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Determination of progesterone and some of its neuroactive ring A-reduced metabolites in human serum

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Abstract

A method for the separation and assay of some ring A-reduced metabolites of progesterone (pregnanediones and pregnanolones) is described. Serum was extracted with an organic solvent, and the extract chromatographed using high performance liquid chromatography (HPLC). A total of 50 fractions was collected for each sample and split using a stream splitter so that 30% was collected in counting vials for recovery while 70% was collected in test tubes which were assayed by radioimmunoassay. An antiserum raised in our laboratory to progesterone-3-CMO-BSA cross-reacted with five of these compounds (5α- and 5β-dihydroprogesterone, 3α- and 3β-5α-tetrahydroprogesterone, and 3β,5β-tetrahydroprogesterone). Since pregnenolone eluted with 5α,3β-tetrahydroprogesterone, pregnenolone was assayed separately and its effect subtracted. Using this method it was shown that picogram to nanogram/ml amounts of these metabolites are present in all human sera. Levels in men were comparable to those of women in the follicular phase of the menstrual cycle. 5α-Dihydroprogesterone and 3α,5α-tetrahydroprogesterone rose substantially in the luteal phase of the menstrual cycle and all rose considerably during pregnancy.

Keywords: Pregnanolone; Pregnenolone; Pregnanedione; Progesterone; Progesterone metabolites; Radioimmunoassay; High performance liquid chromatography (HPLC); Neuroactive steroids

1. Introduction

Although the ring A-reduced metabolites of progesterone have been known for many years to have potent anaesthetic properties [1,2], little is known about their possible effects on behaviour. As anaesthetics, they act rapidly — within seconds to minutes — too fast to depend on genomic mechanisms. With few exceptions they have never been measured in peripheral blood. Levels of 5α-dihydroprogesterone have been measured by Milewich et al. [3] and by Backström et al. [4] who both found levels in plasma of the same order of magnitude as those of progesterone in the follicular phase and $\sim 1/3$ those of progesterone in the luteal phase. Backström et al. also showed that its concentration in plasma from a vein draining an ovary containing the corpus luteum was 22-fold higher than that from the contralateral ovarian vein, indicating that the corpus luteum secretes significant amounts of 5α-dihydroprogesterone. High levels of 5α-dihydroprogesterone were observed in pregnancy [5,6].

Recently there has been considerable interest in allopregnanolone (3α,5α-tetrahydroprogesterone) because it binds strongly to the $GABA_A$ receptor [7]; levels in men and in non-pregnant women in the follicular phase were shown to be in the range of 0.5–1 nmol/l; those of women in the luteal phase rose to 3–5 nmol/l [8–10].

This study was aimed at measuring levels of five of the ring A-reduced metabolites of progesterone simultaneously in human peripheral plasma or serum. The steroids studied and their abbreviations used here are given in Table 1.

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2. Experimental

Subjects were healthy men and women, aged 18–60 years. Cycling women had regular menstrual cycles; pregnant women were at 30–38 weeks gestation. Blood samples were taken at 09:00–11:00 h.

Tritiated progesterone ([1,2,6,7-^{3}H](N), 50–100 Ci/mmol) was obtained from New England Nuclear, Boston, MA, and immediately diluted to 50 μCi/ml in ethanol. A portion of 10 μl (0.5 μCi) was further diluted to 10 ml in hexane to give ~3000 cpm in 50 μl for recovery studies. Non-radioactive steroids were obtained from Sigma, St. Louis, MO or from Steraloids, Wilton, NH, USA.

Norit A charcoal was obtained from Fisher Scientific, Montreal; dextran T-70 from JT Baker, Philipsburg, NJ, USA. A stock solution containing 2.5 g Norit A, 0.25 g dextran, and sodium azide 0.2 g per 200 ml was diluted 1/100 with phosphate buffer (0.075 M, pH 7.0) to separate the unbound fractions in the radioimmunoassays. Optiphase and later Scintisafe 30% were obtained from Fisher Scientific, Montreal, Canada.

