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SEPARATION AND COMPUTERIZED GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF UNCONJUGATED NEUTRAL STEROIDS IN PLASMA

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SUMMARY

A method is described for analysis of unconjugated neutral steroids in plasma. It is based on extraction of the sample with Amberlite XAD-2 at elevated temperature, purification of the extract on lipophilic ion exchanging and neutral Sephadex derivatives, and gas chromatography–mass spectrometry–computer analysis of O-methyloxime-trimethylsilyl ether derivatives purified on hydrophobic Sephadex. Examples of the application of the method to analysis of steroids in plasma from pregnant women are given.

INTRODUCTION

Many methods have been described for analysis of unconjugated steroids in plasma. In these methods the steroid to be analyzed has to be selected prior to analysis. However, knowledge of the nature of steroids present in samples from patients may be too incomplete to permit an appropriate choice of steroid and important information about steroid metabolism may be lost. The aim of the present study has been to devise methods for an unbiased search for unconjugated neutral steroids in plasma from patients. Computerized gas chromatography–mass spectrometry (GC–MS) has been used as a final analytical step, and purification of steroids as a group has been based on the use of neutral and ion exchanging derivatives of lipophilic Sephadex [1–3].

EXPERIMENTAL

Glass-ware was washed in an ultrasonic bath. All solvents were redistilled.

Tritium-labelled steroids were obtained from the Radiochemical Centre (Amersham, England) or NEN Chemicals (Dreieichenhein, Germany). The specific activities were between 20 and 300 mCi/mmol. Radioactivity was determined in a Packard liquid scintillation spectrometer model 3003, using Instagel^{R} as scintillation liquid.

Unsieved, coarse *Amberlite XAD-2* was from Rohm and Haas (Philadelphia). It was washed in batches in columns with large volumes of water, ethanol, acetone and water and was stored in water until used.

Lipidex^{R}-5000 was from Packard–Becker (Groningen). Its polarity corresponds to that of 50% (w/w) alkylated Sephadex LH-20[2]. It was stored in dark bottles in ethanol containing 0·1% of BHT antioxidant. Immediately before use it was washed on a Buchner funnel with 30 ml of ethanol and with 10 ml/g gel of the solvent to be used in the chromatography.

Diethylaminohydroxypropyl Sephadex–LH-20 (DEAP–LH-20; capacity about 1·5 meq/g) was prepared from chlorohydroxypropylated Sephadex LH-20 [3, 4] and was kindly supplied by Dr. B. Almé. It was washed with 0·2 M NaOH in 75% aqueous ethanol and 75% ethanol, and was then dried. It was stored at −18°C and was used in the OH^- form.

O-Methyloxime–trimethylsilyl ether (MO–TMS) derivatives were prepared with methoxyamine hydrochloride (Eastman Organic Chemicals) and trimethylsilylimidazole (Pierce Chemical Co.) at elevated temperatures[5].

Hexamethyldisilazane (Applied Science Laboratories) was redistilled prior to use in chromatography.

Gas chromatography–mass spectrometry was carried out using an LKB 9000 instrument equipped with units for automatic time programmed magnet scanning and repetitive accelerating voltage scanning[6]. A 3 m × 3·5 mm glass column packed with 1·5% SE-30 on Chromosorb WHP, 80–100 mesh, was used at 225°C. Temperatures of molecule separator and ion source were 260°C and 290°C, respectively. The ionization energy was 22·5 eV; current 60 μA. Spectra (m/e 1–660, taken every 6·3 s) were recorded on an incremental tape recorder[7]. This mode of recording

could also be used with repetitive accelerating voltage scanning (scan rate 2 s) when values taken at a rate of 10 kHz were first added to give about 10 readings per mass spectrometric peak before being written on the tape. This interface unit was designed by Mr. R. Reimendal and Mrs. A. Lindow. The computer programs for presentation and evaluation of results recorded on tape were those described previously [6, 8, 9].

