Mass Spec Testing for Steroid Hormone Profiles: Making an Impact on Patient Care

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Mayo Clinic

Objectives

• Congenital Adrenal Hyperplasia (CAH)
• Sex Steroids
• Cushing’s

CAH
New Born Screening
Basics of MS Method

Lack of Standardization

$y = 2121.2x + 67.563$
$r^2 = 0.9759$

Figure 3
Correlation Between Two Sites

Table 4: Correlation between Steroid Analyses from Two Sites

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/DHEA</td>
<td>0.98</td>
<td>-123.6</td>
<td>0.98</td>
<td>0.96</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.01</td>
<td>-57.9</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>Estradiol</td>
<td>1.03</td>
<td>-45.2</td>
<td>0.98</td>
<td>0.96</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.99</td>
<td>-69.3</td>
<td>0.98</td>
<td>0.96</td>
</tr>
</tbody>
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Mayo = Mayo Clinic; DHEA = Dehydroepiandrosterone; E2 = Estradiol; Testosterone = Testosterone; Progesterone = Progesterone.

Available online at www.sciencedirect.com

Simultaneous determination of 12 steroids by isotope dilution liquid chromatography–photodiode array tandem mass spectrometry

Tielong Gu et al.

Steroid Analysis

RI兰 vs. LC-MS/MS

Correlation Between Two Sites

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Mayo = Mayo Clinic; DHEA = Dehydroepiandrosterone; E2 = Estradiol; Testosterone = Testosterone; Progesterone = Progesterone.
Bland Altman Plot (N=76)

Mean difference = 10.1

- 1 SD = 385.6

+ 1 SD = 405.8

- 2 SD = 781.4

+ 2 SD = 801.4

Average 17-OHPG Values 2.122*RIA and LC

Biosynthesis of Steroids

Monthly Test Volumes of Testosterone
Androgens in Health and Disease

- General well-being
  - Energy
  - Mood
- Libido and sexual satisfaction
- Bone physiology
- Muscle mass
- Hot flashes


Methodologies for Endocrine Analytes:

- RIA
- CIA
- ELISA
- FIA
- ICMA
- GC-FID
- LC-UV/EC
- GC-MS
- LC-MS
- LC-MS/MS

Low Male Testosterone (<60 ng/dL):
ACS-Enhanced vs. LC-MS/MS

\[ y = 0.8306x - 2.5493 \]
\[ R^2 = 0.8482 \]

Testosterone by Enhanced ACS (ng/dL)
Testosterone by LC-MS/MS (ng/dL)
Correlation between Immunoassays and Gold standard GC-MS method

GC-MS method is labor intensive and less sensitive compared to LC-MS/MS method

Quality of The T Lab Test

<table>
<thead>
<tr>
<th>Instrument</th>
<th>No.</th>
<th>Mean</th>
<th>S.D.</th>
<th>C.V.</th>
<th>Median</th>
<th>Low Value</th>
<th>High Value</th>
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<tr>
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<td>10</td>
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<td>37.1</td>
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<td>636</td>
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<td>1007</td>
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<td>920</td>
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<td>Vitros ECi 80</td>
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<td>890.3</td>
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<tr>
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<td>145.0</td>
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<td>334</td>
<td>56.7</td>
<td>590</td>
<td>336</td>
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</table>

Shcp Takings = 10000/Day with Coef. of Var. of 0.1 and 0.01
Laboratory proficiency testing should be based on the ability to measure accurately and precisely samples containing known concentrations of testosterone, not only on agreement with others using the same method.
Instrument Parts to TLX4 – MS/MS 4000

TLX4 (Liquid Chromatography-LC) MS/MS (Tandem Mass Spectrometer)

Computer

TX Inj. ports

Injector

LK Inj. ports

LC System 1

LC System 2

LC System 3

LC System 4

8 value VIM

SPE Disk Cartridge
Dynamic range of competitive immunoassays

Sex Hormone-Binding Globulin
- SHBG is the carrier protein for estrogen and testosterone
- SHBG-bound fraction is unavailable for biological activity
- Production regulated by estrogen-testosterone balance
- Estrogen stimulates SHBG production
- Testosterone decreases SHBG synthesis


