A SPECIFIC, NON-CHROMATOGRAPHIC RADIOIMMUNOASSAY

FOR HUMAN PLASMA CORTISOL*

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Received: 4/30/75

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ABSTRACT

A radioimmunoassay technique has been developed for the measurement of cortisol in a single methylene chloride extract of human plasma without chromatography. The antiserum, obtained by immunizing rabbits with cortisol-3-carboxymethyl-oxime conjugated to bovine serum albumin, had a high affinity ($K_A = 1.8 \times 10^9$ l/mole) and capacity (2.3×10^{-6} moles/L undiluted serum) for cortisol. The minimum detectable amount determined at the lower 95% confidence limit of the buffer control tubes was 8.3 ± 4.7 pg/tube and a log dose – logit response standard curve was linear between 20 pg and 20 ng/tube. The antiserum was highly specific for cortisol with only corticosterone, cortisone, 11-deoxycortisol and 21-deoxycortisol showing significant cross-reaction (12.4, 6.6, 3.8 and 3.7%, respectively). The cross-reaction for the other tested naturally occurring and synthetic steroids did not exceed 1%. Regression analysis of cortisol concentration estimates obtained on 20 samples before and after Sephadex LH-20 column chromatography gave a coefficient of correlation (r) of 0.995 and a regression coefficient (b) of 1.04. Recovery of cortisol added to plasma samples was quantitative. The intra-assay error was 8.5% and the inter-assay error averaged 5.7%. The method is simple requiring a single solvent extraction of plasma, therefore permitting large numbers of samples to be handled efficiently by a single technician.

INTRODUCTION

Cortisol in plasma and urine is usually measured with either the competitive protein binding (CPB) technique, originally described by Murphy (6,7), the Porter-Silber color reaction (8) or fluorometry (9). Cortisol binding globulins (CBG) from various species have high affin-

ity (10) but relatively low specificity for cortisol. To make the CPB

technique more specific, prior chromatographic systems may be employed;

but, in the absence of considerable care, these approaches can give

rise to high plasma and urinary blank values. The Porter-Silber method

and fluorometry also lack in specificity, unless prior chromatography

of the samples is undertaken. Further, many drugs interfere with the

Porter-Silber color reaction which makes this method unsuitable for

random use (11). Ruder et al. (1972) have reported a radioimmunoassay

procedure for plasma and urinary free cortisol, using antibodies

against cortisol-21-hemisuccinate-bovine serum albumin (BSA) conjugate.

The antiserum had a high affinity ($K_A = 2 \times 10^9$ 1/mole) and binding ca-

pacity (2×10^{-6} moles/1 of undiluted serum) but a low specificity for

cortisol (12).

This communication describes the development of a sensitive, accu-

rate and specific, non-chromatographic method for radioimmunoassay of

cortisol in a single methylene chloride extract of plasma. The proce-

dure uses an antiserum raised against a BSA conjugate of cortisol-3-

carboxymethyl-oxime and tritiated cortisol (^{3}H-cortisol) as the tracer.

METHODS AND MATERIALS

All solvents used in the assay were glass distilled (Burdick-Jack-
son Laboratories) and were used without further purification. Corti-
sol-1,2,6,7,^{3}H(n), purified by thin layer chromatography, with a speci-
fic activity of 107 Ci/m mole, was purchased from the Radiochemical
Centre, Amersham. The cortisol standard was prepared by dissolving
crystallized cortisol (Hydrocortisone, Sigma Chemical) in ethanol (1 mg
/ml). The ethanol solution was diluted with 0.1% gel-PBS (0.1% gelatin
in 0.14 M NaCl, 0.01 M NaPO$_4$ buffer and pH 7 with 1:1000 merthiolate)
to a final concentration of 40 ng/ml, dispensed in 5 ml aliquots, quick
ly frozen by immersion in a dry ice-ethanol mixture, and stored at -20°
C. Other steroid preparations used in validation of the procedure were
identically prepared, dispensed and stored at final concentrations of
40 and 800 ng/ml. Prior to use, each solution was thawed by gently agi
tating the vial in lukewarm water. A small fraction of ^{3}H-cortisol in
benzene-ethanol (1:1 v/v) was pipetted into a 500 ml brown flask, the

solvent evaporated to dryness under nitrogen and the ^{3}H-cortisol recon-
stituted in 0.1% gel-PBS to a final concentration of approximately 20,
000 cpm/100 μl.

