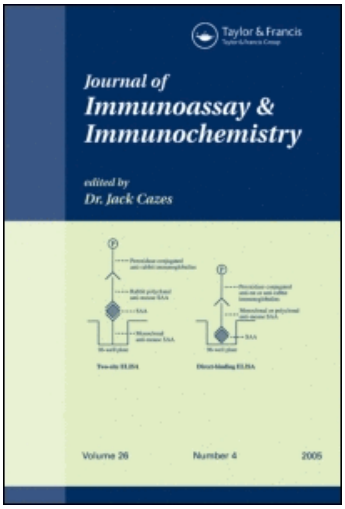

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Beverley E. Pearson Murphy[a]; Doreen Lobo Vedady[a]
[a] Reproductive Physiology Unit and McGill University Medical Clinic, Montreal General Hospital, Montreal, Quebec

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RADIOENZYMATICASSAY FOR SOME SUBSTRATES AND INHIBITORS OF HUMAN PLACENTAL 11-HYDROXYSTEROID DEHYDROGENASE

Beverley E. Pearson Murphy and Doreen Lobo Vedady
Reproductive Physiology Unit and McGill University Medical Clinic, Montreal General Hospital, 1650 Cedar Av., Montreal, Quebec H3G 1A4

ABSTRACT

A radioenzymaticassay (REA) is described, using labelled cortisol and a homogenate of human placenta. Steroids which are able to compete with the tracer for conversion to the 11-keto form can be measured. Of these, 11β and 11α-hydroxyprogesterone (11β-hydroxypregn-4-ene-3,20-dione), 3β,11β-dihydroxypregn-5-ene-20-one, 11β,20α-dihydroxypregn-4-ene-3-one and corticosterone are the most active, while cortisol and prednisolone are moderately active. All other steroids tested competed less than 10% as effectively as 11β-hydroxyprogesterone at 50% conversion with no added substrate. The sensitivity was 0.8 ng of 11β-hydroxyprogesterone and 5 ng cortisol. Using this REA, preliminary studies were carried out to investigate the endogenous substrates and inhibitors of 11-hydroxysteroid dehydrogenase in human fetal tissues.

Key Words: radioenzymaticassay, 11-hydroxysteroid dehydrogenase, human placenta, 11-hydroxysteroids.

INTRODUCTION

Radioenzymaticassays were introduced in 1965 by Rothenberg (1) who employed folate reductase to measure folic acid and its antagonists. Many enzymes have specificities and affinities comparable to those of antibodies, transins, and receptors and can therefore be used in similar fashion for assay purposes. The mathematical models, i.e. Scatchard and Michaelis-Menten equations are formally identical. However while the Scatchard equation deals with binding at equilibrium, the Michaelis-Menten describes the rate of the reaction. Despite the potential of this type of assay for the measurement of steroid substrates, it has so far found few applications. Bleau et al (2) have used an REA to measure cholesterol sulfate.

11β-Hydroxysteroids are of great biological significance since they include cortisol, corticosterone and aldosterone, corticoids which are necessary for life in man and many other species. Cortisol and corticosterone are reversibly altered to essentially inactive 11-keto products by the enzyme 11β-hydroxysteroid dehydrogenase (11-HSD)(E.C. 1.1.1.146). Our recent studies showing that the affinity of the enzyme for these hormonal steroids is less than that for 11β-hydroxyprogesterone and some of its analogs suggested that other substrates might also be important (3). The present REA was developed to measure these compounds, thereby providing a means of investigating the endogenous substrates for this enzyme in serum and tissues.

The principle of the assay is similar to that of radioimmunoassay and other competitive protein-binding procedures except

that in the REA the binding protein is an enzyme and the entity measured is the product rather than the binding of a ligand. The tracer substrate (here ^{3}H-cortisol) competes with other substrates in the sample for sites on the enzyme which converts the labelled substrate to a product (^{3}H-cortisone). The amount of labelled product is therefore inversely related to the amount of competing unlabelled substrate. The amount of competing substrate is derived from a calibration curve obtained using known amounts of the same substrate. In these studies ^{3}H-cortisol was the tracer since it can be easily separated from ^{3}H-cortisone.

