

Development of a Highly Selective LC/MS Assay for Vitamin D Metabolites

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The accurate quantification of vitamin D metabolites from serum samples by LC/MS is very challenging, especially when considering the presence of isobaric metabolites. This application has become increasingly important to allow the quantification of markers for a variety of diseases, and due to the pressure for the development of a high throughput assay, a method development project was initiated in our laboratories to provide a fast and optimum method. This was performed in conjunction with the development of a more efficient extraction of the vitamin D metabolites from serum to create a complete solution.

Introduction

Increased clinical interest in vitamin D has been well publicised. Its well-known role in the regulation of calcium and phosphate metabolism supports the maintenance of bone density but its importance has been extended as a biomarker for a variety of diseases, including Alzheimer's, immune diseases and renal failure and so its measurement has broad ranging importance. Recent studies have also indicated that it may be involved in the regulation of the cell cycle and is therefore implicated in the development of certain cancers [1].

Vitamin D₃ (cholecalciferol) is made in the human body on exposure of the skin to sunlight (a reaction of 7-deoxycholesterol), while in contrast Vitamin D₂ (ergocalciferol) is synthesised by plants and so is present in the body only through the consumption of food supplements. They both metabolise to 25-hydroxy vitamin D and to the biologically active 1 α ,25 dihydroxy vitamin D. The best indicator of vitamin D levels was recognised as 25-hydroxy vitamin D as it has a longer half-life of 2-3 weeks, compared to 1 α ,25 dihydroxy vitamin D, which has a half-life of 4-6 hours [2]. There are further metabolites of varying degrees of biological activity. The structures of all seven principal metabolites are shown in Figure 1. One of these, the C3-epimeric form, 3-epi-25-hydroxy vitamin D, is present, although its role is still unclear. This metabolite can be present at relatively high concentrations in sera from infants and at lower concentrations in adults [3].

Analysis

The ability to separate the 25-hydroxyvitamin D₃ (25-OH D₃) and 25-OH D₂ metabolites is important in order to distinguish between natural and supplemental levels of vitamin D. Current ELISA methods cannot distinguish between these [1], resulting in a reporting of total 25-hydroxyvitamin D. Moreover, 25-OH D₃ and 3-epi-25 hydroxyvitamin D₃ (and their D₂ analogues) are isobaric meaning that MS alone would not distinguish between these analogues. Therefore, chromatographic resolution is also required to ensure that the correct quantification is performed; otherwise measurement of higher levels than are actually present would result. Additionally, the separation

of the active 25-hydroxyvitamin D₃ from the 3-epi-25-hydroxyvitamin D₃ may provide more accurate information for treatment and prevention of associated diseases [1,3]. LC/MS has the highest potential for high throughput, compared to alternative radioimmunoassay (RIA) methods [2]. The aim of this study was to identify an HPLC stationary phase that would resolve vitamin D homologues, with short analysis time and high efficiency giving special consideration towards the chromatographic resolution of the isobaric compounds for accurate quantification.