The antiserum was obtained by immunizing each of 6 New Zealand
White female rabbits, approximately 4 months of age, in the foot pads
with 2.5 mg of cortisol-3-carboxymethyl-oxime BSA conjugate (18-20 mol-
ecules of cortisol/molecule of albumin) emulsified in complete Freunds
adjuvant (13). The adjuvant was prepared by mixing 15.5 mg cortisol-
BSA conjugate in 3.1 ml of phosphate-buffered-saline (pH 7.4), 0.5 ml
of Arlacel A (Atlas Chemical Industries) and 2.8 ml of Bayol F (Esso
Petroleum Co.), together with 15 mg of desiccated M. tuberculosis, in
the barrel of a 20 ml glass syringe using a high speed Vertis Macro-
Homogenizer, Model 45. The initial immunization was followed by 2
booster immunizations (M. tuberculosis normally not included in booster
adjuvant) given at approximately 3 week intervals and a final booster
given 2 1/2 months later. The rabbits were bled biweekly, from the
marginal ear vein, following the second booster immunization. The
bleeding selected for use in the assay system was obtained 3 weeks after
the final immunization (22 weeks following the initial immunization).
This bleeding showed the highest titer and since it proved to be highly
specific, other bleedings from the same rabbit or other rabbits were
not evaluated further. The antiserum(#891, 2-8-71), used in the radio-
immunoassay at a final dilution of 1:20,000 in 0.1% gel-PBS, bound 38.5%
of 88 pg of ^{3}H cortisol (B/F = 0.63).

The dextran-coated charcoal suspension, prepared by adding 250 mg
of doubly washed charcoal (Mallinckrodt Chemical Works) and 25 mg of
dextran-80 (Pharmacia Corp.) to 100 ml of de-ionized water, was used
for separation of antibody-bound and free ^{3}H-cortisol.

All glassware used in the radioimmunoassay was heated in the self-
cleaning cycle of a Frigidaire self-cleaning oven prior to use. The
entire assay procedure was carried out in 12 x 75 mm disposable glass
tubes. Scintillation counting was done by using a solution containing
toluene (analytical grade, Mallinckrodt Chemical Works), Triton X-100
and Permaflour (Packard Instruments) (1.0:0.95:0.16) in a refrigerated
Packard Tricarb scintillation counter with a counting efficiency for
^{3}H of 35.4%. Nitrogen used to evaporate the solvents was passed over
Drierite, soda lime and a thick pad of glass wool.

<u>Assay Procedure</u>. The general outline of the assay is as follows:
Freshly thawed plasma(100μl), de-ionized water(500μl) and ^{3}H-cortisol
(5000-6000 cpm) in 100 μl of 0.1% gel-PBS were pipetted into a 16 x
150 mm culture tube and extracted with 3 ml of methylene chloride by
thoroughly mixing the contents on a vortex mixer for about 2 min. The
methylene chloride phase was transferred to a 12 x 75 mm tube and the
solvent evaporated under nitrogen in a water bath kept at 45°C. The
residue was then reconstituted in 3 ml of 0.1% gel-PBS. Aliquots (100
μl) of this were taken for assay and recovery estimates of ^{3}H-cortisol.
All assay results were corrected for procedural losses using calculated
recovery rates of ^{3}H-cortisol. The low levels of ^{3}H-cortisol used for
recovery were neglected in later calculations.

