Adrenal Gland

Adrenal gland

Kidney

Chemical structure of Adrenal Hormone
The Adrenal Gland

Anatomy was first described in 1563.
Is located above (or attached to) the upper pole of the kidney.
Is pyramidal in structure and weighs about four grams.
Consists of the adrenal cortex and adrenal medulla
Activities are regulation of fluid volume and stress response
Adrenal Histology
Adrenal Cortex: Steroid Hormone Production

Figure 23-2: Synthesis pathways of steroid hormones
Investigation of adrenal function

- Requires knowledge of:
  - Biological rhythms
  - Concept of feedback controls

- Limitations of analytical techniques.
**Circadian rhythm in serum cortisol** Circadian rhythm in serum cortisol concentrations in two normal subjects. Blood samples were drawn every 20 to 30 minutes. The shaded areas indicate the hours of the day during which the lights were turned out. To convert serum cortisol values to nmol/L, multiply by 27.6. Data from Weitzman, ED, Fukushima, DK, Nogeire, C, et al, J Clin Endocrinol Metab. 1971; 33:14.
Control of Cortisol Secretion: Feedback Loops

- External stimuli
- Hypothalamic
- Anterior Pituitary
- Adrenal cortex
- Tissues

Figure 23-3: The control pathway for cortisol
Cushing’s Syndrome
Clinical Features

• **Skin**
  – Thin Skin
  – Hirsutes
  – Acne
  – Striae
  – Bruising

• **Cardio-vascular**
  – Hypertension

• **Psychiatric**
  – Depression

• **Musculoskeletal**
  – Moonface
  – Buffalo hump
  – Truncal obesity
  – Thin Limbs
  – Proximal weakness

• **Metabolic**
  – Hyperglycaemia
  – Osteoporosis
  – Hypo-kalaemia
Urinary Free Cortisol:

- 5-10% of plasma cortisol is non protein bound
- Any increase of cortisol rapidly saturates the remaining protein binding sites and thus increase free cortisol
- 24 hour collection to give an integrated result
- Reference values vary with assay:
  - <75 µg/24 hours (UZ Leuven)
- Assays are cumbersome, often involving extraction's.
Urinary Free Cortisol:

- variability of normal ranges
- inaccurate urine collections
- 3 or more determinations needed
- Creatinine to assess adequacy
- No value if renal function is seriously impaired
- Sensitivity 95%-100%
- Specificity 94%-98%
Low dose dexamethasone suppression test

- Procedure:
  - 1mg dexamethasone 23.00-24.00h
  - Serum cortisol 08.00h following day.
  - Cortisol should = <5 µg/dl

- Interpretation: -
  - Cortisol should = < 5 µg/dl
  - (method dependant? 3.6 µg/dl ?)

- Requirement to measure dexamethasone (>5.6 nmol/L)
- Why dexta? Does not interfere!
Low dose dexamethasone suppression test.

- Drug effects = false positive:
  - Oestrogen & tamoxifen due to effect on Cortisol Binding Globulin.
  - Nasal decongestants and oral contraceptives
- Obesity = false positives
- Alcohol = false positives
- Depressive illness = 30 - 50% false positives
Two-day dexamethasone suppression test:

- 0.5 mg dexamethasone 6 hourly for 48 hours
- Serum cortisol 08.00h day 3 or 24 hour Urinary free cortisol.
- Failure to suppress < 5μg/dl indicates Cushing.
  Specificity = 97-100% versus 87.5% for overnight procedure
  • Higher dextro dose of 8 mg per day suppresses cortisol in Cushing disease (where the axis is only moderately insensitive to feedback inhibition)
  • High dextro dose does NOT suppress cortisol in ectopic ACTH syndrome or adrenal tumor
Dexamethasone-CRH test

- 0.5 mg dexamethasone 6 hourly for 48 hours
- CRH (1 µg/kg body weight IV after last dose)
- eventually measure dexamethasone
- Serum cortisol > 1.4 µg/dl supports diagnosis of Cushing syndrome

- Cumbersome
- Requires sensitive cortisol assay
- Exploits high dexam suppression AND low CRH response in the pseudo Cushing group
Diurnal variation

– Serum Cortisol at 8:00 and 20:00.
  • Avoid stress.

– Normal:-
  • 08.00h - 10.00h    10 - 22 µg/dl
  • 20.00h - 24.00h    < 10 µg/dl

  or 50% of 08.00h value

  Check local reference values

– Cushings:-
  • Loss of diurnal variation.
Diurnal variation

• Single morning or evening value hard to interpret
• Minimum 2 samples per 24 h
• Cortisol day profile: every 4 hours
• Single midnight value during sleep  
  – High capability to exclude Cushing  
  – Impractical in ambumulatory setting  
  – (some advocate salivary cortisol)
Pituitary v Ectopic ACTH

