A GAS-CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF PROGESTERONE IN HUMAN PLASMA

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INTRODUCTION

Until recently, the endogenous production of progesterone by the adrenal cortex, the ovary and the placenta has been evaluated indirectly through the determination of its major metabolite, pregnanediol.

In the last few years, biological[1] and chemical[2–5] methods for the determination of progesterone in peripheral venous blood have been developed. The chemical methods are suitable for routine assays, especially for the study of ovarian and placental function both in physiological and pathological conditions. Recently WOOLEVER AND GOLDFIEN[6] have described a double isotope derivative technique with a high degree of specificity, sensitivity and accuracy. This method has been used to investigate plasma progesterone levels in non-pregnant women[7], but it cannot be easily employed in every laboratory.

In this paper a thin-layer and gas–liquid chromatographic technique for the determination of plasma progesterone is described.

Gas–liquid chromatography has already been applied to the separation and determination of several hormonal steroids in biological materials. In particular, we have applied this technique to the quantitation of urinary oestrogens in normal[8] and pregnant women[9] and young boys[10].

Gas-chromatographic methods have also been described for progesterone evaluation in biological materials. However, the amounts of hormone which are required could be easily detected by colorimetry or spectrophotometry[11].

Methods employing progesterone-7-^{3}H have been used for the determination of plasma progesterone in pregnant women[12].

Our method for the determination of plasma progesterone consists of extraction with ether, solvent partition, thin-layer chromatography to purify the extract and separate the progesterone fraction, and finally gas–liquid chromatography for the identification and quantitation of progesterone.

APPARATUS AND MATERIALS

A gas-chromatographic apparatus, Fractovap, model C (Carlo Erba, Milan), was used. It was equipped with a hydrogen flame ionization detector, and with U-shaped

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micro-glass columns 80 to 90 cm long whose inside diameter was 2 mm. The columns were packed with silanized Chromosorb W, and coated to 3 % with SE-30, or packed with NPG, and coated to 1 % with QF-1.

The columns were preconditioned by heating at 250° in a nitrogen atmosphere for 12 h before use. All analyses were carried out at a column temperature of 200° and a nitrogen carrier pressure of 0.45 atm. The vaporization chamber was kept at 285°.

The samples, dissolved in acetone, were introduced by means of a 1 or 10 μl Hamilton syringe. Samples were usually run at a detector voltage of 1,500 V, and at recorder attenuation of 10.

For the quantitative determination, the area of the progesterone peak was calculated by means of the planimetric and triangulation methods, and then compared with the peak areas of known amounts of authentic standard of progesterone run on the same day.

For the purification of the extract, horizontal thin-layer chromatography was carried out using the "B.N.-Kammer" (Desaga, Heidelberg). The horizontal thin-layer technique permits the flow of solvents for a very long time, so that a greater purification of the extracts can be achieved[13].

All the solvents were of A.R. grade, and were redistilled immediately before use. The diethyl ether was peroxide free. For thin-layer chromatography silica gel G (Merck, Darmstadt) was used. Progesterone, obtained from Mann Research (New York) was prepared as standard solution in absolute ethanol.

PROCEDURE

The plasma, obtained by centrifugation of heparinized blood immediately after collection, was stored at —18° for at least 12 h.

Two extractions with 3 volumes of diethyl ether were performed by shaking gently for 10 min. The ether extracts were collected in a centrifuge tube with a conical base and then dried on a water-bath under nitrogen. The dry residue was dissolved in 10 ml of warm 70 % methanol and then kept overnight at —18° in order to precipitate lipids. The tube was spun in a refrigerated centrifuge for 25 min at 3,000 r.p.m. The upper liquid phase was carefully transferred to another tube, and the precipitate washed with 3 ml of cold 70 % methanol, which was decanted to the same tube. The pooled methanol extracts were then shaken with an equal volume of heptane. The heptane phase was separated and re-extracted with $^1/_2$ volume of 70 % methanol, which was added to the pooled methanol extracts. Twenty ml of distilled water was added to the combined methanol extracts, which were then extracted 3 times with 15 ml of petroleum ether, b.p. 35–40°. The combined petroleum ether extracts were then dried as previously described.