The samples were assayed in duplicate at two dose levels. The
volume was brought to 500 μl by adding the requisite amount of 0.1%
gel-PBS. Upon addition of 200 μl of a 1:2000 dilution of antiserum and
100 μl of ^{3}H-cortisol (approx. 20,000 cpm) in 0.1% gel-PBS, the tubes

were mixed and incubated overnight (15-18 hrs) at 4°C (5). The boun
and free ^{3}H-cortisol were separated by addition of 1 ml dextran-coat
charcoal suspension to each tube in batches of 30-40 and the incubat
was continued for another 10 min. The tubes were then centrifuged a
600 x g in a refrigerated centrifuge (Model PR-J, International Equi
ment Co.) for 10 min. The supernatant was decanted into scintillati
vials containing 10 ml of scintillation fluid and counted for one mi
ute. An eleven point standard curve (0.02 - 20 ng/tube) was constru
ted for each assay and run in triplicate (at the beginning, middle a
end of samples). Standard curves and unknown samples were processed
simultaneously with a computer program which uses weighting procedur
and logit-log dose transformation to obtain a linear regression curv
The program tests all sample curves for linearity and parallelism wi
the standard and then computes the weighted mean concentration ± 1 SE
per tube (14). The values obtained for unknown samples were correct
for procedural losses based on the recovery of ^{3}H-cortisol.

Sephadex LH-20 column chromatography. Sephadex LH-20 (lot 2764
Pharmacia Corp.) was equilibrated in a methylene chloride: methanol
(98:2) mixture for 24 hrs. and then packed into 5 x 100 mm columns.
Floating of the gel in the solvent system was eliminated by placing
fine glass wool between 2 filter paper plates on the top of the gel
columns. The solvent eluted from the column at a rate of 0.35 - 0.4
ml/min. Prior to use, each column was washed with 20 ml of the elue
The samples were prepared by extracting 300 µl of freshly thawed pla
and approximately 15,000 cpm ^{3}H-cortisol in 100 µl of 0.1% gel-PBS w
3 ml of methylene chloride. Eight hundred µl of the methylene chlor
extract was evaporated under nitrogen in a water bath at 45°C and th
residue dissolved in 500 µl of the elution solvent for column chroma
tography. The samples were eluted in 500 µl fractions. The content
of the 3-4 tubes containing cortisol were pooled, dried and reconsti
ted in 2 ml of 0.1% gel-PBS. Another 800 µl of the methylene chlori
extract was dried and the contents reconstituted in 2 ml of 0.1% gel
PBS for assay. This allowed a comparison to be made of estimates of
cortisol in a methylene chloride extract of a sample and the same ex
tract after it had been subjected to chromatography.

Following use, the columns were washed with 40 ml of a methylen
chloride: methanol (98:2) mixture to remove any residual radioactivi
and stored in air-tight glass jars containing 150-200 ml of the chro
tography solvent system.

RESULTS

Immunoreactivity of the antiserum against cortisol-3-BSA. The

specificity of the antiserum used in the cortisol radioimmunoassay w

assessed by examining the dose response curves for 19 different ster

oids. Commonly used glucocorticoids, dexamethasone and triamcinolon

showed extremely low cross-reactivity (< .01%) in the assay system.

Similar, nearly parallel dose response curves were obtained with pre

nisolone, cortisone, corticosterone, 11-deoxycortisol, 21-deoxycortisol

and prednisone (Fig. 1). However, the relative activity of these com-
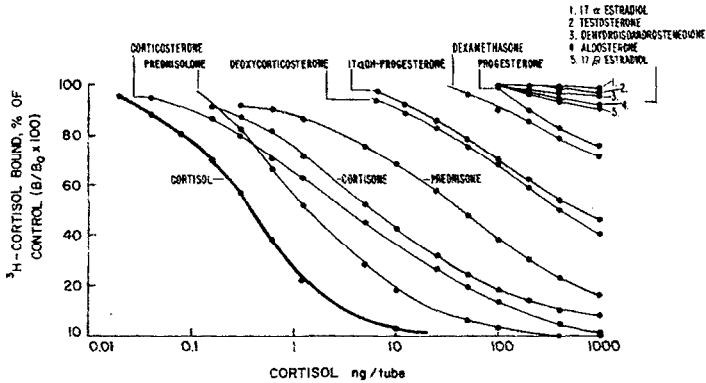

Fig. 1 Inhibition curves of various steroids, naturally occur-
ring and synthetic, in cortisol-3-BSA radioimmunoassay.

pounds as determined at the 50% displacement of ^{3}H-cortisol were negli-

gible for all except prednisolone (28%), corticosterone (12.5%), corti-

sone (6.6%), 11-deoxycortisol (3.8%) and 21-deoxycortisol (3.7%) (Table

1).

