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SELECTIVE LIQUID CHROMATOGRAPHIC ISOLATION PROCEDURE FOR GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF 3-KETOSTEROIDS IN BIOLOGICAL MATERIALS

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SUMMARY

A method for the isolation of 3-ketosteroids based on the positive charge of their oximes is described. The biological extract is filtered through a column of sulphoethyl Sephadex LH-20 (H^+). Steroids in the filtrate are converted into oximes and the dried reaction mixture is filtered through a column of diethylaminohydroxypropyl Sephadex LH-20 (OH^-) in methanol. After evaporation of the solvent and hydroxylamine, the oximes are taken up by and separated on a column of sulphoethyl Sephadex LH-20 (H^+) in methanol. Following elution of other steroids, the oximes of 3-ketosteroids are eluted as a group with methanol–pyridine (20:1, v/v), and are converted into trimethylsilyl ethers. Removal of reagents and further purification of the sample is achieved by rapid filtration through Lipidex 5000 in *n*-hexane–pyridine–hexamethyldisilazane–dimethoxypropane (97:1:2:10). The derivatives are then analyzed by computerized gas chromatography–mass spectrometry using open-tubular glass capillary columns. Recoveries of picogram amounts of ^3H-labelled steroids carried through the entire isolation procedure are 80–90%. The purification achieved in analyses of plasma permits solid injection of the equivalent of 1–2 ml of plasma without overloading of the capillary column. The principle of the isolation procedure might be applicable to other groups of compounds that possess keto or aldehydo groups.

INTRODUCTION

Most of the biologically active neutral steroids possess a 3-keto group. Many methods exist for the analysis of individual 3-ketosteroids but there is no method which permits the isolation of this entire group of steroids for further analysis by procedures that separate the individual compounds. Such a method would be of obvious value as it would permit an unbiased analysis of 3-ketosteroid profiles in biological materials under different physiological conditions.

In the course of studies on the use of ion exchangers for the purification of steroids and steroid derivatives from biological materials[1,2], it was observed that the positive charge of O-methyloximes[3] of some 3-keto-Δ^4-steroids was sufficiently strong to permit sorption by a cation exchanger. This paper describes a quantitative isolation

and purification procedure for picogram amounts of 3-ketosteroids based on this principle. The final analytical step consists in repetitive scanning gas chromatography–mass spectrometry using open-tubular glass capillary columns.

EXPERIMENTAL

Glassware, solvents and reagents

All glassware was silanized with dimethyldichlorosilane in toluene. Cleaning was carried out in an ultrasonic bath.

All solvents were of reagent grade and were re-distilled twice in an all-glass system with 1-m distillation columns. Methanol was stored over sodium hydroxide prior to re-distillation to remove contaminating acids. Hexamethyldisilazane and trimethylchlorosilane (Applied Science Labs., State College, Pa., U.S.A.) were re-distilled, and trimethylsilylimidazole (Supelco, Bellefonte, Pa., U.S.A.) was used as supplied. Hydroxylammonium chloride (Fluka, Buchs, Switzerland) was recrystallized from 70% aqueous methanol.

Column packing materials

Amberlite XAD-2 (Rohm and Haas, Philadelphia, Pa., U.S.A.) was washed in the following way: 200 g was kept in 2 l of 2 *M* sodium hydroxide solution in 50% aqueous ethanol overnight at 39°; it was then washed in a chromatographic column with 2 l each of water, 2 *M* hydrochloric acid, water, acetone, ethanol and water, and was then refluxed for 4 h in ethanol and 4 h in water. Fines were removed by decantation or by back-washing of the column. The resin was finally washed with re-distilled ethanol and was stored in this solvent until used.

Lipidex® 5000 (Packard, Downers Grove, Ill., U.S.A.) was washed prior to use. Amounts of 50–100 g were stirred for 1 h at 70° in 1 l of 20% aqueous ethanol. After filtration on a Büchner funnel, similar extractions were carried out with 50% aqueous ethanol and ethanol. The gel was finally washed and stored in re-distilled methanol in dark bottles at 4°. Immediately before use, the appropriate amount of gel was washed on a Büchner funnel with absolute ethanol (20 ml per gram of gel) and *n*-hexane (20 ml/g), 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[4]. The capacity of the gel was about 0.10 mequiv./g. The Na^+ form of the gel was washed as described above for Lipidex 5000 and was stored at 4° in methanol. Prior to use, a few grams were washed on a Büchner funnel with 100 ml each of 0.3 *M* hydrochloric acid in 72% aqueous ethanol, 20% aqueous ethanol (until neutral), absolute ethanol and re-distilled methanol.

Diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20) was prepared and washed as described previously[4,5]. The Cl^- form of the gel, prepared by washing the gel with 0.25 *M* hydrochloric acid in 72% aqueous ethanol, was washed as described above for Lipidex 5000 and was then converted back into the acetate form and stored in a dry form at −20°. Prior to use, the gel was converted into the OH^- form by washing 1–3 g on a Büchner funnel with 100 ml each of 0.3 *M* sodium hydroxide solution in 72% ethanol, 20% aqueous ethanol (until neutral), absolute ethanol and re-distilled methanol.

Steroids

Unlabelled steroids were purchased from Ikapharm (Ramat-Gan, Israel) and kindly donated by Dr. J. Babcock (Upjohn, Kalamazoo, Mich., U.S.A.) and the MRC Reference Steroid Collection (Professor W. Klyne and Dr. D. N. Kirk).

Tritium-labelled steroids (1,2,6,7-$^3H_4$, 80–110 Ci/mmole except for 17α-hydroxyprogesterone, which was 7-^3H, 10 Ci/mmole) were obtained from the Radiochemical Centre (Amersham, Great Britain). Radioactivity was determined in a Packard Model 3003 liquid scintillation spectrometer using Instagel as the scintillation liquid.

Preparation of oximes

Oximes were prepared as described by Thenot and Horning[6,7]. A 5-mg amount of hydroxylammonium chloride and 50 μl of pyridine were added to the dried sample in a glass-stoppered centrifuge tube and heated at 60° for 30 min. The pyridine was then removed under a stream of nitrogen.

The residue was dissolved in 1 ml of methanol and applied to a column of DEAP-LH-20 (OH^-), *ca.* 400 mg, 150 × 4 mm packed in methanol under gravity flow. The sample was allowed to pass through the column at a flow-rate of 10 ml/h. The reaction tube was washed with 1-ml portions of methanol, which were also passed through the column until 5 ml of effluent had been collected in a round-bottomed flask. The solvent was removed *in vacuo*.

As an alternative to the DEAP-LH-20 column step, the reaction mixture can be diluted with 1 ml of water and extracted three times with 1 ml of ethyl acetate. However, in work with biological extracts, the DEAP-LH-20 filtration procedure is preferred as extraction losses do not occur and sample purification is achieved.

Chromatography of oximes on SE-LH-20

The residue from the DEAP-LH-20 column effluent was dissolved in 1 ml of methanol and applied to a column of SE-LH-20 (H^+), *ca.* 250 mg, 100 × 4 mm, packed in methanol under gravity flow. The flask was rinsed with methanol, which was also passed through the column, and elution with methanol was continued at a flow-rate of about 12 ml/h with collection of 0.5–1-ml fractions. When oximes of 3-ketosteroids were to be isolated as a group, the first 10 ml of effluent were discarded and the column was eluted with 3 ml of methanol–pyridine (20:1), to recover these steroids. The solvent was evaporated under a stream of nitrogen.

Preparation and purification of oxime trimethylsilyl ether derivatives

A 5-mg amount of hydroxylammonium chloride (as catalyst), 50 μl of pyridine and 50 μl of trimethylsilylimidazole were added to the sample of steroid oximes, and the mixture was heated at 100° for 2.5 h, as described by Thenot and Horning[6,7]. The solution was then diluted with 1.0 ml of *n*-hexane–pyridine–hexamethyldisilazane–dimethoxypropane (97:1:2:10) and rapidly filtered through a column of Lipidex 5000 by applying pressure of 0.5 kp/cm^2. The column, *ca.* 250 mg, 70 × 4 mm, was packed under gravity flow and washed with 10 ml of solvent prior to sample application. The reaction tube was rinsed with the same solvent in 0.5-ml portions and elution was continued at a flow-rate of 3 ml/min. The steroid derivatives were recovered in the first 2.5 ml of effluent, which were collected and stored at room

temperature in a stoppered centrifuge tube until required for analysis by gas chromatography and mass spectrometry.

As an alternative to this silylation procedure, when hindered hydroxyl groups are not present in the steroids, the oxime derivatives can be dissolved in pyridine–hexamethyldisilazane–trimethylchlorosilane (3:2:1), and left to react for 30 min at room temperature. The reagents are then removed under a stream of nitrogen.

Gas–liquid chromatography (GLC)

GLC was carried out using a Pye 104 gas chromatograph equipped with a flame-ionization detector, and housing a 20 m × 0.34 mm open-tubular glass capillary column coated with SE-30[8]. Solid injection in an all-glass system[9] was employed. 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. The oven temperature was 260°.

Computerized gas chromatography–mass spectrometry (GC–MS–COM)

GC–MS–COM was carried out using a modified LKB 9000 instrument[10]. A 25-m open-tubular glass capillary column coated with OV-1 was connected to the ion source via a single-stage adjustable jet separator. Other details of the connection between the capillary column and the ion source will be published elsewhere.