Extraction of plasma steroids. A column bed, 140 × 7·5 mm, of 4g Amberlite XAD-2 is prepared in a jacketed glass column containing 50 ml of 1 M hydrochloric acid. The column is left overnight and is then washed with water until neutral. Before application of the sample it is washed with 30 ml of ethanol and 50 ml of water. Water at 64°C is then circulated through the jacket. Ten ml of plasma is diluted with 10 ml of saline and is passed through the column at a rate of 17 ml/h. The column is then rinsed with 15 ml of water at the same flow rate, and the flow of water through the jacket is then stopped. The water contained in the column void volume is displaced with 12 ml of hexane. The steroids are eluted with 12 ml of methyl acetate–methanol, 2:1 (v/v), followed by 12 ml of methanol at a flow rate of 25 ml/h. The effluent is collected in a round-bottomed flask, solvents are removed *in vacuo* and the residue is dissolved in 0·5–1 ml of methanol–water–chloroform, 9:1:2 (by vol.) for filtration through the ion exchange column.

Purification of steroids

A DEAP-LH-20 column, 1·5 g, 410 × 4·3 mm, is packed by gravity flow in methanol–water–chloroform, 9:1:2 (by vol.). Prior to application of the sample it is washed with mobile phase. The sample is applied and is rinsed through the column at a flow rate of 4 ml/h. A total of 7·5 ml of effluent is collected in a round-bottomed flask and is evaporated *in vacuo*. The residue is dissolved in 0·5 ml of methanol–water–chloroform, 9:1:2 (by vol.) for filtration through Lipidex^{R}-5000.

Lipidex^{R}-5000 is packed under gravity flow in methanol–water–chloroform, 9:1:2 (by vol.), to give a column of 110 × 4·3 mm (this corresponds to about 0·5 g of dry gel). The column is washed with 30 ml of the solvent, and the sample is applied and is rinsed through the column at a flow rate of 5 ml/h. A total of 3·5 ml of effluent is collected in a Teflon test tube and solvents are removed under a stream of nitrogen.

Pyridine, 50 μl, and methoxyamine hydrochloride, 5 mg, are added. After heating at 60°C for 15 min the pyridine is evaporated and 50 μl of trimethylsilylimidazole is added[5]. After 2 h at 100°C 0·5 ml of hexane–pyridine–hexamethyldisilazane, 98:1:1 (by vol.), is added and the sample is filtered through a Lipidex column.

This column is prepared by gravity flow in hexane–pyridine–hexamethyldisilazone, 98:1:1, to give the dimensions 70 × 4 mm (corresponding to 0·25 g of dry gel), and is washed with 10 ml of the solvent prior to application of the sample. The sample is rinsed through the column at a flow rate of 6 ml/h. A total of 2 ml of effluent is collected in a small centrifuge tube, solvents are removed under a stream of nitrogen and the residue is dissolved in an appropriate volume of hexane for GC–MS analysis.

RESULTS

Extraction and purification of steroids

Conditions for extraction of unconjugated steroids from plasma by columns of Amberlite XAD-2 were studied by varying plasma dilution, ionic strength, pH, and temperature. Recoveries of 0·03–0·9 ng amounts of ^{3}H-labelled steroids dissolved in 2 ml of

Table 1. Recoveries of ^{3}H-labelled steroids added to 2 ml of plasma and extracted with 0·5–2 g columns of Amberlite XAD-2 at 64°C

Steroid[a]	Amount added (ng)	Number of experiments	Recovery, % of added ^{3}H ± SD	Loss in H_2O and hexane, % ± SD
A^{4}-17β-ol-3-one	0·04–0·15	6	92·2 ± 5·0	0·9 ± 0·2
A^{5}-3β-ol-17-one	0·37	8	96·0 ± 2·1	1·2 ± 0·3
P^{4}-3,20-one	0·20–0·60	11	90·4 ± 4·7	1·4 ± 1·4
P^{4}-20α-ol-3-one	0·10	7	93·4 ± 6·6	2·0 ± 1·1
P^{4}-17α-ol-3,20-one	0·03–0·21	7	92·9 ± 7·7	1·2 ± 0·9
P^{5}-3β-ol-20-one	0·30	7	84·1 ± 4·0	3·2 ± 1·7
5βP-3α,20α-ol	0·90	6	93·9 ± 4·4	0·3 ± 0·2
P^{4}-11β,21-ol-3,20-one	0·03–0·17	8	95·7 ± 3·8	1·5 ± 1·0
P^{4}-11β,17α,21-ol-3,20-one	0·03–0·17	6	92·0 ± 3·3	1·6 ± 1·0