The dry residue is quantitatively spotted on silica gel G plates, using three times 15 μl of acetone, according to a previously described technique[14]. A standard solution of progesterone is also spotted on the plate. Horizontal development was carried out, at room temperature, by employing the "B.N.-Kammer", with 70 % methanol (v/v) saturated heptane. In this system progesterone does not move from the origin, while the interfering substances are displaced; therefore good purification of the plasma fraction containing progesterone can be obtained. Other solvent systems, *e.g.* methanol–benzene (1:9, v/v), which causes progesterone displacement

TABLE I

R_F AND R_{Oe_1}* VALUES OF AUTHENTIC AND PLASMA PROGESTERONE

Horizontal thin-layer chromatography in a cyclohexane–ethyl acetate (1:1, v/v) system. S.D. = standard deviation.

Compound	$R_F \pm$ S.D.	$R_{Oe_1} \pm$ S.D.
Authentic progesterone	0.550 ± 0.01	0.851 ± 0.01
Plasma progesterone	0.548 ± 0.03	0.846 ± 0.04
Oestrone	0.640 ± 0.01	— —

* Oe_1 = oestrone.

(R_F 0.60), do not reach the degree of purification of our proposed solvent system. On completion of the first development, the plate was left in the tank and the solvents were allowed to evaporate. A second development was then performed, using the cyclohexane–ethyl acetate (1:1, v/v) system. In this system, progesterone migrates and can be characterized by its mobility. The R_F values and the R_{Oe_1} values (R_F values of progesterone relative to oestrone) are reported in Table I.

The progesterone was visualized by spotting the plates with a hexane-saturated solution of iodine (yellowish brown spots), or an ethanolic solution of H_2SO_4 (spots fluorescent under U.V. light), or a Zimmermann reagent (violet spots).

The progesterone separated and purified is eluted from the silica gel by shaking in a centrifuge tube with absolute ethanol. After centrifugation, the ethanol extract was separated and dried. The residue was dissolved in acetone and quantitatively introduced into the gas-chromatographic apparatus as previously described.

RESULTS

Determinations of progesterone were carried out, by means of the method described above, on plasma obtained from 10 normally menstruating women at various stages of the cycle and from a pregnant woman. The results are reported in Table II.

TABLE II

PLASMA LEVELS OF PROGESTERONE IN NORMALLY MENSTRUATING AND PREGNANT WOMEN

Plasma volume extracted: 10 or 20 ml.

Subject	*Duration of cycle (days)*	*Day of cycle*	*Plasma progesterone (µg/100 ml)*
1	28	5th	0.25
2	27	6th	0.45
3	28	8th	0.50
4	30	15th	1.9
5	28	16th	2.5
6	28	22nd	3.5
7	28	26th	2.6
8	33	26th	2.4
9	30	26th	3.7
10	33	29th	1.1
11	Twenty-eighth week of pregnancy		15.5

Figs. 1 and 2 show, respectively, typical gas chromatograms of authentic progesterone and of plasma from a normal woman, which had been subjected to the thin-layer purification. The retention time of progesterone in both chromatograms was calculated by the time in minutes elapsing from the introduction of the sample into the vaporizing block to the highest point of the peak.

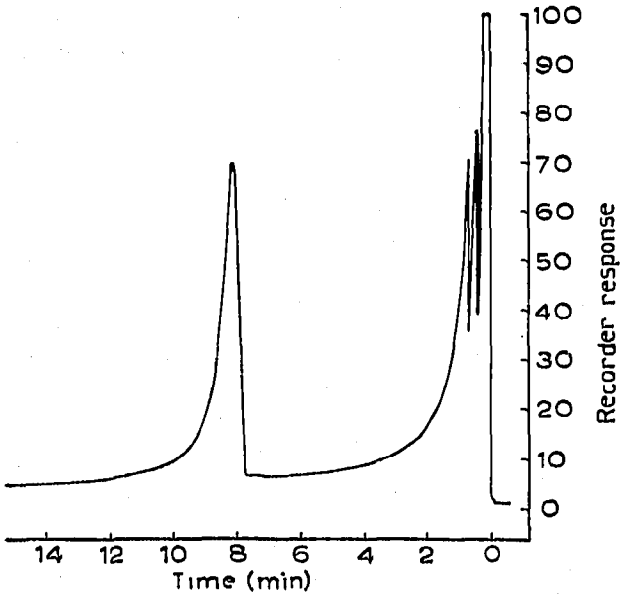

Fig. 1. Typical gas chromatogram of authentic progesterone. The retention time is 8.42 min (Attenuation 10)

