects and in the urine of a non-pregnant women. It was also possible to measure these estrogens in the unconjugated and conjugated fractions of a 20-ml pool of pregnancy plasma. It was observed that mass fragmentographic determination of estrogens is superior to conventional gas chromatographic techniques, especially with regard to sensitivity and specificity.

INTRODUCTION

The use of a mass spectrometer as a highly sensitive detector in gas chromatography (G.C.) was first demonstrated by Henneberg[1] and single or multiple ion monitoring, known as mass fragmentography (MF) [2], is becoming increasingly important as a technique of quantitative analysis in biochemical medicine[3, 4, 5].

The aim of the present study was to explore the usefulness of MF in the analysis of a number of estrogen metabolites in pregnant and nonpregnant subjects. MF was applied to the analysis of some selected samples of various body fluids containing concentrations of estrogens which are difficult to measure by conventional G.C. techniques. The fractionation procedure previously developed for estrogen determinations[6, 7] was followed but the mass spectrometer was used in place of the hydrogen flame ionization detector thus allowing MF quantitation of eleven estrogens in plasma, bile and urine. In addition, preliminary experiments suggested that considerable shortening of the purification procedure is possible without decreasing the reliability of the mass fragmentographic method.

EXPERIMENTAL

Material

Estrogens were determined in 50 ml urine samples from a woman on the 14th, 17th and 20th day of the menstrual cycle, in 100–150 ml samples of bile from a 53-year-old man and three postmenopausal women as well as in a 20 ml pool of late pregnancy plasma obtained from 10 women.

Methods

Estrogen fractionation. The estrogens were fractionated according to the method of Adlercreutz and Luukkainen[6, 7] with the following modifications: (a) No sodium chloride was added at any stage of the procedure; (b) The acid alumina (Merck AG, Darmstadt, Germany) used for chromatography was pre-washed with ethyl acetate (Merck) and reactivated[8]; (c) Acetonides were formed according to the method of McCloskey and McClelland[9]. A detailed description of the method will be published in a forthcoming methodological monograph[10]. With this method the following five estrogen fractions are obtained: I: estriol fraction (estriol)*, II: nonpolar ketonic fraction

* The following trivial names and abbreviations were used in this text: *Estrogens*: estriol (E_3) = 1,3,5(10)-estratriene-3,16α,17β-triol; estrone (E_1) = 3-hydroxy-1,3,5(10)-estratrien-17-one; 2-methoxy-estrone ($2MeE_1$) = 2-methoxy,3-hydroxy-1,3,5(10)-estratrien-17-one; estradiol-17β (E_2-17β) = 1,3,5(10)-estratriene-3,17β-diol; estradiol-17α (E_2-17α) = 1,3,5(10)-estratriene-3,17α-diol; 16-epiestriol (16epiE_3) = 1,3,5(10)-estratriene-3,16β,17β-triol; 17-epiestriol (17epiE_3) = 1,3,5(10)-estratriene-3,16α,17α-triol; 16α-hydroxyestrone (16αOHE_1) = 3,16α-dihydroxy-1,3,5(10)-estratrien-17-one; 16β-hydroxyestrone (16βOHE_1) = 3,16β-dihydroxy-1,3,5(10)-estratrien-17-one; 16-oxoestradiol-17β (16oxoE_2) = 3,17β-dihydroxy-1,3,5(10)-estratrien-16-one; 15α-hydroxyestrone (15αOHE_1) = 3,15α-dihydroxy-1,3,5(10)-estratrien-17-one; 11-dehydroestradiol-17α = 1,3,5(10) 11-estratetraene-3,17α-diol. *Abbreviations*: MF = mass fragmentography; g.c. = gas chromatography; MS = mass spectrometry; TMSi = trimethylsilyl ether.

(estrone and 2-methoxyestrone), III: estradiol fraction (estradiol-17α and estradiol-17β as methyl ethers), IV: epiestriol fraction (16-epiestriol and 17-epiestriol as acetonides), V: polar ketonic fraction (16α-hydroxyestrone, 16β-hydroxyestrone, 16-oxoestradiol-17β and 15α-hydroxyestrone). The reliability of the fractionation method has already been extensively investigated[6, 7, 10].

