Unique Liquid Chromatography Separation of Calcifediol and its 3-epimer analog using Dimethylpentafluorophenyl Propyl Column on a LCMSMS

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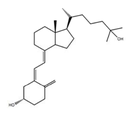
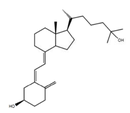
Introduction

Calcifediol, also known as 25-hydroxyvitamin D_3 is a prehormone that is produced in the liver by hydroxylation of vitamin D_3. It is acknowledged that both calcifediol and its 3-epimer account for a significant part of the circulating vitamin D_3. In order to have an adequate characterization of calcifediol, the chromatographic separation of calcifediol and its 3-epimer was deemed necessary.



25-hydroxyvitamin D_3



3-epi-25-hydroxyvitamin D_3

Method

Several available stationary phases were tested to accomplish the separation between calcifediol and its 3-epimer analog with an acceptable run time. The separation of calcifediol and 3-epi-calcifediol was performed on a Supelco Ascentis Express F5. Mobile phase consisted of a mixture of water, methanol and formic acid at a flow rate of 0.5 mL/min. The analytical column was heated to 40°C to decrease the run time while keeping good separation between vitamins. A positive ESI LCMSMS method was developed in MRM mode for the quantification of Calcifediol. The extraction procedure involves a liquid-liquid extraction with a mixture of hexanes and dichloromethane using only 50 µL of human serum without requiring tedious and time consuming derivatization procedure.

Extraction Procedure

Add 50 µL of human serum
↓
Add 100 µL of Internal Standard
↓
Add 500 µL of Buffer Solution
↓
Add 6 mL of Hexanes/Dichloromethane
↓
Shake for 15 minutes at 240 excursions/minute
↓
Centrifuge for 5 minutes at 3000 rpm
↓
Flash Freeze in a methanol/dry ice bath for 5 minutes
↓
Poor organic phase and evaporate to dryness
↓
Add 200 µL of reconstitution solution

LC-MS/MS Analysis

Chromatographic mode:	Reversed Phase
Analytical Column:	Ascentis Express F5, 100 x 3 mm, 2.7 µm
Elution mode:	Isocratic
Mobile Phase A:	Water/Methanol and Formic acid
Mobile Phase B:	100% Acetonitrile
Flow Rate:	0.500 mL/minute
Injection volume:	15 µL
Retention Times:	7.15 minutes for 25-hydroxyvitamin D_3 7.85 minutes for 3-epi-25-hydroxyvitamin D_3 7.10 minutes for the Internal Standard
Acquisition time:	9.00 minutes
Detector:	AB Sciex API 5000
Source:	TurboIonSpray, Positive mode
Ion Monitored:	401.3 → 159.1 amu for 25-hydroxyvitamin D_3 (Calcifediol) 401.3 → 159.1 amu for 3-epi-25-hydroxyvitamin D_3 417.3 → 381.3 amu for 24,25-dihydroxyvitamin D_3 417.3 → 381.3 amu for 1,25-dihydroxyvitamin D_3 (Calcitriol) 407.3 → 159.1 amu for the Internal Standard

Results

The retention times were 7.15 minutes for Calcifediol and 7.85 minutes for 3-epi-25-hydroxyvitamin D_3 with a baseline separation between these vitamins. The mass transitions of 401.5→159.1 for calcifediol and its 3-epimer for better selectivity and sensitivity was used. This assay was validated over a dynamic range of 5-200 ng/mL for calcifediol while proving the separation with the epimer. The recovery of the liquid-liquid extraction was higher than 70% either in surrogate matrix and human serum. Since remaining endogenous level of calcifediol is still too high in matrix and cannot be removed by stripping process, standard curve and quality control samples were prepared in 2% bovine serum albumin (BSA) in phosphate buffer saline (PBS). Selectivity, matrix effect, hemolysis effect and lipemic effect were evaluated and all met acceptance criteria.

