Improved Sample Preparation and HPLC/MS Analysis of Vitamin D Metabolites from Human Plasma

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Abstract

Analysis of Vitamin D metabolites has continued to be a topic of interest in recent publications, primarily as biomarkers for possible disease states and vitamin sufficiency. While Vitamin D presents two forms, Vitamin D$_2$ and Vitamin D$_3$, current ELISA methods cannot distinguish D$_2$ and D$_3$ forms of the vitamin metabolites resulting in a reporting of total 25-hydroxylated D$_2$. In this study, an LC-MS method for the analysis of Vitamin D metabolites is expanded to include dihydroxy metabolites along with the diox-homologs. Chromatographic resolution is utilized for the quantification of hydroxy and dihydroxy Vitamin D$_2$ and D$_3$ metabolites, including the isotopic isomers. In addition, sample preparation techniques are evaluated for the impact of biological matrix interferences effects.

Introduction

This study describes the development process for accurate quantification of vitamin D metabolites from serum samples. Column selection, screening, method optimization for LC-MS detection and sample preparation optimization aspects are detailed. In this study, special consideration was given to chromatographic resolution of isobaric compounds for accurate quantitation. Here, chromatographic method development consisted of screening columns, Phenyl Hexyl and polar zirconia phases (ZIC) for resolution of isobaric compounds. Method optimization was conducted using LC-MS detection along with sample preparation optimization. The conditions described enable the direct quantitation of isobaric metabolites 25-hydroxy vitamin D$_3$, 3α-25-dihydroxy vitamin D$_3$, 3α-hydroxy vitamin D$_3$, and 25-hydroxy vitamin D$_2$, along with 25-hydroxy vitamin D$_2$, 3α-25-dihydroxy vitamin D$_2$, and 3α-hydroxy vitamin D$_2$. In addition, human serum samples were processed using standard protein precipitation and a new method, also referred to as phospholipid depletion phase for the comparison of matrix interference impact. All studies were conducted using UHPLC/TOF instrumentation.

Experimental

Objective / Experimental Design

Objective is to review chromatographic resolution of isobaric compounds for accurate quantitation. Here, chromatographic method development involved screening columns, Phenyl Hexyl and polar zirconia phases (ZIC) for resolution of isobaric compounds. Method optimization was conducted using LC-MS detection along with sample preparation optimization.

Experimental Conditions

<table>
<thead>
<tr>
<th>Component</th>
<th>Mobile Phase</th>
<th>Minimum Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
<td>Mobile Phase</td>
<td>Minimum Resolution</td>
</tr>
<tr>
<td>Vitamin D$_2$</td>
<td>Acetonitrile</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin D$_3$</td>
<td>Ethanol</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin D$_2$</td>
<td>Methanol</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 1. Summary of Minimum Isocratic Resolution by Phase and Organic Combination

<table>
<thead>
<tr>
<th>Column</th>
<th>Organic Modifier</th>
<th>Minimum Resolution (isobaric pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascentis Express ES-CN</td>
<td>Methanol</td>
<td>2.5</td>
</tr>
<tr>
<td>Ascentis Express Phenyl-Hexyl</td>
<td>Ethanol</td>
<td>2.5</td>
</tr>
<tr>
<td>Ascentis Express Phenyl-Hexyl</td>
<td>Acetonitrile</td>
<td>2.5</td>
</tr>
<tr>
<td>Ascentis Express Phenyl-Hexyl</td>
<td>Methanol</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Figure 1. Vitamin D Metabolites

- 3-epi-25-hydroxyvitamin D3 (400.33 Da)
- 25-hydroxyvitamin D3 (400.33 Da)
- 3-epi-25-hydroxyvitamin D2 (412.33 Da)
- 25-hydroxyvitamin D2 (412.33 Da)
- 25-dihydroxyvitamin D3 (428.33 Da)
- 1α-25-dihydroxyvitamin D2 (428.33 Da)
- 1α-hydroxy vitamin D3 (428.33 Da)
- 25-hydroxy vitamin D$_3$ (401.33 Da)
- 1α-hydroxy vitamin D2 (413.33 Da)

Chromatographic Conditions

- System: Agilent 1290, 6210 TOF
- MS detector: ESI+, 100-1000m/z

Figure 3. Separation of Vitamin D Metabolites on Ascentis Express ES-CN

- For LC-MS method, goal was to develop single method with sub-5 minute run time.
- Mobile phase additives were also evaluated for impact on resolution/interference of unknowns under acidic conditions.
- Both ammonium formate and formic acid were both evaluated as the aqueous portion of the mobile phase.
- There was minimal impact on selectivity between the ammonium formate and formic acid additive. There is however a slight difference in analyte solvation between the two additives.
- Analyte response using ammonium formate is nearly twice that of formic acid additive.
- The ammonium formate condition was then used for the optimized LC-MS method as depicted in Figure 3. This method was utilized for the analysis of vitamin D$_2$ and vitamin D$_3$ metabolites.

