CHROM. 4531

SEPARATION AND DETERMINATION OF STEROIDS IN OIL SOLUTION

IV. GLC ANALYSIS OF ANABOLIC, ANDROGENIC, ESTROGENIC AND PROGESTATIONAL STEROIDS

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SUMMARY

In earlier papers we described the gas chromatographic determination of mono- and di-esters of estradiol and estrone esters in oil solutions at a low concentration level (2 mg/ml). In this case a preliminary separation by means of quantitative thin-layer chromatography was necessary in order to separate the steroids from the oil.

In order to make the separation step simpler and more generally applicable, we experimented with the partition between hexane and 85% ethanol, and obtained a steroid fraction suitable for gas chromatographic analysis.

The present method has been applied to oil solutions of steroids which are representative of the anabolic, androgenic, estrogenic and progestational classes, at concentration levels between 10 and 50 mg/ml; precision and accuracy values for each experiment are reported.

The procedure is simple, rapid and suitable for multisample analysis.

INTRODUCTION

Many applications of gas chromatography to the analysis of hormonal steroids in pharmaceutical preparations have been described in the last few years. Gas chromatography has been used mostly when the steroid to be analysed was present in small quantities.

This is the case with ethynyl estradiol and its methyl ether in estrogen–progestogen preparations for oral use[1–8] or when mixtures of various steroids which are difficult to separate by other techniques[9–14] have to be analysed.

Another case where gas chromatography is useful is for the analysis of steroids which are difficult to isolate owing to the nature of the vehicle or to their rather low concentration in the solution[15–18]; for example, of oil solutions of steroids. This has

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been discussed earlier[16,17] when the low concentrations (2 mg/ml) of estrogen were subjected to a preliminary separation, by means of thin-layer chromatography, in order to separate the steroid from the main components of the oil, before analysis by gas chromatography.

Recently we have observed, for more concentrated solutions (10 mg/ml and more) that the method of partition between hexane and 85% ethanol according to NF XII[19] with reciprocally saturated solvents[20] isolates the steroids in such a way that they can be determined directly by gas chromatography.

The results of experiments reported here for solutions of steroids of different classes demonstrate the various possibilities of this simple and convenient method.

EXPERIMENTAL

Reagents

Ethanol was purified by distillation over alkaline silver nitrate; hexane, heptane, carbon disulphide and benzyl alcohol were analytically pure reagents. A mixture composed of anhydrous pyridine, hexamethyldisilazane, and trimethylchlorosilane (9:3:1) was used for the preparation of the trimethylsilyl ethers. Olive oil for pharmaceutical use was refined and deacidified.

Reference compounds

Estradiol-17β-valerate, testosterone-17β-propionate* and cyclopentylpropionate, progesterone, 19-nortestosterone-17β-propionate, phenylpropionate and decanoate, estrone-3-benzoate, estradiol dipropionate and 5α-cholestane-3β-ol-acetate were analytical grade and if necessary recrystallised.

Preparation of the solutions for analysis

(a) Reference solutions. The steroids to be analysed were dissolved in ethanol at a concentration of 1 mg/ml and diluted to the range indicated in Table I under "steroid calibration range" so that the mean quantity refers to 1 ml of the diluted solution.

(b) Solutions in oil. These were prepared in 50-ml tared vessels by dissolving 100 mg of the steroid in 0.5 ml of benzyl alcohol and 10 ml of olive oil* and then diluting with heptane to the final volume (steroid concentration 2 mg/ml, oil concentration 0.2 ml/ml).

(c) Blank (oil only). A solution of oil in heptane at a concentration of 0.2 ml/ml was prepared.

(d) Solutions of internal standards for GLC. These were prepared in heptane; suitable quantities are reported in Table I under "Internal standard" and refer to 1 ml of the solution.

Separation of the steroid from the oil solution

The following procedure was used: 50 ml of 85% ethanol (previously saturated with hexane) and 5 ml of the sample diluted as indicated in (b) were introduced into a 100-ml separator (with a teflon stopcock) and shaken. 35 ml of hexane (previously

* 500 mg of testosterone-17β-cyclopentylpropionate were used in order to obtain a concentration of 50 mg/ml of the steroid in oil.

