

OPTIMIZATION OF SOLID PHASE EXTRACTION FOR THE LC-MS/MS ANALYSIS OF VITAMIN D METABOLITES IN CLINICAL RESEARCH

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INTRODUCTION

Matrix interferences are challenging when analyzing vitamin D metabolites by LC-MS/MS for clinical research. In particular lysophosphatidylcholines (LysoPCs 16:0, 18:1 and 18:0), which have similar hydrophobic properties to 25-hydroxyvitamin D (25OHD), despite being structurally very different, have been shown to cause ion suppression effects in mass spectrometry. This is due to difficulties in removing the LysoPCs during sample preparation and challenges in obtaining chromatographic separation from the vitamin D metabolites.

Here we describe an approach using Waters® Oasis® HLB and Oasis PRiME HLB µElution solid phase extraction (SPE) plates to assess the reduction of such interferences compared to protein precipitation. Chromatographic separation of 25OHD₂, 25OHD₃, 24,25diOHD₃ and C3-epi-25OHD₃ enabled peak area profiles to be compared with targeted LysoPCs for the sample extraction methods. Precision, accuracy and analytical sensitivity testing were performed using an ACQUITY UPLC I-Class Xevo TQ-S micro system to assess feasibility of the optimized method.

METHODS

Materials

- Certified solutions; 25OHD₂, 25OHD₃, 24,25diOHD₃ and C3-epi-25OHD₃ were obtained from IsoSciences (King of Prussia, PA).
- Certified internal standard solutions; [²H₃]-25OHD₂, [²H₃]-25OHD₃, [²H₃]-24,25diOHD₃ and [²H₃]-C3-epi-25OHD₃ were obtained from IsoSciences (King of Prussia, PA).
- Human serum and stripped human serum were obtained from Golden West Biologicals (Temecula, CA).
- EQA samples were obtained from DEQAS (London, UK) and NIST SRM972a samples were obtained from the National Institute of Standards and Technology (Gaithersburg, MD).

Sample Preparation and Analysis

- A pooled serum sample was supplemented with 25OHD₂, 24,25diOHD₃ and C3-epi-25OHD₃ for elution profile testing.
- The stable labeled internal standards were added to 100µL aliquots and protein precipitation performed using a methanol/zinc sulphate_(aq) solution.
- Following centrifugation, the supernatant was transferred to a Waters Oasis HLB or Oasis PRiME HLB µElution plate.
- Elution profile testing was performed by passing different concentrations (0-100%) of methanol_(aq) or acetonitrile_(aq) through each SPE sorbent and the eluate collected for analysis.
- Using a Waters ACQUITY UPLC® I-Class system, precipitated samples or diluted eluate were separated using a 2.1 x 100mm Waters HSS PFP column using a water, methanol, ammonium acetate and formic acid gradient and analyzed on a Waters Xevo TQD using the MRM transitions and precursor scan conditions listed in Table 1.
- To assess whether improvements to the optimized method could allow for a clinical research method to be developed, precision, accuracy and analytical sensitivity were assessed using the following calibration ranges:
25OHD₃ & 25OHD₂: 2.5 — 370nmol/L (1 — 150ng/mL)
24,25diOHD₃ & C3-epi-25OHD₃: 0.5 — 75nmol/L (0.2 — 30ng/mL)
- Samples were extracted and injected onto a Waters ACQUITY UPLC I-Class FTN system and quantified on a Waters Xevo TQ-S micro using the MRM transitions listed in Table 1.

Analyte	Transition (m/z)	Cone (V)	Collision (kV)
25OHD ₃ & C3-epi-25OHD ₃	401.3 > 159.1 (Quan)	20	24
	401.3 > 365.3 (Qual)	20	10
25OHD ₂	413.3 > 355.3 (Quan)	20	10
	413.3 > 83.1 (Qual)	20	24
24,25diOHD ₃	417.3 > 159.1 (Quan)	20	24
	417.3 > 381.3 (Qual)	20	8
LysoPCs	Parents of 184 Scan	20	20

Table 1. MRM transitions and Precursor Scan conditions

RESULTS

Chromatography

Separation of the C3-epi-25OHD from 25OHD was achieved for 25OHD₂ and 25OHD₃. An additional isobaric peak present in both the quantifier and qualifier transitions for 24,25diOHD₃ was also separated and confirmed to be 25,26diOHD₃ using standard addition and confirmed to be 25,26diOHD₃ using standard addition of a 25,26diOHD₃ solution into pooled serum. An example chromatogram is shown in Figure 1 demonstrating a runtime of <8 minutes injection-to-injection, providing a reasonable analysis time for vitamin D metabolites in clinical research.