In some experiments with pregnancy urine samples a shortened version of the fractionation procedure was used. In the shortened procedure the following steps were omitted: several solvent partitions, all chromatographies on alumina and paper, as well as methylation of the estradiols. Estriol and both estradiols were included in the same fraction (I + III). The shortened procedure is presented in Flow diagram 1.

Mass fragmentography. The samples for MF determination were prepared as follows: Each fraction obtained from the fractionation procedure was evaporated to dryness in a stream of nitrogen. The dry residue was then silylated in pyridine (Merck) with hexamethyl-disilazane (Fluka AG, Buchs, Switzerland) and trimethylchlorosilane (Fluka), the ratio of the three solvents being 100:10:1, at room temp overnight. The reagents were evaporateed and the silylated steroid fraction extracted with n-hexane (Merck). A known amount of standard steroid (see below) was silyated simultaneously with hexamethyl-d_{18}-disilazane (Merck Sharp & Dohme of Canada Ltd., Montreal, Canada) and trimethyl-d_9-chlorosilane (Merck Sharp & Dohme). The n-hexane extracts of the silylated deuterostandards which serve as internal standards during MF were then combined with the n-hexane extracts of the corresponding samples in graduated microtubes.

For MF, a Varian MAT CH7 G.C.-MS instrument equipped with peak matching system was used. Two ions were monitored simultaneously and their intensities recorded on a two-pen recorder: in most instances one was the molecular ion of the steroid derivative being measured and the other was that of the corresponding deuterated internal standard derivative. The deuterated internal standards as well as the ions that were monitored are listed, together with other conditions for MF, in Table 1. The molecular ions were monitored except in the case of the nonpolar ketonic fraction where the M-30 fragment (m/e 342) of the TMSi derivative of 2 methoxyestrone was chosen in order to allow simultaneous measurement of the TMSi derivative of estrone (molecular ion, *m/e* 342).

Since non-derivatized deuterated steroids were not available the results were corrected for losses incurred during fractionation by carrying out a parallel duplicate recovery experiment with known amounts of all estrogens (with the exception of two: 16β-hydroxyestrone and estradiol-17α) added to the plasma, bile or urine samples. The recovery of 16α-hydroxyestrone was used for correcting the 16β-hydroxyestrone value, and that of estradiol-17β was used for correcting the estradiol-17α value.

For calibration purposes five silylated estrogen standard mixtures corresponding to the five fractions were prepared and the deuterated internal standard derivative of one estrogen (see line 1, Table 1) added. These standard mixtures were repeatedly injected between the unknown samples (minimum four times). The amounts were chosen so that the concentration of the standard estrogens was similar to that of the unknown samples. It was not necessary to use estradiol-17α or 16β-hydroxyestrone standards because the fragmentation pattern of their epimers is exactly the same with the electron energy used for ionization in MF.

The MF determinations after the shortened procedure were carried out in the same way as after the original fractionation procedure (see Flow diagram 1). The conditions used are described in Table 1.

The results were calculated according to the formula:

$$X = K \times dIS \times \frac{A_X}{A_{dIS}} \times \frac{100}{a} \times \frac{1000}{b}\ \mu g/l.$$

where

$$K = \frac{\sum\left(\frac{S \times A_{dS}}{dS \times A_S}\right)_n}{n}$$

Symbols:

X = μg/l. of estrogen in biological fluid (= unknown)

dIS = μg (prior to derivatization) of deuterated derivative of internal standard added to the sample before MF (see Table 1)

A_X = peak area of unknown

A_{dIS} = peak area of deuterated derivative of internal standard

S = μg of calibrating standard

dS = μg (prior to derivatization) of deuterated derivative of internal standard used for calibration (see Table 1)

A_S = peak area of calibrating standard

A_{dS} = peak area of deuterated derivative of internal standard used for calibration

a = calculated percentage recovery

b = volume (ml) of original sample of biological fluid

n = number of injections of calibrating standard mixtures ($\geqq 4$).