TABLE I

OPERATING CONDITIONS FOR GAS CHROMATOGRAPHIC ANALYSIS

Steroid	*Column*	*Derivative*	*Column temp. (°C)*	*Injector temp. (°C)*	*Detect temp. (°C)*
Estradiol-17β-valerate	N. 1: 3% JXR on silanised 100–120 mesh Gas-Chrom P; 2.20 m length	mono TMSE	230	270	250
Testosterone-17β-propionate	N. 2: 3% QF-1 on 100–120 mesh Gas-Chrom Q 1.80 m length	none	240	270	250
Progesterone	N. 3: 1% OV-17 on 100–120 mesh Gas-Chrom Q 2.20 m length	none	250	270	260
Testosterone-17β-cyclopentyl-propionate	N. 2	none	240	270	250
19-Nortestosterone-17β-phenylpropionate	N. 1	none	240	270	250
19-Nortestosterone-17β-phenylpropionate	N. 3	none	270	290	280
19-Nortestosterone-17β-decanoate	N. 1	none	240	270	250
19-Nortestosterone-17β-decanoate	N. 3	none	270	290	280
19-Nortestosterone-17β-propionate	N. 1	none	210	270	240
19-Nortestosterone-17β-propionate	N. 3	none	270	290	280

[a] (A) indicates calculation made by area of the peaks; (H) indicates calculations made by heig the peaks.
[b] The amount used is reported in parenthesis.
[c] The number of determinations is indicated in parenthesis.

saturated with 85% ethanol) were then added and the mixture was thoroughly shaken, the phases were allowed to separate until clear, and the alcohol phase was transferred into another (250 ml) separator which already contained 25 ml of hexane (ethanol saturated). The hexane phase in the first separator was extracted three times, successively, with 25 ml of 85% ethanol (hexane saturated), and all the alcohol phases were collected in the second separator. This was then shaken and the two phases were carefully separated. The alcohol phase together with two 10-ml 85% ethanol washings of the hexane layer were transferred into a tared 200-ml flask and filled up with 95% ethanol (solution C_P).

Procedure for the gas chromatographic analysis

A calibration curve was first obtained for each steroid, referring the steroid to an internal standard. The calibration ranges are given in Table I. Normally volumes of 0.5, 1.0 and 1.5 ml of the reference steroid solution to be analysed are transferred

;en :in)	*Attenuation*	*Steroid calibration range (μg)*[a]	*Internal standard*[b]	R_M *value*[c]	*Retention time of the steroid (min)*	*Retention time of the standard (min)*
	× 50	50–150 (H)	5 α-cholestane 3 β-ol-acetate (200 μg)	1.559 ± 0.032 (± 2.0%) (5)	11	17
	× 50	50–150 (A)	estrone-3-benzoate (200 μg)	0.897 ± 0.035 (± 3.9%) (6)	8	21
	× 50	50–150 (A)	estradiol dipropionate (150 μg)	1.092 ± 0.26 (± 2.4%) (6)	8	13
	× 50	250–750 (A)	estrone-3-benzoate (250 μg)	0.928 ± 0.034 (± 3.66) (6)	30	21
	× 20	50–150 (H)	5 α-cholestane-3β-ol-acetate (30 μg)	0.264 ± 0.008 (± 3.0%) (6)	20	9
	× 20	—	—	—	22	—
	× 20	125–375 (H)	5 α-cholestane-3β-ol-acetate (30 μg)	—	20	9
	× 20	—	—	—	15	—
	× 20	25–75 (H)	5 α-cholestane-3β-ol-acetate (150 μg)	—	9	33
	× 20	—	—	—	3	—

into conical glass-stoppered test tubes, 1 ml of the internal standard is added and the solution is evaporated to dryness in a water bath under dry nitrogen. The tubes are then stored in a vacuum desiccator over KOH and silica gel. The residue is dissolved in 50–100 μl of carbon disulphide and 1–3 μl are injected. When the preparation of the trimethylsilyl derivate is necessary (in the case of estradiol-17β-valerate) the procedure described in an earlier paper[17] is used. The value $R = A/B \times C/D$ (A = reference steroid area or height, B = internal standard area or height, C = internal standard amount, D = reference steroid amount) is calculated, and the linear relationship of A/B with respect to D/C is verified. The mean value (R_M) is calculated with its S.D. and used for the determinations.

For the analysis of the samples, 2 ml of the solution C_P were taken, the procedure as described for the calibration curve being used. Each sample analysis was repeated and the mean value taken. The quantity of steroid per sample was obtained by using the formula $D = A/B \cdot C/R_M$ where A is the sample area or height, B is the

internal standard area or height, C is the internal standard amount and R_M is defined as above. A Perkin-Elmer gas chromatograph Model 801 with a hydrogen flame ionisation detector and a 5 mV recorder, with helical glass columns, I.D. 2.5 mm, was used. The lengths of the columns, the packings, and the operating conditions are given in Table I.

