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MASS SPECTROMETRIC AND MASS FRAGMENTOGRAPHIC DETERMINATION OF NATURAL AND SYNTHETIC STEROIDS IN BIOLOGICAL FLUIDS

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SUMMARY

One of the most significant advances in steroid methodology in the last decade has been the development of gas chromatograph–mass spectrometric (GC–MS) procedures. In this report a series of methods for steroid determination and detection, in which an LKB 9000 gas chromatograph–mass spectrometer linked to a Hewlett–Packard minicomputer (HP 2100A) or a Varian MAT CH7 with the data system Spectrosystem 100 MS has been used, are described. A mass fragmentographic (MF) method for the determination of 11 estrogens in various biological fluids is outlined. The levels of these steroids in late pregnancy plasma pools and in male and female bile are discussed. A method for the determination of unconjugated estriol in pregnancy plasma, for use in the monitoring of fetal well-being, and a second, for measuring unconjugated and conjugated estriol in nonpregnancy plasma are described. The latter method has been used in a pharmacological study of the luteolytic effects of administered estriol in humans. After the administration of single oral doses of megestrol acetate (MA) plasma levels were monitored by both m.f. and radioimmunoassay (RIA). The better specificity of the m.f. method unmasks interference by other compounds, probably metabolites, with the RIA. Studies on the mass spectrometrical characteristics of human urinary MA metabolites are also described.

The use of m.f. facilitated the measurement of conjugated 17-ketosteroids in small portal venous plasma pools from postmenopausal women. Their concentrations are compared to those in a peripheral pool from the same subjects.

INTRODUCTION

Recent methodological advances have resulted in the development of four ultra-sensitive techniques for quantitative steroid determination: gas chromatography (GC) with electron capture detection (sensitivity: 10^{-12} M), radioimmunological techniques (sensitivity: 10^{-11}–10^{-13} M), enzymatic techniques (sensitivity: 10^{-14} M) [1] and mass fragmentography (MF) (sensitivity: 10^{-12}–10^{-14} M).

Mass fragmentography, however, offers a number of advantages over the other techniques mentioned. In addition to providing highly specific and sensitive detection the mass spectrometer is linked to a very efficient chromatographic system. After preparatory purification steps at least one additional chromatographic step would be required to reach comparable specificity using any of the other methods. In general, preliminary purification is necessary before analysis, but usually it need not be as extensive as for equivalent gas chromatographic methods [2]. The gas chromatograph–mass spectrometer has also wide applicability in the area of compound detection and identification when used either in the "gas chromatography + mass spectrum" mode or "gas chromatography + single or multiple ion detection" mode.

In this report we would like to outline the results of recent experiments carried out using both the LKB 9000 and Varian MAT CH7 instruments on the measurement of estrogens in pregnancy and nonpregnancy body fluids, megestrol acetate in plasma and urine and neutral steroid conjugates in peripheral and portal vein blood.

EXPERIMENTAL

Instrumentation

Most experiments, which will be presented, have been carried out using an LKB 9000 gas chromatograph–mass spectrometer (GC–MS). However, significant amounts of data have also been obtained using a Varian MAT CH7 through the courtesy of the Varian MAT company in Bremen, who permitted us to use the instrument for a week on several occasions.

Our *LKB 9000 instrument* belongs to the first series and was installed in this laboratory in 1966. In 1970 the mass marker and the accelerating voltage alternator (AVA) system were added, and because the U.V.-recorder could not be used in combination with the AVA system, the paper speed was too high, the signals were taken to an ordinary single-pen recorder. However, in order to allow different degrees of background suppression (zeroing) and also different amplification settings for signals differing greatly in intensity, two of the three signals obtainable with the AVA

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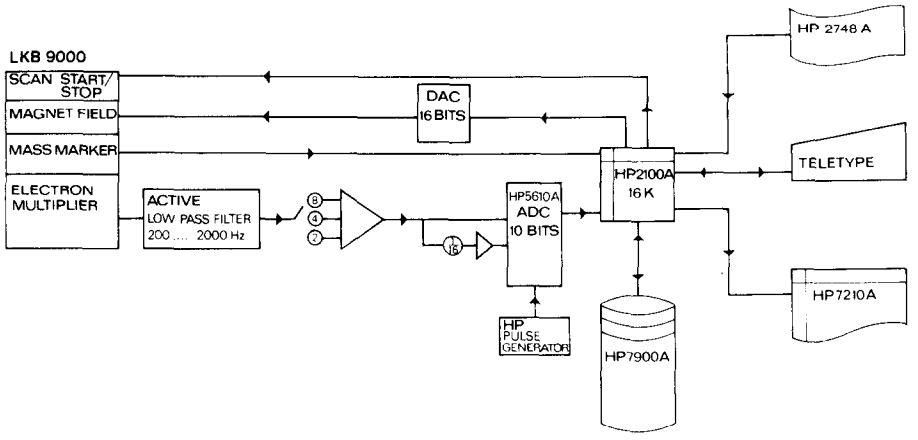

Fig. 1. Block diagram of the computer system and interface to the LKB 9000 GC–MS used in the Department of Clinical Chemistry, University of Helsinki.

system were fed to separate recorders. This was performed in a simple way by means of a Reed relay, which, synchronously with the change in accelerating voltage caused by the AVA, switched the multiplier signal alternatively to one of two active low pass filters (cut-off frequency about 0·3 Hz). Each of the filters was connected to the input of a multirange recorder with an adjustable zero. The coil of the Reed relay (12 V) was connected in parallel with the coil (L 1) of one of the relays switching the high voltage divider in the AVA unit. A DC coupled operational amplifier with an amplification factor of 25 was connected between the Reed relay and the multiplier-preamplifier for the purpose of reducing the effects of contact potentials and resistances of the relay upon the measurement.

Computer and interface to LKB 9000. A block diagram of the system is presented in Fig. 1. The LKB 9000 gas chromatograph–mass spectrometer is connected on-line with a data processing system, that consists of HP 2100A minicomputer, HP 7900A disc drive, HP 2748A punched tape reader, HP 7210A digital plotter, and teletype printer. HP 2100A has a 16 k core memory of 16 bit words and a memory cycle of 980 ns. The HP 7900A disc unit has the storage capacity of 5 M words of 16 bits per word.

Two kinds of information come from the mass spectrometer to the computer. The mass marker provides the mass numbers as binary coded decimal numbers, in that way they can be sent directly to the computer. The mass abundance from the electron multiplier comes, instead, as an analog signal and it is converted to the digital form by passing the signal from the electron multiplier first through an active low pass filter, which filters high frequency noise out and then, to one of the three channels with different sensitivity. The user chooses this channel externally. Finally the mass abundance signal is split into two channels, with a relative gain of 16:1. This is necessary because the ten-bit analog to digital converter only has a dynamic range of 1000:1. By using the two channels, the range is extended to 16,000:1. Signals from these two channels are then converted to digital form in the HP 5610A analog to digital converter. The sampling rate can be externally controlled by the HP pulse generator. The maximum sampling rate is 100 kHz.

The computer can control the mass spectrometer in two ways. It can start a scan by sending a pulse to the scan start circuit. It can also regulate the magnet field directly through a 16 bit digital to analog converter.