The antiserum used to measure progesterone and its metabolites was raised in rabbits by injecting 4-pregnene-3,20-dione 3-*o*-carboxymethyloxime:bovine serum albumin (Steraloids, Wilton, NH, USA) in Freund's adjuvant into four 2-kg rabbits weekly for 1 month, then twice the following month, and monthly thereafter for 6 months. The antiserum having the highest cross-reactivity for the metabolites was used here. Sodium azide 0.1% was added. A titre of 1/5000 was used.

The antiserum to pregnenolone was obtained from Medicorp, Montreal, Canada. The antiserum was raised in rabbits using pregnenolone-3-hemisuccinate-BSA as antigen, and was used at a titre of 1/1 000 000 in phosphate buffer 0.1 M, pH 7.4, containing 0.25% BSA, and 0.1% sodium azide.

HPLC was carried out using pumps (Gilson model 302), a manometric module (Gilson 802B), a dynamic mixer (Gilson 811), a stream splitter (model ES, Radiomatic Instruments and Chemical), and two fraction collectors (Gilson 202), all obtained from Mandel Scientific, Montreal. An autosampler (SP8780XR) was obtained from Spectra-Physics, Ottawa, Canada, through Technical Marketing, Montreal. The columns used were Nova-Pak Silica (5 × 100 mm) from Waters, Mississauga, Ont., Canada.

Protein-tracer solution for the progesterone metabolite RIA was made up as follows: 26 μl [^{3}H]progesterone in ethanol was evaporated and redissolved in 1.0 ml phosphate buffer, 50 μl antiserum (dilution 1/10), and 9.0 ml gelatin water (0.5 g gelatin/l).

2.1. Procedure for the determination of progesterone and its metabolites

2.1.1. Extraction

After adding 2000–4000 cpm [^{3}H]progesterone in hexane to an aliquot (0.1–3.0 ml) of serum or heparinized plasma, it was extracted twice with 5 vol. of hexane or toluene in a Pyrex extraction tube. For non-pregnant subjects, 3.0 ml was used; for pregnant subjects 0.1 ml was used and the eluates containing progesterone (P) were further diluted 1/10 in order to have values on the curve.

Table 1
Cross-reactivities of progesterone and related compounds with the antisera, estimated at 50% displacement of tracer

Compound	Abbreviation	Cross-reactivity (%)	Cross-reactivity (%)
Pregn-4-ene-3,20-dione (progesterone)	P	100	0.015
3β-Hydroxypregn-5-ene-20-one	Preg	15	100
5α-Dihydroprogesterone	5α-DHP	155	≤0.1
3α-Hydroxy-5α-pregnan-20-one	3α,5α-THP	68	<0.1
3β-Hydroxy-5α-pregnan-20-one	3β,5α-THP	250	<0.1
5β-Dihydroprogesterone	5β-DHP	96	<0.1
3α-Hydroxy-5β-pregnan-20-one	3α,5β-THP	9	<0.1
3β-Hydroxy-5β-pregnan-20-one	3β,5β-THP	102	<0.1
20α-Dihydroprogesterone		15	
20β-Dihydroprogesterone		36	
17-Hydroxyprogesterone		1.3	<0.1
Cortisol		0.6	<0.1
Corticosterone		0.6	<0.1
Cortisone		0.5	<0.1
11-Desoxycortisol		0.6	<0.1
11-Desoxycorticosterone		1.9	<0.1
Estradiol		0.6	<0.1
Testosterone		0.6	<0.1
Dehydroepiandrosterone		0.5	<0.1

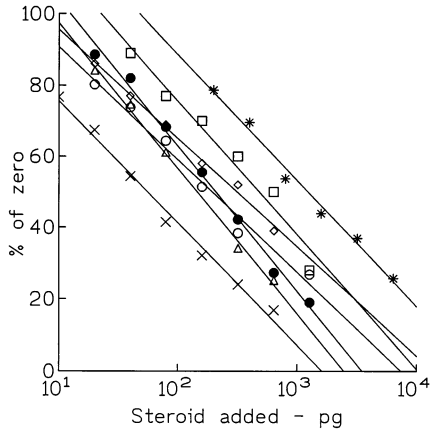

Fig. 1. Log plots of progesterone and competing compounds in the progesterone assay. ●, Progesterone; *, pregnenolone; △, 5α-DHP; ○, 5β-DHP; X, 3β,5α-THP; ◇, 3β,5β-THP; □, 3α,5α-THP.