TLC control of the steroid separation

This was carried out on 20 × 20 cm Silica Gel G layers, 0.5 mm thick, with hexane–ethyl ether–acetic acid (HEAA) (70:30:1) as solvent with a normal development. The 85% ethanol and the hexane fractions were chromatographed as well as the hexane used for washing of the ethanol fraction.

RESULT AND DISCUSSION

Fig. 1 shows the separation of testosterone propionate from an oil solution by the partition procedure between hexane and 85% ethanol. It can be seen that the testosterone propionate was completely transferred into the ethanol fraction; while it is accompanied by small quantities of mono- and di-glycerides and sterols, originally contained in the oil vehicle, it remains separated from triglycerides. A small loss of the steroid is visible in the hexane washings*; on the other hand this step of the procedure permits better purification of the steroid.

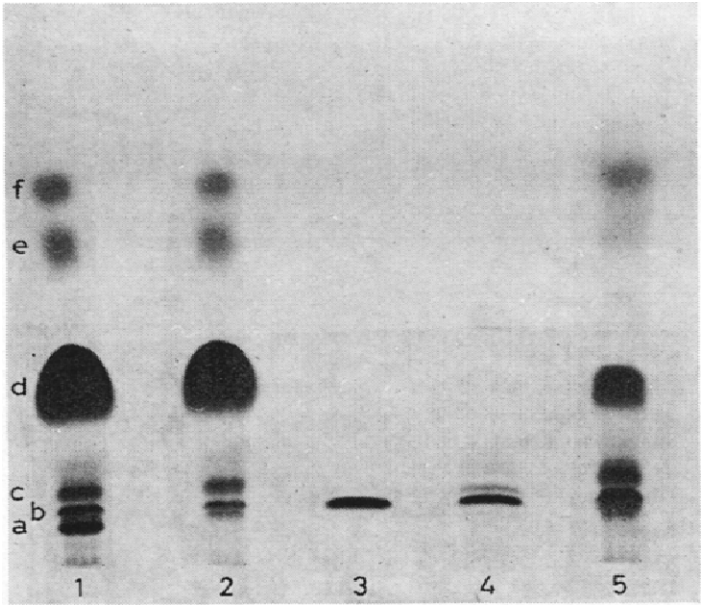

Fig. 1. Thin-layer chromatography with solvent HEAA (70:30:1) normal development: (1) Oil solution of testosterone propionate 10 mg/ml, 4 μl. (2) Hexane phase after extraction (quantity corresponding to 4 μl of oil solution). (3) Testosterone propionate 40 μg (quantity corresponding to 4 μl of oil solution). (4) Ethanol phase after extraction (quantity corresponding to 4 μl of oil solution and to 40 μg testosterone propionate. (5) Hexane washings, quantity corresponding to 25 times the ethanol phase chromatographed under (4) (quantity corresponding to 100 μl of oil solution). (a) = Testosterone propionate; (b) = diglycerides; (c) = natural sterols of the oil; (d) = triglycerides; (e) = natural sterol esters; and (f) hydrocarbons. Detection: 50% H_2SO_4 and heating.

* Testosterone propionate stains dark-green and is clearly distinguishable from other substances with close R_F values, which stain brown.

The behaviour of oil solutions of the other steroids studied in this paper are similar; they are listed in Table I.

All the oil solutions considered in this paper had a steroid concentration of 10 mg/ml except for testosterone-17β-cyclopentylpropionate, when a 50 mg/ml solution was prepared in accordance with the therapeutic doses of these steroids.

The gas chromatographic behaviour of the steroids studied is summarised in Table I where the working conditions, the columns, the internal standards and the most convenient concentration ranges for quantitative analysis are reported.

The aim of this work was primarily to demonstrate that this extraction and gas chromatographic analysis procedure is valid for the quantitative determination of each individual steroid in an oil solution.

Therefore the most suitable column for each case was chosen; on the other hand it can be seen from Table I that the columns of JXR (methyl silicone polymer) and of OV-17 (methylphenyl silicone polymer) permit the analysis of many of the steroids studied.

