NOTE

The Simultaneous Assay of Progesterone, 17-Hydroxyprogesterone, and Deoxycorticosterone in Human Plasma by Competitive Protein Binding

MASANOBU HONDA

*The 2nd Department of Internal Medicine,
Nihon University School of Medicine,
Ooyaguchi, Itabashi-ku, Tokyo 173*

Synopsis

A method is described for the simultaneous measurement of progesterone (P), 17-hydroxyprogesterone (17-OHP) and deoxycorticosterone (DOC) in plasma by competitive protein-binding assay. After extraction, these steroids were purified by Sephadex LH-20 columns. The method described here is sensitive enough to detect the above-mentioned steroids in as little as 5 ml of plasma under physiological conditions. The mean physiological plasma level (ng/100 ml) of P, 17-OHP and DOC are 18.2 ± 1.6 (SE), 136.5 ± 11.7 , and 8.6 ± 1.0 in men, 14.7 ± 1.7 , 61.2 ± 6.9 and 6.0 ± 1.2 in women in the follicular phase, and 645.0 ± 108.1 , 155.2 ± 16.9 , and 7.5 ± 1.2 , in the luteal phase of the menstrual cycle.

By this method and others, the responses of plasma levels of 7 adrenal steroids (P, 17-OHP, DOC, corticosterone, deoxycortisol, cortisol and aldosterone) to ACTH infusion (25U i.v. over 8 hours) were investigated in healthy men. The increase of DOC was more pronounced than that of the other steroids.

The steroids, progesterone (P), deoxycorticosterone (DOC) and 17-hydroxyprogesterone (17-OHP) are especially important precursors in the biosynthesis of adrenal steroid hormones. However, the simultaneous measurement of these three steroids has never been presented because of difficulties in separating and purifying plasma DOC from the other steroids by thin-layer or paper chromatography. Therefore, we have developed a sensitive, precise and reproducible method based on competitive protein-binding assay (Murphy, 1967) for the simultaneous measurement of plasma P, 17-OHP and DOC by the use of Sephadex LH-20.

In addition, this method and others (Newsome *et al.*, 1972; Nowaczynsky *et al.*, 1974) the plasma levels of P, 17-OHP,

corticosterone, deoxycortisol, cortisol and aldosterone were measured under physiological conditions and in response to ACTH infusion.

Materials and Methods

Subjects:

Nineteen healthy male and 24 female normotensive subjects (mean age: 31.2 ± 1.4 (SE) years) were served as controls. All female subjects were selected after their menstrual cycles had been monitored for the last two months.

An 8-hour intravenous infusion of 25 units of ACTH (Acthar, Armour Pharmaceutical Co.) in 1 liter of 5% dextrose was given to 7 healthy male volunteers (mean age: 42 ± 4.5 (SE) years). A baseline sample was taken in the recumbent position between 8 and 9 a.m. and two more samples were collected for 4 and 8 hours, respectively, after the advent of the infusion. All subjects studied were on a diet containing 135 mEq of sodium and 90 mEq of potassium, daily.

Reagents and equipment:

a) *Steroids*: All tritiated steroids (30–53 Ci/mmol) were obtained from Amersham Searle. Crystalline steroids were used without further purification.

b) *Solvents*: All solvents were of reagent grade and purified before use (Shao *et al.*, 1970). Pyridine and acetic anhydride were of analytical reagent grade and were not any further purified.

c) *Florisil* (Fisher Scientific Co.): 60–100 mesh was used as described before (Sasaki *et al.*, 1972).

d) *Liquid scintillation solution* (Bray's solution) was prepared as described before (Nowaczynski *et al.*, 1974).

e) *Sephadex LH-20* was obtained from Pharmacia, Uppsala.