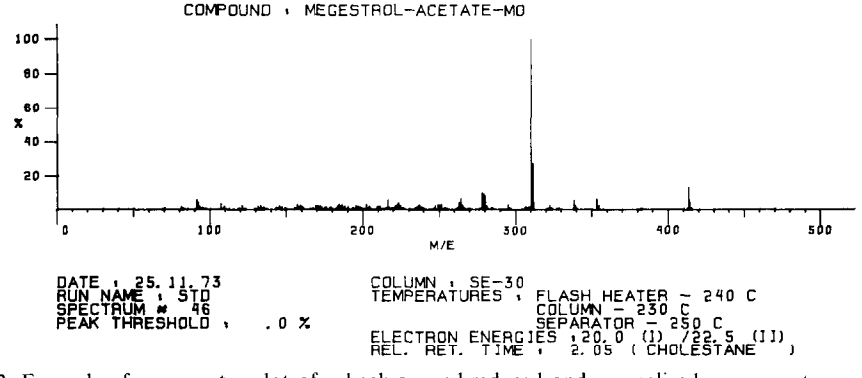

Fig. 2. Example of a computer plot of a back-ground-reduced and normalized mass spectrum of the monomethoxime derivative of megestrol acetate (reference standard). LKB 9000 GC-MS coupled on-line to a HP 2100A minicomputer.

Programs for the computer connected to the LKB 9000. At this stage programs are ready to read, normalize, print, plot, and store mass spectra. The program MASP transmits spectra from the mass spectrometer to the data system. It reads mass numbers from the mass marker and follows the mass abundance signal from the A/D-converter. Program recognizes the peaks in the spectrum, calculates their intensity and the corresponding mass numbers. All this happens during the scan. Finally, a reduced spectrum is transmitted for storage in the disc memory, where space has been reserved for 256 mass spectra in the primary file. Program MASP also reads and stores all environmental parameters like date, run temperatures, retention times etc.

With the program MASH you can handle mass spectra stored in the primary file. Normally it is used to print and/or plot mass spectra. Program automatically subtracts the background and then normalizes the intensities. It uses a special routine to convert the mass numbers to the right nominal masses. Spectra can be presented in a tabular form using the teletype or plotted as bar graphs using the plotter. Either absolute or relative intensities can be used.

The system has also a library of mass spectra with storage space for 512 spectra. Spectra can be transmitted for storage in the library both from the primary file and from external sources such as the literature. In the latter case teletype input is used. A special plot is also available with the library as its source. Using this a plot is obtained containing the mass spectrum and all the information concerning the circumstances under which it has been taken (example in Fig. 2). The mass spectra in the library and in the primary store are protected.

The *Varian MAT CH7 instrument* was equipped with a peak-matching device capable of simultaneously monitoring two ions, whose intensity could be recorded and amplified independently on a two-pen recorder. The instrument was coupled to the data system Spectrosystem 100 MS and the programs used were those commercially available with this system.

METHODS

Method for the determination of 11 *estrogens in various biological fluids*

The method is based on the original gas chromatographic procedure described by Adlercreutz and

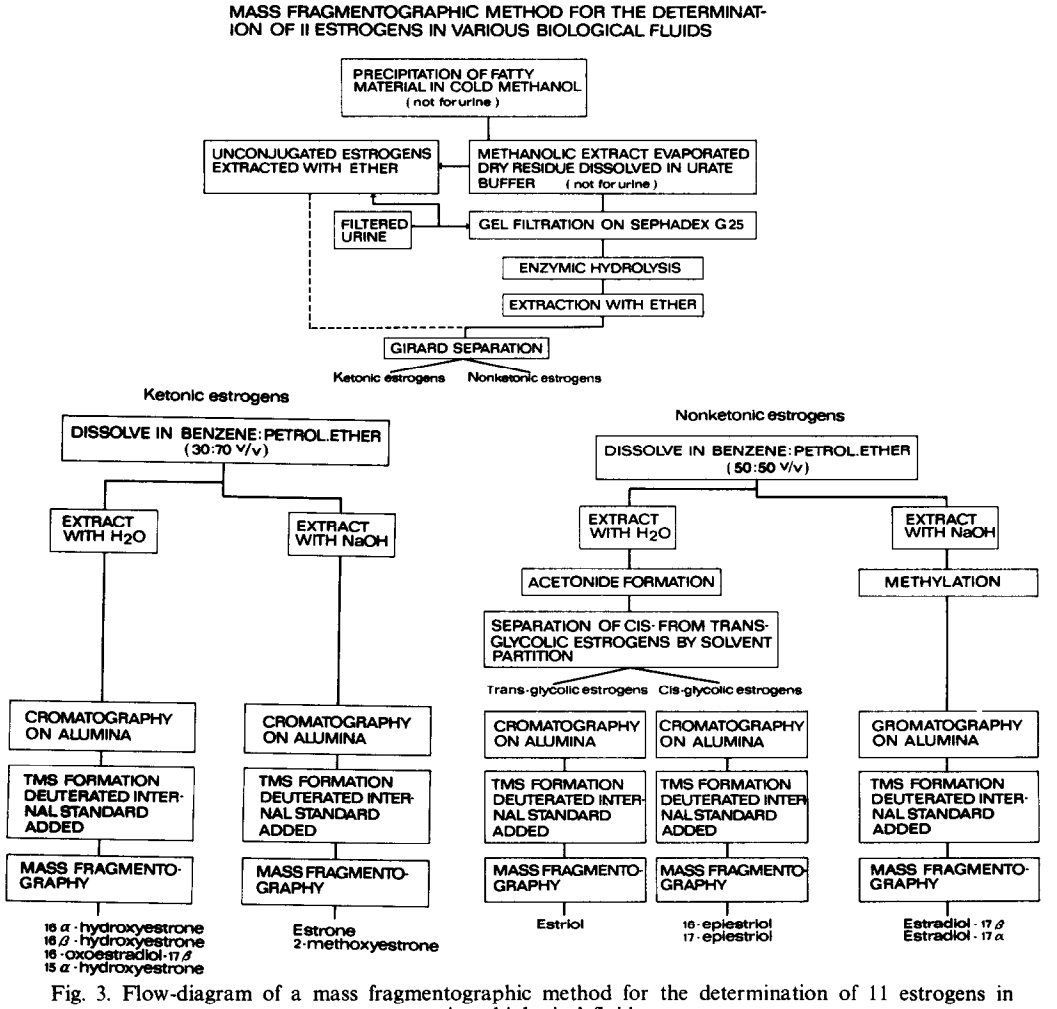

Fig. 3. Flow-diagram of a mass fragmentographic method for the determination of 11 estrogens in various biological fluids.

Luukkainen[3, 4] with some slight modifications. The complete GC method with all details will be presented in a methodological monograph [5] the publication of which has been delayed more than 3 years*. In the new procedure the quantitation is carried out by mass fragmentography instead of GC with hydrogen flame ionization detection [6]. In the first experiments no other modifications were made and it could be demonstrated that highly sensitive and specific measurements of 11 estrogens in plasma, bile and urine could be carried out [2, 6]. Some attempts were also made to simplify the very long procedure by excluding a number of chromatographic steps [2]. However, further experience with the shortened procedure showed that some estrogens could not be measured and that for some biological fluids the measurements did not have the required specificity. Finally the method shown in the flow-diagram in Fig. 3 was adopted. When compared to the original procedure [5] the new one does not contain the two paper chromatographic steps used for the purification of the labile ring D-ketolic estrogens. The MF determination was carried out as described previously using deuterated trimethylsilyl ether derivatives of standard estrogens as internal standards [2]. However, peak heights instead of peak areas were used for quantitation.

