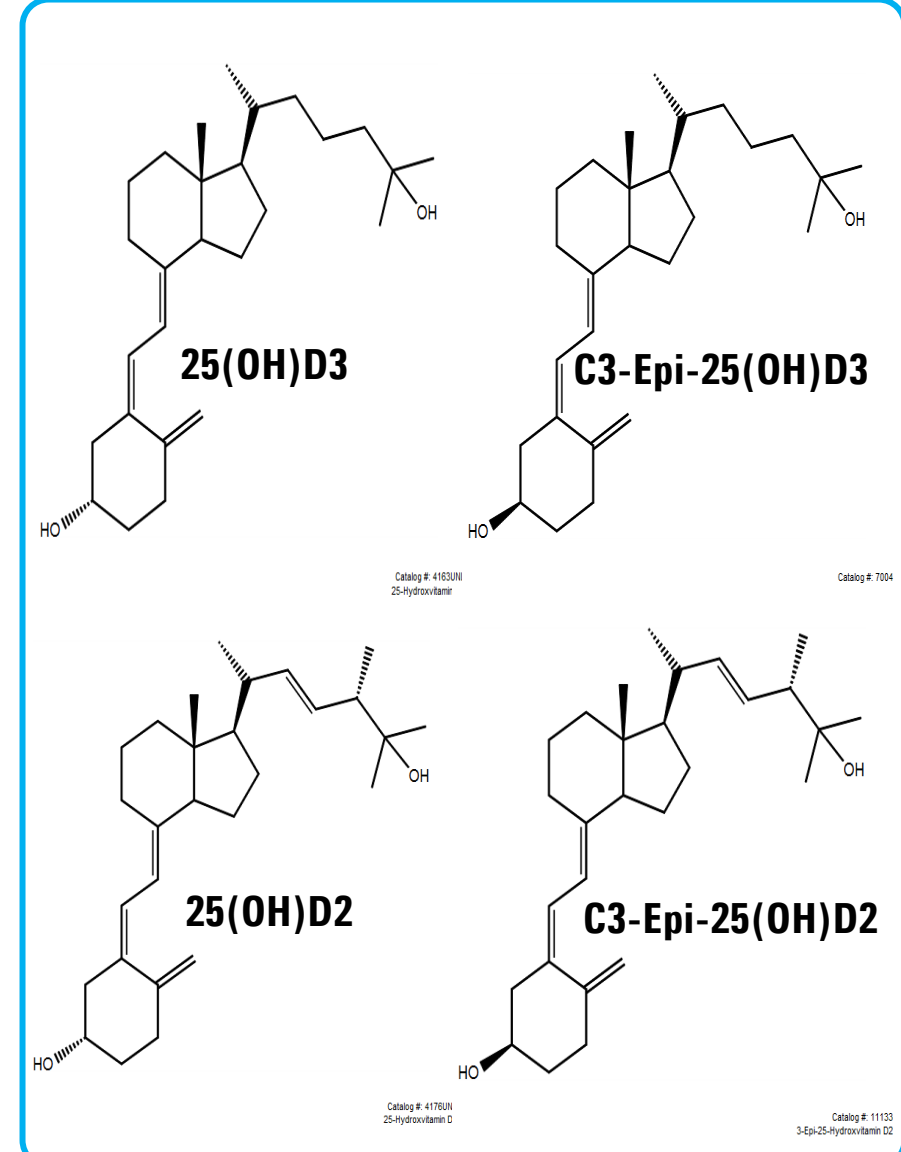


Introduction

Analysis of 25-Hydroxy-Vitamin D (25(OH)D) has become an important tool for the clinical diagnosis of vitamin D deficiency and supplementation monitoring. The different forms of 25(OH)D (D3 and D2) can be easily measured by LC-MS/MS with high sensitivity. Interferences from co-eluting isomeric compounds of identical elemental composition but of different structure can result in the over-estimation of 25(OH)D concentration as is the case with C3-epimer of 25(OH)D. The C3-epimer of 25(OH)D structure only differs from 25(OH)D in that a hydroxyl group at position C3 is arranged asymmetrically making chromatographic separation and mass spectrometric characterization difficult due to similar MRM transitions.

C3-epi-25(OH)D has been shown to be more significant in infants than adults. In this study, we developed a method for the analysis of 25(OH)D and C3-Epi-25(OH)D for both D2 and D3. We were able to isocratically separate isomers chromatographically to baseline resolution using a Pursuit 3 PFP column on an Agilent 1260 HPLC and 6460 Mass Spectrometer system in under 10 minutes. Other types of chromatographic columns such as SB-CN, Diphenyl, etc were also evaluated which did not show the same separation capability.



