Quantitative Analysis of Underivatized 1,25-Dihydroxyvitamin D_3 and D_2 in Blood by UHPLC and Triple Quadrupole Mass Spectrometer Utilizing Ion Funnel Technology

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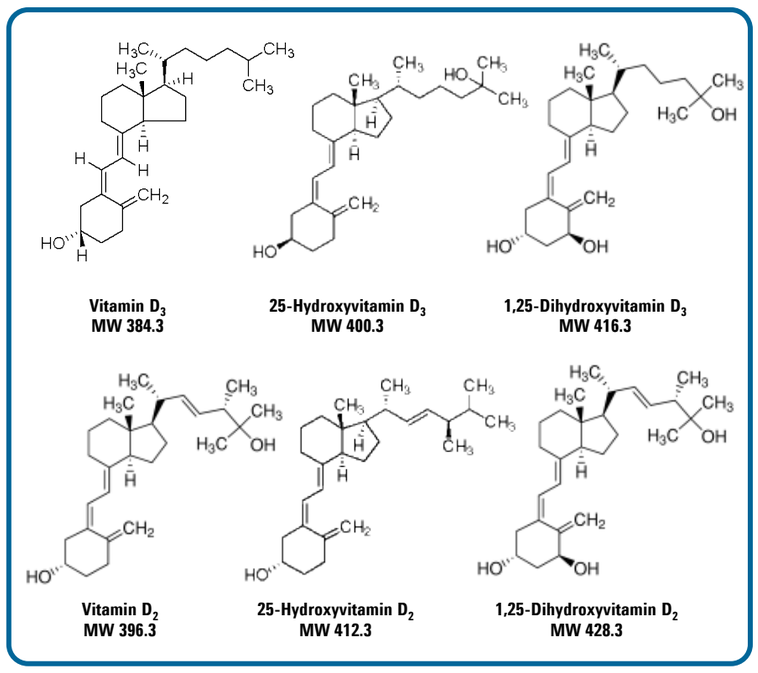
Introduction

1,25-dihydroxyvitamin D exists in two forms – D_2 and D_3 – and while they are not isobaric to each other, both have several isobaric compounds which can be separated through liquid chromatography to achieve accurate quantitation using triple quadrupole mass spectrometry. A highly sensitive, selective and robust LC-MS/MS method for determining the concentration of these two analytes in plasma is a powerful tool for clinical researchers.

Figure 1: Structures



Matrix-stripping liquid chromatography has been implemented in in order to minimize throughput of sample matrix to the mass spectrometer, allowing for increased sensitivity and reproducibility without the addition of any off-line sample preparation.

Experimental

Sample Preparation

Standards: 1,25-(OH)2D3 standards were prepared neat in 1:1, methanol:water with 100 mM ammonium acetate.

Calibration curve: 1-100 pg/mL for neat standards and 5-500 pg/mL for serum extracted standards

Serum samples: 20 µL of IS solution was added to 200 µL of serum sample and vortexed. 380 µL of acetonitrile was added, vortexed for 30 seconds, then incubated at room temperature for 10 min. Samples were centrifuged at 10,000 RPM for 5 min. and 500 µL of supernatant were transferred to autosampler vial for injection to the LC-MS.

LC Method

Agilent 1290 Infinity UHPLC series binary pump, well plate sampler, temperature controlled column compartment with 2 position/6 ports switching valve, 1260 binary pump

Columns
- Loading: Eclipse Plus C18, 4.6x12.5mm 3.5mm
- Analytical: Eclipse Plus C18, 2.1x50mm 1.8mm

Column temperature: 50 °C
Injection volume: 100 µL
Autosampler temp: 4 °C
Needle wash: methanol:water (75:25), 10 sec
Switching Valve: Initial in position 1, at 1 min in position 2, at 12 min in potion 1

Mobile phases
- A = 5 mM ammonium formate in water
- B = 5 mM ammonium formate in methanol

Flow rate: 0.3 mL/min

Gradient

Pump 1: Analytical

Time	Flow	% Solvent B
0.0	0.3	85
12.0	0.3	85
13.0	0.3	85
13.1	0.3	95
15.0	0.3	95
15.3	0.3	50

Pump 2: Loading

Time	Flow	% Solvent B
2.0	0.4	50
2.1	0.1	50
9.0	0.1	50
9.1	0.4	95
13.0	0.4	95
13.1	0.4	50

Experimental

MS Method

Agilent 6490 triple quadrupole mass spectrometer with Agilent JetStream in ion positive mode (ESI).

