A Semi-Automated Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Method for the Measurement of Plasma Aldosterone

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Introduction

• Hypertension is an important chronic disease
• Public health challenge – high frequency and concomitant risks of cardiovascular and kidney disease
• A recent analysis of worldwide data predicts that in 2025 there will be 1.6 billion adults with hypertension¹

Introduction

• The detection of underlying endocrine causes of hypertension, such as primary aldosteronism (PAL) gives the opportunity to cure or achieve improved control through specific treatment.

• PAL was once thought to be rare (1 to 2%) but has been found to be more common (5-10%)\textsuperscript{1}.

\textsuperscript{1}Stowasser M, et al. \textit{J Hypertens} 2003;21:2149-57
Why Should We Look For PAL?

• The longer the duration of hypertension before PAL is detected and treated, the more severe and difficult to control the hypertension will become.

• Hypertensive patients who are found to have PAL, especially the surgically correctable form, are either cured of hypertension (50 to 60%) or show significant improvement.
Diagnosis of PAL
Hypertension

Aldosterone/renin ratio

Repeat aldosterone/renin ratio at least 1-2 times

Fludrocortisone suppression test

Hypokalemia Normokalemia

Hybrid gene test

CT scanning

Mass ≥ 2.5 cm Mass < 2.5 cm No mass

Adrenal venous sampling

Unilateral Bilateral

Unilateral adrenalectomy Spironolactone or amiloride

Treatment

Screening

Confirming diagnosis

Determining subtype

FH-I
Screening for PAL

- Aldosterone/renin ratio (ARR)

- Aldosterone - pg/mL, plasma renin activity - ng/mL/hr; Cut-off value for ARR = 300

- Accurate measurement is required for a correct diagnosis

1Stowasser M, Gordon RD. The Endocrinologist 2004;14:267-276
Aldosterone Measurement

- Analytical range – 25 to 2000 pg/mL
  (70 to 5540 pmol/L)

- **Gas chromatography** – complex sample preparation with chemical derivatisation

- **Immunoassay** – currently the most routinely used
Immunoassays

• RIA – typically rabbit polyclonal antisera

• Questionable specificity

• Lack of adequate standardisation

• Poor inter-laboratory reproducibility
Immunoassays

- Limited comparability of different immunoassays – difficulty in defining cut-off values for PAL → each laboratory establish their own reference interval

- Automated chemiluminescence-immunoassay withdrawn from market – questionable analytical performance
Comparison of Four Immunoassays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Adalts Aldosterone Mala</th>
<th>DSL Active Aldosterone</th>
<th>Nichols Advantage Aldosterone</th>
<th>In-house assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracer</td>
<td>$^{125}$I</td>
<td>$^{125}$I</td>
<td>(chemiluminescence.)</td>
<td>$^3$H</td>
</tr>
<tr>
<td>Antibody</td>
<td>Polyclonal</td>
<td>Polyclonal</td>
<td>Monoclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Sample serum or</td>
<td>50</td>
<td>100</td>
<td>450</td>
<td>250–500</td>
</tr>
<tr>
<td>plasma, μL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Working range,</td>
<td>6–2500</td>
<td>2–1600</td>
<td>15–1200</td>
<td>10–2000</td>
</tr>
<tr>
<td>ng/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraassay</td>
<td>3.5–5.4</td>
<td>3.6–8.3</td>
<td>2.9–14.0</td>
<td>3.5–8.5</td>
</tr>
<tr>
<td>variability, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interassay</td>
<td>3.6–6.4</td>
<td>7.3–10.4</td>
<td>4.9–18.6</td>
<td>9.6–12.2</td>
</tr>
<tr>
<td>variability, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-reactivity, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldosterone 100</td>
<td>Aldosterone 100</td>
<td>Aldosterone 100</td>
<td>Aldosterone 100</td>
<td>Neiblable since cross-reacting steroids are removed by chromatographic purification</td>
</tr>
<tr>
<td>Progesterone 0.0004</td>
<td>Progesterone &lt;0.0001</td>
<td>Corticosterone 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone 0.0005</td>
<td>Testosterone &lt;0.0001</td>
<td>18-OH–corticosterone 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosterone 0.002</td>
<td>Corticosterone &lt;0.0001</td>
<td>Cortisol &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol 0.00007</td>
<td>Corticosterone &lt;0.0001</td>
<td>Cortisol &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrone 0.00002</td>
<td>Estrone &lt;0.0001</td>
<td>Desoxycorticosterone 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol 0.00004</td>
<td>Estradiol 0.00004</td>
<td>Dexamethasone 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estriol 0.00003</td>
<td>Estriol undetectable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Dihydroaldosterone 14.1</td>
<td>18-hydroxy corticosterone 0.42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3α,5β-Tetrahydroaldosterone 1.1</td>
<td>Dexamethasone &lt;0.00001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference interval</td>
<td>Serum: not given</td>
<td>Serum: 30–340</td>
<td>Serum: 38–313</td>
<td>Serum and plasma: 60–300 standing</td>
</tr>
<tr>
<td>(standing) ng/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma: 70–350</strong></td>
<td></td>
<td><strong>Plasma: 30–220</strong></td>
<td></td>
<td>20–100 recumbent</td>
</tr>
</tbody>
</table>