Steroids with Mol. Wt. of 288.4
- 5, 7-ANDROSTADEN-3β, 17ß-DIOL
- 5α-ANDROSTAN-3, 16-DIONE
- 5β-ANDROSTAN-3, 17-DIONE
- 1, (5α)-ANDROSTEN-17ß-OL-3-ONE
- 2, (5α)-ANDROSTEN-11α-OL-17-ONE
- 4-ANDROSTEN-3α-OL-17-ONE
- 4-ANDROSTEN-3ß-OL-17-ONE
- 4-ANDROSTEN-17ß-OL-3-ONE
- 4-ANDROSTEN-17ß-OL-17-ONE
- 5-ANDROSTEN-3ß-OL-16-ONE
- 5-ANDROSTEN-3α-OL-17-ONE
- 5-ANDROSTEN-3ß-OL-17-ONE
- 5-ANDROSTEN-17α-OL-3-ONE
- 5-ANDROSTEN-17α-OL-17-ONE
- 9(11), (5α)-ANDROSTEN-3ß-OL-17-ONE
- 9(11), (5ß)-ANDROSTEN-3α-OL-17-ONE
**Compound** | **Amount Added** | **% Cross-reactivity**
--- | --- | ---
5α-dihydrotestosterone | 100 ng/mL | 5.4
Androsterone | 100 ng/mL | 0.94
Methyltestosterone | 100 ng/mL | 0.68
Estradiol-17β | 100 ng/mL | 0.02
Androstenedione | 1 µg/mL | < 0.1
Cortisol | 1 µg/mL | < 0.1
Corticosterone | 1 µg/mL | < 0.1
Cypionate | 100 ng/mL | < 0.1
Danazol | 1 µg/mL | < 0.1
DHEA-sulfate | 1 µg/mL | < 0.1
11-deoxycortisol | 1 µg/mL | < 0.1
Dexamethasone | 1 µg/mL | < 0.1
Estrone | 100 ng/mL | < 0.1
Oxymetholone | 100 ng/mL | < 0.1
Progesterone | 1 µg/mL | < 0.1

**Reference Ranges for Testosterone by LC-MS/MS from Two Different Endocrine Laboratories**

<table>
<thead>
<tr>
<th>N</th>
<th>Ts1</th>
<th>Ts2</th>
<th>Ts3</th>
<th>Ts4</th>
<th>Ts5</th>
<th>Reference Range (ng/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ts1</td>
<td>147</td>
<td>&lt;0.009 (31)</td>
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<td></td>
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<tr>
<td>Ts2</td>
<td>66</td>
<td>0.108-1.39 (4-39)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ts3</td>
<td>67</td>
<td>0.106-1.47 (4-39)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ts4</td>
<td>71</td>
<td>0.277-2.19 (8-62)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Ts5</td>
<td>125</td>
<td>0.038-1.37 (1.1-44)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ts1</td>
<td>148</td>
<td>&lt;0.067 (59)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ts2</td>
<td>94</td>
<td>0.089-2.38 (1.1-62)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ts3</td>
<td>60</td>
<td>0.085-2.97 (1.1-64)</td>
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<tr>
<td>Ts4</td>
<td>98</td>
<td>0.719-12.04 (2-348)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ts5</td>
<td>82</td>
<td>0.711-23.09 (2-573)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Inter-individual Variability**

Testosterone – Representative Sample

Testosterone – Comparison Curve – 1216 Samples

0 500 1000 1500 2000 2500
0 500 1000 1500 2000 2500

y = 0.9951x - 26.305
R² = 0.9879

Poor Inter-Laboratory Agreement of Testosterone Measurements
Biosynthesis of Steroids

Estrogens Undergo Extensive CYP Enzymatic Modifications

Limitations of Steroid Determination by Direct Immunoassay

Zhu BT. Lee AJ. NADPH-dependent metabolism of 17beta-estradiol and estrone to polar and nonpolar metabolites by human ovarian and breast cancer cell lines. Steroids. 70(4):225-44, 200

