Analytical measurement of serum 25-OH-vitamin D$_3$, 25-OH-vitamin D$_2$ and their C3-epimers by LC-MS/MS in infant and pediatric specimens

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ABSTRACT

Objective: To develop a simple and sensitive LC-MS/MS procedure for quantification of serum 25-OH-vitamin D$_3$ (25-OH-D$_3$), 25-OH-vitamin D$_2$ (25-OH-D$_2$), and their C3-epimers.

Methods: Serum 25-OH-vitamin D$_3$ metabolites were extracted with MTE and cleaned-up with C$_18$- cartridges. Analytical measurement of serum 25-OH-vitamin D$_3$ and D$_2$ was performed using the Analyst spectrometer. Vitamin D metabolites were separated on a PFP HPLC column (100 x 3.0 mm, 2.6 µm) and their C3-epimers with a simple extraction procedure and short analytical run time.

25-OH VITAMIN D LC-MS/MS METHODOLOGY

The LC-MS/MS method is described in Figure 1. The extraction procedure and LC-MS/MS protocol provided adequate separation of vitamin D metabolites with their C3-epimers and can be applied to other congeners.

Figure 1. Representative LC-MS/MS spectra of serum spiked with C3-25-OH-D$_3$ metabolites.

LIMIT OF QUANTIFICATION

The limit of quantitation (LOQ) was determined from four replicate measures of serum pools diluted with water. The cut-off criteria for LOQ was either a CV of 20% or a linear range.

Table 1. Serum 25-OH-D$_3$ metabolite limit of quantification

<table>
<thead>
<tr>
<th>Serial</th>
<th>LOQng/mL</th>
<th>S.D. ng/mL</th>
<th>CV%</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH-D$_3$</td>
<td>0.1</td>
<td>0.05</td>
<td>5.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Medium</td>
<td>2.0</td>
<td>0.5</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>High</td>
<td>20.0</td>
<td>0.5</td>
<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

ACCURACY

Accuracy was established by quantifying vitamin D external quality assessment materials (DOQAAS). NIST 1927, NIST 1928, and NIST 1929 were used for all 25-OH-D$_3$ and 25-OH-D$_2$ standards.

Table 3A. Analysis of vitamin D external quality assessment materials

<table>
<thead>
<tr>
<th>Vitamin D</th>
<th>Proposed method</th>
<th>NIST mean (n=6)</th>
<th>NIST SD (n=6)</th>
<th>CV% (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH-D$_3$</td>
<td>100.0</td>
<td>98.0</td>
<td>80.0</td>
<td>8.0</td>
</tr>
<tr>
<td>25-OH-D$_2$</td>
<td>100.0</td>
<td>98.0</td>
<td>80.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

RESULTS

Conclusions: The proposed LC-MS/MS procedure is suitable for quantifying 25-OH-D$_3$ and 25-OH-D$_2$ metabolites. The C3-epimer is present in all pediatric subjects, but is significantly elevated in individuals 1 year of age and 12 months of age.

INTRODUCTION

Quantitation of 25-hydroxyvitamin D by the clinical laboratory continues to receive considerable attention due to an ever-growing body of evidence suggesting it may be implicated in a number of disorders including osteoporosis, cancer, multiple sclerosis, diabetes and cardiovascular disease.

Liquid vitamin D supplements are commonly prescribed to infants <1 year of age. Although standard vitamin D$_3$ supplements are unlikely to be a significant source of C3-epimer, the evidence for a potential role of the metabolite demands its quantification.

METHODS

A volume of 100 µL of serum was spiked with 25 µL Internal standard (25-D$_3$-OH-vitamin D$_3$ and 25-D$_2$-OH-vitamin D$_2$). Samples were vortexed (15 s) and then centrifuged (10 min at 10,000 g). The supernatant was evaporated to dryness under N$_2$ gas and the residue was re-dissolved in 50 µL of 50% acetonitrile in water and filtered through a 0.2 µm syringe filter. The LC-MS/MS system consisted of an Applied Biosystems API 4000 QTRAP triple quadruple mass spectrometer linked to an Agilent 1200 series HPLC and an Agilent 1200 series autosampler. The LC column was a Waters X Terra MS C$_18$ (100x3.0 mm, 2.6 µm). Mass spectrometry was conducted in positive AP mode. The LC system was linked for each metabolite as follows: 401-2.9436-2.6279, 432.2-3.8577 and 432.2-3.8577 for 25-OH-D$_3$ and C3-25-OH-D$_3$; 432.2-3.8577 for 25-OH-D$_2$ and C3-25-OH-D$_2$ respectively. The source temperature was set at 300°C and the nebulizing gas at 40 psi. Data acquisition and quantification was performed using the Analyst software.

INTERFERENCES

Matrix effect and ion suppression were investigated by analyzing serum extracts from four pediatric subjects. The proposed assay to quantify vitamin D$_3$ and D$_2$ was examined to address whether they contain appreciable amounts of C3-epimer.

CONCLUSION

The proposed LC-MS/MS is suitable for routine use in a clinical laboratory as it provides the following:

• A limit of detection and linear range suitable for clinical samples (2.8 - 400 nmol/L).

• Acceptable imprecision, even for C3-epimers (<20% for low C3-epimer concentrations).

• More accurate method than other vitamin D$_3$ and D$_2$ epimeric methods.

• Better detection and linearity for clinical samples.

• The proposed LC-MS/MS was suitable for quantifying vitamin D$_3$ and vitamin D$_2$ epimeric methods.

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