Materials

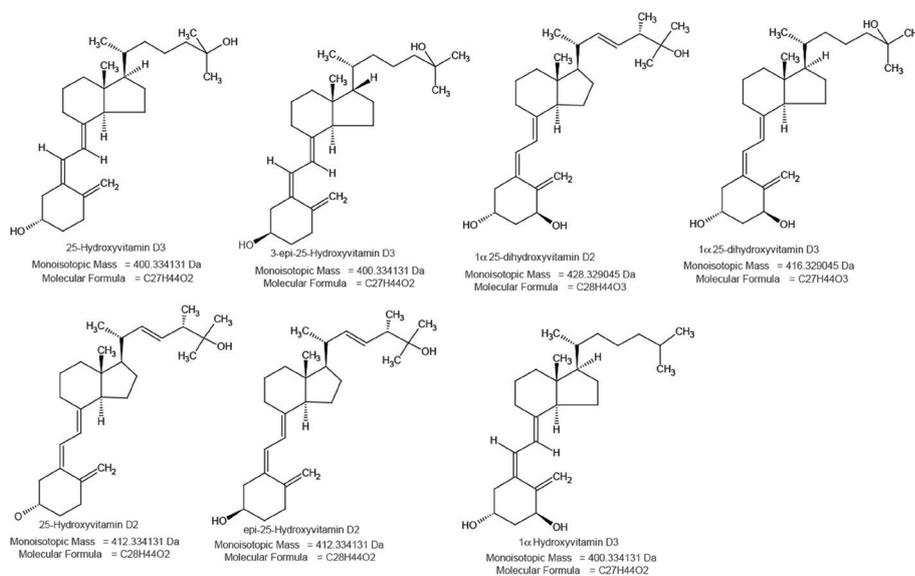


Figure 1. Structures for the principal vitamin D metabolites

All products used in this study were obtained from Sigma-Aldrich under the following brands noted below. Ascentis® Express columns, 10 cm x 3.0 mm I.D., 2.7 µm were used in Pentafluorophenyl F5, Phenyl-Hexyl and ES-Cyano chemistries from Sigma-Aldrich / Supelco (Bellefonte, PA). Formic acid, ammonium formate, acetonitrile and methanol were HPLC Chromasolv grade from Fluka, while the water used was Chromasolv Ultra grade (Fluka, Buchs, Switzerland). All vitamin D standards used were from Cerilliant, with exception to the 3-epi-25-hydroxy vitamin D isomers (Aldrich, St Louis, MO). HybridSPE Plus 96-well plates comprising a 50mg bed were obtained from Sigma-Aldrich / Supelco (Bellefonte, PA). Chromatographic methods are detailed in Tables 1 and 2.

HPLC Method Development

Since high throughput is critical in a clinical environment, especially for vitamin D assays where analyses can exceed 1500 samples per day, the speed of the assay is important. To enable this, Ascentis Express Fused Core® columns in combination with UHPLC were chosen to provide optimum efficiency and fast elution. Since C18 had been previously eliminated [internal study at Supelco] because of the coelution of 25-hydroxyvitamin D₃ and 3-epi-25-hydroxyvitamin D₃, Ascentic Express Pentafluorophenyl F5, Phenyl-Hexyl and ES-Cyano were screened to provide alternative selectivities.

UHPLC with UV detection was used for the initial screening, and the following sample mixes chosen to enable simple highlighting of any possible coelution of the isobaric pairs during the method development phase.

Sample 1, Vitamin D Mix 1, 5 µg/mL in methanol

3-epi-25-hydroxyvitamin D₂ (412.33 Da)

3-epi-25-hydroxyvitamin D₃ (400.33 Da)

Sample 2, Vitamin D Mix 2, 5 µg/mL in methanol

1-alpha-25-dihydroxyvitamin D₂ (428.33 Da)

1-alpha-25-dihydroxyvitamin D₃ (416.33 Da)

Sample 3, Vitamin D Mix 3, 5 µg/mL in methanol

25-hydroxyvitamin D₂ (412.33 Da)

25-hydroxyvitamin D₃ (400.33 Da)

1-alpha-hydroxyvitamin D₃ (400.33 Da)

The experimental conditions used are shown in Table 1

To demonstrate the different HPLC selectivities, Figure 2 shows the co-elution of the isobaric pairs 25-hydroxyvitamin D₃ and the 3-epi-25-hydroxyvitamin D₃ and also of 25-hydroxyvitamin D₂ and the 3-epi-25-hydroxyvitamin D₂ on the Phenyl-Hexyl column, while Figure 3 shows the same metabolites on the F5 HPLC phase. The latter was chosen because of its ability to rapidly resolve the vitamin D homologs tested especially the 25-hydroxyvitamin D₃ and the 3-epi-25-hydroxyvitamin D₃. The coelution of 25-hydroxyvitamin D₂ and 3-epi-25-hydroxyvitamin D₃ is not an issue because they are resolved by mass.

Since the goal was to develop an isocratic method with sub-5 minute run time, mobile phase additives were also evaluated for impact on resolution/ionisation of analytes. It was found that the use of ammonium formate resulted in almost twice the MS response compared to formic acid. Figure 4 shows the optimised method selected.