• Petrosal sinus sampling:
  • Ratio Petrosal/peripheral ACTH >3:1 if pituitary.
  • Lateralisation of microadenoma:
  • CRH can be used to improve
    – the gradient
    – the lateralisation.
<table>
<thead>
<tr>
<th>Tijdstip</th>
<th>IPS rechts</th>
<th>IPS links</th>
<th>perifere vene</th>
</tr>
</thead>
<tbody>
<tr>
<td>basaal 1</td>
<td>275.4</td>
<td>40.8</td>
<td>30.3</td>
</tr>
<tr>
<td>basaal 2 (3 minuten later)</td>
<td>302.3</td>
<td>43.9</td>
<td>27.6</td>
</tr>
<tr>
<td>basaal 3 (3 minuten later)</td>
<td>232.9</td>
<td>33.7</td>
<td>31.1</td>
</tr>
<tr>
<td>Injectie CRH 1 µg/kg IV op tijdstip 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 minuten post CRH</td>
<td>1211.1</td>
<td>40.1</td>
<td>50</td>
</tr>
<tr>
<td>6 minuten post CRH</td>
<td>1350.0</td>
<td>72.4</td>
<td>54.9</td>
</tr>
<tr>
<td>9 minuten post CRH</td>
<td>793.5</td>
<td>79.8</td>
<td>70.0</td>
</tr>
</tbody>
</table>

Besluit:
1. Basale ratio IPS/perifeer > 7.5 (rechts) en < 2 (links)
2. Post CRH ratio IPS/perifeer > 11 (rechts) en < 3 (links)
3. Ratio IPS rechts/IPS links > 6
Differential Diagnosis of Cushing’s Syndrome

• Imaging:
  – Adrenal CT: Adenoma, carcinoma
  – Pituitary CT/MRI: Adenoma
  – Lung CT: Small cell carcinoma
Adrenal Failure
Clinical Features

• Early:
  – Anorexia, lethargy & weakness.

• Skin:
  – Hyperpigmentation
    • Sun exposed areas
    • Buccal cavity
    • Scars

• Cardiovascular:
  – Hypotension

• Gastrointestinal:
  – Nausea & vomiting

• Acute:
  – Addisonian Crisis:
    • Post surgery/trauma
    • Infection
  – Hypotension, nausea, vomiting, weakness, hypovolaemic shock

• Autoimmune Disease:
  – thyroid, ovary, pancreas
Adrenal Failure. Endocrine Investigation.

• Random and “normal” 08.00h serum cortisol may be misleading.
  – Normal 10-20 µg/dL (sens 62% - spec 77%)
  – <3 µg/dL is suspect for insufficiency

• Urine cortisol assay has no place in the diagnosis of adrenal failure
  – Low normal values hard to establish
Adrenal Failure.
Endocrine Investigation.

• Establish Deficiency: -
  – Short Synacthen test: needed in all clinically suspect
  – Can be performed at any time of day.
  – Non-stressed patient
  – 250 µg “high dose” tetracosactrin (1-24 ACTH)i.m. (equivalent to 1 µg).
  – Blood for cortisol at 0, 30, and 60 mins.

• Interpretation:-
  – Serum cortisol should rise by 7-10 µg/dl to a concentration of > 17-22.5 µg/dl (no consensus)
Adrenal Failure.
Endocrine Investigation.

- **Low dose ACTH test**

- **Insulin Tolerance Test**
  - Hypoglycemia = stress situation
  - $< 5 \, \mu g/dl$
  - Gold standard
Case 1

- Thin, anxious 64 y old male
- hypotension

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Serum Cort.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10-20 µg/dl at 8:00)</td>
</tr>
<tr>
<td>0</td>
<td>&gt;60</td>
</tr>
<tr>
<td></td>
<td>250 µg Synacthen</td>
</tr>
<tr>
<td>30</td>
<td>&gt;60</td>
</tr>
<tr>
<td>60</td>
<td>&gt;60</td>
</tr>
</tbody>
</table>
Case 1

• grossly elevated levels of cortisol
• rules out adrenal insufficiency
• general stress of the patient's illness

Diagnosis: Severe stress
Case 2

- 34 year old female
- insulin-dependent diabetes
- thyroid nodules
- ACTH test (autoimmune process)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Serum Cort. (10-25 μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>250 μg Synacthen</td>
</tr>
<tr>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>
Case 2

- post-stimulation samples exceeds an absolute value of 20 µg/dl
- Cortisol rose above the basal level by more than 7 µg/dl

Diagnosis: Normal adrenal function
Case 3

- 68 year old male
- thyroid hormone replacement therapy for primary hypothyroidism
- admitted for investigation of extreme tiredness

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Serum Cort. µg/d1</th>
<th>Urine Cort. µg/24u</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td>30</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

250 ug Synacthen
Case 3

- Cortisol levels fail to exceed the basal level by 7 µg/dl
- Cortisol levels fail to surpass a value of 20 µg/dl in either of the post-stimulation samples

Diagnosis: adrenal insufficiency

PS: urinary cortisol estimation was valueless, as was the basal serum cortisol, emphasizing the need to perform a Stimulation test
Case 4

- 39 year old female
- 4 days of vomiting
- history of tiredness, poor appetite, salt craving and dizziness over the previous 4 months
- marked pigmentation in all exposed areas and palmar grooves
Case 4

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Na</td>
<td>115</td>
<td>mmol/l</td>
<td>(132-144)</td>
</tr>
<tr>
<td>K</td>
<td>7.2</td>
<td>mmol/l</td>
<td>(3.1-4.8)</td>
</tr>
<tr>
<td>Serum Cort.</td>
<td>0.9</td>
<td>µg/dl</td>
<td>(10-20)</td>
</tr>
</tbody>
</table>

