Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 683–692. Pergamon Press. Printed in Great Britain

ANALYSIS OF UNCONJUGATED STEROIDS IN PLASMA BY LIQUID–GEL CHROMATOGRAPHY AND GLASS CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

M. AXELSON and J. SJÖVALL
Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

(*Received* 29 *November* 1976)

SUMMARY

A method is described for analysis of metabolic profiles of unconjugated steroids in plasma. After extraction by Amberlite XAD-2 at 64°, the steroids are purified by filtration through a 0.5 g column of sulphoethyl Sephadex LH-20 in methanol, and by chromatography on a 0.5 g column of triethylaminohydroxypropyl-hydroxyalkyl Sephadex LH-20. The latter column is used in a reversed-phase system which separates neutral steroids as a group from less polar lipids, and yields phenolic steroids in a separate fraction. The neutral steroids are converted into MO-TMS derivatives which are purified by rapid filtration through a 0.25 g column of Lipidex 5000 in hexane containing pyridine, hexamethyldisilazane and dimethoxypropane to prevent hydrolysis of TMS ethers. Estrogens are converted into TMS ethers. The recoveries of added [^{3}H]-labelled neutral steroids and estrogens are about 85%. Sample sizes corresponding to 1–2 ml plasma can be injected onto glass capillary columns.

The derivatives are analyzed by repetitive scanning GC–MS using a glass capillary column connected *via* a single-stage adjustable jet separator. Metal surfaces are deactivated with water glass and benzyltriphenylphosphonium chloride, and a Teflon coupling device is used for glass–glass capillary connections. Characterization and quantitation of steroids is based on computer construction of fragment ion current chromatograms.

When the method was applied to the analysis of unconjugated steroids in plasma from pregnant women, 27 steroids in the concentration range 1–300 ng/ml could be identified. The 13 major pregnane derivatives were quantitated. The coefficients of variation were 5–13% when 5 analyses of the same sample were performed.

INTRODUCTION

In a previous paper [1] a method was described for analysis of metabolic profiles of unconjugated neutral steroids in plasma. The final analytical step of this method consisted of repetitive scanning gas chromatography–mass spectrometry (GC–MS), which is the only existing technique that permits detailed analysis of complex and partly unknown steroid mixtures. However, packed columns were used so that sensitivity was limited by the background from the stationary phase, the low column efficiency and losses of steroids due to adsorption in the gas chromatographic system [2, 3].

In order to increase the sensitivity and specificity of the analyses, an open-tubular glass capillary column was connected to the mass spectrometer. However, since the capacity of this type of column is low, more effective procedures were required for removal of interfering compounds. The present paper describes an improved and simplified method for purification of unconjugated steroids in plasma. A capillary column–mass spectrometer connection giving relatively small adsorptive losses is also described.

EXPERIMENTAL

Glassware, solvents and reagents. All glassware was silanized with dimethyldichlorosilane and cleaning was carried out in an ultrasonic bath. All solvents were of reagent grade and were redistilled twice before use. Hexamethyldisilazane and trimethylchlorosilane (Applied Science Labs., State College, PA, U.S.A.) were redistilled and trimethylsilylimidazole (Supelco, Bellefonte, PA, U.S.A.) was used as supplied. Methoxyamine hydrochloride (Eastman Organic Chemicals, Rochester, NY, U.S.A.) was recrystallized from methanol.

Column packing materials. Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) was extensively

The following trivial names and abbreviations are used: Dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one; tetrahydrocortisol, 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one. The terms pregnanolone, pregnanediols etc. indicate general structures and do not imply a specific stereochemistry. A, P and E (used in tables and figures), androstane, pregnane and estrane, respectively. FIC, fragment ion current; FID, flame ionization detector; GC–MS, gas chromatography-mass spectrometry; GLC, gas–liquid chromatography; *m/e*, mass/charge ratio; MO, O-methyloxime; RIA, radioimmunoassay; SE-LH-20, sulphoethyl Sephadex LH-20; TEAPHA-LH-20, triethylaminohydroxypropyl-hydroxyalkyl Sephadex LH-20; TMS, trimethylsilyl.

washed with acid, base, water and solvents and was then stored in ethanol [4]. *Lipidex® 5000* (Packard Instrument Co., Downers Grove, IL, U.S.A.) was washed and stored at 4° in methanol [4]. Immediately before use, the appropriate amount of gel was washed on a Büchner funnel with absolute ethanol (20 ml per gram gel) and hexane (20 ml $\times$ g^{-1}), dried at 60° for 15 min and allowed to swell in the solvent used for chromatography.

Sulphoethyl Sephadex LH-20 (*SE-LH*-20) was prepared as described previously [5]. The capacity of the gel was about 0.10 meq $\times$ g^{-1}. After washing of the gel, it was stored in Na^+ form at 4° in methanol [4]. Prior to use, a few grams were converted into H^+ form by washing on a Büchner funnel with 100 ml each of 0.3 M hydrochloric acid in 72% aqueous ethanol, 20% ethanol (until neutral), ethanol and methanol.

Triethylaminohydroxypropyl-hydroxyalkyl Sephadex LH-20 (*TEAPHA-LH*-20). Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden), 50 g, was reacted with Nedox 1114 [6] to give a product with a hydroxyalkyl group content of 55% (w/w). This hydrophobic gel was converted into a chlorohydroxypropyl derivative (14% (w/w) substitution) [7, 8]. After washing with 85% aqueous ethanol, ethanol, chloroform and ethanol, the derivative was dried at room temperature and used for the synthesis of TEAPHA-LH-20. The gel, 25 g, was allowed to swell at room temperature in 85 ml of isopropanol under continuous stirring for 30 min and 85 ml of water were then added. After one h, 1.6 g of sodium hydroxide in 125 ml of isopropanol–water, 1:1 (v/v), were added and the mixture was stirred for 30 min. Triethylamine, 100 ml, was then added slowly (3 ml $\times$ min^{-1}). The temperature was increased to 55° and the mixture was stirred for 4 h. The product was washed on a Büchner funnel with 0.5–1 l of the following series of solutions: 50% aqueous ethanol, 0.3 M sodium hydroxide in 72% ethanol, 20% ethanol (until neutral) and 72% ethanol. The ion exchanger was then converted into acetate form by washing with 0.2 M acetic acid in 72% ethanol, and was washed to neutrality with 20% ethanol and ethanol. After drying overnight at room temperature, it was stored at $-20°$. Under these conditions there was no indication of decomposition. When kept at room temperature in OH^- form the gel was not stable.

