Root Cause Analysis of 1α,25-Dihydroxyvitamin D Overestimation in LC-MS/MS Assay

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INTRODUCTION

The 1α,25-dihydroxyvitamin D (DHVD) test is used to assess the level of the biologically active form of vitamin D in patients with chronic renal disease or for diagnostic differentiation of hypercalcemia. The DHVD assay is challenging because there are numerous isomers present in patient specimens, and the active form has very low circulating concentrations (picomolar). Our hospital is currently using a Cookson-type triazolinedione derivatization protocol followed by LC-MS/MS quantification to determine 1α,25-dihydroxyvitamin D2 and 1α,25-dihydroxyvitamin D3 levels in serum specimens. While investigating a positive bias in a DHVD method comparison study, we found that two diastereoisomers were produced in the reaction between 1α,25-(OH)2VitD2 and PTAD. Separation of diastereoisomer peaks offers improved quantification of DHVD.

MATERIALS & METHODS

• Sample preparation
  Serum samples (0.5 mL) were mixed with equal volumes of internal standard (in acetonitrile) to precipitate protein. After centrifugation, vitamin D metabolites in the supernatant were purified by solid phase extraction and dried under nitrogen.

• PTAD derivatization
  • LC-MS/MS method
    Analysis was performed on a Qtrap5500 mass spectrometer coupled with a Shimadzu HPLC. In the current method (6 min), analytes were separated on an Acquity BEH 1.7 µm C18 column 2.1X100 mm (Waters) using acetonitrile and water with 0.1% formic acid as mobile phase at a flow rate of 0.6 mL/min.
    In a longer method (9 min), a Kinetex 1.7 µm Phenyl-hexyl column, 2.1X100 mm (Phenomenex), was used. MRM transitions and ion source parameters were set according to the previous report (3). Quantitation was performed in MassHunter with a six-point calibration based on the ratio of light vs. heavy labeled internal standard (δ48-25-(OH)2VitD).

RESULTS

Positive bias in a DHVD method comparison study

<table>
<thead>
<tr>
<th>No.</th>
<th>Reference method</th>
<th>Short LC-MS/MS method</th>
<th>Total DHVD bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59.7</td>
<td>82.3</td>
<td>39%</td>
</tr>
<tr>
<td>2</td>
<td>74.6</td>
<td>93.3</td>
<td>25%</td>
</tr>
<tr>
<td>3</td>
<td>113</td>
<td>139.0</td>
<td>23%</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>51.1</td>
<td>50%</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>76.5</td>
<td>70%</td>
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</table>

Challenges in the quantification of DHVD: other vitamin D metabolites, such as 4β,25-(OH)2VitD3 and 3-epi-1α,25-(OH)2D5, may be co-eluted with 1α,25-(OH)2VitD3 peak in a short separation method (2,3).

Investigating possible DHVD interferences using an optimized LC-MS/MS method

Figure 1. A: 1α,25-(OH)2VitD3 peak in the short method. B: Noise 1α,25-(OH)2VitD3 peaks in the long LC-MS/MS method. C: Internal standard δ48-25-(OH)2VitD also has two peaks in the longer method. D: Noise 4β,25-(OH)2VitD3 has only one peak in the longer method. E: Mixture of 1α,25-(OH)2VitD3 and 4β,25-(OH)2VitD3 (ratio: 1:1) shows three peaks.

Figure 2. Extracted ion chromatogram of 1α,25-(OH)2VitD3 in serum specimens without (A) and with (B) interference peaks.

RESULTS continued

• We found that two diastereoisomers were produced in the DHVD method comparison study between 1α,25-(OH)2VitD2 and PTAD.
• Separation of diastereoisomer peaks offers improved quantification of DHVD.

CONCLUSIONS

• We found that two diastereoisomers were produced in the Diels-Alder reaction of 1α,25-(OH)2VitD2 with PTAD.
• The presence of 4β,25-(OH)2VitD3 may cause positive bias when using a short LC-MS/MS assay of 1α,25-(OH)2VitD3.
• Separation of two diastereoisomer peaks provides improved quantification of DHVD.
• We developed a LC-MS/MS method to quantify 1α,25-(OH)2VitD3 and 1α,25-(OH)2VitD2 without using antibody enrichment.

REFERENCES