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A SIMPLE GAS–LIQUID CHROMATOGRAPHIC METHOD WITH ELECTRON CAPTURE DETECTION FOR THE DETERMINATION OF PROGESTERONE IN THE BLOOD OF HUMANS AND DOMESTIC ANIMALS

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SUMMARY

A simple routine method is reported for the determination of progesterone in blood by gas–liquid chromatography. An unlabelled internal standard, testosterone acetate, was added to plasma samples. Following preliminary purification by thin-layer chromatography, progesterone in pregnancy plasma was evaluated as the free steroid with a flame ionization detector. The hormone in the plasma of women during the menstrual cycle and of cycling domestic animals was determined as the 3-enol ester heptafluorobutyrate by electron capture detection.

The validity of testosterone acetate as an internal standard was proved by simultaneous processing of [7α-^{3}H]progesterone and [4-^{14}C]testosterone acetate and the determination of the isotope ratio in samples. The results of control experiments and normal values are also presented.

INTRODUCTION

The progesterone (PROG) level in blood is very low and requires extremely sensitive methods for its determination. Although an increasing number of assay methods utilizing competitive protein binding or radioimmunological principles has been reported, laboratories equipped with gas chromatographs will use gas–liquid chromatographic (GLC) procedures. The introduction of a GLC method appears to be particularly justified when, in addition to quantitation, the identification of one hormone or the quantitative determination of several steroids is also necessary.

In our simplified method, unlabelled testosterone acetate (TAc) is used as the internal standard for the correction of methodological loss throughout the procedure and for quantitative determinations. The higher hormone levels in the systemic blood

of pregnant women and domestic animals were determined by using a flame ionization detector (FID), and the lower progesterone concentrations during the cycle by using an electron capture detector (ECD).

EXPERIMENTAL

Instrumental

A Pye Unicam Series 104 gas chromatograph equipped with an FID and an ECD was used. The glass column, 7 ft. × 4 mm I.D., was packed with 1 % XE-60 on Gas-Chrom Q (100–120 mesh) and operated at 205° (ECD) or 230° (FID). The carrier gas was nitrogen at a flow-rate of 60 ml/min. A nickel-63 ECD was maintained at 250°, and every 150 μsec a 47-V pulse of duration 0.75 μsec (standing current $1.7 \cdot 10^{-9}$ A) was applied with an attenuation setting for analysis producing a full-scale deflection (f.s.d.) for $2 \cdot 10^{-9}$ A. The FID was maintained at 260° with an attenuation setting of 10^{-10} A. The samples were injected directly on to the top of the column. The detector response was recorded on a Honeywell Electronic 196 Lab Chart Recorder with a chart speed of 0.1 in./min.

Isolation of progesterone from plasma

To a 5.0-ml aliquot of the heparinized plasma, 0.1 or 1.0 μg of TAc in ethanol was added as internal standard. The plasma was diluted with an equal volume of 0.3 *N* sodium hydroxide solution. The sample was extracted with two 10-ml volumes of peroxide-free diethyl ether and the combined extract was washed with two 5-ml volumes of water, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was dissolved in 3 ml of methanol–water (7:3), 3 ml of *n*-hexane were added to the solution and the mixture was shaken and centrifuged. The upper phase was separated and discarded. The aqueous phase containing progesterone was evaporated. The residue was dissolved in distilled chloroform, and half of the volume applied to a 1.5-cm strip coated with a silica gel G layer (Merck, Darmstadt, G.F.R.) (size of chromatoplate 200 × 200 × 0.2 mm). The adjacent strips contained pregnenolone dinitrophenylhydrazone as marker. Thin-layer chromatography (TLC) was performed in benzene–ethyl acetate (8:2). In this solvent system, the mobilities of PROG, TAc and the marker steroid were identical ($R_F = 0.40$). Following development, the zone corresponding to the yellow spot was scraped into a test-tube.