Next step was to compare sample preparation techniques for recovery of vitamin D metabolites from human serum, evaluate analyte response and matrix effects.

Sample Preparation

- Sample: Human serum spiked at 25 ng/mL vitamin D metabolites
- Sample Prep: 1. HybridSPE® PluS 50 mg 96-well plate (57569-U) 2. standard protein precipitation
- Process: Human serum was spiked at 25 ng/mL with vitamin D metabolites. Protein precipitation was performed offline by adding 100 µL of spiked serum to 900 µL of 1% formic acid acetonitrile. Samples were mixed by performing the 250 µL ultracentrifuge cycles using digital pipette, then centrifuged for 3 minutes before removing 900 µL of precipitate into the HybridSPE-Plus plate by applying 10 Lg at room temperature for 4 minutes. The resulting filtrate was analyzed. As a comparison, spiked human serum was also processed using standard protein precipitation by adding 100 µL of serum into 2 mL centrifuge tube followed by 500 µL of 1% formic acid acetonitrile. Samples were vortexed and centrifuged, and the resulting supernatant was collected and analyzed directly.

Figure 4. Sample Process for HybridSPE Plus 96-Well Plate

Table 2. Vitamin D Metabolite Recovery Comparison

<table>
<thead>
<tr>
<th>Sample Preparation</th>
<th>Recovery %</th>
<th>1α-25-dihydroxyvitamin D2</th>
<th>25-dihydroxyvitamin D3</th>
<th>3α-25-dihydroxyvitamin D3</th>
<th>25-hydroxyvitamin D2</th>
<th>25-hydroxyvitamin D3</th>
<th>1α-hydroxy vitamin D2</th>
<th>1α-hydroxy vitamin D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HybridSPE® PluS</td>
<td>92.0%</td>
<td>98.9%</td>
<td>98.4%</td>
<td>96.8%</td>
<td>97.0%</td>
<td>98.8%</td>
<td>98.8%</td>
<td>98.5%</td>
</tr>
<tr>
<td>PPT</td>
<td>82.3%</td>
<td>91.9%</td>
<td>95.0%</td>
<td>90.5%</td>
<td>76.8%</td>
<td>92.8%</td>
<td>90.8%</td>
<td>87.3%</td>
</tr>
</tbody>
</table>

- The HybridSPE Plus demonstrated excellent recovery of all analytes in both standard solutions along with serum samples, as depicted in Table 2.
- Sample processed using standard protein precipitation (PPT) demonstrated significantly reduced recovery for 25-hydroxy vitamin D2 and 25-hydroxy vitamin D3.
- Comparison of phospholipid matrix interference is depicted in Figure 5.

Figure 5. Phospholipid Matrix Monitoring

Conclusions

- Sample processed using the HybridSPE-Plus technique demonstrated no matrix interference resulting in lower limits of quantitation for the method.
- Direct overlap of phospholipid matrix interference was observed using the standard protein precipitation technique resulting in 40% reduction in signal response for three of the metabolites.
- Chromatographic resolution of analytes still plays an important role in LC-MS applications when dealing with biological samples.
- The unique selectivity of the Ascentis Express ES-CN enable a fast and efficient method for the analysis of vitamin D metabolites from serum samples.
- The selectivity extraction of phospholipids is achieved using zirconia-coated particle technology.
- The high selectivity towards phospholipids is achieved utilizing Lewis acid/base interactions between the phosphorus group of the phospholipids and the zirconia surface.
- The zirconia-coated particle is not is Lewis “acidic” as pure zirconium oxide, thus enabling highly efficient extraction of phospholipids while retaining non-selectivity towards a broad range of basic, neutral, and acidic compounds.

Interaction represents a representative phospholipid and the zirconia surface of the HybridSPE-Plus particle via Lewis acid-base interaction.