Rapid and Effective Cleanup of Vitamin D2 and D3 from Human Plasma using Phree™ Phospholipid Removal Plates and Kinetex® Core-Shell HPLC/UHPLC Columns

Shahana Huq and Erica Pike Safan
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Introduction

Vitamin D was first discovered when researching rickets, the childhood form of osteomalacia. It was found that populations who have rickets were lacking the compound vitamin D[1]. Further studies have revealed that vitamin D is not scientifically a vitamin. By definition, a vitamin cannot be synthesized in sufficient quantities by an organism and must be consumed through their diet. Vitamin D, however, can be synthesized by most mammals when they are exposed to sunlight. Vitamin D consists of two compounds, vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) which each play a role in the intenstinal absorption of calcium and phosphate. These unique compounds have been widely studied in clinical settings however the link to disease states due to vitamin D deficiencies have shown inconsistent results, prompting a large interest in the research of these compounds.

Vitamin D levels are often analyzed in plasma or serum samples. In high-throughput labs, protein precipitation has become increasingly popular when working with plasma samples because it is rapid and requires little to no method development. However, protein precipitation does not remove phospholipids which cause many negative effects on chromatographic results including ion suppression, reduced HPLC column lifetime, and an increased need for mass spec maintenance due to build up on the mass spec source. More effective cleanup techniques, such as Solid Phase Extraction (SPE), provide much cleaner samples and allow for concentration of the target analyte however SPE requires method development and requires more time as compared to protein precipitation. Recent advances in technology have included phospholipid removal products which produce cleaner samples than a protein precipitation step yet require little to no method development and very little time as compared to SPE. Using this technology, our goal was to develop a rapid cleanup method for vitamin D (25-OH D2 and 25-OH D3) from plasma using Phree Phospholipid Removal Plates that yielded good precision and accuracy yet was sensitive enough to analyze down to the 5 ng/mL level.

Experimental Conditions

Phree Phospholipid Removal Procedure

1. Dispense 300 µL of ACN/MeOH (85:15) into the wells of the Phree plate
2. Add 100 µL of spiked (analyte and internal standard) Human Plasma (stripped) directly into the ACN/MeOH mixture within the wells of the Phree plate
3. Aspirate and dispense the solvent/plasma mixture 2-3x in order to ensure complete mixing of solvent and plasma (or vortex the plate for 2 minutes at maximum possible speed, taking care not to spill the solvent)
4. Wait 30 seconds to allow precipitation to complete
5. Apply vacuum for 1-2 minutes at 15-20" Hg and collect filtrate in a 96-well collection plate
6. Dispense another 200 µL of ACN/MeOH (85:15) then vortex for 2 minutes at the maximum possible speed, taking care not to spill the solvent
7. Apply vacuum for 1-2 minutes at 15-20" Hg and collect filtrate in the same collection plate used in step 5
8. The sample is now ready for direct injection onto the HPLC column

LC/MS/MS Conditions

Column: Kinetex 2.6 µm C18
Dimensions: 30 x 3.0 mm
Part No. : 00A-4462-Y0
Mobile Phase: A: 0.1% Formic acid acid in Water
B: 0.1% Formic acid in methanol
Flow Rate: 0.6 mL/min
Gradient:

Time (min)	% B
0.00	60
0.50	95
2.00	95
2.01	60
3.50	60

Injection Volume: 50 µL
Temperature: Ambient
Detection: API™ 5000 MS/MS, ESI Positive (ESI+)

Gas Parameters	
CUR:	25.0 psi
TEM:	360 °C
GS1:	40.0 psi
Ihe:	ON
CAD:	10.0
NC:	5.0 µA

Note: Gas 2 is not available in APCI source

MRM Transitions and Mass Dependent Parameters

Compound	Q1, Da	Q3, Da	Dwell, msec	DP, V	EP, V	CE, V	CXP, V
Vit D2 (1)	395.3	209.3	45	69	10	25	10
Vit D2 (2)	395.3	269.2	45	69	10	25	10
IS (D3-3H2)	386.2	257.2	45	69	10	25	10
Vit D3 (1)	383.2	257.2	45	69	10	23	10
Vit D3 (2)	383.2	211.1	45	69	10	34	10

Results and Discussion

When analyzing vitamin D2 and D3 from human plasma, phospholipid removal was chosen as the ideal sample preparation method because it was rapid, provided enough cleanup so that column lifetime was not negatively affected, and required very little time and method development to perform. Using phospholipid removal we were also able to avoid a dry down step, which helped to save even more time. Overall, the entire sample preparation procedure using Phree™ Phospholipid Removal Plates took about 4 minutes to complete which is comparable to a protein precipitation procedure.

