

Supported Liquid Extraction of Vitamin D Metabolites: 25-hydroxy and 1 α , 25-dihydroxy Vitamin D2/D3 using PTAD Derivatization prior to LC-MS/MS Analysis.

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Introduction

Vitamin D deficiency can result in various health issues such as osteoporosis, liver and kidney problems and is associated with an increased risk of cancers and multiple sclerosis. From this standpoint vitamin D related analysis has extremely important clinical relevance. Traditional analysis has been of the 25-hydroxy metabolites because of their high concentrations. Optimum prediction of Vitamin D deficiency can be performed by measuring the much lower concentrations of the biologically active 1 α ,25-dihydroxy Vitamin D. This poster presents a novel method for the simultaneous extraction, derivatization and subsequent detection of both the traditional 25-hydroxy and the biologically active 1 α ,25-dihydroxy vitamin D metabolites in serum.

Experimental

Reagents

Formic acid, 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD), methylamine, bovine serum albumin, phosphate buffered saline tablets, metabolites and d₆-25-OH-vitamin D3 internal standard were purchased from Sigma Chemical Co. (Poole, UK). Human serum was obtained through Sera Labs International (West Sussex, UK). IPA, heptane and ethyl acetate were purchased from Fisher Scientific (Loughborough, UK). Methanol, acetonitrile and d₆-1 α , 25-di-OH Vitamin D3 internal standard were purchased from LGC (Shepperton, UK). Milli-Q water was used throughout (Millipore, Watford, UK).

Sample Preparation

Supported Liquid Extraction Procedure (Figure 2.)
Plate: ISOLUTE[®] SLE+ 400 Supported Liquid Extraction Plate, part number 820-0400-P01.

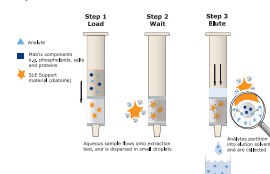
Matrix pre-treatment:

Serum (200 μ L) or spiked substitute matrix (phosphate buffered saline containing 50 mg/mL bovine serum albumin), was spiked with 10 μ L of ISTD solution in water: methanol (50:50). Solutions were left to stand for an hour to equilibrate. Samples were pre-treated with 200 μ L of water:IPA (50:50) to disrupt Vitamin D from its binding sites.

Sample Application: A 300 μ L aliquot was transferred onto the ISOLUTE[®] SLE+ plate. A pulse of positive pressure or vacuum was applied to initiate flow and the samples were left to absorb for 5 minutes.

Analyte Extraction: A 700 μ L aliquot of heptane was applied to the plate and allowed to flow under gravity for 5 minutes. A second 700 μ L aliquot of the heptane was applied to the plate and allowed to flow under gravity for 5 minutes. Vacuum or positive pressure was applied for 10-20 seconds to pull through any remaining solvent. Solvents were collected in a 2 mL collection plate containing 100 μ L of a 0.25 mg/mL solution of PTAD in a mixture of 92/8 heptane/EtOAc.

Figure 1. Schematic of ISOLUTE[®] SLE+ Supported Liquid Extraction procedure.



Post Extraction: The block was capped and shaken in the dark for 2 hours at room temperature (this time can be significantly reduced but may result in slightly lower derivatization). The eluate was then evaporated to dryness at 40 °C and reconstituted in 100 μ L of the t zero mobile phase.

UPLC Conditions

Instrument: Waters Acquity UPLC (Waters Assoc., Milford, MA, USA).

Column: Phenomenex Kinetex 50 x 2.1 mm, 1.7 μ m (Macclesfield, UK).

Mobile Phase: A: Water: Acetonitrile (80:20, v/v) containing 0.1% formic acid and 0.01 mM methylamine

Mobile Phase B: Methanol

Injection Volume: 15 μ L

Flow Rate: 0.4 mL/min

Column Temp: 40 °C

Sample Temp: 15 °C

Table 1. Gradient Parameters.

Time (min)	Mobile Phase (A)	Mobile Phase (B)
0	30	70
2	30	70
3	0	100
4	0	100
4.01	30	70
5	30	70

Mass Spectrometry

Instrument: Quattro Premier XE triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis.

Desolvation Temperature: 450 °C

Ion Source Temperature: 120 °C

Collision Gas Pressure: 3.5 x 10⁻³ mbar

Table 2. Waters Quattro Premier XE parameters.