2.1.2. HPLC

After evaporating the solvent to dryness under air (caution: do not overdry), the residue was dissolved in 40 μl methylene dichloride. Then 25 μl was injected into the HPLC column, and eluted using 0.18% ethanol in methylene dichloride (modified after Lin et al. [11]. After flushing with 10% ethanol for 5 min, the column was re-equilibrated for 1 h. A portion of the eluate (30%) was separated using the stream splitter and collected in counting vials to determine recovery, while the remainder was collected in test tubes for assay.

2.1.3. RIA

After evaporating the eluate fractions to dryness, 100 μl protein-tracer solution was added to each tube. Tubes containing 0, 20, 40, 80, 160, 320, 640 and 1280 pg progesterone as standards were treated similarly. The rack containing the eluate fractions and the standards was incubated at 37°C for 10 min and at 4°C for 90 min. Keeping the tubes in the bath, 1.0 ml dextran-charcoal suspension was added to each tube. After 4 min, the tubes were shaken vigorously for 1.0 min and then centrifuged at 4°C for 5 min at 3000 rpm. They were returned to the cold water bath and 0.50 ml of each supernatant was transferred to a counting vial along with 2.0 ml scintillator.

The samples were counted in an LKB Rackbeta liquid scintillation counter (Fisher Scientific, Montreal) to 10 000 counts or 10 min. The cpm of the standards were plotted versus pg progesterone and the results computed using the on-line LKB Rackbeta RIA program. The concentrations of the fractions were graphed, the peaks identified and quantitated, and then corrected for recovery and cross-reactivity. Each sample was carried through the whole procedure at least twice.

In addition to their retention times, the identities of the serum peaks were also verified by multiple competitive binding [12] to avian, human and guinea pig corticosteroid-binding globulins and to another antibody to progesterone. The identities of 5α-DHP, 3α,5α-THP, and 3β,5α-THP were also confirmed when it was shown that they are formed from progesterone by lymphocytes [13].

However, the determination of 3β,5α-THP was complicated by the fact that it eluted exactly with pregnenolone, which competes in the progesterone assay. Although pregnenolone competes 15% as strongly as progesterone for sites on the antibody (Table 1), it competes only 5% as strongly as 3β,5α-THP for these sites in the ranges used. Progesterone and the progesterone metabolites compete negligibly in the pregnenolone assay. The results for 3β,5α-THP were therefore corrected for the pregnenolone contribution.

2.2. Pregnenolone radioimmunoassay

A volume of 20 μl of pregnancy serum or 100 μl of non-pregnant serum was extracted with toluene (recovery 96%), and dried in a test tube to which was added 100 μl protein-tracer solution. The remainder of the procedure was identical to that for the progesterone RIA. Samples were assayed at least twice, giving an intra-assay coefficient of variation of $\pm 7\%$ for samples in pregnancy, and $\pm 16\%$ for values below 1 ng/ml.

3. Results

3.1. Specificity of the antisera

Table 1 shows the competition for binding of [^{3}H]progesterone by various steroids relative to that of P. The activity of each 5α compound was greater than that of its 5β analog. Competition of 3α,5β-THP and the hexahydro compounds was low. The competition of the more polar steroids including 17-hydroxyprogesterone, cortisol, estradiol, and testosterone was very low, and they were all effectively separated from the progesterone metabolites being measured.

The antiserum for pregnenolone was highly specific for pregnenolone and showed no significant cross-reactivity with the other steroids of interest, which were all separated by HPLC except for 3β,5α-THP.

3.2. Characteristics of the assay for progesterone and its metabolites

A log plot for progesterone is shown in Fig. 1. Parallelism of the metabolites was satisfactory. The sensitivity was 20 pg progesterone, the intra-assay reproducibility $\pm 4\%$ and the inter-assay reproducibility $\pm 12\%$.