For determination of ion exchanging capacity, 1 g gel in OH^- form was suspended in 20 ml of 0.25 M sodium chloride in 72% ethanol and titrated with 0.1 M hydrochloric acid in 72% ethanol. The capacity was usually about 0.7 meq $\times$ g^{-1} dry gel.

Prior to use, the ion exchanger was converted into OH^- form. Between 1–3 g were washed on a Büchner funnel with 100 ml of the following series of solutions: 0.3 M sodium hydroxide in 72% ethanol, 20% ethanol (until neutral), ethanol and methanol. The gel was then allowed to swell in the solvent used for chromatography.

In addition to the washing procedures described above, all gels were extracted at 70° with 20% and 50% aqueous ethanol and ethanol prior to storage and use [4]. These extractions were carried out with the anion exchanger in Cl^- form and the cation exchanger in Na^+ form to avoid possible decomposition.

Steroids. Unlabelled steroids were purchased from Ikapharm (Ramat-Gan, Israel) or kindly donated by Dr. J. Babcock (Upjohn Co., Kalamazoo, MI, U.S.A.).

Tritium-labelled steroids were obtained from the Radiochemical Centre (Amersham, England) or NEN Chemicals (Dreieichenhain, Germany). The specific radioactivities were between 10 and 110 Ci/mmol. Radioactivity was determined in a Packard Model 3003 liquid scintillation spectrometer using Instagel as the scintillation liquid. The purity of steroids was evaluated by t.l.c., chromatography on Sephadex LH-20 [9] and gas–liquid chromatography (G.L.C.).

Procedure for analysis of unconjugated steroids in plasma. Four g of Amberlite XAD-2 are packed in ethanol to give a column bed of 130 $\times$ 8 mm which is washed at 64° with 50 ml of water. Ten ml of plasma are diluted with 10 ml of saline and slowly (17 ml $\times$ h^{-1}) passed through the column at 64°. The column is then washed with 20 ml of water and, after cooling to room temperature, with 8 ml of hexane. The steroids are eluted with 12 ml of methyl acetate–methanol, 2:1 (v/v), followed by 12 ml of methanol [1].

The extract is taken to near dryness *in vacuo* at 30–40° and 1 ml of methanol is added. The sample is applied to an SE-LH-20 (H^+), column (500 mg, 200 $\times$ 4 mm), packed in methanol under gravity flow. The steroids are recovered in 6 ml of methanol at a flow rate of 7 ml $\times$ h^{-1} [4]. After evaporation *in vacuo* to near dryness, 1 ml of methanol–water–chloroform, 80:30:15 (by vol.), is added. The sample is applied to a column of TEAPHA-LH-20, about 0.5 g, 150 $\times$ 4 mm, packed by gravity flow in the same solvent. Neutral steroids are eluted with 7 ml of the methanol–water–chloroform mixture at a flow rate of 30 ml $\times$ h^{-1}, produced by applying a pressure (N_2) of 0.3 kp $\times$ cm^{-2}. Neutral lipids are then removed from the column by washing with 6 ml of methanol–chloroform, 80:15, using the same flow rate. Finally, unconjugated estrogens are eluted with 8 ml of the methanol–chloroform mixture saturated with carbon dioxide. During this elution CO_2 is used instead of N_2 to produce the flow rate of 30 ml $\times$ h^{-1}. Since CO_2 evaporates readily, it is important that leaks at the top of the glass column are prevented.

The neutral steroid and estrogen fractions are taken to dryness *in vacuo*, transferred with methanol to glass-stoppered centrifuge tubes and derivatized.

Preparation and purification of derivatives for GC–MS analysis. After addition of 1 μg of 7-ketocholesterol as internal standard, the neutral steroids are converted to *O*-methyloxime-trimethylsilyl ether (MO–TMS) derivatives according to Thenot and

Horning [10]. A 5 mg amount of methoxyamine hydrochloride and 50 μl of pyridine are added to the dried steroid extract. After heating for 30 min at 60°, 50 μl TSIM are added and heating is continued at 100° for 2.5 h. The reaction mixture is then diluted with 1 ml of hexane–pyridine–hexamethyldisilazane–dimethoxypropane, 97:1:2:10 (by vol.), and rapidly filtered through a column of Lipidex 5000 [1]. The column, about 250 mg, 70 × 4 mm, is packed under gravity flow and washed with 10 ml of solvent prior to sample application. The reaction tube is rinsed with the same solvent in 0.5 ml portions, and elution is continued at a flow rate of 3 ml × min^{-1} by application of a pressure of about 0.5 kp × cm^{-2}. The steroid derivatives are recovered in the first 3.5 ml of effluent, which are collected and stored at room temperature in a stoppered centrifuge tube until required for analysis by GC–MS.

To the estrogen fraction, 0.3 μg of 5β-cholestan-3α-ol is added as internal standard. TMS ethers are formed in pyridine–hexamethyldisilazane–trimethylchlorosilane, 3:2:1 (by vol.), at 60° for 30 min. After removal of the reagents under a stream of nitrogen, the sample is dissolved in hexane for G.L.C. and GC–MS analyses. If required, the derivatized estrogens may be further purified (after removal of the reagents) as described above for the MO-TMS derivatives of neutral steroids.

g.l.c. Analysis. G.L.C. Was carried out using a Pye 104 gas chromatograph housing a 20 m × 0.34 mm open-tubular glass capillary column coated with SE-30 [11]. Solid injection in a silanized all-glass system was used [12]. The column was connected to the flame-ionization detector (FID) *via* a deactivated steel capillary tubing (0.20 × 150 mm) [3]. The column connections were made with thermo-shrinkable Teflon tubing. Nitrogen was used as the carrier gas at an inlet pressure of 0.5 kp × cm^{-2}, giving a flow-rate of about 1 ml × min^{-1}. The oven temperature was 260°.