- Synachthen test: 0.8  3.5  4.5

→ Adrenal failure with loss of both aldosterone and cortisol secretion
Case 6

- 10 year old girl
- investigated for obesity

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>16.00</th>
<th>20.00</th>
<th>24.00</th>
<th>04.00</th>
<th>08.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Cort.</td>
<td>13</td>
<td>2.1</td>
<td>1.0</td>
<td>5.8</td>
<td>21</td>
</tr>
</tbody>
</table>
Case 6

- normal circadian rhythm
- midnight plasma cortisol: 1.2 µg/dl
  - absolute value of the midnight sample is a better test
  - also raised in alcoholic patients
  - acutely ill (severe stress)
  - depression
Case 7

- obese 25 year old female
- amenorrhoea
- red patchy facial skin
- overnight dexamethasone suppression:
  - 1 mg dexamethasone at 23 u
  - cortisol at 8:00 = < 1 µg/dl
Case 7

- Excludes Cushing syndrome
- False positive dexamethasone suppression:
  - Severe stress—acutely ill patients.
  - Endogenous depression, obesity, alcoholism
  - Oestrogen therapy—excess oestrogen stimulates CBG
  - Phenytoin therapy—can enhance the hepatic metabolism of dexamethasone
Case 8

- 31 year old male
- muscle weakness in his left leg
- 'mooning of the face' and raised blood pressure
- headaches and nausea
- poor healing of damaged skin
- visual field disturbances
Case 8

- Urine cortisol: 500 µg/24h
- Serum cortisol na dexta: 37 µg/dl
- Prolonged dexta suppression test

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
<th>Serum Cort. (µg/dl)</th>
<th>Urine Cort. (µg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19/05</td>
<td>08.30</td>
<td>29</td>
<td>470</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 mg/6 h dexamethasone</td>
<td></td>
</tr>
<tr>
<td>20/05</td>
<td>08.00</td>
<td>25</td>
<td>427</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 mg/6 h</td>
<td></td>
</tr>
<tr>
<td>21/05</td>
<td>08.15</td>
<td>35</td>
<td>450</td>
</tr>
</tbody>
</table>
Pituitary tumour

ACTH

Bilateral adrenal hyperplasia
Case 9

- 32 year old female
- marked hirsutism
- weight gain and a 3 month history of amenorrhoea
- Testosteron: 244 ng/dl (15-45)
- DHEAS: 441µg/dl (80-350)
- Urine cortisol: 630 µg/24u (< 75)
## Case 9

**prolonged suppression test:**

<table>
<thead>
<tr>
<th>Date</th>
<th>Time (h)</th>
<th>Plasma Cort. (µg/d1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13/01</td>
<td>08.30</td>
<td>0.5 mg/6 h dexamethasone</td>
</tr>
<tr>
<td>14/01</td>
<td>08.30</td>
<td>30</td>
</tr>
<tr>
<td>15/01</td>
<td>08.30</td>
<td>0.5 mg/6 h dexamethasone</td>
</tr>
<tr>
<td>16/01</td>
<td>08.15</td>
<td>2.0 mg/6 h dexamethasone</td>
</tr>
<tr>
<td>17/01</td>
<td>08.30</td>
<td>2.0 mg/6 h dexamethasone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.2</td>
</tr>
</tbody>
</table>
Case 9

Both low (2 mg/day) and high (8 mg/day) doses of dexamethasone failed to suppress the level of cortisol, suggesting the presence of either an ectopic ACTH-secreting tumour or an adrenal tumour.
Case 10

- 72 year old female
- epigastric pain and abdominal distension
- hepatomegaly
- oedema
- widespread skin pigmentation
- blood pressure of 180/90.
Case 10

- Na 142 mmol/l (132-144)
- K 2.1 mmol/l (3.1-4.8)
- Glucose 290 mg/dl
- Cortisol > 60 µg/dl
- ACTH 450 pg/ml
Case 10

- High Cort. — high mineralocorticoid activity — Na+ retention — water retention — hypertension
- K+ loss — hypokalemia
- high glucocorticoid activity — anti-insulin effect — hyperglycaemia
11β,17α, 21-Trihydroxy-4-pregnene-3,20-dione
Cortisol in blood

- 10 % free
- 90 % bound to proteins
  - CBG (high affinity)
  - Albumin (low affinity, high capacity)
Cortisol metabolism

- Reduction yields tetrahydrocompounds
- Conjugation yields glucurononides and sulfates (water soluble)
- Metabolites have no biological activity
- In urine
  - 1 % free cortisol
  - 95% glucurononides
  - 4 % sulfates
Cortisol metabolism

- When binding capacity of CBG is exceeded, the amount of free cortisol in serum and urine increases.
- Fenytoin, primidone, rifampicine and fenobarbital induce cortisol degrading enzymes.
Cortisol methods: colorimetric Porter-Silber and Zimmerman reaction
Cortisol assays: ligand assays

- Competitive protein binding
  - CBG used as binder
- Radio-immunoassay and Enzyme-immunoassay
  - Remove binding proteins (ANS, salycilate, pH, heat)
  - Eventually: extraction with dichlormethane
  - Over-estimates cortisol due to cross-reaction (with cortisone and others)
Cortisol assays: chromatographic methods