MATERIALS AND METHODS

(1,2,6,7-^{3}H-HN) cortisol (SA 98 Ci/mmol) was obtained from the New England Nuclear Corp., Lachine, Que. Unlabelled steroids were purchased from the Sigma Chemical Co., St. Louis, Mo., or Steraloids Inc., Wilton, N.H., or were provided through the courtesy of Prof. Kirk from the Steroid Reference Collection, London, England; 1α,11β,21-trihydroxypregn-4-ene-3,20-dione was kindly donated by Prof. D.R. Idler, Memorial University, Nfld. Steroids were stored in ethanol. Sephadex LH-20 was obtained from Pharmacia, Montreal.

Placentas were obtained from the operating room immediately after delivery by Caesarean section and were placed at 4 C until dissected (usually immediately, occasionally the next day). Pieces of tissue, 1-2 g, were dissected from the middle layer (avoiding the chorionic plate and the 'maternal' placenta) and

frozen in individual containers at -20 C, or lower, until required for assay.

Assay procedure

When the sample tubes were ready to assay, placental tissue from one container (0.5 g or more) was weighed, thawed and homogenized in 12 vol 0.9% saline using a glass hand homogenizer. After passage through 2 layers of gauze, 0.1 ml homogenate was added immediately to each dry assay tube as quickly as possible. Each tube contained either standard or sample, plus tracer ^{3}H-cortisol, which had been mixed and taken to dryness under air. The rack holding the tubes was immediately placed into a gently shaking bath at 37 C for 15 min, then into a freezer at -20 C. When convenient, 4 tubes at a time were removed from the freezer, and 0.5 ml ethyl acetate added. After thawing, the tubes were vortexed 2 min, and centrifuged 4 min at 2000 g. After re-freezing the aqueous phase, the organic phase was poured into a centrifuge tube, evaporated to dryness, redissolved in 0.2 ml methylene chloride, transferred to the top of a Sephadex LH-20 column (40 x 0.5 cm) and eluted with methylene chloride:methanol 98:2 to separate the cortisone and cortisol fractions (4). The columns gave highly reproducible results and could be re-used many times without repacking. The cortisol and cortisone fractions were evaporated to dryness in disposable plastic counting vials and 2 ml toluene scintillator (Econoflor, New England Nuclear Corp.) was added to each vial. After shaking thoroughly, each vial was counted for 1 min. The percentage conversion was

calculated as: cpm cortisone x 100, divided by the cpm (cortisol + cortisone). Values for the unknowns were read from the appropriate standard curve (Fig. 1) and were expressed as ng/ml or ng/g steroid or equivalent.

Sample preparation

To prepare tissue extracts, an appropriate aliquot (e.g. 0.1 ml cord serum, 10 mg liver or adrenal) was weighed, homogenized and extracted twice with 5 vol ethyl acetate. After centrifugation, the organic phase was transferred to an assay tube (Kimax, 16 x 100 mm) and evaporated to dryness.

To prepare chromatographed samples, tissue extracts to which tracers of various steroids (20,000 cpm, 80 pg) had been added, were chromatographed on Sephadex LH-20 columns (40 x 0.8 cm) using methylene chloride:methanol 98:2 as above. Fractions of 2 ml were collected, evaporated to dryness and redissolved in 2.0 ml ethanol (because ethanol is less volatile). Aliquots of 0.2 ml were counted to give the location of the tracers while another aliquot of each fraction was taken to dryness and assayed by REA, and suitable aliquots were assayed by radiotransinassay for cortisol, corticosterone and progesterone as described previously (5) using horse or dog serum and Florisil.