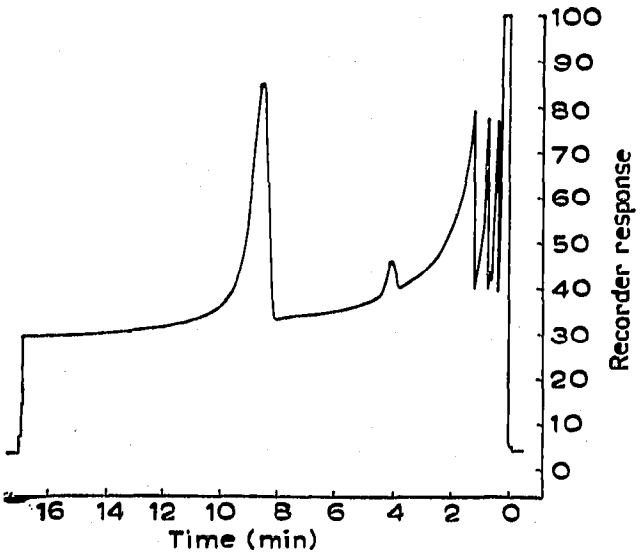

Fig. 2. Gas chromatogram obtained by the analysis of 10 ml of plasma from a subject in the follicular phase of the menstrual cycle. The retention time of progesterone is 8.42 min. (Attenuation 1)

The retention time of authentic and plasma progesterone was, under our conditions, 8.42 ± 0.07 (S.D) and 8.42 ± 0.15 (S.D.) min, respectively. As already mentioned, for the quantitative determination of progesterone the area of the authentic compound was compared with the area of the peak of the unknown sample showing the same retention time.

The amounts of progesterone determined by our method in plasma from normal women in the luteal phase of the menstrual cycle were comparable to those reported by other authors[5,7]. Very recently[15], similar results have been obtained by the application of two-dimensional thin-layer and gas–liquid radiochromatography.

DISCUSSION

Our gas-chromatographic method appears to be useful for determining amounts of progesterone in plasma ranging from 0.23 to 3.1 μg. Since the sensitivity of our gas-chromatographic apparatus can be greatly increased, it might be anticipated that smaller quantities (about 0.025 μg) of progesterone could be assayed with adequate reproducibility. This has, in fact, been confirmed by estimating very small amounts of authentic progesterone (≥ 0.025 μg). For the gas-chromatographic analysis of such small quantities of steroids it is necessary to use short columns in order to obtain sharp and easily measurable peaks. The efficiency of short columns, on the other hand,

is somewhat limited and only highly purified extracts can be applied to them. This can be achieved with a series of preliminary steps, namely cold methanol precipitation, solvent partition and thin-layer chromatography. The first two steps are used to remove the major part of the lipidic contaminants; horizontal thin-layer chromatography with 70% methanol-saturated heptane permits a further purification of the progesterone fraction from interfering material, and the second development with cyclohexane–ethyl acetate (1:1, v/v) separates it from other steroids of similar mobility. The usefulness and efficiency of this procedure is apparent from Figs. 3 and 4, where