After extraction, the vitamin D2 and D3 were separated and quantified via LC/MS/MS using a Kinetex® core-shell 2.6 µm C18 HPLC/UHPLC column coupled to an API™ 5000 MS/MS (AB SCIEX, Framingham, MA, USA). The Kinetex core-shell column was selected for this separation because the design of the core-shell particles allows for rapid separation and provides excellent efficiencies as compared to a traditional 3 µm HPLC column.

Stripped human plasma was spiked with standards at a concentration of 75 ng/mL, which was then prepared using the Phree protocol. 50 µL of the resulting eluent was injected directly onto the LC/MS/MS. Vitamin D2 and D3 were measured using appropriate quantitation and confirmation ions for each compound (see MRM transitions) and were separated in under 1.8 minutes (**Figure 1**).

Because we were working with human plasma, it was important to also measure the endogenous levels of vitamin D2 and D3 in order to accurately quantify the spiked vitamin D2 and D3. Blank human plasma samples were subjected to the Phree protocol and 50 µL was directly injected onto the LC/MS/MS to measure the concentration of endogenous vitamin D2 and D3 within our plasma samples. **Figure 2** shows that there is very little vitamin D2 present in the plasma samples however the plasma appeared to contain approximately 2-3 ng/mL of endogenous vitamin D3 which must be accounted for when calculating recoveries in the spiked plasma.

Calibration curves for extracted samples across a concentration range of 5-100 ng/mL showed good linearity with correlation coefficient values of 0.997 and 0.998 for vitamin D2 and vitamin D3, respectively (**Figures 3** and **4**). Precision and accuracy of our analysis was also determined at 25 ng/mL and 75 ng/mL. Both vitamin D2 and vitamin D3 were extracted at accuracies greater than 89 % while the precision (% CV) was less than 5, proving that our method is not only accurate but reproducible (**Tables 1** and **2**).

Figure 1.
Phree Extracted Human Plasma (Stripped) Spiked with Vitamin D2 and D3 at 75 ng/mL by Measuring both the Quantitation Ion (red) and Confirmation Ion (blue).

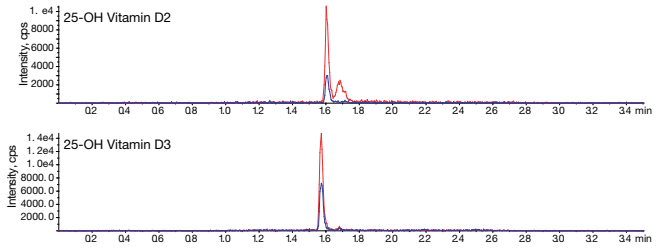

Figure 2.
Endogenous Levels of Vitamin D2 and D3 in Phree Extracted Plasma by Measuring both the Quantitation Ion (red) and Confirmation Ion (blue).

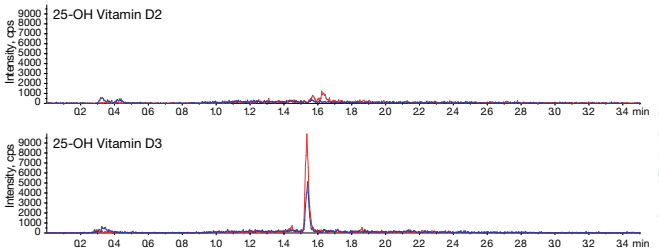

Figure 3.
Phree Extracted Linearity Curve of 25-OH Vitamin D2 from Human Plasma at a Concentration Range of 5 to 100 ng/mL.