[a] A = androstane, P = pregnane, superscript indicates position of double bond, greek letter denotes configuration of hydroxyl group.

plasma were optimal, 90–95%, when the column temperature was about 64°C (Table 1). About 0·4 g of Amberlite XAD-2 was required per ml of plasma, and the flow rate should be kept low during application of the sample.

Recoveries of neutral steroids during chromatography on DEAP–LH-20 has been found to be better than 95% (B. Almé and J. Sjövall, unpublished data). The average recovery of ^{3}H-labelled progesterone, 3β-hydroxy-5-pregnen-20-one, 5β-pregnane-3α,20α-diol and corticosterone added to extracts of bovine corpora lutea and subjected to chromatography on Lipidex^R-5000 was found to be 93·2 ± 7·7% in 20 experiments (M. Axelson *et al.*, to be published).

Various solvents were tested for purification of MO–TMS derivatives by chromatography on Lipidex^R-5000. Hydrolysis of trimethylsilyl ethers of 3β-hydroxy-5-pregnen-20-one and estradiol was not observed when hexane containing 1% of pyridine and 1% of hexamethyldisilazane was used. With this solvent the methyloxime of progesterone was somewhat retarded compared to other steroids studied. However, a small column could be used which permitted collection of the derivatives of all steroids in a small effluent volume before the appearance of contaminating plasma compounds and reagents. Recoveries of subnanogram amounts of pure ^{3}H-labelled progesterone methoxime were 85–90% in 5 experiments. Three experiments with 0·1 ng of pure ^{3}H-labelled estradiol TMS ether gave recoveries of 90–91%. Difficulties were experienced with the conversion of 0·3 ng of ^{3}H-labelled cortisol into an MO–TMS derivative. Only about 60% of the radioactivity appeared in the appropriate fraction from the Lipidex^R column. When the experiment was repeated with 14 μg, and when 10 μg of progesterone were present, the recoveries were about 90%. The loss in experiments with tracer amounts may be due to solubility problems which are presently being studied.

The recovery of 1·2 ng of ^{3}H-labelled progesterone added to 10 ml of plasma from a woman in the third trimester and carried through the entire procedure was 82 and 84% in two experiments. Corresponding figures in two experiments with 0·8 ng of 3β-hydroxy-5-pregnen-20-one were 79 and 90%.

Gas chromatography–mass spectrometry–computer analysis

The purity of the final sample containing the steroid MO–TMS derivatives is high, and aliquots corresponding to an original plasma volume of 5 ml can readily be injected without overloading a packed column. Figure 1 shows an analysis of steroids in plasma from a woman in the 29th week of pregnancy. A search was made for precursors and metabolites of progesterone, and the computer constructed fragment ion current (FIC)[9] chromatograms of m/e values characteristic of derivatized structures present in progesterone, pregnenolones, pregnanolones and pregnanediols. The presence of progesterone and 20α-hydroxy-4-pregnen-3-one is clearly seen from coinciding peaks in FIC chromatograms characteristic of 20-MO, 20-TMS and 3-MO-Δ^4 structures. There are derivatives of at least two pregnanolone and two pregnanediol isomers and of a pregnenolone which does not have the retention time of the MO–TMS derivative 3β-hydroxy-5-pregnen-20-one. By repetitive acceleration voltage scanning of the m/e range 415–420, at a 100 times higher amplification, the low intensity molecular ions of pregnanolone (m/e 419) and pregnenolone (m/e 417) derivatives could be clearly seen.