• HPLC
  – Reversed phase column
  – UV detection or MS detector

• Gas chromatography
  – Derivatisation required
  – MS detector
  – Reference method
Cortisol assays: UZ Leuven

• Serum:  **Bayer Immuno1** (500-700 per month)
  – Good analytical quality (UK NEQAS)
  – Disappeared in 2005

• Urine:  **Immunotech RIA** (180 – 220 per month)
  – Most specific cortisol assay
  – Good performance; suitable for urine

• In development: **LC/MS/MS**
Immuno 1 versus Elecsys

P/B Regression

\[ Y = 1.044 \times X - 0.075 \]

\( N = 60, \ r = 0.391 \)
Immuno 1 vs Elecsys (2 outliers)

P/B Regression

\[ Y = 1.032 \times X - 0.007 \]

N = 58, r = 0.970
Reproducibility was determined using Elecsys reagents, pooled human sera, and controls in accordance with a modified protocol (EP5-A) of the NCCLS (National Committee for Clinical Laboratory Standards): 6 times daily for 10 days (n = 60); within-run precision on E170, n = 21. The following results were obtained:

<table>
<thead>
<tr>
<th>Elecsys 2010 Sample</th>
<th>Within-run precision</th>
<th>Total precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean nmol/L</td>
<td>Mean µg/dL</td>
</tr>
<tr>
<td>HS&lt;sup&gt;c&lt;/sup&gt; 1</td>
<td>208</td>
<td>7.53</td>
</tr>
<tr>
<td>HS 2</td>
<td>561</td>
<td>20.3</td>
</tr>
<tr>
<td>HS 3</td>
<td>1268</td>
<td>46.0</td>
</tr>
<tr>
<td>PC U&lt;sup&gt;d&lt;/sup&gt; 1</td>
<td>363</td>
<td>13.2</td>
</tr>
<tr>
<td>PC U 2</td>
<td>865</td>
<td>31.4</td>
</tr>
</tbody>
</table>

c) HS = Human serum  
d) PC U = PreciControl Universal

Excellent precision
Analytical sensitivity (lower detection limit)
< 1.00 nmol/L (< 0.036 μg/dL)
The detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying two standard deviations above that of the lowest standard (master calibrator, standard 1 + 2 SD, within-run precision, n = 21).

Functional sensitivity
< 8.0 nmol/L (< 0.29 μg/dL)
The functional sensitivity is the lowest analyte concentration that can be reproducibly measured with a between-run coefficient of variation of 20%.
Analytical specificity
For the antibody derivate used, the following cross-reactivities (%) were found:

a) substance added per 10 μg/mL:
corticosterone 5.8
cortisol-21-sulfate 0.04
cortisone 0.30
11-deoxycorticosterone 0.69
11-deoxycortisol 4.1
dexamethasone 0.08
17-α-hydroxyprogesterone 1.50
prednisone 0.28
progesterone 0.35

b) substance added per 1 μg/mL:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-deoxycortisol</td>
<td>45.4</td>
</tr>
<tr>
<td>6-β-hydroxycortisol</td>
<td>158</td>
</tr>
</tbody>
</table>

(c) substance added per 0.1 μg/mL:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>allotetrahydrocortisol</td>
<td>165</td>
</tr>
<tr>
<td>prednisolone</td>
<td>171</td>
</tr>
<tr>
<td>6-α-methylprednisolone</td>
<td>389</td>
</tr>
</tbody>
</table>
Immuno 1 versus Centaur

**GRAFIEKEN Lineaire regressie (orthogonaal)**

A. Regressielijnen

![Graph showing cortisol levels for Immuno 1 and Centaur](image)

**A. Regressie vergelijking**

\[ Y(\mu g/dL) = a(\mu g/dL) + \frac{b(\mu g/dL)}{X(\mu g/dL)} \]

<table>
<thead>
<tr>
<th>Est</th>
<th>S_{Est}</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>60</td>
</tr>
<tr>
<td>a</td>
<td>-1.788</td>
</tr>
<tr>
<td>b</td>
<td>1.450</td>
</tr>
<tr>
<td>r^2</td>
<td>0.8915</td>
</tr>
</tbody>
</table>
**Precision**

Five samples were assayed 6 times, in each of 24 runs, on 6 systems, \((n = 144\) for each sample), over a period of 2 days. The following results were obtained:

<table>
<thead>
<tr>
<th>Mean ((\mu g/dL))</th>
<th>Mean ((nmol/L))</th>
<th>Within-run % CV</th>
<th>Run-to-run % CV</th>
<th>Total % CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.88</td>
<td>107.05</td>
<td>3.69</td>
<td>5.45</td>
<td>6.58</td>
</tr>
<tr>
<td>5.63</td>
<td>155.33</td>
<td>3.09</td>
<td>3.83</td>
<td>4.92</td>
</tr>
<tr>
<td>14.17</td>
<td>390.95</td>
<td>2.89</td>
<td>3.07</td>
<td>4.22</td>
</tr>
<tr>
<td>27.53</td>
<td>759.55</td>
<td>3.82</td>
<td>1.86</td>
<td>4.25</td>
</tr>
<tr>
<td>37.15</td>
<td>1024.97</td>
<td>2.98</td>
<td>3.99</td>
<td>4.98</td>
</tr>
</tbody>
</table>
Sensitivity and Assay Range