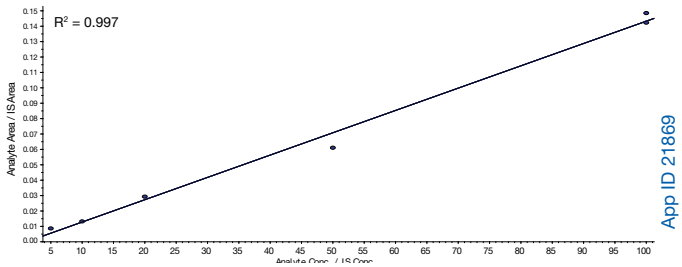

Figure 4.
Phree Extracted Linearity Curve of 25-OH Vitamin D3 from Human Plasma at a Concentration Range of 5 to 100 ng/mL.

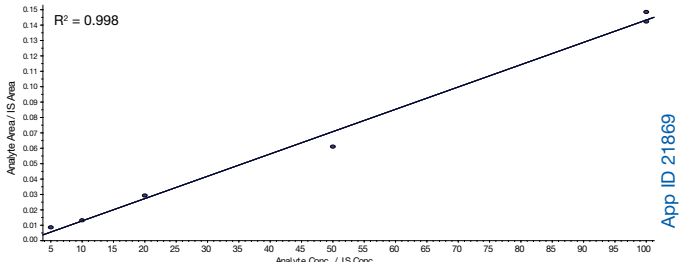

Table 1.
Precision and Accuracy of Phree Extracted 25-OH Vitamin D2 from Plasma.

Expected Concentration (ng/mL)	% CV	Accuracy
25.0	4.767	103.74
75.0	4.610	89.78

Table 2.
Precision and Accuracy of Phree Extracted 25-OH Vitamin D3 from Plasma.

Expected Concentration (ng/mL)	% CV	Accuracy
25.0	3.316	105.77
75.0	3.469	97.82

Conclusion

When analyzing vitamin D2 and D3 from plasma, phospholipid removal using Phree™ Phospholipid Removal plates proved to be the ideal cleanup method because it was rapid, did not require extensive method development and produced acceptable recoveries and precision. Using Phree, the total sample preparation time required was approximately 4 minutes and the cleanup resulted in accuracies greater than 89 % and precisions (% CV) of less than 5 for both vitamin D2 and D3 at concentration levels as low as 25 ng/mL. Linearity of the method was 0.997 and 0.998 for vitamin D2 and D3, respectively, across a concentration range of 5 to 100 ng/mL. Coupled with a rapid separation using a Kinetex® core-shell 2.6 µm C18 HPLC/UHPLC column which was able to resolve both vitamin D2 and D2 in under 1.8 minutes, our method has proven to be rapid, accurate, and reproducible and can be easily implemented into a high-throughput laboratory.

References

1. Wolf G (June 2004). "The discovery of vitamin D: the contribution of Adolf Windaus". *J Nutr* **134** (6): 1299–302.

Ordering Infomation

Phree Phospholipid Removal Products

Part No.	Description	Unit
8B-S133-TAK	Phree Phospholipid Removal 1 mL Tube	100/box
8E-S133-TGB	Phree Phospholipid Removal 96-Well Plates	2/box

Accessories

Collection Plates (deep well, polypropylene)		
AH0-7192	Strata® 96-Well Collection Plate 350 µL/well	50/pk
AH0-7193	Strata 96-Well Collection Plate 1 mL/well	50/pk
AH0-7194	Strata 96-Well Collection Plate 2 mL/well	50/pk
AH0-8635	Strata 96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AH0-8636	Strata 96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AH0-7279	Strata 96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifolds		
AH0-6023*	SPE 12-Position Vacuum Manifold Set, for tubes	ea
AH0-6024*	SPE 24-Position Vacuum Manifold Set, for tubes	ea
AH0-8950	Strata 96-Well Plate Manifold, Universal with Vacuum Gauge	ea

*Manifolds include: Vacuum-tight glass chamber, vacuum gauge assembly, polypropylene lid with gasket, male and female luers and yellow end plugs, stopcock valves, collection rack assemblies, polypropylene needles, lid support legs. Waste container included with 12-positive manifold.

guarantee

If Phree Phospholipid Removal products do not perform as well or better than your current phospholipid removal product, return the product with your comparative data within 45 days for a FULL REFUND.

APPLICATIONS

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: 02 503 4015 (French)
t: 02 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: 09 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

India
t: 040-3012 2400
f: 040-3012 2411
indiainfo@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 001-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Norway
t: 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

Sweden
t: 08 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

United States
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

All other countries:
Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

www.phenomenex.com

Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com

TN13900513_W