An additional purification of the PROG zone was effected by acetylation of impurities[1] in the presence of silica adsorbent. To the tube, 1.0 ml of pyridine and 1.0 ml of acetic anhydride–benzene (2:8) were added, and the suspension was incubated at 37° for 16 h. The reaction was stopped by adding 5 ml of ethanol–water (2:8) and the steroids were extracted with 10 ml of distilled chloroform. The extract was washed with 2 ml of 1 *N* sodium hydroxide solution, twice with 2 ml of water, dried over sodium sulphate and evaporated.

Re-chromatography of steroids was performed on highly purified silica gel G adsorbent. The adsorbent was refluxed twice with methanol, separated, extracted with distilled chloroform, dried and then the coated chromatoplate was re-activated at 105°. The residue of the above steroid extract and androstenedione bisdinitrophenylhydrazone as marker were applied on the purified silica layer and chromatographed in *n*-hexane–ethyl acetate (8:2). The R_F value of PROG, TAc and the marker was 0.35.

This zone was aspirated with an applicator, eluted with five 1-ml volumes of chloroform, the combined eluate evaporated, and the residue transferred quantitatively with peroxide-free diethyl ether into a capillary tube and evaporated.

When flame ionization detection was used, the residue was dissolved in 5.0 μl of dioxane and 0.5–1.0 μl aliquots of the solution were injected into the gas chromatograph.

Preparation of steroid heptafluorobutyrate 3-enol esters

When a PROG level in plasma below 1.0 μg per 100 ml was expected, enol ester heptafluorobutyrates were formed, and ECD employed for detection. The method recommended by Exley and Chamberlain[2] with slight modification[5] was used for derivative formation. To a sample in a capillary tube containing isolated PROG and TAc as internal standard, 10 μl of absolute tetrahydrofuran, 25 μl of distilled benzene and 25 μl of heptafluorobutyric anhydride (Fluka, Buchs, Switzerland) were added and the mixture was heated at 65° for 30 min. The excess of reagent was re-

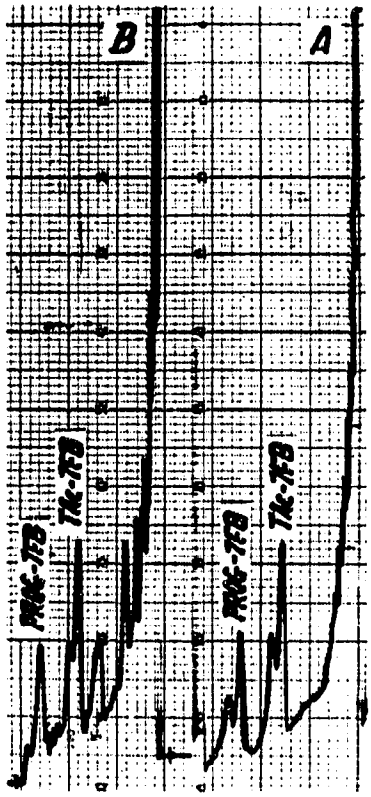

Fig. 1. Gas chromatograms of authentic (A) and plasma (B) progesterone 3-enol ester heptafluorobutyrate (PROG-7FB). Conditions: Column, 1% XE-60, Gas-Chrom Q, 205°, nitrogen carrier gas at a flow-rate of 60 ml/min. Nickel-63 detection at 250°, 150 μsec, 47-V pulse of 0.75-μsec duration. TAc-7FB, testosterone acetate 3-enol ester heptafluorobutyrate as internal standard. Each steroid derivative represents peaks of two isomers. Amount of authentic steroids chromatographed, 200 pg (A). Human plasma was obtained on the 20th day of the menstrual cycle (B).

moved under vacuum in a desiccator at 65°. The residue containing 3-enol esters of progesterone heptafluorobutyrate (PROG-7FB-3E) and testosterone acetate heptafluorobutyrate (TAc-7FB-3E) was dissolved in 10.0 μl of *n*-heptane, and 0.5–1.0-μl aliquots were injected.

The formation of enol esters by PROG and TAc in benzene medium according to the method originally proposed by Exley and Chamberlain[2] resulted in two isomers, which gave two distinct peaks in the XE-60 column (Fig. 1). The proportion of the isomers was about 2:1 in favour of the more polar component.

