

Quantitative Analysis of 1,25-Dihydroxyvitamin D₂ and D₃ by LC-MS/MS Utilizing Ion Funnel Technology



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Introduction

A highly sensitive, and selective LC-MS/MS method for determination of 1,25-dihydroxyvitamin D₂ and D₃ is a powerful tool for clinical researchers. While 25 hydroxy vitamin D is found in the ng/ml concentration range, 1,25-dihydroxyvitamin D₂ and D₃ are typically found in the low pg/ml range, making quantitative analysis challenging except when employing highly sensitive analytical techniques. Additionally, extraction is a critical step for this analysis as removal of interfering analytes is required to quantify at the low pg/ml concentration range. Where previously published work (Casetta et al., 2010) demonstrates good sensitivity, such approaches require a complex 2D LC set-up, with post column infusion. The work presented in this poster illustrates the quantitative analysis of 1,25-dihydroxyvitamin D₂ and D₃ using the Agilent 1260 UHPLC & 6490 QQQ with Ion Funnel technology coupled with ImmunoTube LC-MS/MS Kit (an extraction kit from ImmunoDiagnostic) for the extraction of 1,25-dihydroxyvitamin D₂ and D₃ from plasma samples.

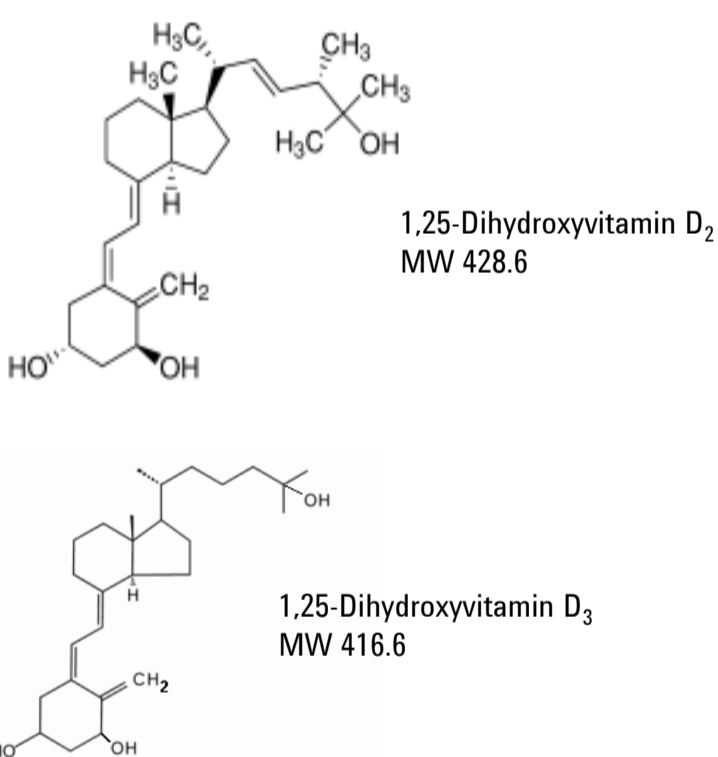


Figure 1. Structures and molecular weights of 1,25-dihydroxyvitamin D₂ and D₃

Sample Preparation

ImmunoTubes were spun down to ensure all the suspension was forced to the bottom of the tube. 500µl of calibrator / sample / control was added followed by 10µl of IS and mixed gently. ImmunoTubes were then mixed in a spiral rotator for 1hr at RT. The closed ImmunoTubes were then placed in a micro tube and centrifuged for 1min at 550 x g. Subsequently, the cover and the outlet of the ImmunoTubes were removed. The ImmunoTubes were then placed back into the micro tubes for centrifugation a further 2min at 550 x g. The waste collected in the micro tubes was discarded.

Sample Preparation

Continued...

500uL of WASHSOL was added to the ImmunoTubes and centrifuged for 2 min at 550 x g. This step was repeated twice. Each micro tube was replaced by a glass vial and 250µL of ELUREAG were added to each ImmunoTube which was centrifuged for 2min at 550 x g. The recovered eluent was evaporated under N₂ at 37°C. Samples were reconstituted with 165µL of activated Solution A prior to analysis.