GC–MS analysis. Computerized GC–MS was carried out using a modified LKB 9000 instrument [13]. An open-tubular glass capillary column (25 m × 0.34 mm) coated with OV-1 [11], was connected to the direct inlet side of this instrument *via* a single stage adjustable jet separator [14]. A sliding valve [14] was inserted between the jets. Glass and steel parts of the separator with which the steroids were likely to come into contact were deactivated [3]. A schematic drawing of the connection is shown in Fig. 1. For optimal flow conditions a silanized glass capillary resistance, 70 × 0.05 mm, was used between the column and separator. This resistance was attached to the separator in the same way as a packed column. Since thermo-shrinkable Teflon tubing did not provide leak-free glass–glass connections, a special Teflon coupling device was constructed that could tolerate high vacuum and temperature variations between room temperature and 280° (Fig. 1). It consists of a Teflon rod with a hole having the appropriate diameters at both ends to fit the dimensions of the glass capillaries to be connected. The rod is then compressed from both ends in a metal capsule with a screw cap. The advantage of using Teflon in these connections is the relative softness of the material which prevents breakage of the glass capillaries. An injection system identical to that in the gas chromatograph was used. It was attached to the column using the Teflon coupling device. The inlet pressure of the carrier gas was 0.5 kp × cm^{-2} giving a flow rate of about 0.5 ml × min^{-1}. Leaks were detected by placing a piece of dry ice in the oven, and monitoring m/e 44. When leaks were present, they could readily be localized by determination of the time lag between introduction of the dry ice and the appearance of CO_2 in the mass spectrometer.

The temperature of the column was programmed from 220° to 275° at a rate of 1.2° × min^{-1}. The temperatures of the separator and ion source were 240° and 290°, respectively. The energy of the bombarding electrons was 22.5 eV, ionizing current 60 μA and accelerating voltage 3.5 kV. Spectra, m/e 0–730, 10 scans/min, or m/e 250–480, 15 scans/min, were recorded after a suitable delay following injection of the sample.

Identification and quantitation of steroids. Methods for computer evaluation of the mass spectral data recorded on magnetic tape have been described [15].

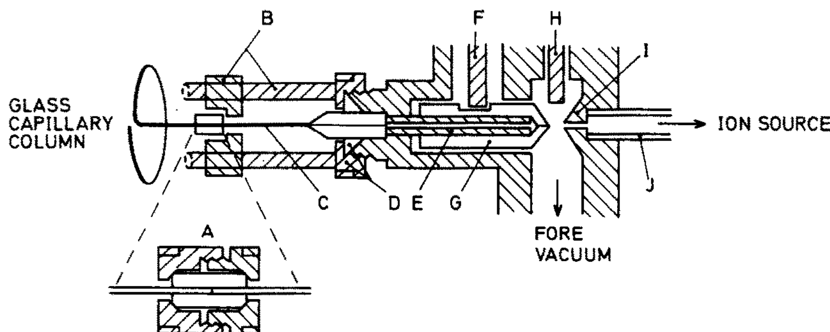

Fig. 1. Schematic drawing of the coupling of a glass capillary column to the mass spectrometer. A. Glass–glass coupling device. B. Supporting rods and sleeve for coupling device. C. Glass capillary resistance (70 × 0.05 mm). D. Swagelok connection (1/4 inch) with Teflon ferrule. E. Glass-lined steel capillary. F. Jet adjustment rod. G. Adjustable jet. H. Sliding valve. I. Collecting jet. J. Glass tube.

The identification of a steroid was based on the retention time, the complete mass spectrum and partial mass spectra obtained from fragment ion current (FIC) chromatograms for characteristic ions given by the steroid derivatives.

Quantitative analyses were based on determination of peak areas in specific FIC chromatograms. An internal standard was added to the samples and to a mixture of reference steroids. Following GC–MS analysis, peak areas were calculated in FIC chromatograms using the 2–10 most intense or diagnostically significant *m/e* values for each compound. The area of a peak given by the internal standard served to normalize the results so that areas given by known amounts of reference steroids and unknown amounts of steroids in the samples could be compared [15].

RESULTS AND DISCUSSION

Extraction and purification of steroids

Columns of Amberlite XAD-2 have been widely used for extraction of steroids in urine [16]. If conditions are selected which minimize protein binding, this method can also be used for extraction of steroids in plasma [1, 17]. High recoveries are obtained by performing the extraction at 64° [1]. Under this condition plasma only has to be diluted twice, which is an advantage since the flow rate through the Amberlite XAD-2 column has to be kept low. In order to shorten the time for further processing of the sample, interstitial water in the column is displaced by elution with hexane, which also removes some lipid contaminants. Steroids are eluted with a mixture of methyl acetate and methanol which is more volatile than methanol alone. The recoveries of [^{3}H]-labelled steroids (0.03–0.9 ng) added to plasma are about 90–95% [1] (see below).

The purification of steroids is based on chromatography on neutral and ion exchanging lipophilic/hydrophobic Sephadex derivatives. These gels are inert, have high loading capacity, are simple in operation and provide rapid ion-exchange in organic solvents [18]. A previous procedure, which gave a sufficiently clean extract for analysis on packed G.L.C. columns [1] had to be improved to permit analysis of the same amount of sample on glass capillary columns with a low loading capacity. Contaminating compounds may be divided into four main groups: less polar and more polar than steroids, organic acids and bases. Removal of a sufficiently large amount of these compounds is achieved by filtration through columns of Sephadex derivatives.

Chromatography on SE-LH-20. A lipophilic, strong cation exchanger, SE-LH-20, is used for removal of organic bases. The weaker carboxymethyl Sephadex LH-20 [19] had negligible effect on the purity of the sample. The use of SE-LH-20 has also been found essential for quantitative sorption of organic acids on a weak anion exchanger [5], probably because it removes cations which form ion-pairs with the acids.