It can also be seen from Table I that for the analysis of the three esters of 19-nortestosterone studied two different columns have been proposed. The column of 1% OV-17 permits the separation and identification of the three esters at 270°, which is not easy under other conditions (Fig. 2). The other column (3% JXR) is

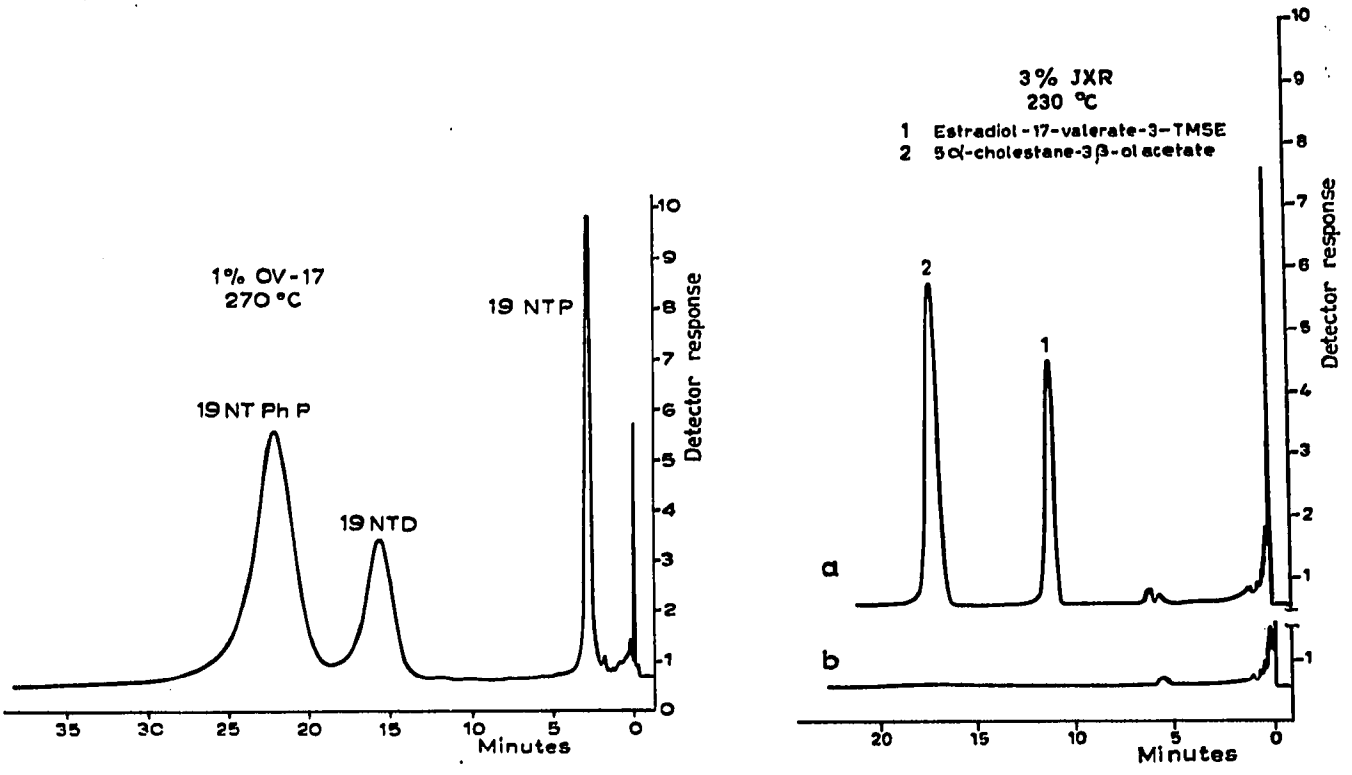

Fig. 2. Gas chromatogram of 19-nortestosterone-17β-esters:propionate (19 NT P); decanoate (19 NT D); phenylpropionate (19 NT Ph P). Column: 1% OV-17 on 100–120 mesh Gas-Chrom Q, 2.20 m length, 270°.

Fig. 3. (a) Gas chromatogram of estradiol-17β-valerate separated from an oil solution and of its internal standard 5α-cholestane-3β-ol acetate. Column: 3% JXR on silanised 100–120 mesh Gas-Chrom P, 1.80 m length, 230°. (b) Gas chromatogram of the reference oil blank subjected to the same procedure.

