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SIMPLE AND RAPID METHOD FOR THE DETERMINATION OF PROGESTERONE IN RAT PLASMA BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A method for estimating progesterone in rat plasma using gas–liquid chromatography (GLC) with electron-capture detection is described. By means of a column chromatographic technique for the extraction of progesterone from plasma, it is possible to isolate the steroid in such a specific way that, contrary to previously published methods, no additional chromatographic purification and separation steps are needed prior to the final GLC analysis. As a derivative for electron-capture detection we used the 3,20-di-O-pentafluorobenzyloxime of progesterone. This derivative is superior to those used hitherto with regard to sensitivity and stability. The high overall recovery (mean value, 89.4%) makes this method especially convenient for research work when only little plasma is available, or when the concentration of progesterone is very low.

INTRODUCTION

The determination of progesterone in plasma requires extremely sensitive analytical techniques. Methods have been developed based upon double isotope derivative formation[1–3], gas–liquid chromatography (GLC)[4–12] and competitive protein binding[13–15]. Because of their complex nature, double isotope techniques are no longer in use. GLC, for a long time the method of choice, is being more and more displaced by radioimmunochemical assays (RIA)[16–20]. By means of RIA, it is possible to determine even picogram amounts of steroid hormones in plasma. Results can be obtained more simply and rapidly than by other methods.

The performance of RIA, however, is not as easy as it may seem at first sight. Recently published papers[21–23] refer to the numerous possibilities of error involved in radioimmunochemical procedures. Some of the commercial kits for assaying progesterone have turned out to be unsuitable in metabolic research, which requires a very high accuracy[12]. A new study of Röhle *et al.*[24] shows that results obtained for progesterone by RIA in different laboratories are not comparable and that the within-laboratory variance can also be high. Therefore, in our opinion, there is still a need for other highly sensitive and specific progesterone assay methods.

Gas–liquid chromatography in combination with electron-capture detection (ECD) offers nearly equal sensitivity. The detection limits are in the low picogram range. Owing to the complexity of plasma, however, considerable purification is needed to eliminate interfering substances. Methods for assaying progesterone with GLC–ECD developed up to now[7,9,11] include several purification and separation steps (thin–layer, paper or column chromatography) prior to the final GLC analysis. The great expenditure of time and labour thus required makes these methods inconvenient for routine analyses.

In this paper, we describe a simple and rapid method for estimating progesterone by means of GLC–ECD, requiring no additional chromatographic separation steps after extraction from plasma. Procedural simplicity and speed are achieved by using a column chromatographic extraction technique allowing a "selective" separation of progesterone from plasma.

EXPERIMENTAL

Materials

All solvents were of analytical-grade quality and were fractionally distilled prior to use. Dichloromethane was additionally purified by washing with concentrated sulphuric acid, sodium hydroxide solution and water before distillation. Pyridine was refluxed over potassium hydroxide pellets for 2 h and then distilled through a fractionating column.

Extrelut was obtained from E. Merck (Darmstadt, G.F.R.). The commercial product was washed with *n*-hexane in a Soxhlet apparatus for 6 h, taken to dryness in a rotary evaporator and stored in dust-free conditions. About 300 g of the Kieselguhr were purified simultaneously. Sephadex LH-20 was from Pharmacia (Uppsala, Sweden).

Progesterone and testosterone were obtained from Fluka (Neu-Ulm, G.F.R.), pentafluorobenzoyl chloride from EGA (Steinheim, G.F.R.). O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (Fluka) was recrystallized from ethanol. Progesterone 3,20-di-O-pentafluorobenzyloxime (di-O-PFBO) was prepared according to Koshy *et al.*[25]. Testosterone 3-O-pentafluorobenzyloxime-17-pentafluorobenzoate (O-PFBO-PFB) was prepared from testosterone 17-pentafluorobenzoate[26] in the same way. [4-^{14}C]Progesterone, specific activity 55 mCi/mmol, was purchased from Amersham-Buchler (Braunschweig, G.F.R.). It was purified prior to use by chromatography on Sephadex LH-20.