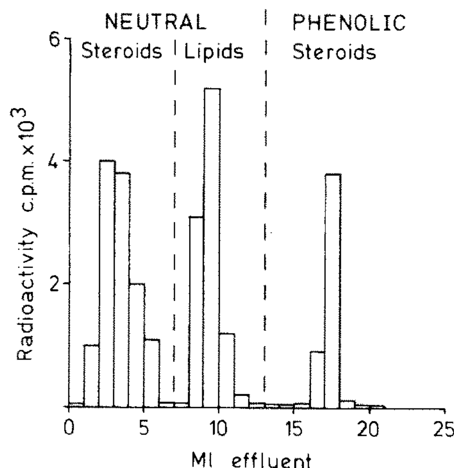

Fig. 2. Separation on a column of TEAPHA-LH-20 (0.5 g) in OH^- form of radioactive steroids added to plasma. Neutral steroids (progesterone and tetrahydrocortisol) were eluted with methanol–water–chloroform, 80 : 30 : 15 (by vol.); neutral lipids (cholesterol) with methanol–chloroform, 80 : 15; and phenolic steroids (estrone, estradiol and estriol) with methanol–chloroform, 80 : 15, saturated with CO_2.

SE-LH-20 is used in the H^+ form since this was found to give the highest degree of purification and because the combination with an anion exchanger in OH^- form will yield water. Nonaqueous methanol was selected as solvent since it was found that oximes of 3-ketosteroids, which behave like weak bases, are not sorbed by SE-LH-20 in the presence of water [4]. In spite of the low ion exchanging capacity of SE-LH-20, a 0.5 g column was found to be sufficient for extracts of 10 ml plasma.

Chromatography on TEAPHA-LH-20. A gel containing alkyl and triethylamino groups, TEAPHA-LH-20, is used for removal of nonpolar lipids and organic acids. Since these compounds constitute a large part of the contaminating material, this step gives a marked purification of the extract. In the previous study, lipids were removed on a column of Lipidex 5000 and acids on a column of a lipophilic weak anion exchanger [1]. Estrogens were only partly retained on the latter column, indicating incomplete sorption of weak acids. TEAPHA-LH-20 was therefore synthesized. This gel retains the estrogens and can be simultaneously used in a reversed-phase system for removal of nonpolar lipids. The solvent system was selected to give optimal separation of cholesterol from progesterone while keeping the elution volume of progesterone low. In the system used (methanol–water–chloroform, 80:30:15 (by vol.)), the cholesterol–progesterone separation factor is about 3–4, and progesterone is eluted at about 2–3 column bed volumes (Fig. 2). After the elution of progesterone and other neutral steroids in one fraction, neutral lipids are removed from the column by deletion of the water from the solvent so that these compounds will not interfere with subsequent separation of acids. Unconjugated estrogens are then eluted by saturation of the solvent with CO_2 (Fig. 2). About 4 column

bed volumes are used for the latter elution, since the concentration of CO_2 may vary. A procedure for isolation of estrogens based on the use of an anion exchange resin in carbonate form has previously been described by Eberlein [20]. Since TEAPHA-LH-20 is a strong base, transformations of alkali-labile compounds may occur. This possibility was tested with 16α-hydroxyestrone and it was found that about 15% of this steroid was converted to 16-ketoestradiol during the chromatography. A detailed study of the separation and analysis of estrogens will be published elsewhere [21].

Following the elution of estrogens it may be possible to fractionate acidic steroids in a way analogous to that previously described for a lipophilic weak anion exchanger [5]. Thus, cholic acid is eluted when acetic acid is added to the solvent.

Chromatography of MO–TMS derivatives on Lipidex. The purpose of this step is to remove nonvolatile reagents and polar contaminants which are not converted into nonpolar derivatives during the derivatization reactions. In the previous study, variable and low recoveries were obtained when the MO–TMS derivative of cortisol was filtered through a 70 × 4 mm column of Lipidex 5000 in hexane–pyridine–hexamethyldisilazane, 98:1:1 (by vol.), at a flow rate of 0.1 ml × min^{-1} [1]. Studies of the behaviour of MO-TMS derivatives of different steroids on Lipidex 5000 in different solvents showed that the losses were due to hydrolysis and that an *O*-methyloxime group at C-3 and a readily hydrolyzable TMS group (e.g. at C-21) had to be present for hydrolysis to occur. Incorporation of about 10% dimethoxypropane into the solvent and use of a high flow rate, 3 ml × min^{-1}, prevented the loss caused by hydrolysis and did not decrease the degree of purification achieved.

Estrogens are analyzed as TMS ethers which are prepared with the use of volatile reagents. Purification of the derivatives by filtration through Lipidex 5000 was not required when the samples consisted of plasma from pregnant women. However, if further purification is necessary, this may be carried out as described for the MO–TMS derivatives of neutral steroids without loss of estrogens containing one keto group.

The procedure for isolation and purification of steroids in plasma can be carried out in one day. The purity of the sample is such that the equivalent of 1–2 ml plasma can be injected onto a glass capillary column. The estrogen fraction may be analyzed by g.l.c. or GC–MS on the same day while preparation and purification of MO-TMS derivatives of neutral steroids are carried out on the second day. The major impurities interfering in the GC–MS analysis are those arising from solvents, chemicals and column packing materials.

With minor modifications, the combined use of SE-LH-20 and TEAPHA-LH-20 may be applicable to the purification of a wide range of naturally occurring and synthetic compounds present in biological materials.

Recovery of added steroids

Recoveries were studied by addition of tracer amounts of individual or mixtures of [^{3}H]-labelled steroids to plasma. Results of experiments with mixtures of representative neutral steroids and estrogens are shown in Table 1. The mean recoveries of neutral steroids and estrogens carried through the entire procedure were 84% and 86%, respectively. The loss of estrogens in the nonpolar lipid fraction was about 1%, and about 1% could be recovered from the TEAPHA-LH-20 column by additional elution with acetic acid added to the solvent. Filtration of the derivatized estrogens through a Lipidex column gave a recovery of 87.9 ± 2.1% in five experiments. Selective losses were not observed, neither for neutral nor for phenolic steroids.