Because of the elimination of the paper chromatographic steps the recovery of unconjugated estrogens added to extracts of biological samples after hydrolysis increased as compared to the original GC procedure, for 16α-hydroxyestrone from 53 to 82%, for 16-oxoestradiol from 50 to 76% and for 15α-hydroxyestrone from 59 to 79%. Because the recoveries of the other estrogens remained the same as before it may be concluded that the *accuracy* of the method increased significantly in the new procedure because no mean recovery was lower than 73% (for 17-epiestriol) and all fell within the range 73–82%.

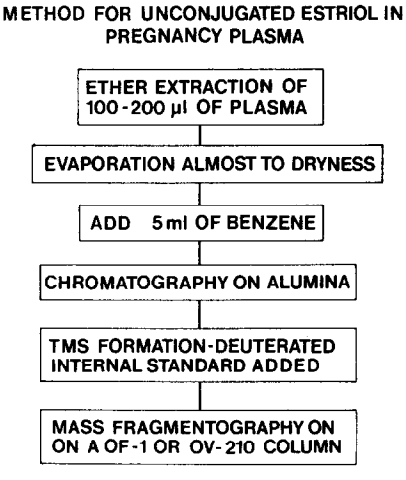

Fig. 4. Flow-diagram of a mass fragmentographic method for the determination of unconjugated estriol in pregnancy plasma.

* Complete procedure available upon request.

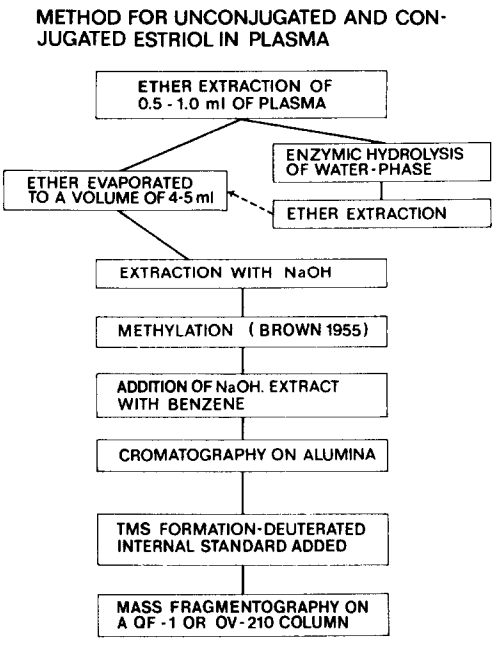

Fig. 5. Flow-diagram of a mass fragmentographic method for the determination of unconjugated and conjugated estriol in plasma.

The *sensitivity* of the method is good, in "routine" work levels of estrogens down to 20 pg/ml of biological fluid have been determined [2, 6] and if optimal conditions are employed amounts as low as a few picogram per injection may be analyzed [7].

The *specificity* of the original GC procedure has been extensively studied with regard to various biological fluids [2–5, see also references in 8]. When the MF technique was applied to pregnancy urine it was observed that the measurement of 16-epiestriol had probably not been absolutely specific in the original GC method because a non-steroidal impurity with no characteristic MF peaks interfered to some extent [2]. The MF measurements of this estrogen are not disturbed by this impurity. Generally the specificity of MF as compared to GC is better, because a characteristic ion is monitored instead of the total ion current measured by flame ionization detection.

Methods for the determination of estriol in plasma

One mass fragmentographic method for the assay of unconjugated estriol in pregnancy plasma and another for the measurement of unconjugated and conjugated estriol in plasma after estrogen administration have been developed [9]. The methods are presented in flow-diagrams in Figs. 4 and 5. The reliability of the procedures has been evaluated and the results have been presented previously [9].

Method for the determination of megestrol acetate in plasma

The procedure is presented in a flow-diagram shown in Fig. 6. The details of the procedure and the results of the evaluation of its reliability have been presented previously [10].

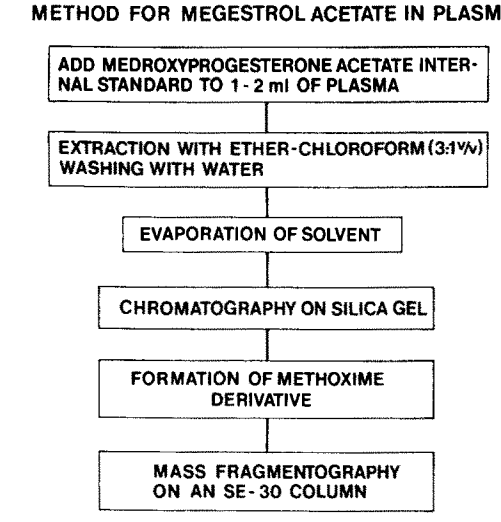

Fig. 6. Flow-diagram of a mass fragmentographic procedure for the determination of megestrol acetate in plasma.

Method for the isolation of megestrol acetate and its metabolites from urine

Megestrol acetate and its metabolites were isolated from a 10-day urine pool collected from a postmenopausal woman who had received 100 mg of the drug per day over the collection period. A 6 l sample from the pool was analyzed according to the flow-diagram presented in Fig. 7. Two litre portions were chromatographed on Amberlite XAD-2 according to the method of Bradlow[11]. The combined residues were chromatographed in two equal parts on 100 g Amberlyst 15 columns (15 mm dia.), the resin was prepared and elution based on the method of Viinikka and Jänne[12]. The material was then chromatographed on two 20 g Sephadex LH-20 columns [13] and unconjugated plus glucuronide, monosulphate and disulphate fractions collected [14]. The appropriate fractions were pooled and further purified by a second Sephadex LH-20 chromatography. The unconjugated plus glucuronide fraction was then fractionated between 8·4% (w/v) $NaHCO_3$ and ethyl acetate [14]. Thereafter the glucuronide fraction was hydrolyzed with ketodase and the mono- and disulphate fractions were solvolyzed as described by Jänne *et al.*[15]. All the fractions were then fractionated by silicic acid chromatography on 3 g columns (210 × 6 mm) [13]. The silicic acid fractions obtained from the unconjugated and glucuronide conjugated steroids were further purified by thin-layer chromatography (20 × 20 cm. silica gel $60F_{254}$ precoated plates, E. Merck, Darmstadt, Germany, solvent system: chloroform: acetone, 9:1 (v/v), 3 developments, running distance 12 cm.). After development each plate was divided into four 3 × 20 cm. areas, starting just behind the origin and each area scraped and the gel eluted with chloroform and methanol. O-Methoxime trimethyl silyl ethers of all fractions were now prepared and the fractions screened by GC–MS on a 1% SE-30 column, temperature 230–240°C.

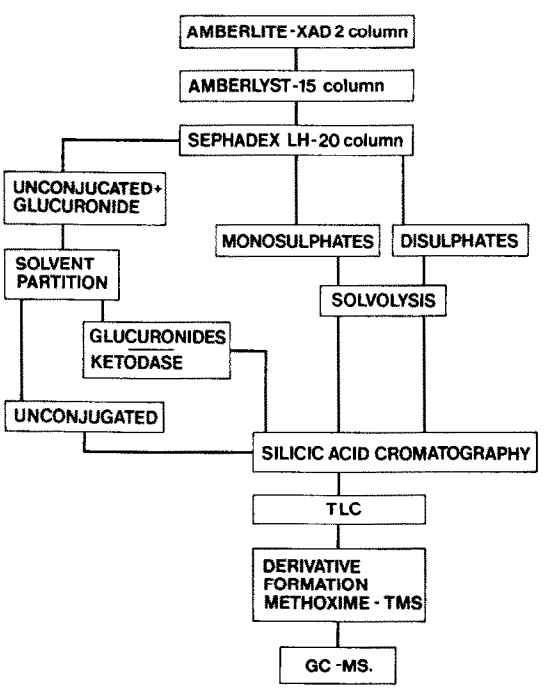

Fig. 7. Flow-diagram of the method used for the isolation and identification of megestrol acetate and its metabolites in urine.