Methods for computerized evaluation of the mass spectral data recorded on tape have been described[11]. The temperature of the column was programmed from 235° to 275° at 0.6°/min, and the temperatures of the separator and ion source were 225° 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* 0–800, 10 scans/min) were recorded after a suitable delay following injection of the sample.

Procedure for analysis of 3-ketosteroids in plasma

The steroids in 10 ml of plasma were extracted on a 4-g Amberlite XAD-2 column at 64° as described previously[2]. The column was packed in ethanol and was then washed with 50 ml of water prior to sample application. When the sample had passed through the column the latter was washed with 20 ml of water and 8 ml of *n*-hexane. The steroids were eluted according to the original procedure[2].

After evaporation of the solvents, the extract was dissolved in 1 ml of methanol and passed through a column of SE-LH-20 (H^+), *ca.* 500 mg, 200 × 4 mm, packed by gravity flow in methanol. A flow-rate of 7 ml/h was used. The flask containing the extract was rinsed with methanol, which was also passed through the column until 6 ml of effluent had been collected. The methanol was evaporated *in vacuo*.

The residue was transferred to a stoppered centrifuge tube and oximes were prepared using the DEAP-LH-20 column for purification. Oximes of 3-ketosteroids were isolated as a group by chromatography on SE-LH-20, and trimethylsilyl ether derivatives were prepared using trimethylsilylimidazole and purification on Lipidex 5000 as described above. The sample was then analyzed by GC–MS–COM.

RESULTS AND DISCUSSION

Separation of oximes of ketosteroids on sulphoethyl Sephadex LH-20

Originally, it was observed that the O-methyloxime of progesterone was taken

up by the strong cation exchanger Amberlyst A-15 (H^+) in methanol. However, O-methyloximes of more polar 3-keto-Δ^4-steroids were only incompletely sorbed, and quantitative conditions could not be achieved. Further studies were therefore carried out in which different ion exchangers, solvents and oxime derivatives were tested. It was found that a strong cation exchanger was required and that a non-aqueous solvent had to be used. Simple (unsubstituted) oximes were most strongly sorbed and the method was therefore based on this derivative. As an organic solvent had to be used, the lipophilic ion exchanger sulphoethyl Sephadex LH-20 was selected. Previous work has shown that adsorptive losses on lipophilic Sephadex derivatives are minimal and ion-exchange processes in organic solvents are rapid[12]. The selection of methanol as the solvent was based on the solubility properties of steroids, the swelling of lipophilic Sephadex and the chromatographic behaviour of the oximes.

The mobilities of the oximes of a number of steroids on columns of SE-LH-20 in methanol are given in Table I. Based on the elution volumes of their oximes, the monoketosteroids can be divided into three main groups in the following order of retention: 3-keto-Δ^4 > 3-keto (saturated) > 20-keto, 17-keto and 7-keto. The simultaneous presence of two oxime groups increases the retention. The elution volume is also influenced by the presence of neighbouring functional groups and by configuration. Thus, the oximes of 20-ketosteroids are less retarded when a 16,17-double bond or 17α- and 21-hydroxyl groups are present. In the absence of such effects, oximes of the 7-, 17- and 20-ketosteroids studied are eluted in this order.

As expected from previous work on simple and substituted oximes of ketosteroids[13,14], geometric isomers of the *syn/anti* type are formed from the 3-ketosteroids. The isomeric O-methyloximes of 3-keto-Δ^4-steroids can be separated by thin-layer chromatography[13] and the present study has shown that the isomeric oximes are well separated on SE-LH-20 columns in methanol. The latter separation seems to depend on the presence of a 4,5-double bond, as the two isomers formed from the saturated 3-ketosteroids were not separated. The influence of the double bond is also seen in the relative proportions of the two isomers formed (Table I). It was therefore of interest to study the effect of the presence of a hydroxyl group at C-2. It can be seen from Table I that there was no indication of the formation of two isomeric oximes from 2α-hydroxytestosterone, and one predominant isomer was formed from 2β-hydroxytestosterone. The oxime of 2α-hydroxytestosterone was retained much longer than the major oxime from 2β-hydroxytestosterone on the SE-LH-20 column, and the retention volumes of these two oximes were about the same as those of the *syn/anti* isomers formed from testosterone. However, the minor isomer of 2β-hydroxytestosterone was eluted rapidly and this isomer will therefore be lost in the procedure for the analysis of 3-ketosteroids in biological materials. As the separations are most likely based on an ion-exchange process, the differences in mobility of the isomers may be due to interactions between the hydroxyl group of the oxime and neighbouring functional groups, resulting in differences in the positive charge of the two isomers. As the *syn/anti* isomers of the oxime of testosterone can now be separated on a preparative scale, it may be possible to use NMR spectrometry to reveal interactions between the hydroxyl group of the oxime and neighbouring protons in the two isomers.