f) *Sudan III* (Chroma-Gesellschaft Schmit & Co.) was separated into two bands on a Sephadex LH-20 column as described before (Lebel *et al.*, 1975). The less polar fraction (about 3 ml) was designated as the first part of Sudan III, and the other fraction (about 3 ml) as the second part. 0.1 ml aliquots of these two dye fractions were used as indicators after evaporation.

g) *Sea sand* (Fisher Scientific Co.) was washed with methylene chloride before use.

h) *Corticosteroid binding globulin (CBG)*: Plasma obtained from a male bilaterally adrenalectomized dog was used as the source of CBG (Murphy, 1967). CBG solution for the assays of P and 17-OHP was prepared by the following procedure: Before each assay, an aliquot of 1, 2-³H-corticosterone stock solution, to give a final concentration of approximately 21,000 cpm/ml, was evaporated in a flask. One ml of the plasma was then diluted with 100 ml of 0.05 M phosphate buffer (pH 8.0) and incubated for 5 minutes at 40°C by intermittent shaking and the mixture was stored at 4°C until required.

In the assay for DOC, an amount of 1, 2-³H-DOC stock solution, to give a final concentration of approximately 60,000 cpm/ml, was evaporated. One ml of plasma was then added and the solution was gently mixed up for 2 minutes at 40°C before 36 ml of 0.05 M phosphate buffer (pH 8.0) was added. The mixture was stored at 4°C until needed.

i) *Equipment*: Chromatographic columns were of brosilicate glass, either 20 cm, 50 cm or 70 cm in length, 0.9 cm in inside diameter and their solvent reservoirs 80 ml in capacity.

A Packard liquid scintillation spectrometer (model 3375) was used for radioactive counting. The counting efficiency (³H) was 48.6%.

*Methods:**Extraction:*

Internal recovery standards (approximately 1,000 cpm for P and 17-OHP, and 600 cpm for DOC)

were added to 5–10 ml of plasma and the steroids were extracted with methylene chloride and then evaporated. The residue was then dissolved in 3 ml of 50% aqueous methanol and partitioned against 7 ml isooctane. The aqueous methanol phase (containing DOC and 17-OHP) and the isooctane phase (containing P) were separated and evaporated.

Column chromatography for P (Fig. 1):

Sephahex LH-20 (7 g) was equilibrated with a mixture (200 ml) of isooctane: benzene: methanol (85:10:5) and poured into columns 20 cm in length as described before (Lebel *et al.*, 1975). After being packed, each sample was applied in 0.1 ml of the eluant containing the first part of Sudan III as a marker. After the complete elution of the dye, 4 ml of the eluate containing P was collected and evaporated. This residue was dissolved in 2 ml of methanol and 0.5 ml of this solution was used for recovery determinations by radioactive counting. The remainder was dried and then used for the binding assay.

Column chromatography for 17-OHP and DOC:

a) *The first column chromatography* (Fig. 2): The solvent system was methylene chloride: methanol (98:1). As described for the P column, column beds of 65 cm were packed in 70 cm columns and a layer of sand (0.5 cm) was added to the column bed to prevent flotation of the Sephadex beads. The residue of the adequate methanol phase from the initial partition step was applied to the column with the first part of Sudan III dye in the same way as described for the P column chromatography. After elution of the dye, 3 ml was collected for DOC

Fig. 1. Elution profile of some steroids compared to that of progesterone. Approximately 5,000 cpm of each steroid were chromatographed. P: Progesterone; 1: androstenedione; 2: pregnenolone; 3: dihydrotestosterone; 4: 20 α -dihydroprogesterone; 5: testosterone; 6: deoxycorticosterone; 7: 17-hydroxyprogesterone; 8: dehydrocorticosterone; S-III: the first part of Sudan III.

Fig. 2. Elution profile of some steroids compared to those of DOC and 17-OHP. P: progesterone; DOC: deoxycorticosterone; 17-OHP: 17-hydroxyprogesterone; 1: androstenedione; 2: dehydrocorticosterone; 3: 20 α -dihydroprogesterone; 4: dihydrotestosterone; 5: androsterone; 6: testosterone; 7: pregnenolone; 8: corticosterone; 9: deoxycortisol; 10: aldosterone; 11: 17-hydroxypregnenolone; 12: cortisone; 13: cortisol. S-III: the first part of Sudan III.