The cortisol standard curve. Figure 2 shows the standard dose re-

sponse curve for cortisol based on data from 8 assays (24 curves). The

average index of precision (λ) based on a weighted least squares linear

regression for the logit-log transformation of standard curves was 0.018

(range 0.012 - 0.026). The assay median variance ratio (14) ranged

from 0.012 to 0.055 with an average of 0.025. The non-specific back-

ground (NSB) as a percent of the total counts added (T) ranged from 0.5-

0.9% (0.6 $\pm$ 0.01%) and the radioactivity bound in the absence of unla-

beled steroid (buffer control, B_0) expressed as a percent of the total

activity added (B_0-NSB)/(T-NSB) ranged from 32.7% - 40.4% (38.5 $\pm$ 2.6%).

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Regression analysis of the concentration of cortisol bound (from the

TABLE 1. %Cross reactivity* (CR) of Selected Steroids in
Two Different Radioimmunoassay Systems

Steroid	-21 Hemisuccinate (Ruder et al, 1972) (K_A=2 x 10^9 1/mole)	-3 Carboxy-methyl-oxime (Present Study) (K_A=1.8 x 10^9 1/mole)
Cortisol	100%	100%
Prednisolone	-	28.5%
Corticosterone	-	12.5%
Cortisone	40%	6.6%
11-deoxycortisol	-	3.8%
21-deoxycortisol	-	3.7%
Prednisone	16%	0.7%
11-dehydrocorticosterone	46%	0.1%
11-deoxycorticosterone	46%	0.06%
6 β-cortisol	-	0.05%
Tetrahydrocortisol	-	0.01%
Progesterone	28%	0.001%
17 α-OH-progesterone	56%	0.06%
Dexamethasone	-	0.01%
Triamcinolone	-	0.01%
Testosterone	13%	0.001%
Estradiol-17 β	0.01%	0.001%
Aldosterone	0.01%	0.001%

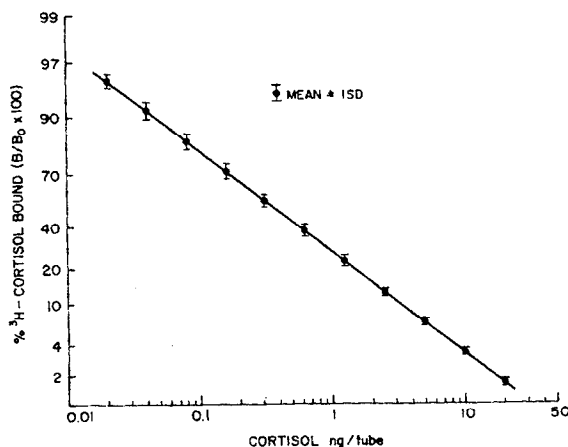*Determined at 50% displacement = $\dfrac{\text{ng of cortisol}}{\text{ng of the steroid}}$ X 100

Fig. 2 Standard dose response curve, mean ±1 standard
deviation from 24 individual standard curves.

above results) versus the bound to free ratio, according to the method

of Scatchard (15), revealed a linear relationship and indicated that
the antiserum possessed an equilibrium association constant of 1.8 x
10^9 1/mole and a capacity of 2.3 x 10^{-6} moles/l of undiluted serum.

Sensitivity. The smallest quantity of cortisol which could be
distinguished from "no cortisol" determined at the lower 95% confidence
limit of the buffer control tubes, was 8.3 $\pm$ 4.7 pg/tube. The logit-
log transformation was linear between 20 pg and 20 ng with a useful
range of 80 pg to 10 ng/tube.

Precision. The intra-assay error, determined as the coefficient
of variation of 58 assay replicates of 40 plasma samples, (each run in
duplicate at two levels), was 8.5%. The inter-assay error measured as
coefficient of variation of reference plasma included in each assay
averaged 5.7% (n=6).

Recovery of ^{3}H-cortisol. Recovery of ^{3}H-cortisol added to the un-
known samples prior to extraction with 30 volumes of methylene chloride
averaged 93.5 $\pm$ 4.3%.

Accuracy.