The ADVIA Centaur Cortisol assay measures serum cortisol concentrations up to 75 μg/dL (2069 nmol/L) with a minimum detectable concentration (analytical sensitivity) of 0.20 μg/dL (5.5 nmol/L). Analytical sensitivity is defined as the concentration of cortisol that corresponds to the RLUs that are two standard deviations less than the mean RLUs of 20 replicate determinations of the Cortisol zero standard.
<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross-reactivity</th>
<th>Compound</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous steroids</strong></td>
<td></td>
<td><strong>Endogenous steroids</strong></td>
<td></td>
</tr>
<tr>
<td>Aldosterone</td>
<td>ND*</td>
<td>11β-hydroxyprogesterone</td>
<td>0.50</td>
</tr>
<tr>
<td>Allotetrahydrocortisol</td>
<td>4.60</td>
<td>17α-hydroxyprogesterone</td>
<td>0.50</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>ND</td>
<td>17β-hydroxypregnanolone</td>
<td>ND</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>2.80</td>
<td>11-keto-androsterone</td>
<td>ND</td>
</tr>
<tr>
<td>Cortisone</td>
<td>7.40</td>
<td>11-keto-etiocholanolone</td>
<td>ND</td>
</tr>
<tr>
<td>α-cortol</td>
<td>ND</td>
<td>Pregnanetriol</td>
<td>ND</td>
</tr>
<tr>
<td>α-cortolone</td>
<td>ND</td>
<td>Pregnenolone</td>
<td>ND</td>
</tr>
<tr>
<td>β-cortol</td>
<td>ND</td>
<td>Progesterone</td>
<td>ND</td>
</tr>
<tr>
<td>β-cortolone</td>
<td>ND</td>
<td>Spironolactone</td>
<td>ND</td>
</tr>
<tr>
<td>Dehydrocorticosterone</td>
<td>0.50</td>
<td>Testosterone</td>
<td>ND</td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
<td>0.40</td>
<td>Tetrahydrocortisol</td>
<td>ND</td>
</tr>
<tr>
<td>11-deoxycortisol</td>
<td>7.30</td>
<td>Tetrahydrocortisone</td>
<td>0.50</td>
</tr>
<tr>
<td>21-deoxycortisol</td>
<td>4.50</td>
<td>Tetrahydro-11-deoxycortisol</td>
<td>0.20</td>
</tr>
<tr>
<td>20α-dihydrocortisol</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20β-dihydrocortisol</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20α-dihydrocortisone</td>
<td>0.40</td>
<td>Prednisolone (100 µg/dL)</td>
<td>27.00</td>
</tr>
<tr>
<td>20β-dihydrocortisone</td>
<td>ND</td>
<td>6-methyl-prednisolone</td>
<td>20.90</td>
</tr>
<tr>
<td>11β-hydroxyandrostosterone</td>
<td>ND</td>
<td>Dexamethasone</td>
<td>0.20</td>
</tr>
<tr>
<td>6β-hydroxycortisol</td>
<td>2.40</td>
<td>Prednisone</td>
<td>6.60</td>
</tr>
<tr>
<td>11β-hydroxyetiocholanolone</td>
<td>ND</td>
<td>Canrenone</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND = Not Detectable
The expected results for the ACS:180® Cortisol assay were previously established. Data was obtained on serum samples from 249 apparently healthy individuals. Based on a central 95% interval, the following reference ranges were established:

<table>
<thead>
<tr>
<th>Sample Category</th>
<th>N</th>
<th>Cortisol Range (μg/dL)</th>
<th>Cortisol Range (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.m. serum (7–9 a.m.)</td>
<td>125</td>
<td>4.30–22.40</td>
<td>118.6–618.0</td>
</tr>
<tr>
<td>p.m. serum (3–5 p.m.)</td>
<td>124</td>
<td>3.09–16.66</td>
<td>85.30–459.6</td>
</tr>
</tbody>
</table>

A study was performed on 116 direct urine samples from apparently healthy males and females. Based on this population, the 95% reference interval for these samples is shown below:

<table>
<thead>
<tr>
<th>Sample Category</th>
<th>N</th>
<th>Cortisol Range (μg/24 hours)</th>
<th>Cortisol Range (nmol/24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>direct urine</td>
<td>116</td>
<td>28.5–213.7</td>
<td>78.6–589.6</td>
</tr>
</tbody>
</table>

These results were confirmed for the ADVIA Centaur Cortisol assay by analyzing 129 direct urine samples in the range of 12.68 to 263.17 μg/24 hours (349.84 to 7260.72 nmol/24 hours). Refer to Method Comparison.
Immuno 1 versus Access

Correlatie Cortisol Immuno 1 - Unicel DxI 800

\[ y = 0.8354x + 4.7208 \]

\[ R^2 = 0.8073 \]
Imprecision
This assay exhibits total imprecision of less than 12% at approximately 5 μg/dL (138 nmol/L) and less than 10% for higher concentrations of cortisol. Using commercially available human serum based control material, 20 assays with three replicates per assay were generated to provide the following data on precision. The data were analyzed via analysis of variance (ANOVA) \(^{17,18}\):