Mean overall recovery was 35 ± 6%, ranging from 22 to 51%. Values were corrected individually for recovery. It was found that toluene was more efficient for extraction than was hexane and that extraction by inverting the tubes 30 times by hand was more efficient (87.8% mean recovery for toluene) than extraction by vortexing for 10 s (68% mean recovery for toluene), so that toluene and inversion were used routinely.

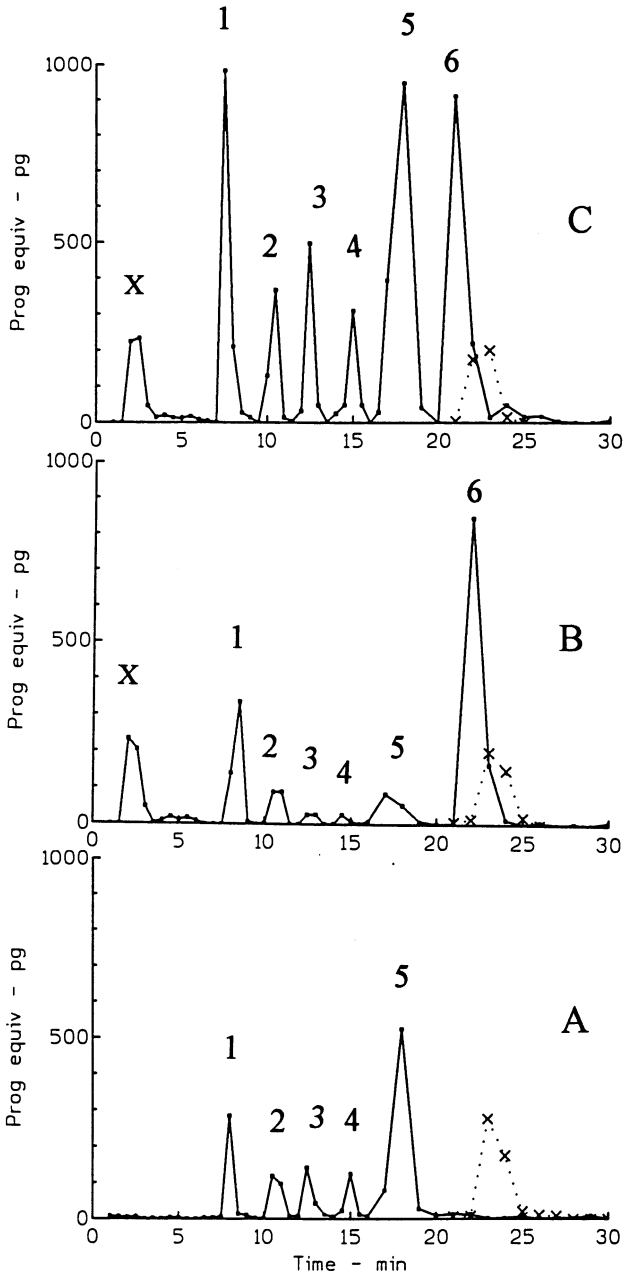

Fig. 2. Raw data showing patterns of steroids determined by RIA after HPLC. (A) Pure standards (1 ng) of the metabolites with tracer P* (recovery 39.5%). (B) Serum pattern in a patient in early pregnancy (recovery 30.5%). Only 1/10th of the samples containing progesterone was assayed. (C) Pattern in the same patient after adding 1 ng of each of the metabolites (recovery 51.1%). The pattern of the tracer P is shown as broken lines --- x ---. The tracer elutes slightly later than the non-radioactive P. X, still unidentified material less polar than 5α-DHP; 1, 5α-DHP; 2, 5β-DHP; 3, 3β,5β-THP; 4, 3α,5α-THP; 5, 3β,5α-THP + Preg; 6, progesterone.

The overall precision of the method depends on the details of the collection program. For a single determination, the CV was ± 29% at levels below 0.5 ng/ml ($n = 20$) and ± 16% at levels above 40 ng/ml ($n = 15$). Since each sample was carried through the whole procedure twice, the CVs of individual mean values were ± 20 and ± 11%, respectively.

