APPLICATION

The Development of an Automated Online SPE-LC/MS/MS Analysis of 25-OH-Vitamin D2, 25-OH-Vitamin D3, and 3-Epi-25-OH-Vitamin D3 from Human Serum

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Introduction

Vitamin D refers to a group of fat-soluble secosteroids responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphate, and zinc. In humans, the most important compounds in this group are Vitamin D3 and Vitamin D2. For many years, Vitamin D has been the subject of many clinical investigations for a range of potentially beneficial health effects. Vitamin D metabolites, 25-hydroxy (25-OH) and 1,25-dihydroxy are often used as markers to determine Vitamin D deficiency. However, faulty metabolism in children less than 12 months of age can lead to the formation of the inactive 3-Epi-25-monohydroxy (3-Epi-25-OH) form. This inactive form is an epimer of the primary metabolite, and thus is impossible to separate via tandem mass spectrometry alone. In this study, we will demonstrate the development of an automatable online SPE extraction and a LC/MS/MS method for the analysis of these challenging metabolites to avoid the overestimation of vitamin D in clinical labs.

Due to the similarity in chemical structure of 25-OH-Vitamin D3 and 3-Epi-25-OH-Vitamin D3, chromatographic separation is one of the challenges of the assay. The method developed here permitted the independent measurement of all three compounds. The assay dynamic range is 2.5 - 100 ng/mL for all compounds, with precision less than 9.62 % and accuracy less than 13 %, respectively.

Experimental Conditions

Automated Online SPE Extraction Method Development Strategies

The method development of an online extraction method using a Thermo Cohesive® TLX system has three major steps:

1. LC/MS/MS method development
   - Separation of compounds, peak shape, and sensitivity, etc.
2. Online SPE extraction method development
   - Online SPE extraction column screening
   - Determine the wash solvent and efficient elution solvent
3. Combine above methods into a final online SPE-LC/MS MS method
   - Needle wash, flow rate, gradient, etc.

Step 1. LC/MS/MS Method Development

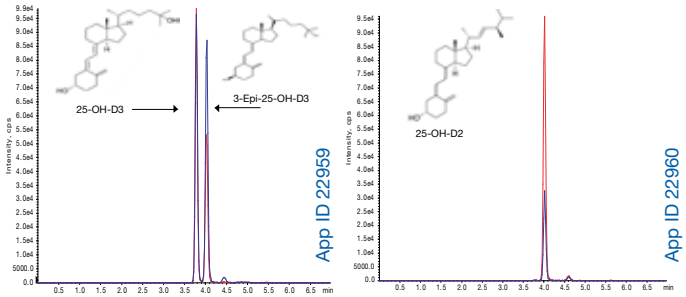
LC/MS/MS was performed on a Thermo Cohesive TLX system in LX injection mode and interfaced with a 4000 QTRAP® mass spectrometer (SCIEX). The ionization source was APCI in positive ion mode.

Column: Kinetex® 2.6 µm F5
Dimensions: 100 x 4.6 mm
Part No.: 00D-4723-E0
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Methanol
Isocratic: A / B (15:85)
Flow Rate: 750 µL/min

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	CE
25-OH-D2 1	395.4	269.1	150	28
25-OH-D2 2	395.4	209	150	36
25-OH-D3 1	383.6	257.2	150	23
25-OH-D3 2	383.6	159.1	150	36
3-Epi-25-OH-D3 1	383.6	257.2	150	23
3-Epi-25-OH-D3 2	383.6	159.1	150	36
25-OH-D3-$^2H_3$ 1	383.6	257.2	150	23
25-OH-D3-$^2H_3$ 2	383.6	368.4	150	20

1. Quantitation mass transition
2. Confirmation mass transition

Figure 1.
Representative Chromatograms with Analytical Column Only (LX Injection Mode)



Step 2. Online SPE Extraction Method Development

In this study, we evaluated a variety of silica-based and polymer-based online SPE sorbents, including Strata™–X 25 µm 20 x 2.0 mm, Strata® C8 20 µm 20 x 2.0 mm, and Luna® C18 5 µm 20 x 4.0 mm column.