<table>
<thead>
<tr>
<th>Sample</th>
<th>Grand Mean (n=60) (μg/dL)</th>
<th>Within Run (% CV)</th>
<th>Total Imprecision (% CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>6.0</td>
<td>6.7</td>
<td>7.9</td>
</tr>
<tr>
<td>Level 2</td>
<td>24.1</td>
<td>4.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Level 3</td>
<td>38.4</td>
<td>4.4</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Analytical Sensitivity
The lowest detectable level of cortisol distinguishable from zero (Access Cortisol Calibrator S0) with 95% confidence is 0.4 μg/dL (11 nmol/L). This value is determined by processing a complete six point calibration curve, controls, and 10 replicates of the zero calibrator in multiple assays. The analytical sensitivity value is interpolated from the curve at the point that is two standard deviations from the mean measured zero calibrator signal.
<table>
<thead>
<tr>
<th>Substance</th>
<th>Analyte Added (µg/dL)</th>
<th>Cross-Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>100</td>
<td>4.7</td>
</tr>
<tr>
<td>Cortisone</td>
<td>100</td>
<td>14.5</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>1000</td>
<td>1.4</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>100</td>
<td>21.6</td>
</tr>
<tr>
<td>17-α Hydroxyprogesterone</td>
<td>1000</td>
<td>1.9</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1000</td>
<td>0.5</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>20</td>
<td>40.3</td>
</tr>
<tr>
<td>Tetrahydrocortisone</td>
<td>1000</td>
<td>0.6</td>
</tr>
<tr>
<td>Prednisone</td>
<td>1000</td>
<td>2.8</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1000</td>
<td>0.3</td>
</tr>
</tbody>
</table>
**Axsym cortisol**

## Product Information

### Precision

<table>
<thead>
<tr>
<th>Panel</th>
<th>Mean Conc. (µg/dL)</th>
<th>Total % CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5 - 5.0</td>
<td>12.8 - 15.0</td>
</tr>
<tr>
<td>2</td>
<td>14.9 - 15.2</td>
<td>6.4 - 6.6</td>
</tr>
<tr>
<td>3</td>
<td>38.1 - 40.9</td>
<td>5.7 - 6.5</td>
</tr>
</tbody>
</table>

### Assay Range (µg/dL)

≤ 1.1 - 60.0

### Sensitivity (µg/dL)

≤ 1.1

### Throughput

Up to 57 tests per hour

### Time to First Result

12 minutes

### Reagents, Calibrators and Controls

**Ready to Use**

### Calibrators (µg/dL)

0.0, 2.5, 5.0, 10.0, 25.0, 60.0

### Calibration Curve Stability

Typically 2 weeks

### Reagent Stability Onboard

336 cumulative hours onboard

### Method

Fluorescence Polarization Immunoassay (FPIA)

### Sample Type

Serum, Plasma, Urine

### Expected Normal Values (95% confidence limit)

- Serum morning (7:00 - 9:00 a.m.): 4.2 - 38.4 µg/dL
- Serum afternoon (4:00 - 6:00 p.m.): 1.7 - 16.6 µg/dL
- Urine: 32 - 243 µg/24 hours
Immuno 1 versus Immunotech RIA

P/B Regression

\[ Y = 1.120 \times X + 1.480 \]

N = 60, \ r = 0.955
### Specificity

Cross-reactivity with 44 steroids was tested, and mainly:

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-deoxycortisol</td>
<td>18 %</td>
</tr>
<tr>
<td>21-deoxycortisol</td>
<td>7.5 %</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>6 %</td>
</tr>
<tr>
<td>Dihydrocortisol</td>
<td>2.4 %</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.8 %</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>1.1 %</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>8.4 %</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>7.3 %</td>
</tr>
<tr>
<td>17α-hydroxyprogesterone</td>
<td>3.5 %</td>
</tr>
<tr>
<td>5α-dihydrocortisone</td>
<td>2.3 %</td>
</tr>
<tr>
<td>Cortisone</td>
<td>1.5 %</td>
</tr>
<tr>
<td>Allotetrahydrocortisone</td>
<td>0.8 %</td>
</tr>
</tbody>
</table>

With most of the other 32, cross-reactivity was less than 0.1 %.
# Improved Specificity of a New Direct Assay for Urinary Cortisol: Application in Corticoid Treated Patients

Rémy Sapin¹, Jean Louis Schlienger², Françoise Gasser³, Alain Pradigues² and Daniel Grucker¹

## Table 2: Urinary free cortisol (nmol/24h) in the different patient groups: ex cortisolic controls, controls before and after Synacthen stimulation and patients treated with prednisone.