3.3. Comparison of standards with serum patterns

Fig. 2 compares the HPLC patterns obtained by measuring standards alone (A) with those obtained in plasma alone (B) and with standards added (C). The plasma was obtained from a patient in early pregnancy. The pattern of peak location in plasma corresponded exactly with that of the standards. Several still unidentified peaks were also found, which were less polar than 5α-DHP and are indicated as X. Recovery of standards added varied from 92 to 110%.

3.4. Concentrations in human plasma (Table 2)

Values for the steroids measured in men, and in women in the follicular phase of the menstrual cycle or in menopausal women, were similar except for higher values of 3β,5α-THP in men ($P = 0.020$); progesterone values also tended to be higher in men ($P = 0.052$). Mean values for all the steroids were higher during the luteal phase, although the differences for 5β-DHP and 3β,5β-THP were borderline ($P = 0.05–0.06$). Levels for all the steroids were much higher in pregnancy ($P \leq 0.001$).

4. Discussion

To our knowledge this is the first time that measurements of 5β-DHP, 3β,5β-THP, and 3β,5α-THP have been made in plasma. They were lower than those for 3α,5α-THP and 5α-DHP.

Our mean level (± S.D.) of 5α-DHP in follicular phase plasma (0.18 ± 0.07 nmol/l) was lower than that found by Milewich et al. (0.51 ± 0.14 nmol/l) [3], much lower than that by Backström et al. (1.2 ± 0.65 nmol/l) [4], and very much lower than that found by Wang et al. (2.2 ± 0.57) nmol/l [9] (Table 3). All groups used similar methods involving a crude separation of progesterone and 5α-DHP on celite-proplylene glycol columns with isooctane. Milewich acknowledged that his fraction of 5α-DHP was 'slightly contaminated' with an oily substance. The antigen of Milewich et al. was conjugated at the C1α position while ours was conjugated at the C3 position, and cross-reactivities of the

Table 2
Concentrations of P and related compounds in human plasma (mean ± S.D. nmol/l)[a]

	5α-DHP	5β-DHP	3β,5β-THP	3α,5α-THP	3β,5α-THP	Preg	P
Men ($n = 5$)	0.23 ± 0.10	0.14 ± 0.08	0.20 ± 0.12	0.27 ± 0.05	0.24 ± 0.13	2.03 ± 1.08	1.57 ± 1.21
Women							
Follicular ($n = 9$)	0.18 ± 0.07	0.14 ± 0.07	0.14 ± 0.07	0.36 ± 0.24	0.09 ± 0.04	1.90 ± 1.09	1.17 ± 1.05
Luteal ($n = 9$)	1.07 ± 0.57	0.27 ± 0.27	0.20 ± 0.18	1.84 ± 1.18	0.14 ± 0.14	4.24 ± 2.36	34.9 ± 17.9
Pregnant ($n = 6$)	28.9 ± 8.7	2.32 ± 1.23	2.21 ± 1.37	13.6 ± 5.8	5.01 ± 2.63	57.1 ± 13.3	657 ± 178

[a] P, progesterone; Preg, pregnenolone.

two antibodies were fairly similar. Since we showed that very significant amounts of cross-reacting substances, still unidentified, were eluted just before 5α-DHP, it seems likely that cross-reaction with such substances may have increased the values they obtained. These are likely fatty acid esters; fatty acid esters of 5α-DHP and 3β,5α-THP have been identified in preparations of bovine corpora lutea [14], where they were present in amounts comparable to or exceeding the concentrations of the unesterified steroids in luteal tissue. The antibody used by Backström et al. and by Wang et al. was raised to progesterone conjugated at C11, and their cross-reactivity data were limited to only nine steroids (given by Backström et al. [4]). It is not clear why their values were so much higher.

In pregnancy, the levels found by Parker et al. [5] and by Lofgren et al. [6] were four to five times higher than those found here. These authors also used the same chromatographic method as described by Milewich et al. [3]. The interfering material of very low polarity, which we found cross-reacted with our antibody, also rose considerably in pregnancy and may account for this large discrepancy.

