Identification of 1α,25(OH)₂-Vitamin D₂ and D₃ in Serum Samples Using the AB SCIEX Triple Quad™ 5500 System

Simplified sample processing and analysis

Adam Latawiec¹ and Bruno Casetta²
¹ AB SCIEX, Canada; ² AB SCIEX, Italy

Introduction

Over the years Mass Spectrometry (MS) techniques have spread to an ever wider range of clinical analyses supplanting traditional analytical techniques such as immunoassays and UV-Vis spectroscopic techniques. The challenges are to expand the high sensitivity and specificity of tandem mass spectrometry (MS/MS) to simple instrumental techniques for the preparation and analysis of real life samples.

Among the large number of papers published by various authors on steroid analysis, we have recently presented a paper¹ dealing with the measurement of 1α,25(OH)₂-vitamin D₃, a secosteroid which normally presents several challenging features to the mass spectrometrist. The low level of observed ionization means a low intrinsic sensitivity at the mass spectrometer level which, when coupled with a very low concentration in the plasma, creates difficulties in obtaining adequate functional sensitivity. As well, attempts to improve sensitivity by derivatization lead to longer and more complex sample preparation steps and reductions in instrumental throughput.

This application note describes a novel LC/MS/MS method for the determination of dihydroxyvitamin D₃ and D₂ (DHVD₃ and DHVD₂) from extracted human serum samples. The key features of the method are a 2D chromatographic separation followed by MRM detection of the DHVD species as their lithium adducts. The use of the lithium DHVD adduct allows for low levels of detection without the use of derivatizing agents thereby greatly simplifying the analytical workflow.

Key Features of the Method

- The sensitive and selective Multiple Reaction Monitoring (MRM) scan function on the AB SCIEX Triple Quad™ 5500 and QTRAP® 5500 provides the highest level of sensitivity over a wide linear dynamic range.
- The use of dual Phenomenex Onyx Monolithic C-18 columns enhances the separation of complex biological matrices while providing extremely low back pressures. This allows the use of conventional LC equipment and standard fittings throughout the system.
- Sample clean-up is performed using a POROS R1/10 column providing direct in-line sample clean-up from the protein precipitated sample. Minimal sample clean-up increases analytical throughput.
- Preparative and analytical column flows are regulated by use of a Valco 10-port valve assembly, allowing the use of different mobile phase compositions from the dual binary LC pump configuration.
Experimental Conditions

Serum and plasma samples were processed using a simple acetonitrile protein precipitation step followed by centrifugation. The extraction procedure is described in Table 1.

For the measurement of 1α,25(OH)2-vitamin D2 and 1α,25(OH)2-vitamin D3, the layout of the HPLC system is shown in Figure 1.

Table 1. DHVD extraction procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliquot 200 µL Sample/Standard/QC into a 1.5mL polystyrene centrifuge tube</td>
<td></td>
</tr>
<tr>
<td>Add 20 µL of Internal Standard (DHVD3-d6 and DHVD2-d6 at 100 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Add 400 µL of acetonitrile to each tube and vortex mix for at least 30 seconds to precipitate proteins</td>
<td></td>
</tr>
<tr>
<td>Let stand for 10 minutes in water/ice bath</td>
<td></td>
</tr>
<tr>
<td>Centrifuge samples at 14000 rpm for 10 minutes</td>
<td></td>
</tr>
<tr>
<td>Transfer clean supernatant to HPLC autosampler vial</td>
<td></td>
</tr>
<tr>
<td>Inject 100 µL onto HPLC-MS/MS system</td>
<td></td>
</tr>
</tbody>
</table>

The basis of the 2D-LC method is the “clean-up” of a large sample volume (100 µL of protein precipitated sample) by way of a perfusion column (POROS R1/10, 4.6 x 50 mm, Applied Biosystems) with a binary gradient from the “loading” pump at a high flow rate of 1.25 mL/min. The 10-port switching valve diverts the POROS effluent to waste. During this time the analytical column (2 x Onyx C18, 3 x 100 mm – Phenomenex) is equilibrated at a low flow rate (0.10 mL/min) with a solution containing 0.5 mM Lithium acetate from the “separation” pump. As the target material begins to elute from the POROS column, the 10-port valve is switched and the target analytes are flushed on to the analytical column as the flow from the “loading” pump is reduced to 0.30 mL/min. After all of the target material has transferred to the analytical column, the 10-port valve is again switched to the waste position and the “separation” pump gradient and flow increased to achieve separation on the analytical column.

The AB SCIEX API 5000™, Triple Quad™ 5500, or QTRAP® 5500 LC/MS/MS system is operated with the Turbo V™ source in the electrospray mode. The targeted lithiated ions are monitored in the Multiple Reaction Monitoring (MRM) mode exploiting the transition m/z 423/369 for the 1α,25(OH)2-vitamin D3 and 435/399 for the 1α,25(OH)2-vitamin D2. Transitions m/z 429/393 and m/z 441/405 are used for the 1α,25(OH)2-vitamin D3-d6 and 1α,25(OH)2-vitamin D2-d6 internal standards, respectively.

Figure 2 shows the attainable performance for real-life samples containing 57 pg/mL of dihydroxyvitamin D3 and 25 pg/mL of dihydroxyvitamin D2 analyzed on the AB SCIEX QTRAP® 5500 system. Under the conditions described an LOQ of approximately 15 pg/mL (36 pmol/L) for 1α,25(OH)2-vitamin D3 is readily achieved. The useful linear range is from 0 to 250 pg/mL, and is shown in the calibration curve for 1α,25(OH)2-vitamin D3 (Figure 3).

Figure 2. Extracted Ion Chromatogram (XIC) of DHVD3 and DHVD2. XIC of DHVD3 (57 pg/mL) and DHVD2 (25 pg/mL), with DHVD3-d6 internal standard, acquired on the AB SCIEX QTRAP® 5500.
Conclusions

This method has been demonstrated to be robust and reproducible. Using this approach, 1α,25(OH)₂-vitamin D₂ and 1α,25(OH)₂-vitamin D₃ can be effectively separated from other matrix components with minimal interferences (e.g. ion suppression) in the ion source. With proper set up and calibration an LOD as low as 10 pg/mL is achievable. The lithium adduct provides additional sensitivity which eliminates the time consuming requirement of derivatization and concomitant clean-up which is a feature of many other analytical tandem MS procedures.

References