Journal of Steroid Biochemistry, Vol. 9, pp. 671–676

0022-4731/78/0701-0671$02.00/0

RADIOIMMUNOASSAY OF CORTICOSTERONE, CORTISOL AND CORTISONE: THEIR APPLICATION TO HUMAN CORD AND MATERNAL PLASMA

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(*Received* 26 *September* 1977)

SUMMARY

A critical analysis of the applicability of corticosterone, cortisone and cortisol radioimmunoassays in human plasma is presented. Rabbits immunized with corticosteroid-3-CMO-BSA conjugates yielded specific antisera. Yet, their specificity may become insufficient when the concentrations of weakly cross-reacting steroids are much higher than that of the steroid to be assayed. In these particular instances, appropriate and simple purification steps, such as hexane extraction or celite chromatography, must be added. Normal values for pregnant women at delivery and their babies are presented.

INTRODUCTION

In the newborn, the concentrations of various corticosteroids measured in the umbilical cord plasma are strikingly different from those of adults [1, 8]. It is now well established that cortisone is a significant component of human umbilical cord plasma [2, 4, 7, 9, 10] whereas cortisol is the most important 17-hydroxy-corticosteroid in the maternal circulation [11, 12]. The plasmatic concentrations of corticosterone and 11-deoxycorticosterone (DOC) are not negligible [5, 13, 14]. At delivery, the corticosteroid concentration rises in the plasma of the mother and her baby [9, 11, 15, 19]. Until now, various techniques have been used for the measurement of these steroids: chromatography, fluorimetry and competitive-protein-binding assay [10, 11, 14, 20, 21]. Usually, these methods require large volumes of plasma and/or many steps of purification. This report describes simple, sensitive and specific radioimmunoassay of corticosterone, cortisol and cortisone. The specificity of each procedure was assessed by comparison of the values obtained with and without a previous chromatographic purification.

MATERIALS AND METHODS

Solvents and reagents. *N*-hexane (Merck, Darmstadt) and methylene chloride (Carlo Erba, Milano) were used without redistillation. All solvents were analytical grade.

Natural and synthetic steroids were obtained from Sigma Chemical Corporation (St Louis, MO). 1,2-[^{3}H]-cortisol (49 Ci/mmol), 1,2-[^{3}H]-cortisone (48 Ci/mmol) and 1,2,6,7-[^{3}H]-corticosterone (102 Ci/mmol) were purchased from the Radiochemical Center (Amersham).

Before use, Celite analytical Filter Aid (Johns Mansville, Product Corp., Lompoc, CA) was heated at 600°C for 16 h as described by Abraham[22]. Disposable 5 ml glass pipettes were used as support for the celite. Glass beads (3 mm diam.) were laid at the bottom of each pipette prior to celite packing.

Sephadex LH-20 (Pharmacia, Uppsala) column chromatography was performed to check the purity of the labeled steroids and to improve the method of corticosteroid separation [35]. Glass columns, 12 cm long and 0.7 cm internal diameter, were fitted with a porous filter P2 and a Teflon stopcock. Elution was performed with a mixture of benzene–methylene chloride–methanol (63:35:2).

Aminooxy-acetic acid (Sigma Chemical Corporation, St Louis, MO) and crystallized bovine serum albumin (Armour Co., Eastbourne) were used without purification. Freund's complete adjuvant was obtained from Difco Laboratories (Detroit, MI).

0.15 M NaCl, 0.1 M phosphate buffer pH 7.0 containing 0.1% gelatine was used throughout the radioimmunological procedure. Dextran-coated charcoal was prepared by adding 0.5 g charcoal (Norit A, Fisher Scientific Co., Fair Lawn, NJ) and 0.05 g dextran-T70 (Pharmacia, Uppsala) to 100 ml assay buffer.