As mentioned above, the water content of the solvent used in the SE-LH-20 chromatography has to be kept low. The marked influence of the addition of water

TABLE I

ELUTION VOLUMES OF STEROID OXIMES ON COLUMNS OF SULPHOETHYL SEPHADEX LH-20 (CAPACITY ABOUT 0.10 mequiv./g) IN METHANOL AND RETENTION TIMES RELATIVE TO THAT OF CHOLESTANE OF THE OXIME TRIMETHYLSILYL ETHER DERIVATIVES ON AN OPEN-TUBULAR GLASS CAPILLARY COLUMN (SE-30) AT 260°

Position of oxime	*Steroid**	*Column length*** *(mm)*	*Peak elution volume*		*Relative retention time*	*Relative amounts of isomers (%)*
			ml	*Col. vols.****		
—	Cholesterol	100	1.0– 2.0	0.8– 1.6	—	—
—	Estradiol	100	1.0– 2.0	0.8– 1.6	—	—
C-20	5β-P-3α,11β,17α,21-ol-20-one	100	1.0– 2.0	0.8– 1.6	1.93	—
C-20	P^5-3β,17α-ol-20-one	100	1.5– 2.0	1.2– 1.6	1.49	—
C-20	$P^{5,16}$-3β-ol-20-one	100	1.5– 2.0	1.2– 1.6	1.16	—
C-7	C^5-3β-ol-7-one	100	2.0– 2.5	1.6– 2.0	3.30	—
C-17	A^5-3β-ol-17-one	100	3.0– 3.5	2.4– 2.8	0.78	—
C-20	5β-P-3α,21-ol-11,20-one§	100	4.0– 4.5	3.2– 3.6	1.79	—
C-20	P^5-3β-ol-20-one	100	4.5– 5.0	3.6– 4.0	1.24	—
C-3	5α-A-17β-ol-3-one	100	10.0–11.0	8.0– 8.8	0.88	49
			10.0–11.0	8.0– 8.8	0.84	51
C-3, C-17	5β-A-3,17-one	20	6.0– 7.0	24.0–28.0	0.89	48
			6.0– 7.0	24.0–28.0	0.86	52
C-3, C-20	5α-P-3,20-one	100	>30.0	>24.0	1.78	40
			>30.0	>24.0	1.68	60
C-3	A^4-17β-ol-3-one	100	25.0–30.0	20.0–24.0	0.91	70
			>30.0	>24.0	0.88	30
C-3	A^4-17β-ol-3-one	20	4.0– 5.0	16.0–20.0	0.91	70
			12.0–13.0	48.0–52.0	0.88	30
C-3	A^4-2β,17β-ol-3-one	20	1.0– 2.0	4.0– 8.0	1.32	15
			3.0– 4.0	12.0–16.0	1.17	85
C-3	A^4-2α,17β-ol-3-one	20	11.0–12.0	44.0–48.0	1.01	—
C-3	P^4-20α-ol-3-one	20	5.0– 6.0	20.0–30.0	1.50	70
			12.0–13.0	48.0–52.0	1.43	30
C-3, C-20	P^4-17α-ol-3,20-one	23	9.0–10.0	31.3–34.8	2.15	70
			>20.0	>69.6	1.96	30
C-3, C-20	P^4-11β,17α,21-ol-3,20-one	23	12.0–13.0	41.7–45.2	4.18	70
			>20.0	>69.6	3.99	30
C-3, C-17	A^4-3,17-one	25	14.0–15.0	44.8–48.0	1.13	70
			>20.0	>64.0	1.09	30
C-3, C-20	P^4-11β,21-ol-3,20-one	23	14.0–15.0	48.7–52.2	3.51§§	45
			>20.0	>69.6	3.51§§	40
					3.69	15
C-3, C-20	P^4-3,20-one	23	>20.0	>69.6	1.88	70
			>20.0	>69.6	1.81	30

* A = androstane; P = pregnane; C = cholestane; superscript indicates position of double bonds; Greek letter denotes configuration of hydroxyl groups.

** Column diameter 4 mm; a length of 100 mm corresponds to about 250 mg of SE-LH-20.

*** Elution volume (ml) divided by total column volume.

§ 11-Keto group underivatized. A minor isomer is not included.

§§ The major GC peak of corticosterone is eluted as two peaks on the SE-LH-20 column.

to the methanol on the mobility of the isomeric oximes of testosterone is shown in Table II. It is evident that the water content must be kept low for separations to occur. For comparison, mobilities in ethanol and acetone are also given. Although the oximes are retarded by the column, in acetone the peak is broad and no separation of the isomers is obtained.

If acids or bases are present in the methanol, there is no retention of the oximes. The addition of 5% of pyridine to the methanol provides a rapid means of recovering the oximes of 3-ketosteroids after the other steroids have been eluted.

