Improved Radioimmunoassay for 11-Deoxycorticisol (Compound S) in Plasma

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We describe a radioimmunoassay for 11-deoxycorticisol (Compound S) that involves preliminary chromatography on Sephadex LH-20, to isolate 11-deoxycorticisol from crossreacting structurally related steroids. Pools with means of 3.4, 12.9, and 69.9 µg/L gave intra-assay coefficients of variation of 9.7, 9.6, and 11.4%, respectively; pools with means of 4.6, 27, and 95 µg/L gave inter-assay CV’s of 10.1, 9.7, and 6.2%, respectively. A normal range (mean ± 2SD) of 0.5 to 12.0 µg/L was obtained. This procedure is useful for the specific determination of 11-deoxycorticisol, commonly determined in blood as part of the metyrapone test for assessing pituitary corticotropin reserve.

Additional Keyphrases: metyrapone-challenge test • steroids • liquid column chromatography • pituitary-reserve assessment • normal range • adrenal status • Cushing’s syndrome • metyrapone test

The “metyrapone challenge test” is used both to test for pituitary corticotropin reserve and to establish the diagnosis of Cushing’s syndrome (1–3). This test, to be of great value, must measure both Compound S (11-deoxycorticisol) and Compound F (cortisol), separately. In the past, competitive protein-binding radioassay has been used in conjunction with solvent partitioning to determine these compounds separately (3–5). With the advent of more specific antibodies to measure these compounds selectively, this procedure has become more useful in clinical diagnosis (6–8). Although antibody specificity has been improved, solvent partitioning as well as chromatographic separation of these steroids is still required by virtue of their close structural and chemical similarities.

Here we report an improved rapid separation of 11-deoxy cortisol from crossreacting steroids by liquid chromatography on Sephadex LH-20. Progesterone and 17-hydroxyprogesterone were completely separated from 11-deoxycorticisol with use of the solvent system isooctane/benzene/methanol (80/10/10 by vol). Analytical recoveries after plasma extraction and liquid column-chromatographic separation averaged 65%.

Materials and Methods

Apparatus

Borosilicate glass columns, 8 × 1 cm (i.d.), for liquid chromatography were obtained from New England Nuclear, Boston, MA 02118. Fiber paper (Balston Ltd., Maidstone, England) was used at the column ends. Radioactivity was determined with a Delta 300 liquid scintillation counter (Searle Analytic, Des Plaines, IL 60019) with a 60% efficiency for tritium.

Reagents

Glass-distilled benzene and methanol were purchased from Burdick and Jackson, Muskegon, MI 49442, and isooctane from Baker Chemical Co., Phillipsburg, NJ 08865. The assay buffer consisted of phosphate buffer (50 mmol/L, pH 7.4) containing 5 g of bovine serum albumin per liter. The mixture used to separate free from antibody-bound 11-deoxycorticisol consisted of 0.25 g of Norit A charcoal, 25 mg of Dextran T-70, and 0.9 g of sodium chloride, all dissolved in and diluted to 100 mL with assay buffer. The chromatographic material, Sephadex LH-20, was obtained from Pharmacia Fine Chemicals, Piscataway, NJ 08854. Tritiated steroids with specific activities ranging from 40 to 60 mCi/mol, from New England Nuclear, were purified by liquid chromatography on a column of Sephadex LH-20. The antibody we used was obtained from Radioassay Systems, Carson, CA 90746; it had been raised against 11-deoxycorticisol-21-monohemisuccinate coupled to human serum albumin. The lack of complete specificity of available antisera for 11-deoxycorticisol necessitates the use of Sephadex LH-20 chromatography to isolate it from crossreacting steroids before radioimmunoassay. Several reports (9, 11–14) cite progesterone and 17-hydroxyprogesterone as the principal interfering steroids. The antisera we used showed similar crossreactivity problems, and we thus found it necessary to include a liquid column-chromatographic step for steroid isolation before radioimmunoassay.

Further to substantiate this, we compared results for patients’ samples by the 11-deoxycorticisol radioimmunoassay with and without the preliminary chromatography.

