High Throughput Reliable Quantitation of 25-hydroxyvitamin D in Serum by Offline Sample Preparation and a LC-MS/MS Instrument

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INTRODUCTION

Vitamin D is a group of fat-soluble hormones, which have the two major forms: D2 (ergocalciferol) and D3 (cholecalciferol). The metabolites of vitamin D have a critical physiological function to maintain calcium and phosphate homeostasis. Vitamin D deficiency can be best diagnosed using 25(OH) vitamin D versus the other vitamin D metabolites because 25(OH) vitamin D levels in serum reflect the body's storage levels of vitamin D and correlate with the clinical symptoms of vitamin D deficiencies.[1,2] A simple and fast offline sample preparation coupled to a sensitive LC-MS/MS tandem mass spectrometer has been developed to simultaneously measure 25(OH) vitamin D3 and 25(OH) vitamin D2 over a commercial level I to IV analytical concentration range in human serum.

METHOD

Chemicals and Solvents

25(OH) vitamin D was purchased from Sigma (Milwaukee, WI) and vitamin D free human serum was purchased from Golden Western Biologicals (Temecula, CA). Serum level I to IV and Recipe 25(OH) vitamin D quality controls were purchased from IRIS (Olathe, KS). All of the chemicals were stored in the freezer. No IS was used.

Sample Extraction

Sample preparation was carried out with the Orochem (Naperville, IL) PURITY Phospholipid Depletion Kit 96-well plate. The eluting step was performed with an Orochem Ezpress™ positive pressure manifold. Refer to **Table 1** for the steps used.

Table 1: Steps & Procedure

Step	Procedure
Load 1	300 µL of Vitamin D commercial precipitation reagent
Load 2	100 µL of serum sample, wait for 5 minutes,
Elution	apply a few pressure pulse until all solution passes through

Mass Spectrometry Conditions

The LC-MS/MS analysis was performed using IONICS 3Q 220 triple quadrupole mass spectrometer. **Table 2** outlines the MS instrumental source parameter settings. The optimized MRM transition parameters for 25(OH) vitamin D are shown in **Table 3.**

Table 2: MS Conditions

ESI Voltage (V)	5050
HSID Temp (°C)	175
Nebulizer Gas Setting	450
Drying Gas Setting	120
Source Temp (°C)	350

Table 3: Optimized MRM Parameters

Compound Name	Precursor (m/z)	Fragment (m/z)	CCL 2	CE
25(OH) VD3	401.3	257.2	-51	23
	401.3	383.2	-60	13
25(OH) VD2	413.3	355.2	-55	16
	413.3	395.2	-60	13

LC Conditions

Shimadzu UFLCxr system was used with a Imtakt Cadenza C18 –HT (2.1X 50mm) 3 µm particle size column. The LC was run with a gradient flow with a run time of 5min and the following conditions:

Mobile Phase: A (H_2O, 0.1% Formic Acid, 5mM NH_4OAc)
B (MeOH,0.1% Formic Acid, 5mM NH_4OAc)
Flow Rate: 0.6 mL/min
Injection Volume: 10 µL
Column Temperature: 32 °C

Time(min)	0.1	0.5	2.8	3.1	3.2	5
B%	10	70	100	100	10	10

RESULTS

Sample Extraction Results

The extraction recovery rate on samples using PURITY Phospholipid Depletion Kit 96-well plate are about 60 and 65% for 25(OH) VD3 and 25(OH) VD2, respectively. Overall the extraction efficiency is about 50% for serum samples. Summary of the extraction performance is shown in **Table 4**:

Table 4: Sample Extraction Performance.

%	25-(OH)-VD_3	25-(OH)-VD_2
Recovery rate	57.9	64.6
Matrix effect	87.5	74.9
Process efficiency	50.6	48.4

Extracted Ion Chromatograms (EICs)

EIC Chromatogram in serum blank and spiked one with 0.25 and 0.5 ng/mL 25 -Hydroxyvitamin D_3 and D_2 Is shown **in Figure 1a-b** & **2a-b**.

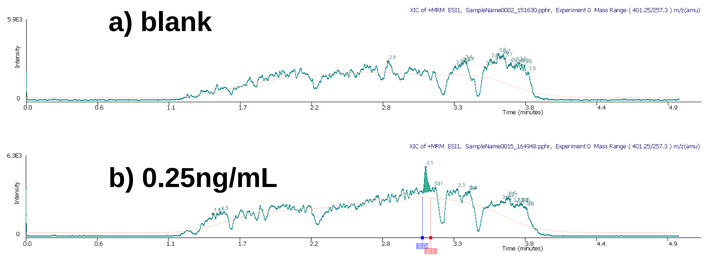

Fig. 1. Chromatograms of 25 -Hydroxyvitamin D_3 for blank and 0.25ng/mL.