Specificity studies

Varying amounts of competing steroids were assayed and the curves obtained compared with that for 11β-hydroxyprogesterone. The competition at 50% of the conversion with no added substrate was calculated as: ng 11β-hydroxyprogesterone x 100/ng competitor.

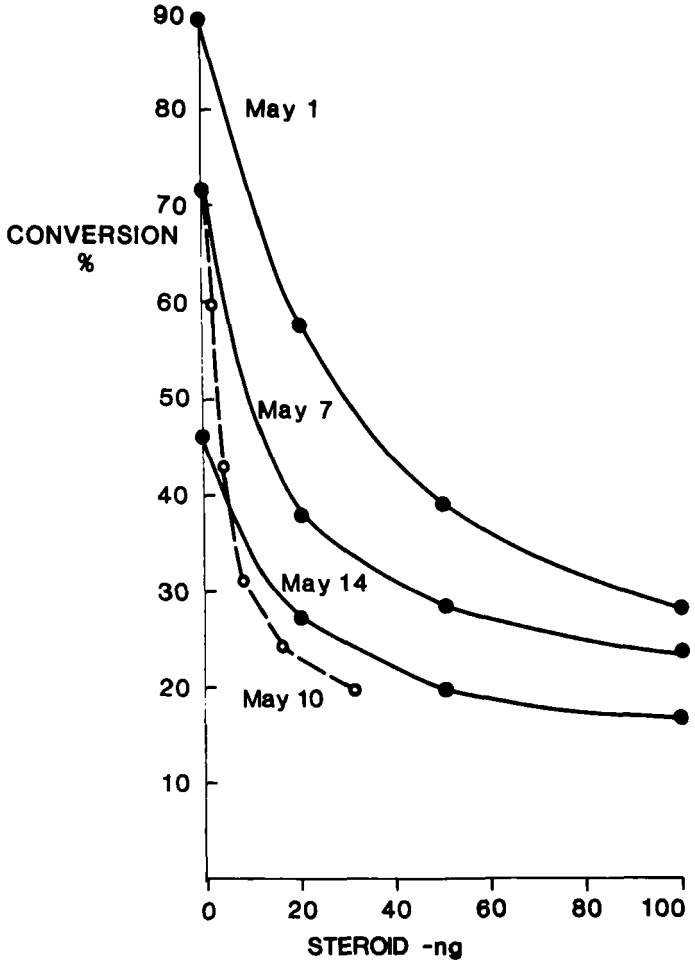

Fig. 1. Standard curves for cortisol (May 1,7,14) and for 11β-hydroxyprogesterone (May 10). Placental tissue frozen at -20 C April 30th.

RESULTS

Stability of the enzyme

Enzyme activity in the homogenate fell rapidly so that it could not be kept overnight. Refrigerated placental tissue could be used for up to a week but allowance had to be made for falling

activity. Frozen placental tissue retained activity up to several months, with large individual variations which seemed to be reduced by rapid freezing in liquid nitrogen and storage at -80 C. Standard curves for cortisol and 11β-hydroxyprogesterone, obtained over a 2-week period, are shown in Fig. 1.

The percentage conversion with no added substrate was usually 70 to 80%; if less than 70%, the placenta was discarded. Previous studies (6) have suggested that NADP is a cofactor for this reaction; however under the present assay conditions, addition of NADP did not enhance the reaction and was therefore omitted.

Sensitivity

The smallest amount distinguishable from zero under the conditions used was 0.8 ng 11β-hydroxyprogesterone or 5 ng cortisol, calculated as 2 x S.D. at zero concentration of added substrate.

Precision

When the placental homogenate was added with the tubes in an ice bath, between-assay variability for a pooled cord serum with a mean value of 311 ng/ml cortisol equivalents on 19 different days was ± 16%. Within-assay variability was ± 8%.

Specificity

Table 1 lists the relative inhibition of a variety of steroids. Although this enzyme has been termed '11β-hydroxysteroid dehydrogenase' the 11α form competed almost as strongly, and progesterone itself had a significant inhibitory effect.

