Evaluation of 25-hydroxy Vitamin D Extraction using Phospholipid Depletion Plate Technology and Method Comparison using Automated Sample Preparation.

Helen Lodder[1], Kerry Challenger[1], Alan Edgington[1], Lee Williams[1], Rhys Jones[1], Katie-Jo Teehan[1], Adam Senior[1], Geoff Davies[1], Steve Jordan[1], Claire Desbrow[1], Paul Roberts[1], Victor Vandell[2], Elena Gairloch[2]

[1]Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Cardiff, CF82 7TS, UK
[2]Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte North Carolina 28269, USA

Introduction

Vitamin D deficiency can result in various health issues such as osteoporosis, liver and kidney problems and is associated with increased risk of cancers and multiple sclerosis. From this standpoint vitamin D analysis has extremely important clinical relevance. Many sample preparation approaches to the extraction of 25-hydroxy vitamin D have been employed prior to LC-MS/MS analysis. Simple protein precipitation, supported liquid extraction and complex SPE methodology are all in routine use. This poster demonstrates the use of a novel protein and phospholipid depletion plate, for the extraction of 25-hydroxy vitamin D. The extraction protocol was ultimately transferred to an SPE automation platform and method performance versus manual processing was compared.

Experimental

Reagents

Formic acid (FA), ammonium formate and 25-hydroxy vitamin D metabolites and internal standards were purchased from Sigma Chemical Co. (Dorset, UK). Human serum and Bovine serum albumin was obtained through Sera Labs International (West Sussex, UK). 25-hydroxyvitamin D serum calibrators were purchased from Chromsystems (Munich, Germany). Test serum samples were obtained from the DEQAS scheme, Imperial College London. All solvents were HPLC grade from Fisher Scientific (Loughborough, UK) and Milli-Q (Merck Millipore, Germany) water used throughout.

Sample Preparation

ISOLUTE® PLD+ Optimized Procedure (*Figure 1.*)

96-well Plates: ISOLUTE® PLD+ 50mg; P/N: 918-0050-P01.

Apply Solvent: Apply 400 µL of MeCN into the wells.

ISTD: d6 25-OH D3: 30 ng/mL.

Sample Application: Apply 100 µL of serum with 10 µL ISTD and mix thoroughly via repeat aspirate/dispense steps.

Elution: Apply vacuum -0.2 bar or 3 psi positive pressure for approximately 5 minutes. For highly particulate laden samples increased processing conditions may be required.

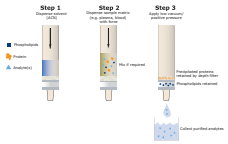

Figure 1. *Schematic of ISOLUTE® PLD+ Phospholipid Depletion Plate Procedure.*

Extrahera™ Automated Sample Preparation Platform

The optimized extracton protocol was transferred to an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. The Extrahera™ platform is shown in ***Figure 2***.

Sample Mixing: Apply 100 µL of serum with 10 µL ISTD and mix thoroughly via repeat aspirate/dispense steps. Heights and volumes were dependent on matrix and precipitant volumes.

Elution: Apply 0.4 bar positive pressure for approximately 5 minutes.

Figure 2. *Picture of the Extrahera™ automated sample preparation platform.*

Post Extraction: Extracts were evaporated using a SPEDry 96 at 40 °C and reconstituted in 100 µL of 30/70% mobile phase.

UPLC Conditons

Instrument: Waters Acquity UPLC (Waters Assoc., Milford, MA, USA).
Column: ACE EXCEL 2 C18-PFP, 100 mm x 2.1 mm id 2 µm, (ACT, UK).
Mobile Phase A: 2 mM Ammonium Formate (aq) with 0.1% FA.
Mobile Phase B: 2 mM Ammonium Formate methanol with 0.1% FA.
Flow Rate: 0.4 mL/min
Gradient: 75-100% B over 3 min; hold 1 min: Resume 75%B at 4 min
Injection Volume: 15 µL
Column Temperature: 40 °C

Mass Spectrometry

Instrument: Quattro Premier XE triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. ***Table 1***. shows the MRM transition details for the quantifier and qualifier ions (parentheses).
Desolvation Temperature: 450 °C
Ion Source Temperature: 150 °C
Collision Gas Pressure: 3.5 x 10^{-3} mbar

Table 1. *Quattro Premier XE mass spectrometer parameters.*

Analyte	MRM Transition	Cone V	Collision Energy eV
25-OH D_2	395.5 > 269.5 (395.5 > 119.2)	30	18 26
25-OH D_3	383.5 > 257.5 (383.5 > 107.2)	30	17 25
d6-25-OH D_3	389.6 > 263.5	30	16

Results

Method development involved optimization of organic solvent composition and crash ratio for efficient precipitation of proteins, phospholipid removal and maximum analyte recovery. Various proportions of MeOH and MeCN with and without 1 % formic acid were investigated. Serum precipitation with MeCN and 1% FA/MeCN in a 1:4 ratio demonstrated recoveries greater than 70 %, while MeOH and MeOH/MeCN solvent combinations demonstrated lower recoveries and higher RSDs. ***Figure 3.*** demonstrates vitamin D recoveries using various combinations of extraction conditions.