When we analyzed 15 samples after a hexane wash and a simple extraction with ethyl acetate, a mean value of 15.9 ± 9.1 µg/L was obtained. When these samples were also subjected to the column-chromatographic step, a mean of 9.3 ± 9.7 µg/L was observed. Standard deviations for each set of data were large, owing to the small sample volume we used. By Student’s t-test, there was a significant difference (p < 0.001) between these values. Parallelism between the 50- and 500-µL aliquots of the extracted plasma without column chromatography was extremely poor, further evidence of the presence of interfering substances in these samples and the need for additional purification.

Procedures

Extraction. To 1.0 mL of patient’s plasma, add 100 µL of assay buffer containing 1500 cpm (0.001 µCi) of [3H]11-deoxycorticisol, for estimating recovery. Vortex-mix the mixture and add 5 mL of n-hexane, allow to stand for 5 min, and then discard the n-hexane. Add 4 mL of ethyl acetate to the aqueous layer and shake the mixture for 5 min. Remove the ethyl acetate layer, which now contains the 11-deoxycorticisol,
to a clean 13 × 100 mm acid-washed test tube and evaporate it under a stream of filtered air.

Liquid column chromatography. Progesterone and 17-
hydroxyprogesterone cross reacted 100% with Radioassay
Systems' 11-deoxycortisol C21 antibody. With all other major
C21 steroids the cross reactivity was less than 10%; with C18
and C19 steroids it was less than 2%

Prepare columns for liquid chromatography by adding 5 mL
of a mixture of isocane/benzene (85/15 by vol) to 1.0 g of
Sephadex LH-20, placing the slurry on a magnetic stirrer,
and stirring continuously.

Place 5 mL of the slurry in a 8 × 1 cm (i.d.) glass column
containing a fiber glass disc to retard loss of the column ma-
terial. Allow the solvent to drain, wash once with 20 mL of
isocane/benzene/methanol (80/10/10 by vol), and discard the
washings. Dissolve the dried sample extracts in 200 μL of
the same solvent and add to the top of the column bed with
a Pasteur pipette. Repeat the above process with the solvent
to ensure quantitative removal of 11-deoxycortisol. Allow the
solvent containing the same steroids into the column bed,
add 14 mL of the solvent, and discard the effluent. This
fraction contains the progesterone and 17-hydroxyprogester-
one. Then add an additional 9 mL of the solvent to the
column and collect the effluent in a 20-mL scintillation vial.
Evaporate this fraction, which contains an average of 65% of
the 11-deoxycortisol, under filtered air, redissolve in 2.0 mL
of ethanol, and evaporate aliquots for radioimmunoassay.

For analytical recovery determinations, 500 μL of the
ethanolic solution containing the extract was pipetted into a
scintillation vial and dried in a stream of filtered air; its ra-
dioactivity was counted after adding liquid scintillation
cocktail (ACS scintillation fluid; Amershain/Searle, Arlington
Heights, IL 60004). All recovery-assessment samples were
thoroughly vortex-mixed and their radioactivity was counted
for 5 min in the liquid scintillation counter.

Radioassay Procedure

We prepared standards containing 39, 78, 156, 313, 625, and
1250 pg of 11-deoxycortisol in 100 μL of ethanol from a 1.0
mg/L stock solution of 11-deoxycortisol and constructed the
standard curve from data on duplicate 0.1-mL aliquots of
these standards.

From the ethanolic extract of serum containing 11-deox-
cortisol, transfer 0.5 and 0.05 mL, in duplicate, to 12 × 75 mm
glass acid-washed test tubes. Evaporate both samples and
standards in a rotary evaporator under filtered air, and add
0.1 mL of phosphate buffer (50 mmol/L, pH 7.4), containing
50 g of bovine serum albumin per liter, to each tube. Add 1,
0.2, and 0.1 mL of assay buffer to the total count, nonspecific
binding, and maximum binding tubes, respectively. Excep-
t for total and nonspecific binding tubes, add 0.1 mL of diluted
antisem at a titer of 1:15 000 to each tube, followed by 0.1
mL of [3H]11-deoxycortisol (approximately 10 000 counts per
minute) to all tubes. Mix the contents of each tube well and
incubate at 4 °C overnight. Transfer the tubes to an ice bath,
incubate, and then rapidly stirring, add 0.8 mL of dextran-
coated charcoal suspension to all but the total-count tubes.
Vortex-mix the contents of the tubes briefly and centrifuge
the tubes for 15 min in a refrigerated (4 °C) centrifuge. Decant
the supernate into scintillation vials containing 10 mL of the
scintillation fluid and count the radioactivity for 2 min in a
liquid scintillation counter.