Fig. 3 (left). Elution profile of some acetylated steroids compared to that of 17-OHP. 17-OHP: 17-hydroxyprogesterone; 1: 20 α -dihydroprogesterone acetate; 2: testosterone acetate; 3: dehydrocorticosterone acetate; 4: dihydrotestosterone acetate; S-III: the first part of Sudan III.

Fig. 4 (right). Elution profile of some steroids compared to that of DOC. DOC: deoxycorticosterone; 1: progesterone; 2: androstenedione; 3: 17-hydroxyprogesterone; 4: dehydrocorticosterone. S-III: the second part of Sudan III.

Fig. 5. Standard curves for progesterone (A), 17-hydroxyprogesterone (B) and deoxycorticosterone (C). The abscissa is the log dose. The ordinate is the percentage of bound steroids which was calculated from the formula: $\frac{B-N}{B_0-N} \times 100$ where B: the bound counts, B₀: the counts at 0 ng, and N: the non-specific counts.

(Fig. 2). Then 1 ml of eluate was allowed to pass through the column before 4 ml was collected for 17-OHP (Fig. 2). These fractions were collected and then evaporated. The residue containing 17-OHP was acetylated as described before (Strott and Lipsett, 1968). After the reaction, the tube was rinsed with methanol and the mixture evaporated. This residue was rechromatographed on the same column and 17-OHP, which remained unchanged, was collected as before (Fig. 3). The binding assay and the estimation of recovery are as described above.

b) *The second column chromatography* (Fig. 4): The solvent system was isooctane: methylene chloride: ethanol (50:50:0.25). The column was 50 cm in length, with gel 45 cm in height. The dried residue containing DOC was chromatographed in the same way as described above except that the second part of Sudan III was used as the marker. After elution of the dye, 4 ml was collected for DOC, evaporated and processed as described above.

Competitive protein-binding assay:

Standard binding curves for P, 17-OHP and DOC were prepared simultaneously with each assay. In order to estimate the non-specific binding of the ligand to the above mentioned steroids, 1 μ g of these unlabeled steroid standards was used in each series of assays.

In the protein-binding assay for P and 17-OHP, 1 ml of ^3H -corticosterone-CBG solution was added to the dried standards and unknowns. The tubes were processed as described before (Sasaki, *et al.*, 1972).

For the assay of DOC, 0.2 ml of the ^3H -DOC-CBG solution was added to each tube and the contents were processed as described before (Brown and Strott, 1971).

Calculations:

The standard dose-response curve was made by plotting the percentage of the total bound cpm versus the log of the amount of the added unlabeled steroid. Typical standard curves for each steroid are shown in Fig. 5. The results, expressed in ng/100 ml, were calculated by means of the following equation:

$$\text{ng/100 ml} = A \times \frac{2}{B} \times \frac{100}{C} \times \frac{100}{D}$$

where A: ng of each steroid read off the standard curve

B: eluate aliquot used for the assay

C: percentage of recovery

D: plasma aliquot used for analysis

Results

Recovery: The mean recovery ($n=50$) for P, 17-OHP and DOC was 44.2 ± 1.2 (SE)%, 56.1 ± 1.3 and 40.2 ± 1.1 , respectively.

Blank: Five ml of distilled water ($n=10$) or charcoal treated steroid-free plasma ($n=5$) was used as blanks for the procedure.

All these samples gave values indistinguishable from zero.

Sensitivity: The useful range of the standard curve (Fig. 5) was 0.05 to 5 ng for P and 17-OHP, and 0.05 to 2 ng for DOC.

