Method Optimization for LC-MS Analysis of Vitamin D Metabolite Critical Pairs in Serum

Using HybridSPE®-Phospholipid, Ascentis® Express, and Other LC-MS Workflow Components

The unique combination of column selectivity using Ascentis® Express F5 and phospholipid removal using HybridSPE®-PLus provides a robust and accurate LC-MS method for accurate and sensitive analysis of vitamin D metabolites, including isobaric critical pairs.

Introduction: Interest in Vitamin D

Analysis of vitamin D metabolites has continued to be a topic of interest in recent publications, primarily as biomarkers for possible disease states and vitamin deficiency. While vitamin D is present in two forms, vitamin D_3 and vitamin D_2, current ELISA methods demonstrate different cross-reactivities and cannot distinguish between D_2 and D_3 forms of the vitamin metabolites resulting in erroneous reporting of total 25-hydroxyvitamin D concentrations. Further, there is interest in an analytical means to differentiate the D_2 and D_3 forms from the D_2 and D_3 epimers because of their different degrees of bioactivity.

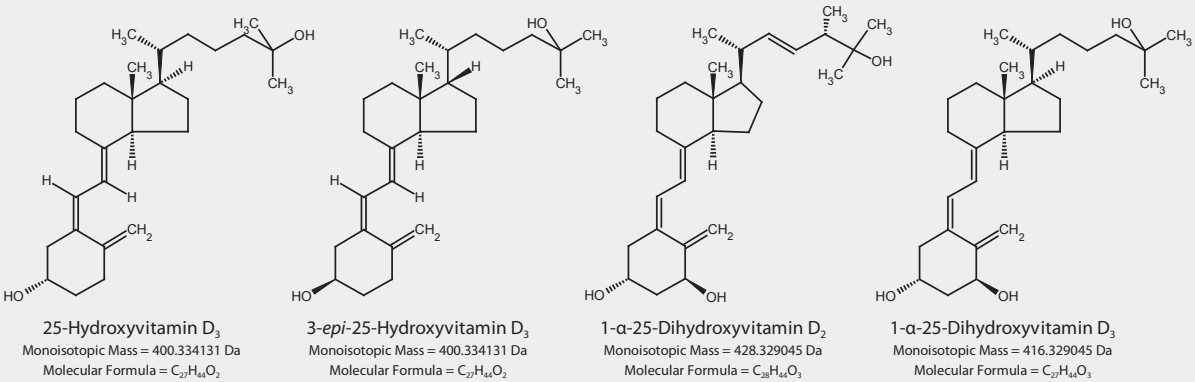
Study Aim and Overview

In this study, chromatographic screening studies for the analysis of vitamin D metabolites were expanded to include dihydroxy metabolites along with 25-*epi*-homologs (**Figure 1**). Screening consisted of evaluating various column stationary phases, organic modifiers and buffers for the resolution of critical pair isobaric compounds. The goal was to develop chromatographic conditions for the quantitation of hydroxy and dihydroxy vitamin D_2 and D_3 metabolites, including the isobaric epimers, from human serum samples. In addition to chromatographic method optimization, sample preparation techniques were evaluated to determine the impact of biological matrix on the sensitivity and accuracy of the LC-MS method.

Analytical Challenges with Vitamin D Metabolites

The particular difficulty in vitamin D metabolite analysis is the differentiation between the epimer isoform of the D_2 and D_3 metabolites. The epimers are isobaric to the D_2 and D_3 metabolites

Figure 1. Structures of Vitamin D Metabolites

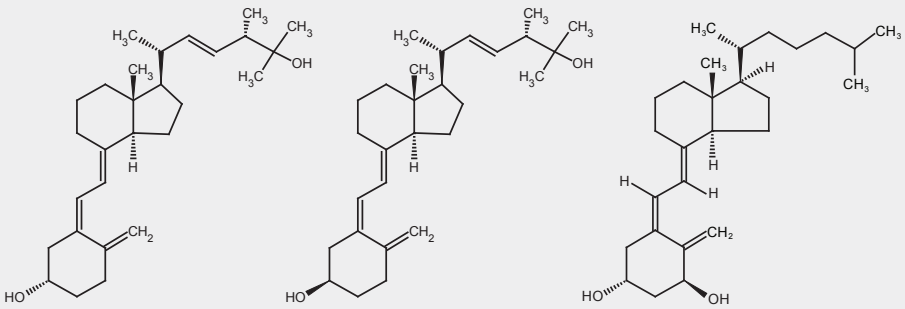


25-Hydroxyvitamin D_3
Monoisotopic Mass = 400.334131 Da
Molecular Formula = $C_{27}H_{44}O_2$