A simplified picture of the occurrence of structures mentioned above is obtained by conversion of each set of FIC chromatograms characteristic of a certain structure into a CIC (combined ion current) chromatogram. This involves multiplication of the intensity values in each scan of *n* characteristic FIC chromatograms and extraction of the *n*th root of the product. This yields a single chromatogram where simultaneous occurrence of peaks in the original FIC chromatograms is emphasized[9]. The CIC chromatograms obtained from the FIC chromatograms shown in Fig. 1 are seen in Fig. 2, which also shows the CIC chromatograms obtained in an analysis of a plasma sample from a woman in the 25th week of pregnancy. The results of the two analyses are similar. However, it

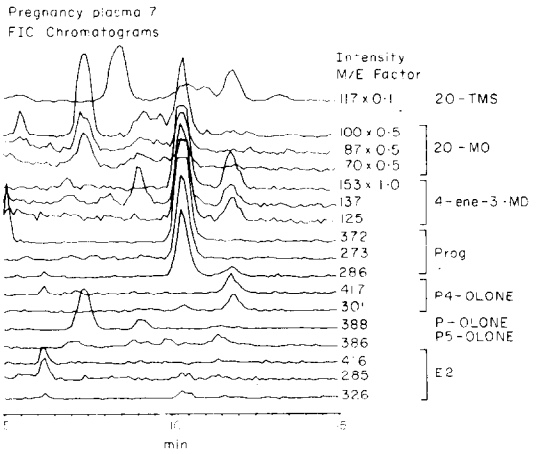

Fig. 1. Fragment ion current (FIC) chromatograms constructed by the computer in an analysis of steroids in plasma from a woman in the 29th week of pregnancy. M/e values characteristic of structures in progesterone and some of its precursors and potential metabolites were selected. For purposes of illustration the intensities of some chromatograms were multiplied by the factor shown to the right of the m/e values.

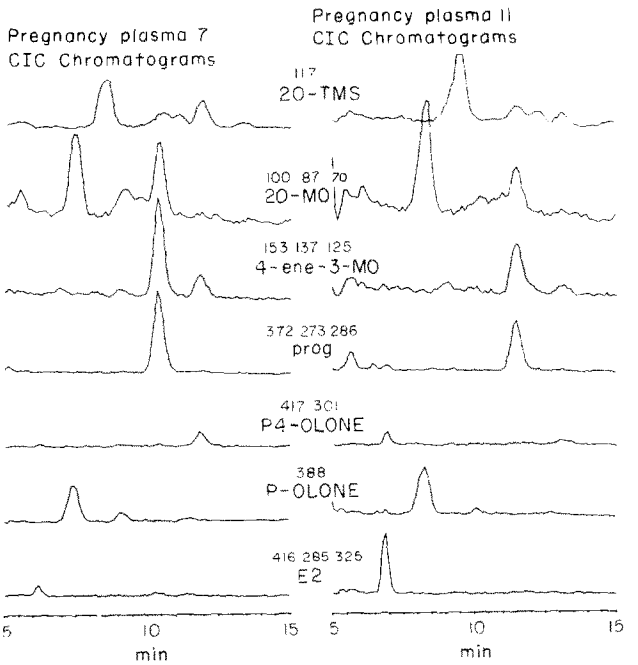

Fig. 2. Combined ion current (CIC) chromatograms (see text) constructed by the computer from the FIC chromatograms shown in Fig. 1 (left curves), and from the FIC chromatograms obtained in an analysis of steroids in plasma from a woman in the 25th week of pregnancy (right curves). The m/e values of ions included in the calculation of each chromatogram are given above the abbreviation of the structural feature for which they are characteristic.

is clear that the relative concentration of pregnanolone(s) is higher in the latter sample (no. 11).