Microgram amounts of three representative neutral steroids and three estrogens were added to male plasma and carried through the procedure. The reco-

Table 1. Recoveries of [^{3}H]-labelled steroids added to plasma (10 ml) and carried through the steps of extraction, purification and derivatization described in Experimental

Steroid mixtures*	Amount added		Recovery from columns (%)†		
	pg	c.p.m.	Amberlite XAD-2 + SE-LH-20 + TEAPHA-LH-20	Lipidex 5000	Total
A^4-17β-ol-3-one	8	2000	90.8 ± 4.0 (n = 20)	92.3 ± 5.0 (n = 20)	83.7 ± 3.7 (n = 20)
A^5-3β-ol-17-one	80	2000			
P^5-3β-ol-20-one	80	2000			
P^4-3,20-one	8	2000			
P^4-17α-ol-3,20-one	80	2000			
P^4-11β,17α,21-ol-3,20-one	8	2000			
5βP-3α,11β,17α,21-ol-20-one	25	2000			
E$^{1,3,5(10)}$-3-ol-17-one	25	6000	85.7 ± 4.4 (n = 20)		
E$^{1,3,5(10)}$-3,17β-ol	25	6000			
E$^{1,3,5(10)}$-3,16α,17β-ol	40	6000			

* A = androstane, P = pregnane, E = estrane. Superscript indicates position(s) of double bond, Greek letters denote configuration of hydroxyl groups. † Percent of added radioactivity ±S.D.; *n* = number of experiments.

Table 2. Recoveries of unlabelled steroids added to 5 ml of plasma and carried through the steps of extraction, purification and derivatization described in Experimental

Steroid mixture*	Amount added μg	Recovery from columns (%)†			
		Amberlite XAD-2 + SE-LH-20 + TEAPHA-LH-20		Amberlite XAD-2 + SE-LH-20 + TEAPHA-LH-20 + LIPIDEX 5000	
		I	II	I	II
P^4-3,20-one	3.58	—	—	92.1	92.8
P^4-20α-ol-3-one	1.51	—	—	98.5	98.3
P^4-11β,21-ol-3,20-one	3.04	—	—	96.4	93.8
$E^{1,3,5(10)}$-3-ol-17-one	3.04	93.7	96.2	86.9	91.4
$E^{1,3,5(10)}$-3,17β-ol	2.18	90.6	90.3	89.6	87.4
$E^{1,3,5(10)}$-3,16α,17β-ol	2.74	95.7	95.1	94.0	88.7

* For abbreviations see Table 1. † Percent of added steroids as calculated from peak areas in the G.L.C. analysis.

veries were determined by G.L.C. analysis of the final extracts. The results are shown in Table 2. The recoveries were between 92 and 99% for the neutral steroids and 90–96% for the estrogens. Additional purification of the estrogen TMS ethers on Lipidex gave a total recovery of 87–94%. Selective losses were not observed.

Glass capillary GC–MS analysis

The use of packed columns in GC–MS analyses of plasma steroids does not permit a separation of certain steroid isomers present in the samples [1]. The necessary separations can be achieved by the use of glass capillary columns. In addition, the reduction of peak width and column bleed results in an enhancement of sensitivity. Two main techniques have been used for the connection of glass capillary columns to a mass spectrometer. The column may be connected directly to the ion source *via* a platinum capillary resistance, or extra carrier gas may be added at the end of the column to permit the use of a molecule separator for packed columns [22, 23]. The direct coupling prevents losses that may occur in the separator, but high pumping capacity is required to maintain the vacuum in the ion source, and the lack of a valve between the column and mass spectrometer is inconvenient. When a separator and extra gas are used, the flow through the column is easy to control and a simple valve can be employed. However, loss of steroids may occur due to adsorption or chemical transformation in the separator. Thus, the presence of steel surfaces in a GC–MS system causes severe loss of MO derivatives of steroids with a 3-keto-4-ene structure [3]. Based on these considerations a single-stage adjustable jet separator [14] with a sliding valve [14] was selected as interface between the column and the ion source (Fig. 1). Metal surfaces were deactivated with water glass and benzyltriphenylphosphonium chloride and glass surfaces were silanized [3]. Commonly used glass capillary–metal connections were avoided by development of a vacuum tight, temperature resistant Teflon coupling device (Fig. 1).

Using this system, the response for progesterone *bis-O*-methyloxime, one of the steroids lost to the greatest extent, was linear down to the detection limit (100 pg) using repetitive magnetic scanning (see below).

Although large sample sizes can be injected due to the efficient purification procedure, the sensitivity of repetitive magnetic scanning over the entire mass range (*m/e* 0–730) is often insufficient (1–5 ng) since a high scan rate has to be used due to the narrow G.L.C. peak width obtained with capillary columns. Scanning over a limited mass range (*m/e* 250–480) at a lower rate was therefore employed to permit use of filters and higher amplification. This increased sensitivity about 10-fold (100 pg–1 ng). Relatively little information is lost, since MO-TMS derivatives of steroids usually give diagnostically significant ions of high relative intensity in the mass range covered by the partial scan. Obviously the sensitivity and accuracy may be further increased by the use of multiple ion detection techniques for analyses of selected steroids.

Steroids in plasma from pregnant women

The unconjugated steroids in plasma from a 29-year old pregnant woman were analyzed by the method described. The steroids identified in the sample collected in the 38th week of pregnancy are listed in Table 3. Major previously unidentified steroids include 3α,5α, 3α,5β and 3β,5α isomers of 3-hydroxypregnan-20-one and pregnane-3,20α-diol. The presence of these compounds is also seen from the analysis shown in Fig. 3. The approximate concentrations of individual pregnanolones and pregnanediols in late pregnancy were 10–20 ng × ml^{-1} and 2–10 ng × ml^{-1}, respectively (Table 4). These steroids are present in much higher concentrations as sulphates, but in this case pregnanediols are the predominant compounds [24].

