Extraction of 1,25 di-OH Vitamin D2, 1,25 di-OH Vitamin D3, 25 OH Vitamin D2 and 25 OH Vitamin D3 from serum Using ISOLUTE® SLE+ prior to LC-MS/MS Analysis

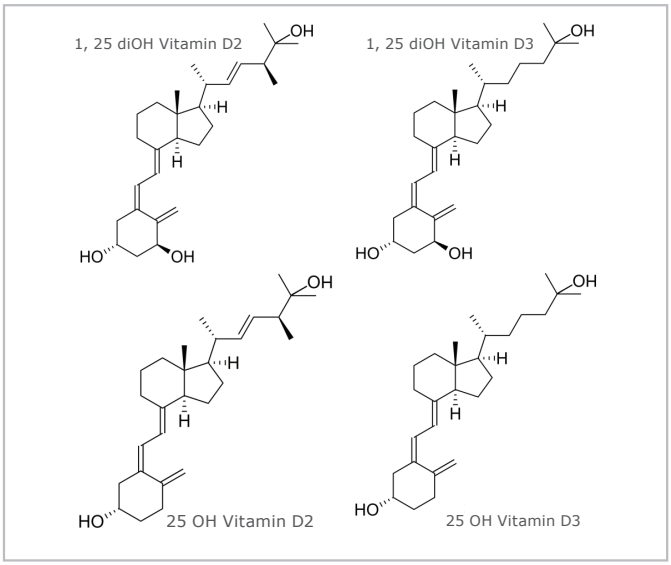

Figure 1. Structure of Vitamin D metabolites

This application note describes a Supported Liquid Extraction (SLE) protocol for the extraction of both forms of 1,25 diOH Vitamin D metabolite and both forms of 25 OH metabolite in serum using ISOLUTE®SLE+ plates with LC-MS/MS detection.

Introduction

The method described in this application note achieves high recoveries of the 1,25 diOH and 25 OH metabolites of vitamins D2 and D3 from serum. The method is versatile enough to measure both the commonly measured 25 hydroxy metabolites and sensitive enough to also measure the better predicting 1,25 dihydroxy metabolites. The method involves SLE extraction followed by a simple PTAD derivatization.

ISOLUTE® SLE+ products provide clean, rapid, robust and efficient extraction solutions for a wide range of analytes.

Analytes

1,25 diOH Vitamin D3, 25 OH Vitamin D3, 1,25 diOH Vitamin D2, 25 OH Vitamin D2

Sample Preparation Procedure

Format: ISOLUTE® SLE+ 400 µL Supported Liquid Extraction Plate, part number 820-0400-P01

Spiking of Calibration Standards: A stock solution containing all four (4) vitamin D analytes was prepared in methanol: water (50:50, v/v) from the individual pure stock standards at a concentration of 500 ng/mL for each analyte. An internal standard solution of D6-25-OH vitamin D3 was prepared at a concentration of 500 ng/mL in methanol: water (50:50, v/v). Two **sets** of "working solutions" were prepared from the stock vitamin D solution and the stock d6-25,OH vitamin D3 solution at concentrations of 10 ng/mL and 5 ng/mL.

A total volume of 300 µL of vitamin D stripped serum (purchased from Golden West Biologicals, Temecula, CA.) was spiked with a calculated aliquot of one of the working standards to give eight (8) calibrant level solutions ranging from 0.01 ng/mL to 1.0 ng/mL. An aliquot of the internal standard of D6-25-OH vitamin D3 was added to each sample at a spiking concentration of 0.4 ng/mL.

Sample Pre-treatment:

1. Calibrant, blanks and patient samples (300 µL) were combined with the required level of internal standard and left to stand for 0.5 hour to reach binding equilibrium.
2. The samples and standards were then combined with no more than 100 µL of water: propan-2-ol (50:50) and left to stand for an additional 15 minutes for consistent protein disruption. Note: addition of water: propan-2-ol should be added in such a manner as to not exceed the 400 µL maximum load volume for the ISOLUTE SLE+ plate.

Supported Liquid Extraction

Sample Loading: Load pre-treated sample (0.4 mL) onto each well. Apply a pulse of vacuum (VacMaster-96 Sample Processing Manifold, **121-9600**) or positive pressure (Pressure+ Positive Pressure Manifold, **PPM-96**) to initiate flow. Allow the sample to absorb for 5 minutes.