Figure 2.
Online SPE Extraction Column Screening: Chromatograms of Strata-X 25 µm 20 x 2.0 mm

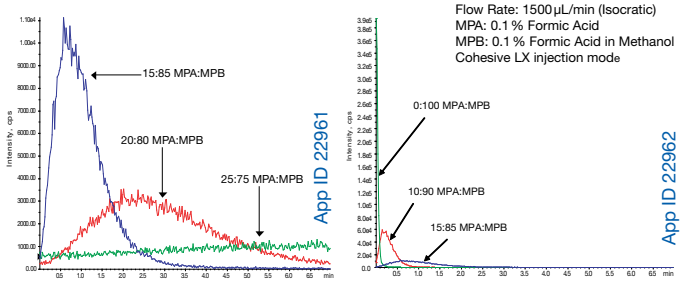


Figure 3.
Online SPE Extraction Column Screening: Chromatograms of Luna® C18 5 µm 20 x 4.0 mm

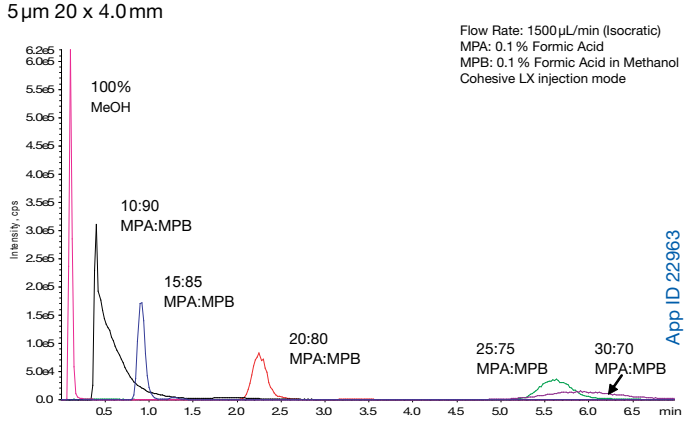
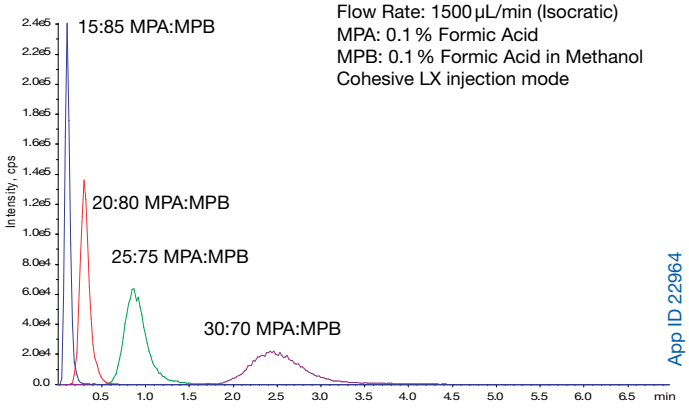


Figure 4.
Online SPE Extraction Column Screening: Chromatograms of Strata® C8 20 µm 20 x 2.0 mm



Step 3. Finalized Online SPE-LC/MS/MS Method

In all samples, including calibration standards and QCs, a protein precipitation of the human serum sample was performed. A calibration curve from 2.5-100 ng/mL was established. Due to the high concentration of the 25-Vitamin D3 and 3-Epi-OH-Vitamin D3 in purchased human serum blank matrix (**Figure 5**), double charcoal-stripped human serum was used to prepare all standards and QCs.

Sample pretreatment: 100 µL of double charcoal-stripped human serum was precipitated with 200 µL of 5:2:1 Methanol:Acetonitrile:2% $ZnSO_4$ 30 µL of 1 µg/mL working internal standard in water and mixed. The sample was centrifuged for 10 minutes at 14000 rpm, and 200 µL supernatant was transferred to a vial/plate for LC/MS/MS analysis.

Automated online SPE-LC/MS/MS was performed using an online SPE extraction column (Strata C8 20 µm 20 x 2.0 mm), followed by LC/MS/MS analysis using a Kinetex® F5 2.6 µm 100 x 4.6 mm analytical column on a Thermo Cohesive® TLX system in TX injection mode, coupled with an API 4000™ QTRAP (SCIEX). The ionization source was APCI and analyzed in positive ion mode.