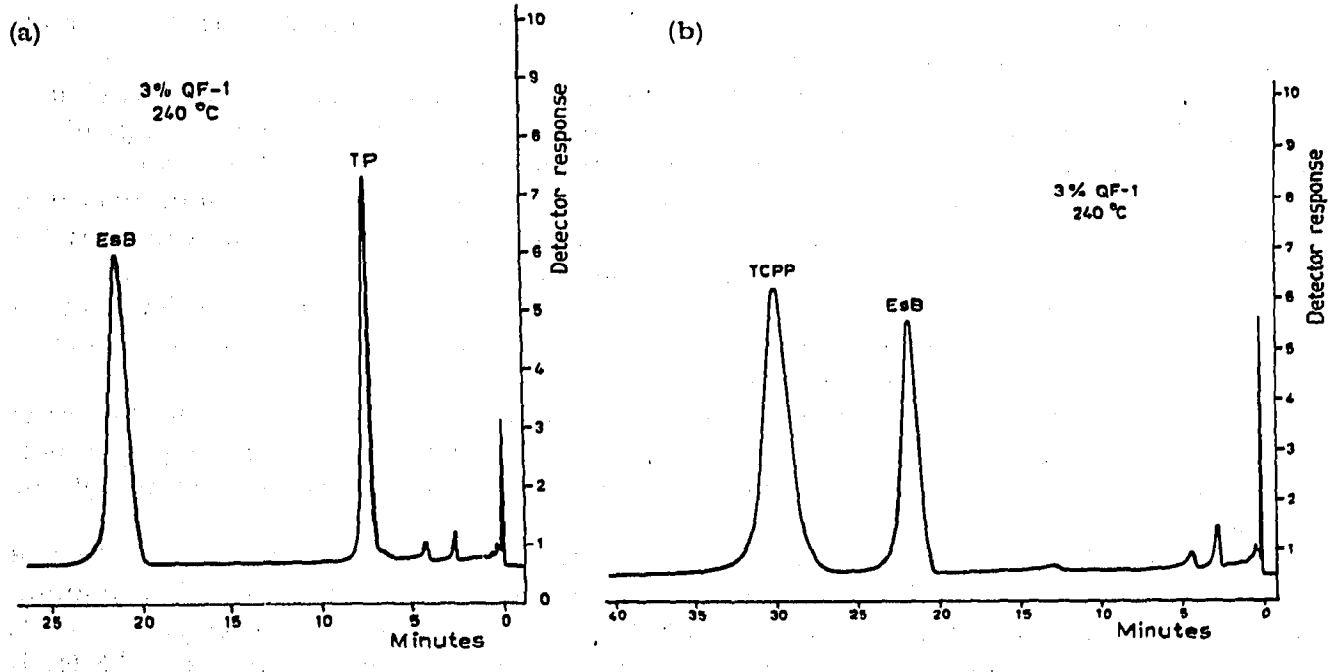

Fig. 4. (a) Gas chromatogram of testosterone-17β-propionate (TP) separated from an oil solution and of its internal standard, estrone benzoate (EsB). Column: 3% QF-1 on 100–120 mesh Gas-Chrom Q. 1.80 m length, 240°. (b) Gas chromatogram of testosterone-17β-cyclopentylpropionate (TCPP) separated from an oil solution; internal standard and operating conditions as in (a).