Analyte	MRM	Cone Voltage (V)	Collision Energy (eV)
1 α ,25 diOH Vit D2	635.3 > 314.3	27	21
1 α ,25 diOH Vit D3	623.3 > 314.3	29	21
d ₆ -1 α ,25 diOH Vit D2	629.3 > 314.3	26	22
25 diOH Vit D2	619.2 > 298.4	28	21
25 diOH Vit D3	607.2 > 298.4	27	21
d ₆ -25 diOH Vit D3	613.2 > 298.4	26	22

Results

Initial investigations focused on addition of the PTAD derivatizing agent into the extraction solvent for the ISOLUTE SLE+ procedure. Data suggested that this was non-optimal due to the protic environment of the loading mixture resulting in incomplete derivatization. As a result PTAD solution was added to the collection plate prior to elution. Derivatization efficiency was found to be solvent dependent and aided from improved mixing with the eluent and a non-polar environment. During method development the hydroxy and the dihydroxy metabolites were found to give significantly different optimum conditions regarding the pre-extraction preparation, the elution solvent volume and the PTAD concentration (shown in **Figure 2**).

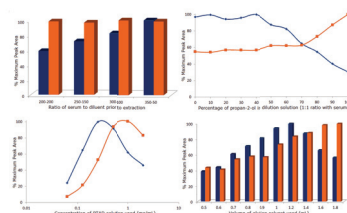


Figure 2. Extraction properties during method development between 1 α ,25 diOH Vit D3 (blue) and 25 OH Vit D3 (orange): matrix dilution (top left), IPA treatment (top right), PTAD levels (bottom left) and total elution volume (bottom right).

Ultimately compromise conditions had to be found that favoured the low level dihydroxy metabolites but still gave an acceptable hydroxy metabolite conversion. Extraction recoveries were calculated as 69% for 1 α ,25 diOH Vitamin D2, 66% for 1 α ,25 diOH Vitamin D3, 89% for OH Vitamin D2 and 84% for OH Vitamin D3 (**Figure 3**).

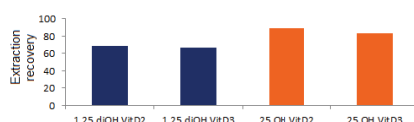


Figure 3. Extraction recoveries with final extraction method.

To provide enhanced sensitivity methylamine was incorporate as a mobile phase modifier (**Figure 4**).

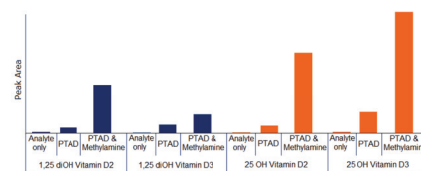


Figure 4. Peak area comparison: no derivatization; PTAD derivatization; PTAD derivatization with methylamine as a mobile phase modifier.

Good chromatographic separation was obtained between the 1 α ,25 diOH Vitamin D3 and the isobaric 24,25 diOH Vitamin D3. Linear calibration lines were recorded for each 1 α ,25 diOH metabolite between 5 and 320pg/mL and for each 25 OH metabolite between 1 and 64ng/mL. Calibrations for the D2 and D3 metabolites in serum are shown in **Figure 5**.

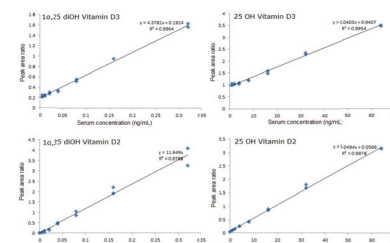


Figure 5. Example calibration lines: Vitamin D metabolites in serum.

To improve the sensitivity of the assay simple method modifications were implemented and tested using an AB SCIEX 4000 QTRAP. The modified extraction procedure involved increased serum volumes of 300 μ L. This was spiked with ISTD, pre-treated with 100 μ L H₂O:IPA (50:50) and 400 μ L loaded on to the plate. Extraction was again performed using 2 x 700 μ L aliquots of heptane. Recoveries greater than 75% were returned with corresponding RSDs below 10%. Calibration data again demonstrated linearity and coefficients of determination > 0.99 for all analytes (data not shown). Final methods were then tested for phospholipid removal from the samples. **Figure 6.** demonstrates excellent PL removal for both methods when compared to a protein precipitated samples.

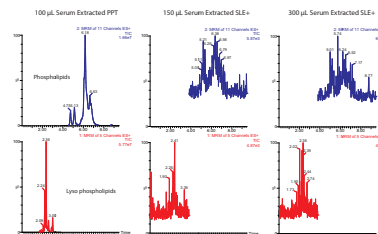


Figure 6. Phospholipid MRM TICs compared to protein precipitated samples.

Conclusion

- This poster demonstrates a simple high sample throughput approach to the extraction of both 1 α ,25-dihydroxy and 25-hydroxy vitamins D2 and D3
- High analyte recoveries and low ion suppression were demonstrated, allowing limits of quantitation at low pg/mL levels for the di-OH metabolites and < 1 ng/mL levels for the OH metabolites
- The method demonstrated good linearity and coefficients of determination from calibration curves
- Excellent PL removal was afforded