Recovery of added steroids

The recovery of radioactivity from the columns as cortisone

Table 1
Specificity of 11-HSD*

Trivial name	Chemical name	% of 11OH-P
11βOH-progesterone	11βhydroxypregn-4-ene-3,20-dione	100
11αOH-progesterone	11αhydroxypregn-4-ene-3,20-dione	85
11βOH-pregnenolone	3β,11β-dihydroxypregn-5-ene-20-one	66
11βOH,20α-dihydroprogesterone	11β,20α-dihydroxypregn-4-ene-3-one	50
corticosterone	11β,21-dihydroxypregn-4-ene-3,20-dione	50
cortisol	11β,17α,21-trihydroxypregn-4-ene-3,20-dione	15
prednisolone	11β,17α,21-trihydroxypregn-1,4-diene-3,20-dione	12
11βOH-testosterone	11β,17β-dihydroxyandrost-4-ene-3-one	7
11βOH,20β-dihydroprogesterone	11β,20β-dihydroxypregn-4-ene-3-one	6
progesterone	pregn-4-ene-3,20-dione	3
11βOH-androstenedione	11β-hydroxyandrost-4-ene-3,17-dione	2
11βOH-DHEA	3β,11β-dihydroxyandrost-5-ene-17-one	2
desoxycorticosterone	21-hydroxypregn-4-ene-3,20-dione	1.6
11βOH-androstanolone-5α	3β,11β-dihydroxy-5α-androstane-17-one	1.5
cortisone	17α,21-dihydroxypregn-4-ene-3,11-dione	1
corticosterone sulfate	11β-hydroxypregn-4-ene-3,20-dione-21-sulfate	0.9
11βOH-androsterone	3α,11β-dihydroxy-5α-androstane-17-one	0.7
11βOH-androstanolone-5β	3β11β-dihydroxy-5β-androstane-17-one	0.7
11β,16αdiOH-DHEA	3β,11β,16α-trihydroxyandrost-5-ene-17-one	0.7
cortisol sulfate	11β,17α-dihydroxypregn-4-ene-3,20-dione-21-sulfate	0.6
6αOH-progesterone	6α-hydroxypregn-4-ene-3,20-dione	0.5
12αOH-progesterone	12αhydroxypregn-4-ene-3,20-dione	0.5
6βOH-cortisol	6β,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione	< 0.1
aldosterone	11β,21-dihydroxy-18-oxo-pregn-4-ene-3,20-dione	< 0.1
15αOH-progesterone	15α-hydroxypregn-4-ene-3-one	< 0.1
16αOH-progesterone	16α-hydroxypregn-4-ene-3-one	< 0.1
16-dehydroprogesterone	pregn-4,16-diene-3,20-dione	< 0.1
pregnanediol	5β-pregnane-3α,20α-diol	< 0.1
DHAS	androst-5-ene-17-one-3β-sulfate	< 0.1
betamethasone	9αfluor-11β,17α,21-trihydroxy-16β-methylpregn-1,4-diene-3,20-dione	< 0.1
11βOH-etiocholanolone	3α,11β-dihydroxy-5β-androstane-17-one	< 0.1

*calculated at 50% of conversion with no added substrate; inhibition due to 11βOH-progesterone has been arbitrarily set at 100.

and cortisol consistently exceeded 80%, indicating that no other major metabolic conversion of cortisol occurred in placental tissue.

Recovery of non-radioactive cortisol and 11β-hydroxyprogesterone added to pooled cord serum averaged 92% and 97% respectively.

Use of cortisol standards to estimate the amount of competing substrate in umbilical cord serum, fetal liver and fetal adrenal

The accurate determinations of pure analyte by any competitive binding method requires that it be measured using standards identical to the analyte itself. In this instance, since the identity of the material in fetal tissues is still in part unknown, cortisol was used as a standard and the results were therefore expressed as cortisol equivalents. This approach is capable of yielding consistent data since increasing amounts of cord serum or extracts of liver or adrenal gave curves that were essentially parallel with the cortisol curve on a semilogarithmic plot (Fig. 2)(Table 3).