Our mean ± S.D. levels of 3α,5α-THP (0.36 ± 0.24 nmol/l) in women in the follicular phase are higher than those initially reported by Purdy et al. (less than 0.1 ng/ml (0.3 nmol/l) [15] if $P \leq 1.91$ nmol/l) [6] and somewhat lower than those recently described by Genazzani et al. (0.79 ± 0.30 nmol/l) [7]. Values in the luteal phase were higher than those in the follicular phase of the menstrual cycle, as found by all authors for both steroids.

We were unable to find data for 3α,5α-THP in pregnancy, nor for plasma levels of the other three ring A-reduced compounds.

Progesterone is known to be metabolized mainly in the ovary, adrenal and liver. Some degree of peripheral conversion occurs also, particularly to the 5α-reduced form, since the 5α-reductase is present in many tissues. We have shown that metabolism at both the C5 and C3 positions takes place in the lymphocytes [3].

Although sensitive methods for pregnenolone determination have been available for more than 20 years, we found surprisingly few data in the literature. Aedo et al. [16] showed a marked diurnal rhythm in serum levels, the highest occurring at 06:00 h. McNeil et al. [17] found a mean level of 1.21 nmol/l in men at 18:00 h, while Nishida et al. [18] found a mean level of 4.36 nmol/l in men at 09:00 h, values in accord with the range of 0.96–6.37 nmol found by McKenna et al. [19] and with our mean level of 2.29 ± 0.76 nmol/l. Gennarelli et al. [20] found levels of 1.7–2.3 nmol/l in women in the follicular phase, similar to our level of 1.90 ± 1.09 nmol/l. We were unable to find any values for pregnenolone in pregnancy in the literature; our values rose very significantly.

Although at pharmacological doses many progesterone metabolites have been known for decades to exert immediate and profound effects on the brain [1,2], there has been very little work on their possible physiological significance. However because of their extremely high potency — some of them are more powerful anaesthetics than barbiturates or ketamine [2] — it seems reasonable to expect that they would affect the functioning of the brain at lower concentrations as well. Evidence for this was our report of the effects of 5α- and 5β-dihydroprogesterone on motor activity in rats [21]; when administered for several weeks to ovariectomized rats, the 5α compound increased motor activity while the

Table 3
Values obtained by various authors for 5α-DHP and 3α,5α-THP in plasma of healthy women (nmol/l)

Reference	5α-DHP		3α,5α-THP	
	Follicular	Luteal	Follicular	Luteal
Milewich et al., 1977 [3]	0.5 ± 0.1	~6		
Backström et al., 1986 [4]	1.2 ± 0.6	9.5 ± 3.7		
Purdy et al., 1990 [15]			<0.3	up to 8.0
Schmidt et al., 1994 [8]				3.5 ± 1.5
Wang et al., 1996 [9]	2.2 ± 0.6	5.8 ± 0.8	1.8 ± 0.3	3.6 ± 0.6
Genazzani et al., 1998 [10]			0.79 ± 0.30	3.7 ± 1.0
This study	0.18 ± 0.07	1.1 ± 0.6	0.36 ± 0.24	1.8 ± 1.2

5β compound decreased it. Majewska et al. [7] have also shown effects of some of these steroids, particularly 3α,5α-THP, on the GABA receptor. Further evidence of the possible importance of such observations in plasma is our recent demonstration of higher levels of some of these steroids in women suffering from chronic fatigue syndrome [22], and in pregnant women suffering from depression [23].

While the increases in plasma levels in the luteal phase and pregnancy are likely related mainly to increased ovarian production, there is also adrenal production occurring, and in addition there is convincing evidence for synthesis of most of these steroids within the brain itself [24]. Although 5β-reduction has been shown in dogs [25], it has not so far been demonstrated in human brain tissue. Brain levels of these steroids are higher than plasma levels and there may well be regional differences, so that plasma may not be an accurate reflection of the brain levels of particular areas such as the hippocampus. However, since steroids pass freely into and out of brain tissue, plasma levels may afford useful clinical markers of brain function.

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