Direct Quantitation of 1,25-$(OH)_2$ Vitamin D_3 in Serum Using a Highly Sensitive LC-MS/MS

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INTRODUCTION

LC-MS/MS has emerged as a reliable quantitation 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-hydroxy (OH) vitamin D. Further hydroxylation takes place in the kidney to yield 1,25-dihydroxy vitamin D (1,25-$(OH)_2$-VD). 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 to the low pg/mL level. Most current LC-MS/MS method require derivatization of 1,25-$(OH)_2$-VD to improve the ionization efficiency, and therefore enhancing sensitivity.[1,2] However, direct analysis of 1,25-$(OH)_2$-VD using a highly sensitive IONICS 3Q 320 triple quadrupole mass spectrometer simplifies the process by eliminating the need for derivatization. Direct analysis of 1,25-$(OH)_2$-VD by LC-MS/MS is however susceptible to endogenous interferences from serum due to the hydrophobic character of 1,25-$(OH)_2$-VD. The present study evaluates 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-MSMS analysis of 1,25-$(OH)_2$-VD. The SLLE (Supported Liquid-Liquid Extraction) in plate format is packed with flux calcined diatomaceous earth which is chemically inert but adsorbs sample load. The 1,25-$(OH)_2$-VD can be eluted using an organic solvent after the serum sample is fully adsorbed. 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 ammonium adduct is used to enhance the ionization efficiency. Because the 3Q 320 is capable of ultra-sensitive detection, no additional derivatization is required. 1,25 $(OH)_2$-VD_3 was purchased from Cerilliant (Round Rock, TX). Vitamin D free serum was purchased from Golden West Biologicals (Temecula, CA). All the solvents are HPLC grade.

Sample Extraction Methods:

Method 1 utilizing Orochem's Aquamatirx Supported Liquid-Liquid Extraction (SLLE) plate (using diatomaceous earth as the support) and 400 mg/well:

- Pre-mix 100 µL of serum sample and 100 µL of 50:50 water:IPA, vortex for 30 seconds
- Load the pre-mix solution onto 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 100µL mobile phase

Method 2 utilizing Orochem's RubyPro 96-Well Protein Precipitation Filter (PPF) plate:

- Add 300 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 100µL mobile phase

Method 3 using a conventional Protein Precipitation (PPT) technique:

- Mix 100µL of serum with 200µL of acetonitrile, vortex for 10 minutes
- Centrifuge for 15 minutes
- Transfer and inject the supernatant directly into the LC-MS/MS system

Time (min)	Flow (µL/min)	Solvent B %
0.1	600	10
0.7	600	65
4.5	600	98
4.7	600	98
4.8	600	10
6.0	600	20

Table 1: LC chromatographic elution time program

LC Conditions:

The serum samples were injected and separated using a Shimadzu Nexera system (Kyoto, Japan) using the gradient shown in Table 1 and the following LC conditions:
Column: Waters BEH C18 (50 x 2.1, 1.7 µm)
Mobile phase:
A: H_2O with 0.1% formic acid & 10mM NH_4OAc
B: MeOH with 0.1% formic acid & 10mM NH_4OAc
Flow Rate: 0.6 mL/min
Injection Volume: 50 µL
Column Temperature: 30°C

MS Conditions:

MS/MS sensitivity of protonated (m/z=417, $[M+H]^+$) and ammonium adduct (m/z=434, $[M+NH_4]^+$) precursor ions was investigated. Both the protonated and ammonium adduct precursor ions were present in electrospray. However, the ammonium adduct precursor ion was more intense than that of protonated precursor ion and was therefore chosen as the precursor ion for the quantitation analysis. The m/z of 363 (or loss of three water fragment ion) was selected for quantification calculations and extraction efficiency evaluation. Positive mode ESI at 5000V was used for the multiple reaction monitoring (MRM) data collection. Instrument parameters including collision energy had been optimized using the required standards.

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 plate (PPF) were surveyed for a set of samples by measuring the difference between a) human serum spiked with standard, followed by the sample prep method "Pre-Spiked"; and b) human serum that has undergone the sample prep method, and then spiked with standard "Post-Spiked". The recovery rate is defined as the ratio of the sensitivity of "Pre-Spiked" and "Post-Spiked". Similarly the matrix effect is the ratio of the "Post-Spiked" with a neat standard; and the process efficiency is the ratio of the "Pre-Spiked" with neat standard.[3] 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 are 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 loss due to matrix effect is about 40%.

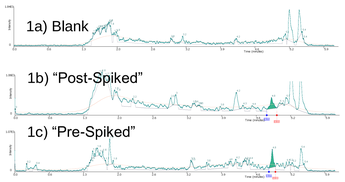

Figure 1: Comparison of supported liquid-liquid extraction method a) Blank b) "Pre-Spiked" and b) "Post-Spiked" sample, showing a recovery rate of 70%.

%	SLLE	PPF	PPT
Recovery rate	71.0	112.2	79.9
Matrix effect	89.8	57.1	68.2
Process efficiency	63.7	64.1	70.5

Table 2: Extraction Performance Results for 1,25-$(OH)_2$-VD_3

Pre-Spiked and Post Spiked PPT LC-MS/MS

The 1,25-$(OH)_2$-VD_3 LC-MS/MS chromatograms displayed in Figure 2 show results of a "Pre-Spiked" and "Post-Spiked" assay using PPT. The ratio of the integrated intensity demonstrates a recovery rate of 80%, displaying little loss due to the PPT extraction process.

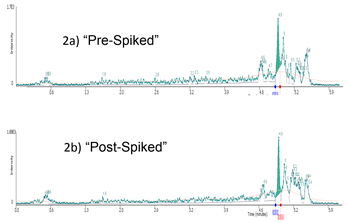

Figure 2: Comparison of the conventional protein precipitation method of a) "Pre-Spiked" and b) "Post-Spiked" sample, showing a recovery rate of 80%.

Calibration Curve and Limit of Quantitation:

A calibration curve using "post-spiked" serum was generated for 434/363 MRM transition using 1/x weighting , as shown in Figure 3. Linearity of R^2 > 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%.

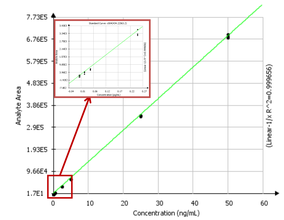

Figure 3: Standard calibration curve for MRM transition of 434/363 m/z over a concentration range of 5-50,000pg/mL.

SUMMARY

The present study demonstrates direct quantitation of 1,25-$(OH)_2$-Vitamin D_3 using a highly sensitive LC-MS/MS triple quadrupole mass spectrometer (IONICS 3Q 320). A simple PPT method for the determination of 1,25-$(OH)_2$-VD_3 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, suggesting an LOQ of 6-7 pg/mL for clinical samples. The plate format of the SLLE and PPF methods render them compatible with robotic liquid handling devices and are expected to facilitate the adoption of automated extraction protocols which would be a viable alternative to traditional liquid-liquid and protein precipitation extraction techniques for high throughput requirements.

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