All glassware was cleaned by soaking it overnight in a detergent solution (LM3; Weigert, Hamburg, G.F.R.) and washing in a laboratory glassware washing-machine; it was rinsed with deionized water and double-distilled acetone immediately before use. The derivatization tubes were silanized with a 5% solution of dimethyldichlorosilane in toluene and then rinsed with toluene and acetone.

Instrumental

GLC was carried out using a Packard–Becker gas chromatograph (Model 417), equipped with a ^{63}Ni electron-capture detector. The detector response was recorded on a PM 8000 (Philips) recorder. The glass column (2 m × 2 mm I.D.) was silanized and packed with Chromosorb G AW DMCS (100–120 mesh), coated with

3% OV-17. The carrier gas used was argon, containing 5% methane, at a flow-rate of 60 ml/min (inlet pressure 38 p.s.i.). No purge gas was used. Chromatography was carried out at 285° with detector and flash-heater temperatures of 310° and 300°, respectively. The detector was used in the pulsed mode (pulse period, 200 μsec; pulse width, 0.5 μsec; pulse amplitude, 50 V ± 10%).

Radioactive counting was performed on a Packard Tri-Carb liquid-scintillation spectrometer (Model 3380). Samples were dissolved in 10 ml toluene scintillator (Packard–Becker, Groningen, The Netherlands) containing 5 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP) per litre of toluene. The counting of samples was continued for a period sufficient to achieve a standard deviation of less than 2% of the estimate. Efficiency for ^{14}C-counting was *ca.* 92%. Quench corrections were not needed.

Peak measurements were made with the DP 88 (Pye Unicam) computing integrator.

Sample preparation

Rats were anaesthetized with diethyl ether, and blood obtained by puncture of the abdominal aorta was collected into tubes containing sodium heparin. Plasma was separated by centrifugation and stored at −20° until it was assayed.

Glass columns (52 cm × 14 mm I.D.), fitted with a fritted-glass filter and a PTFE stopcock, were filled with 13 g of purified Extrelut. To achieve settling of the material a slight vacuum was applied to the bottom of the column. Extrelut ready-to-use columns were not used, because contaminants from the plastic material interfered with the final GLC–ECD analysis. Moreover, we found that better separations could be attained by using the narrower glass columns.

Plasma (2–3 ml) was introduced into a 50-ml test-tube containing *ca.* 6000 cpm [4-^{14}C]progesterone. After dilution to 10 ml with 0.1 *N* NaOH solution and vigorously shaking on a Vortex mixer, the specimen was poured onto the column. The tube was rinsed with two 5–ml volumes of 0.1 *N* NaOH solution, which were also transferred to the column. After distribution of the aqueous phase on the matrix (*ca.* 15 min were required) the column was first washed with 100 ml of *n*-hexane (this wash was discarded) and then eluted with 50 ml of *n*-hexane–dichloromethane (1:1). On the basis of preliminary recovery experiments with radioactively labelled progesterone, the 10–50-ml fraction was collected in a flask and evaporated *in vacuo*. The dry residue was transferred with 4 × 0.3 ml acetone to a small reaction tube with PTFE-lined screw cap and taken to dryness under a stream of nitrogen at 56°.

For derivatizing we used the method recommended by Koshy *et al.*[25] with a slight modification. We added 50 μl of a solution of pentafluorobenzylhydroxylamine hydrochloride in pyridine (10 mg in 4 ml) to the steroid extract and kept the mixture at 65° for 30 min. After removal of pyridine under nitrogen at 56°, the residue was dissolved in 1 ml of 0.1 *N* NaOH solution and extracted three times with 1 ml of *n*-hexane. The organic layer, containing the progesterone derivative, was transferred to an other tube and again evaporated to dryness under nitrogen.

Depending on the anticipated concentration of progesterone in the unknown plasma sample, the residue was dissolved in 0.2–0.8 ml of *n*-hexane, containing a known amount of testosterone O-PFBO-PFB as internal standard. Two aliquots (each one-tenth of the chosen volume) were taken for measuring the radioactivity to

determine losses during preparation. Portions of 3 to 4 μl of the solution were injected into the gas chromatograph. Peak areas were evaluated with a computing integrator. The retention times of progesterone di-O-PFBO and testosterone O-PFBO-PFB were 15.5 and 11.2 min, respectively.