Investigation of competing substances in umbilical cord serum

The chromatographic mobility of the material measured in 2 cord sera is shown in Fig. 3. The caculated equivalent concentrations of corticosterone, progesterone and cortisol for serum #1 are given in Table 4. One prominent unidentified peak occurred between corticosterone and cortisol in a position close to that of cortisone. A similar peak also occurred in chromatographs of adrenal and liver. Further chromatography showed that this material comprised at least 3 peaks. These did not correspond to any of the steroids listed in Table 1, and cross-reacted poorly with antisera for 11-hydroxyandrostenedione, 11-hydroxydehydroepiandrosterone

Table 2
Recovery of cortisol added to pooled cord serum 0.1 ml

Cortisol added ng	Cortisol recovered %	11βOH-Progest. added ng	11βOH-Progest. recovered %
10	83	5	106
20	93	10	92
40	97	20	92
80	95	30	100

Each value is the mean of results obtained in 3 different assays.

Table 3
Assays of enzyme-competing material - ng/ml or ng/g*

Tissue	n	Total 11-HCS	Cortisol	Corticosterone	NGCS@
Cord serum, delivery	19	269 ± 17	91 ± 10	10 ± 5	169 ± 18
Neonatal adrenal	1	30,000	1100	700	24,150
Fetal adrenal - 29 wk	1	3,900	315	85	3,500
Neonatal liver	1	3,120	870	200	2,050

*mean of 2 or more samples ± S.E. @non-glucocorticoid steroids expressed in cortisol equivalents.

Table 4
Assays of chromatographed material in cord serum #1

Peak ml	REA ng C.E.*	REA ng/ml	RTA ng/ml	Steroid
14	42	220	200	Progesterone
22	18	8	7	Corticosterone
28	32	-	-	Unidentified
42	154	-	-	Unidentified
58	146	146	49	Cortisol

*C.E. = cortisol equivalents; values obtained using a cortisol standard curve.
REA = radioenzymaticassay; RTA = radiotransinassay.

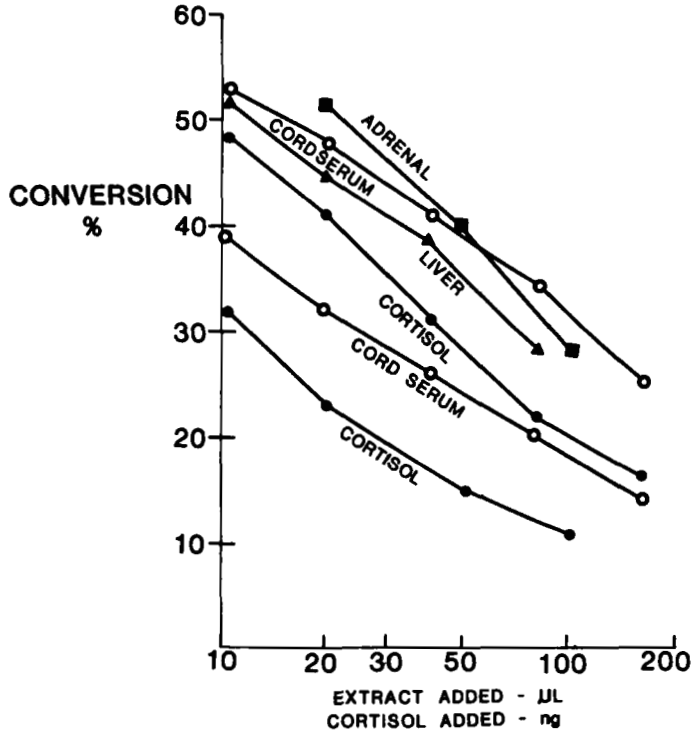

Fig. 2. Curves obtained using increasing amounts of cortisol and tissue extracts plotted on a semi-logarithmic scale.

and 11-hydroxyprogesterone. Cross-reaction with an antiserum to pregnenolone conjugated at C-16 was moderately strong, suggesting that the likely structures are C21 steroids with a 3βOH,Δ5 configuration. Larger amounts of these compounds have now been isolated and further studies to identify them are in progress.