Gas temperature	130 °C
Drying gas (nitrogen)	20 L/min
Nebulizer gas (nitrogen)	15 psi
Sheath gas (nitrogen)	200 °C
Sheath flow	11 L/min
Capillary voltage	3500 V
Nozzle voltage	300 V
Q1/Q2 Resolution	1.2/0.7 unit
Dwell time	60 msec
Delta EMV	400V
Collision Accelerator	2 V

Multiple Reaction Monitoring (MRM) transition information is listed in Table 1. All precursor ions are ammonium adducts of Vitamin D metabolites.

Table 1. MRM Setup

Compound Name	ISTD?	Prec Ion	Prod Ion	Dwell	CE (V)
1,25-DHVD$_2$-d3	☒	449.3	396.3	60	8
1,25-DHVD$_2$	☐	446.3	411.3	60	6
1,25-DHVD$_2$	☐	446.3	393.3	60	8
1,25-DHVD$_2$	☐	446.3	375.3	60	8
1,25-DHVD$_3$-d3	☒	437.3	384.3	60	8
1,25-DHVD$_3$	☐	434.3	399.3	60	6
1,25-DHVD$_3$	☐	434.3	381.3	60	8
1,25-DHVD$_3$	☐	434.3	363.3	60	8

Figure 2. Ion Funnel Technology

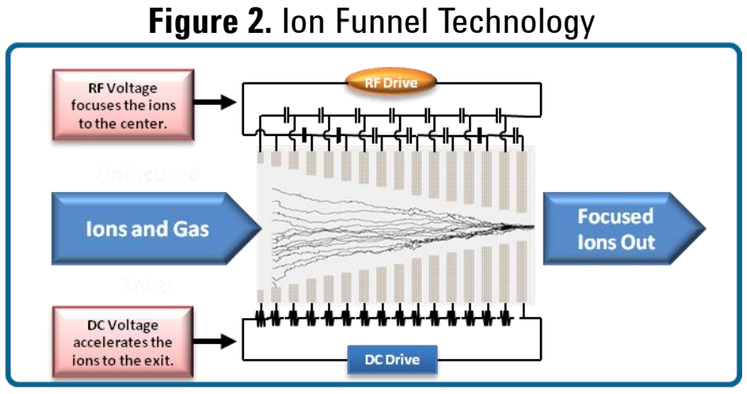
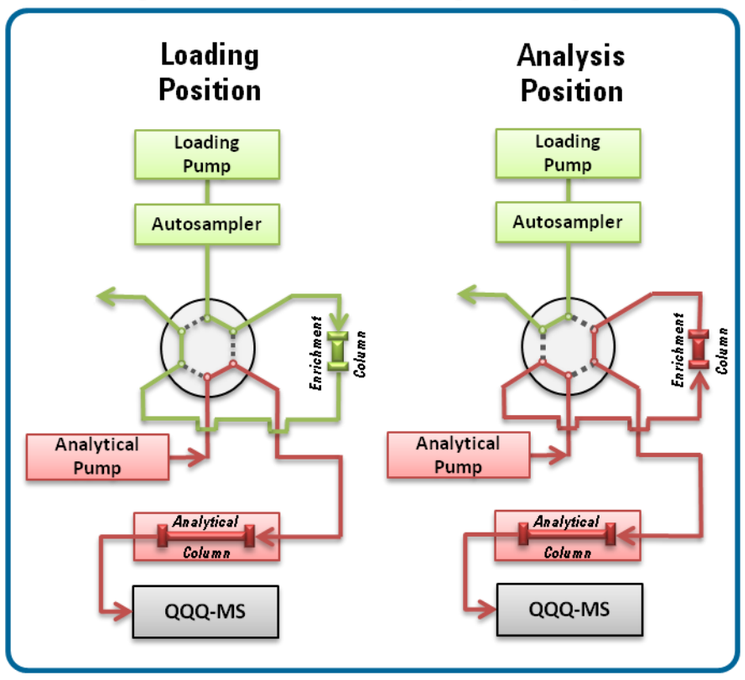