1. Recovery of unlabelled cortisol. To 100 µl aliquots of plasma
from a fasting male and a female in the second trimester of pregnancy,
0, 5, 10, 20 and 40 ng of unlabelled cortisol were added, and incubated
for one hour at room temperature. The samples were then extracted with
methylene chloride for radioimmunoassay. Figure 3 shows the correla-
tion between the "observed" and the "expected" cortisol concentration/
tube. The aggreement between the two was assessed by linear regression
analysis. The correlation coefficient (r) and slope (b) were 0.999 and
0.94 for male plasma and 0.998 and 1.10 for pregnancy plasma respective-
ly. This indicates no influence of increased levels of cortisol bind-

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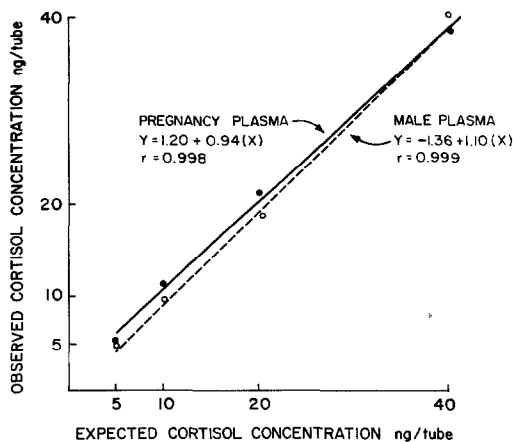ing globulin (CBG) on recovery of unlabeled cortisol.

Fig. 3 Recovery of known amounts of cortisol added to human plasma. Observed values were calculated by differ- ence between enriched samples and original sample.

2. Comparison of the estimates of cortisol by the present method

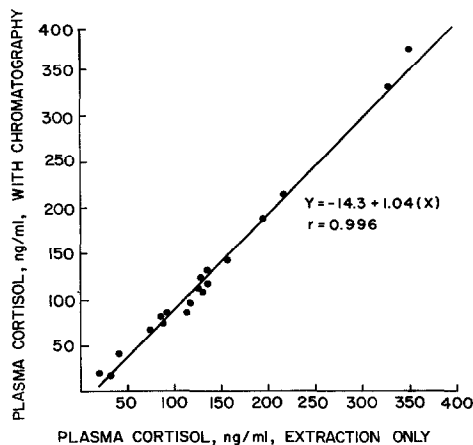with and without chromatography. Plasma samples with high, normal and

Fig. 4 Comparison of cortisol concentration estimates in the same samples with and without chromatography.

low cortisol levels obtained from 3 normal subjects at 8 a.m. (fasting)

and 4 p.m., 2 patients with controlled Cushing's Syndrome before and

after ACTH infusion, 5 subjects with normal pregnancy and 5 newborns of

different gestational age were processed by (a) the present method (sim-

ple methylene chloride extraction) and (b) a methylene chloride extrac-

tion followed by Sephadex LH-20 column chromatography. Under the chrom-

atographic conditions approximately 70% of cortisol could be eluted in

fractions 10-12 (5-6.5 ml). Figure 4 shows the correlation between the

estimates of cortisol obtained with and without chromatography, all es-

timates were corrected for recovery of ^{3}H cortisol; the correlation co-

efficient (r) was 0.995.

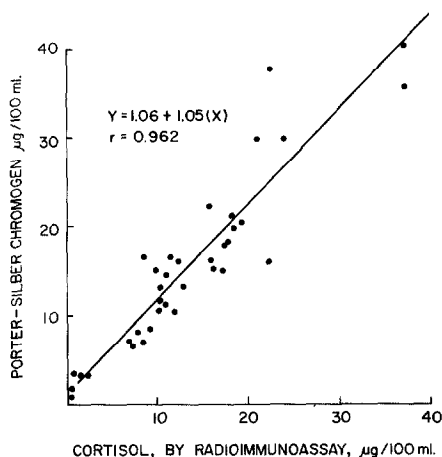3. Comparison of the present method with the Porter-Silber method.

Fig. 5 Comparison of corti-
sol Radioimmunoassay
with Porter-Silber
method.

Forty-one plasma samples with a wide range of cortisol concentrations

were measured by radioimmunoassay and the Porter-Silber method (16).