WASHSOL and ELUREA are solutions from the ImmunoDiagnostic extraction kit.

LC Method

An Agilent 1260 HPLC series binary pump with 56 vial sample tray, sampler with thermostat, temperature-controlled column compartment, 2 position/6 ports switching valve, was used.

Column : Zorbax Eclipse Plus 2.1x100 mm 1.8µm
Column temperature: 50 °C
Injection volume: 100 µL
Autosampler temperature: 4 °C
Needle wash: 3:1 MeOH:H₂O, 10 seconds

Mobile Phase

A: ImmunoDiagnostic Mobile Phase A
B: ImmunoDiagnostic Mobile Phase B

Gradient	Flow	% Solvent B
0.00	0.3	0
6.00	0.3	100
6.50	0.3	100
6.51	0.3	0
8.00	0.3	0

Table 1. LC conditions

Compound	Prec Ion	Prod Ion	CE (V)
1,25(OH) ₂ Vitamin D ₂	411.1	150.7	20
1,25(OH) ₂ Vitamin D ₂	411.1	132.9	18
1,25(OH) ₂ Vitamin D ₃	399.1	150.9	12
1,25(OH) ₂ Vitamin D ₃	399.1	134.9	12
1,25(OH) ₂ Vitamin D ₃ -d ₆	405.1	150.6	12
1,25(OH) ₂ Vitamin D ₃ -d ₆	405.1	134.6	12

Table 2. MRM Parameters

Results and Discussion

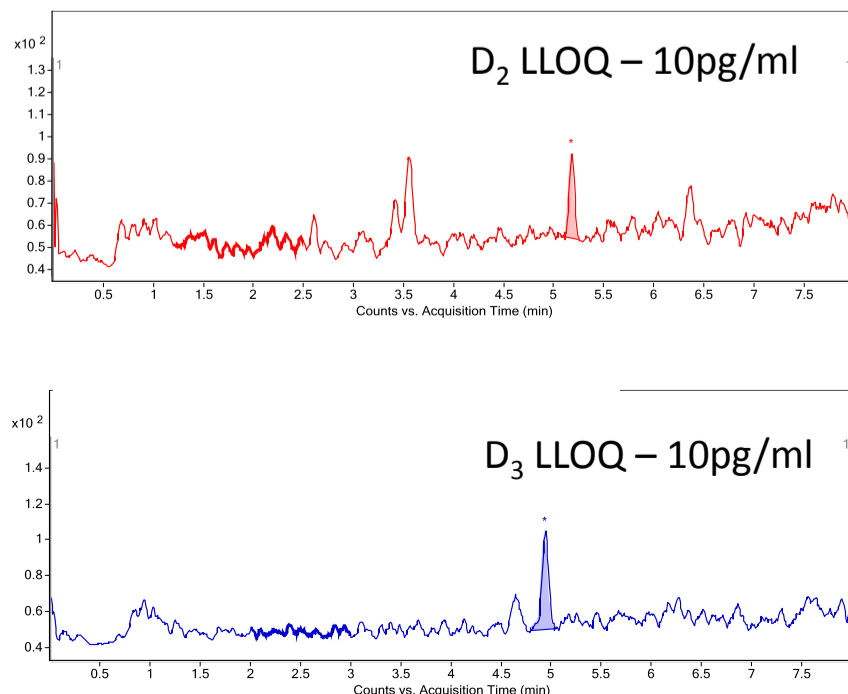


Figure 2. Lower limits of quantification (LLOQ) for neat, unextracted 1,25-dihydroxyvitamin D₂ and D₃.