Mobile Phase: MPA: 0.1 % Formic Acid in Water
MPB: 0.1 % Formic Acid in Methanol
Needle Wash: Wash 1: Methanol/Water (50:50)
Wash 2: 0.1 5 Formic Acid in Water
Injection Volume: 40 µL

LC Conditions

Loading Pump **Eluting Pump**

Focus mode configuration

Figure 5.
Representative Chromatogram of Blank Human Serum

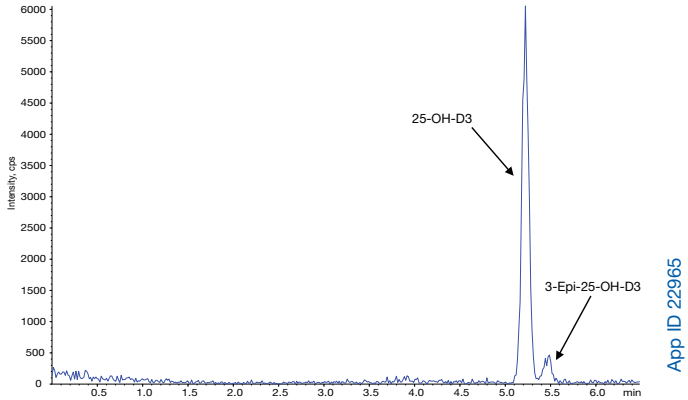


Figure 6.
Representative Chromatograms of LLOQ (2.5 ng/mL) in Human Serum

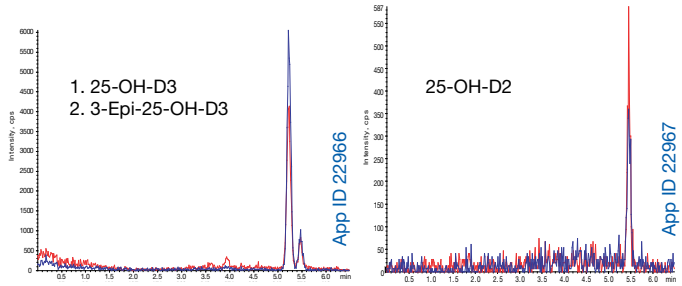


Figure 7.
Representative Chromatograms of ULOQ (100 ng/mL) in Human Serum

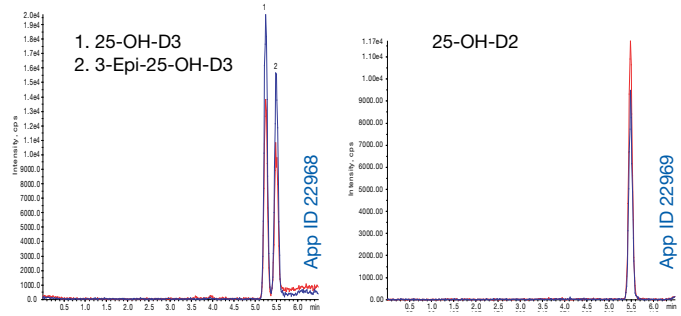


Figure 8.
Representative Curve in Human Serum

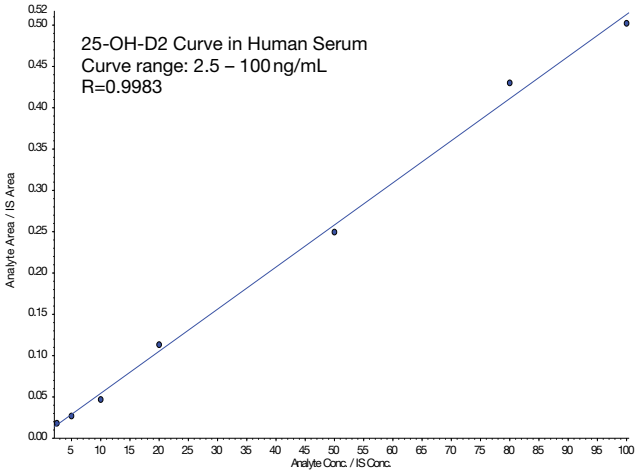


Table 2.
Accuracy and Precision

Target Conc. (ng/mL)	QCL	QCM	QCH
	7.5	50	80
	25-OH-D2		
Mean Conc. Found (ng/mL)	8.47	56.3	90.8
STDV	0.170	0.945	1.35
CV%	2.01	1.68	1.49
Accuracy (%)	113	113	113
	25-OH-D3		
Mean Conc. Found (ng/mL)	7.26	56.4	97.0
STDV	0.098	1.86	4.72
CV%	9.62	3.30	4.87
Accuracy (%)	96.8	113	97.0
	3-Epi-25-OH-D3		
Mean Conc. Found (ng/mL)	7.57	55.7	88.3
STDV	0.672	1.24	3.59
CV%	8.89	2.23	4.06
Accuracy (%)	101	111	110