TABLE II

EFFECT OF WATER ON THE MOBILITIES OF THE TWO ISOMERIC OXIMES OF TESTOSTERONE ON SE-LH-20 IN METHANOL

Mobilities in ethanol and acetone are given for comparison.

Solvent	*Water content (%)*	*Peak elution volume**	
		ml	*Col. vols.***
Methanol	<0.1	4.0– 5.0	16.0–20.0
		12.0–13.0	48.0–52.0
Methanol	0.1	3.0– 4.0	12.0–16.0
		>10	>40
Methanol	1.0	1.0– 2.0	4.0– 8.0***
		4.0– 5.0	16.0–20.0***
Methanol	5.0	0.0– 1.0	0.0– 4.0***
Ethanol	<1.0	1.0– 2.0	4.0– 8.0***
Acetone	<0.1	5.0– 6.0	20.0–24.0***

* The column was 20 × 4 mm, requiring 50 mg of gel in methanol or ethanol and 70 mg in acetone.

** See Table I.

*** Incomplete or no separation of the isomers.

Recoveries of tritiated steroids

The recoveries of steroids in the preparation of oximes and filtration through DEAP-LH-20, in the chromatography on SE-LH-20, and in the preparation of trimethylsilyl ether derivatives and filtration through Lipidex 5000 were tested with tracer amounts of ^{3}H-labelled steroids used individually or in mixtures. The results of experiments with a mixture of six 3-keto-Δ^4-steroids are shown in Table III. The mean recovery was 87% for the entire procedure, and experiments with individual steroids showed no selective losses.

A comment should be made concerning the purification of trimethylsilyl ethers of oxime and O-methyloxime derivatives of steroids on Lipidex 5000. In a previous study, difficulties were experienced when the O-methyloxime trimethylsilyl ether derivative of cortisol was filtered through a Lipidex 5000 column at a flow-rate of 6 ml/h, using as the solvent *n*-hexane–pyridine–hexamethyldisilazane (98:1:1). Filtration of the O-trimethylsilyl oxime trimethylsilyl ether derivative of the same steroid in this system gave a considerably higher yield (about 80%). The losses seem to be due to hydrolysis of trimethylsilyl ether groups in the derivatives. However, when dimethoxypropane was added to the solvent and the sample was rapidly filtered through the Lipidex column, hydrolysis was prevented. Trimethylsilyl ethers of oxime and O-

TABLE III

RECOVERIES OF ^{3}H-LABELLED 3-KETO-Δ^4-STEROIDS IN THE CONVERSION INTO OXIMES, FILTRATION THROUGH DEAP-LH-20, SEPARATION ON SE-LH-20, CONVERSION INTO TRIMETHYLSILYL ETHER DERIVATIVES AND FILTRATION THROUGH LIPIDEX 5000

*Steroid mixture**	*Amount added*		*Recovery from columns (%)**			*Loss in first 10 ml from SE-LH-20 (%)***
	pg	*cpm*	*DEAP-LH-20 + SE-LH-20*	*Lipidex 5000*	*Total*	
A^4-3,17-one	4	1000				
A^4-17β-ol-3-one	4	1000				
P^4-3,20-one	4	1000	92.2 ± 4.6	96.7 ± 2.8	87.4 ± 5.1	3.5 ± 0.4
P^4-17α-ol-3,20-one	40	1000	(n = 10)	(n = 10)	(n = 10)	(n = 10)
P^4-11β,17α,21-ol-3,20-one	4	1000				
P^4-17α,21-ol-3,11,20-one	4	1000				

* For abbreviations see Table I.

** Per cent of added radioactivity ± S.D.; n = number of experiments.

methyloxime derivatives of cortisol were recovered quantitatively and essentially the same purification of the sample was achieved.

Application to the analysis of 3-ketosteroids in plasma

The extraction of steroids in plasma using Amberlite XAD-2 at elevated temperature has been described previously[2]. Recoveries of 3-keto-Δ^4-steroids by this method were better than 90%. In order to remove cations that might have interfered in the subsequent separation of oximes and in the GC–MS analysis, the extract from Amberlite XAD-2 was filtered through a column of SE-LH-20 in methanol. Recoveries of ^{3}H-labelled steroids in this step were quantitative and a 500-mg column was sufficient for an extract of 10 ml of plasma.

If large amounts of non-polar lipids contaminate the sample, they can be removed by filtration through a column of Lipidex 5000 in the reversed-phase system methanol–water–chloroform (9:1:2)[1,2]. Hormonal steroids are eluted early, while cholesterol and neutral lipids are retained on this column.

After the reaction with hydroxylammonium chloride, the sample was filtered through a column of DEAP-LH-20. This step served two purposes: to remove chloride ions so that hydroxylamine could be evaporated, and to remove contaminating anions from the sample.