Results and Discussion

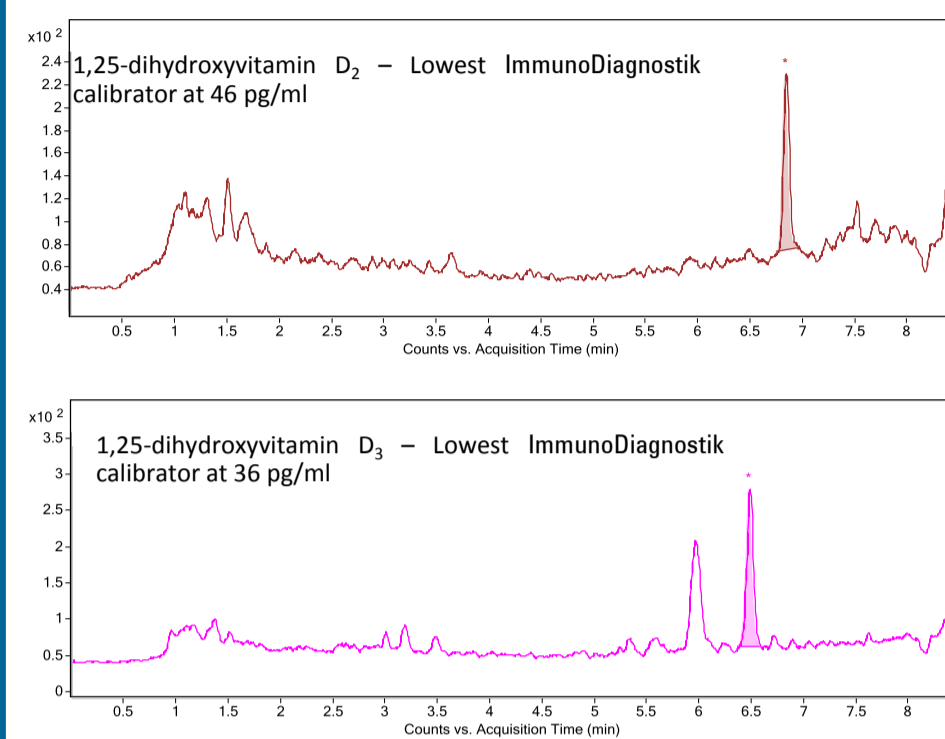


Figure 3. Chromatograms of extracted calibrators from ImmunoDiagnostic

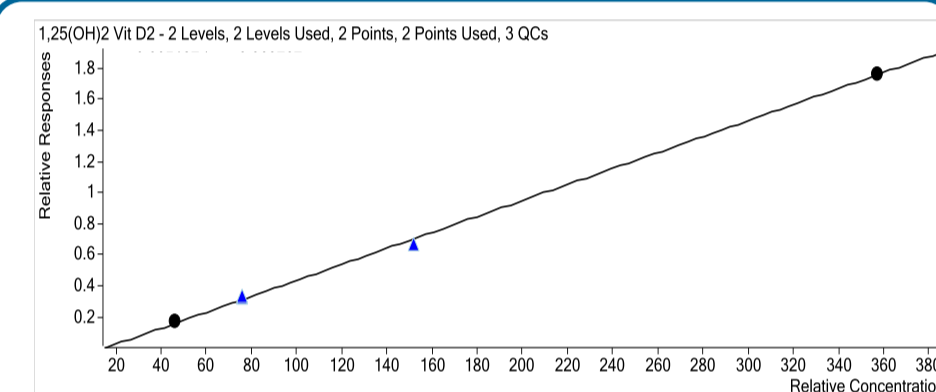


Figure 4. Calibration curve for 1,25-dihydroxyvitamin D₂

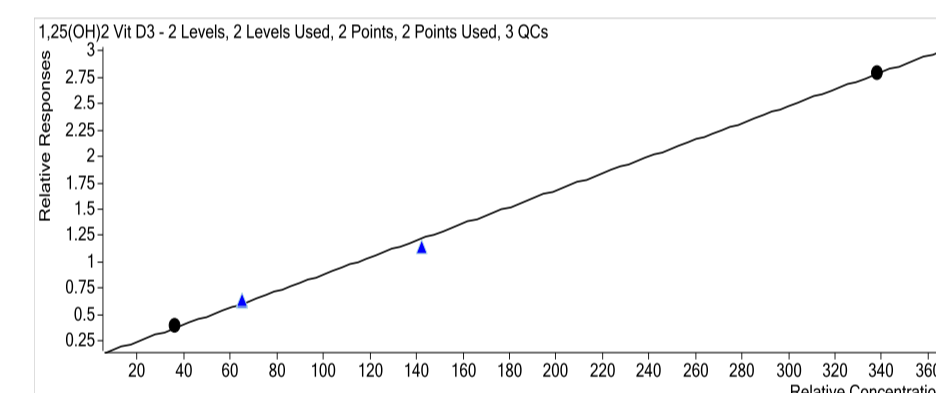


Figure 5. Calibration curve for 1,25-dihydroxyvitamin D₃

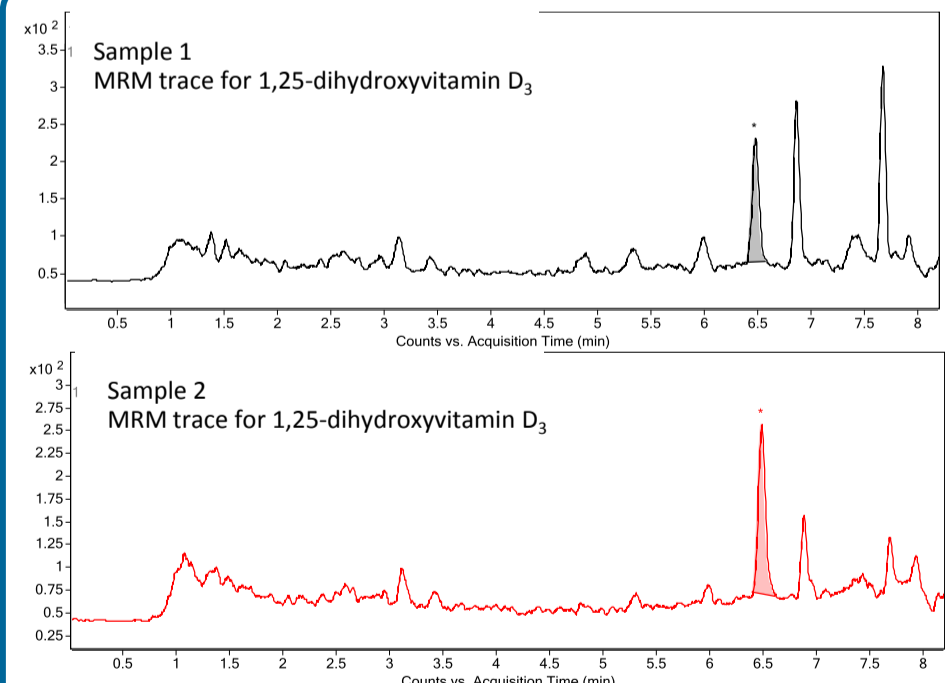


Figure 6. 1,25-dihydroxyvitamin D₂ and D₃ analyzed in 2 human samples. 1,25-dihydroxyvitamin D₂ was not detected in either sample but 1,25-dihydroxyvitamin D₃ was measured at 22 and 36 pg/ml in sample 1 and 2 respectively. Quantification of sample 1 required extrapolation of the calibration curve

Conclusion

A highly sensitive and selective method for quantifying 1,25-dihydroxyvitamin D₂ and D₃ from human plasma has been optimized. By combining the sensitivity of the 6490 QQQ with Ion Funnel Technology and the ImmunoDiagnostic extraction method, quantification at low pg/ml levels has been achieved.

Agilent MS systems are for research use only. Not to be used in diagnostic procedures.

Casetta A, Jans I, Billen J, Vanderschueren, Bouillon R. (2010) Development of a method for the quantification of 1,25(OH)₂-vitamin D₃ in serum by liquid chromatography tandem mass spectrometry without derivatization *Eur. J Mass Spectrom* 16(1):81-9.