Direct Quantification of 1,25-(OH)₂Vitamin D₃ in Serum Using a Highly Sensitive LC-MS/MS

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INTRODUCTION
LC-MS/MS has emerged as a reliable quantitative approach in clinical research to assess the vitamin D status in patients, due to its high sensitivity and specificity. Vitamin D is rapidly metabolized in the liver to form 25-hydroxyvitamin D (25(OH) vitamin D). Further, hydroxylation takes place in the kidney to yield 1,25-dihydroxyvitamin D₁(25(OH)₂D₃). This biologically active form of vitamin D, playing a role in numerous disease pathways, circulates in the blood at a very low concentration range, typically below the low pg/mL level. Most current LC-MS/MS method require derivatization of 1,25-(OH)₂D₃ to improve the ionization efficiency, and therefore enhancing sensitivity.[1,2] However, direct analysis of 1,25-(OH)₂D₃ using a highly sensitive LCQ 300 triple quadrupole mass spectrometer simplifies the process by eliminating the need for derivatization. Direct analysis of 1,25-(OH)₂D₃ by LC-MS/MS is however susceptible to interferences arising from endogenous interferents and matrix interferences characteristic of 1,25-(OH)₂D₃. The presence of Supported Liquid-Liquid Extraction (SLLE) and Protein Precipitation Filter (PPF) sample extraction methodologies against a conventional protein precipitation technique without derivatization, all of which are suitable for high throughput reliable LC-MS/MS analysis of 1,25-(OH)₂D₃. The SLLE (Supported Liquid-Liquid Extraction) in conjunction with protein precipitation, is a protocol which is fast, and avoids the co-extraction of matrix interferences/generation of co-eluting byproducts. The 1,25-(OH)₂D₃ can be eluted using an organic solvent after the serum sample is fully digested. The PPF in plate format makes use of flow-through filter microplates with traditional protein precipitation techniques without leakage of organic solvent.

EXPERIMENTAL METHODS
Sample preparation methods were evaluated using an IONICS 3Q 320 triple quadrupole mass spectrometer. Direct detection of the amminod acid is used to enhance the ionization efficiency. Because the 3Q 320 is capable of ultra sensitive detection of 1,25-(OH)₂D₃ (OH)₂D₃), was purchased from Cerilliant (Round Rock, TX). Vitamin D free serum was purchased from Gemini Biochemicals (Torrance, CA). All the solvents are HPLC grade.

Sample Extraction Methods:
Method 1: utilizing Orochem’s Aquamix Supported Liquid-Liquid Extraction (SLLE) plate (using dithiothreitol as the support) and 400 µg/mL:
• Pre-mix 100 µL of serum sample and 100 µL of 50:50 water/IPA, vortex for 30 seconds
• Load the pre-mix solution into the SLLE plate, wait for 5 minutes
• Add 1 mL of ethyl acetate to the SLLE plate, wait for 5 minutes, and apply positive pressure to elute the analyte
• Evaporate the elution solution at 37°C under nitrogen flow to dryness and reconstitute it with 100 µL mobile phase

Method 2: utilizing Orochem’s RubyPro 96-Well Protein Precipitation Filter (PPF) plate:
• Add 30 µL of Vitamin D commercial precipitation reagent
• Load 100 µL of serum sample, wait for 5 minutes, then apply positive pressure to elute the analyte
• Evaporate the elution solution at 37°C under nitrogen flow to dryness and reconstitute it with 100 µL mobile phase

Method 3: using a conventional Protein Precipitation (PPF) technique:
Mix 100 µL of serum with 200 µL of acetonitrile, vortex for 10 minutes
Centrifuge for 15 minutes, 1,300 g, 4°C
Transfer and inject the supernatant directly into the LC-MS/MS system

RESULTS
Comparison of Sample Extraction Methods

Recovery rate, matrix effect and process efficiency of protein precipitation (PPT), supported liquid-liquid extraction (SLLE) and protein precipitation filter (PPF) were surveyed for a set of samples by measuring the difference between a) human serum spiked with standard, followed by the sample preparation method “Pre-Spiked” and b) human serum that has undergone the sample prep method, and then spiked with 1,25-dihydroxyvitamin D₃. This is referred to as the ratio of the “Post-Spiked” to “Pre-Spiked”.

In a similar manner, matrix effect is the ratio of the “Post-Spiked” with a neat standard, and the process efficiency is the ratio of the “Post-Spiked” with neat standard.

Chromatograms using the SLLE method are shown in Figure 1 and results for all methods are shown in Table 2. The principle of the PPF and PPT methods is similar and are expected to produce similar results. The SLLE extraction method, more effective at removing phospholipids, yields less matrix effect as expected. Overall the extraction performance is comparable with the largest sensitivity is about 40%.

Table 1: LC chromatographic elution time program

Table 2: Extraction Performance Results for 1,25-(OH)₂D₃

Figure 1: Comparison of supported liquid-liquid extraction method to (a) "Pre-Spiked" and (b) "Post-Spiked" sample, showing a recovery rate of 70%.

SUMMARY
The present study demonstrates direct quantification of 1,25(OH)₂D₃ using a highly sensitive LC-MS/MS triple quadrupole mass spectrometer (IONICS 3Q 320). A simple PPT method for the quantitation of 1,25(OH)₂D₃ in serum or plasma provides an LOQ of 5 pg/mL, and a linear range from 5 to 50,000 pg/mL. Excellent recovery rates of 70-100% were observed for a number of sample preparation methods, improving to 100% for 97.7 pg/mL, for different samples. The plate format of the SLLE and PPF methods render them compatible with robotic liquid handling devices and expected to facilitate the adoption of automated protocols which would be a viable alternative to traditional liquid-liquid and protein precipitation extraction techniques for high throughput requirements.

REFERENCE:

Calibration Curve and Limit of Quantitation

A calibration curve using "post-spiked" serum was generated for 434/363 MRM transition using 1x standards, as shown in Figure 3. Linearity of R² = 0.999 was obtained for a concentration range of 5 to 50,000 pg/mL, with an accuracy of 82.6 to 111.9%. The LOQ obtained is 5 pg/mL and corresponding CV at LOQ is less than 16.1%.

Figure 3: Standard calibration curve for MRM transition of 434/363 across a concentration range of 0.1-50,000 pg/mL.