The scintillation fluid was a solution of 16.5 g P.P.O., 0.3 g P.O.P.O.P. in 1 liter of Triton X-100 (Packard) and 3 liters of toluene. Radioactivity was measured using a Tri-Carb Liquid Scintillation Spectrometer (Packard, Model 3380, Chicago, IL) with 37% counting efficiency.

Antisera. Cortisol, cortisone and corticosterone were reacted with aminooxy-acetic acid as described by Cook *et al.* [23]. Coupling to BSA was achieved

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by the mixed anhydride method [24]. 1 mg of conjugate, dissolved in 0.5 ml 0.9% NaCl solution and emulsified with 0.5 ml Freund's complete adjuvant, was subcutaneously injected once a week for the first six weeks and every other week for the next eight weeks. Three rabbits were immunized against each conjugate. All produced antibodies to their respective hapten; the most specific antiserum was selected and its properties described in this report.

Extraction and celite chromatography. Plasma samples (20 μl for cortisol and cortisone; 200 μl for corticosterone) were pipetted in duplicate into glass tubes in which [^{3}H]-reference steroid had been added (approximately 3,000 d.p.m.) and previously dried. The plasma aliquots were diluted with water (0.5 ml). After 1 or 2 h at room temperature, 5 ml of hexane were added to the plasma and the steroids extracted by vigorously shaking for one min. The tubes were centrifuged and the organic phase, containing progesterone and its derivatives, was aspirated and discarded. Five ml of methylene chloride were then added to the aqueous phase. After thorough mixing and centrifugation, the plasma layer was removed. The dichloromethane fractions were assayed with and without a prior chromatographic step. In the direct assay, 1 ml of evaporated dichloromethane extract was used. In the other procedure, the total dicloromethane extract was evaporated and submitted to appropriate celite chromatography, according to Abraham[25].

Corticosterone. 0.5 g of celite was mixed with 250 μl of ethylene glycol (stationary phase) and packed in a pipette. Each column was washed with 2 × 3.5 ml of ethyl acetate–isooctane (20:80). The dried residue of plasma extracts was transferred to the celite column by rinsing twice with 0.5 ml of ethyl acetate–isooctane (20:80). The first 1 ml fraction was discarded and elution was performed with 3.5 ml of mixtures of ethyl acetate in isooctane of increasing polarity (20:80 followed by 30:70, 35:65 and 40:60). Corticosterone was eluted in the second and third fractions. 0.5 and 1 ml of each of these fractions was submitted to the radioimmunoassay. Additional 1.6 ml aliquot samples were used to determine the percentage of recovery.

Cortisone. The stationary phase was ethylene glycol–water (80:20). The elution system was similar to that described for corticosterone. Cortisone was eluted in the second fraction ethyl acetate–isooctane (30:70).

Cortisol. The stationary phase also was ethylene glycol–water (80:20). The same elution was used. Five ml fractions were collected. Cortisol was estimated in the third fraction ethyl acetate–isooctane (35:65).

LH-20 column chromatography (35). In this procedure, a large volume of plasma (10 ml) was extracted, as previously described, with dichloromethane, with or without the hexane extraction step. The dried residue of plasma extracts was transferred to the LH-20 column with two 0.2 ml aliquot samples of the eluting mixture (Benzene 63:methylene chloride 35:methanol 2). Forty 1 ml fractions were collected and evaporated to dryness. Calibration was performed by chromatography of reference labeled steroids.

Radioimmunoassay procedure. The dried samples (obtained with and without a chromatographic step) were mixed with 0.1 ml of radioactive tracer (about 10,000 dpm) and 0.1 ml of antiserum dilution. The volume was adjusted to 0.5 ml with saline phosphate buffer. The tubes were kept at 37°C for one h and incubated at 4°C overnight. Separation of free from antibody-bound steroid was performed by adding 0.5 ml of ice-cold dextran-charcoal suspension. The tubes were mixed on a vortex mixer and allowed to stand on ice for 15 min before centrifugation for 10 min at 4°C (2,000 *g*). After centrifugation, the supernatant was transferred to a counting vial to which 10 ml of scintillation solution were added.

