Introduction
Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency affects around 1 in 10,000 births and results in decreased production or absence of the adrenal steroids aldosterone and cortisol. Patients may present with a severe salt-wasting crisis early in the neonatal period and require replacement therapy for life. Analysis of 17α-hydroxyprogesterone (17OHP) in dried blood spots (DBS) by radioimmunoassay for monitoring therapy has been performed at Mater Pathology since 1997. Due to the kit being discontinued and suspected cross-reactivity with other endogenous steroids in 17OHP immunoassays, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed. 17OHP DBS analysis by LC-MS/MS is not uncommon, but is generally used in newborn screening in which 17OHP levels in affected newborns are markedly elevated and assay sensitivity is not a consideration. DBS analysis offers a profile of results to be developed over a 24 hour period which assists in optimising frequency and size of glucocorticoid dose and avoids over- and under-treatment (Figure 1). Monitoring CAH treatment by DBS analysis is typically undertaken in newborn screening in which 17OHP levels in affected newborns have been developed. 17OHP measurement using DBS for monitoring treatment of Congenital Adrenal Hyperplasia (CAH) due to 21-hydroxylase deficiency affects around 1 in 10,000 births and results in decreased production or absence of the adrenal steroids aldosterone and cortisol. Patients may present with a severe salt-wasting crisis early in the neonatal period and require replacement therapy for life.

Method Development
Validation according to NPAAC IVD requirements1 was performed, including sample size (number of discs punched from the DBS card), extraction method, comparison with RIA using affected, unaffected and fortified specimens, linearity, matrix effects, interference, punch carry-over, extraction vs elution, reconstitution volume and injection volume.

Due to the significant bias, reference intervals for the new method were established using DBS prepared from typical cases from unaffected children and adults. However, the early morning treatment target interval for CAH is higher than the reference interval for unaffected individuals, therefore this was established using 170HP-fortified steroid-stripped DBS comparison data between RIA and LC-MS/MS.

 Calibration and QC Preparation
Fortified steroid-stripped DBS for use as calibrators and QC were prepared by mixing heparinised plasma with activated charcoal on a rotational mixer overnight. Stripped plasma was mixed back into washing red cells and volumes adjusted to give a final haematocrit of 0.4 L/L, then fortified with 17OHP working standard. The blood was spotted onto Whatman 903 collection cards and left to dry overnight.

Sample Preparation
In a microwave tube, 4 x 3 mm discs were punched from each DBS. Distilled H2O (0.5 mL) was added and tubes were vortexed and left for 30 min. After addition of 30 µL of deuterated internal standard and 280 µL of 50% acetonitrile/methanol, tubes were sonicated for 30 min and centrifuged for 5 min. Supernatant was dried under N2 flow and the residue reconstituted with 0.1% formic acid in 50% methanol.

Chromatography Conditions
Software: Analyst Version 2.6 and Multiquant Version 2.1
Instrument: Shimadzu Prominence UFLC
MS Detector: ABSciex 4000 QTRAP®
Column: Phenomenex Kinetex Biphenyl 2.6μm 2.1 x 100mm
Guard Column: SecurityGuard Ultra Biphenyl for 2.1mm columns
Mobile Phase A: H2O + 0.1% formic acid
Mobile Phase B: Methanol + 0.1% formic acid
Gradient: Mobile Phase B 870 – 98% over 7 min
Flow Rate: 0.20 mL/min
Injection Volume: 40 µL
Column Temp: 50°C
Sample Temp: 8°C
Run Time: 12.0 min

Table 1: Results of fortified plasma samples analysed by established LC-MS/MS method.

<table>
<thead>
<tr>
<th>Expected Concentration (nmol/L)</th>
<th>Result* (nmol/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>9.6</td>
<td>96</td>
</tr>
<tr>
<td>30</td>
<td>28.6</td>
<td>95</td>
</tr>
</tbody>
</table>

*R* result from established LC-MS/MS method

Table 2: Limit of Detection and Limit of Quantification

<table>
<thead>
<tr>
<th>Expected Concentration (nmol/L)</th>
<th>Limit of Detection (nmol/L)</th>
<th>Limit of Quantification (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>0.0236</td>
<td>0.0203</td>
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</table>

Table 3: Within-Run Precision

<table>
<thead>
<tr>
<th>Expected Concentration (nmol/L)</th>
<th>CV (%)</th>
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<tbody>
<tr>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2.0</td>
<td>1.1</td>
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<tr>
<td>10</td>
<td>1.2</td>
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Table 4: Matrix Studies

<table>
<thead>
<tr>
<th>Slope Matrix</th>
<th>Slope Matrix</th>
<th>Slope Matrix</th>
<th>Slope Matrix</th>
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<tbody>
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<td>0.0236</td>
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<td>0.0203</td>
<td>0.0218</td>
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</table>

Method Development (cont’d)

Comparison of steroid-stripped material and patient samples suggests there is significant bias between the calibration standards of the two assays. The better accuracy of the LC-MS/MS method was established by validation of the calibration standards with an accredited LC-MS/MS plasma 17OHP assay. The increased scatter of results between the two assays in high-concentration specimens suggests cross-reactivity of related endogenous steroids in the RIA method in patients with CAH whose treatment was not optimised. The DBS matrix has made compliance with NPACC IVD requirements difficult but not impossible, with techniques more suited to the matrix and LC-MS/MS being employed where appropriate and when supported by additional sources of literature.

A sensitive, accurate and robust LC-MS/MS method for quantification of 17OHP in DBS for monitoring glucocorticoid therapy in CAH has now been developed. This method is less influenced by the presence of related steroids, and appears to have superior accuracy to the assay it is replacing.

Discussion

Comparison of steroid-stripped material and patient samples suggests there is significant bias between the calibration standards of the two assays. The better accuracy of the LC-MS/MS method was established by validation of the calibration standards with an accredited LC-MS/MS plasma 17OHP assay. The increased scatter of results between the two assays in high-concentration specimens suggests cross-reactivity of related endogenous steroids in the RIA method in patients with CAH whose treatment was not optimised. The DBS matrix has made compliance with NPACC IVD requirements difficult but not impossible, with techniques more suited to the matrix and LC-MS/MS being employed where appropriate and when supported by additional sources of literature.

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Acknowledgements

We acknowledge the assistance of ABSciex and Phenomenex® in supplying information regarding LC-MS/MS setup conditions and HPLC column selection.

References


Figure 1: Example of DBS profile collected by patients and the 4 discs punched from the DBS.

Figure 2: Comparison of 17OHP in 54 patient samples.

Figure 3: Comparison of 17OHP in 7 fortified steroid-stripped DBS samples.

Figure 4: Comparison of 17OHP in 7 fortified steroid-stripped DBS samples.