The values obtained by radioimmunoassay ranged from 0.4 - 62.3 μg/100 ml

and the corresponding values, without recovery corrections by the Por-

ter-Silber method (approximately 80% recovery) 0.9 - 63.7 μg/100 ml. In

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general, the values obtained by the Porter-Silber method (Y) were
slightly higher than those of radioimmunoassay (X), Y = 1.05 (X), the
correlation coefficient (r) being 0.962 (Fig. 5).

4. Plasma cortisol levels in normal subjects and patients with
treated Cushing's Syndrome. Figure 6 shows the diurnal rhythm of corti-
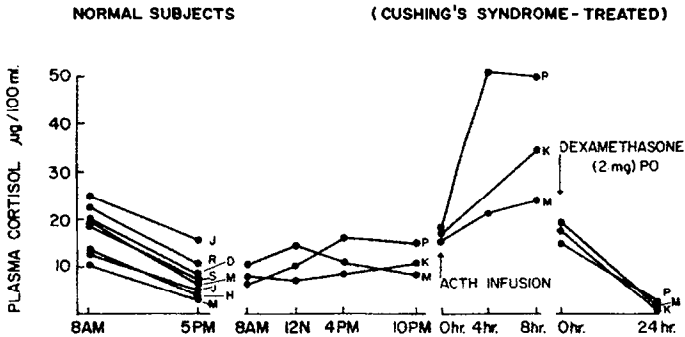

Fig. 6 Plasma cortisol levels in normal subjects and
in patients with treated Cushing's Syndrome.

sol secretion in 8 normal subjects and 3 patients with Cushing's Syn-
drome (ACTH dependent) before and after treatment with Lysodrine (0'P'
DDD; 2, 2-bis (2-chlorophenyl-4-chlorophenyl)-1, 1-dichlorethane 2 mg
orally). In normal fasting subjects the 8 a.m. plasma cortisol ranged
from 10.6 - 25.0 µg/dl (17.7 ± 5.0) and 4 p.m. estimates were 3.0 -
16.1 µg/dl (7.7 ± 4.2). These values are similar to those reported in
the literature (12,17). In patients adequately treated for Cushing's
Syndrome, the resting plasma cortisol levels were normal, but the ex-
pected diurnal rhythm was absent. These patients, however, did respond
normally to ACTH infusion (25 U ACTH over 8 hrs) and dexamethasone sup-
pression.

DISCUSSION

Radioimmunoassays have the potential of being used for quantita-
tion of steroids in raw or extracted plasma or serum samples without
additional chromatography and have the advantage over competitive-pro-
tein-binding (CPB) assays, provided a truly specific antiserum is avail-
able. Earlier data have shown that specificity of antisera against
steroids are largely dependent on the site at which the carrier protein
is conjugated (17). Conjugation of the protein to a site remote from
the sites of the unique functional groups on the steroid molecule re-
sults in an increase in antibody specificity. This statement is amply
documented by the observations that specificity of the antibodies a-
gainst testosterone-3, progesterone-11, and 17β -estradiol-11 BSA con-
jugates are much greater than those of antibodies against testosterone-
17, progesterone-3, or 17β -estradiol-3 or 17 BSA conjugates (18-20).
Cortisol differs structurally from other steroids by the presence of
hydroxy groups at C-11, C-17 and C-21. Therefore, an antibody prepared
against a cortisol-21-BSA conjugate should be relatively less specific
than one against a cortisol-3-BSA conjugate, since in the latter the
unique functional groups are available for antibody induction and recog-
nition. This is further supported by comparing the specific activity
of 11 different steroids in two different radioimmunoassay procedures
(Table 1). While the majority of steroids cross-reacted with the cor-
tisol-21-BSA antiserum, few did so against cortisol-3-BSA antiserum (the
cross-reaction of prednisolone was expected as it is structurally anal-
ogous to cortisol with only the insertion of a double-bond at C 1).
Extraction of serum samples is required, however, to remove endogenous
binding protein and cross-reacting water soluble compounds including

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cortisol-3-conjugates. It should be apparent that the assay has not been validated for sera from animals, nor for sera from patients with certain rare diseases such as congenital adrenal hyperplasia with 17-hydroxylase deficiency.