3-*epi*-25-Hydroxyvitamin D_3
Monoisotopic Mass = 400.334131 Da
Molecular Formula = $C_{27}H_{44}O_2$

1-α-25-Dihydroxyvitamin D_2
Monoisotopic Mass = 428.329045 Da
Molecular Formula = $C_{28}H_{44}O_3$

1-α-25-Dihydroxyvitamin D_3
Monoisotopic Mass = 416.329045 Da
Molecular Formula = $C_{27}H_{44}O_3$



25-Hydroxyvitamin D_2
Monoisotopic Mass = 412.334131 Da
Molecular Formula = $C_{28}H_{44}O_2$

epi-25-Hydroxyvitamin D_2
Monoisotopic Mass = 412.334131 Da
Molecular Formula = $C_{28}H_{44}O_2$

1-α-Hydroxyvitamin D_3
Monoisotopic Mass = 400.334131 Da
Molecular Formula = $C_{27}H_{44}O_2$

rendering them indistinguishable in the MS and thus requiring chromatographic separation to resolve them.

Chromatographic Method Development

Traditional reversed-phase chemistries, e.g., C18, do not resolve the critical epimeric pairs of vitamin D metabolites. Therefore, the initial phase of chromatographic method development consisted of screening Ascentis® Express cyano, phenyl hexyl and pentafluorophenyl (F5) stationary phases for the efficient resolution of 1-α-25-dihydroxyvitamin D_3, 1-α-25-dihydroxyvitamin D_2, 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, 3-*epi*-25-hydroxyvitamin D_3, 3-*epi*-25-hydroxyvitamin D_2 and 1-α-hydroxyvitamin D_3. Gradient elution was conducted using several organic modifiers (**Table 1**). Initial organic modifier concentrations were adjusted to provide equivalent eluotropic strength.

The column screening criteria was used to determine which stationary phase/organic modifier combination produced the highest resolution of the critical isobaric pairs 25-hydroxyvitamin D_2 and *epi*-25-hydroxyvitamin D_2 (observed mass m/z 413.33) and 25-hydroxyvitamin D_3 and *epi*-25-hydroxyvitamin D_3 (observed mass m/z 401.33). **Table 2** presents the minimum resolution table of these isobaric pairs. Results indicated that methanol produced the highest resolution between isobaric pairs on all stationary phases compared to acetonitrile and ethanol. While both the cyano and F5 stationary phases demonstrated some resolution of the isobaric pairs, baseline resolution was only achieved on the F5.

Table 1. Gradient Screening Conditions for Analysis of Vitamin D Metabolites

column:	Ascentis® Express, various phases, 10 cm x 3.0 mm I.D., 2.7 µm particles
flow rate:	0.6 mL/min
column temp:	35 °C
injection:	5.0 µL
mobile phase:	(A) 1% formic acid; (B) water; (C) as listed below
gradient acetonitrile (C):	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 methanol (C):	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 (C):	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

Table 2. Summary of Minimum Isobaric Resolution by Column Phase and Organic Modifier

Ascentis® Express Column	Organic Modifier	Minimum Resolution Between Isobars
F5	Methanol	2.0
ES-CN	Methanol	1.2
F5	Acetonitrile	1.2
F5	Ethanol	1.1
ES-CN	Acetonitrile	0.8
ES-CN	Ethanol	0.5
Phenyl-Hexyl	Ethanol	0.3
Phenyl-Hexyl	Acetonitrile	0
Phenyl-Hexyl	Ethanol	0