RESULTS

1. *Percentage of recoveries after extraction and chromatography*

The recovery of tritiated-reference steroids added to a pool of plasma prior to hexane and methylene chloride extraction was 95 ± 3% (mean ± S.D.) (n = 10). The percentages of [^{3}H]-reference steroids recovered after celite chromatography are indicated in Table 1.

2. *Standard curves*

The competition curves of the labeled ligand with increasing concentrations of corticosterone, cortisone or cortisol are illustrated in Fig. 1. Antisera dilutions which bound 50% of the tracer were 1:2,000 for corticosterone, 1:1,500 for cortisol and 1:10,000 for cortisone respectively. The equilibrium constant (K), the concentration of binding sites (undiluted serum) (Ab_0) and the index of heterogeneity (α) were calculated from the radioimmunological data using the Sips formula [26].

$$B/F^{\alpha} = K^{\alpha}(Ab_0 - B)$$

$0 < \alpha \leq 1$; B: bound antigen; F: free antigen.

The experimentally determined values are reported in Table 2.

3. *Precision*

The precision of direct cortisone assay was evalu-

Table 1. Percentage of recovery in the most specific fraction for each steroid

	Number of determination n	Mean ± S.D.
Corticosterone	16	48 ± 11
Cortisol	14	67 ± 7
Cortisone	11	49 ± 11

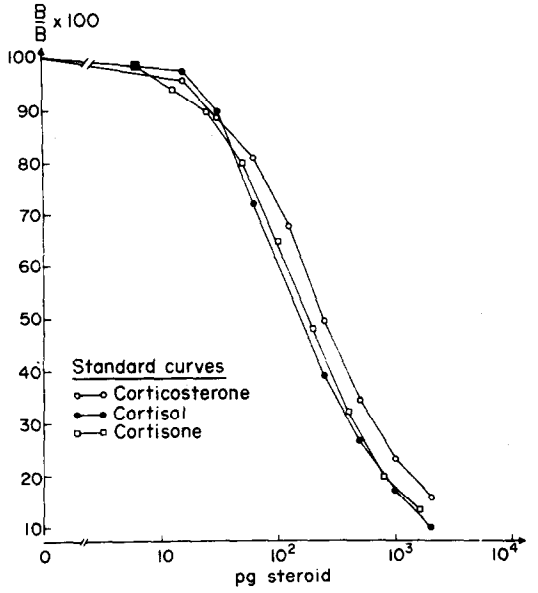

Fig. 1. Standard curves of corticosterone, cortisol and cortisone obtained with their respective specific antiserum. Each point represents the mean of three consecutive duplicate assays.

ated by multiple measurements of cortisone in a pool of plasma. The mean value (± S.D.) of twelve determinations on a pool of cord plasma was 24.8 ± 1.2 μg per 100 ml (coefficient of variation: 7%). A similar intra-assay coefficient was found for cortisol. The inter-assay coefficient of correlation (cortisone or cortisol) was 0.95.

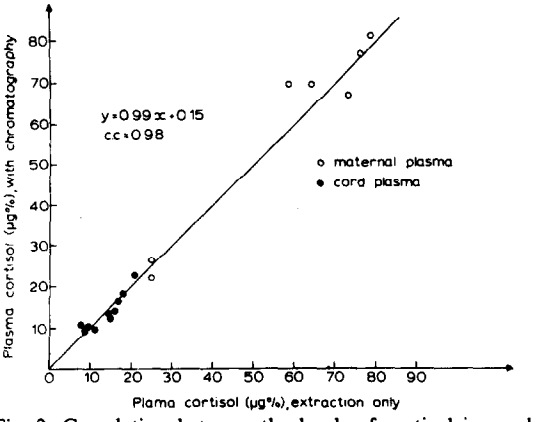

Fig. 2. Correlation between the levels of cortisol in cord and maternal plasma determined by radioimmunoassay with and without prior celite chromatography.