Calculations

Estimation was made by comparing the peak areas of progesterone di-O-PFBO with those of the internal standard. The ratio of area responses of progesterone di-O-PFBO and testosterone O-PFBO-PFB was found to be linear from 0.05 to 2 ng (Fig. 1).

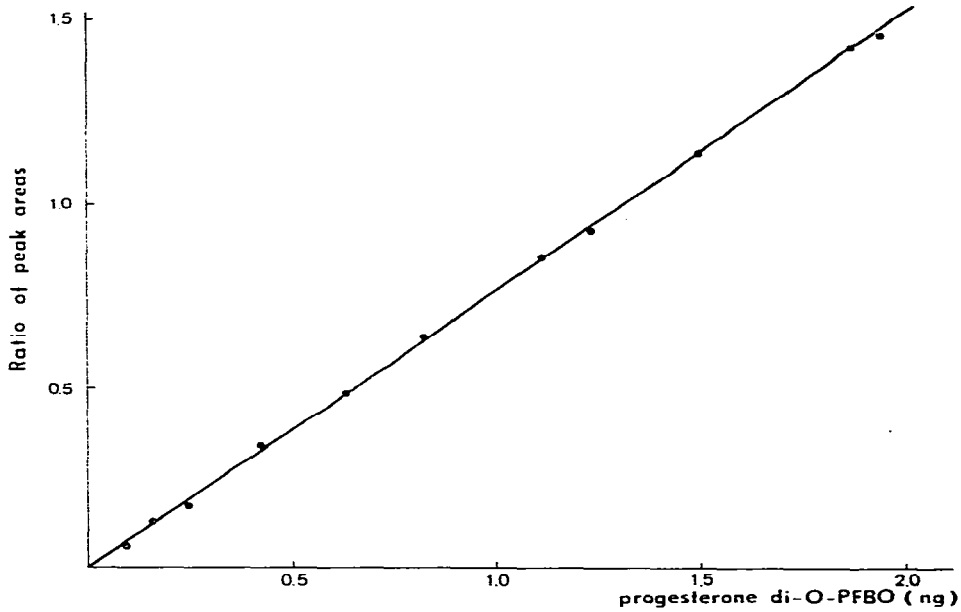

Fig. 1. Ratio of peak area of progesterone di-O-PFBO to that of internal standard (testosterone O-PFBO-PFB) plotted against amount of progesterone di-O-PFBO.

The total amount (P) of progesterone in the plasma sample was calculated as follows:

$$P(\text{ng}) = \left(\frac{A_P}{A_T} \cdot F_R \cdot [\text{T}] \cdot V \cdot 0.446 - P^*\right) \frac{100}{R} \quad (1)$$

where

A_P = peak area of progesterone di-O-PFBO in sample

A_T = peak area of testosterone O-PFBO-PFB in sample

[T] = concentration (ng/μl) of testosterone O-PFBO-PFB standard solution

V = volume (μl) of internal standard solution in which the derivatized steroid extract is taken up

$$0.446 = \frac{\text{molecular weight of progesterone}}{\text{molecular weight of progesterone di-O-PFBO}} = \frac{314.5}{704.5}$$

P^* = amount of [4-^{14}C]progesterone added to the plasma sample

R = per cent recovery of [4-^{14}C]progesterone prior to GLC analysis

F_R, the relative response factor, was determined prior to analysis by injection of standard solutions containing known amounts of progesterone di-O-PFBO and testosterone O-PFBO-PFB (m_P and m_T) and was calculated as follows:

$$F_R = \frac{A_T \cdot m_P}{A_P \cdot m_T} \tag{2}$$

RESULTS

Recovery

To estimate losses during the column extraction procedure, five plasma samples (2 ml) were supplemented with [4-^{14}C]progesterone and processed by the described method. The mean yield of the tracer was 93.8 ± 4.7%. The overall recovery (up to gas–liquid chromatography) of 28 plasma samples averaged 89.4% (76.3–97.6%).

Accuracy and precision

Replicate determinations were done on known amounts of progesterone dissolved in 3 ml water and assayed by our method. The amounts of progesterone added (50, 100 and 150 ng) were in the "physiological range". The results are shown in Table I.