Note: Minimum resolution is defined as resolution between two sets of isobaric compounds
25-hydroxyvitamin D_2 and *epi*-25-hydroxyvitamin D_2, 413.33 m/z
25-hydroxyvitamin D_3 and dpi-25-hydroxyvitamin D_3, 401.33 m/z

Sensitivity Optimization and Buffer Selection

The second phase of the study focused on conversion of the optimum screening method into an isocratic method that could ultimately be used for the analysis of human serum samples. The F5 column was selected based on its ability to provide the highest minimum resolution of isobaric pairs using methanol as the organic modifier in the mobile phase. At this stage, the impact of the buffer modifier was evaluated for the analyte responses. Because the vitamin D metabolites are neutral compounds, it was anticipated that the buffer would have minimal impact on selectivity, although it might impact ionization in the LC-MS source.

Figure 2 and **Table 3** demonstrate the impact of mobile phase buffer on the ionization of the vitamin D metabolites when a 300 ng/mL standard of vitamin D metabolites is injected onto the F5 column. **Figure 2** depicts the full scan comparison of analyte response differences between analysis conducted using the ammonium formate buffer and the formic acid modified mobile phase. As can be observed in **Table 3**, using ammonium formate as the mobile phase buffer nearly doubles the response of the vitamin D metabolites over the formic acid additive. The final optimized chromatographic method using ammonium formate buffer and the Ascentis® Express F5 stationary phase is shown in **Figure 3**. Note that baseline resolution of isobaric vitamin D metabolites is achieved in less than 6 minutes.

Figure 2. Comparison of Buffer Effect on Vitamin D Metabolite Response on Ascentis® Express F5

column:	Ascentis® Express F5, 15 cm x 2.1 mm I.D., 2.7 µm particles (53571-U)
mobile phase:	buffer as listed, (A) water, (B) methanol, (25:75, A:B)
flow rate:	0.4 mL/min
temp:	40 °C
injection:	2.0 µL
detection:	ESI+, 100-1000m/z
instrument:	Agilent 1290, 6210 TOF

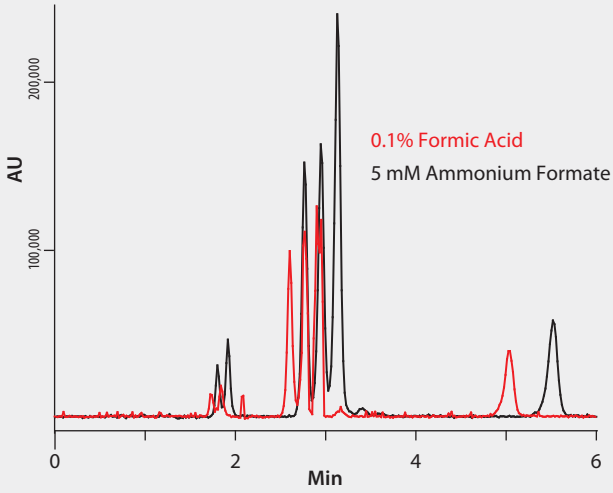
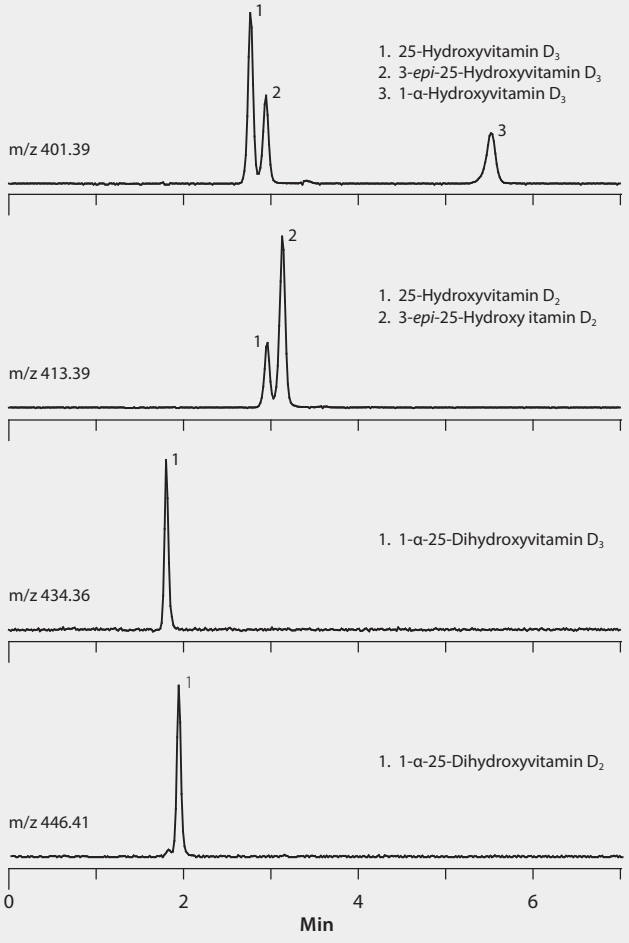