Results and Discussion

Figure 1 shows the chromatographic separation of 25-OH-Vitamin D3 and 3-Epi-OH-Vitamin D3 on the Kinetex® F5 2.6 µm 100 x 4.6 mm analytical column. In **Figure 2**, **Figure 3**, and **Figure 4** we compare the online SPE extraction columns by screening Phenomenex Strata™–X 25 µm 20 x 2.0 mm (polymer-based); Luna® C18 5 µm 20 x 4.0 mm, and Strata® C8 20 µm 20 x 2.0 mm (silica-based), under isocratic conditions with a flow rate of 1500 µL/min in Thermo Cohesive® LX injection mode. Based on the analytical sensitivity, elution time, elution time duration, and particle size of the media, Strata C8 20 µm 20 x 2.0 mm was selected as the online SPE extraction column. From the data in **Figure 4**, we were able to determine optimal wash and eluting solvents for the online SPE method. In addition, analyte transfer time from loading pump to eluting pump was optimized to maximize the recoveries of all compounds.

Figure 6 and **Figure 7** show representative chromatograms of analyte concentration at LLOQ (2.5 ng/mL) and ULOQ (100 ng/mL) after online SPE extraction, showing that both the Phenomenex Strata C8 20 µm 20 x 2.0 mm online SPE extraction column and Kinetex® F5 2.6 µm 100 x 4.6 mm analytical column provided good sensitivity and selectivity, especially for 25-OH-Vitamin D3 and its epimer of 3-Epi-25-OH-Vitamin D3.

Table 2 shows the accuracy and precision of three QC levels in human serum. The results showed that the assay was accurate, precise, and reproducible. Linearity was determined to be acceptable from 2.5-100 ng/mL for all analytes. **Figure 8** shows the representative curve of 25-OH-D2 in human serum.

Conclusion

In this study, we demonstrate how to move an offline, labor intensive solid phase extraction sample preparation technique to an automatable, higher throughput online SPE format. Through three online SPE extraction method development strategies, we were able to minimize the stress of the online extraction method development. We evaluated three online SPE extraction sorbents, and showed how to screen online extraction columns, establish wash and elution solvents, and how to determine the flow rate for both the online SPE extraction column and the analytical column in focus mode to ensure the chromatographic resolution and mass spectrometer sensitivity. The developed assay provides a dynamic range of 2.5 - 100 ng/mL across all compounds to meet clinical relevance; three levels of QCs (n=6) show the assay precision within 1.49 – 9.62 % and accuracy within 92.6 - 113 % by using a Thermo Cohesive TLX system and a 4000 QTRAP® mass spectrometer (SCIEX). We demonstrated an accurate, precise, reproducible and automated online SPE-LC/MS/MS method. It is a great format to increase sample throughput in the laboratory.

Acknowledgements

We would like to thank Seyed Sadjadi and all PhenoLogix members for their technical assistance.

Ordering Information
Online SPE Extraction Columns

Description	Dimensions	Part Number	Unit/Box
Strata® C8 20 µm	20 x 2.0 mm	00M-S101-B0-CB	ea
Strata C18 20 µm	20 x 2.0 mm	00M-S039-B0-CB	ea
Strata™-X 25 µm, polymeric reversed phase	20 x 2.0 mm	00M-S033-B0-CB	ea
Strata-X-C 25 µm, polymeric strong cation-exchange	20 x 2.0 mm	00M-S048-B0-CB	ea
Strata-X-CW 25 µm, polymeric weak cation-exchange	20 x 2.0 mm	00M-S036-B0-CB	ea

Requires cartridge holder, Part No. CH0-5845

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Kinetex Core-Shell
HPLC/UHPLC Columns

2.6 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard™ ULTRA Cartridges‡ 3/pk
F5	00A-4723-AN	00B-4723-AN	00D-4723-AN	00F-4723-AN	AJ0-9322 for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)

Phases	50 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard ULTRA Cartridges‡ 3/pk
F5	00B-4723-Y0	00D-4723-Y0	00F-4723-Y0	AJ0-9321 for 3.0 mm ID

2.6 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard ULTRA Cartridges‡ 3/pk
F5	00B-4723-E0	00D-4723-E0	00F-4723-E0	AJ0-9320 for 4.6 mm ID

1.7 µm Minibore Columns (mm)

Phases	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges‡ 3/pk
F5	00B-4722-AN	00D-4722-AN	00F-4722-AN	AJ0-9322 for 2.1 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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