Analyte Elution: Elute with heptane, (2 x 0.7 mL) and allow solvent to flow under gravity into a 2 mL collection plate already containing 100 µL of 0.5 mg/mL PTAD solution in ethyl acetate: heptane (8:92, v/v). Apply vacuum or positive pressure to elute any remaining extraction solvent.

Post Elution: Let collection plate sit for 5 minutes at room temperature. Dry the wells in a stream of air or nitrogen using a SPE Dry (40 °C, 20 to 40 L min-1 for 10 minutes) or TurboVap 96 (15 bar at 40 °C for 20 minutes). Reconstitute samples in 100 µL of acetonitrile: water (50:50, v/v).

HPLC Conditions

Instrument: Applied Biosystems 4000 QTrap

Column: Restek Allure PFP Propyl, 5 µm, 50 x 2.1 mm

Mobile Phase: A: Water containing 10% acetonitrile, 0.1% formic acid, 50 µL of methylamine per liter
B: Acetonitrile, containing 50 µL methylamine per liter

Flow Rate 0.8 mL min^{-1}

Injection: 40 µL

Gradient:

Time (min)	% B
0	40
0.5	40
0.6	80
2.0	80
2.1	40
4.0	40

Column Temperature: 25 °C

Sample Temperature: 5 °C

Compound	Retention time (min)
1,25 diOH Vitamin D2	0.92
1,25 diOH Vitamin D3	0.80
25 OH Vitamin D2	1.81
25 OH Vitamin D3	1.63

Table 1. Typical retention times for vitamin D using the LC-MS/MS method described.

MS Conditions

Ions were selected in order to achieve maximum sensitivity using multiple reaction monitoring.

Instrument: Applied Biosystems 4000 Qtrap

Ionization mode ESI+

Source Temperature: 700 °C

MRM transition	Compound ID	DP, V	CE, V	EP, V
635.3 > 314.3	1,25 diOH Vit D2	60	35	10
623.3 > 314.3	1,25 diOH Vit D3	60	27	10
619.2 > 298.4	25 OH Vit D2	60	29	10
607.2 > 298.4	25 OH Vit D3	60	29	10
613.2 > 298.4	D6 25 OH Vit D3	60	28	10

Table 2. Positive Ion Mode - MRM Parameters

Results

The PTAD derivatized vitamin D analytes and deuterated standard were eluted in less than two (2) minutes from the Restek Allure PFP column. **Figure 2** shows a typical extracted ion chromatogram for all four derivatized analytes and the internal standard as single peaks. While the peaks are not fully chromatographically resolved for each analyte, they are fully resolved as groups representing the mono-hydroxy and di-hydroxy vitamin D2/D3. Earlier method development work noted that both of the extracted derivatized mono-hydroxy vitamin D2/D3 analytes gave a significant split peak on several different C18 columns (data not shown here) which made it hard to quantitate accurately. This problem was resolved when analytes were evaluated on the Restek PFP propyl column.

Recovery of analytes from spiked matrix was determined by direct comparison to a fortified blank. The recoveries for all four derivatized analytes ranged from 75.5% to 92% with RSDs ‹10%. Table 3 shows observed recoveries for six (6) individually prepared samples. The quantitation of patient samples can be performed using generated calibration curves derived from extracted calibrants.

An eight point calibration curve was generated from extracted calibrant solution. The calibrant levels for the 1,25 di-hydroxy vitamin D2/D3 analytes were 0.01, 0.02, 0.04, 0.10, 0.20, 0.40, 0.80 and 1.0 ng/mL. Typical calibration curves for the mono and di-hydroxy vitamin D analytes post extraction from stripped serum are shown in **Figure 3** and **Figure 4**. The calibration curves that were generated had linear regression values of r^2 ›0.99 for each curve (**Table 4**). Two in-house quality control standards were prepared and extracted as per protocol at 0.1 and 0.8 ng/mL and verified against the generated calibration curves. Pooled human serum containing endogenous vitamin D was prepared as per sample preparation protocol and was run as “mock patient” samples to determine concentration levels of endogenous target analytes. The mock patient came from two different lots of pooled human serum. Each pooled sample was prepared and extracted in triplicate and quantitatively evaluated against the generated calibration curves. A typical quantitative results table is shown below for 1,25 di-OH Vitamin D3 (**Table 5**).

Analyte	Recovery %	% RSD (n=6)
1,25 diOH Vitamin D2	89.3	5.2
1,25 diOH Vitamin D3	92.0	5.9
25 OH Vitamin D2	75.5	7.5
25 OH Vitamin D3	82.5	5.7

Table 3. Performance and recovery data for vitamin D and metabolites.

