APPLICATION NOTE

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High Throughput, Reliable Quantitation of 25-hydroxyvitamin D in Serum Using Offline Sample Preparation and PerkinElmer QSight® 220 mass spectrometer

Introduction

Vitamin D is a group of fat-soluble hormones, which have the two major forms: D_2 (ergocalciferol) and D_3 (cholecalciferol). The metabolites of vitamin D have a critical physiological function to maintain calcium and phosphate homeostasis. In order to determine levels vitamin D, both 25-Hydroxy Vitamin D2 and D3 are assessed. 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 D_3 and 25(OH) vitamin D_2 in human serum.

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2. Method

25-hydroxyvitamin D was purchased from Sigma-Aldrich (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.

2.1. 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 & Procedures

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

2.2. Mass Spectrometry Conditions

The LC-MS/MS analysis was performed using the QSight® 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

Quick Facts:

- Quantitative method for 25-Hydroxyvitamin D_2 and D_3 in serum using offline sample prep.
- LLOQs of 0.5 and 0.25 ng/mL for 25-OH D_2 and 25-OH D_3 respectively.
- Simple 2 step method with no interference and excellent linearity.

Table 3: Optimized MRM Parameters

Compound	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.	-55	16
	413.3	395.2	-60	13

2.3. LC Conditions

HPLC separation 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_2$0, 0.1% Formic Acid, 5mM NH_4OAc)
B (MeOH, 0.1% Formic Acid, 5mM NH_4OAc
Flow rate: 0.6mL/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

3. Results

3.1. 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 date	57.9	64.6
Matrix effect	87.5	74.9
Process efficiency	50.6	48.4

3.2. Extracted Ion Chromatograms (EICs) of Analytes

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 Figures 1a-b & 2a-b.

Figure 1: Chromatograms of 25 -Hydroxyvitamin D_3 for blank and 0.25ng/mL

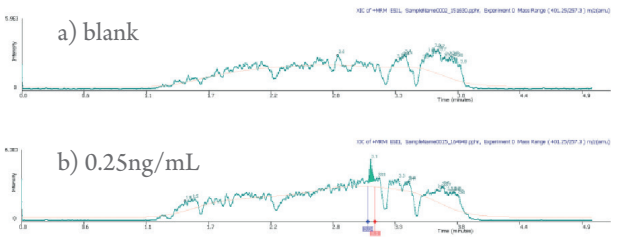
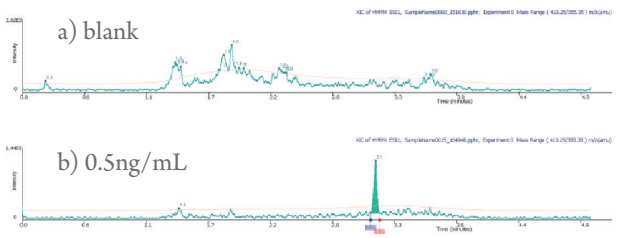


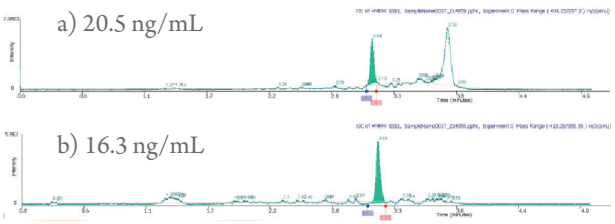
Figure 2:Chromatograms of 25-Hydroxyvitamin D_2 for blank and 0.5ng/mL



3.3. Extracted Ion Chromatograms (EICs) of QC Samples

Representative chromatograms of 25 -Hydroxyvitamin D3 and D2 for a Recipe level I serum control (20.5 and 16.3 ng/mL, respectively) in this study, are shown in Figure 3a-b.

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



3.3. 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 (R2>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.

Figure 4:Calibration curves of 25-Hydroxyvitamin D_2 and D_3

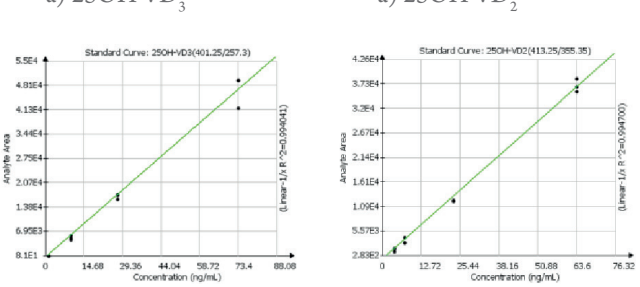


Table 5:Level I and II QCs Quantification Results (n=3)

25-Hydroxyvitamin D_3			25-Hydroxyvitamin D_2		
Cong. (ng/mL)	Avg. accu-racy (%)	CV (%)	Cong. (ng/mL)	Avg. accu-racy (%)	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

4. 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 in human serum using the PerkinElmer QSight® 220 mass spectrometer for 25-OH-D3 and 25-OH-D2 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. This research study demonstrates that offline sample preparation for this LC-MS/MS method is simple and could be suited for analysis of 25(OH) vitamin D.

5. Contact Information

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

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