Limitations of Steroid Determination by Direct Immunoassay

J. Taieb, C. Benattar, A. S. Birr, and A. Lindenbaum
Impact of Drugs

![Diagram showing the circulation of testosterone and estrogen in the body]

- Approximately 30% of testosterone is free to albumin.
- Approximately 5-2% of testosterone is free.
- Approximately 85% of testosterone is bound to SHBG.
- Approximately 60% of estrogen is bound to albumin.
- Approximately 1.8% of estrogen is free.
- Approximately 35% of estrogen is bound to SHBG.

**Table 1:**

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Impact on Testosterone</th>
<th>Impact on Estrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug 1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Drug 2</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*Note: ++ indicates a significant impact, + indicates a minor impact.*

**References:**
- Simon JA. Fert Steril. 2002;77:S77-S82.

**Epilepsia,** 32(Suppl. 6):S60-S67, 1991
Anti-aromatase Compounds

Chromatographic Separation of Estrogens

Anastrozole vs. Placebo

Mauras N et al. JCEM 2008;93:823-831

©2008 by Endocrine Society
Potential Steroid-Based Molecules of Ion Pair 279.1/149.2

Biosynthesis of Steroids

Feedback Inhibition
Cushing's Syndrome

- "Cushing's Syndrome" refers to the symptoms and signs of hypercortisolism regardless of etiology
- "Cushing's Disease" is Cushing's Syndrome due to pituitary hypersecretion of ACTH.
Pseudo-Cushings Disease:

- Condition that mimics the biochemical laboratory results of Cushing's Syndrome
  - Stress
  - Morbid obesity
  - Marathon running
  - Alcoholism
  - Depression
Lab Tests & Cushing’s

- Urine Free Cortisol
- Plasma Cortisol
- Plasma Free Cortisol
- Midnight Plasma Cortisol
- Midnight Salivary Cortisol

Steroid Hormone

- Total: $S + S\text{-BG} + S\text{-A}$
- Bio-available: $S + S\text{-A}$
- Free: $S$
- Conjugates: $S\text{-G} + S\text{-S}$

Salivary Cortisol ng/dL

Normals: $n = 186$
Cushing’s: $n = 32$
Methods for Urine Cortisol Test:

- Immunoassay: Cortisol 24-108 µg/24 hrs
- HPLC-UV: Normal Cortisol 5-55 µg/24 hrs
- LC-MS: Cortisone 16-128 µg/24 hrs

Steroid interference in Immunoassay

<table>
<thead>
<tr>
<th>Substance Added</th>
<th>Amount (ng/mL)</th>
<th>Reactivity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>250</td>
<td>0.30</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>10000</td>
<td>0.91</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>10000</td>
<td>5.33</td>
</tr>
<tr>
<td>Cortisol</td>
<td>10000</td>
<td>17.8</td>
</tr>
<tr>
<td>Cortisone</td>
<td>10000</td>
<td>5.33</td>
</tr>
<tr>
<td>Dehydrocorticosterone</td>
<td>10000</td>
<td>0.46</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>200</td>
<td>7.60</td>
</tr>
<tr>
<td>Tetrahydrocortisol</td>
<td>10000</td>
<td>0.54</td>
</tr>
<tr>
<td>Prednisone</td>
<td>10000</td>
<td>3.05</td>
</tr>
<tr>
<td>Desamethasone</td>
<td>10000</td>
<td>0.04</td>
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</table>

Cortisol – Serum Access

Protocol Known Interferences

<table>
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<tr>
<th>Substance Added</th>
<th>Amount (ng/mL)</th>
<th>Reactivity %</th>
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<td>Corticosterone</td>
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**Conclusion**

- Using MS methods sometimes is not a choice but is necessity.
- Doing a thorough method validation can be tedious, but the consequences of not doing it right are wasted time, money, and resources.
Thank you
singh.ravinder@mayo.edu