Experimental

Standards, Calibrators and Controls

C3-Epi-25(OH)D3 /D2-Stock: 50µg/ml in Ethanol (Isosciences)
 25(OH)D3 /D2-Stock: 50µg/ml in Ethanol (Isosciences)
 25(OH)D3 /D2-²H₃-ISTD: 50µg/ml in Ethanol (isosciences)
 NIST SRM 972: Control Levels 1 to 4
 UTAK: Level L, 1 and 2
 Patient Samples: 5 Adult samples

Sample Preparation

- 150 µl of serum sample, calibrators, controls + 150 µl ZnSO₄ + 300 µl Methanol and 7.5 µl ISTD at 1000 ng/ml was added to tubes and vortexed for 1 min
- 1.2 ml HPLC grade Heptane was added and vortexed for 1 min prior to centrifugation
- Organic layer (Upper) was transferred to another tube and dried down under nitrogen at room temperature
- Reconstituted in 120 µl 75% Methanol:25% 0.1% Formic Acid in water
- All in-house calibrators were prepared in Vitamin D Free delipidized Serum-VD-DDC Mass Spec Gold (Golden West Biological's)

Method

HPLC Conditions

Agilent 1260 Infinity HPLC series binary pump, well plate, thermostatted column compartment
 Column: Agilent Technologies Pursuit PFP 3 µm, 3 x 100 mm
 Column Temperature: 45 °C
 Injection Volume: 10 µl
 Autosampler Temperature: 4 °C
 Needle Wash: Flush port (50%Methanol:50%Water) 3 seconds

Mobile Phase A: 0.1% Formic Acid in Water
 Mobile Phase B: 0.1% Formic Acid in Methanol
 Flow Rate: 0.8 ml/min
 Gradient: Isocratic- 30%A:70%B
 Run time: 10 minutes

MS Conditions

Agilent 6460 Triple Quadrupole Mass Spectrometer
 Ion mode: Agilent Jet Stream Positive Mode
 Gas Temperature: 250°C
 Gas Flow: 5 L/min
 Nebulizer: 45 psi
 Sheath Gas Temperature: 325°C
 Sheath Gas Flow: 11 l/min
 Capillary Voltage: 5000V
 Nozzle Voltage: 1500V
 Q1/Q2 Resolution: 0.7/0.7 unit
 Dwell time: 50 msec
 Delta EMV: +400V