<table>
<thead>
<tr>
<th>Group</th>
<th>DCA°</th>
<th>ECA°</th>
<th>Dim♭</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=35)</td>
<td>387</td>
<td>127</td>
<td>108</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>387</td>
<td>127</td>
<td>108</td>
</tr>
<tr>
<td>Min-Max</td>
<td>171–654</td>
<td>39–249</td>
<td>45–228</td>
</tr>
<tr>
<td>2.5–97.5 percentiles</td>
<td>201–648</td>
<td>55–196</td>
<td>47–188</td>
</tr>
<tr>
<td>Synacthen stimulation (n=7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before test median</td>
<td>309</td>
<td>113</td>
<td>83</td>
</tr>
<tr>
<td>After test median</td>
<td>3590</td>
<td>1915</td>
<td>1796</td>
</tr>
<tr>
<td>Median difference</td>
<td>3288</td>
<td>1851</td>
<td>1752</td>
</tr>
<tr>
<td>Patients treated with prednisone (n=14)</td>
<td>1559</td>
<td>1004</td>
<td>59</td>
</tr>
</tbody>
</table>

° DCA: direct INCSTAR assay  
♭ ECA: INCSTAR assay after methylene chloride extraction 

Clinical Assays direct
Clinical Assays MeCl
Immunotech direct
Performance characteristics of five automated serum cortisol immunoassays

Richard F. Roberts\textsuperscript{a,\*} and William L. Roberts\textsuperscript{b}

\textsuperscript{a} ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT 84103, USA
\textsuperscript{b} Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, UT 84132, USA

Received 26 August 2003; received in revised form 19 January 2004; accepted 19 January 2004

The reason for the two discordant results that were unique to the Elecsys 2010 method is unknown. It is most likely due to a cross-reacting substance(s) in the subjects’ serum. This substance(s) could be either an endogenous or exogenous steroid. Additional information about these samples including age, gender, diagnosis, and medications is not available. When one considers endogenous substances, it is known that patients with 21-hydroxylase deficiency can exhibit elevated 21-deoxycortisol concentrations [10]. A review of assay package inserts revealed that the Elecsys 2010 cortisol method demonstrates 45.4% cross-reactivity with 21-deoxycortisol compared to the Advia Centaur method which only shows 4.5% cross-reactivity with this endogenous steroid (Table 3). Unfortunately, cross-reactivity data for the Access, AxSYM, and IMMULITE 2000 methods were lacking for this compound. Previous work
Specificity of 7 cortisol assays  
(own data on 10 exogenous steroids)

<table>
<thead>
<tr>
<th>% CROSS-REACTIVITY</th>
<th>Immuno 1</th>
<th>Immunotech RIA</th>
<th>Elecsys E 170</th>
<th>Access Dxi</th>
<th>Centaur</th>
<th>DPC Immulite</th>
<th>DPC RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone</td>
<td>3.4</td>
<td>1</td>
<td>0.5</td>
<td>7.8</td>
<td>26.5</td>
<td>6.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Fludrocortisone</td>
<td>1</td>
<td>27.5</td>
<td>6.8</td>
<td>8.3</td>
<td>5.4</td>
<td>&lt; 0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>1</td>
<td>0.2</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>0.7</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>7.8</td>
<td>0.7</td>
<td>125.4</td>
<td>5</td>
<td>18.6</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>Cortisone</td>
<td>2.2</td>
<td>3.7</td>
<td>0.4</td>
<td>10.4</td>
<td>28</td>
<td>1</td>
<td>6.8</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>55.8</td>
<td>11.2</td>
<td>68.1</td>
<td>43.8</td>
<td>31.5</td>
<td>62</td>
<td>67.8</td>
</tr>
<tr>
<td>Bethamethasone</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>20-alfa-dihydrocortisone</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>0.5</td>
<td>1</td>
<td>1.2</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Flumethasone</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>
INTERFERENCE OF EXOGENOUS STEROIDS IN CORTISOL IMMUNO-ASSAYS: AN UNDERESTIMATED PROBLEM?

**Conclusion:** Cross reactivity with exogenous steroids is a serious source of interference in cortisol assays. Moreover, these steroids are converted in vivo into cortisol and prednisolone. Prednisolone (Deltacortril®) cross reacts in all tested cortisol assays. Methylprednisolone (Medrol®) strongly affects cortisol measurements in the Roche Elecsys assay. The Immunotech RIA is the most specific assay. Triamcinolone, dexamethasone and bethamethasone don’t interfere in any of the tested assays.
Serum free cortisol

• Free fraction is the biologically active fraction
• Regulates the feedback mechanism

• Methods
  – Direct measurement (equilibrium dialysis)
  – Free cortisol index
  – Mathematical modeling
Mathematical modeling

• Method used in GHB
• Calculation of [Free Cortisol] using [Total Cortisol] and [Cortisol Binding Globulin] ‘law of mass action’
• Rapid, simple and convenient
Measurements of Serum Free Cortisol in Critically Ill Patients

Amir H. Hamrahian, M.D., Tawakalitu S. Oseni, M.D., and Baha M. Arafah, M.D.

**Conclusions** During critical illness, glucocorticoid secretion markedly increases, but the increase is not discernible when only the serum total cortisol concentration is measured.

In this study, nearly 40 percent of critically ill patients with hypoproteinemia had subnormal serum total cortisol concentrations, even though their adrenal function was normal.