TABLE I

RECOVERY OF DIFFERENT AMOUNTS OF PROGESTERONE, ADDED TO 3 ml WATER AND PROCESSED BY OUR METHOD

	Progesterone added (ng)		
	50	*100*	*150*
Found (ng)	48.7	108.4	147.9
	52.3	96.5	153.6
	45.2	98.2	155.1
	46.9	105.2	142.5
	51.0	107.8	156.5
Mean (ng)	48.8	103.2	151.1
S.D. (ng)	2.9	5.5	5.8
Precision (%)	6.0	5.4	3 9

Precision was estimated by performing replicate analyses on a plasma pool, obtained from female rats. A mean value of 113 ng per 3 ml plasma was found with a standard deviation of 5.3. ng (n = 5). Thus, at a concentration of 38 ng/ml, the precision of the assay is *ca.* 4.7%.

Specificity

Samples were analyzed using liquid phases of different polarity (SE-30 and OV-17). In both cases, peaks were observed at the same retention time as standard

progesterone di-O-PFBO. On the SE-30 column, we obtained two peaks corresponding to the *syn* and *anti* forms of the oxime[25], while on the more polar stationary phase OV-17 the retention times of the isomers were identical.

The absence of detectable progesterone in the plasma from adrenal and ovariectomized rats is additional evidence for the high degree of specificity achieved. Fig. 2 demonstrates that gas–chromatographic tracings of such samples show no peaks in either the progesterone O-PFBO area or the testosterone O-PFBO-PFB area.

GLC–mass fragmentography data also confirmed the identity of peaks obtained in plasma extracts with authentic progesterone di-O-PFBO.

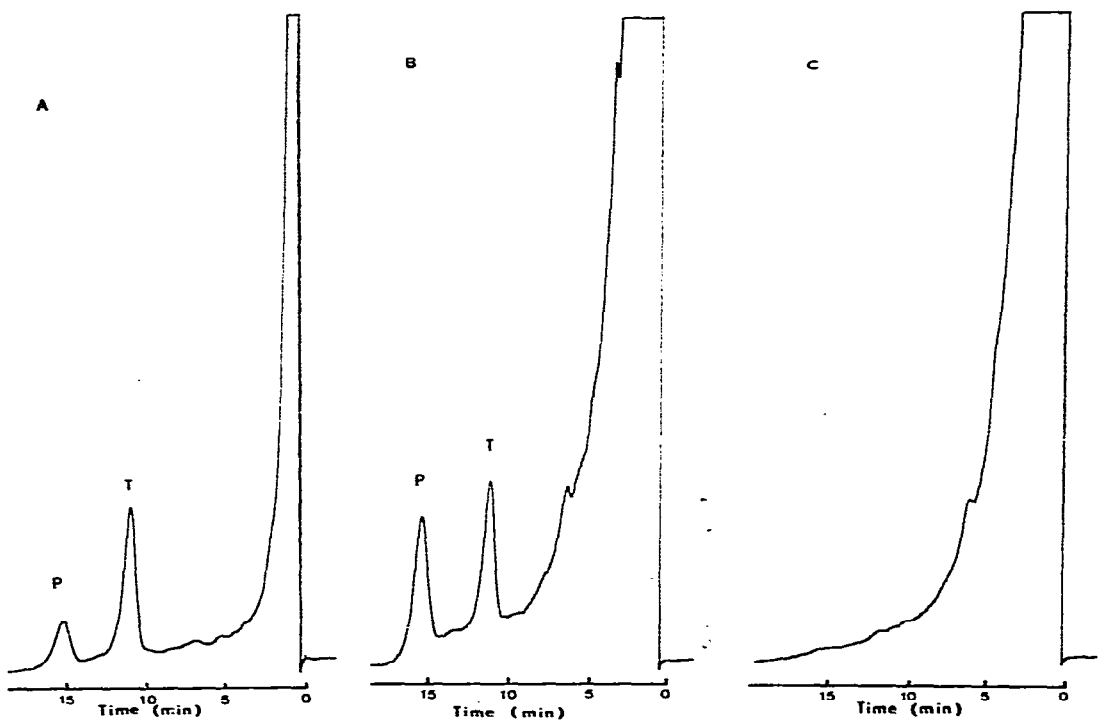

Fig. 2. Gas chromatographic tracings of A, standard mixture of 360 pg of progesterone di-O-PFBO (P) and 450 pg of testosterone O-PFBO-PFB (T); B, sample of 2.8 ml plasma obtained from a pregnant rat (4th day of pregnancy) and processed by our method (peak area of progesterone di-O-PFBO corresponds to 960 pg); C, sample of 3 ml plasma obtained from an adrenal and ovariectomized female rat and processed by our method without addition of [4-^{14}C]progesterone and internal standard. The conditions were as described under '*Instrumental*'.