Competing substances in adult serum

Increasing amounts of maternal serum gave a non-linear relationship and initially led us to believe that cortisol accounted for most or all the activity present (7). However on chromatography, patterns

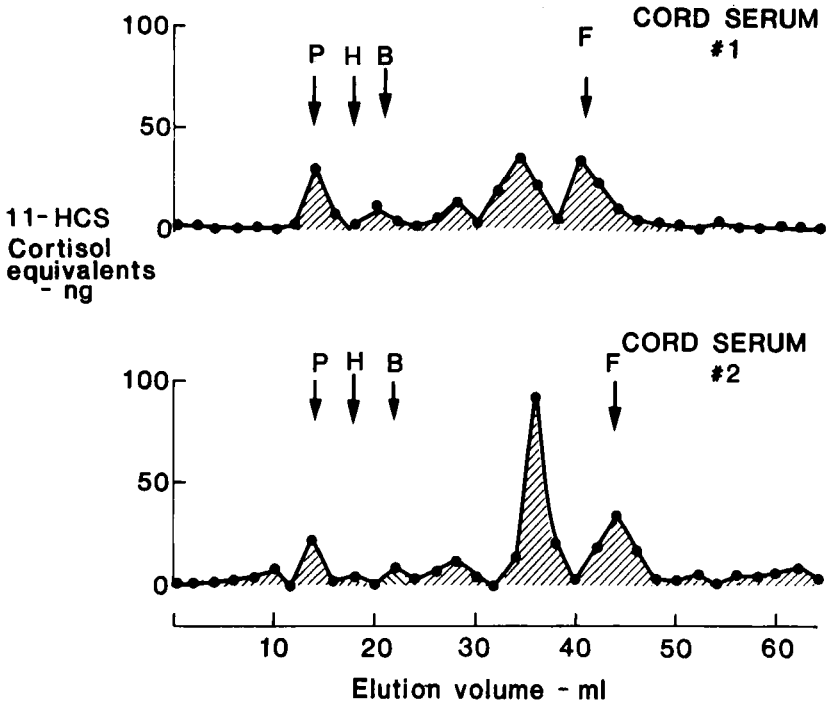

Fig. 3. Chromatographic patterns obtained for 2 cord sera. Values for the various peaks are given in Table 3. Steroids are eluted in order of polarity, the least polar appearing first, the most polar, last. The material shown was measured using the REA. P = progesterone; H = 11ß-hydroxyprogesterone; B = corticosterone; F = cortisol.

similar to those of cord serum were observed; such peaks eluting between corticosterone and cortisol were absent or very low in non-pregnant serum.

DISCUSSION

This study shows that unconjugated steroids can be measured using enzymes in a manner similar to that using antibodies, transins and receptors. The enzyme used here had a specificity comparable to

that of many antibodies to steroids but less than that of most antibodies to 11β-progesterone conjugates. The ability of Δ5 analogs to compete is possibly due to the presence of the 3β-ol dehydrogenase-isomerase in placental tissue.

Relatively little is known about the function of the enzyme 11-HSD. It has been demonstrated in many mammalian tissues and appears to be important in the interconversion of cortisol and cortisone in man (8) and of corticosterone and 11-dehydrocorticosterone in mouse and rat (9), particularly in fetal life. The highest concentration is found in placenta, where oxidative activity is very high and reductive activity negligible, and in the liver, where reductive activity predominates after birth (8). The presence of unidentified peaks in chromatographed tissue extracts suggests that there may be unrecognized substrates or inhibitors present and this REA provides a means of investigating them.

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