When urine samples were analyzed the values were converted from μg/l to μg/24 h urine vol.

Table 1. Conditions for mass fragmentography of estrogens

Fraction*	I	II	III	IV	V	I + III	
Estrogen† used as internal standard in sample or for calibration	E_3	E_1	E_2-17β	16epiE_3	16αOHE_1	E_2-17β	E_3
m/e of ion of measured estrogen (= unknown)	504	342	352	400	430	416	416 or 386
m/e of ion of deuterated estrogen derivatives (= internal standard)	531	351	361	409	448	434	434 or 531
Electron energy (eV)	70	70	70	70	70	70	70
G.C. column	1% QF-1	1% QF-1	1% SE-30	1% SE-30	1% QF-1	1% SE-30	
Flash heater temp (°C)	260	235	260	260	235	260	
Column temp (°C)	250	225	250	250	225	250	
Separator temp (°C)	248	248	248	248	248	248	
Ion source heater (μA)	300	300	300	300	300	300	

* Definition of fractions: see *Estrogen fractionation* (Methods).

† Abbreviations for estrogens: see Footnote to *Estrogen fractionation* (Methods).

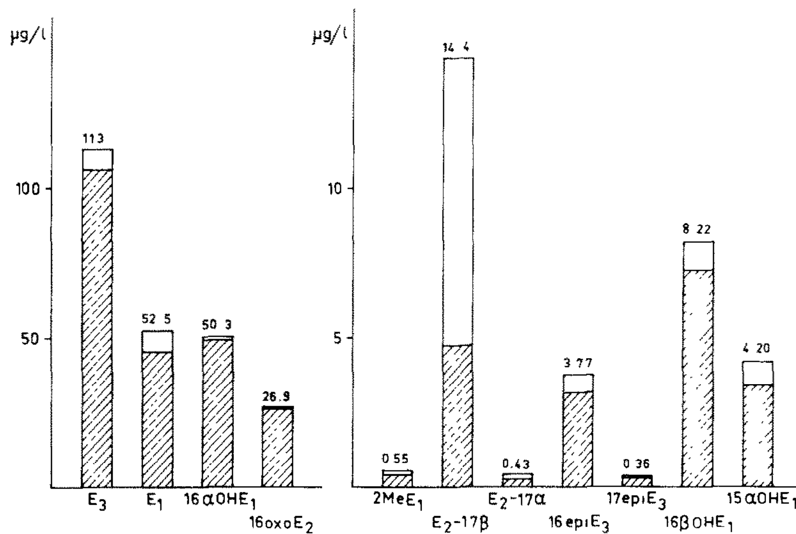


Fig. 1. Concentration of estrogens in a pool of pregnancy plasma: in the conjugated fraction (hatched area) and unconjugated fraction (blank area). For explanation of abbreviations, see Methods: Footnote to *estrogen fractionation*.

RESULTS

Determinations in human body fluids

Twelve estrogens can be determined using the original G.C. method. The twelfth estrogen, 11-dehydroestradiol-17α, could not be included in the present study due to the lack of a synthetic reference standard. Since its fragmentation pattern differs from that of both estradiols[11] the latter cannot be used for calibration as in the G.C. method.

The results of the estrogen determinations in the pooled sample of pregnancy plasma are shown in Fig. 1. Comparison of the unconjugated and conjugated fraction shows that estradiol-17β is the only estrogen present in plasma predominantly in the unconjugated form. Other estrogens present in high concentrations in the unconjugated fraction in addition to estradiol-17β (9·6 μg/l.) were estriol (7·1 μg/l.) and estrone (7·0 μg/l.).

The results of the estrogen determinations in the bile of three post-menopausal women are presented in Table 2 and those obtained in a sample of male bile are shown in Fig. 2. Although the estrogen levels in female bile are higher than in male bile the patterns are rather similar. In these few samples the quantitatively most important metabolite is 16α-hydroxyestrone (55% of total biliary estrogens estimated in females and 45% in the male).