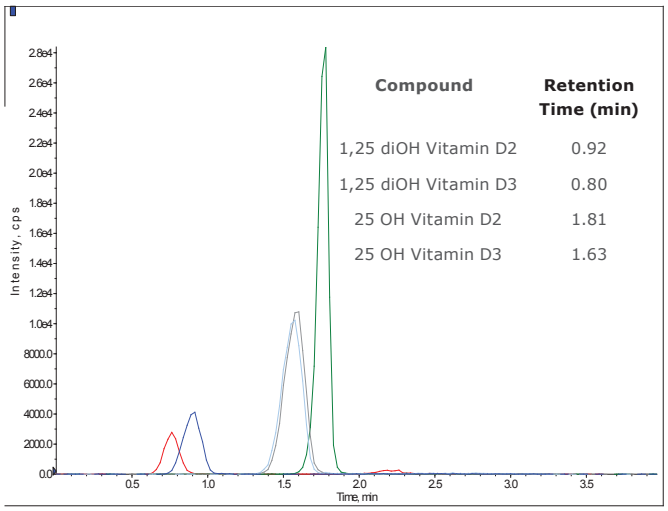

Figure 2. Typical extracted ion chromatogram for all four PTAD derivatized target analytes and the deuterated internal standard at a spike concentration of 5 ng/mL in mobile phase solution.

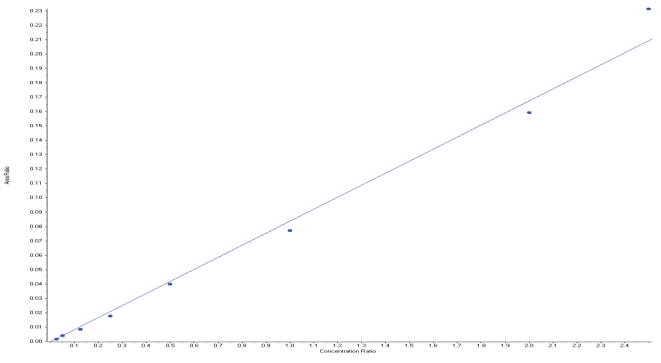

Figure 3. Typical calibration curve for 1,25 di-OH Vitamin D3 expressed on a linear scale.

Analyte	r^2
1,25 diOH Vitamin D2	0.992
1,25 diOH Vitamin D3	0.996
25 OH Vitamin D2	0.999
25 OH Vitamin D3	0.999

Table 4. Summary of calibrant performance extracted from vitamin D stripped serum.

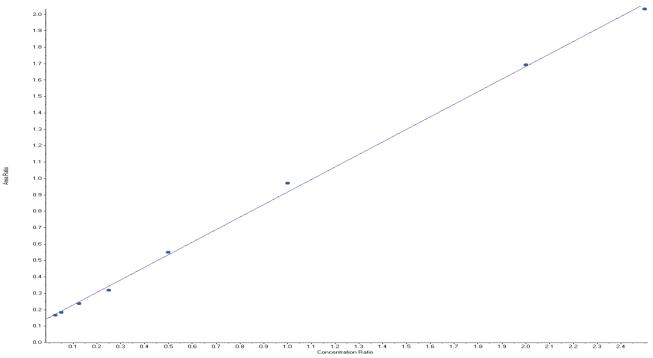

Figure 4. Typical calibration curve for 25, OH Vitamin D3 expressed on a linear scale.

Sample Name	Sample Type	Area (cps)	RT (min)	Target [Conc]. (pg/mL)	[Calculated Conc]. (pg/mL)	Accuracy (%)
0.01ng in Serum	Standard	5.271e+01	0.79	10.0	8.7	87
0.02ng in Serum	Standard	1.467e+02	0.81	20.0	19	96
0.05ng in Serum	Standard	3.037e+02	0.80	50.0	40.0	82
0.10ng in Serum	Standard	6.489e+02	0.79	100.0	85.0	85
0.20ng in Serum	Standard	1.403e+03	0.79	200.0	190.0	95
0.40ng in Serum	Standard	2.699e+03	0.79	400.0	370.0	92
0.80ng in Serum	Standard	5.529e+03	0.79	800.0	760.0	95
1.0ng in Serum	Standard	8.010e+03	0.79	1000.0	1100	111
QC sample (0.1ng)	Q.C.	6.203e+02	0.79	100.0	91	86
QC sample (0.8ng)	Q.C.	4.376e+03	0.80	800.0	650	81
Mock Patient S1a	Unknown	1.187e+03	0.81	N/A	270	-
Mock Patient S1b	Unknown	1.070e+03	0.81	N/A	250	-
Mock Patient S1c	Unknown	1.026e+03	0.81	N/A	250	-
Mock Patient S2a	Unknown	6.458e+02	0.83	N/A	330	-
Mock Patient S2b	Unknown	6.609e+02	0.83	N/A	300	-
Mock Patient S2c	Unknown	6.254e+02	0.82	N/A	320	-