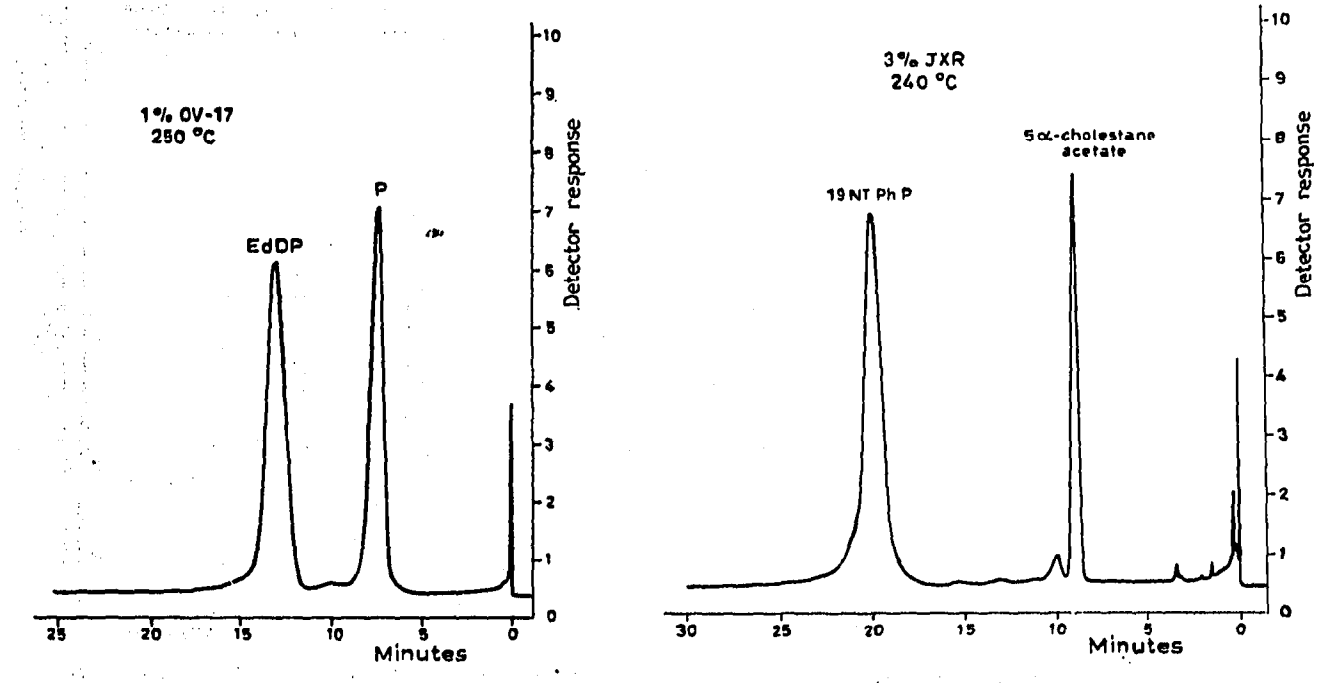

Fig. 5. Gas chromatogram of progesterone (P) separated from an oil solution and of its internal standard, estradiole dipropionate (EdDP). Column 1% OV-17 on 100–120 mesh Gas-Chrom Q, 2.20 m length, 250°.

Fig. 6. Gas chromatogram of 19-nortestosterone-17β-phenyl propionate (19 NT Ph P) separated from an oil solution and of its internal standard, 5α-cholestane-3 β-ol acetate. Column 3% JXR on silanised 100–120 mesh Gas-Chrom P, 2.20 m length, 240°.

TABLE II

DETERMINATION OF PERCENTAGE RECOVERY

Number of determinations indicated in brackets

Steroid	*Quantity of steroid in oil solution used for the analysis (mg)*	*Recovery*	
		Mean ± S.D. (mg)	*Percentage ± S.D.*
Estradiol-17β-valerate	10.0 (5)	9.54 ± 0.05	95.4 ± 0.5
Testosterone-17β-cyclopentyl propionate	50.0 (6)	47.0 ± 1.8	94.0 ± 3.6
Testosterone-17β-propionate	10.0 (6)	10.16 ± 0.14	101.6 ± 1.4
Progesterone	10.0 (10)	10.30 ± 0.16	103.0 ± 1.6
19-Nortestosterone-17β-phenylpropionate	10.0 (6)	9.90 ± 0.18	99.5 ± 1.8

more suitable for the determination of each ester individually but does not permit the separation of the decanoate and phenylpropionate esters, which owing to their close molecular weights have the same retention time.

It should also be noted that except for estradiol-17β-valerate, where the preparation of the trimethylsilyl ether is advisable, all the other steroids studied can be analysed directly by gas chromatography.

The results of the analysis of solutions containing known quantities of the studied steroids are reported in Table II. In all cases the data obtained show satisfactory accuracy and reproducibility for the procedure described. With respect to the gas chromatographic procedure, further data on the reproducibility are given in Table I, *viz.* the R_M values with their S.D., and in Table III; the data reported in both tables are in good agreement. (R_M values are obtained over a wide range of concentrations; values in Table III are obtained for a single concentration level.)

Some gas chromatograms are shown in Figs. 3–6. All the chromatograms show regular and well-separated steroid peaks, without interference from any small peaks originating from the oil. Only one chromatogram of the oil blank is shown: this is representative of the general pattern of the oil vehicle and is the result of many experiments with different steroid solutions.

Finally it can be said that this method permits, with small changes in the working conditions, the analysis of the various solutions of different steroids with good

TABLE III

PRECISION OF ANALYSIS BY GAS CHROMATOGRAPHY

Number of determinations indicated in brackets.

Steroid	*Quantity used for the analysis (μg)*	*Coefficient of variation*
Testosterone-17β-propionate	50.0 (6)	± 1.28
Progesterone	100.0 (6)	± 1.14

accuracy and precision. Thus the analysis of steroids of different chemical properties, otherwise requiring different reactions for their determination and identification, can be carried out by this procedure.

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