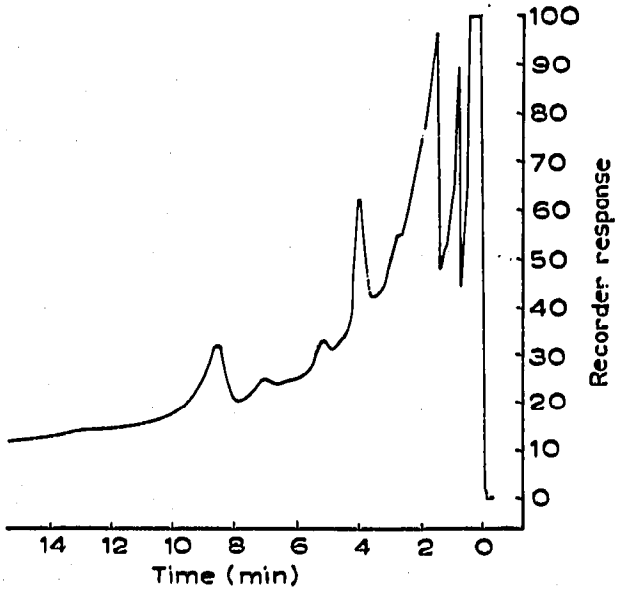

Fig. 3. Gas chromatogram obtained from 20 ml of female plasma without thin-layer purification. The retention time of progesterone is 8.42 min. (Attenuation 10)

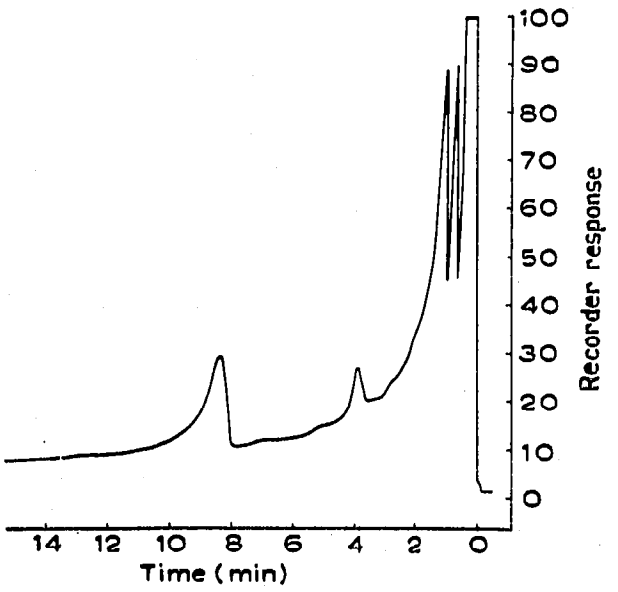

Fig. 4. Gas chromatogram obtained from 20 ml of plasma from the same subject as in Fig. 3, after thin-layer chromatography. The retention time of progesterone is 8.42 min. (Attenuation 10)

gas chromatograms of plasma, with and without purification by thin-layer chromatography, are shown. The peak with a retention time characteristic for progesterone emerges from a very low baseline, which does not interfere with the quantitative analysis. Horizontal thin-layer chromatography is particularly suitable for our purpose, since it permits two successive runs on the same plate with different solvent systems, simply by evaporating the first solvent system, and without moving the plate from the chromatographic tank.

TABLE III

PERCENTAGE RECOVERY OF VARYING AMOUNTS OF PROGESTERONE ADDED TO MALE PLASMA

No. of samples	*Amounts of progesterone added (μg)*	*Recovery % from male plasma (10 ml)*
10	2.5	94.3 ± 3.6*
6	5.0	88.5 ± 4.8*

* Standard deviation.

TABLE IV

ANALYSIS OF DUPLICATE SAMPLES OF PLASMA PROGESTERONE FROM FOUR SUBJECTS
Plasma volume extracted: 20 ml.

Subject	*Concentration of progesterone (μg/100 ml of plasma)*		*Deviation from the mean (%)*
	Sample I	*Sample II*	
1	3.0	3.5	7.7
2	1.6	2.1	13.5
3	2.0	1.7	8.5
4	2.5	2.7	3.8
$\bar{X} \pm$ s.e.m.*	2.3 ± 0.3	2.5 ± 0.4	8.4 ± 2.0
	P = N.S.		

* S.e.m. = standard error of means.

The relationship between different quantities of injected authentic progesterone and the areas of the peaks so obtained is linear and follows the postulate of BEER. This relationship is shown in Fig. 5.

The sensitivity, accuracy, precision and reproducibility of the method have been checked by means of recovery experiments and duplicate analyses.