The separation of steroid oximes on SE-LH-20 has been discussed above. For the analysis of 3-ketosteroids, the oximes of these steroids were eluted as a group with methanol–pyridine following elution of other steroids with methanol. The selectivity of this purification step is high.

Final purification of the sample prior to GC–MS–COM analysis was achieved by filtration of the reaction mixture after formation of trimethylsilyl ether derivatives through a column of Lipidex 5000. This step served two purposes: to remove reagents and to remove interfering compounds that had not become non-polar in the silylation reaction.

The recoveries of ^{3}H-labelled steroids added to plasma and carried through the entire procedure were better than 78% in five experiments (mean: 82.3%).

The degree of purification achieved in the entire procedure was such that the equivalent of 1–2 ml of plasma could be injected without overloading of the glass capillary column in the GC–MS–COM analysis. A high degree of purification is necessary as analyses of steroid metabolite profiles require rapid repetitive scanning of the entire mass range, which reduces the sensitivity compared with detection by single or multiple ion monitoring. Obviously, the ability of the latter methods to detect low concentrations of steroids is also increased with improved sample purification.

The isolation procedure may appear tedious but it is in fact relatively easy to carry out as it is based on simple filtrations through a series of columns. The 3-ketosteroids in a plasma sample can be isolated for GC–MS analysis in 2 days and one technician can run four samples in parallel. Although the present study has been limited to ketosteroids, it should be pointed out that the principle of isolating oximes by cation-exchange chromatography in organic solvents might be more widely applicable to other groups of compounds that possess keto or aldehydo groups.

The GC–MS–COM analyses of 3-ketosteroids in plasma samples from two pregnant women (35th and 38th weeks) are shown in Fig. 1. Fragment (and molecular) ion current (FIC) chromatograms were constructed by the computer for *m/e* values typical of O-trimethylsilyloxime trimethylsilyl ether derivatives of various steroids possessing a 3-keto group. The selection of *m/e* values was based on spectra of reference compounds, the molecular weight of steroids, the known loss of 15 and 89 mass units from the molecular ion and the knowledge of ions typical of specific structural features in the molecules. Spectra of this type of steroid derivative have been discussed by Brooks and co-workers[14,15].

As expected, cortisol (scans 293 and 308, plasma sample 1) and progesterone (scans 60 and 69) are the major 3-ketosteroids in the analyses shown in Fig. 1. The spectrum of the derivative of progesterone is shown in Fig. 2. The molecular ion at *m/e* 488 is seen as well as the peak at *m/e* 211 typical of the derivative of the 3-keto-Δ^4-structure[15]. The peaks at *m/e* 145 and 158 arise from the derivatized 20-keto-21-deoxy structure[14]. A relatively large amount of 20α-hydroxy-4-pregnen-3-one (scans 14 and 21) is also seen. The spectrum of the derivative of this steroid is shown in Fig. 2. The molecular ion at *m/e* 475, the ion at *m/e* 211 and the intense ion at *m/e* 117, arising from the side-chain of the derivatized 20-hydroxy-21-deoxy structure, are seen.

In addition to these steroids, the presence of 17α-hydroxy-4-pregnene-3,20-dione (main isomer scan 85, plasma sample 2) and 16α-hydroxy-4-pregnene-3,20-dione (relative retention times 2.52 and 2.59, scans 100 and 107, plasma sample 2) was noted. 5α-Pregnane-3,20-dione has been identified in plasma from pregnant women[2,16] and its derivatives are seen to be eluted at scans 37 and 51 in plasma 1.

Recently, Murphy *et al.*[17] demonstrated the presence of cortisone in plasma of pregnant women, and the analyses shown in Fig. 1 confirm these results. Relatively large amounts of the derivatives of this compound (relative retention times 3.51 and 3.92) appear at scans 252 and 286 (plasma sample 1), and the spectrum of this steroid derivative is shown in Fig. 2. The concentrations of the different steroids can be calculated from the peak areas in appropriate FIC chromatograms by comparison with known amounts of reference compounds. The following approximate values were found in the analysis of plasma sample 1 shown in Fig. 1 (the equivalent of 0.3 ml plasma was injected): progesterone, 200 ng/ml; 20α-hydroxy-4-pregnen-3-one, 37 ng/ml; 5α-pregnane-3,20-dione, 35 ng/ml; 17α-hydroxy-4-pregnene-3,20-dione, *ca.* 1