Method for the determination of glucuronide and monosulphate conjugated 17-ketosteroids in plasma

Plasma samples 0·5 ml were diluted with 4·5 ml of 0·1 mol/l NaOH in physiological saline, 50 µl aliquots of [^{3}H]-testosterone glucuronide and [^{3}H]-testosterone sulphate (each containing ca. 50,000 c.p.m.) in methanol were added, and the samples sonicated for 15 min (see flow-diagram in Fig. 8). The samples were then applied to 3 cm. × 1 cm. Amberlite XAD-2 columns. The columns were washed with distilled water until the effluent was neutral and the steroids were then eluted in 5 ml of methanol [16]. The dried extract was chromatographed on 20 g Sephadex LH-20 columns in chloroform–methanol (1:1 v/v) containing 0·01 mol/l NaCl [13]. The columns were eluted with the same solvent and fractions between 40 and 140 ml and 140 and 240 ml collected. The fractions were evaporated to dryness and the first, containing the glucuronide conjugated steroids (the unconjugated steroids were also contained in this fraction, but because of their low concentration they were not removed by a solvent partition) was subjected to Ketodase hydrolysis. The second fraction containing the monosulphates was solvolysed [15]. In both cases the liberated steroids were then chromatographed on 200 mg silicic acid columns (0·6 × 45 mm). The steroids were applied to the column in 0·5, 0·5 and 1 ml 4% ethyl acetate in toluene. The column was then eluted with a further 2 ml of the same solvent (fraction 1), 4 ml of 10% ethyl acetate in toluene (fraction 2), in which the 17-ketosteroids were eluted and 4 ml of ethyl acetate (fraction 3), in

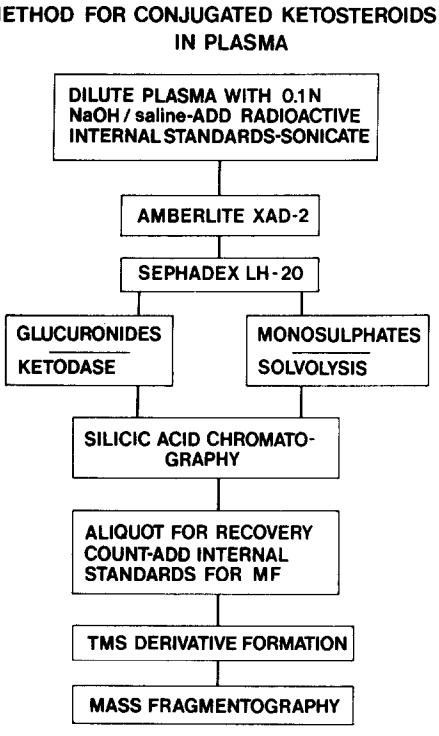

Fig. 8. Flow-diagram of a mass fragmentographic method for the determination of glucuronide and monosulphate conjugated 17-ketosteroids in plasma.

which the [^{3}H]-testosterone was eluted. An aliquot of fraction 3 was counted for recovery correction. Suitable amounts of 5α-dihydrotestosterone and testosterone were then added to fraction 2 as internal standards for mass fragmentography and the samples silylated. The samples were analyzed by mass fragmentography on a 3% OV-210 column by monitoring m/e 347 for androsterone, etiocholanolone, epiandrosterone and 5α-dihydrotestosterone (M-15) and m/e 360 for dehydroepiandrosterone and testosterone (molecular ion). The energy of the bombarding electrons was 22·5 eV.

The *recoveries* of the radioactive internal standards after the silicic acid chromatography step were 65·4 ± 8·8% (mean ± S.D.) for the monosulphates and 67·7 ± 4·5% (mean ± S.D.) for the glucuronides. The *sensitivity* of the method is of the order of 5–10 pg per injection for androsterone and epiandrosterone and 50–100 pg per injection for dehydroepiandrosterone. The *precision* based on the determination of these steroids in six 1 ml samples of plasma from a normal female plasma pool (mass fragmentography: mean of duplicate determinations used for calculation) ranged from 5·9 to 8·4% for the monosulphates and was 11·3% for androsterone glucuronide. The *specificity* of the method is based (1) on the proven Sephadex LH-20 group separation of neutral steroid glucuronides and monosulphates, (2) isolation of the steroids being analyzed as a group by silicic acid chromatography, and (3) their identification on the basis of relative retention time and response when m/e values characteristic of their fragmentation were monitored.

RESULTS AND DISCUSSION

The aim of this presentation is to demonstrate the usefulness of GC–MS and of MF in the identification and especially the quantitation of natural and synthetic steroids in biological material. The methods presented have been and are being used to solve a variety of biological and pharmacological problems. A number of examples of the use of these procedures are given below and the order of presentation is the same as in the methods section.

Estrogens in various biological fluids

Estrone, estradiol-17β, estriol and a variety of "nonclassical" estrogens have been identified by GC–MS and determined in pregnancy urine, cord plasma, amniotic fluid [17, 18], pregnancy bile [8] and plasma [17–19] and placental tissue [20] using GC. However, in order to quantitate some of the estrogens present

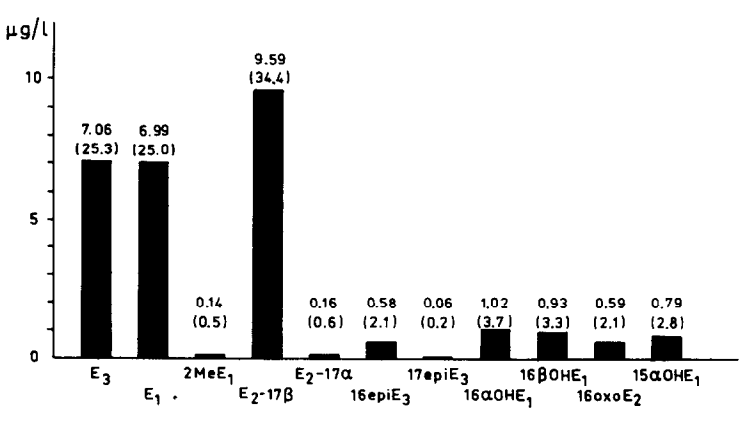

Fig. 9. Pattern of unconjugated estrogens determined in a pool of late pregnancy plasma. The concentration and the relative proportion of each estrogen (in per cent of total estrogens determined, in parenthesis) are shown on the top of the bars. Abbreviations: E_3 = estriol; E_1 = estrone; $2MeE_1$ = 2-methoxyestrone; E_2-17β = estradiol-17β; E_2-17α = estradiol-17α; 16epiE_3 = 16-epiestriol; 17epiE_3 = 17-epiestriol; 16αOHE_1 = 16α-hydroxyestrone; 16βOHE_1 = 16β-hydroxyestrone; 16oxoE_2 = 16-oxo-estradiol-17β; 15αOHE_1 = 15α-hydroxyestrone.