Figure 3. Matrix-Stripping LC Diagram



Results and Discussion

Analysis of 1,25-Dihydroxyvitamin D_3

There is a need for the ability to quantitate 1,25-diydroxyvitamin D_3 at low picogram per milliliters levels. Several existing methods employ sample derivatization, however, in this work an attempt was made to develop a simple method without derivatization. The proposed method utilizes state of the art ion funnel technology (Fig.2) integrated into the Triple Quadrupole Mass Spectrometer (QQQ) in conjunction with best in class, Ultra High Performance Chromatography (UHPLC) system configured in a matrix-stripping setup. Samples are first injected onto an enrichment column to wash away as much matrix as possible. Next, the analytes are eluted onto an analytical column that takes advantage of the chromatographic separation power of sub two micron particles. This results in a limit of detection (LOD) of 1 pg/mL and 10 pg/mL for 1,25-Dihydroxyvitamin D_3 standard in neat solution and extracted biological matrix, respectively.

Signal selection for quantitation

The most intense fragment for the precursor ion, m/z 434 ([M+NH4])+, (Fig.5) was m/z 399 ([M+NH4]-[H2O+NH3])+. However, this fragment had interfering background peaks at the retention time of 1,25-(OH)2D_3.

Results and Discussion

The m/z 381 ([M+NH4]-[2H2O+NH3])+ fragment was about 2 times smaller then m/z 399 fragment but it had no interfering peaks. Since the S/N was equivalent for both fragments(Fig.6), the m/z 381 fragment was selected for quantification calculation. For ion ratio conformation, the m/z 363 ([M+NH4]-[3H2O+NH3])+ fragment was selected.

Limit of Detection (LOD) Determination

The neat standards of 1,25-(OH)2D_3 were dissolved in 50% methanol with 100 mM Ammonium Acetate. The LOD was observed at 1pg/mL (%RSD of 9.99) with 100 µL injction (100fg on column) (Fig.4). In the serum extracted standards, the LOD was observed at 5 pg/mL (500 fg on column) (Fig.4). It was necessary to run a 12 minute gradient in order to separate 1,25-(OH)2-D from its isobaric isomers that were present in the serum extracted samples.

Quantitative Results

Both calibration curves for neat and serum extracted standards had R2 > 0.999, (Fig. 5 & 6).