Table 5. Typical quantitative summary table generated for 1,25 di-OH Vitamin D3. Similar tables were generated for each of the analytes extracted from stripped serum using the ISOLUTE® SLE+ plate.

Additional Notes

The PTAD solution was prepared in a heptane rich solvent to encourage mixing with the eluent as it passed through the ISOLUTE® SLE+ well and dissolved water within the solvent to a minimum. PTAD is insoluble in 100% heptane and so it was dissolved in a small volume of ethyl acetate first to give a concentration of 3.125 mg/mL before a subsequent 1 in 12.5 dilution in heptane. The PTAD in heptane solution was prepared fresh immediately prior to use.

When added to the wells and after sample elution the PTAD solution has a pale pink color however this disappears during the incubation period.

Following the evaporation step the wells had the appearance of 'breakthrough' samples with an apparent discoloration being visible at the bottom of the wells. This was due to the PTAD and not due to serum breakthrough, the discoloration being lost upon reconstitution.

The method used additional reagents of methylamine and PTAD. The combined cost by these reagents (based on pricing at the time of writing this method and not allowing for wastage) was approximately US$0.16 per full 96-well plate.

Dropping the organic composition of the reconstitution solvent was found to lower the ability of the analytes to come back into solution. Therefore an organic rich minimum gradient system was used rather than a large gradient which could have suffered from peak fronting.

The use of PTAD and methylamine are essential to the ability of the method to measure the 1,25 diOH metabolites. The four metabolites were measured in equal concentrations using the method detailed here, without methylamine as a mobile phase modifier and without methylamine or a PTAD derivatization step. Relative peak areas are quoted in the table below.

	1,25 diOH Vit D3	1,25 diOH Vit D2	25 OH Vit D3	25 OH Vit D2
No additives	4.1	8.6	4.9	1
PTAD derivatization	64	42	165	55
PTAD & methylamine	144	379	948	632

Phospholipid Removal

Blank serum samples were prepared and extracted as described in the sample preparation procedure (**page 1**). Residual phospholipid levels were investigated to provide an indication of extract cleanliness. We investigated the most abundant phospholipids (selected from full scan, SIR and precursor ion scanning experiments) using MRM transitions monitoring the common 184 product ion. **Figure 5** demonstrates phospholipid content comparing protein precipitated serum and the final extraction protocol.

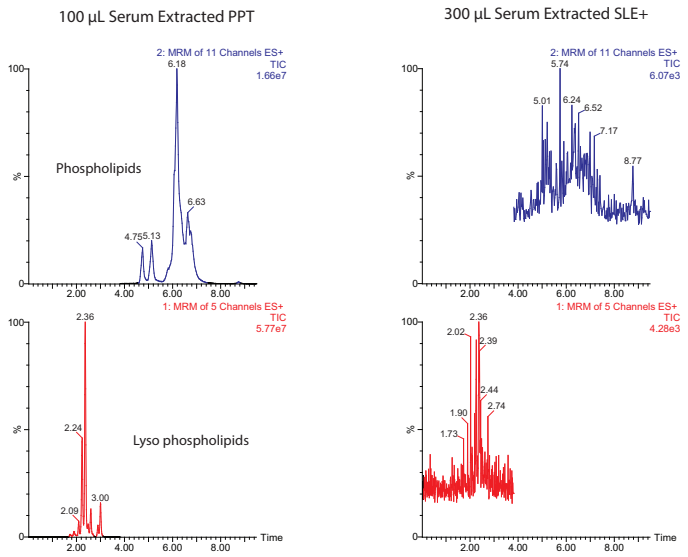

Figure 5. MRM TICs comparing phospholipid content

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE® SLE+ 400 µL Supported Liquid Extraction Plate	1
121-9600	Biotage® VacMaster™ -96 Sample Processing Manifold	1
PPM-96	Biotage® Positive Pressure Manifold 96 position	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1
C103263	TurboVap® 96, 100/120V	1
C103264	TurboVap® 96, 220/240V	1

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