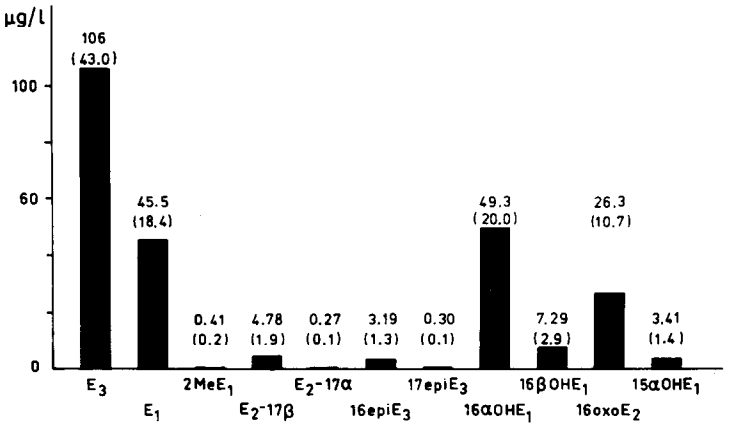

Fig. 10. Pattern of conjugated estrogens determined in a pool of late pregnancy plasma. The concentration and the relative proportion of each estrogen (in per cent of total estrogens determined, in parenthesis) are shown on the top of the bars. For abbreviations, see legend to Fig. 9.

in very low concentration in plasma, by GC, large pools (100–200 ml) are needed [19]. For radioimmunoassay only small amounts of plasma are required but methods have only been described for the determination of two "non-classical" unconjugated estrogens: 2-hydroxyestrone [21] and 15α-hydroxyestriol [22, 23]. The MF method developed has for the first time permitted the analysis of up to 11 unconjugated and conjugated estrogens in 20 ml of pregnancy plasma [2]. The results of the analysis of a pooled specimen obtained from women in late pregnancy are shown in Figs. 9 and 10. In this sample pool the most abundant unconjugated estrogen is estradiol, followed by estriol and estrone. The ring D α-ketolic unconjugated estrogens occur in concentrations about half or less than half of those reported for 2-hydroxyestrone (2 µg/l) [21] and 15α-hydroxyestriol (2 µg/l) [22, 23]. The most abundant conjugated estrogen in pregnancy plasma is estriol, followed by 16α-hydroxyestrone, estrone and 16-oxoestradiol. The pattern is very different from that seen in pregnancy urine [17]. Unfortunately the procedure is still too elaborate to permit serial determination of these estrogens in pathological pregnancies. It would be of great interest to investigate whether specific alterations in the pattern can be seen in selected well-defined pathological cases.

In connection with our continuing studies on the enterohepatic circulation of estrogens we have used the mass fragmentographic method to determine 11 estrogens in human non-pregnancy bile samples (50–100 ml) obtained by T-tube drainage of the main bile duct after cholecystectomy. The mean concentrations of the 11 estrogens in bile from four postmenopausal women are presented in Fig. 11. Two estrogens predominate: 16α-hydroxyestrone and estriol. All 11 estrogens could be detected and all except estradiol-17α were conclusively identified by GC–MS when a larger pool was analyzed. With the exception of the three "classical" estrogens [24], the presence of these other endogenous estrogens has not previously been demonstrated in the bile of non-pregnant individuals.

In Fig. 12 the results of two analyses of the 11 estrogens in two bile samples obtained at different times on the same day from a 66-year-old male patient are given. As can be seen in this case also,

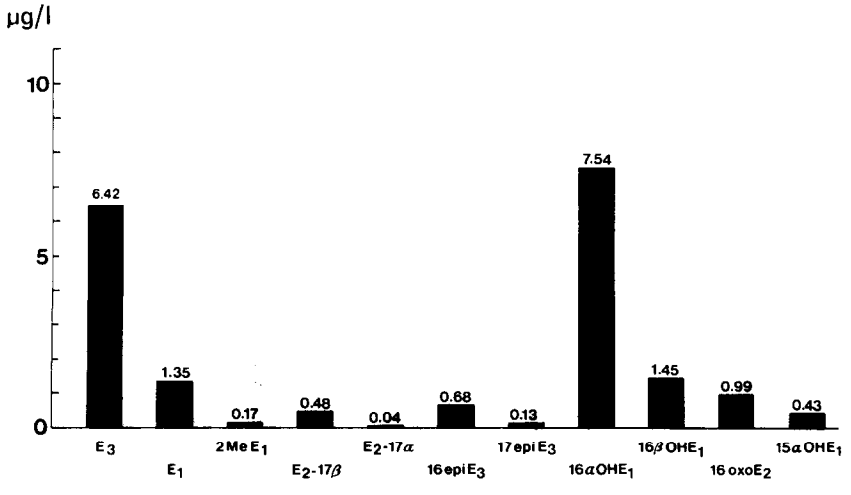

Fig. 11. Pattern of conjugated estrogens in bile of four postmenopausal women (mean values shown on the top of the bars). For abbreviations see legend to Fig. 9.

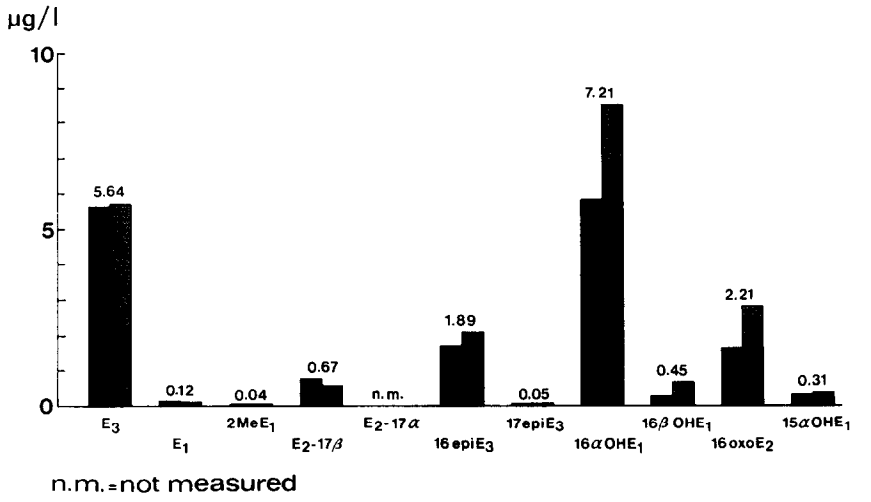

Fig. 12. Pattern of conjugated estrogens in bile from a 66-year-old male. The results of two analyses in separate bile samples obtained during the same day are shown and the mean values indicated on the top of the bars. For abbreviations, see legend to Fig. 9.

the concentration of 16α-hydroxyestrone is higher than that of any of the other estrogens. Estradiol-17α could not be measured on account of the high background noise. The differences between the values in the two samples are not significant.

A remarkably high concentration of estriol (43·8 μg/l) and a relatively low concentration of 16α-hydroxyestrone was found in the bile of a 36-year-old man (Fig. 13). In a previous study using a colorimetric technique the highest concentration of estriol (12·2 μg/l) in male bile was found in a 69-year-old man. In general, the values obtained with MF for estrone, estradiol-17β and estriol in bile from men and postmenopausal women agree surprisingly well with those obtained more than 12 years ago using a colorimetric procedure [24]. At that time 100 ml bile samples were used. With MF it is not necessary to use such large amounts of bile if only the "classical" estrogens are analyzed. However, because some estrogens occur in concentrations 100–1000 times less than estriol it is advantageous to use 50 ml samples for the MF analysis.

Analyses of 11 estrogens have also been carried out on 50 ml samples of urine from non-pregnant women [2]. All 11 estrogens could be determined, many of them for the first time.