The results of estrogen determinations in urine on 3 days during a normal menstrual cycle are presented in Table 3. Characteristic mass fragmentograms of the polar ketonic fractions obtained from bile and urine are shown in Fig. 3.

Table 2. Results of mass fragmentographic determination of biliary estrogens in three postmenopausal women (μg/l.)

Subject no.	1	2	3	Mean
Estriol	1·79	1·63	1·86	1·76
Estrone	0·39	0·60	1·53	0·84
2-methoxyestrone	0·04	0·27	0·87	0·39
Estradiol-17β	0·18	0·11	0·87	0·39
Estradiol-17α	0·02	0·02	0·07	0·04
16-epiestriol	0·53	0·50	1·22	0·75
17-epiestriol	0·07	0·04	0·06	0·06
16α-hydroxyestrone	8·27	3·14	16·3	9·24
16β-hydroxyestrone	1·58	0·51	3·34	1·81
16-oxoestradiol-17β	1·24	0·95	1·43	1·21
15α-hydroxyestrone	0·45	0·28	0·97	0·57

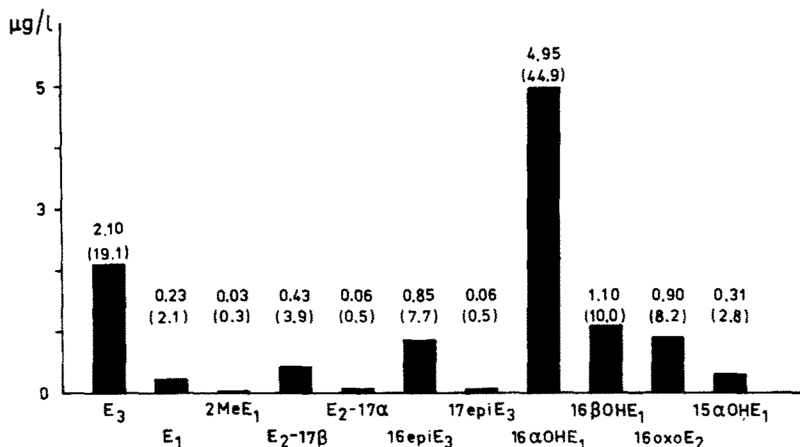
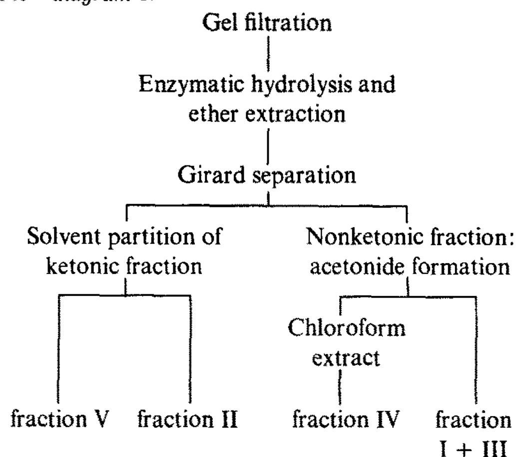


Fig. 2. Concentration of estrogens in the bile of a 53-year-old man. Numbers in brackets indicate percentage of total biliary estrogens. For explanation of abbreviations, see Methods: Footnote to *estrogen fractionation.*

Studies employing the shortened estrogen fractionation procedure

The shortened estrogen fractionation procedure is outlined below:

Flow diagram 1.



When the shortened procedure was applied to the analysis of pregnancy urine samples the mass fragmentograms obtained indicated that the estrogens in fraction II and fraction I + III could be quantitated without difficulty. A mass fragmentogram of fraction I + III is shown in Fig. 4. Since estradiol-17β occurs in relatively small amounts its signal must be amplified more than that of estriol. Attempts to determine estradiol-17α were not made. In the experiment shown in Fig. 4 no internal standard was used; *m/e* 416 (molecular ion for estradiol-17β TMSi derivative) and *m/e* 386 (M-118 for estriol TMSi derivative) were monitored separately. A mass fragmentogram of the polar ketonic fraction *V* is shown in Fig. 5. 16α-hydroxyestrone, 16-oxoestradiol-17β and 15α-hydroxyestrone can be readily quantitated. But in Fig. 5 an additional peak is seen between the 16α-hydroxyestrone and 16β-hydroxyestrone peaks which makes the quantitation of 16β-