DISCUSSION

Owing to the non-specific nature of the commonly used liquid–liquid extraction for isolation of progesterone from plasma, subsequent elaborate purification and separation steps are needed in order to remove lipid material which is also extracted by the solvents used to extract progesterone and further to separate progesterone from other steroids also present in the plasma extract. This is time–consuming and laborious.

Recently, we described a simple column chromatographic method for the extraction of steroid hormones from plasma, allowing a nearly quantitative separation of the steroids in a single step[27]. Further experimentation showed that this technique could be used for a more selective isolation of hormones from plasma when advantage is taken of the different elution behaviour of plasma components with different polarities. By means of this method we were able to isolate progesterone in such a specific way that no additional chromatographic separation steps were necessary before final GLC analysis. Highly lipophilic compounds are removed by washing the column with 100 ml of *n*-hexane. Less than 1% of the total progesterone was found in this effluent. The use of the non-polar solvent mixture *n*-hexane–dichloromethane (1:1) allows elution of progesterone free from more polar hormones, such as corticosterone, estriol and estradiol, which are not at all or only partially extracted from the column under these conditions.

Furthermore, the specific retention behaviour of progesterone di-O-PFBO, recently described by Koshy *et al.*[25], adds to the specificity of the method. The late elution of this derivative from the GLC column guarantees good separation of the progesterone peak from other plasma components still present and from interfering material from glassware, solvents, etc. (see Fig. 2). The progesterone 3-enol heptafluorobutyrate preferred up to now[11,28] coincided with the solvent peak under our preparation conditions, and could not be quantitatively estimated.

An additional advantage of progesterone di-O-PFBO, as compared to the derivatives hitherto used for GLC determination of progesterone with ECD (monochloroacetate[29] and heptafluorobutyrate[30,31], is its greater stability. The greater sensitivity of our method not only results from the higher molar response of the oxime[25] to electron capture but also from the fact that a single peak is obtained on OV-17 in spite of the formation of isomers (*syn* and *anti*) during derivatization. The isomeric forms of progesterone 3-enol heptafluorobutyrate, are however, resolved on the GLC column (on SE-30 as well as on OV-17), and double peaks are less easy to estimate and to see at low concentrations. The high stability of progesterone di-O-PFBO allows easy removal of unreacted reagents and other interfering materials by simple washing of the reaction mixture with NaOH solution. The complete conversion of progesterone in to the dipentafluorobenzyloxime was proven by GLC with flame-ionization detection.

The mean overall recovery of the method is high (89.4%). Previously described similar methods[7,9,11] have recoveries of 51, 60 and 21% up to gas chromatography. The small loss of progesterone during sample preparation makes our method especially convenient for research work when only small amounts of plasma are available, or when the concentration of progesterone is very low. The accuracy and precision of our method are similar to those reported for other progesterone GLC determinations.

All solvents and reagents used for sample preparation must be thoroughly purified. Special care must be taken in the purification of Extrelut; otherwise, the solvent front broadens to such a degree that the steroid peaks under observation come off on a sloping baseline, and thus the sensitivity and accuracy of the method are diminished. Moreover, all glassware must be kept scrupulously clean. Tubes used in the last stage must be silanized to prevent absorption of steroid derivatives on the glass surface.

The method is used by us for estimating progesterone in plasma of female rats of different stages of pregnancy. Progesterone levels vary from 20 to 150 ng/ml, and the plasma volumes available per estimation vary from 1.5 to 3 ml. Plasma samples of non-pregnant rats can also be assayed by our method. Excluding the purification of solvents and column material and cleaning of glassware, a complete assay can be carried out within 3 h. Eight to ten plasma specimens can be analyzed daily, with the final GLC analysis performed in duplicate.

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