**Quantitative Analysis of 1,25-Dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> by LC-MS/MS Utilizing Ion Funnel Technology**

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**Introduction**

A highly sensitive, and selective LC-MS/MS method for determination of 1,25-dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> is a powerful tool for clinical researchers. While 25 hydroxyvitamin D is found in the ng/ml concentration range, 1,25-dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> are typically found in the low pg/ml range, making quantitative analysis challenging except when employing highly sensitive analytical techniques. Additionally, extraction is a critical step for this analysis as removal of interfering analytes is required to quantify at the low pg/ml concentration range. Where previously published work (Casetta et al., 2010) demonstrates good sensitivity, such approaches require a complex 2D LC set-up, with post column infusion. The work presented in this poster illustrates the quantitative analysis of 1,25-dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> using the Agilent 1260 UHPLC & 6490 QQQ with Ion Funnel technology coupled with ImmunoTube LC-MS/MS Kit (an extraction kit from ImmunoDagnostik) for the extraction of 1,25-dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> from plasma samples.

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**Sample Preparation**

Immuno Tubes were spun down to ensure all the suspension was forced to the bottom of the tube. 500 µL of calibrator / sample / control was added followed by 10 µL of IS and mixed gently. Immuno Tubes were then mixed in a spiral rotator for 1hr at RT. The closed Immuno Tubes were then placed in a micro tube and centrifuged for 1min at 550 x g. Subsequently, the cover and the outlet of the Immuno Tubes were removed. The Immuno Tubes were then placed back into the micro tubes for centrifugation a further 2min at 550 x g. The waste collected in the micro tubes was discarded.

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**Results and Discussion**

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500 µL of WASHSOL was added to the Immuno Tubes and centrifuged for 2 min at 550 x g. This step was repeated twice. Each micro tube was replaced by a glass vial and 250 µL of ELUREAG were added to each Immuno Tube which was centrifuged for 2 min at 550 x g. The recovered eluent was evaporated under N2 at 37° C. Samples were reconstituted with 165 µL of activated Solution A prior to analysis.

WASHSOL and ELUREA are solutions from the ImmunoDagnostik extraction kit.

**LC Method**

An Agilent 1260 HPLC series binary pump with 56 vial sample tray, sampler with thermostat, temperature-controlled column compartment, 2 position/6 ports switching valve, was used.

Column: Zorbax Eclipse Plus 2.1x100 mm 1.8µm
Column temperature: 50 °C
Injection volume: 100 µL
Autosampler temperature: 4 °C
Needle wash: 3.1 MeOH:H2O, 10 seconds

**Mobile Phase**

A: ImmunoDagnostik Mobile Phase A
B: ImmunoDagnostik Mobile Phase B

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Flow</th>
<th>% Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>0.50</td>
<td>0.3</td>
<td>100</td>
</tr>
<tr>
<td>0.51</td>
<td>0.3</td>
<td>100</td>
</tr>
<tr>
<td>0.80</td>
<td>0.3</td>
<td>0</td>
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Table 1. LC conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Prec Ion</th>
<th>Prod Ion</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)&lt;sub&gt;2&lt;/sub&gt;Vitamin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>411.1</td>
<td>150.7</td>
<td>20</td>
</tr>
<tr>
<td>1,25(OH)&lt;sub&gt;2&lt;/sub&gt;Vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>411.1</td>
<td>132.9</td>
<td>18</td>
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<tr>
<td>1,25(OH)&lt;sub&gt;2&lt;/sub&gt;Vitamin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>399.1</td>
<td>150.9</td>
<td>12</td>
</tr>
<tr>
<td>1,25(OH)&lt;sub&gt;2&lt;/sub&gt;Vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>399.1</td>
<td>134.9</td>
<td>12</td>
</tr>
<tr>
<td>1,25(OH)&lt;sub&gt;2&lt;/sub&gt;Vitamin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>405.1</td>
<td>150.6</td>
<td>12</td>
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<tr>
<td>1,25(OH)&lt;sub&gt;2&lt;/sub&gt;Vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>405.1</td>
<td>134.6</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2. MRM Parameters

**Figure 3. Chromatograms of extracted calibrators from ImmunoDagnostik**

**Figure 4. Calibration curve for 1,25-dihydroxyvitamin D<sub>2</sub>**

**Figure 5. Calibration curve for 1,25-dihydroxyvitamin D<sub>3</sub>**

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**Conclusion**

A highly sensitive and selective method for quantifying 1,25-dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> from human plasma has been optimized. By combining the sensitivity of the 6490 QQQ with funnel Technology and the ImmunoDagnostik extraction method, quantification at low pg/ml levels has been achieved.

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