An evaluation of the possibility of using plasma estriol MF assays for monitoring fetal well-being has been carried out [9]. It was observed with the limited material investigated (30 patients) that the correlation between fetal well-being and plasma estriol values was good. However, the MF procedure is still not simple enough to be adopted for routine use. A chromatographic step seems necessary in order to allow measurement of low concentrations, rending the method less practical. Some other problems are also

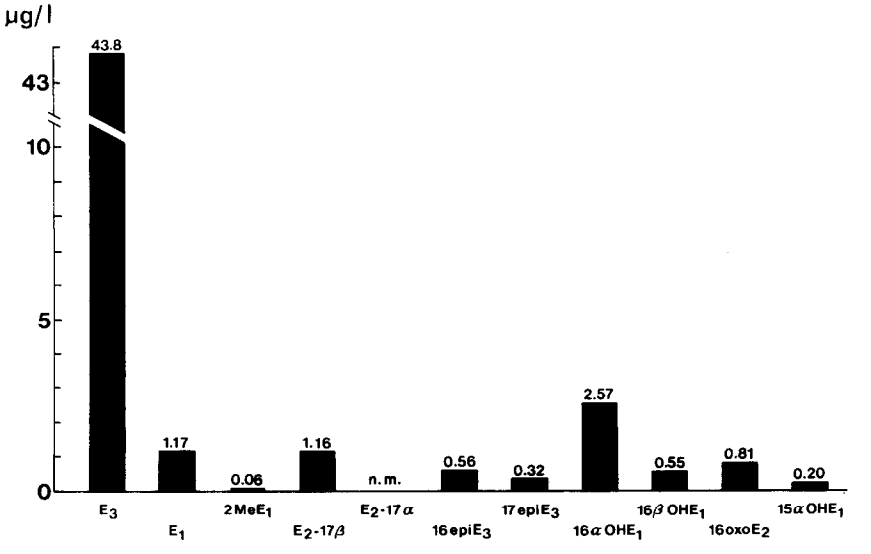

Fig. 13. Pattern of conjugated estrogens in bile from a 36-year-old male. The values are indicated on the top of the bars. For abbreviations, see legend to Fig. 9.

Table 1. Some examples of unconjugated estriol plasma values in pregnancy as measured by mass fragmentography

Stage of pregnancy	Estriol µg/l	Birth weight (g)	Weight of placenta (g)	Diagnosis
36th-40th week	4.3 - 9.3	3200 - 4850	520 - 820	Normal (9 subjects)
36th week	9.9	2780, 3400	1150	Twin pregnancy Hypertension
38th week	11.8	3760	780	Hypertension
37th week	3.7	2230	400	Hypertension (Cesarean section)
31st week	2.7	1740	400	Severe toxemia (Cesarean section) Child died
41st week	2.7	3650	720	Toxemia (Cesarean section)
38th week	11.1	3420	580	Pruritus gravidarum

encountered in such clinical assays, one of them being the fact that no presently available GC–MS can be kept running on a daily basis without interruptions for repairs, an essential requirement when monitoring fetal well-being.

Some examples of the results of our estriol analyses are given in Table 1. As can be seen the values are lower than those reported in the literature with one exception, [32] and obtained using various competitive protein binding methods (see Table 2). The reason for this is most probably the presence of many other estrogens in plasma which may interfere with the latter methods. To present a view of the extent of this problem the approximate concentration of bis- and tri-hydroxylated estrogens in late pregnancy plasma are shown in Table 3.

In our studies on pregnancy plasma the shorter estriol method was utilized. The method including the methylation step has been used in pharmacological studies on the luteolytic effect of estriol after

Table 2. Values for unconjugated estriol in pregnancy plasma obtained using competitive protein binding methods

Stage of pregnancy	Approximate mean concentration µg/l	Reference
Term	16	Den et al. [25] Goebel and Kuss [26]
32nd - 40th week	16.1	Youssefnejadian and Sommerville [27]
Late pregnancy	14 - 16	Wu and Lundy [28] Cleary and Young [29]
Term	11 - 13	Tulchinsky et al. [30] Wilkinson et al. [31]
35th week	6	Loriaux et al. [32]

Table 3. List of estrogens known to be present in pregnancy plasma and which may interfere with estriol assays

Steroid	Approximate concentration in late pregnancy µg/l	Method used for assay[1]	Reference
2-hydroxyestrone	2.0	RIA	[21]
15α-hydroxyestrone	0.8	m.f.	[2]
16α-hydroxyestrone	1.0	m.f.	[2]
16β-hydroxyestrone	0.9	m.f.	[2]
16-oxo-estradiol-17β	0.6	m.f.	[2]
16-epiestriol	0.6	m.f.	[2]
17-epiestriol	0.1	m.f.	[2]
15α-hydroxyestriol	2.0	RIA	[22,23]
Total	8.0		

[1] RIA = radioimmunoassay
m.f. = mass fragmentography

administration of various amounts of this compound (Vähäpassi and Adlercreutz, unpublished). Continuous oral administration of 6, 20 or 40 mg of estriol in the morning to normal women resulted in increasing values for conjugated estriol with increasing dose, but the values for unconjugated estriol measured in morning samples remained remarkably constant (Table 4). In order to investigate this problem further, large single doses of estriol (40 and 60 mg) were administered orally to normal women. It was found that the peak concentration of unconjugated estriol is followed over the next 2–4 h by a rapid decrease to a steady state level but that the conjugated estriol concentrations decreased very slowly and were still high 24 h after ingestion of the dose (example in Figs. 14 and 15). This phenomenon explains why the concentration of unconjugated estriol (measured in the morning before the next dose of estriol) is always similar after continuous oral administration of 6–40 mg doses of estriol per day, despite the fact that the level of conjugated estriol is related to the dose administered.

Megestrol acetate in plasma

Plasma concentrations of megestrol acetate have been determined in female subjects treated with this progestin for carcinoma corporis uteri. These studies have been presented previously [10]. In this connection the use of MF for the evaluation of the specificity

Table 4. Estriol in plasma after oral administration of estriol (according to Vähäpassi and Adlercreutz, unpublished)

Subject	No. of assays	Treatment estriol/day	Unconjugated estriol µg/l	Conjugated estriol µg/l
H.H.	10	6 mg	2.15 ± 0.85	not determined
N.M.	12	6 mg	1.75 ± 0.55	11.6 ± 3.5
M.E.	14	20 mg	1.58 ± 0.27	29.7 ± 18.1
J.R.	3	40 mg	2.10 ± 0.70	25.9 ± 10.4
V.K.	3	40 mg	2.14 ± 0.66	68.8 ± 31.4

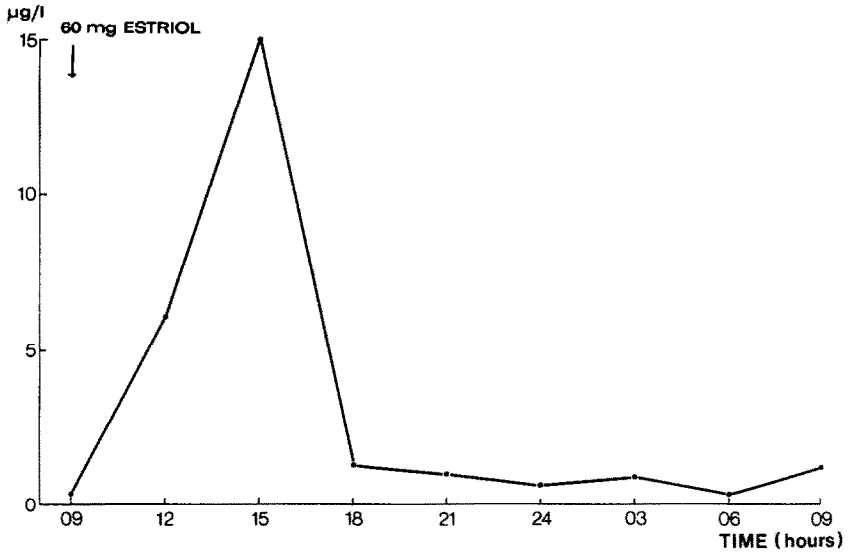

Fig. 14. Plasma level of unconjugated estriol in a normal woman following a single oral dose of 60 mg of estriol (according to Vähäpassi and Adlercreutz, unpublished).

