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COMPARISON OF PLASMA PROGESTERONE ASSAY IN WOMEN BY COMPETITIVE PROTEIN BINDING (CPB) AND RADIOIMMUNOASSAY (RIA)

BERNARDO LEO WAJCHENBERG, SEICO HANADA SUGAWARA, CARLOS HENRIQUE MESQUITA, ANTONIO CARLOS LERÁRIO, HELENA WACHSLICHT, ROMULO RIBEIRO PIERONI and EMILIO MATTAR

Section of Radioimmunoassay, Department of Radiobiology, Instituto de Energia Atômica and Diabetes and Adrenal Unit, Department of Medicine, Hospital das Clínicas, São Paulo (Brazil)

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Summary

Plasma progesterone levels during the follicular and luteal phases were compared when measured simultaneously by competitive protein binding (using guinea pig sera as binding agent) and radioimmunoassay (antiserum against progesterone-11-α-succinyl bovine serum albumin in rabbits), the values obtained were significantly different within each technique, depending on whether previous thin-layer chromatographic purification of the extracts was employed or not.

No significant differences were noticed between CPB and RIA when the chromatographic step was used, but when it was omitted, CPB was greater than RIA at the follicular but not at the luteal phase.

Introduction

An essential component of the saturation assays, particularly competitive protein binding (CPB) and radioimmunoassay (RIA), is the binding protein. The affinity and capacity of the binding protein define the sensitivity of the method.

Murphy [1], Neill et al. [2], Johansson et al. [3], Knobil et al. [4] and Lipsett et al. [5], used corticosteroid-binding globulin (CBG) to measure plasma progesterone. However, many steroids other than progesterone bind with CBG, making this binding agent not greatly specific.

Diamond et al. [6] have demonstrated in guinea pig serum the presence of a protein showing a high binding affinity for progesterone (progesterone binding globulin (PBG)) with increasing concentration during pregnancy, reaching its

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maximum at the time of parturition. Later on, Tan and Murphy [7] studied extensively the specificity of PBG, indicating its high affinity for progesterone. On the other hand, methods for the preparation of antisera to progesterone conjugated to serum albumin and subsequently radioimmunoassay techniques for the assay of progesterone in plasma have been developed [8,9,10].

Our work was intended to compare the measurement of plasma progesterone by competitive protein binding using PGB and by RIA with an antiserum developed in rabbits against progesterone-11α-succinyl bovine serum albumin. The assays were carried out with and without previous thin-layer chromatographic purification of the plasma extract to verify the possible interference of normally occuring steroids, at different levels of plasma progesterone. These steroids have cross-reactivity with the binding agent and so constitute a source of potential interference, even at physiological concentrations.

Material and methods

Solvents and reagents

Analytical grade ethyl ether, methanol, benzene and toluene were used without further purification. Ethanol was redistilled twice before use.

Precoated thin-layer plates (silica gel F-254, E. Merck A.G., Darmstadt, G.F.R.) were washed successively, by ascending chromatography, with methanol/EDTA 0.003 M and ether before use, as suggested by Lipsett et al. [5]. [1,2-^{3}H]Progesterone (spec. act. 50 Ci/mmole) was obtained from the New England Nuclear Corp. and purified by thin-layer chromatography using the solvent system ether/benzene (2 : 1, v/v).

Guinea pig serum was used as a source of PBG, blood being withdrawn by cardiac puncture one day post-partum. The endogenous steroids were removed by charcoal pre-treatment (50 mg Norit A per ml of serum).

The antiserum, prepared in rabbits against progesterone-11α-succinyl bovine serum albumin, was purchased from New England Nuclear Corp.

Nonradioactive progesterone was obtained from Sigma Chemical Comp. and its purity checked by liquid-liquid chromatography using a Porasil-C column and *n*-octane as support, testing simultaneously ultraviolet absorbance and refraction index.

Plasma extraction

One-ml plasma samples were added to 15-ml conical glass tubes containing 1000 dpm of radioactive progesterone to correct for procedural losses.

Extraction was carried out by vortex mixing for 30 seconds with 10 ml of ether. After clear separation of the 2 phases, the lower phase (plasma) was quick frozen by dipping in acetone containing chips of dry ice. The ether was decanted in a 15-ml conical glass tube and aliquots were taken for: recovery, CPB with and without chromatography and RIA with and without chromatography.

The ether aliquots were evaporated to dryness on a 30°C waterbath under nitrogen.