Calculations

For data reduction we used a Hewlett-Packard Model 9831
desk top calculator for weighted least-square regression
analysis of the logit transformation of B/Bo. The following
equation was used to calculate the 11-deoxycortisol content
in plasma:

\[ A = B \times (C/D) \times (10^{-3}/E) \]

where A = final concentration (μg/L), B = picograms of
Compound S from radioassay curve, C = total volume (mL)
of ethanolic extract, D = volume of extract (mL) used in assay,
and E = volume of plasma (mL) extracted.

Analytical Variables

Reproducibility of standard curves. We generated five
standard curves with use of the same lot of antibody. Mean
values and standard deviations of the percent bound (B/B0
× 100) for each standard point were calculated from five
standard curves.

Precision. We then performed inter- and intra-assay pre-
cision studies on three separate pools in the high, medium,
and low range of the standard curve. Standard deviations and
coefficients of variation were calculated, based on 10 separate
determinations of each pool for the intra-assay precision study
and on six separate runs for inter-assay precision.

Parallelism. We tested an extract of patient's serum for
parallelism, using 1/5, 1/4, 1/3, 1, and 1.5 sample volumes,
and compared results with values of a typical standard curve.
Slope and intercept values were calculated on both sets of data
by using the logit transformation of raw data.

Analytical recovery. We measured analytical recovery of
steroid added to an extract of a patient's serum. Steroid in
amounts ranging from 39 to 625 pg per 100 μL of assay buffer
was added to 12 × 75 mm acid-washed test tubes containing
100 μL of evaporated extract. We also evaluated the extract
alone by adding 100 μL of assay buffer to a duplicate set of 12
× 75 mm test tubes. Recovery was plotted as 11-deoxycortisol
(pg/tube) measured vs. 11-deoxycortisol added (pg/tube).

Normal Range

A normal range for the 11-deoxycortisol radioimmunoassay
was evaluated, with use of 57 control subjects, each judged to
be free of disease on the basis of a complete history and
physical examination and normal results from a clinical
chemical profile. A modification (12) of Harding's probability
analysis of frequency distributions (13, 14) was used to de-
terminate if the control population used in this study was nor-
mally or log normally distributed. The normal range calcu-
lated from this rigorous statistical analysis was 0.5 to 12 μg/L.
We then administered 750 mg of metyrapone to 15 control
subjects (10 women, ages 27–43, and five men, ages 31–41) to
test their clinical response to metyrapone. Blood was sampled

![Fig. 1. Chromatographic separation of progesterone, 17-hy-
droxyprogesterone, and 11-deoxycortisol on Sephadex LH-20](image-url)

Each fraction is equivalent to 2 mL of column effluent.
for 11-deoxycortisol radioimmunoassay before and 3 h after the metyrapone challenge.

**Results and Discussion**

Figure 1 depicts a typical steroid separation profile after chromatography on Sephadex LH-20. We added a mixture of tritiated (0.02 μCi of each steroid) 17-hydroxyprogesterone, progesterone, and 11-deoxycortisol to extracted plasma containing unlabeled 11-deoxycortisol just before chromatography. Separation of the 11-deoxycortisol from progesterone and 17-hydroxyprogesterone was essentially complete with use of the solvent system isooctane/benzene/methanol (80/10/10 by vol). Both 17-hydroxyprogesterone and progesterone appear in the first 14 mL, followed by elution of 11-deoxycortisol in the next 9 mL. Recovery averaged 65% for all determinations.

Standard curve reproducibility is shown in Figure 2. Intra-assay and inter-assay precision is shown in Table 1. The methodological CV, both between-run and within-run, approximated 10% at all three concentrations tested.

Figure 3 shows our results for analytical recovery. Recovery values averaged 98%, with the recovery slope close to 1.