* Conversion: ng/L × 2.774 = pmol/L. Cross-reacting steroids are removed by chromatographic purification.
Summary

• There is an ever growing population that require screening for PAL
• Accurate measurement of aldosterone is required for the diagnosis of PAL
• Immunoassays have a number of issues that make them less than desirable

• Can HPLC-MS offer a viable alternative?
Methods
Symbiosis LC – Mass Spectrometer System

- Sample Stacker
- Autosampler
- High Pressure Dispensers
- Binary Pumps
- ACE
- Column Oven/ Switching valve
- Mass Spectrometer
Mass Spectrometry (1)
Mass Spectrometry

- Positive ionisation: \([M+H]^+\) and \([M+Na]^+\)
- Protonated species fragmented through a series of water losses – non-specific
- Negative ionisation: \([M-H]^-\)
Mass Spectrum of Aldosterone

\([\text{M-H}]^-\) 358.9
Mass Spectrometry

- Positive ionisation: \([M+H]^+\) and \([M+Na]^+\)
- Protonated species fragmented through a series of water losses – non-specific
- Negative ionisation: \([M-H]^-\)
- Fragmentation resulted in 2 major ions: 
  \[m/z\ 331.3\ \text{and}\ 189.3\]
CID Mass Spectrum of Aldosterone

![CID Mass Spectrum of Aldosterone](image)

- Mass to charge ratio (m/z): 330.9 [M-H]-
- Intensity (cps): 188.8

**CID Mass Spectrum of Aldosterone**

- Mass to charge ratio (m/z): 330.9 [M-H]-
- Intensity (cps): 188.8
On-Line SPE With a Hysphere C18 HD Cartridge

- Condition: acetonitrile (1 mL) and water (1 mL)
- Load: water (1 mL)
- Wash: 10% acetonitrile in 0.1% ammonium hydroxide
  10% acetonitrile in 0.1% formic acid
  10% acetonitrile in water
- Elution time: 45 sec
Online Extraction of Aldosterone
Water-Based Standard 18.5 pg/mL

150806B117 Smooth(Mn,2x2)
18.5 pg/ml in water
aldosterone

MRM of 2 channels,ES-359.2 > 331.4
6.023e+002
Water-Based Standard Curve

Compound name: aldosterone
Correlation coefficient: \( r = 0.998480, r^2 = 0.996962 \)
Calibration curve: \( 0.589704 \times x + -4.30067 \)
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