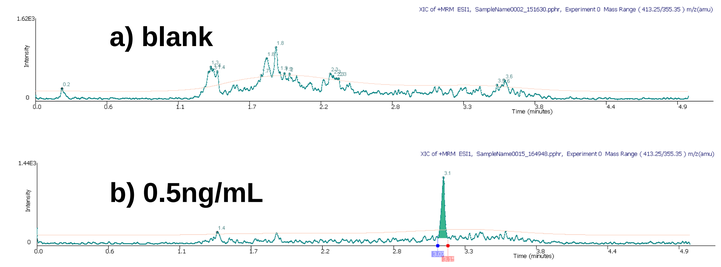

Fig. 2. Chromatograms of 25 -Hydroxyvitamin D_2 for blank and 0.5ng/mL.

Extracted Ion Chromatograms for QC samples

Representative chromatograms of 25 -Hydroxyvitamin D_3 and D_2 for a Recipe level I serum control (20.5 and 16.3 ng/mL, respectively) in this study, are shown in **Figure 3a-b.**

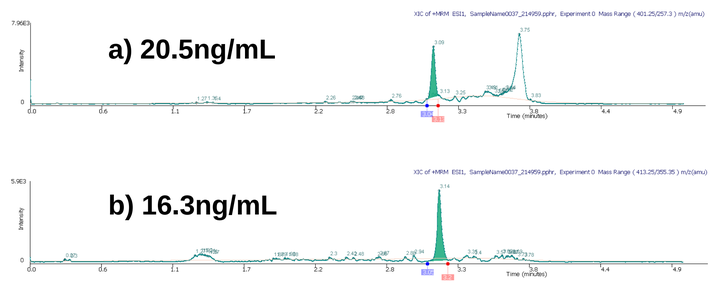

Fig. 3. Chromatograms of 25 -Hydroxyvitamin D quality control level I.

Quantitation Results

The calibration curves generated for 25-hydroxyvitamin D_2 (413.2/355.2) and 25-hydroxyvitamin D_3 (401.3/257.2) show injections which covers a concentration range of nearly 2 orders of magnitude from 1.1 to 73.4 ng/mL for 25-hydroxyvitamin D_3 (413.2/355.2) and from 3.9 to 63.6 for 25-hydroxyvitamin D_2 (401.3/257.2) (**Figure 4a-b**, respectively). The linear regression has a weighting factor, 1/x. Good linearity (R^2>0.994) was found for both analytes. Level I and II Recipe quality controls results with 3 injections were found to be excellent as shown in summary **Table 5**.

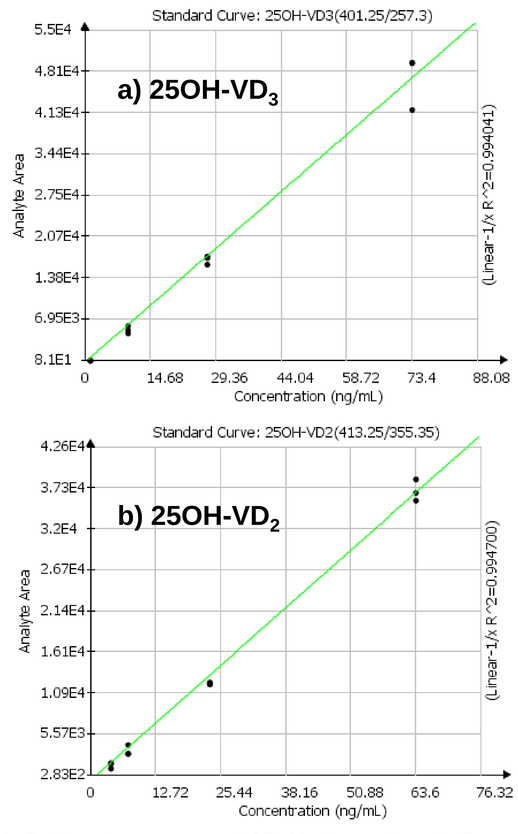

Fig. 4. Calibration curves of 25 -Hydroxyvitamin D_3 and D_2 .

Table 5: Level I and II QCs quantification results (n=3).

25 -Hydroxyvitamin D_3			25 -Hydroxyvitamin D_2		
Conc. (ng/mL)	Avg. accuracy(%)	CV (%)	Conc. (ng/mL)	Avg. accuracy(%)	CV (%)
20.5	92.6	8.3	**16.3**	95.7	4.0
44.3	101.3	1.1	**36.6**	101.0	1.9

CONCLUSION

A 5-min, sensitive, and reliable LC-MS/MS method was developed for quantitative determination of 25(OH) vitamin D in human serum. The LLOQ achieved by IONICS 3Q 220 triple quadrupole mass spectrometer for 25-OH-D_3 and 25-OH-D_2 in human serum are 0.25 and 0.5 ng/mL, respectively. The load, filter two-step simple method showed no signs of interferences. The results show a good linearity and selectivity over level I to IV Recipe calibrators. The offline sample preparation for this LC-MS/MS method is simple and well suited for routine clinical analysis of 25(OH) vitamin D.

REFERENCES

[1] Reinhold Vieth, Am J Clin Nutr 1999;69:842–56.
[2] Robert P. Heaney, Clin J Am Soc Nephrol 3: 1535–1541, 2008.