Purification

The dried extracts to be purified were transferred to the washed, precoated

thin-layer plates developed initially with benzene followed by the solvent system ether/benzene (2 : 1, v/v). Progesterone spots were eluted from the plates with ether/methanol 0.5% and evaporated to dryness under nitrogen.

Competitive protein-binding assay

[1,2-^{3}H]Progesterone-PBG (0.25 ml) was added to the tubes with known amounts of non-radioactive progesterone and also those containing the evaporated, purified and non-purified plasma extracts. The contents were vortexed and incubated for 1 h at 4°C.

Dextran-coated charcoal suspension (0.1 ml of 25 mg of Dextran-70 and 250 mg of Norit A charcoal suspended in 100 ml 0.1 M phosphate buffer pH 7.0) was then added, the contents of the tubes mixed again and allowed to stand in an ice bath for 10 min. Afterwards, the tubes were centrifuged at 2000 × *g* for 10 min in a refrigerated centrifuge. The supernatant was decanted into scintillation counting vials and 10 ml scintillation fluid added (4 g PPO and 50 mg POPOP dissolved in 1 liter toluene). Radioactivity was measured using an LS-150 Beckmann scintillation spectrometer.

Radioimmunoassay

To the assay tubes were added 0.1 ml of assay buffer containing radioactive progesterone (10 000 dpm) and 0.1 ml of the dilute antiserum (1 : 50 000). After the tubes were vortexed they were incubated overnight at 4°C. One ml of dextran-coated charcoal suspension was then added to the incubation media. All tubes were mixed and centrifuged for 10 min at 2000 × *g* in a refrigerated centrifuge (4C). The contents of each tube were decanted into counting vials, 10 ml of scintillation solution were added and counted.

The standard curve was constructed by plotting the logit of $(B—N/B_0—N) \times 100$ * against the logarithm of dose [11], the experimental data being adjusted by linear regression.

To correct for losses of progesterone in processing and the difference between the mass of radioactive progesterone in the standards and in the aliquots of the plasma extracts, the equation suggested by Furuyama and Nugent [8] was used.

Results

The parameters of the linear regression corresponding to the standard curves (log dose × logit $(B—N)/(B_0—N)$, *a* and *b* corresponding to the intercept (linear coefficient) and intersection (angular coefficient slope), respectively, are indicated in Table I.

In Table II are shown the plasma progesterone levels from 20 samples obtained during the follicular and luteal phases, measured simultaneously by the 4 different techniques: CPB and RIA with and without previous thin-layer chromatography (TLC) purification of the extracts.

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* Where: B, dpm bound in the presence of known amounts of unlabeled progesterone.
B_0, dpm bound in the absence of unlabeled hormone.
N, dpm bound in the presence of excessive amounts (250 mg) of unlabeled progesterone.

TABLE I

Parameters of the linear regression a and b corresponding to 5 standard curves of CPB and RIA assays of progesterone.

	CPB		RIA	
	a	b	a	b
	0.48	1.28	5.27	1.08
	0.51	1.12	5.71	1.11
	0.58	1.41	6.02	1.06
	0.53	1.41	5.65	1.17
	0.46	1.04	5.39	1.11
$\bar{X}$	0.51	1.25	5.608	1.106
S.D.	0.047	0.168	0.293	0.042

The results were analyzed by the usual statistical methods and by variance analysis [12] with two-way classification, technique and plasma sample. Sequential differences among means were tested according to Tukey-contrast analysis, with the formula $q = W/S_p \sqrt{n}$, where W is the range of means, S_p^2 is the pooled, or within groups, the mean square obtained in the variance analysis

TABLE II

Plasma progesterone levels during follicular and luteal phases of the menstrual cycle, in 20 samples, measured simultaneously by four different techniques.