Analytical range
18.5 to 3700 pg/mL

\( R^2 = 0.996 \)
Summary: Water-Based Standards

- Sample volume: 250 μL
- Limit of quantification: 18.5 pg/mL
- Linear range: 18.5 to 3700 pg/mL ($r^2 = 0.996$)
Response vs Injection Volume
Neat Plasma – 50 x 2.1 mm

Approximately -70%
Sample Pre-treatment

- Plasma (200 µL) + 1:5 vol:vol 0.3M zinc sulphate: methanol containing d-7 aldosterone (200 µL)
- Mix and centrifuge
- Transfer supernatant (could perform this in plate format and remove transfer step)
Response vs Injection Volume
Protein Precipitation – 50 x 2.1 mm

Aldo Peak Area vs Volume Spiked Plasma (uL)

≈ -40%
Response vs Injection Volume
Protein Precipitation – 50 x 3.0 mm

Volume Spiked Plasma (uL)

Aldo Peak Area

≈ -15%
HPLC Conditions

- Analytical column: Sunfire C18 (50 x 3.0 mm, 3 μm, Waters)
- Mobile phase A: water
- Mobile phase B: acetonitrile
- Column temperature: ambient
- Isocratic elution (35% B at 0.3 mL/min) with an organic wash (100% B at 1.0 mL/min)
## HPLC Conditions – Gradient

<table>
<thead>
<tr>
<th>Time (min:sec)</th>
<th>%B</th>
<th>Flow rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35</td>
<td>0.3</td>
</tr>
<tr>
<td>2:28</td>
<td>35</td>
<td>0.3</td>
</tr>
<tr>
<td>2:30</td>
<td>100***</td>
<td>1.0</td>
</tr>
<tr>
<td>4:30</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>4:35</td>
<td>35</td>
<td>0.3</td>
</tr>
<tr>
<td>6:59</td>
<td>35</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Results
Selectivity

- Screened 5 patients with Addison’s disease and observed no interferences at the retention times of aldosterone or the internal standard in their respective mass transitions
Addison’s Patient Sample

358.9 → 330.9
Aldosterone LLOQ (25 pg/mL)

S/N = 17:1

358.9 → 330.9
Internal Standard (LLOQ)

365.9 → 337.9
Patient Sample (52.7 pg/mL)

358.9→330.9
Linearity

- 25 to 2000 pg/mL
- $r^2 > 0.996$ (n = 16)
# Inter-Day Accuracy and Imprecision

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nominal aldosterone concentration (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Mean Concentration (pg/mL)</td>
<td>23.6</td>
</tr>
<tr>
<td>S.D. (pg/mL)</td>
<td>2.22</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>94.5</td>
</tr>
<tr>
<td>Imprecision (%)</td>
<td>9.4</td>
</tr>
</tbody>
</table>
Frequency Distribution of Aldosterone Results Obtained from Upright (Seated) Normotensive Subjects (n = 97)
External Quality Controls DGKL (Germany)

\[ y = 0.986x + 4.2 \]

\[ R^2 = 0.9997 \]

\[ n = 5 \]
Influence of Collection Tube on Results

- **Plasma:**
  - EDTA
  - Li Heparin

- **Serum:**
  - Plain clot tube
  - Serum separator tube (SST)
Comparison of Aldosterone Results Obtained from Plain Clot Versus EDTA Collection Tubes

\[ y = 1.0393x + 0.7078 \]
\[ r^2 = 0.993 \]
\[ n = 66 \]
Confirmation of PAL by Fludrocortisone Suppression Test
Fludrocortisone Suppression Test

- Testing involved the collection of blood samples for aldosterone measurement at 0800 h (after overnight recumbency) and 1000 h (after 2 h of upright posture) basally and after 3 and 4 days of fludrocortisone and oral salt loading. A day 4 upright plasma aldosterone concentration >165 pmol/L was considered diagnostic of PAL.
Aims

• To compare aldosterone results obtained by a commercial immunoassay with a our newly developed LC-MS/MS method, using samples obtained from patients undergoing FST