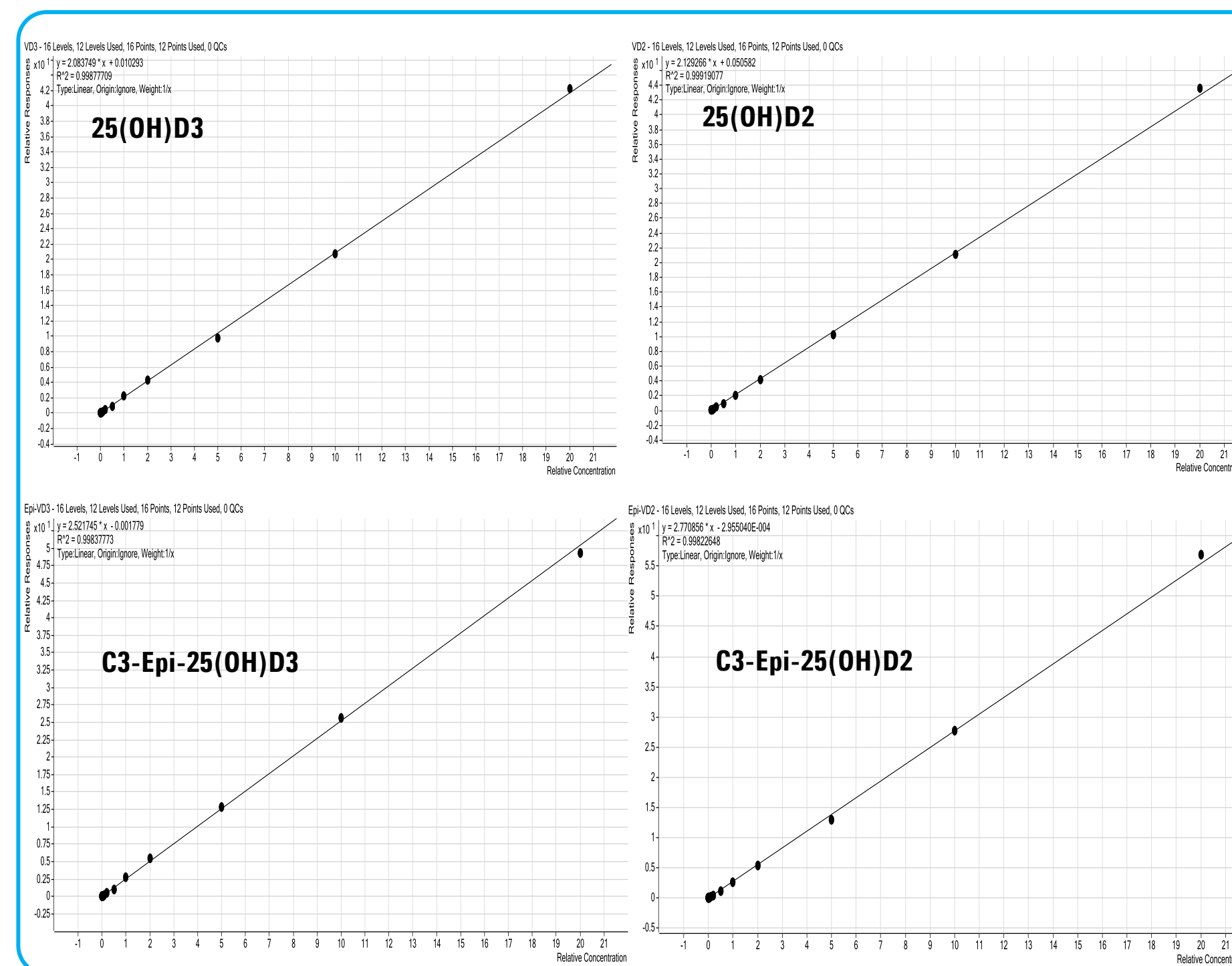
Results and Discussion

Table 1: MRM Acquisition Table

Compound	RT (Min)	MRM	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage
25-Hydroxy-Vitamin D3	6.66	401.3 > 383.2	112	4	2
	6.66	401.3 > 365.2	112	4	2
25-Hydroxy- Vitamin D2	7.19	413.4 > 395.3	121	4	2
	7.19	413.4 > 355.2	121	4	2
C3-Epi-25-Hydroxy-Vitamin D3	7.06	401.3 > 383.2	112	4	2
	7.06	401.3 > 365.2	112	4	2
C3-Epi-25-Hydroxy-Vitamin D2	7.55	413.3 > 395.3	121	4	2
	7.55	413.4 > 355.2	121	4	2
25-Hydroxy-Vitamin D3-2H3	6.61	404.3 > 386.2	112	4	2
25-Hydroxy-Vitamin D2-2H3	7.12	416.3 > 398.2	121	4	2

Linearity

The assay was linear over the range of 0.5 – 500 ng/ml for 25(OH)D3, 25(OH)D2, C3-Epi-25(OH)D3 and C3-Epi-25(OH)D2 with a mean of coefficient of determinations (R²) > 0.998.