Table III shows the results obtained for recovery experiments of 2.5 and 5.0 μg of authentic progesterone added to 10 ml of male plasma.

Further evidence of the accuracy of this method has been obtained by recovering progesterone from silica gel after thin-layer chromatography. During these trials a negligible amount (about 2 %) is lost.

The results of the analyses in duplicate are shown in Table IV. It will be seen that there is satisfactory agreement, and that the difference is not statistically significant (P = N.S.). This confirms the reproducibility of the method.

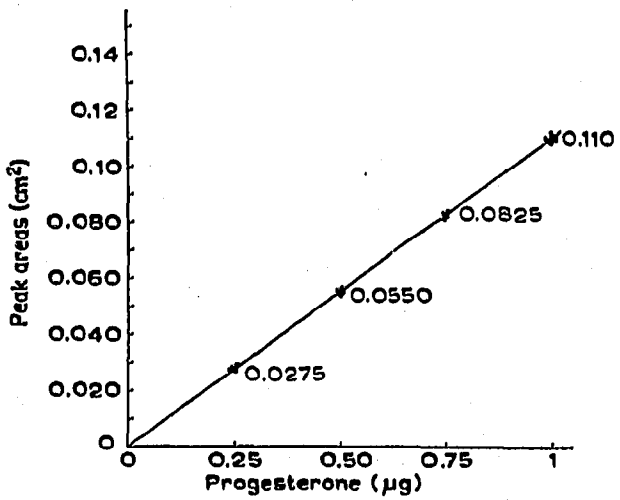

Fig. 5. Linear relationship between peak area and quantity of progesterone injected. The experimental conditions are described in the text.

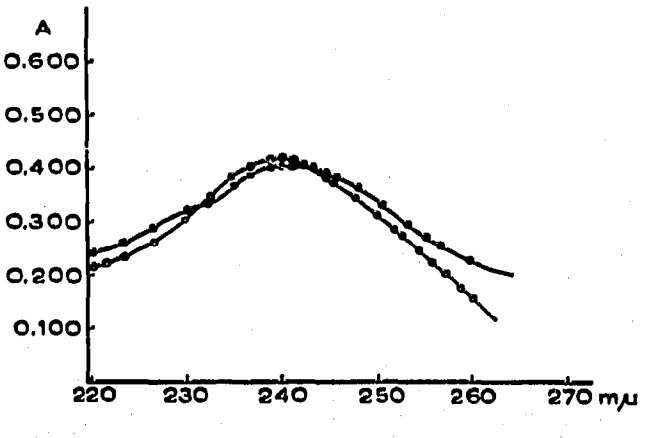

Fig. 6. U.V. absorption spectra of an ethanolic solution of crystalline progesterone (open circles) and of the progesterone fraction of a pool of plasma from women (filled circles) after purification by horizontal thin-layer chromatography.

The specificity of the method depends on a preliminary purification and on the separation of progesterone from other Δ^4-3-ketosteroids. In fact, no other peaks, apart from that with the same retention time as progesterone, have been identified when extracts of a large pool of plasma were chromatographed on a 90 cm micro-glass column packed with 1 % QF-1. Further evidence of specificity of the method has been obtained by comparing the U.V. spectra of plasma fractions containing progesterone (after thin-layer chromatography) with the spectrum of the authentic compound. The spectra are recorded in Fig. 6.

Concluding, the results reported indicate that gas–liquid chromatography can be applied to the qualitative and quantitative determination of plasma progesterone in non-pregnant women. The importance of a preliminary purification of the extracts by means of horizontal thin-layer chromatography is shown, in order to obtain well defined and measurable peaks.

With some changes in the preliminary extraction and chromatographic separation, it is possible to apply this general technique to the quantitative determination of various steroid hormones in both tissue and biological fluids.

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SUMMARY

A method for the quantitative determination of progesterone in human plasma is described. This method consists of extraction with ether, solvent partition, thin-layer chromatography and finally gas–liquid chromatography for the quantitation of progesterone. The method is suitable for application to peripheral blood levels at all stages of the menstrual cycle and pregnancy. Data are presented on the sensitivity, accuracy, precision and reproducibility of the method.

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