• (1) address whether the two methods would give similar diagnoses (i.e. "PAL confirmed" versus "PAL excluded") among this patient cohort

• (2) compare the absolute concentrations obtained by each method
Aldosterone Measurement

• A commercial immunoassay (DPC Coat-a-Count™ aldosterone kit, Diagnostic Products Corporation, Los Angeles, CA, USA)

• Our in-house LC-MS/MS method
Patients

- A total of 19 patients undergoing FST were investigated in this study.
- 16 patients had been diagnosed with PAL based on aldosterone immunoassay results.
- 3 patients had undergone post-operative FST following unilateral adrenalectomy for aldosterone producing adenoma.
Results

- The aldosterone results (>165 pmol/L) obtained by LC-MS/MS confirmed the diagnosis of PAL in all 16 patients
Results

• In the 3 patients undergoing post-operative FST following unilateral adrenalectomy for aldosterone-producing adenoma, both immunoassay and LC-MS/MS day 4 upright aldosterone concentrations were <70 pmol/L, confirming biochemical cure of PAL
Deming regression analysis gave the equation LC-MS/MS = 0.80*RIAl + 43.
Bland Altman Plot Comparing Aldosterone Results

mean bias = -10.6%
95% confidence intervals = -85.8% to 64.3%
## Comparison of Aldosterone Results Stratified According to Concentration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration range (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 165</td>
</tr>
<tr>
<td>Number of samples</td>
<td>11</td>
</tr>
<tr>
<td>Mean bias (pmol/L)</td>
<td>9.8</td>
</tr>
<tr>
<td>95% Limits of agreement (pmol/L)</td>
<td>-43 to 63</td>
</tr>
</tbody>
</table>

# Mean bias is based on immunoassay minus LC-MS/MS
DPC Immunoassay Binding Curve

Percent bound vs. Aldosterone concentration (pg/mL)
Discussion

• The results obtained in this study confirm the clinical validity of our currently used immunoassay for the diagnosis of PAL.
Discussion

• The underestimation of aldosterone results by the immunoassay at higher concentrations may be an important consideration for some clinical applications (i.e. adrenal venous sampling)

• Ongoing studies are addressing this issue
Case Report

- A 44 year old male with congenital adrenal hyperplasia had an aldosterone of 694 pg/mL as measured by immunoassay (DPC)
Case Report

- A 44 year old male with congenital adrenal hyperplasia had an aldosterone of 694 pg/mL as measured by immunoassay (DPC)
- Result of 92 pg/mL
- Greater than 6-fold overestimation of result
Chromatograms From CAH Patient

090807B204 Smooth(Mn,2x2)  
S_N PATIENT High

MRM of 2 channels, ES-  
365.9 > 337.9  
3.051e+004

Aldosterone; 2.14; 243.64; 1976  
2.41

090807B204 Smooth(Mn,2x2)  
S_N PATIENT High

MRM of 2 channels, ES-  
358.9 > 330.9  
3.246e+003

d7-Aldosterone; 2.11; 4518.09; 30117  
1.52  
1.74  
1.90

Discussion

- The semi-automated method described is a world-first for the analysis of aldosterone.

- Excellent accuracy and precision across the clinically important concentration range.

- Selective/specific method for aldosterone.

- Sample throughput of 7 min/sample – suited to screening large numbers of patients.
Discussion

- The results obtained using this method will allow the establishment of reference ranges for the aldosterone/renin ratio

- Translate across laboratories – harmonisation

- Improved diagnosis

- Will provide an invaluable tool for future research into endocrine disorders
Discussion

• This analytical approach has the potential to be used for the measurement of other steroids (e.g. testosterone, estradiol, etc)

• Potential for simultaneous measurement of a panel of steroids (e.g. aldosterone and cortisol)
Conclusion

• It can be envisaged that this approach will become the “gold standard” for screening of primary aldosteronism and other aldosterone related diseases