Results and Discussion

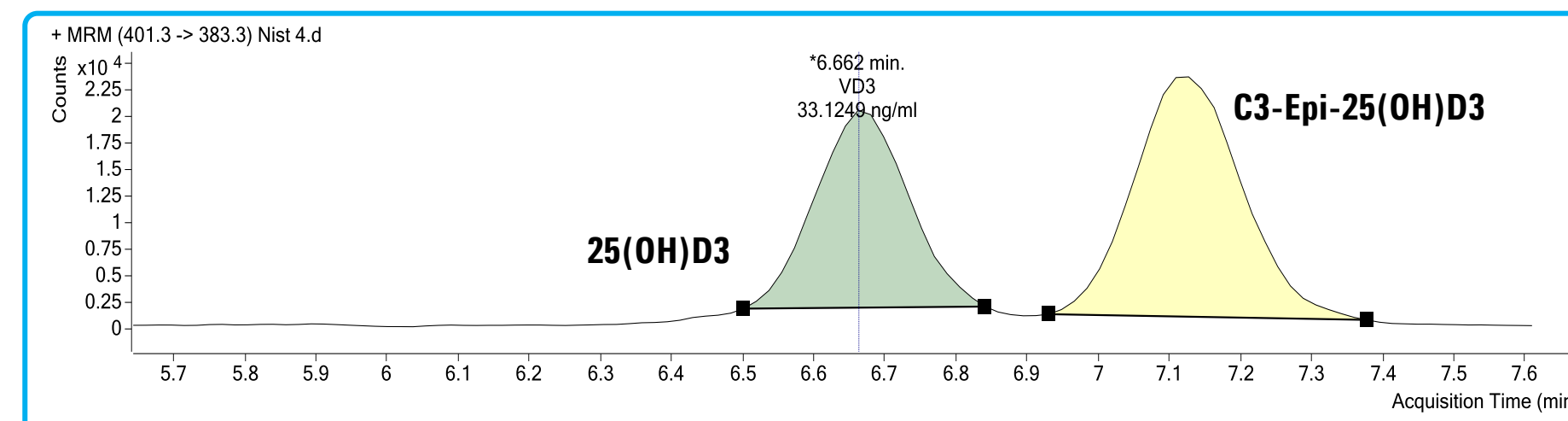
Precision

The inter-assay precision for 25(OH)D3 and 25(OH)D2 was determined by extracting and quantifying five replicates of the tri-level QC material from UTAK with %CV for 25(OH)D2 of 5.7, 5.8, 2.2 and for 25(OH)D3 of 3.6, 3.8, 1.8.

Sample	UTAK Low	UTAK 1	UTAK 2
25(OH)D2	11.89 ng/ml	35.49 ng/ml	83.26 ng/ml
Expected (ng/ml)	11 - 15	27 - 37	62 - 84
25(OH)D3	12.86 ng/ml	33.43 ng/ml	82.86 ng/ml
Expected (ng/ml)	12 - 16	27 - 37	64 - 86

Specificity and Sensitivity

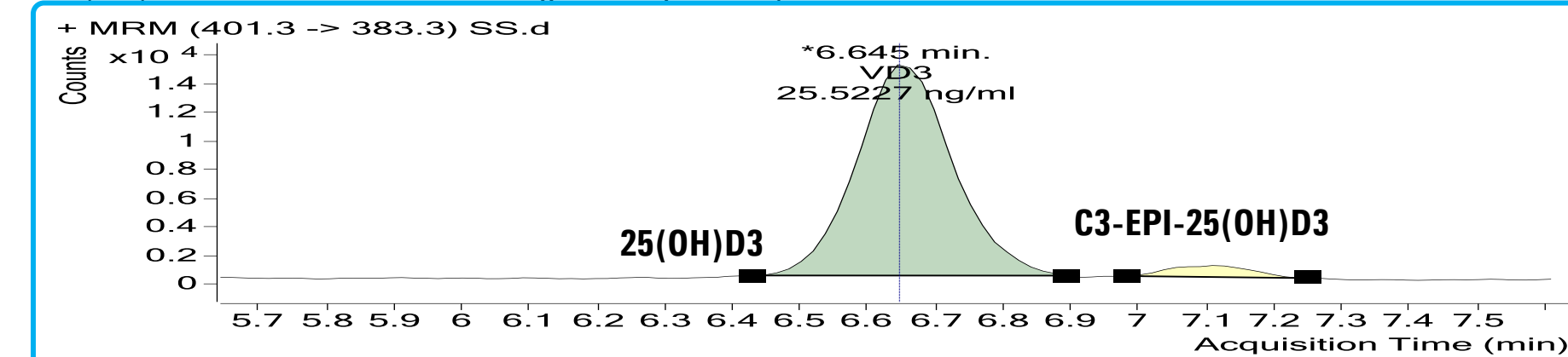
The chromatographic separation of 25(OH)D3 from C3-Epi-25(OH)D3 for NIST Level 4 by five replicates resulted in mean 33.1 ng/ml and 37.3 ng/ml levels with %CV of 5.83 and 6.42 respectively.



The LOD (S:N- 3:1) was determined using serum samples to be 0.25 ng/ml and LLOQ at 0.5 ng/ml.

Sample Analysis

The calculated mean of the 5 adult samples for Total 25(OH)D concentration was 27.4 ng/ml and C3-Epi-25(OH)D concentration was 0.75 ng/ml respectively.



Conclusion

Baseline separation of 25(OH)D and C3-Epi-25(OH)D in under 10 minutes using isocratic chromatography. Excellent linearity (>998) of calibration curves with great accuracy, precision and reproducibility. Potential over-estimation of 25(OH)D levels in adults due to similar mass transitions. Evaluate the method using child samples and to speed up analysis time.

References

- The C3 epimer of 25-Hydroxy-Vitamin D is present in Adult serum
 J. Clin. Endocrin Metab, 97(1), 1-6
 Fast separation of 25-Hydroxy-Vitamin D3 and C3-Epi-25-Hydroxy-Vitamin D3 in human serum by LC-MS/MS
 Clinchem, 57(11), 1618-1619