The possibility that the unconjugated steroids were formed by hydrolysis of the sulphate during the extraction and purification procedure was considered. Therefore, a series of experiments was carried out

Table 3. Unconjugated steroids found in plasma from a woman in the 38th week of pregnancy

Steroids†	Ions‡ (m/e)	Relative retention time§	Approximate concentrations in plasma (ng/ml)∥
5αA-3α-ol-17-one	360,270	0.47	*
5βA-3α-ol-17-one	360,270	0.49	*
A^5-3β-ol-17-one	358,368	0.57	***
5αP-3α-ol-20-one	404,388	0.79	***
5βP-3α-ol-20-one	404,388	0.81	***
A^5-3β,16α-ol-17-one	462,446	0.83 + 0.85¶	*
5αP-3α,20α-ol	449,269	0.89	**
5βP-3α,20α-ol	449,269	0.92	**
P^5-3β-ol-20-one	402,386	0.96	**
5αP-3β-ol-20-one	404,388	0.98	***
5αP-3,20-one	343,288	1.01 + 1.04	***
P^5-3β,20α-ol	462,447	1.07	*
5αP-3β,20α-ol	449,269	1.11	**
P^4-3,20-one	372,286	1.12	*****
P^4-20α-ol-3-one	417,386	1.28	****
P^4-17α-ol-3,20-one	460,429	1.38 + 1.43	**
5αP-3α,20α,21-ol	449,269	1.66	*
5αP-3β,16α,20α-ol	462,447	1.66	*
P^4-16α-ol-3,20-one	429,339	1.67 + 1.69	**
P^4-17α,21-ol-3,11,20-one	459,441	2.78 + 2.86	****
P^4-11β,17α,21-ol-3,20-one	425,361	3.36 + 3.42	*****
$E^{1,3,5(10)}$-3-ol-17-one	342,327	0.56	**
$E^{1,3,5(10)}$-3,17β-ol	416,285	0.67	***
$E^{1,3,5(10)}$-2-OMe-3-ol-17-one	372,342	0.77	*
$E^{1,3,5(10)}$-3,16α-ol-17-one	430,286	0.87	*
$E^{1,3,5(10)}$-3,17β-ol-16-one	430,285	0.96	*
$E^{1,3,5(10)}$-3,16α,17β-ol	414,386	1.15	***

† For abbreviations see Table 1. ‡ Intense and/or significant ions for the derivatized steroids in the mass range m/e 250–480. § 5α-cholestane = 1.00. Glass capillary column, SE-30, 260°. ∥ * = 0.1–1 ng/ml; ** = 2–10 ng/ml; *** = 11–30 ng/ml; **** = 31–50 ng/ml; ***** ≥ 50 ng/ml. ¶ Due to formation of syn/anti isomers some MO derivatives give rise to two peaks.

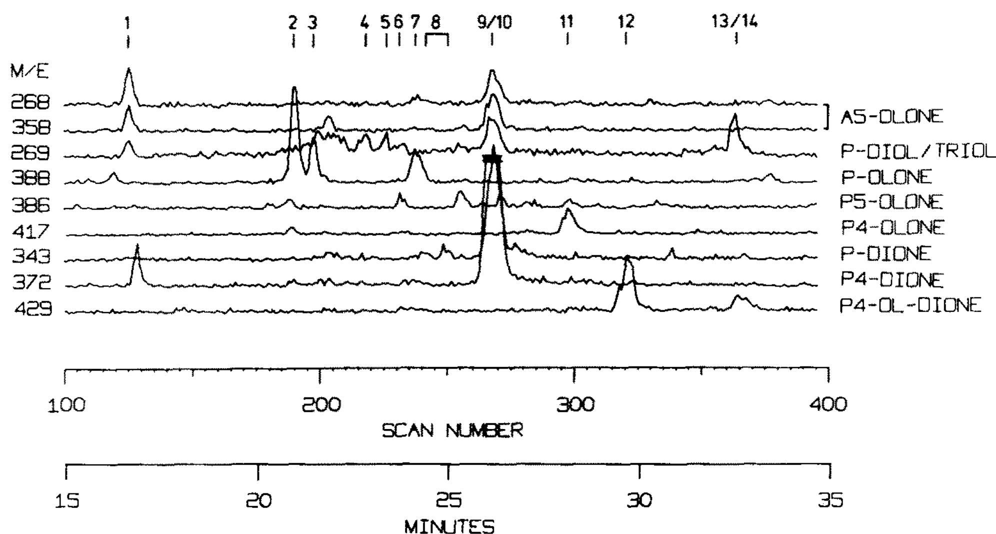

Fig. 3. Fragment ion current chromatograms constructed by the computer in an analysis of MO-TMS derivatives of unconjugated neutral steroids in plasma from a 27 year old woman in the 37th week of pregnancy. M/e values typical for the derivatives of progesterone and some of its potential metabolites as well as for dehydroepiandrosterone were selected. The numbers indicate retention times of the derivatives of (see Table 3): 1. A^5-3β-ol-17-one. 2. 5αP-3α-ol-20-one. 3. 5βP-3α-ol-20-one. 4. 5αP-3α,20α-ol. 5. 5βP-3α,20α-ol. 6. P^5-3β-ol-20-one. 7. 5αP-3β-ol-20-one. 8. 5αP-3,20-one. 9. P^4-3,20-one. 10. 5αP-3β,20α-ol. 11. P^4-20α-ol-3-one. 12. P^4-17α-ol-3,20-one. 13. P^4-16α-ol-3,20-one. 14. 5αP-3α,20α,21-ol.

Table 4. Concentrations, ng/ml, of some C_{21} steroids in plasma from a pregnant woman

Steroids*	Week of pregnancy 20	24	28	32	37	38
5αP-3α-ol-20-one	4.2	5.3	6.0	10.3	11.4	15.3
5βP-3α-ol-20-one	2.3	3.1	5.0	7.5	8.9	12.5
5αP-3β-ol-20-one	3.6	9.2	7.8	13.8	16.4	20.6
P^{5}-3β-ol-20-one	7.3	5.6	5.6	9.6	7.2	8.5
5αP-3α,20α-ol	0.8†	2.1	2.0	2.6	3.1	2.5
5βP-3α,20α-ol	1.0†	2.4	2.2	4.0	3.7	5.3
5αP-3β,20α-ol	0.9†‡	6.4‡	4.0‡	7.9‡	5.3‡	6.1‡
5αP-3,20-one	9.6†	5.2†	7.4†	11.2	14.4	25.6
P^{4}-3,20-one	25.4	39.4	51.5	78.1	92.8	98.9
P^{4}-20α-ol-3-one	11.2	12.0	17.2	22.6	30.3	37.7
P^{4}-17α-ol-3,20-one	—	4.6	9.6	4.7	6.2	7.0
P^{4}-17α,21-ol-3,11,20-one	—	18.5	29.8	24.3	36.0	44.9
P^{4}-11β,17α,21-ol-3,20-one	124	97	212	224	244	295

