Direct Quantitation of 1α,25-dihydroxyvitamin D_3 in Serum using a Highly Sensitive LC-MS/MS System

Quick Facts:

- Highly sensitive method for quantitation of underivatized 1α,25-dihydroxyvitamin D_3 using IONICS 3Q 320 triple quadrupole mass spec.
- Calibration curve generated with R > 0.997 over the concentration range from 5 to 50,000pg/mL
- Quantitation as low as 5pg/mL

1. Introduction

Vitamin D (VD) promotes intestinal calcium absorption, bone matrix mineralization, normal muscle function and regulates absorption of phosphorus and suppresses PTH release from the parathyroid gland. Vitamin D is rapidly metabolized in the liver to form 25-hydroxy (OH) vitamin D. Further hydroxylation takes place in the kidney to yield 1α,25-dihydroxyvitamin D (1α,25-$(OH)_2$-VD). This biologically active form of vitamin D, circulates in the blood at a very low concentration range, typically to the low pg/mL level and plays a role in numerous disease pathways. Most current LC-MS/MS methods require derivatization of 1α,25-$(OH)_2$-VD to improve the ionization efficiency, and therefore enhancing sensitivity.[1,2] However, direct analysis of 1α,25-$(OH)_2$-VD using a highly sensitive LC-MS/MS system simplifies the process by eliminating the need for derivatization. This study provides a much simplified approach for the direct quantitation of 1α,25-$(OH)_2$-VD_3, without the need for derivatization.

2. Sample Preparation Method

1α,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. Protein precipitation method was used by mixing one volume of serum with two volumes of acetonitrile. The mixture was vortexed for 10 minutes followed by centrifugation for 15 minutes. The supernatant was transferred and injected directly into the LC-MS/MS system.

2.1 Mass Spectrometry Conditions

Measurements are made using an IONICS 3Q 320 triple quadrupole mass spectrometer. The 3Q 320 has a patented HSID interface which efficiently samples ions while removing chemical interferences and small particles, providing high sensitivity, and maintaining an exceptionally low noise level. Optimized parameters are shown in **Tables 1** and **2**.

Table 1: 3Q 320 Instrument Settings

ESI Voltage (V)	5000
HSID Temp (°C)	175
Nebulizer Gas Setting	450
Drying Gas Setting	120
Source Temp (°C)	350
Dwell Time (ms)	50
Pause Time (ms)	5

Table 2: Optimized MRM Parameters

Compound Name	Precursor	Fragment	CCL2	CE
1α,25-$(OH)_2$-VD_3	434.2	399.2	-50	-15
	434.2	381.2	-50	-15
	434.2	363.2	-50	-17
	434.2	245.1	-50	-20
	434.2	135.1	-50	-24

2.2 LC Conditions

This method utilized a Shimadzu Nexera LC system. Sample volume of 50µL was loaded onto an ACQUITY UPLC BEH C18 (50x2.1, 1.7µm) column using the gradient as shown below in **Table 3** at a flow rate of 0.6mL/min with the column temperature set at 30°C. The composition of the two mobile phases was: Mobile phase A: H_2O with 0.1% Formic Acid, 10mM NH_4OAc; Mobile phase B: MeOH with 0.1% Formic Acid, 10mM NH_4OAc.

Table 3: LC Cycle Time

Time (min)	Solvent B %	Time (min)	Solvent B %
0.0	10	**4.8**	10
0.7	65	**6.0**	10
4.5	98		

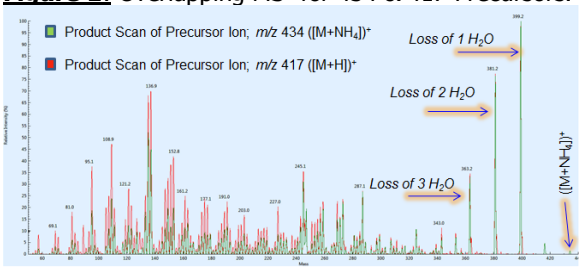
3. Results

3.1 Precursor and Fragment Ion Selection

It was found that both the protonated (m/z=417, $[M+H]^+$) and more intense ammonium adduct (m/z=434, $[M+NH_4]^+$) precursor ions are present in

electrospray. MS/MS scan for both precursor ions are rich and almost identical as shown in **Figure 1**.