Gas chromatography and calculation of values

To plasma samples of pregnant women or domestic animals in the second and third trimesters, 1.00 μg of TAc as internal standard was added, and the final samples were evaluated by means of the FID. Plasma specimens obtained during the menstrual cycle of women or from cycling animals contained 100.0 ng of TAc only, and ECD was used for quantitation under the conditions described above.

The PROG concentration of plasma was calculated by measuring the peak heights of PROG and TAc on the chromatogram according to the following equation:

$$\text{PROG } (\mu\text{g per 100 ml}) = \frac{h_{\text{PROG}}}{h_{\text{TAc}}} \cdot \frac{100}{V} \cdot \frac{R_{\text{TAc}}}{R_{\text{PROG}}}$$

where

h_{PROG} = peak height of PROG, mm;
h_{TAc} = peak height of the internal standard, mm;
V = volume of plasma processed, ml;
$R_{\text{TAc}}/R_{\text{PROG}}$ = peak height ratio of equal amounts of authentic PROG and TAc in the working range of measurements by either the FID or the ECD.

This equation was found to be valid if, into plasma samples with a possible PROG level of $>$ 1 μg per 100 ml, 1.00 μg of TAc standard was added, a 0.5-μl portion of the 5.0 μl of dioxane solution injected and the steroid was detected by the FID. It was also valid when 100 ng of internal standard were processed with plasma samples containing PROG at a concentration $<$ 1 μg per 100 ml, and a 1.0-μl aliquot of the 10.0 μl of *n*-heptane solution was gas chromatographed with electron capture detection. As indicated, the chromatography of PROG-7FB-3E and TAc-7FB-3E in the XE-60 column resulted in two peaks each. Calculation of the PROG value gave the best results, when the peak heights of the two isomers were summed.

Radioactivity measurements

The addition of TAc as an internal standard in each plasma sample was justified by the determination of the ^3H:^{14}C ratio in the final plasma extracts following simultaneous processing of labelled PROG and TAc.

[7α-^{3}H]Progesterone (specific activity 5 Ci/mmole) and [4-^{14}C]testosterone (58.2 mCi/mmole) were purchased from The Radiochemical Centre (Amersham, Great Britain). The former was purified by TLC before use. [4-^{14}C]Testosterone was acetylated as described previously[3], and the radiochemical purity of the acetate checked by TLC in *n*-hexane–ethyl acetate (8:2) and chloroform–acetone (95:5). The stock

solution of the purified tracers contained 12,600 cpm of [^{3}H]PROG and 18,400 cpm of [^{14}C]TAc, giving an isotope ratio of 0.69.

The radioactivity was measured following addition to the samples of 10.0 ml of Liquifluor (NEN Chemicals Dreieichenhain, G.F.R.) containing 4 g of PPO and 50 mg of POPOP in 1000 ml of distilled toluene in a Packard Tri-Carb Model 2420 liquid scintillation spectrometer operating at an efficiency of 87% for [^{14}C] and 32% for [^{3}H]. The counting of samples was continued for a period sufficient to achieve a standard deviation of less than 5% of the estimates.

TABLE I

RESULTS OF CONTROL EXPERIMENTS

In this table, n = number of estimates, $\bar{x}$ = mean, S_x = standard error (%) and S.D. = standard deviation (μg).

Parameter	*Amount injected (ng)*	*n*	*Mode of detection*	R_{TAc}/R_{PROG}
Detector response	10	5	FID	
	25	5	FID	1.4 ± 0.1
	50	8	FID	
	100	9	FID	1.5 ± 0.2
	0.2	6	ECD	
	0.5	6	ECD	1.0 ± 0.1
	5.0	6	ECD	1.1 ± 0.1

	Start	*Final*
^{3}H:^{14}C ratios of samples after adding [^{3}H]PROG and [^{14}C]TAc to plasma	0.68	0.69
	0.68	0.64
	0.70	0.64
		0.62
	$\bar{x}$ = 0.69	0.64
		0.69
		0.68
		0.64
		$\bar{x}$ = 0.65