Table 3. Comparison of Buffer Effect on Vitamin D Metabolite Response on Ascentis® Express F5

Analyte	5 mM Ammonium Formate Peak Area	0.1% Formic Acid Peak Area
1-α-25-Dihydroxyvitamin D_3	95414	25517
1-α-25-Dihydroxyvitamin D_2	181755	59337
25-Hydroxyvitamin D_3	659087	344920
3-*epi*-25-Hydroxyvitamin D_3	351227	178107
25-Hydroxyvitamin D_2	495520	238007
3-*epi*-25-Hydroxyvitamin D_2	1331699	724588
1-α-Hydroxyvitamin D_3	351885	234494

Figure 3. Separation of Vitamin D Metabolites on Ascentis® Express F5

Conditions as in Figure 2. 5 mM ammonium formate



Comparison of Sample Prep Techniques to Reduce Serum Matrix Effect

The final phase of the study involved implementing the optimized chromatographic method for the analysis of spiked human serum samples. Particular attention was paid toward sample preparation and its impact on analytical results. Serum sample preparation requires protein precipitation with organic solvents or strong acids. This approach results in gross depletion of proteins from the sample, but leaves high levels of matrix interference from coextracted phospholipids. Coextracted phospholipids can cause quantitative irregularities and decrease sample throughput due to gradient column washing requirements.

Human serum samples were spiked with vitamin D metabolites and processed using two sample preparation techniques: standard protein precipitation alone and standard protein precipitation followed by phospholipid depletion with HybridSPE®-PLus plates. (Note that protein precipitation can also be performed directly in the HybridSPE® plates for a truly one-step sample prep method. However, users may prefer to carry out the protein precipitation as a separate step.) Results obtained from sample processed using the two sample preparation techniques were evaluated to determine analytical impact of phospholipid matrix interferences, both in terms of chromatographic overlap and impact on analyte response.

Sample Prep Methodologies

Human serum samples were spiked at 25 ng/mL with vitamin D metabolites. Protein precipitation was performed offline by adding 100 µL of spiked serum followed by 300 µL of 1% formic acid acetonitrile to a 96-well collection plate. Samples were thoroughly mixed by performing five 300 µL draw/dispense cycles using a digital pipette. Samples were then left to sit for 5 minutes before transferring 200 µL of the resulting supernatant into a HybridSPE®-PLus 96-well plate. Samples were passed through the HybridSPE®-PLus plate by applying 10" Hg vacuum for 4 minutes and analyzing the resulting filtrate directly. As a comparison, spiked human serum was also processed using standard protein precipitation alone by adding 100 µL of serum followed by 300 µL of 1% formic acid acetonitrile into 2 mL centrifuge vials. Samples were then vortexed and centrifuged, and the resulting supernatant was analyzed directly.

Improved Analyte Response after Phospholipid Removal

Figure 4 depicts the phospholipid selected ion chromatograms from samples processed via standard protein precipitation (red trace) and samples processed using the HybridSPE®-PLus plate (black trace). A significant amount of phospholipid matrix from samples processed via protein precipitation alone was observed eluting in the 2–4 minute window where several vitamin D metabolites also elute. Conversely, samples processed by the HybridSPE®-PLus plate displayed no detectable phospholipid matrix.