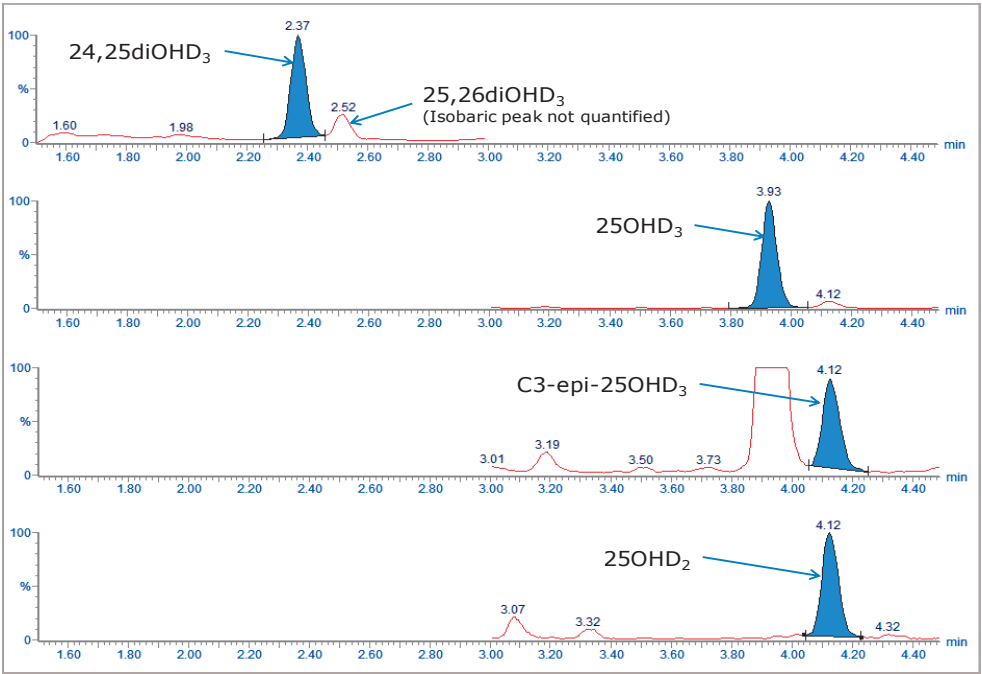


Figure 1. Chromatogram of an extracted serum sample containing endogenous 25OHD₂, 25OHD₃, 24,25diOHD₃, C3-epi-25OHD₃ and 25,26diOHD₃

Elution Profile Testing

Mean peak areas for each vitamin D metabolite and LysoPCs tested were plotted on scatter charts to view their elution profiles (Figure 2). The wash solvent strength for each extraction method was selected at the highest concentration of methanol or acetonitrile without eluting vitamin D metabolites from the SPE sorbent. The elution solvent strength was selected at the concentration with the largest peak areas for vitamin D metabolites and minimizing LysoPC peak areas where possible, to limit interferences.