This method can measure as little as 2.1 ng P, 1.9 ng 17-OHP and 1.9 ng DOC/100 ml plasma in a 5 ml plasma sample.

Accuracy: When 0.2 ng, 1 ng and 2 ng of P ($n=4$), 17-OHP ($n=4$) and DOC ($n=4$) were added to 5 ml of distilled water and carried through in the entire procedure, the mean values were 0.20 ± 0.02 (SE) ng, 0.98 ± 0.09 and 2.0 ± 0.11 for P, 0.23 ± 0.03 , 1.13 ± 0.05 , and 2.0 ± 0.07 for 17-OHP and 0.21 ± 0.03 , 1.0 ± 0.07 and 2.1 ± 0.11 for DOC.

Precision: The precision was assessed by serial determinations of plasma pools from healthy men and women. The coefficients of variation for the three serum samples, one from males and the other two from females in the follicular and luteal phases of the menstrual cycle respectively, were: 17.1, 16.3 and 5.8% for P, 4.3, 9.3 and 6.3% for 17-OHP and 11.5, 13.6 and 6.1% for DOC.

Specificity: The specificity of the method was attained by chromatographic purification of extracts and by the relative binding of steroids by CBG (Table 1). Steroids as listed in Table 2, which seemed to overlap slightly with P, 17-OHP and DOC was tested for their cross reactivity. Two hundred nanograms of all steroids and 500 ng of corticosterone were added to 5 ml of distilled water or charcoal-treated steroid-free plasma and carried through in the entire procedure. None of the steroid tested caused a displacement of P, 17-OHP or DOC in the binding assay.

Normal values: The mean normal plasma levels (ng/100 ml) of P, 17-OHP and DOC are 18.2 ± 1.6 (SE) (range: 6.6–28.9), 136.5 ± 11.7 (range: 72.6–286.3) and 8.6 ± 1.0 (range: 2.2–15.5) in men ($n=19$),

Table 1. Relative affinity of the corticosteroid binding globulin to the progesterone, 17-hydroxyprogesterone and dexamethasone assay for some steroid ligands.

Steroid	Relative affinity	
	P, 17-OHP Assay	DOC Assay
Progesterone	53.68	48.0
17-hydroxyprogesterone	100.0	114.29
Deoxycorticosterone	71.83	100.0
20 α -dihydroprogesterone	0.72	2.92
Pregnenolone	0.65	2.50
Dehydrocorticosterone	0.46	1.46
Testosterone	0.54	2.43
Dihydrotestosterone	0.48	2.33
Dehydroepiandrosterone	0.65	1.59
Androsterone	0.52	1.75
Androstenedione	0.52	1.84

14.7 ± 1.7 (range: 5.7–30.7) ($n=19$), 61.2 ± 6.9 (range: 29.9–113.4) ($n=11$) and 6.0 ± 1.2 (range: 2.7–15.0) ($n=13$) in women in the follicular phase, and 645.0 ± 108.1 (range: 31.0–1791.8) ($n=20$), 155.2 ± 16.9 (range: 35.9–240.5) ($n=16$) and 7.5 ± 1.2 (range: 2.7–14.0) ($n=13$) in women in the luteal phase. There was no significant difference between plasma levels of P in men and those in women during the follicular phase of the menstrual cycle. Furthermore, plasma levels of DOC in men did not differ significantly from those in women throughout the menstrual cycle.

Plasma steroid levels in response to ACTH:

The mean baseline plasma levels were: P, 15.9 ng/100 ml; 17-OHP, 121.8 ng/100 ml; DOC, 8.9 ng/100 ml; corticosterone, 478.0 ng/100 ml; deoxycortisol, 48.7 ng/100 ml; cortisol, 15.4 μ g/100 ml; aldosterone, 6.9 ng/100 ml. The increase after 4 and 8 hours of ACTH infusion, respectively was: P, 10.1 and 14.5 times more than the baseline level; 17-OHP, 2.6 and 3.4; DOC, 14.8 and 18.0; corticosterone, 10.6 and 13.7; deoxycortisol, 6.7 and 10.7; cortisol, 2.9 and 3.1; aldosterone, 2.0 and 1.6. The increase of DOC was more pronounced than that of the other steroids.