Column	10 cm x 3.0 mm I.D., 2.7 µm Acentis Express F5, Phenyl-Hexyl and ES-Cyano
Mobile Phase:	(A) 1% (v/v) formic acid; (B) water; (C) as listed in Figures
Gradient Methanol:	10% A constant; 40% B, 50% C held for 1 min; to 0% B, 90% C, in 8 min; 0% B, 90% C, held for 3 min
Gradient Ethanol:	10% A constant; 50% B, 40% C held for 1 min; to 0% B, 90% C, in 10 min; 0% B, 90% C, held for 3 min
Gradient Acetonitrile:	10% A constant; 50% B, 40% C held for 1 min; to 0% B, 90% C, in 10 min; 0% B, 90% C, held for 3 min
Flow Rate	0.6 mL/min
Column Temperature	35°C
Detector	UV at 265 nm
Injection:	5 µL
Sample 1	vitamin D epi mix, 5 µg/mL in methanol
Sample 2	vitamin D alpha di hydroxyl mix, 5 µg/mL in methanol
Sample 3	vitamin D alpha hydroxy + hydroxy mix, 5 µg/mL in methanol

Table 1: Experimental conditions for method development screening

System:	Agilent® 1290, 6210 TOF MS
Column:	Ascentis Express F5, 15 cm x 2.1 mm, 2.7 µm
Mobile phase:	25:75, (A) 5 mM ammonium formate; (B) methanol
Flow rate:	0.4 mL/min
Column temp.:	40 °C
MS detector:	ESI+, 100-1000m/z
Injection:	2 µL

Table 2: Chromatographic Conditions

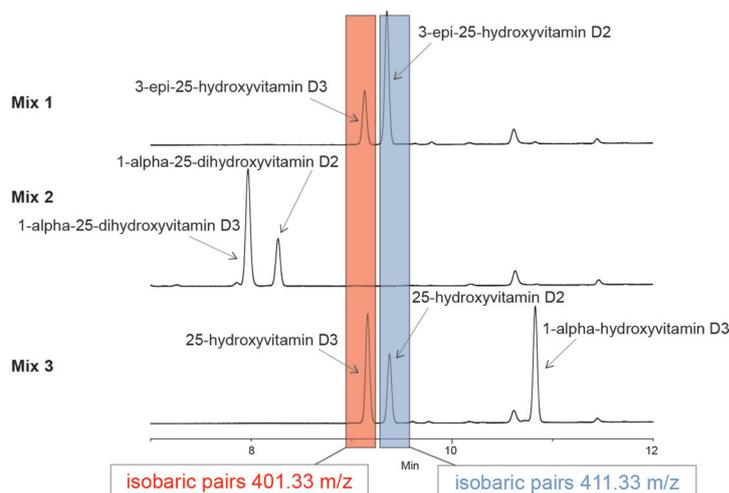


Figure 2: Results of the method development screen on Ascentis Express Phenyl Hexyl with EtOH

The Importance of Sample Preparation

In addition to the optimisation of the chromatographic resolution of the vitamin D analogues, sample preparation effects were investigated. Human serum samples were processed using protein precipitation along with novel phospholipid depletion techniques to ensure the minimisation of matrix effects (see below). Since high throughput screening was the aim of this study, it was important to also consider the possible interferences that can arise from the matrix.