Phospholipid co-elution with analytes of interest has the potential to cause sensitivity and reproducibility issues resulting in irregularities in quantitation. **Table 4** compares analyte recovery of the two sample preparation techniques. The direct overlap in the elution windows of vitamin D metabolites and the phospholipid matrix interference for samples processed with standard protein precipitation resulted in a 40% reduction in signal response for several of the metabolites. As a result, samples processed using the HybridSPE®-PLus plate, which demonstrated no co-eluting matrix interference, demonstrated a higher analyte response.

Figure 4. Phospholipid Matrix Monitoring (selected ion)

Conditions as in Figure 2.
5 mM ammonium formate

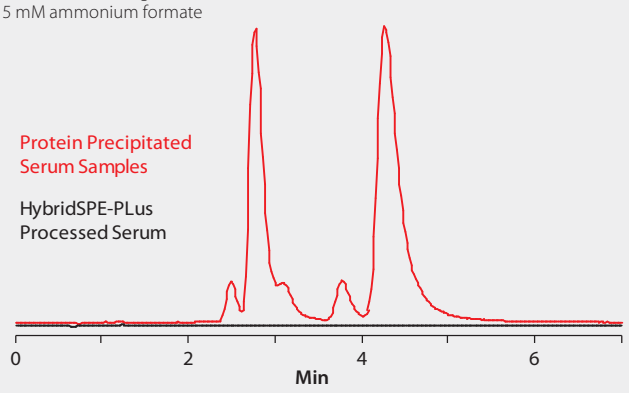


Table 4. Vitamin D Metabolite Recovery

Sample	HybridSPE®-PLus Processed Serum 25 ng/mL Average n=16	Protein Precipitated Serum 25 ng/mL Average n=8
1-α-25-Dihydroxyvitamin D_3	21.1	17.1
1-α-25-Dihydroxyvitamin D_2	19.7	19.0
25-Hydroxyvitamin D_3	24.3	15.5
3-*epi*-25-Hydroxyvitamin D_3	21.3	15.3
25-Hydroxyvitamin D_2	29.8	21.4
3-*epi*-25-Hydroxyvitamin D_2	24.5	23.0
1-α-Hydroxyvitamin D_3	27.7	21.0

Summary

Chromatographic resolution still plays an important role in LC-MS applications when dealing with isobaric compounds. The unique selectivity of the Ascentis® Express F5 column gave a fast and efficient analytical method for 25-hydroxyvitamin D and related forms from serum samples. In addition, phospholipid depletion using the HybridSPE®-PLus 96-well plate enabled efficient sample cleanup increasing method reproducibility and accuracy. As demonstrated in this study, this novel sample prep technique coupled with the unique selectivity of the Ascentis® Express F5 column enables a fast and simplified bioanalytical method for associated vitamin D metabolites. Likewise, this approach demonstrates how selectivity, in both chromatographic and sample preparation steps, allows for efficient analysis that would otherwise be unattainable with traditional reversed-phase approaches.

Featured Products

Description	Cat. No.
HPLC Column	
Ascentis® Express F5, 15 cm x 2.1 mm I.D., 2.7 µm particles	53571-U
Sample Prep Plates & Accessories	
HybridSPE®-PLus 96-Well Plates, bed wt. 50 mg	575659-U
Round Well Cap Mat, Pierceable for HybridSPE®-PLus	575680-U
96 Round/Deep-Well Collection Plate, polypropylene	Z717266
96 Well-Plate Pre-cut Sealing Films	Z721581
Supelco PlatePrep Vacuum Manifold	57192-U
Standards & Reagents	
Water LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS	14263
Methanol LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS	14262
Ammonium formate, eluent additive for LC-MS	70221
1-α-25-Dihydroxyvitamin D_3 (Cerilliant Certified Reference Material)	H-089
1-α-25-Dihydroxyvitamin D_2 (Cerilliant Certified Reference Material)	H-090
25-Hydroxyvitamin D_3 (Cerilliant Certified Reference Material)	H-083
25-Hydroxyvitamin D_2 (Cerilliant Certified Reference Material)	H-073
3-*epi*-25-Hydroxyvitamin D_3	705993
3-*epi*-25-Hydroxyvitamin D_2	753556
1-α-Hydroxyvitamin D_3	17946

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