* For abbreviations, see Table 1. † Value uncertain due to noisy baseline. ‡ Correction was made for the contribution to m/e 269 from the MO derivative of progesterone.

using 3β-hydroxy-5-[4-^{14}C]-androsten-17-one sulphate (55 mCi/mmol), which had been purified on Sephadex LH-20 [25] and by t.l.c.. When 13,000 d.p.m. (80 ng) of this compound were chromatographed on a TEAPHA-LH-20 column, 0.4% of the radioactivity was recovered in the neutral fraction. This was considered to represent the amount of unconjugated steroid present in the sample. When the same amounts were added to 20 ml of saline and extracted on columns of Amberlite XAD-2 at 64° or at room temperature and then chromatographed on SE-LH-20 and TEAPHA-LH-20, 0.6% and 0.3%, respectively, were found in the neutral fraction. In five experiments, where the labelled steroid sulphate was added to 10 ml of plasma and then extracted and purified as described, $0.6 \pm 0.2\%$ of the radioactivity was recovered in the neutral fraction. Furthermore, storage of plasma at room temperature for several hours prior to extraction did not increase the amount of radioactivity appearing in the neutral fraction. Thus, hydrolysis of dehydroepiandrosterone sulphate during processing of the sample is negligible. This is further supported by the reproducibility of the quantitative method as shown in Table 5.

However, hydrolysis of sulphates may occur if the eluates from the Amberlite XAD-2 and SE-LH-20 columns are taken to complete dryness at 40–50°. In this case, hydrolysis is particularly noticeable for the estrogen fraction. Thus, when evaporation to complete dryness is avoided, the estrone/estradiol ratio in late pregnancy plasma has been less than 1 in the samples studied. When completely dried extracts were left at an elevated temperature in air, a ratio of 2–4 was obtained due to an increase of the concentration of estrone, probably caused by hydrolysis of estrone sulphate.

Seven of the neutral steroids in pregnancy plasma possessed a 3-keto group. These steroids include progesterone, cortisol and related 3-ketosteroids, which have been subject to many previous studies by RIA [26–29]. The concentrations found for these steroids are within the range of levels previously reported.

Analysis of the fraction of unconjugated estrogens confirmed the presence of estrone, estradiol, estriol,

Table 5. Reproducibility of the method as determined by analysis of 5 aliquots from a pooled sample of plasma collected from women in the 35th–40th week of pregnancy

Steroids*	Concentration, ng/ml 1	2	3	4	5	Mean ± S.D.†	CV†
A^{5}-3β-ol-17-one	25.8	29.5	32.6	32.6	33.5	30.8 ± 3.2	10
5αP-3α-ol-20-one	18.7	19.3	17.0	15.0	16.0	17.2 ± 1.8	10
5βP-3α-ol-20-one	15.0	15.8	17.3	14.4	17.3	16.0 ± 1.3	8
5αP-3β-ol-20-one	38.5	30.9	30.9	30.9	34.5	33.1 ± 3.4	10
5αP-3α,20α-ol	6.9	7.4	6.7	6.5	6.7	6.8 ± 0.3	5
5βP-3α,20α-ol	10.5	12.5	11.8	12.2	10.5	11.5 ± 1.0	8
5αP-3β,20α-ol	13.8	13.0	14.0	13.4	15.8	14.0 ± 1.1	8
P^{4}-3,20-one	138	133	129	117	117	126.8 ± 9.8	8
P^{4}-20α-ol-3-one	55.7	57.4	48.8	52.5	54.1	53.7 ± 3.3	6
P^{4}-17α-ol-3,20-one	16.8	17.3	18.1	14.4	13.4	16.0 ± 2.0	13
P^{4}-17α,21-ol-3,11,20-one	43.9	40.3	33.0	45.1	39.1	40.3 ± 4.8	12
P^{4}-11β,17α,21-ol-3,20-one	239	259	267	290	239	258.8 ± 21.7	8
E$^{1,3,5(10)}$-3,17β-ol	25.1	25.5	24.7	19.7	23.1	23.6 ± 2.4	10

* For abbreviations, see Table 1. † S.D. = standard deviation; CV = coefficient of variation.

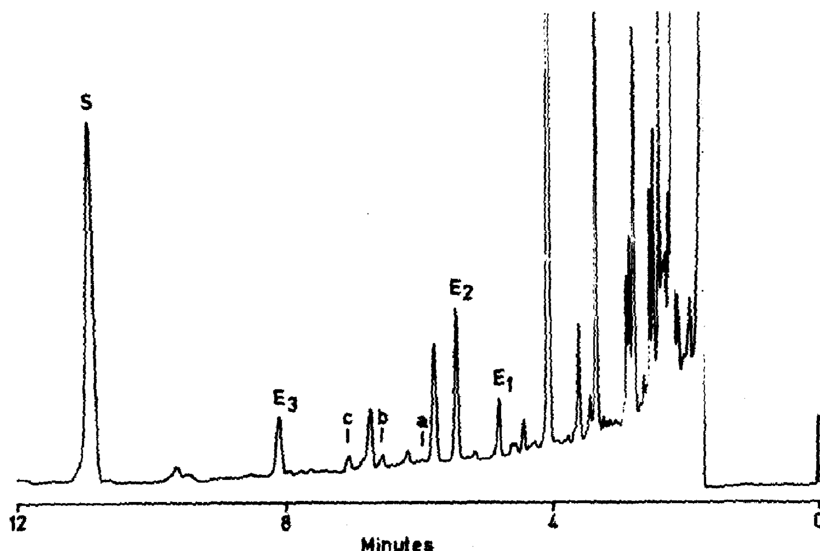

Fig. 4. Gas chromatographic analysis of TMS ethers of unconjugated estrogens from plasma of a pregnant woman (40th week). Peaks E_1, E_2 and E_3 indicate the derivatives of estrone, estradiol and estriol, respectively, and the letters a, b and c the retention times of the derivatives of 2-methoxyestrone, 16α-hydroxyestrone and 16-ketoestradiol. The steroids were identified by GC–MS analysis, and the concentrations of estrone, estradiol and estriol were 11.1, 25.8 and 16.8 ng/ml, respectively. The concentrations of 16α-hydroxyestrone and 16-ketoestradiol were about 1 and 2 ng/ml, respectively. Only trace amounts (<0.2 ng/ml) of 2-methoxyestrone were detected. Glass capillary column, 20 m × 0.3 mm, SE-30, 260°, carrier gas inlet pressure 0.5 kp/cm². S = internal standard.