4. *Specificity*

The cross-reactivity of each antiserum with a variety of natural and synthetic steroids is indicated in Table 3. The percentage cross-reactivity was calculated at the 50% displacement of the labeled steroid. The specificity of each antiserum was also examined by comparing the measurements of corticosterone, cortisol and cortisone concentrations obtained with and without previous celite chromatography. The correlation between the levels of cortisol (n = 18), experimentally determined by both methods in cord and maternal plasma, is depicted in Fig. 2. A similar overall correlation was obtained for cortisone (Fig. 3) although the correlation was not as good for the

Table 2. Affinity constant (K), concentration of binding sites (Ab_0) and heterogeneity index (α) of used antisera

	K (l/mole × 10^8)	Ab_0 (mol/l × 10^{-6})	α
Anti-corticosterone serum	0.76	0.54	0.81
Anti-cortisone serum	5.32	8.44	0.74
Anti-cortisol serum	5.66	1.72	0.92

Table 3. Specificity of antisera described in this report: percentage of cross-reactivity at 50% displacement

Steroid	Anti-cortisol	Anti-corticosterone	Anti-cortisone
Cortisol	100	0.95	3.7
Cortisone	0.2	0.001	100
Corticosterone	10	100	0.06
Progesterone	1	2.7	0.001
Deoxycorticosterone	1.6	3.7	0.12
Aldosterone	0.001	0.001	0.001
17-OH-Progesterone	0.8	0.02	0.01
11-Deoxycortisol	10	0.02	3
Testosterone	0.1	0.01	0.001
11-Dehydrocorticosterone	—	0.001	—
Estradiol	0.001	0.001	0.001
Prednisone	0.01	0.001	100
Prednisolone	100	0.33	1.85
Dexamethasone	1	0.01	0.001

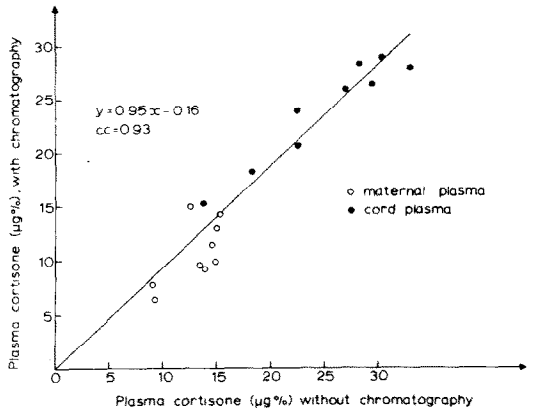

Fig. 3. Correlation between the levels of cortisone in cord and maternal plasma determined by radioimmunoassay with and without prior celite chromatography.

plasma of the mothers than for that of their babies. No correlation was found for plasma corticosterone, due to the high concentration of steroids weakly cross-reacting with the anti-corticosterone-3-CMO-BSA serum. In order to demonstrate further the specificity of the anti-cortisol and -cortisone serums, a large pool of cord plasma of which progesterone had been extracted, was chromatographed on sephadex LH-20.

The steroid content of each fraction was measured with both antisera. From Fig. 4, it appears that two peaks were obtained, the first one in the elution volume of a [^{3}H]-cortisone standard and the second one, in the elution volume of a [^{3}H]-cortisol standard. Cortisone of the first peak cross-reacted slightly with the anti-cortisone serum (total immunoreactivity measured with anti-cortisol serum, 850 ng; ratio = 11.3%). The second peak, containing the cortisol, also cross-reacted slightly with the anti-cortisone serum (total immunoreactivity measured with anti-cortisol serum, 5100 ng; total immunoreactivity measured with anti-cortisone serum, 640 ng; ratio = 12.5%). In both cases, cross-reactivity did not exceed 15%.