Figure 4. LOD 1,25-$(OH)_2$-D_3 Neat & Extracted

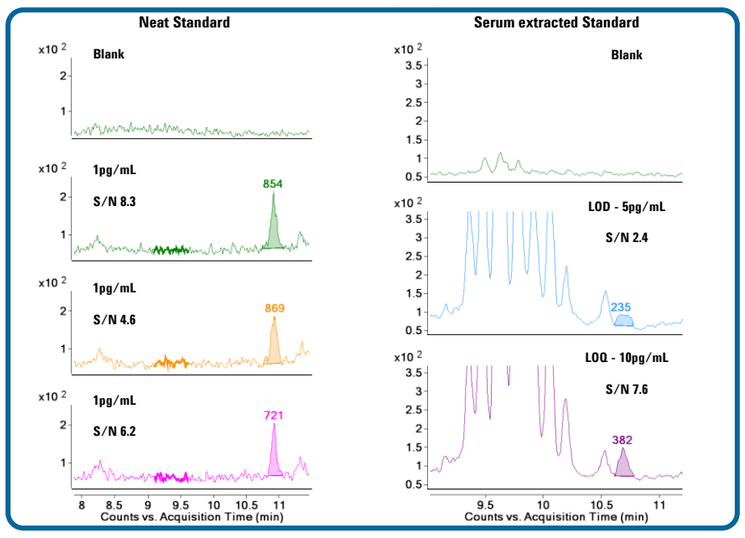
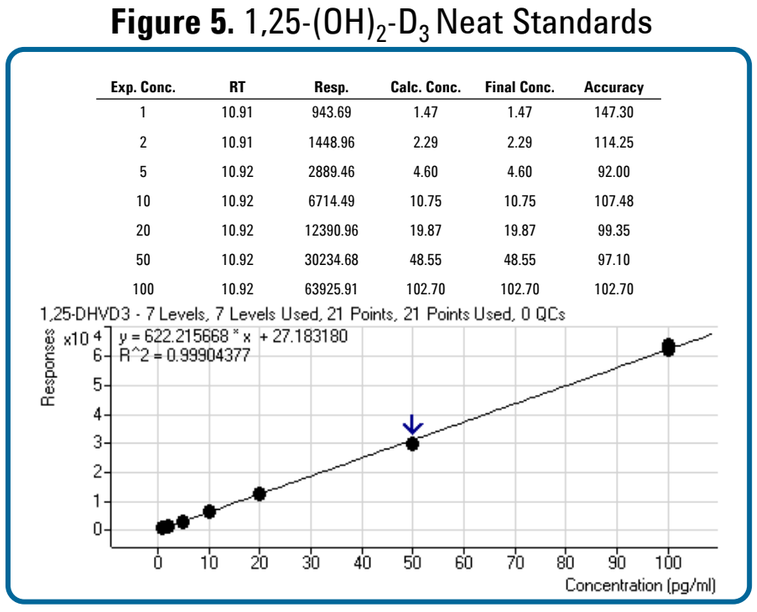
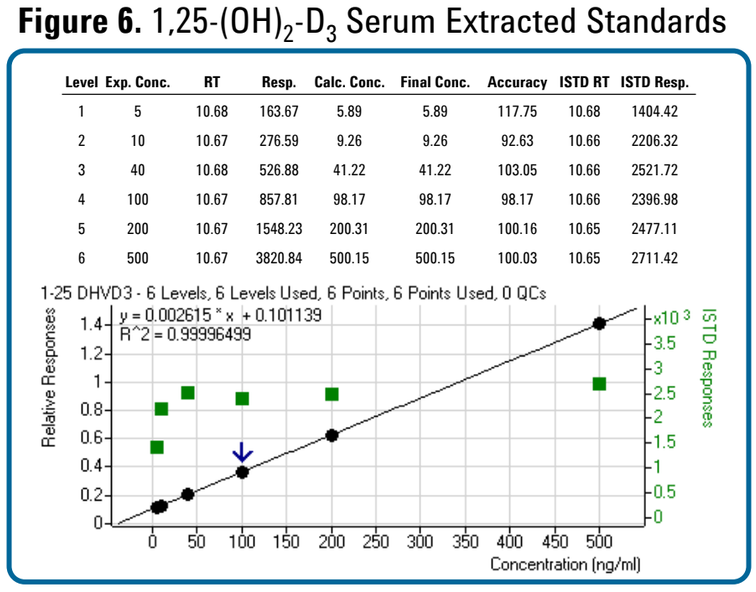


Figure 5. 1,25-$(OH)_2$-D_3 Neat Standards



Exp. Conc.	RT	Resp.	Calc. Conc.	Final Conc.	Accuracy
1	10.91	943.69	1.47	1.47	147.30
2	10.91	1448.96	2.29	2.29	114.25
5	10.92	2889.46	4.60	4.60	92.00
10	10.92	6714.49	10.75	10.75	107.48
20	10.92	12390.96	19.87	19.87	99.35
50	10.92	30234.68	48.55	48.55	97.10
100	10.92	63925.91	102.70	102.70	102.70

Figure 6. 1,25-$(OH)_2$-D_3 Serum Extracted Standards



Level	Exp. Conc.	RT	Resp.	Calc. Conc.	Final Conc.	Accuracy	ISTD RT	ISTD Resp.
1	5	10.68	163.67	5.89	5.89	117.75	10.68	1404.42
2	10	10.67	276.59	9.26	9.26	92.63	10.66	2206.32
3	40	10.68	526.88	41.22	41.22	103.05	10.66	2521.72
4	100	10.67	857.81	98.17	98.17	98.17	10.66	2396.98
5	200	10.67	1548.23	200.31	200.31	100.16	10.65	2477.11
6	500	10.67	3820.84	500.15	500.15	100.03	10.65	2711.42

Conclusions

The limit of detection for underivatized 1,25-Dihydroxvitamin D_3 was observed at 1 pg/mL (100 fg on column) for neat samples and 5 pg/mL for serum extracted sample using ammonium adduct as the precursor ion. Matrix-stripping LC was implemented to help deal with a complex biological matrix. A long chromatographic separation of 12 minutes was necessary to separate 1,25-(OH)2D_3 from isobaric compounds that were present in serum extracted sample. Linearity of calibration curves for both samples were found to have R^2 better then 0.999. Future work will include vitamin D_2 metabolites, more biological samples for statistical analysis and a comparison to analysis of derivatized samples.