	Tracer	*n*	$\bar{x} \pm S_x$ (%)
Recovery of tracers from plasma	[^{3}H]PROG	7	21.02 ± 2.11
	[^{14}C]TAc	7	19.60 ± 1.47

	Subject	*Time*	*n*	*Mode of detection*	$\bar{x} \pm$ S.D. (μg per 100 ml)
Duplicate estimates	Human	20–24th day of cycle	6	ECD	0.90 ± 0.06
		Pregnancy, 5–9th month	3	FID	4.87 ± 0.47
	Cow	Oestrus	4	ECD	0.83 ± 0.08
		Early pregnancy	5	FID	1.87 ± 0.11

RESULTS

Control experiments

TLC of authentic steroids revealed the identical migration of PROG, TAc and the markers in our control experiments, and an effective separation of PROG from the naturally occurring C_{19} and C_{21} steroids with a Δ^4-3-one moiety in the plasma of mammals, as shown by Van der Molen and Groen[4]. An additional acetylation of impurities still present resulted in effective elimination of interfering materials before GLC[1].

The results of our control experiments (Table I) showed TAc to be highly satisfactory as an internal standard throughout the whole procedure. The FID response (R_{TAc}/R_{PROG}) of the two steroids was linear between 10 and 50 ng, resulting in a ratio of 1.4. The ECD responses of the 3-enol ester heptafluorobutyrate isomers of the two steroids were almost identical between 0.2 and 5.0 ng, resulting in a peak height ratio near to unity, when the peak heights of the two isomers were summed either for PROG-7FB-3E or TAc-7FB-3E.

The most reliable and simple control of the requirement of a GLC method—that the internal standard should have physical and chemical properties similar to those of the steroid to be analyzed— was made by using isotopes. As shown in Table I, the

TABLE II

PROGESTERONE IN PLASMA OF NORMAL WOMEN AND COWS DURING CYCLE AND GESTATION

Source	*Condition**	*Mode of detection*	*Progesterone (μg per 100 ml)*
Human	8–12th d.c.	ECD	0.10
			0.15
	20–26th d.c.	ECD	0.75
			0.92
			1.32
	2nd–3rd m.p.	ECD	1.3
			2.0
			2.8
	4–5th m.p.	FID	3.8
			4.2
	6–7th m.p.	FID	3.8
			4.9
			5.2
	8–9th m.p.	FID	7.5
			8.4
Cow	Oestrus	ECD	0.75
			1.10
	7th d.p.	ECD	0.80
			1.15
	10–12th d.p.	FID	0.95
			1.32
			1.90
	20th–22nd d.p.	FID	1.66
			4.7

* d.c. = day of cycle; m.p. = month of pregnancy; d.p. = day of pregnancy.

^{3}H:^{14}C ratio throughout the procedure did not change significantly, and showed TAc to be satisfactory. Recovery experiments with the two tracers revealed an identical 80% loss of both PROG and TAc from the beginning of plasma processing until injection of the samples into the gas chromatograph. Duplicate determination of the plasmas of either non-pregnant (ECD) or pregnant (FID) individuals indicated a satisfactory precision of the method. The sensitivity of the GLC assay of PROG was determined from the 2 S.D. values of parallel estimates. This value was significantly different from the water blank above 150 pg per 100 ml by electron capture detection. With use of the ECD under the conditions described, the lower limit of detection (f.s.d./100) was found to be 5 pg when an additive evaluation was made of the two enol ester isomers.