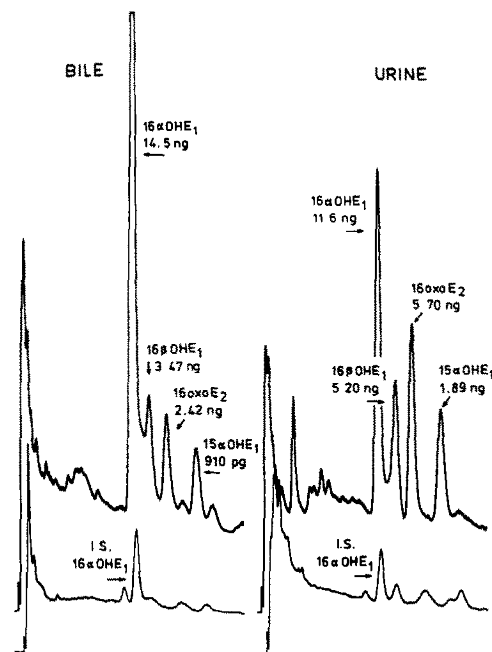


Fig. 3. Mass fragmentograms of the polar ketonic estrogen fractions obtained from bile (left) and urine (right). Abbreviations, upper traces: 16αOHE$_1$ = TMSi (trimethylsilyl ether) derivative of 16α-hydroxyestrone; 16βOHE$_1$ = TMSi derivative of 16β-hydroxyestrone; 16oxoE$_2$ = TMSi derivative of 16-oxoestradiol-17β; 15αOHE$_1$ = TMSi derivative of 15α-hydroxyestrone; lower traces: I.S. = deuterated TMSi derivative of 16α-hydroxyestrone internal standard.

Table 3. Results of mass fragmentographic determination of urinary estrogens in a normal woman (μg of estrogen/24 h)

Day of menstrual cycle	14	17	20	Mean
Estriol	4·12	4·07	3·45	3·88
Estrone	8·69	3·44	5·34	5·82
2-methoxyestrone	2·92	1·87	1·99	2·26
Estradiol-17β	3·40	1·26	1·80	2·15
Estradiol-17α	0·09	0·07	0·07	0·08
16-epiestriol	1·03	0·99	1·42	1·15
17-epiestriol	0·21	0·21	0·34	0·25
16α-hydroxyestrone	2·32	3·25	0·95	2·17
16β-hydroxyestrone	2·74	1·45	0·43	1·54
16-oxoestradiol-17β	1·55	1·96	1·11	1·54
15α-hydroxyestrone	0·20	0·29	0·29	0·26

hydroxyestrone unsatisfactory. This peak was also seen occasionally in samples processed through the original fractionation procedure, which suggests that its presence was not related to the shortening of the procedure. The quantitation of 16-epiestriol in fraction IV was not possible due to interfering peaks. Thus the shortened fractionation procedure seems to allow the quantitation of at least eight estrogens without any modifications. Slight modifications are needed to allow 16-epiestrol and possibly 16β-hydroxyestrone and estradiol-17α measurement.

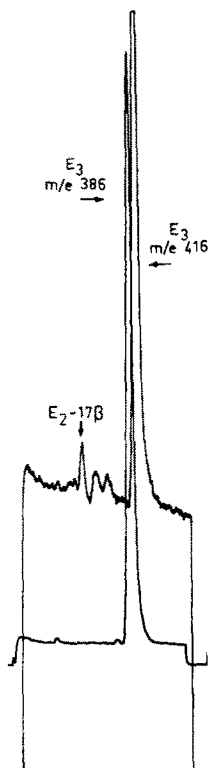


Fig. 4. Mass fragmentogram of the combined estriol–estradiol fraction from pregnancy urine obtained by the shortened fractionation procedure. Abbreviations: E_2-17β = TMSi derivative of estradiol-17β; E_3 = TMSi derivative of estriol. The ion m/e 416 is the molecular ion of the TMSi derivative of E_2-17β and the (M-90) + 2 ion of the TMSi derivative of E_3.

