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

Vitamin D exists in two major forms – vitamin D_3 and D_2 (Figure 1). Vitamin D_3 is obtained from the diet and produced by the skin after exposure to ultraviolet (UV) light from the sun. Vitamin D_2 is obtained from a diet containing plants and fungi. Vitamin D is first metabolized in the liver to form 25-Hydroxyvitamin D (25-OHD) and subsequently in kidneys to 1,25-Dydroxyvitamin D (Figure 2). The analysis of vitamin D levels in blood is done by the measurement of those two major metabolites, 25-OHD and 1,25-$(OH)_2$D.

Figure 1: Structures

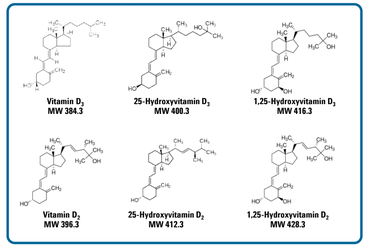
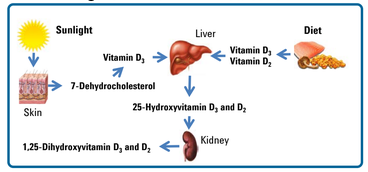


Figure 2: Vitamin D Metabolism



Experimental

Sample Preparation:

Standards: 1,25-$(OH)_2D_3$ standards were prepared neat in 1:1, methanol:water with 100 mM ammonium acetate.

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

Serum sample preparation: 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 (cont.)

Experimental

Sample Preparation(cont.)

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 (1), well plate sampler, thermostatted column compartment with 2 position/6 ports switching valve, 1260 binary pump (2)

Columns

Loading: Eclipse Plus C18, 4.6x12.5mm 3.5µm

Analytical: Eclipse Plus C18, 2.1x50mm 1.8µm

Column temperature: 50 °C

Injection volume: 20 and 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

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

Experimental

Table 1: MRM's Setup

Compound Name	ISTD?	Prec Ion	Prod Ion	Dwell	CE (V)
1,25-DHVD2-3d	☒	449.3	396.3	60	8
1,25-DHVD2	☐	446.3	411.3	60	6
1,25-DHVD2	☐	446.3	393.3	60	8
1,25-DHVD2	☐	446.3	375.3	60	8
1,25-DHVD3-3d	☒	437.3	384.3	60	8
1,25-DHVD3	☐	434.3	399.3	60	6
1,25-DHVD3	☐	434.3	381.3	60	8
1,25-DHVD3	☐	434.3	363.3	60	8

Figure 3: Ion Funnel Technology

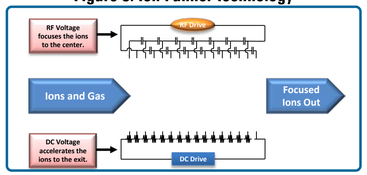
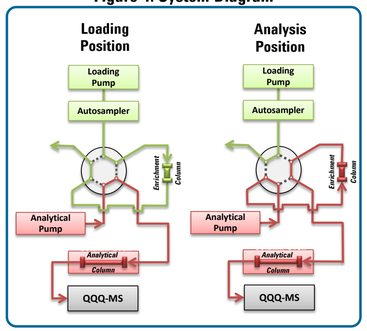


Figure 4: System Diagram



Results and Discussion

Analysis of 1,25-Dihydroxyvitamin D_3

The challenging part of this assay is that in many biological samples, 1,25-Diydroxyvitamin D_3 is in low picogram per milliliters levels and most instruments can not meet this requirement. There are several existing methods that 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.3) integrated into the Triple Quadrupole Mass Spectrometer (QQQ) in conjunction with best in class, Ultra High Performance Chromatography (UHPLC) system, taking advantage of the chromatographic separation power of the column packed with sub two micron particles to achieve the 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 $([M+NH_4]^+$, (Fig.5) was m/z 399 $([M+NH_4]$-$[H_2O+NH_3])^+$. However, this fragment had interfering background peaks at the retention time of 1,25-$(OH)_2D_3$. The m/z 381 $([M+NH_4]$-$[2H_2O+NH_3])^+$ 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 363 m/z $([M+NH_4]$-$[3H_2O+NH_3])^+$ fragment was selected.

Figure 5 : Product Ion Spectra

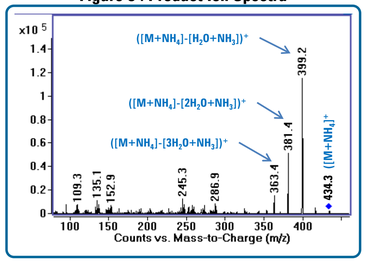
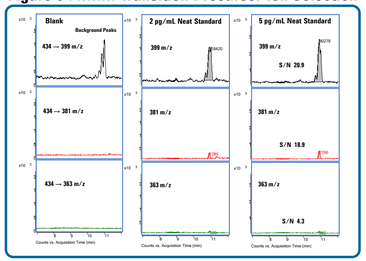
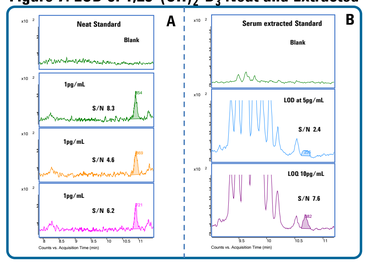


Figure 6 : MRM Transition Precursor Ion Selection



Results and Discussion

Figure 7: LOD of 1,25-$(OH)_2$-D_3 Neat and Extracted



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.7A).

In the serum extracted standards, the LOD was observed at 5 pg/mL (500 fg on column) (Fig.7B). 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. 8 & 9).

Figure 8: Quantitative Results of 1,25-$(OH)_2$-D_3 Neat Standards

1,25 (OH)2-D3 Neat Results

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

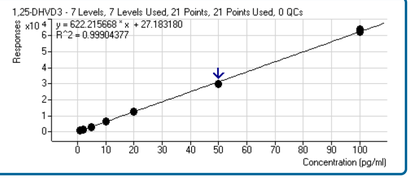
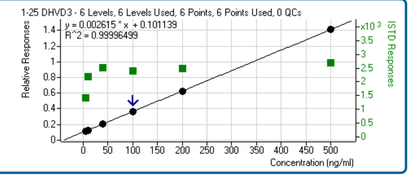


Figure 9: Quantitative Results of 1,25-$(OH)_2$-D_3 Serum Extracted Standards

Sample		1-25 DHVD3 Extracted Results					ISTD Results	
Level	Exp. Conc.	RT	Resp.	Calc. Conc.	Final Conc.	Accuracy	RT	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. Long chromatographic separation of 12 minutes was necessary to separate 1,25-$(OH)_2D_3$ from isobaric isomers 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 D2 metabolites, more biological samples for statistical analysis and a comparison to analysis of derivatized samples.

References

1. A-M. Kissmeyer, K. Sonne, *J. of Chrom. A* 935 (2001) 93-103
2. Z. Maunsell, D.J. Wright, S.J. Rainbow, *Clinical Chem.* 51:9 (2005) 1683-1690
3. R.J. Singh, R.L. Taylor, G.S. Reddy, S.K. Grebe, *J. Clin. Endocrinol. Metab.* 91(8) (2006) 3055-3061

Note: The method described in this presentation is for research purposes only and not approved for diagnostic use