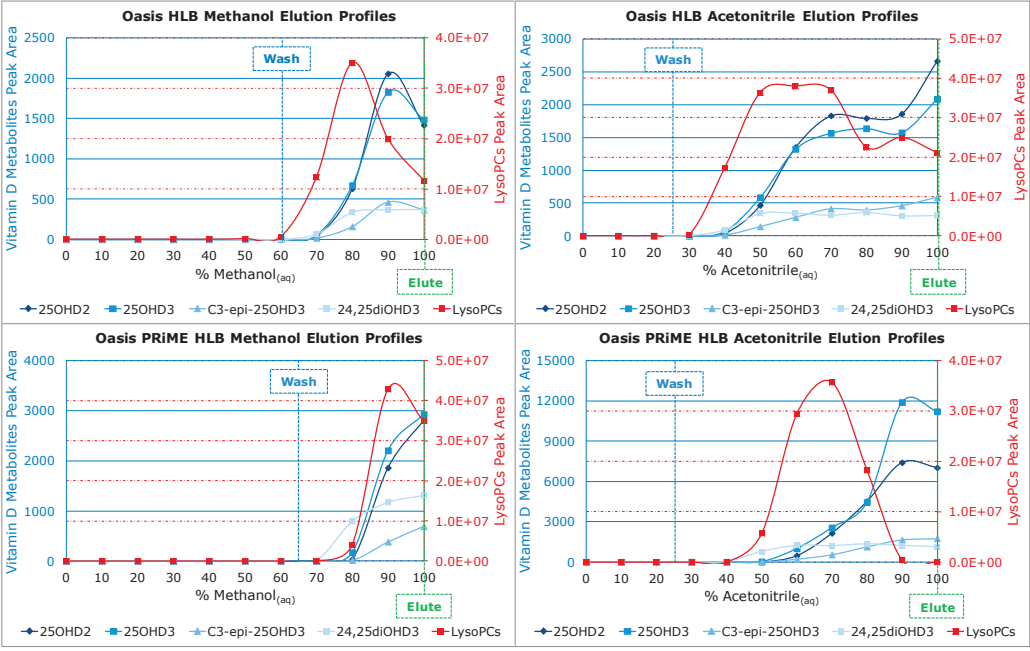


Figure 2. Elution profile plots for vitamin D metabolites and LysoPCs using Oasis HLB and Oasis PRiME HLB with different concentrations (0-100%) of methanol_(aq) and acetonitrile_(aq)

The elution profiles demonstrate that LysoPCs elute from the SPE sorbents at similar organic concentrations to the vitamin D metabolites, due to very similar hydrophobic properties. Using Oasis HLB, LysoPCs were retained at methanol_(aq) concentrations <60% and >90%, and vitamin D metabolites retained at <60% methanol_(aq), therefore, 60% methanol_(aq) wash and 100% methanol elution conditions were selected. Methanol was selected over acetonitrile when using Oasis HLB due to improved retention of LysoPCs. Alternatively, using Oasis PRiME HLB, almost all LysoPCs were retained when using <40% or >90% acetonitrile_(aq) and vitamin D metabolites retained at <30% acetonitrile_(aq), therefore, 25% acetonitrile_(aq) wash and 100% acetonitrile elution conditions were selected. Acetonitrile was selected over methanol due to improved retention of LysoPCs.

Sample Preparation Method Comparison

Mean peak areas obtained from optimized Oasis HLB and Oasis PRiME HLB protocols were compared to a simple protein precipitation extraction and are summarized in Figure 3.

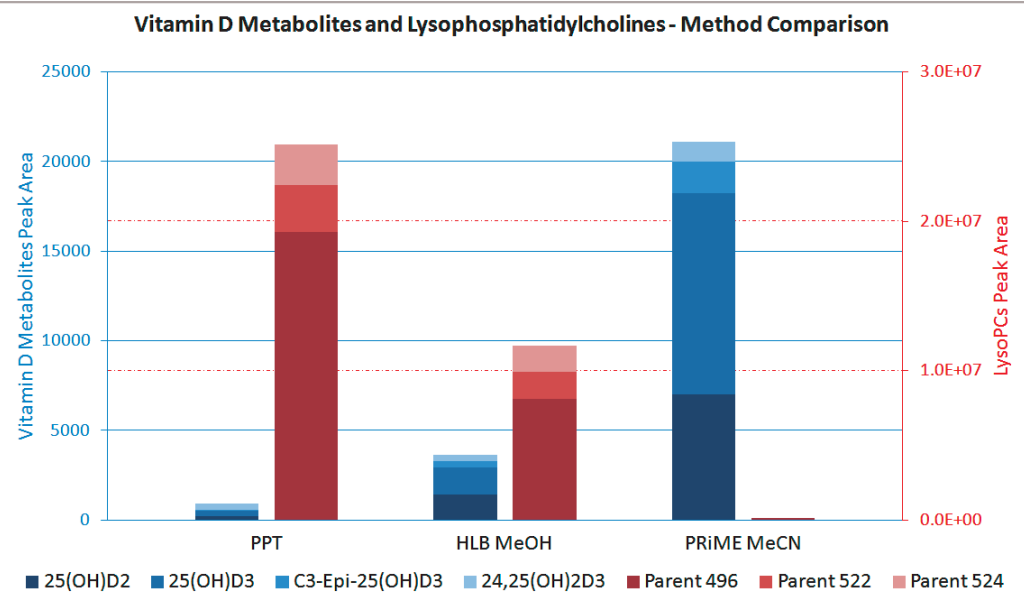


Figure 3. Comparison of vitamin D metabolites and LysoPC peak areas from samples prepared by protein precipitation (PPT), Oasis HLB and Oasis PRiME HLB