Measuring serum free cortisol concentrations in critically ill patients with hypoproteinemia may help prevent the unnecessary use of glucocorticoid therapy.
Urine

- Immuno 1 + chromatography versus RIA
- HPLC versus RIA
- LC/MS/MS versus RIA
Oasis HLB solid phase extraction

- Condition with 1 ml methanol and 1 ml water
- Add 3ml of urine to the column and aspirate
- Rinse with 3 ml DMSO/water (60/40)
- Rinse with 3 ml methanol/water 35/65 with 1% HCl 6N
- Rinse with 3 ml methanol/water 35/65 with NH3 25%
- Rinse with 3 ml methanol/water 40/60
- Rinse twice with ethyl-acetate/n-heptane 10/90
- Elute with 2.5 ml ethyl-acetate/n-heptane 60/40
The graph shows a regression line with the equation $y = 1.0097x + 3.5334$. The identity line is also plotted as $Y = X$. The x-axis represents Immuno I 24u (na chrom zuivering), and the y-axis represents Immunotech 24u.
$y = 1.21x + 10.3$
LC/MS/MS

Waters Alliance 2795 HDVC gekoppeld aan de Micromass quattro micro API massa spectrometer
Een extractie uitvoeren op urinestalen heeft zowel zijn voor- als nadelen. Een extractie zal de gevoeligheid verhogen maar het vergt tijd, werk en dus kosten.

**LC/MS/MS: extractie**
Omgekeerde fase kolommen met een partikelgrootte van 1.5-4 µm, een ID van 0.050-4.6 mm en een lengte van ongeveer 50 mm worden gebruikt. Solventen met een pH tussen 2.0 en 7.5 dienen aangewend te worden omdat een te hoge pH de silicapartikels ontbinden en een te lage pH hydrolyse veroorzaakt. Ook extreem hoge kolomtemperaturen beschadigen de silicapartikels.
Twee massaspectrometers werken in serie. In de eerste massaspectrometer (MS1) wordt cortisol geïoniseerd waardoor moederionen ontstaan die geselecteerd worden op basis van een m/z ratio. Deze ionen gaan vervolgens naar de fragmentatieregio (collision cell) waar dissociatie optreedt onder invloed van het fragmentatiegasp, nl. argon. Bij de botsingen tussen de moederionen en de gasmoleculen wordt kinetische energie van de ionen getransformeerd naar energie dat fragmentatie in dochterionen mogelijk maakt (collision induced decomposition). De dochterionen worden geanalyseerd in een tweede massaspectrometer (MS2)
LC/MS/MS: interne standaard

De IS en cortisol zullen beide blootgesteld worden aan dezelfde interferenties. Kwantificatie is gebaseerd op de ratio van de piekoppervlakte van de targetcomponent op die van de IS.
363.3 moederion

120.9 dochterion

367.2 Int Std
Urine: RIA versus LC/MS/MS

N = 38
Y = 2.60 X + 0.33

<table>
<thead>
<tr>
<th>INTRA-ASSAY</th>
<th>INTER ASSAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortisol µg/L</td>
<td>SD</td>
</tr>
<tr>
<td>15.56 (n = 21)</td>
<td>1.99</td>
</tr>
<tr>
<td>43.74 (n = 21)</td>
<td>5.46</td>
</tr>
<tr>
<td>174.30 (n = 17)</td>
<td>9.38</td>
</tr>
</tbody>
</table>
Cortisol in saliva: sample collection

• Timing of collection:
  – Thorough understanding of diurnal cycle
  – Collection at standardized times (23:00)

• Contamination issues:
  – No meals prior to sample (1 hour)
  – No alcohol during 1 day (interferes)
  – No dairy products (bovine hormones)
  – No acidic or high sugar foods (low pH)
  – No blood contamination!!!
Cortisol in saliva: sample collection

• Sample volume
  – Better use no stimulants
  – Chewing gum
  – Lemon drops, sugar

• Collection devices
  – Cryo-straws
  – Salivette
Fig. 1. Correlation of salivary cortisol measured by RIA (x axis) vs EIA (y axis) in samples with RIA results ≤ 10 nmol/L.

Regression statistics: slope = 1.2 (SE, 0.1); y-intercept = −0.3 (SE, 0.2) nmol/L; $r^2 = 0.93$; $F(1,75) = 1004.5$; $P < 0.001$; $S_{yy} = 1.0$ nmol/L; $n = 77$. 

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Children’s Hospital of Pittsburgh
Pittsburgh, PA 15213
Midnight salivary cortisol for the initial diagnosis of Cushing's syndrome of various causes.

Yaneva M, Mosnier-Pudar H, Dugue MA, Grabar S, Fulla Y, Bertagna X.

Service des Maladies Endocriniennes et Metaboliques, Centre Hospitalier d'Universite Cochin, Universite Paris 5-Rene Descartes, 75014 Paris, France.
**TABLE 1.** Characteristics of the studied inpatient groups (n = 117)

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 54)</th>
<th>Cushing’s syndrome (n = 63)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)(^2)</td>
<td>45.6 ± 13.8</td>
<td>39.2 ± 14.5</td>
<td>0.013</td>
</tr>
<tr>
<td>Gender, F/M (% F)</td>
<td>46/8 (85.2)</td>
<td>55/8 (87.3)</td>
<td>0.740</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>38.3 ± 8.2</td>
<td>28.4 ± 6.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>UFC (µg/d)(^2)</td>
<td>22.4 ± 14.3</td>
<td>632.8 ± 961.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Midnight salivary cortisol (ng/ml)(^2)</td>
<td>0.8 ± 0.6</td>
<td>12.3 ± 20.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Measuring midnight salivary cortisol is an easy and noninvasive means of diagnosing hypercortisolism.

Its diagnostic accuracy is identical to, if not better than, that of previously described gold standards.