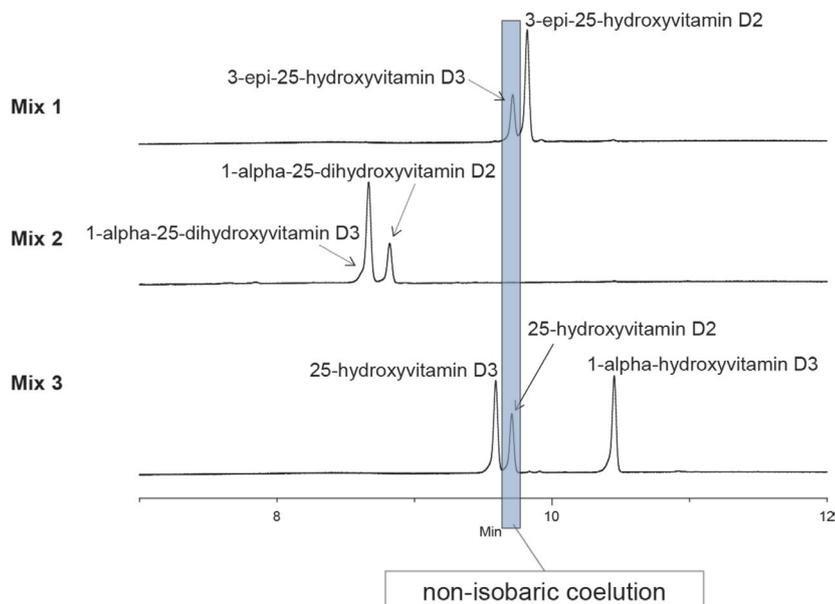


Figure 3: Results of the method development screen on Ascentis Express F5 with MeOH Conditions: Ascentis Express F5 2.7 μm 15 cm x 2.1 mm i.d.; 75:25 (methanol: 5 mM ammonium formate), 0.4 mL/min, 40 $^{\circ}\text{C}$

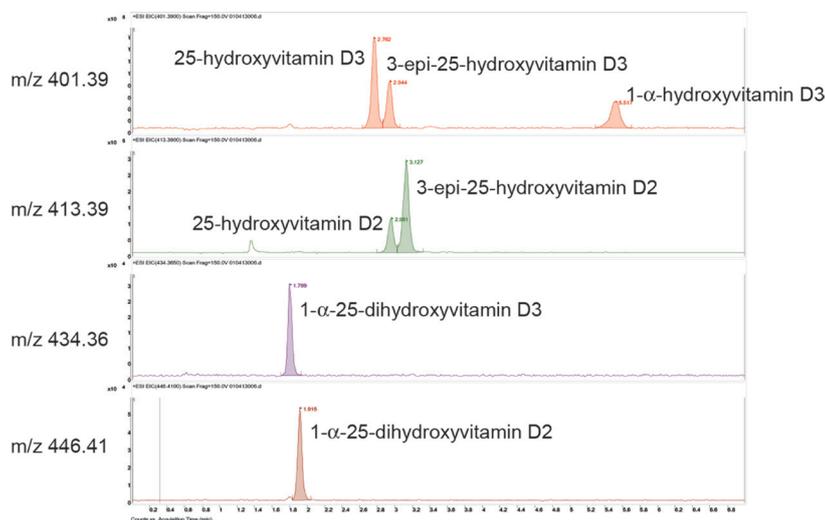


Figure 4. LC-MS/MS Chromatogram of Vitamin D Metabolites on Ascentis Express F5

The hydrophobic character of vitamin D and its metabolites requires mobile phases with high concentrations of organic modifier, conditions that also elute endogenous interferences such as phospholipids. This phospholipid extraction coelution causes ion suppression and/or enhancement in the MS detector that reduces sensitivity and accuracy when the phospholipids elute in the same retention time window as the analyte of interest, either from the current, or a previous injection. This effect has been frequently reported [4,5]. Additionally, serum phospholipids and proteins foul HPLC and UHPLC columns and can cause delayed re-equilibration time [6]. It is therefore important to remove them prior to analysis. In this study, protein precipitation and solid phase extraction methods were chosen for the comparison of matrix interference impact and to improve detection accuracy and precision. The phospholipid removal technique used here utilises zirconia-coated particles in a 96-well plate in a new design HybridSPE® Plus plate. High selectivity towards phospholipids is achieved utilising Lewis acid/base interaction between the phosphate group of the phospholipids and the zirconia surface, whilst remaining non-selective under the conditions used towards a broad range of basic, neutral and acidic compounds.

Human serum was spiked at 25 ng/mL with vitamin D metabolites. Protein precipitation was performed offline by adding 100 μL of spiked serum into a 500 μL 96 well collection plate followed by 300 μL of 1% (v/v) formic acid in acetonitrile. Samples were mixed, set aside for 5 minutes before transferring 300 μL of precipitate into the HybridSPE-Plus 96 well plate. Samples were passed through the HybridSPE-Plus plate by applying a vacuum for 4 minutes, and the resulting filtrate was analysed directly. As a comparison spiked human serum was also processed using standard protein precipitation by adding 100 μL of serum to 2 mL centrifuge vials followed by 300 μL of 1% (v/v) formic acid in acetonitrile. Samples were vortexed and centrifuged, and the resulting supernatant was collected and analysed directly.