Using Oasis PRiME HLB with acetonitrile, >99% of all targeted LysoPCs were removed when compared to Oasis HLB with methanol and protein precipitation. This resulted in an increase (5x) for vitamin D metabolites peak areas when compared to Oasis HLB and 10x when compared to protein precipitation.

Imprecision Testing

Imprecision was assessed by extracting 5 replicates of a low, mid and high serum pools and analyzing them across 5 days (n=25). Data was processed in Analyse-it. Repeatability and total imprecision were <10%CV for all vitamin D metabolites at all concentration levels (Table 2).

25OHD ₃				25OHD ₂			
Sample	Mean (nmol/L)	Repeatability (%CV)	Total (%CV)	Sample	Mean (nmol/L)	Repeatability (%CV)	Total (%CV)
Low	4.89	4.6	5.9	Low	4.66	3.7	5.4
Mid	24.5	3.8	4.0	Mid	23.8	3.4	3.4
High	243	2.3	3.7	High	235	2.3	3.2

24,25diOHD ₃				C3-epi-25OHD ₃			
Sample	Mean (nmol/L)	Repeatability (%CV)	Total (%CV)	Sample	Mean (nmol/L)	Repeatability (%CV)	Total (%CV)
Low	0.967	4.3	4.8	Low	1.02	7.0	8.2
Mid	4.44	3.4	4.2	Mid	4.57	4.6	4.6
High	47.3	2.6	3.1	High	49.0	3.0	3.8

Table 2. Imprecision data summary for vitamin D metabolites

Accuracy Testing

Accuracy was assessed by extracting DEQAS and NIST SRM972a samples for 25OHD₃. Calculated concentrations were compared to the NIST assigned values and described by the Deming regression equation y=1.03x-1.62 and an Altman Bland analysis (Figure 4) demonstrated good agreement with minimal bias (-0.7%).

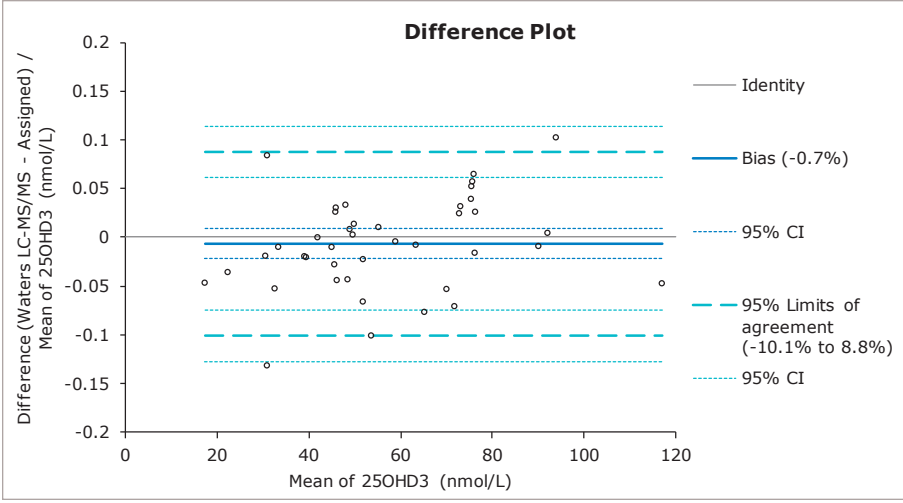


Figure 4. Altman Bland plot for 25OHD₃

Analytical Sensitivity

Analytical sensitivity was assessed by extracting 10 replicates of stripped serum samples from low to high concentrations over 3 days (n=30). Imprecision of <20%CV and S:N (ptp) of >10:1 were obtained at 1nmol/L for 25OHD₃, 25OHD₂ and 0.5nmol/L for 24,25diOHD₃ and C3-epi-25OHD₃.