Figure 4 shows the results of parallelism studies of an above-normal sample from a patient. Parallelism was good over the wide range of dilutions tested, with values quite comparable to the slope of the standard curve. Slope and intercept values were virtually identical between standards and patient's sample dilutions, indicating the absence of substances interfering with the radioimmunoassay of 11-deoxycortisol.

Results for the samples from 57 normal control subjects are shown as a histogram plot in Figure 5. A normal range (mean ± 2 SD) of 0.5–12.0 μg/L was found.

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**Table 1. 11-Deoxycortisol Precision Data**

<table>
<thead>
<tr>
<th>Pool</th>
<th>No.</th>
<th>Mean ± SD, μg/L</th>
<th>CV, %</th>
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<tr>
<td><strong>Intra-assay</strong></td>
<td></td>
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<tr>
<td>1</td>
<td>10</td>
<td>3.4 ± 0.33</td>
<td>9.7</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>12.9 ± 1.24</td>
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<tr>
<td>3</td>
<td>10</td>
<td>69.9 ± 8.00</td>
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<tr>
<td><strong>Inter-assay</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
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<td>4.56 ± 0.46</td>
<td>10.1</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>26.96 ± 2.62</td>
<td>9.7</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>95.15 ± 5.87</td>
<td>6.2</td>
</tr>
</tbody>
</table>

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**Fig. 2. Standard curve reproducibility**

Each point represents the mean and standard deviation for data from five standard curves.

**Fig. 3. 11-Deoxycortisol recovery study**

Different concentrations of the steroid were added to an extract of patient's sample and assayed for 11-deoxycortisol.

**Fig. 4. 11-Deoxycortisol parallel study**

Different concentrations of a high value patient's sample were assayed and compared to standards run at similar concentrations.

**Fig. 5. Data on 11-deoxycortisol for 57 samples from normal individuals**

Normal ranges for 11-deoxycortisol by other methods have been described. Spark (3), using a competitive protein-binding method, published a normal range of 0.0 to 16 μg/L. Lee et al. (10), using a similar assay procedure, reported a normal range of 5.6–13.8 μg/L. Brown et al. (15), using a radioimmunoassay method, reported a normal range of 1 to 20 μg/L. Mason and Frazer (16) published a normal range of 0.4–4.0 μg/L; they used gas chromatography.
We tested the effects of metyrapone administration to patients, using our radioimmunoassay to monitor the response. Metyrapone produces a chemical block of the 11-hydroxylase enzyme in the adrenal glucocorticoid synthetic pathway. This block elicits an immediate decline in blood cortisol concentration, which in turn provokes a pronounced increase in pituitary corticotropin (ACTH) release in normal subjects. This in turn causes an increase in adrenal steroidogenesis up to the point of 11-hydroxylation. Serum 11-deoxycorticisol concentrations increase within 3 h when pituitary corticotropin production is normal. Metyrapone administration to 15 normal subjects resulted in a significant increase in the serum 11-deoxycortisol (from a mean of 0.76 to 124 µg/L) when samples were analyzed 3 h after the subjects received the metyrapone.

These findings agree well with two previously published studies on metyrapone-induced increases in serum 11-deoxycortisol. Seppo (17) reported an increase in serum 11-deoxycortisol to a mean of 137.1 ± 46 µg/L in 51 subjects 3 h after treatment with metyrapone. Spark (3) noted values of 168 ± 44 µg/L 4 h after metyrapone. Our serum 11-deoxycortisol values averaged 124 ± 42 µg/L 3 h after metyrapone.

None of the previous studies cited, however, described a defined protocol for isolation and purification of 11-deoxycortisol from serum by liquid column chromatography.

We found liquid chromatography on Sephadex LH-20 to work well as a purification step before radioimmunoassay. No studies to date have been published describing an antibody for 11-deoxycortisol free from cross reactivity with progesterone. Thus, chromatography is still an obligatory step when 11-deoxycortisol is determined in serum by radioimmunoassay.

Our approach to measurement of this steroid has the apparent advantage of fewer false-positives, a frequent problem encountered when solvent partitioning alone is used to prepare serum samples for 11-deoxycortisol measurements, and also increased sensitivity and specificity over previous methods for 11-deoxycortisol. This assay further improves the clinical reliability of the metyrapone challenge test commonly used to test for pituitary–adrenal abnormalities.

References