DISCUSSION

In the present study the potentialities of MF were investigated by applying a MF method to the quantitative analysis of estrogens in some selected samples of biological fluid where conventional G.C. methods prove unsatisfactory.

The results obtained for the pregnancy plasma sample (Fig. 1) agree for the most part with those obtained previously using the original G.C. method (11).

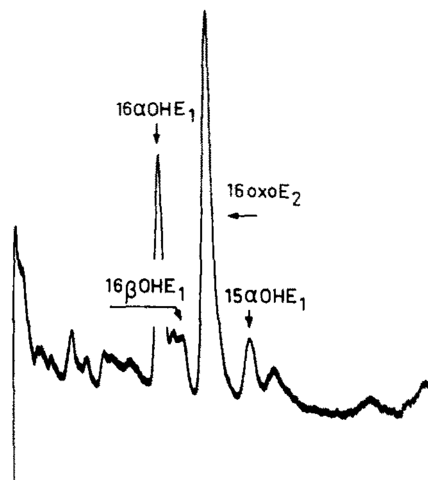


Fig. 5. Mass fragmentogram of the polar ketonic estrogen fraction from pregnancy urine obtained by the shortened fractionation procedure. Abbreviations: see legend to Fig. 3.

except that four new estrogen metabolites (estradiol-17α, 16-β-hydroxyestrone, 17-epiestriol and 15α-hydroxyestrone) could now be determined in the unconjugated fraction and one (17-epiestriol) in the conjugated fraction. Nonclassical estrogens have been determined in bile in pregnancy[12] and after administration of milligram amounts of precursor[6] but otherwise their quantitation in bile in the nonpregnant human has not been possible. This has now been accomplished by MF in bile from both a male (Fig. 2) and postmenopausal female subjects (Table 2). Except for 16-epiestriol[13], the separate quantitative determination of the nonclassical estrogens in urine during the normal menstrual cycle has not been reported. The present results show (Table 3) that all 11 estrogens could be quantitated using the MF method. It is evident from the foregoing that a 100 times greater sensitivity is easily achieved by the MF method when compared to conventional g.c. techniques. Moreover, the sensitivity can be readily enhanced by optimizing conditions[14], including choice of optimal ionization energy, columns (with minimal bleeding), and ions to be monitored. A low temperature in the ion source can further increase the sensitivity[15].

The specificity of the original fractionation procedure has been extensively studied with regard to various biological fluids ([6, 7, 10], for additional references see[12]), and the g.c. peaks were shown by conventional G.C.–MS to be free from significant interfering substances. In a recent study (Aldercreutz and Hunneman, to be published) the five estrogen fractions in a pregnancy urine sample were investigated by automatic repetitive MS scanning of the G.C. effluents with a Varian CH7 mass spectrometer combined with the data system Spectrosystem 100 MS. The preliminary evaluation of several hundred computer-plotted mass fragmentograms revealed (in addition to some new estrogens) only insignificant amounts of steroids interfering with the G.C. peaks. But a nonsteroidal impurity was found in the G.C. peak of 16-epiestriol. Since this impurity does not interfere with MF the latter proves more specific in this case. The specificity of the MF method as compared to G.C. is considerably better since a characteristic ion is monitored instead of the total ion current measured by flame ionization detection and the maximum response should occur exactly at the retention time of the G.C. peak. Moreover, preliminary experiments in the present study seemed to indicate that the coupled separation power of the gas chromatograph and the mass spectrometer allows reliable measurements even in the presence of larger amounts of impurities; the results suggested that at least eight out of 11 estrogens can be assayed without difficulty after the very much shortened fractionation procedure.

In summary, MF will be an important tool in quantitative estrogen studies in the future. Its advantages lie not only in the extremely increased sensitivity but also in the increased speed of measurement obtained from more limited sample purification.

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