2-methoxyestrone, 16α-hydroxyestrone, and 16-ketoestradiol in pregnancy plasma (Table 3) [30]. The concentrations of estrone, estradiol and estriol in plasma from a pregnant woman (40th week) were about 11, 26 and 17 ng/ml, respectively (Fig. 4). These values are within previously reported concentration ranges for these steroids [31]. As shown by Fig. 4, the purity of the estrogen fraction is sufficiently high to permit analysis of the major estrogens by G.L.C. using an FID.

The application to analysis of unconjugated steroids in plasma from pregnant women shows that the method described is capable of providing new information about the composition of circulating steroids. The physiological role of the saturated pregnane derivatives found in this study cannot yet be evaluated. However, the presence of relatively high concentrations of 3β-hydroxy-5α-pregnan-20-one is of importance since this steroid interferes in analyses of pregnenolone by RIA [32]. This shows the importance of GC–MS as a complement to RIA analysis. GC–MS should be used to define the steroid composition of a sample and RIA may then be used to study variations of selected steroids in a larger number of samples.

Acknowledgements—The technical assistance of Mrs. B. Mörk, Mr. T. Pettersson and Mr. R. Reimendal is gratefully acknowledged. Plasma samples from pregnant women were kindly collected by personnel at the Solna Prenatal Clinic. The studies were supported by grants from the Swedish Medical Research Council (grant No. 13X-219), Magn. Bergvalls Stiftelse, Karolinska Institutet, Svenska Sällskapet för Medicinsk Forskning and the World Health Organization.

REFERENCES

1. Axelson M. and Sjövall J.: *J. steroid Biochem.* **5** (1974) 733–738.
2. Horning E. C., Maddock K. C., Anthony K. V. and VandenHeuvel W. J. A.: *Analyt. Chem.* **35** (1963) 526–532.
3. Axelson M.: *J. steroid Biochem.* **8** (1977) 597–602.
4. Axelson M. and Sjövall J.: *J. Chromatogr.* **126** (1976) 705–716.
5. Setchell K. D. R., Almé B., Axelson M. and Sjövall J.: *J. steroid Biochem.* **7** (1976) 615–629.
6. Ellingboe J., Nyström E. and Sjövall J.: *J. lipid Res.* **11** (1970) 266–273.
7. Ellingboe J., Almé B. and Sjövall J.: *Acta chem. scand.* **24** (1970) 463–467.
8. Almé B. and Nyström E.: *J. Chromat.* **59** (1971) 45–52.
9. Setchell K. D. R. and Shackleton C. H. L.: *Clin. chim. Acta* **47** (1973) 381–388.
10. Thenot J. P. and Horning E. C.: *Analyt. Lett.* **5** (1972) 21–33.
11. Rutten G. A. F. M. and Luyten J. A.: *J. Chromat.* **74** (1972) 177–193.
12. Van den Berg P. M. J. and Cox T. P. H.: *Chromatographia* **5** (1972) 301–304.
13. Reimendal R. and Sjövall J.: *Analyt. Chem.* **44** (1972) 21–29.
14. Ryhage R.: *Q. Rev. Biophys.* **6** (1973) 311–335.
15. Axelson M., Cronholm T., Curstedt T., Reimendal R. and Sjövall J.: *Chromatographia* **7** (1974) 502–509.
16. Bradlow H. L.: *Steroids* **11** (1968) 265–272.
17. Makino I. and Sjövall J.: *Analyt. Lett.* **5** (1972) 341–349.
18. Nyström E. and Sjövall J.: In *Methods in Enzymology* (Edited by J. M. Lowenstein). Academic Press, New York (1975) pp. 378–395.
19. Nyström E.: *Arkiv Kemi* **29** (1968) 99–106.
20. Eberlein W. R.: *Steroids* **14** (1969) 553–573.
21. Axelson M., Sjövall J., Adlercreutz H., Taylor N. and Setchell K. D. R.: (in preparation).

22. Schulze P. and Kaiser K. H.: *Chromatographia* **4** (1971) 381–387.
23. Maume B. F. and Luyten J. A.: *J. chromat. Sci.* **11** (1973) 607–610.
24. Sjövall K.: *Ann. clin. Res.* **2** (1970) 393–408.
25. Sjövall J. and Vihko R.: *Acta chem. scand.* **20** (1966) 1419–1421.
26. Chew P. C. T. and Ratnam S. S.: *J. Endocr.* **69** (1976) 163–164.
27. Sribastava L. S., Werk Jr. E. E., Thrasher K., Sholiton L. J., Kozera R., Nolten W. and Knowles Jr. H. C.: *J. clin. Endocr. Metab.* **36** (1973) 937–943.
28. Støa K. F. and Bessesen A.: *J. steroid Biochem.* **6** (1975) 21–25.
29. Apter D., Jänne O. and Vihko R.: *Clin. chim. Acta* **63** (1975) 139–148.
30. Adlercreutz H., Martin F., Wahlroos Ö. and Soini E.: *J. steroid Biochem.* **6** (1975) 247–249.
31. Jakowicki J., Ervast H.-S. and Adlercreutz H.: *J. steroid Biochem.* **4** (1973) 181–191.
32. Brenner P. F., Guerrero R., Cekan Z. and Diczfalusy E.: *Steroids* **22** (1973) 775–794.