Ion Suppression

Ion suppression was assessed from 6 individuals. Injections were made whilst performing a post-column infusion of the analytes. Chromatograms from the extracted serum samples were compared to a water extract. Minimal ion enhancement or ion suppression effects caused by matrix were observed in all samples at the retention times of the vitamin D metabolites (Figure 5).

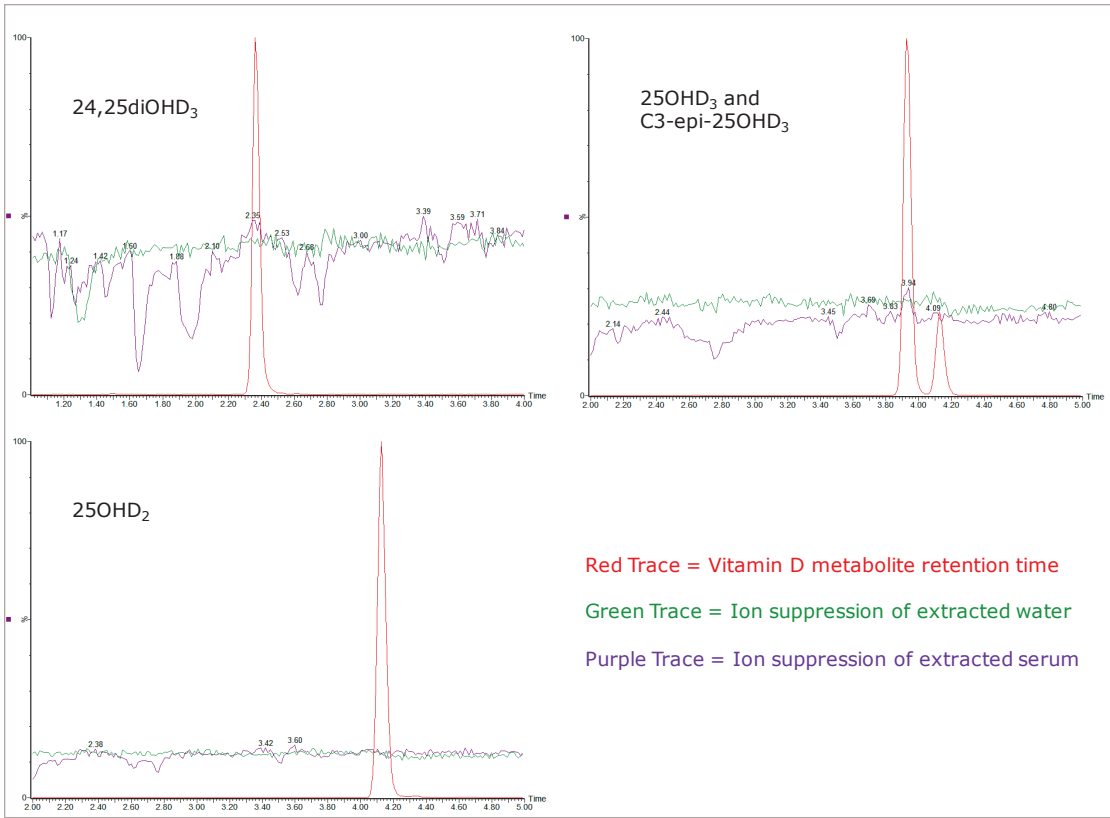


Figure 5. Ion suppression chromatograms of vitamin D metabolites

CONCLUSIONS

- Elution profile testing has proven to be a useful technique in optimizing SPE conditions for the analysis of vitamin D metabolites by LC-MS/MS
- The optimized method using Oasis PRiME HLB provides an increase in analytical sensitivity of greater than 10x when compared to protein precipitation, enabling the potential for a clinical research method to be developed
- Analytical sensitivity gains enabled quantification to 1nmol/L (0.4ng/mL) for 25OHD₃, 25OHD₂ and 0.5nmol/L (0.2ng/mL) for 24,25diOHD₃ and C3-epi-25OHD₃
- Repeatability and total imprecision of ≤8.2% CV was obtained for all vitamin D metabolites
- Excellent correlation and agreement was observed for 25OHD₃ when compared to DEQAS and NIST SRM972a samples