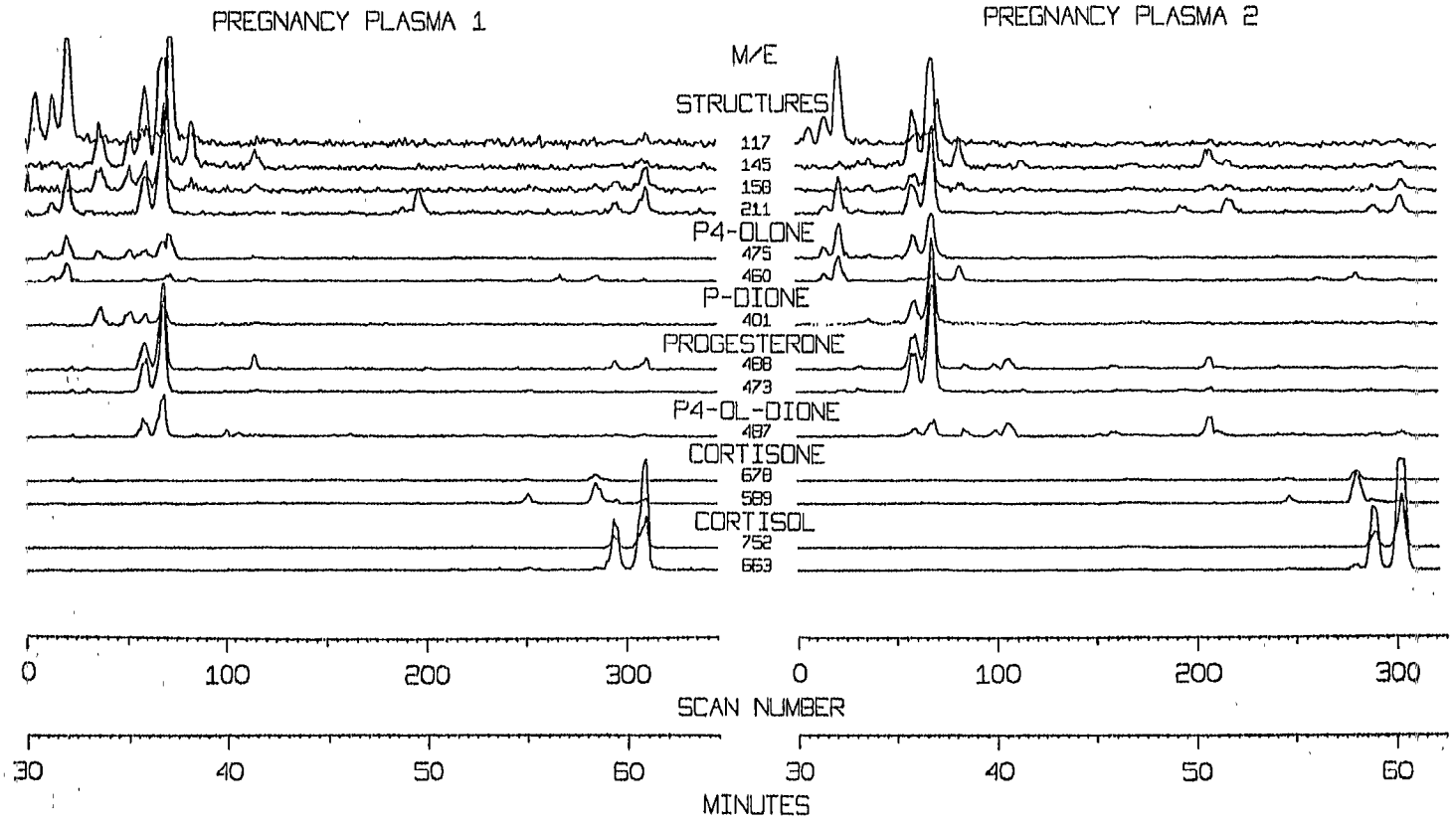

Fig. 1. Fragment ion current (FIC) chromatograms constructed by the computer in analyses of 3-ketosteroids in plasma from women in the 35th (pregnancy plasma 1) and 38th week (pregnancy plasma 2) of pregnancy. The equivalent of about 0.3 ml of plasma was injected into the glass capillary column. *m/e* values characteristic of O-trimethylsilyloxime trimethylsilyl ether derivatives of various steroids possessing a 3-keto group and ions given by specific structural features in the molecules were selected.