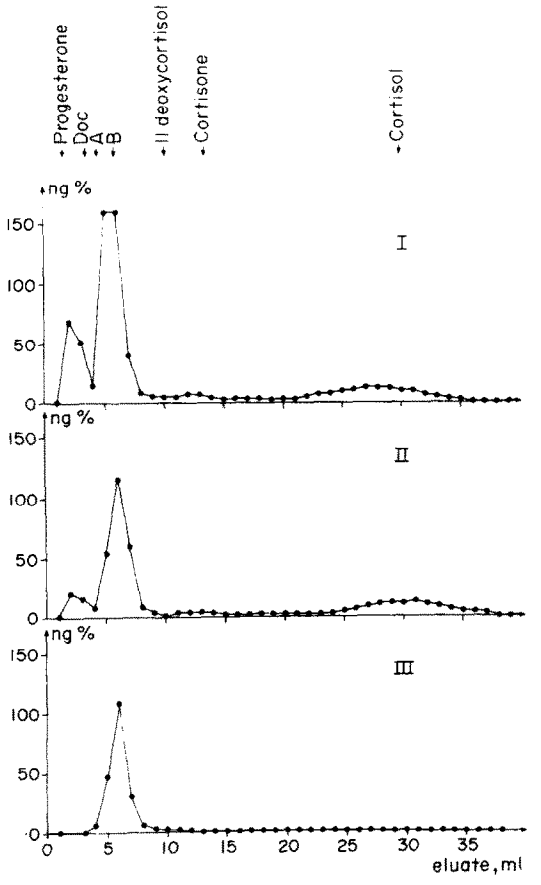

Fig. 5. Corticosterone immunoreactivity in the sephadex LH-20 fractions of a pool of cord plasma extracted with dichloromethane (I), hexane and dichloromethane (II) and dichloromethane followed by celite purification (III).

It is noteworthy that no other fraction contained a significant amount of immunoreactivity as measured with each anti-serum. When a dichlorome-

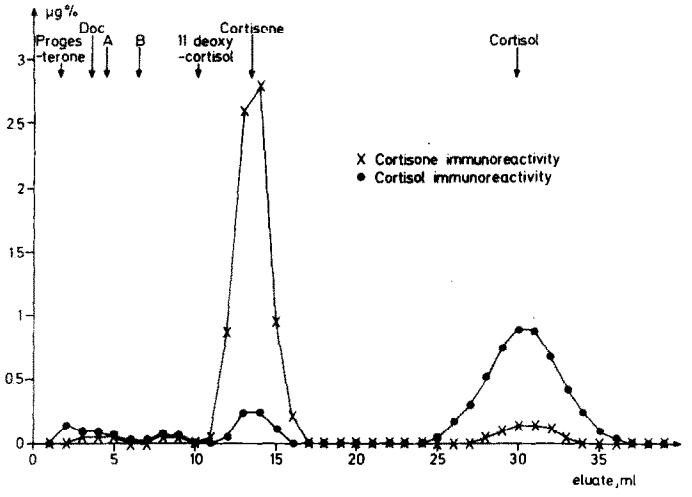

Fig. 4. Cortisone and cortisol immunoreactivity after chromatography of a pool of cord plasma on a sephadex LH-20 column (total immunoreactivity measured with anti-cortisone serum, 7500 ng)/(total immunoreactivity measured with anti-cortisol serum, 850 ng) = 11.3%.

Table 4. Normal values of corticosterone, cortisol and cortisone in maternal, umbilical cord venous and arterial serums (mean ± S.D.) (μg/100 ml)