An internal standard, 5β-cholestane-3β-ol TMS ether, was added to sample 11 and to a series of known amounts of MO–TMS derivatives of reference steroids, and the samples were analyzed by GC–MS. The computer calculated peak areas in the 3–10 most intense (or structurally significant) FIC chromatograms of each reference and plasma compound. The area of a peak by the internal standard served to normalize the results to a common injection volume, and permitted a direct comparison of areas given by known amounts of reference steroids and unknown amounts of steroids in the sample. The following steroid concentrations were obtained (ng/ml, corrected for 15% loss in the 'work-up' of sample): progesterone 61·1, 20α-hydroxy-4-pregnen-3-one 4·7, 3α-hydroxy-5β-pregnan-20-one (tentative configuration, possibly a mixture of 5β and 5α isomers) 15, and 3β-hydroxy-5α-pregnan-20-one 1·8. After the completion of these analyses the concentration of 5α-pregnane-3,20-dione was determined to be 3·8 ng/ml.

As expected, both samples gave prominent peaks in FIC chromatograms characteristic of the MO–TMS derivative of cortisol (Fig. 3). The concentration of cortisol in sample 11, determined as described above, was 110 ng/ml, corrected for a loss of 50% in the 'work-up' of the sample. The reasons for the large losses of cortisol are presently being studied (see above).

DISCUSSION

A variety of solvent extraction methods have been used for the isolation of unconjugated steroids from plasma. In order to recover the polar steroids, polar solvents have to be used which extract large quantities of interfering material from plasma and frequently give emulsion problems. The latter problem is avoided in the method recently described by Stillwell *et al.*[10], and this method also has the advantage that small solvent volumes can be used.

Extraction of urinary steroids with Amberlite XAD-2 [11] is a very convenient method which provides simultaneous purification of the sample. If conditions are chosen which minimize protein binding, bile acids in plasma may be extracted with Amberlite XAD-2[12] and very clean extracts are obtained. In the present method, protein binding of steroids is minimized by the use of a high temperature[13]. Some non-specific loss of steroid occurs (see Table 1), and our method is not as rapid as that of Stillwell *et al.*[10]. However, these disadvantages are counteracted by the fact that a very clean extract is obtained. The necessity of using

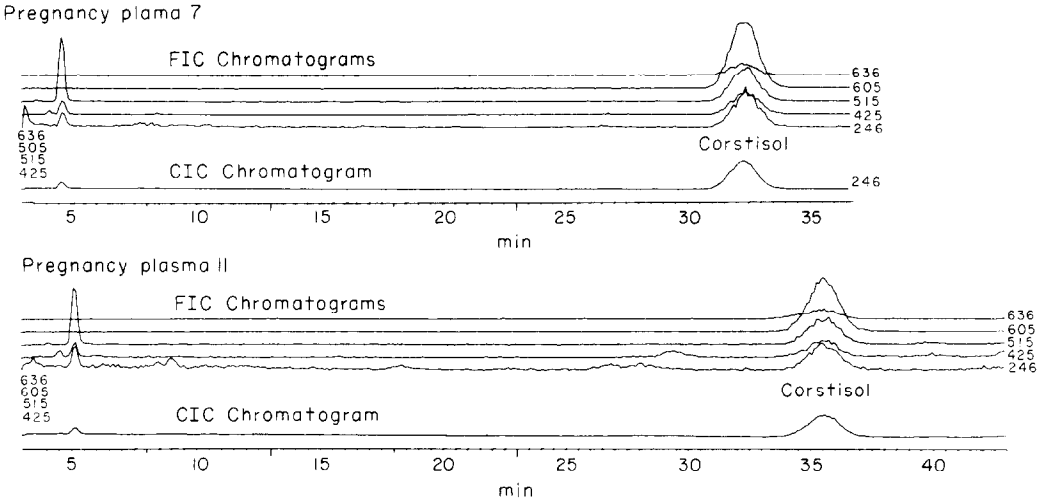

Fig. 3. FIC and CIC chromatograms characteristic of the MO-TMS derivative of cortisol obtained in analyses of steroids in plasma from two pregnant women.

thoroughly washed resin should be emphasized.

Subsequent purification of the extract is based on the use of lipophilic Sephadex derivatives[1–3]. Solvent systems have been selected in which the extracts are readily soluble and in which all steroids are eluted essentially with the front in small solvent volumes, while specific groups of interfering compounds are retained on the columns. This principle of purification provides high speed and simplicity of operation.