Sample No.	CPB		RIA		Tukey's contrast *
	With TLC	No TLC	With TLC	No TLC	
1	1.03	2.61	0.69	0.79	
2	0.80	2.53	0.58	0.60	
3	0.52	2.06	0.20	0.53	
4	1.15	2.69	1.24	1.13	
5	0.48	1.57	0.21	0.55	
6	0.69	1.74	0.44	0.62	
7	0.78	1.77	0.52	0.86	
8	0.64	2.30	0.75	0.74	
9	0.54	1.42	0.50	1.24	
10	0.45	1.78	0.58	1.04	
11	0.39	1.24	0.33	0.63	
12	0.59	2.37	0.31	0.85	
13	0.47	1.65	0.30	0.50	
Mean follicular phase	0.66	1.98	0.51	0.78	0.25 **
14	2.48	4.39	2.11	2.74	
15	4.87	5.86	4.35	5.51	
16	8.41	9.26	8.47	9.51	
17	2.80	3.51	1.79	3.95	
18	8.09	9.37	8.56	10.18	
19	3.21	4.86	3.67	5.16	
20	1.91	2.91	1.37	1.57	
Mean luteal phase	4.54	5.74	4.33	5.52	0.74 **

* Samples No. 1 to 13 are from follicular and the remaining ones from luteal phases.

** Tukey's contrast, minimal significant difference among the means.

TABLE III
Estimates of the parameters of the regression between pairs of techniques for plasma progesterone assays. a, linear coefficient (intercept); b, angular coefficient (slope); r, correlation coefficient.

Parameters	CPB with vs. no TLC	RIA with vs. no TLC	CPB with vs. RIA with TLC
a *	1.34	0.27	−0.24
b **	0.97	1.16	1.04
r	0.989	0.987	0.991

and n is the number of cases. An alpha value of 0.05 was selected as the critical region for rejection of null hypothesis.

There was a significant difference between the results by each technique ($F_{obs.} = 105.18$; $F_{0.05} = 2.90$) and between the plasma samples from the follicular phase ($F_{obs.} = 4.43$; $F_{0.05} = 2.04$). In the same way, the difference between the results obtained by each assay technique ($F_{obs.} = 14.40$; $F_{0.05} = 3.16$) and between the plasma samples from the luteal phase ($F_{obs.} = 140.00$; $F_{0.05} = 2.66$) were significantly different.

The comparison between the results indicated in Table II, are shown in Table III.

Discussion

The specificity of an analytical method applied to such a complex mixture as the plasma depends on the degree of purity which the compound to be measured has when isolated from the sample and/or on the specificity of the measurement itself. Therefore, in relation to the techniques studied, the specificity is determined by 2 factors: (1) the extent to which compounds other than progesterone are extracted from the plasma samples; (2) the displacement of labeled progesterone from the progesterone-binding protein complex by these compounds.

With regard to factor 2, the specificity of the methods employed was limited by the fact that several steroids extracted together with progesterone cross-react with that steroid for its binding sites either for PBG (17α-hydroxy progesterone, 20α-hydroxy-progesterone, cortisol, testosterone and DOC, unpublished data) or for the antiserum (corticosterone, pregnenolone and DOC, unpublished data). Then, because a partially nonspecific detection method was used, adequate specificity could only be expected if the purification steps performed prior to detection removed all the known contaminants that interfere.

As it can be seen in Table I, the mean slope of the dose-response curves defined by the angular coefficient b had a low standard deviation indicating that the curves were parallel. Besides, the intercept a (when l_n concentration = 0) indicated that they also presented a small level of dispersion in relation to the mean value. Both parameters, however, presented smaller deviations from the means with RIA than with PBG.

When the plasma progesterone levels were compared during the follicular and luteal phases measured by both techniques (Table II), it was evident that

there was a significant difference within either method when previous chromatographic purification of the extract was employed or not indicating the presence of an interference with the use of the binding agents as it would be expected considering that the antibodies, still more specific than the PBG, cross-react with various steroids.

However, no significant differences were noticed between the two binding agents after thin-layer chromatography of the plasma extracts either at the follicular or luteal phase. When such a step was omitted, the results were significantly greater with CPB only with the follicular but not with the luteal phase plasma samples, suggesting that the influence of the interfering agents in the measurement of progesterone was different in the 2 phases of the menstrual cycle, related, partially at least, to the quite different progesterone levels in the two phases.

These results indicate that the chromatographic step is a prerequisite for specificity, the interfering factors being estimated as 1.32 ng/ml for the competitive protein binding assay and 0.27 ng/ml for the IRA technique in the follicular phase whereas at the luteal phase the values were 1.20 ng/ml and 1.19 ng/ml, respectively, corresponding to the differences between the mean values of the 2 methods considered (Table II).

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INSTITUTO DE ENERGIA ATÔMICA
Caixa Postal, 11049 – Pinheiros
CEP 05508
01000 – São Paulo – SP

Telefone: 211-6011
Endereço Telegráfico – IEATOMICA
Telex – 011-23592 IENA BR