	Method	Mother (n = 12)	Artery (n = 13)	Vein (n = 13)
Cortisol	Hexane and methylene chloride extraction	66.4 ± 5.6	9.13 ± 1.16	8.95 ± 0.69
Cortisone	Hexane and methylene chloride extraction	13.2 ± 0.8	25.5 ± 2.7	25.1 ± 2.0
	Celite chromatogr.	10.8 ± 0.9	—	24.9 ± 1.3
Corticosterone	Celite chromatogr.	3.54 ± 1.14	0.75 ± 0.27	0.56 ± 0.19

thane extract of pooled cord plasma was chromatographed on sephadex LH-20 and immuno-reactivity was assayed on each fraction using the anti-corticosterone serum, four peaks were obtained (Fig. 5, I) corresponding to progesterone (first peak), compound A* closely followed by corticosterone (2nd peak), cortisone (3rd peak) and cortisol (4th peak). As indicated Fig. 5, DOC is eluted between progesterone and compound A. Hexane extraction removed most of the progesterone immunoreactivity but not the other interfering steroids. The overall corticosterone purification brought about by hexane was less than two fold (Fig. 5, 11). By contrast, after celite chromatography, the interfering steroids were almost completely removed (Fig. 5, III). The specific activity of the corticosterone peak was; 303 d.p.m./ng in Fig. 5, I, 345 d.p.m./ng in Fig. 5, II and 318 d.p.m./ng in Fig. 5, III. The good agreement between these three values suggest that the corticosterone fraction obtained by LH-20 chromatography is indeed fairly pure.

5. *Normal values*

The concentrations of corticosterone, cortisol and cortisone were measured in maternal plasma and in umbilical cord venous and arterial plasma (Table 4). Cortisol was assayed after hexane and methylene chloride extraction only. The values of cortisone concentrations were directly measured after hexane–dichloromethane extraction with and without celite chromatography.

As seen, in the maternal plasma, large amounts of cortisol may interfere with the cortisone assay, making the celite chromatography mandatory. In the cord plasma, containing much less cortisol, the chromatographic step is unnecessary. The values, presented in Table 4, are in good agreement with those found by many authors using various techniques other than radioimmunoasay [14, 18, 20, 27–31].

DISCUSSION

The advantages of the radioimmunological method over the fluorimetric and the competitive-protein-binding assay are the simplicity, rapidity, low-cost, specificity and sensitivity. In the hands of a trained technician, up to 300 unknown samples may be processed in three working days. Except for the antibody and the labeled tracer, the cost of other reagents is trivial. The preparation of the corticosteroid-3-CMO-BSA conjugate is easy and the conjugates seem good antigens for the rabbit. The sensitivity of the antiserums allows the determination of steroid concentration in very small volumes of serum (20 μl for cortisol and cortisone, 200 μl for corticosterone). Although each antiserum demonstrates good specificity for their respective antigen, problems may arise when the concentration of weakly cross-reacting steroids is much higher than that of the steroid to be assayed. For instance, in the case of cortisol assay, progesterone and cortisone may significantly interfere. Progesterone is markedly elevated in pregnancy but may be easily removed by an hexane extraction. The cortisone concentration is normally less than that of cortisol except in the human foetus and the good specificity of the anti-cortisol serum renders the cortisone interference negligible. Even in fetal blood in which cortisone concentration is three fold higher than that of cortisol, a celite purification did not appear necessary. The anti-cortisone serum was less specific since it cross-reacted to a higher extent with cortisol than the anti-cortisol serum did with cortisone. The specificity of the anti-cortisone serum, however, allows the direct assay of this steroid in foetal plasma.

In the maternal plasma, at delivery, in which the cortisol concentration is six fold higher than that of cortisone, a direct assay slightly overestimated the cortisone concentrations and an additional purification step (celite or LH-20 chromatography) may be useful. Despite the good specificity of the anti-corticosterone serum, the low concentration of this steroid, compared to those of cortisol and cortisone, renders the chromatographic purification mandatory.

In conclusion, the radioimmunoassays are very useful methods provided that their limitations are well known and that purification steps are performed when the relative concentration of interfering steroids is much higher than that of the one to be assayed. Attention must be paid to the fact that the relative concentrations of the various steroids may vary in physiological and diseased states. The experimentally determined values of corticosterone, cortisone and cortisol concentration, using the above methods, are in good agreement with those from the literature.

* 11-dehydrocorticosterone.

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