Development of a method for the simultaneous measurement of serum testosterone, 17α-hydroxyprogesterone and androstenedione by liquid chromatography – tandem mass spectrometry (LC-MS/MS)

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Introduction
Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is ideally suited for accurate, rapid analysis of multiple analytes from a single injection.

We developed and validated a sensitive, specific LC-MS/MS method for simultaneous quantitation of testosterone, 17α-hydroxyprogesterone (17OHP) and androstenedione. Biochemical assessment of these steroids is important to investigate disorders such as hyper- and hypogonadism, infertility, poly cystic ovary syndrome, and steroid-secretting tumours, for the diagnosis and monitoring for congenital adrenal hyperplasia and in monitoring prostatic cancer therapy.

By chromatographically separating testosterone, 17OHP and androstenedione from other endogenous compounds (including isomers) (Figure 1), we are able to report the very low concentrations found in women, children and men receiving androgen deprivation therapy with specificity and accuracy not possible with immunnoassay.

The flow on from this is potentially greater confidence in clinical decision making processes.

This assay has entirely replaced our testosterone immunoassay and by introducing in-house testing for 17OHP and androstenedione we have also been able to achieve considerable cost-savings for the laboratory.

Method
Protein precipitation and liquid/liquid extraction with zinc sulphate (ZnSO4) and methyl tertiary butyl ether (MTBE) on 100 µL of serum after the addition of isotope-labelled internal standards. Shimadzu Prominence UPLC and a Phenomenex Kinetex C18, 50 x 2.1 mm, 2.6 µ column. Gradient elution with 2mM Ammonium acetate / 0.1% formic acid mm, 2.6 µ column.

Recovery:

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOQ (nmol/L)</th>
<th>Linearity (r²) (n = 15)</th>
<th>Intralaboratory CV% (n = 30)</th>
<th>Intralaboratory CV% (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>4.6%</td>
<td>0.998</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>17OHP</td>
<td>3.9%</td>
<td>0.995</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>3.7%</td>
<td>0.991</td>
<td>0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Stability: Extracted samples were found to be stable for at least 48 hrs in the instrument autosampler.

Results
Performance characteristics
Established with reference to the NPACC guidelines. Additional consideration was given to the Testosterone Harmonisation Group Guidelines in the method development and selection of calibrators.

Linearity

The assay is linear for testosterone and androstenedione to 125nmol/L, and for 17OHP to 375 nmol/L.

Precision, Limits of Quantitation and Detection (LOQ, LOD): The combined imprecision for all analytes was less than 10% (Table 2). LOD was determined by repeated injections of a low standard (n = 10) with the criterion being a CV% of ≤ 20%. LOD was determined as a signal to noise ratio of ≥ 3:1.

Recovery: The mean absolute recoveries were: 83% for testosterone, 80% for 17OHP and 87% for androstenedione. The mean relative recoveries were: 104% for testosterone, 101% for 17OHP and 98% for androstenedione.

Matrix: The method of Matuzewski was used to assess matrix effect. Six separate patient serum pools each fortified to 5 different concentrations (0, 0.5, 2.5, 10 and 30 nmol/L) were graphed against the fortified target. Each of the slopes were compared using linear regression. A CV% of less than 5 suggests minimal matrix effect. Our results were testosterone 4.6%, 17OHP 4.7% and androstenedione 2.4%.

Results (cont’d)

Interference: Gel vs no-gel (Vacutainer)
Interference from gel in separator tubes was examined by analysing 10 patient samples collected into Vacutainer separator tubes (SST) and trace element tubes without gel. Passing and Bablok fit was used to compare the results. The difference between specimens collected into the 2 different tube types was found to be minimal.

Testosterone:

R²: 0.995; Passing Bablok Fit: y = 1.03x + 0.02
17 OHP:

R²: 0.994; Passing Bablok Fit: y = 1.01x + 0.02
Androstenedione:

R²: 0.928; Passing Bablok Fit: y = 1.05x – 0.08

Method Comparison – Patients and RCPA QAP

Passing and Bablok fit was used to compare methods for each of the steroids.

The results for patient testosterone have been separated into female (n = 75, range 0.1 – 4.1 nmol/L) Figure 3 and males (n = 45, range 0.1 – 81.1 nmol/L) Figure 4. They show very different correlation statistics. This is likely due to cross-reactivity caused by related steroid and steroid-like compounds interfering in immunnoassays.

The 17OHP patients, Figure 5 (n = 38, range 0.10 – 53.2 nmol/L) and androstenedione patients, Figure 6 (n = 21, range 0.7 – 43.0 nmol/L) both compare favourably with the external laboratory LC-MS/MS.

There was also excellent correlation for all three steroids when compared with the RCPA/QAP method means for LC-MS/MS. Shown below:

Testosterone:

R²: 0.990; Passing Bablok Fit: y = 1.12x + 0.12
17 OHP:

R²: 0.976; Passing Bablok Fit: y = 1.06x – 0.35
Androstenedione:

R²: 0.974; Passing Bablok Fit: y = 1.05x – 0.08

Discordant results

Discordant testosterone results in female patients observed during the validation process.

<table>
<thead>
<tr>
<th>Clinical notes</th>
<th>Immuneassay (nmol/L)</th>
<th>LC-MS/MS (nmol/L)</th>
<th>Clinical notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.6</td>
<td>0.6</td>
<td>PCOS</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.7</td>
<td>0.7</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.8</td>
<td>0.8</td>
<td>Early menopause</td>
</tr>
</tbody>
</table>

The falsely elevated immuneassay results, most likely due to cross-reactivity of structurally related compounds in the immunnoassay are likely to have led to unnecessary further investigation of these patients for hypercangonidism.

This underscores the utility of implementing a more specific and accurate routine diagnostic assay for testosterone, now possible with modern mass spectrometry technology.

Conclusion

We have developed and validated a specific, sensitive and accurate LC-MS/MS assay for serum testosterone, 17OHP and androstenedione in-house, we have also been able to achieve considerable cost-savings (and income generation) for our laboratory.

References
2. ASX/AMAS Testosterone Harmonisation Group harmonisation - 2011.
4. Exceptional People. Exceptional Care.

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Figure 1. Baseline resolution of isomers testosterone/epitestosterone and Deoxycorticosterone/17OHP

Figure 2. Typical chromatogram

Figure 3. Comparison of female testosterone results

Figure 4. Comparison of male testosterone results

Figure 5. Comparison of 17OHP patient results

Figure 6. Comparison of Androstenedione patients results

Figure 5. Comparison of 17OHP patient results

Figure 6. Comparison of Androstenedione patients results