Table 2. Steroids proven not to interfere with the simultaneous assay

Progesterone		**	***
Pregnenolone	*	**	***
20 α -dihydroprogesterone	*	**	***
17-hydroxyprogesterone	*	**	
Deoxycorticosterone	*		***
Dehydrocorticosterone		**	***
Corticosterone			***
Testosterone	*	**	***
Dehydroepiandrosterone	*	**	***
Androsterone	*	**	***
Androstenedione	*	**	***
Dihydrotestosterone	*	**	***

* Steroids proven not to interfere with the progesterone assay

** Steroids proven not to interfere with the deoxycorticosterone assay

*** Steroids proven not to interfere with the 17-hydroxyprogesterone assay

Discussion

Since the introduction of the competitive protein-binding method for measuring steroid hormones (Murphy *et al.*, 1963), various assay methods have been developed.

Brown *et al.* (1971) presented a method for measuring plasma DOC using thin-layer chromatography. However, with a sensitivity of 5 ng/100 ml using 10 ml of plasma, detection of DOC was not possible in 40% of normal population. The method reported here was sensitive enough to allow plasma P, 17-OHP and DOC determinations to be made on as little as 5 ml of plasma. Furthermore, by the use of Sephadex LH-20, which has several advantages (Murphy *et al.*, 1971; Newsome *et al.*, 1972; Castro *et al.*, 1973), the procedural blank was reduced to a negligible level and it was shown that the three steroids, as well as corticosterone, deoxycortisol, cortisone, aldosterone, and cortisol, could be separated and collected individually on one column chromatography with only a small proportion of solvent as shown in Fig. 2.

The values obtained for 17-OHP and DOC plasma concentrations were close to those found by Youssefnejadian *et al.* (1972), Jänne *et al.* (1974), Castro *et al.* (1973), and Oddie *et al.* (1972).

With regard to P levels in men and in women in the follicular phase of the menstrual cycle, the present results are in good agreement with the most recent report of Calabresi *et al.* (1974).

Up to this time, studies of plasma levels of DOC during the menstrual cycle have not been performed systematically. In this study, there was no significant difference between the mean values during the follicular phase and the luteal phase of the menstrual cycle.

Several authors have investigated the effect of ACTH on various plasma steroids using different doses and periods of infusion

(James *et al.*, 1968; Strott *et al.*, 1969; Brown and Strott, 1971; Oddie *et al.*, 1972; Varsano-Aharon and Ulick, 1973; Dluhy *et al.*, 1974; Kolanowski, 1974; Nabors *et al.*, 1974). However, no systematic investigation of the responses of the above-mentioned steroids has been made.

The results presented here show that of the seven steroids investigated, the responses of plasma P, DOC, corticosterone, and deoxycortisol were more pronounced. Maybe, these differences could be due, at least to some extent, to a modification of the metabolic clearance rate and the protein bound fraction of the seven steroids by ACTH (Messerli *et al.*, 1975). However, as previously suggested (Nabors *et al.*, 1974), the measurement of plasma P, DOC, corticosterone and deoxycortisol may provide a more useful index for ACTH test of adrenal function than cortisol and 17-OHP.

The method should prove to be reliable and its application useful in the diagnosis and investigation of qualitative changes in adrenal hormones.

Acknowledgement

This work was done at Clinical Research Institute of Montreal, Canada. The author is grateful to Prof. M. Hatano, the 2nd Department of Internal Medicine, Faculty of Medicine, University of Nihon, and Drs. F. H. Messerli, W. Nowaczynski and J. Genest, Clinical Research Institute of Montreal, for their advices and encouragements throughout these studies.

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