Table 1. Within-run Accuracy and Precision in 2% Bovine Serum Albumin in Phosphate Buffer Saline (LLQC, QC1 and QC3) and in Human Serum (QC1S)

	Nominal Concentrations (ng/mL)			
	LLQC	QC1	QC3	QC1S
	5.00	15.00	120.00	13.12
	4.97	14.61	122.71	13.39
	4.96	15.44	118.61	13.54
	5.34	14.97	116.93	13.34
N	3	3	3	3
Mean	5.090	15.007	119.417	13.423
SD(±)	0.2166	0.4162	2.9732	0.1041
CV(%)	4.25	2.77	2.49	0.78
% Nominal	101.80	100.04	99.51	102.31

Chromatography

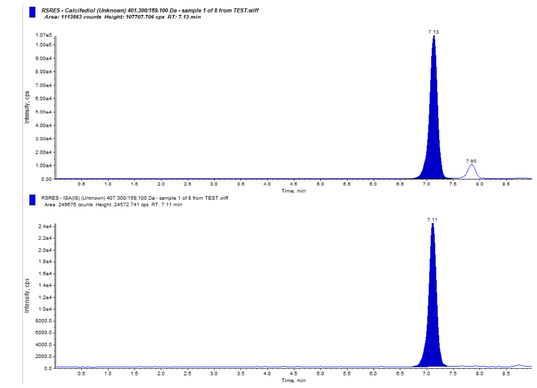


Figure 1. Representative Chromatogram for the Separation of 25-hydroxyvitamin D_3 (50ng/mL) and 3-epi 25-hydroxyvitamin D_3 (5 ng/mL) in Reconstitution Solution. Retention Time for 25-hydroxyvitamin D_3 is 7.13 minutes and 3-epi-25-hydroxyvitamin D_3 is 7.85 minutes

Chromatography

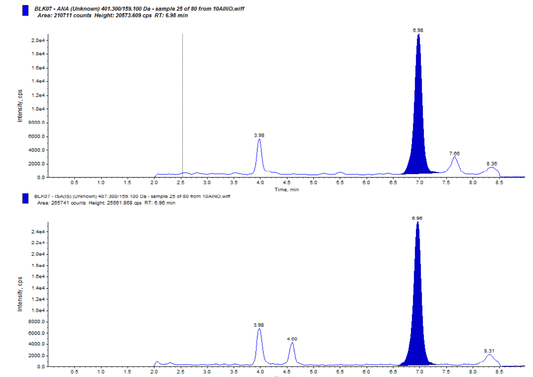


Figure 2. Representative Chromatogram of Human Serum Sample with the Separation of 25-hydroxyvitamin D_3 and 3-epi-25-hydroxyvitamin D_3. Retention Times are 6.98 minutes for 25-hydroxyvitamin D_3 and 7.66 minutes for 3-epi-25-hydroxyvitamin D_3

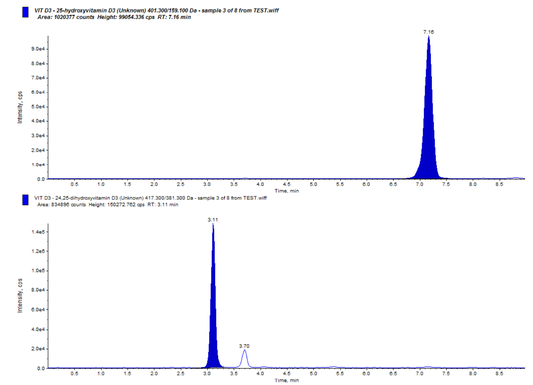


Figure 3. Representative Chromatogram for the Separation of 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3 (Calcitriol) and 24,25-dihydroxyvitamin D_3. Retention Times are 7.16 minutes, 3.70 minutes and 3.11 minutes for 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3 (Calcitriol) and 24,25-dihydroxyvitamin D_3, respectively.

Conclusion

The unique selectivity of the pentafluorophenyl Ascentis Express F5 provided a fast and efficient method without tedious derivatization for the analysis of 25-hydroxyvitamin D_3 from serum samples. Chromatographic resolution is necessary for accurate quantification of isobaric metabolite not distinguishable by mass spectrometer alone.

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