The accuracy of the present method was examined in part by the recovery of various amounts (5-40 ng/tube) of unlabeled cortisol from two different types of plasma, one with normal and the other with an excess of human CBG. Excess CBG in plasma did not interfere with recovery and the coefficient of correlation did not differ between the two plasma specimens. Finding slightly higher values by the Porter-Silber method also lends support to the accuracy of measuring cortisol by the present method. Studies of different physiological states such as variations due to diurnal rhythm, as well as results of ACTH stimulation and dexamethasone suppression, suggests that the plasma cortisol can be reliably estimated over a wide range. Further, the excellent correlation between chromatographed and non-chromatographed cortisol estimates in plasma samples justifies application of this procedure for clinical and research purposes. The procedure prior to radioimmunoassay requires less than one hour for completion, the technique is not complex, and the time used in completion of the assay is reasonable and can be varied to meet individual needs (in emergencies the assay can be completed in about 2 hours using 37°C incubation conditions). The RIA described is more sensitive than the CPB methods, has much greater specificity and is equivalent in ease and convenience of set-up. Most of the synthetic or naturally occurring steroids that might be found in patient specimens (which would invalidate a CPB assay not involving chromatography) do not interfere with the radioimmunoassay described herein. Those steroids

that do cross-react in the RIA include a small number of synthetic ster-

oids (prednisolone, cortisone and to a minor extent prednisone) and only

one of the tested naturally occurring steroids (corticosterone). Other

naturally occurring steroids do not cross-react as shown by the lack of

difference between chromatographed and non-chromatographed samples.

With the described procedure, it is possible for one technician to assay

about 50 different samples in duplicate and at 2 dose levels or a great-

er number of samples at only 1 dilution. The assay can be set up in the

afternoon, allowed to incubate overnight and results obtained the next

day. This schedule would be acceptable in most clinical laboratories

where specimens are collected and run at intervals in large batches.

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     ology, Post-Graduate Institute of Medical Education and Research,
     Chandigarh, India.

(2) Career Development Awardee of the National Institute of Child
     Health and Human Development.

(3) Present address: Department of Physiology, Colorado State Univer-
     sity, Fort Collins, Colorado 80521.

(4) The following trivial names have been used:

     cortisol (11β , 17α , 21-trihydroxypregn-4-ene-3,20-dione),
     corticosterone (11β , 21-dihydroxypregn-4-ene-3,20-dione),
     aldosterone (11β , 21-dihydroxy-3, 20-dioxopregn-4-ene-18-al),
     cortisone (17α , 21-dihydroxypregn-4-ene-3,11,20-trione),
     progesterone (pregn-4-ene-3,20-dione),
     17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3,20-dione),
     deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione),
     17α-estradiol (1,3,5(10)-estratriene-3,17α-diol),

17 β-estradiol (1,3,5,(10)-estratriene-3,17 β-diol),
testosterone (17 β-hydroxyandrost-4-ene-3-one),
dehydroisoandrosterone (3 β-hydroxyandrost-5-ene-17-one),
tetrahydrocortisol (3 α, 11 β, 17 α, 21-tetrahydroxy-5 β-pregnan-
    -20-one),
triamcinolone (11 β, 16 α, 17 α, 21-tetrahydroxy-9 α-fluoro-pregn
    1,4-diene-3,20-dione),
dexamethasone (11 β, 17 α, 21-trihydroxy-9 α-fluoro-16 α-methyl-
    pregn-1,4-diene-3,20-dione),
prednisone (17 α, 21 dihydroxypregn-1,4-diene-3,11,20-trione),
prednisolone (11 β, 17 α, 21-trihydroxypregn-1,4-diene-3,20-dione
6 α-hyrodxycortisol (6 α, 11 β, 17 α, 21-tetrahydroxypregn-4-ene-
    3,20-dione),
6 β-hydroxycortisol (6 β, 11 β, 17 α, 21-tetrahydroxypregn-4-ene-
    3,20-dione),
11-deoxycortisol (17 α, 21-dihydroxypregn-4-ene-3,20-dione)
21-deoxycortisol, (11 β, 17 α-dihydroxypregn-4-ene-3,20-dione).

(5)   The incubation is now being carried out at room temperature for 1
      hours using a 1:4000 dilution of the antiserum. No change in spe
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