Figure 5 shows the phospholipid monitoring chromatograms of coextracted matrix from standard precipitation techniques with that of the eluate from the HybridSPE-Plus plate. The figure shows that the latter selectively depleted the phospholipid matrix and precipitated proteins, providing no observable interference from the serum matrix. In contrast, the protein precipitation technique contained a large amount of co-extracted phospholipid matrix resulting in interference that eluted in the retention range of 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃ and 3-epi-25-hydroxyvitamin D₃. This coelution reduced sensitivity and reproducibility, resulting in irregularities in quantitation as confirmed by the recovery and reproducibility data reported in Table 3.

Conclusion

An LC-MS/MS method have been developed that demonstrated the separation and quantification of vitamin D metabolites, including the isobaric pairs. The conditions described enable the direct quantification of 25 hydroxyvitamin D₃, 3-epi-25 hydroxyvitamin D₃, 1- α hydroxyvitamin D₃, and also that of 25 hydroxyvitamin D₂, 3-epi-25 hydroxyvitamin D₂.

The unique selectivity of the Ascentis Express F5 provided a fast and efficient method for the analysis of 25-hydroxyvitamin D and homologs from serum samples, while selective phospholipid depletion provided by the HybridSPE-Phospholipid method enabled an efficient sample cleanup increasing method reproducibility and accuracy, and enabling a fast and simplified bioanalytical method for associated vitamin D metabolites.

	25-OH Vitamin D ₃	epi-25-OH Vitamin D ₃	25-OH Vitamin D ₂
HybridSPE-Phospholipid Method			
% Recovery	70.4	65.6	55.8
%Standard Deviation	8.2	5.8	12.1
Protein Precipitation Method			
% Recovery	53.0	55.7	33.9
%Standard Deviation	12.1	7.9	118.6

Table 3: Vitamin D Metabolite Sample Preparation Recovery

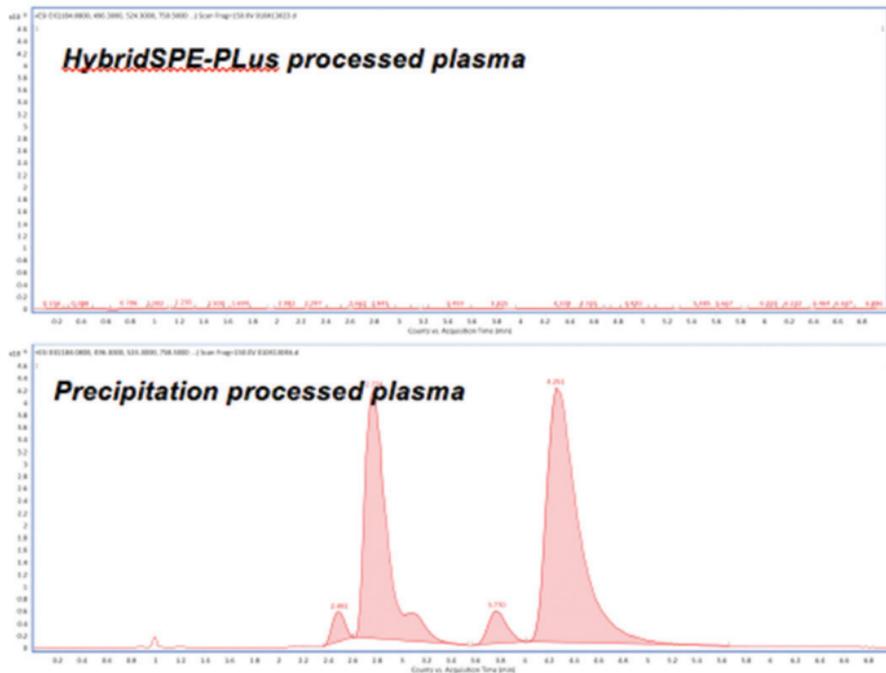


Figure 5: Phospholipid matrix monitoring (+ESI TIC, LC/MS conditions as for Figure 4)

References

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