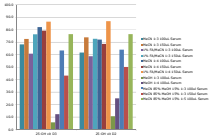

Figure 3. *Recovery comparison using various crash solvents and ratios*

Figure 4. compares protein extraction with a MeCN crash ratio using a forced crash (no mixing) and a vortex mixed crash using the depth filter frit technology. Very little protein was observed in the extracts using a 1:3 serum:MeCN crash ratio or above. MeOH does not provide as good a crash as MeCN.

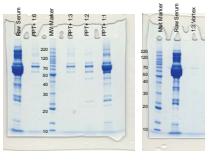

Figure 4. *Gel Electrophoresis protein profiles for ISOLUTE® PLD+ extracts using a forced crash or vortex mixing with MeCN*

The degree of residual phospholipids in the extract for the optimized protocol using 100, 125 and 150 µL of serum is demonstrated in ***Figure 5.*** Phospholipid levels are extremely low when compared to simple protein precipitation techniques. 1% FA/MeCN showed slightly higher PL levels.

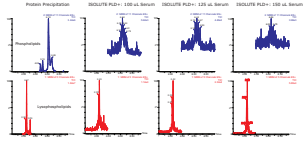

Figure 5. *Phospholipid MRM TIC comparing 100-150 µL serum extractions using ISOLUTE PLD+ to protein precipitation*

The optimized ISOLUTE® PLD+ procedure from a cleanliness and extraction recovery aspect was deemed to be precipitation of 100 or 150uL of serum 1:4 with MeCN. These methods were then transferred to the Extrahera™ automated sample preparation platform. ***Figure 6.*** demonstrates the recovery profile for manual and Extrahera™ processing using 100 and 150 µL of serum.

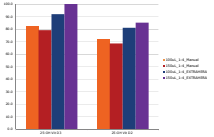

Figure 6. *ISOLUTE® PLD+ recovery comparisons using manual and Extrahera™ processing with 100 or 150 µL of serum*

Calibration curves were constructed extracting 100 µL of PBS/BSA (serum surrogate) from 1-100 ng/mL and are demonstrated in ***Figure 7***. Methods extracting 150 µL of serum or using 1 % formic acid as the crash solvent (1:4 ratio) also returned coefficients of determination > 0.99 (data not shown).

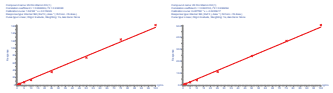

Figure 7. *PBS/BSA Calibration curves for D2 and D3*

Figure 8. demonstrates the chromatography obtained from a Chromsystems calibrated serum sample at 14.8 and 19.6 ng/mL of 25-OH vitamin D2 and D3 respectively.

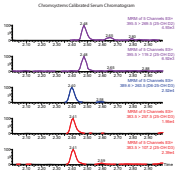

Figure 8. Chromatography for a Chromsystems Calibrated serum sample

The final protocol extracting 100 µL of Chromsystems calibrated serum was then compared using both manual and Extrahera™ processing. Serum calibration lines were constructed from 0-64 ng/mL and are demonstrated in ***Figure 9***.

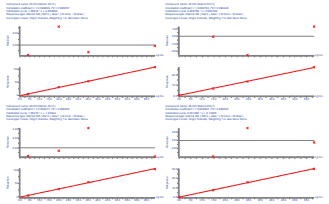

Figure 9. *Chromsystems Calibration curves comparing Manual (top) vs Extrahera™ (bottom) processing using ISOLUTE® PLD+*

Final method testing was performed for 5 DEQAS serum samples. The DEQAS criteria for acceptable performance is that at least 80 % of results should fall within + or – 25 % of the All Laboratory Trimmed Mean. Method performance is shown in ***Table 2***. Units are quoted as ng/mL. All values for both extraction protocols fall within the accepted criteria. The final row of the table contains the results for an unknown pooled patient serum sample (n=10), manually processed compared to automated processing with the Extrahera. Results are shown with % RSD alongside.

Table 2. *DEQAS 25-OH vitamin D results obtained using optimum method.*

DEQAS Sample I.D.	DEQAS LC/MS Mean	PLD+ Manual	PLD+ Extrahera
451	12.9	14.5	13.1
452	46.7	49.1	46.8
453	26.6	28.9	26.6
454	21.4	25.3	21.8
455	22.2	23.7	24.1
Unknown Pooled Serum Sample	-	22.8 ± 7%	24 ± 6%

Conclusion

- This poster demonstrates the applicability of ISOLUTE® PLD+ for the extraction of 25-hydroxy vitamin D from 100 or 150 µL of serum
- Good extraction efficiency, protein and phospholipid removal was afforded by the technique
- Excellent linearity, and r^2 values from PBS/BSA and Chromsystems calibrated serum along with good correlation data to DEQAS reported patient samples
- Successful method implementation was achieved on to the Extrahera™ automated sample preparation platform

ASMS 2015, St Louis
Part Number: P126