of a radioimmunological (RIA) method for this steroid are described. Such comparisons have been carried out previously in connection with developments of a radioimmunological method for estradiol [33] and MF methods for testosterone and progesterone [34] and the results obtained in both investigations showed good agreement between the two types of procedure. However, when megestrol acetate in plasma was measured using an antibody raised against medroxyprogesterone acetate-3-(O-carboxymethyl)-bovine serum albumin it was found that after the administration of a megestrol acetate load the values obtained by RIA were much higher than those obtained by MF especially in later samples. An example of such an investigation is shown in Fig. 16. It should be mentioned that in untreated subjects no cross-reacting steroids were detected in plasma and thus the cross-reacting compound probably is a metabolite of megestrol acetate (see also next paragraph).

Megestrol acetate and metabolites in urine

The higher values obtained by radioimmunoassay (vs MF) suggested that a metabolite of megestrol acetate builds up in the blood especially at later stages after its administration and contributes to the RIA but not to the MF determination. No metabolites of megestrol acetate (MA) have yet been identified in plasma but as a preliminary step to looking for them it was decided to investigate the GC–MS properties of the urinary metabolites. Cooper and Kellie[35] isolated and identified the following metabolites as glucuronide conjugates from urine after oral administration of megestrol acetate to women: 2α-hydroxy MA (17α-acetoxy-2α-hydroxy-6-methyl-4,6-pregnadiene-3,20-dione), 6-hydroxymethyl MA (17α-acetoxy-6-hydroxymethyl-4,6-pregnadiene-3,20-dione) and 2α-hydroxy-6-hydroxymethyl MA (17α-acetoxy-6 - hydroxymethylpregna - 4,6 - diene - 3,20 - dione). No standard samples of these compounds are, how-

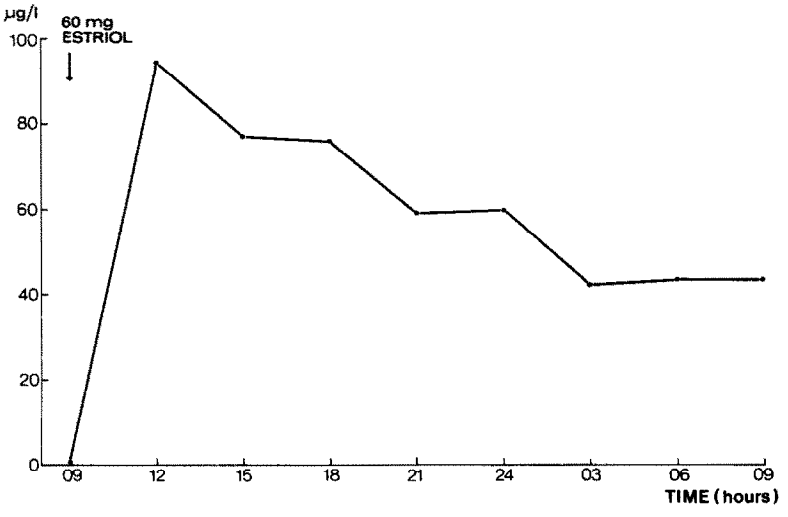

Fig. 15. Plasma level of conjugated estriol in the same woman as in Fig. 14 following a single oral dose of 60 mg of estriol (according to Vähäpassi and Adlercreutz, unpublished).

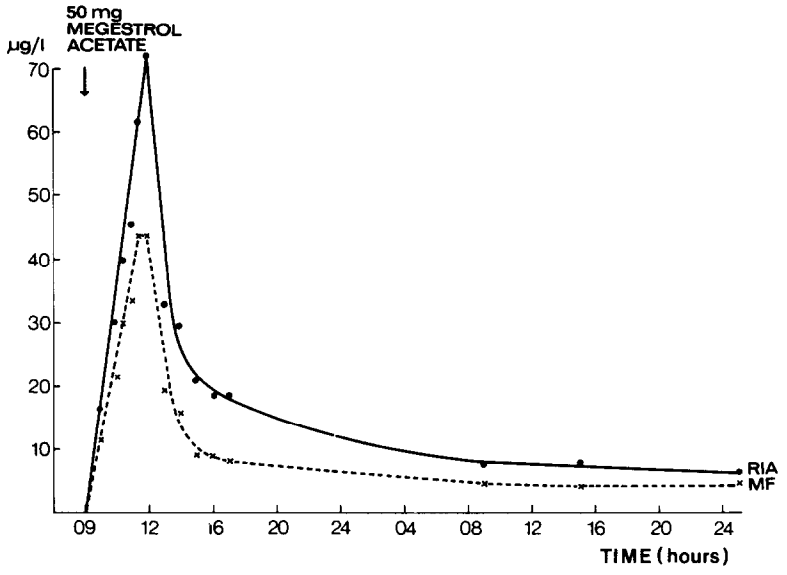

Fig. 16. Plasma concentration of megestrol acetate following a single oral dose of 50 mg of the steroid. Two different methods were used (RIA = radioimmunoassay; MF = mass fragmentography).

ever, available. In addition to the latter three compounds these authors detected two more polar compounds (probably tri- and tetrahydroxy substitued) in the glucuronide fraction and 6-hydroxymethyl MA and other more polar compounds in the unconjugated steroid fraction [35].

In the present study the most prominent compounds detected were megestrol acetate and two monohydroxy substituted metabolites in the unconjugated steroid fraction and the same two monohydroxylated metabolites in the glucuronide fraction. These two metabolites were detected and partially characterized on the basis of the mass spectra of their methoxime-silyl derivatives which are shown in Figs. 17 and 18. Compound I (Fig. 17), the metabolite detected in highest concentrations (unconjugated fraction) has a molecular ion of m/e 501, the expected molecular weight of a monohydroxylated MA-methoxime-trimethylsilyl ether derivative, a base peak at m/e 308 (M-103 + 90) and other significant peaks at m/e 486 (M-15), 470 (M-31, rupture of the N-O bond in the methoxime group), 441 (M-60, loss of the acetate group), 411 (M-90, loss of trimethylsilanol), 398 (M-103, loss of both $C_{17}\alpha + \beta$ side chains (tentative)), 380 (M-90 + 31) and 320 (M-90 + 60 + 31). As the spectra of both megestrol acetate and medroxyprogesterone acetate methoxime derivatives are dominated by M-103 base peaks it is felt that in these and the metabolite spectra shown, this ion represents the fragment left after loss of the $C_{17}\alpha + \beta$ side chains. The spectrum of the second monohydroxylated metabolite detected, compound II contrasts strikingly with that of the first. It has the same molecular ion and base peak but lacks all the fragment ions seen in the first to which the scission of the N-O bond in the methoxime group contributed (M-31, -31 + 90, -31 + 60 + 90) and in their place one sees fragments arising from the loss of a second group with an m/e value of