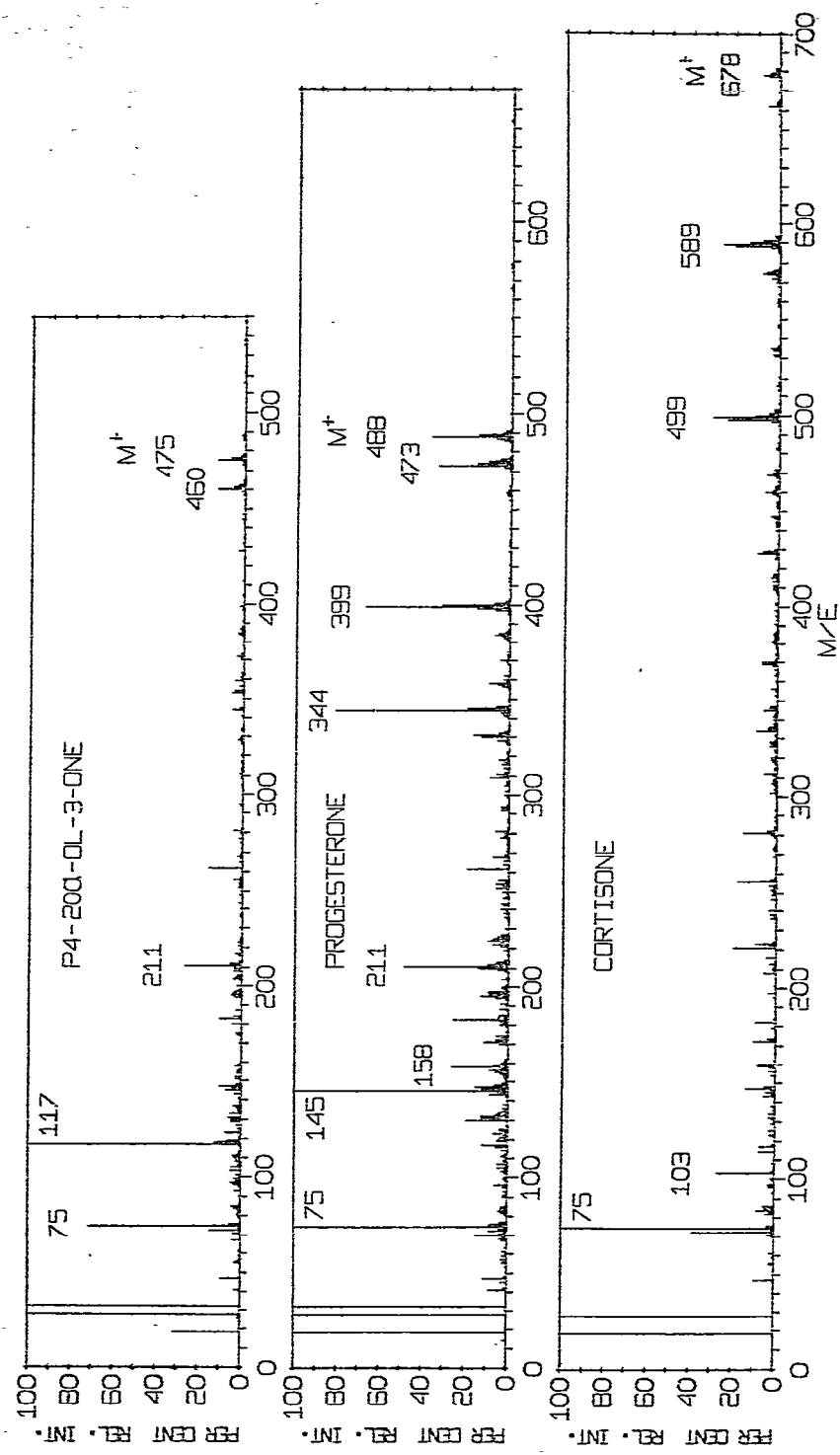

Fig. 2. Mass spectra of O-trimethylsilyloxime trimethylsilyl ether derivatives of steroids found in plasma from a pregnant woman. The spectra of 20α-hydroxy-4-pregnen-3-one, progesterone and cortisone were recorded from the analysis of pregnancy plasma 2, at scans 23, 68 and 280, respectively, shown in Fig. 1.

ng/ml; 16α-hydroxy-4-pregnene-3,20-dione, *ca.* 5 ng/ml; cortisol, 297 ng/ml; and cortisone, 57 ng/ml. These values are in agreement with published values for the concentrations of these steroids in late-pregnancy plasma.

The separation of *syn/anti* isomers of the oxime derivatives of 3-ketosteroids (see Table I) is evident in Fig. 1. This is a disadvantage in quantitative analyses, as it decreases the sensitivity and makes the GLC profiles more complex. However, the formation of two peaks may help to support the identification of the steroids.

The sensitivity of the repetitive scan method using electron-impact ionization and O-trimethylsilyloxime trimethylsilyl ether derivatives is not sufficiently high to detect steroids present in plasma at concentrations below 1 ng/ml. For example, the molecular ion of the progesterone derivative constitutes only 4.6% of the total ionization. In a test in which chemical ionization in isobutane was employed, the value for the quasimolecular ion was 6.5%, *i.e.*, only slightly higher. Attempts to make more suitable derivatives of the oxime groups were unsuccessful. However, recently it has become possible to convert the oximes into the more favourable O-methyloximes and this method is presently being studied[18]. Finally, increased sensitivity is obviously obtained if the ions to be monitored are selected prior to injection of the sample so that multiple ion detection can be employed.

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