Figure 1: Overlapping MS^2 for 434 & 417 Precursors.

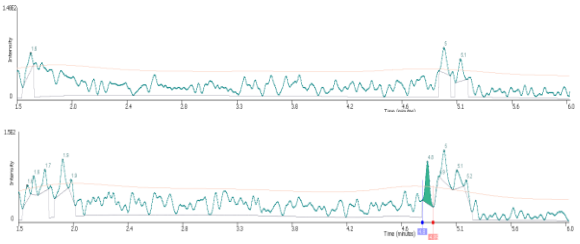


The most intense fragments were the loss of one and two water fragment ions. However, these fragments were found to have relatively high interfering background noise (>500 cps) at the retention time of 1α,25-$(OH)_2$-VD_3. The m/z of 363 (loss of three water fragment ions) was selected for quantitation.

3.2 Extracted Ion Chromatograms (EICs)

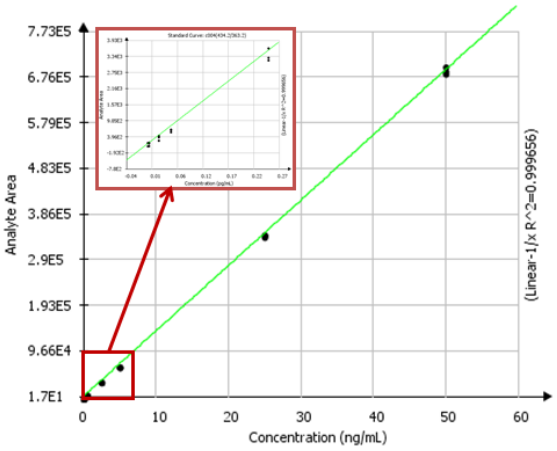
EIC results for 1α,25-$(OH)_2$-VD_3 at blank and 5pg/mL concentration in human serum are illustrated in **Figure 2A** and **2B**, respectively.

Figure 2: A) Reference blank. B) EIC of 1α,25-$(OH)_2$-VD_3 at 5 pg/mL in human serum.



3.3 Linearity

Figure 3: Calibration Curves for 1α,25-$(OH)_2$-VD_3.



The calibration curves generated for 434.2/363.2 transitions using 1/x weighting are shown in **Figure 3**. Linearity (with R^2 > 0.999) was obtained for a concentration range of 5 to 50,000pg/mL with an accuracy of 82.6 to 111.9%.

3.4 Reproducibility

Reproducibility and reliability of the LC-MS/MS run were also accessed. Table 4 shows intra-day assay and inter-day assay variability results. The intra-day assay variability is determined by processing 4 replicates of two QC samples over consecutive days, and the inter-day assay variability is determined with 4 replicates in 3 batches throughout one day.

Table 4: Inter-Day and intra-day assay CVs.

Compound	Conc. (pg/mL)	Intraday imprecision	Interday imprecision
1α,25-OH-VD_3	50	8.1	9.3
	1000	2.6	7.0

4. Conclusion

The present study demonstrates direct quantification of 1α,25-$(OH)_2$-VD_3 using a highly sensitive LC-MS/MS triple quadrupole mass spectrometer (IONICS 3Q 320). A simple protein precipitation method for the determination of 1α,25-$(OH)_2$-VD_3 in serum or plasma provides an LOQ of 5 pg/mL and a linear range from approximately 5 to 50,000 pg/mL. The simplified sample preparation makes this method ideal for routine clinical analysis of 1α,25-dihydroxyvitamin D.

5. References

1. Aronov PA, Hall LM, Dettmer K, Stephensen CB, Hammock BD. Anal Bioanal Chem 2008;391:1917–30.
2. Vreeken RJ, Honing M, van Baar BL, Ghijsen RT, de Jong GJ, Brinkman UA. Biol Mass Spectrom 1993;22:621–32.

6. Contact Information

To learn more about IONICS Mass Spectrometry, our products or services please visit our website or contact us directly.