In a first column, lipids with anionic groups are removed. Besides increasing extract mass, the presence of such compounds may make the final purification of MO–TMS derivatives difficult. Thus, TMS esters are readily hydrolyzed when the silylating reagents are removed and the acids formed catalyze hydrolysis of steroid TMS ethers. Filtration of the extract through an anion exchange column was therefore found to be an essential step. An ion exchanger based on lipophilic Sephadex was chosen since yields of steroids have been found to be better with these materials than with conventional ion exchange resins (including Amberlyst A-26) in organic solvents (B. Almé and J. Sjövall, unpublished results).

Filtration of the extract through a column of Lipidex^{R}-5000 in a reversed phase system[2] removes nonpolar lipids including cholesterol, a small fraction of which is extracted by Amberlite XAD–2.

The method described by Thenot and Horning[5] is superior to other procedures described for preparation of MO–TMS derivatives. It is rapid and appears to give single derivatives (although syn and anti isomers) of all common steroids. This method was therefore chosen. Filtration of the MO–TMS reaction mixture through Lipidex^{R}-5000 in hexane removed the reagents. In addition, interfering material which had followed the steroids in previous purification steps was removed. It is likely that this was material with polarity similar to that of steroids, and that the MO–TMS reaction did not convert it into compounds having the same low polarity as steroid MO–TMS derivatives. The latter could therefore be eluted ahead of interfering material. Recoveries of the cortisol derivative were unsatisfactory and the reasons for the loss of this derivative is presently being studied.

The extraction and purification procedure can be completed in one day. A very clean extract is obtained and sample sizes equivalent to 5 ml of plasma can readily be injected into packed columns without risks of overloading or damage of the column. The low weight of the extract and the absence of high-boiling silylation reagents also makes it possible to use a solid injection system with glass capillary columns [14, 15] for the gas chromatographic analysis (to be published).

Repetitive scanning GC–MS[16] and recording of spectra on magnetic tape provides numerous possibilities for computer evaluation of the results of the analysis[6, 8, 9]. The tape may be processed repeatedly, and rapid searches for specific structures and quantitative determinations based on any specific FIC chromatogram may be performed. The examples presented in this paper of analyses of neutral steroids in plasma from pregnant women point to the presence of pregnanolone and pregnanediol isomers not previously considered in investigations of unconjugated plasma steroids in pregnancy. Steroids of this type are present in high concentration as mono- and disulphates during pregnancy[17]. However, the relative amounts of different compounds are quite different from those found in the present study. Therefore the

unconjugated steroids are unlikely to represent artefacts due to nonspecific hydrolysis of sulphates during 'work-up' of the samples. The physiological importance of these steroids remains to be established. It is obvious that the specificity of radioimmunoassay methods may be reduced by the presence of unexpected steroid metabolites. Unbiased GC–MS–COM analysis of steroids in biological materials can provide positive evidence in favour of the specificity of radioimmunoassay procedures where evidence of a negative type frequently has had to be used.

The sensitivity of the GC–MS–COM analysis depends on the nature of the steroid and its mass spectrum. With the equipment used in the present study repetitive magnetic scanning is usually used with 5–500 ng amounts, and repetitive acceleration voltage scanning of a limited mass range with 10 pg–10 ng samples. This sensitivity is comparable to that obtained by single or multiple ion detection (see [18]). At present the sensitivity is limited by the intensity of stationary phase background peaks and by losses of steroid derivatives in the g.l.c. column and in the interface to the mass spectrometer.

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DISCUSSION

Siekmann:

Why is there less sensitivity when your mass fragmentograph is scanning magnetically than when it is scanning with the high voltage?

Sjövall:

With the acceleration voltage we scan repetitively over a narrow *m/e* range in 2–5 s and have a 10 kHz sampling rate. This allows us to use analog and digital filtration which improves signal/noise ratio so that a higher amplification can be used. With the magnetic scanning we cover the entire *m/e* range in 2–5 s and take only one reading per mass spectrometric peak. This gives a much less favourable signal/noise ratio.