Normal values

As our method was found to be suitable for application, in addition to pregnant and non-pregnant women, to the determination of PROG in the systemic blood of other species, estimates are given in Table II for normal humans and cows. Fig. 1 also shows representative gas chromatograms for the two isomers of authentic PROG-7FB-3E and TAc-7FB-3E (A), and for the two steroids in the plasma extract of a woman on the twentieth day of the menstrual cycle (B). The steroids were detected

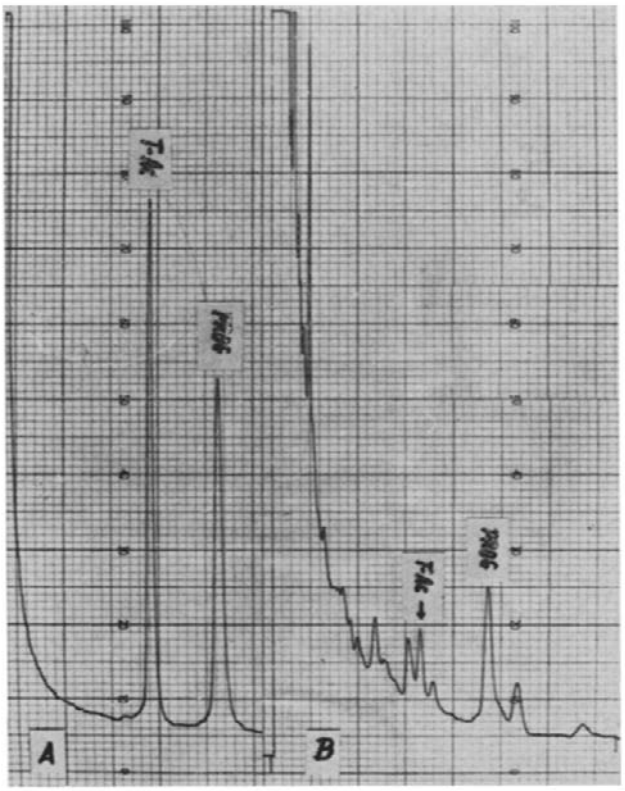

Fig. 2. Gas chromatograms of authentic (A) and plasma (B) free progesterone (PROG). Conditions: Column, 1% XE-60, Gas-Chrom Q, 230°, nitrogen carrier gas at a flow-rate of 60 ml/min. Flame ionization detector 260°. TAc, testosterone acetate as internal standard. Amount of authentic steroids chromatographed, 100 ng (A). Plasma of cow on 22nd day of pregnancy (B).

by the ECD. In Fig. 2, chromatograms for authentic TAc and PROG (A) and for a plasma extract of a cow on the twenty-second day of gestation (B) are shown, obtained by detection with the FID.

DISCUSSION

The method presented here appears to be suitable for the determination of PROG in minimal amounts in the systemic blood of women in the follicular phase of the cycle up to the last stage of pregnancy. TAc was found to be an ideal internal standard either following heptafluorobutyration for electron capture detection or as the free acetate for flame ionization detection. This was proved by comparison of the isotope ratio in initial and final samples when [^{3}H]PROG and [^{14}C]TAc were processed in a series of plasma specimens. Our control experiments showed that the use of progesterone tracer as an internal standard was unnecessary and made the assay more laborious.

In the present method, an ECD was employed for the detection and quantitation when a hormone level of less than 1.0 μg per 100 ml was expected. As indicated, the 3-enol ester heptafluorobutyrates of PROG and TAc were formed according to the general method of Exley and Chamberlain[2] and Clark and Wotiz[5]. Nakagawa *et al.*[6] and Devaux and Horning[7] suggested that this method resulted in instability and poor esterification, yielding two enol ester isomers and unreliable quantitation. The latter authors[7] recommended that the reaction should be performed in pyridine or collidine, which will be suitable for quantitative work as one of the isomers will be produced constantly in negligible amounts. Our control experiments did not confirm the observations of Devaux and Horning[7]. The introduction of an organic base as catalyst gave unidentified peaks in the picogram range of the steroids. However, the possibility that our method for the purification of pyridine by refluxing over sodium hydroxide pellets and re-destillation is inadequate cannot be excluded. On the contrary, the method using benzene and the anhydride in equal amounts without a catalyst resulted, in our experiments, in an almost theoretical yield of derivative and, although the proportion of the isomers varied somewhat, highly reproducible quantitation could be achieved by summing the peak heights of the isomers. Satisfactory result was supported, for example, by parallel estimates and our normal values for humans and cows, which were found to be comparable with the results obtained by other accurate GLC, protein-binding and radioimmunological methods (for reviews, see refs. 8–10).

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