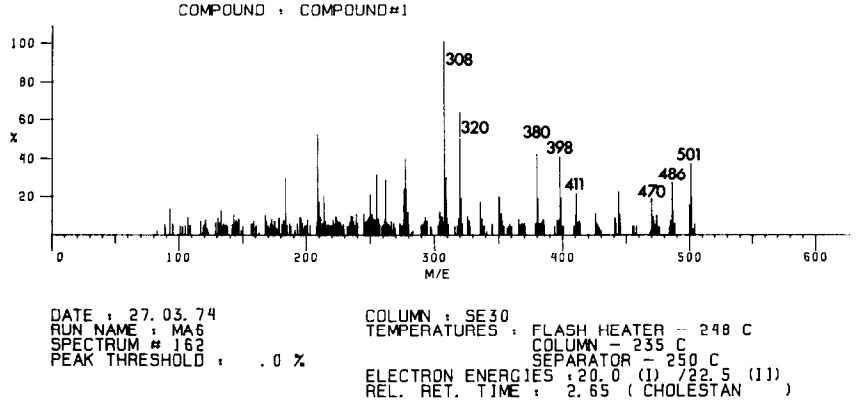

Fig. 17. Mass spectrum of the monomethoxime trimethylsilyl ether of Compound I, a monohydroxylated metabolite of megestrol acetate isolated from both the unconjugated and glucuronide fractions of urine. The subject a postmenopausal woman received 100 mg of megestrol acetate daily for 10 days.

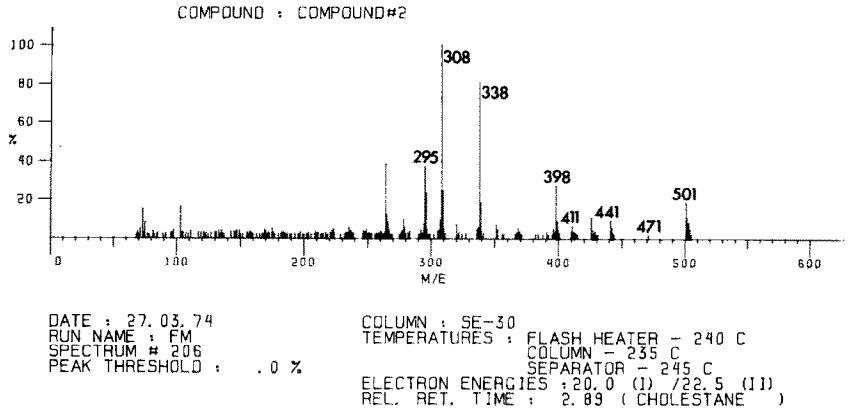

Fig. 18. Mass spectrum of the monomethoxime trimethylsilyl ether of Compound II, a second monohydroxylated metabolite of megestrol acetate isolated from both the unconjugated and glucuronide fractions of urine.

103: m/e 338 (M-103 + 60) and 295 (M-2 × 103) and a more prominent fragment at m/e 103. If compound II was 6-hydroxymethyl MA loss of the $-CH_2-O-Si(CH_3)$ group from C_6 would be a very likely source of the second M-103 fragmentation. Fragments arising from the loss of 103 mass units are seen in the TMS ethers of primary alcohols [36] and of steroids with a C_{21}-hydroxyl group [37] or C_{21} steroids with a C_{19}-hydroxyl group [38]. Peaks at m/e 103 and fragments arising from the loss of 103 mass units are seen in the spectra of 18-hydroxyandrosterone TMS and MO-TMS ethers [39]. Thus it is very possible that compound II is in fact 6-hydroxymethyl MA.

The mass spectrum of compound I contains a significant peak at m/e 486 (M-15) which is absent in the spectrum of compound II. This may be of some diagnostic significance. The TMS ethers of both 2β-hydroxytestosterone [40] and a steroid tentatively identified as 2α,3α-dihydroxy-5ξ-pregnan-20-one [41] both give base peaks at M-15. In the MO-TMS ether of the former, however, the significance of the M-15 fragment is reduced in favour of an M-31 fragment [40]. If the same association exists between the TMS and MO-TMS ethers of compound I it should be of significance and indicate the presence of a silylated 2-hydroxy group.

For a plasma metabolite of megestrol acetate to interfere with the MF determination of megestrol acetate it would have to be unconjugated. It is likely that the two compounds described are eluted with MA in the purifying Adsorbosil chromatographic step. However, the relative retention times (to cholestane) on SE-30 liquid phase of all metabolites so far detected are >2·5 whereas that of MA is about 2·0, in addition the sensitivity of detection for these compounds when monitoring m/e 310 would be extremely low relative to MA and thus they cannot interfere with the MF assay. The possible contribution of such metabolites to the RIA remains open. However, because the antibody used was raised against medroxyprogesterone acetate-3-(O-carboxymethyl)-BSA it could not very easily differentiate against compounds with substitutions at positions close to the 3-oxo group. It is therefore very likely that it cross-reacts with these metabolites if they are present in plasma.

Glucuronide and monosulphate conjugated 17-ketosteroids in peripheral and portal vein plasma

In this study MF was assessed as an alternative to GC in a situation where plasma sample size was limiting. The concentration of dehydroepiandrosterone-, androsterone- and epiandrosterone-monosulphate and androsterone-glucuronide were determined in simultaneously drawn peripheral and portal vein blood samples. The material consisted of samples from five postmenopausal women but on account of its limiting size the material was pooled into portal vein and peripheral vein pools and triplicate determinations carried out on each.

The method used is similar in all respects to those described in the literature for the determination of conjugated steroids in biological fluids [13, 15, 42] with the exception that the Amberlite XAD-2 method described by Makino and Sjövall[16] for bile acid extraction from plasma was successfully used for extracting the $C_{19}O_2$ steroid conjugates. Under the conditions used (3% OV-210, 210-220°C) no etiocholanolone peak was obvious in the monosulphate fractions but etiocholanolone was detected in the glucuronide fractions. Its concentration was of the order of 1/20th or less times the androsterone concentration and no attempt was made to quantitate it. Similarly, the small amounts of dehydroepiandrosterone in the glucuronide fraction were not quantitated. The peripheral and portal vein levels of the conjugated ketosteroids measured are very similar (Fig. 19). The peripheral levels of dehydroepiandrosterone sulphate in the pool were similar to the mean values reported by Buster and Abraham[43] and by Vihko[42] for groups of postmenopausal women. The androsterone- and epiandrosterone-sulphate levels are also similar to those reported by Vihko[42]. Androsterone was the principal steroid found in the glucuronide fraction. No differences were seen between the peripheral and portal pool values. Dehydroepiandrosterone and

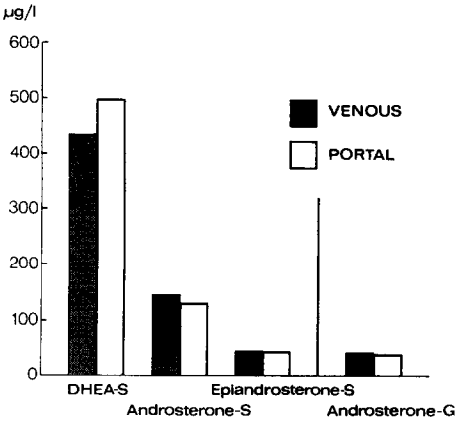

Fig. 19. The levels of 17-ketosteroid monosulphates and androsterone glucuronide in portal and peripheral venous plasma pools from postmenopausal women (according to Martin, Rimér, Sjöberg, Lindström and Adlercreutz, unpublished).

etiocholanolone were also detected in these fractions but were not measured.

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