

Rapid and Simultaneous Analysis of 25-Hydroxy Vitamin D2 and D3 in Plasma by LC-MS/MS with Online Sample Cleanup

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Abstract

This study was developed to simultaneously analyze 25-OH vitamin D3 (25-OHD3) and 25-OH vitamin D2 (25-OHD2) by LC-MS/MS with online sample cleanup utilizing valve switching. By using two LC columns to minimize sample matrix effect, it is possible to reduce the burden of sample preparation to a simple protein precipitation. To ensure data quality, 20 DEQAS samples were analyzed (Vitamin D External Quality Assessment Scheme, www.deqas.org) using LC-MS/MS. Results show good correlation with DEQAS results.

Background

In recent years the demand for 25-hydroxyvitamin D (25-OHD) analysis in serum or plasma has been increasing continuously. Vitamin D exists in two forms, vitamin D2 and vitamin D3. Many clinical research laboratories have now adopted LC-MS/MS based methods to enable the direct and reliable quantification of 25-OHD2 and 25-OHD3 (Figure 1). Traditionally, the analysis of 25-OHD by LC-MS/MS requires pretreatment of serum/plasma samples to release 25-OHD from the vitamin D binding protein and to minimize matrix effects. Often times, these steps can be time-consuming. This study demonstrates the analysis of 25-OHD2 and 25-OHD3 by LC-MS/MS utilizing a 2-column switching valve system for online sample cleanup to reduce matrix effects while simplifying any manual sample preparation.

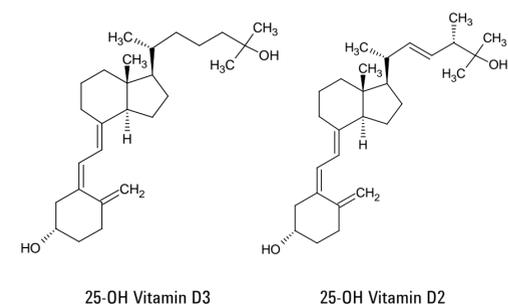


Figure 1. Structures of 25-OH Vitamin D

Sample preparation

1. Pipette 100ul sample/calibrator into 1.5ml reaction vial.
2. Add precipitation reagent (ZnSO4).
3. Add ISTD
4. Vortex for 20sec.
5. Incubate at 4°C.
6. Centrifuge.
7. Transfer clear supernatant into a clean vial.
8. Inject 10ul of supernatant into the LC-MS/MS.

System configuration

An Agilent 1260 LC system with HTC PAL autosampler (DLW option) coupled to an Agilent 6460 triple quadrupole mass spectrometer was used for all analyses. The instrument was operated in positive electrospray ionization mode. Multiple Reaction Monitoring (MRM) was used for quantitation. Agilent MassHunter™ software was used with the automated quantitation function based on batch.

LC Parameters

Source Parameters	
HPLC	Agilent 1260
Autosampler	HTC (DLW option)
Trapping Column	Poroshell 120 EC-C18 2.1 x 50mm, 2.7µm
Analytical Column	Poroshell 120 EC-C18 2.1 x 50mm, 2.7µm
Injection Volume (µl)	10
Column Temp (°C)	40
Flow Rate (ml/min)	0.5
Mobile Phase A	Formic Acid + NH4 Formate in water
Mobile Phase B	Formic Acid + NH4 Formate in ACN
Isocratic Composition	90% B

Online sample cleanup

The LC configuration depicted in figure 2 below allows for cleaner throughput to the mass spectrometer by performing online sample cleanup. Samples are injected onto a trapping column where the vitamin D metabolites are retained and washed, reducing the amount of matrix sent to the mass spectrometer. As the analytes are about to elute off of the first column, a valve is switched and analytes are eluted onto a second column where further chromatography is performed.

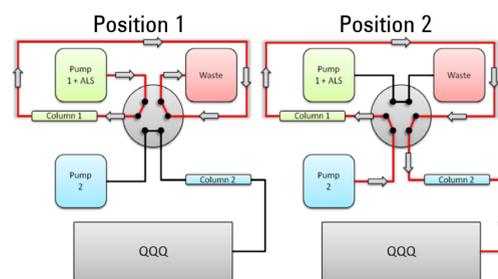


Figure 2. Switching Valve configuration for online sample cleanup

In addition to decreasing the amount of required sample preparation, figures 3 and 4 demonstrate significant advantages to using an LC configured for online sample cleanup.

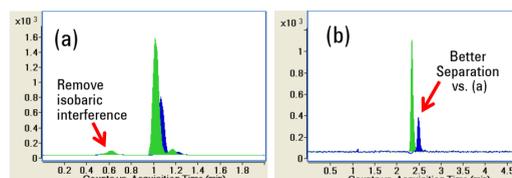


Figure 3. Analysis with (a) and without (b) online sample cleanup.



Figure 4. Source comparison with and without online sample cleanup.

MS Parameters

Source Parameters	
Model	Agilent 6460 QqQ
Dry Gas Temp (°C)	250
Gas Flow (l/min)	4
Nebulizer (psi)	60
Sheath Gas Temp (°C)	400
Sheath Gas Flow (l/min)	10
Capillary (V)	4000
Nozzle (V)	500
Polarity	Positive
MRM Dwell time (ms)	75

MRM transitions (Unit)	Frag (V)	CE (V)
25-OHD3	401.3 - 383.3	100 3
25-OHD2	413.3 - 395.3	100 3
25-OHD3-d6	407.3 - 389.3	100 3

Result

A linear calibration curve was created with a weighting of 1/x. Excellent linearity was achieved with an R² of > 0.999 for both 25-OH vitamin D metabolites. The accuracy of the calibrators was 98.1 to 103.4 for 25-OHD3 and 95.3 to 104.3 for 25-OHD2 across all levels of calibrators. The accuracy of the high and low QC was 93.0 to 107.2 for 25OHD3 and 96.7 to 113.0 for 25OHD2. Figure 4 shows the calibration curve for 25-OHD3 and 25-OHD2.

The sum of 25-OHD3 and 25-OHD2 was used for the total 25-OHD to compare with the LC-MS value by DEQAS (Vitamin D External Quality Assessment Scheme). Figure 6 shows the correlation between DEQAS results and LC-MS/MS results. The R² (0.979), slope close to 1 and small y-intercept suggest excellent correlation of results between the evaluated methods.

Result

Name	Type	Level	25OHD3 Results		25OHD2 Results	
			ng/ml	Accuracy	ng/ml	Accuracy
cal1	Cal	1	3.8	98.9		
cal2	Cal	2	7.8	98.7	5.1	104.3
cal3	Cal	3	20.8	103.4	13.2	95.3
cal4	Cal	4	35.1	100.2	27.1	101.6
cal5	Cal	5	67.2	98.1	50.1	97.3
cal6	Cal	6	107.7	100.6	91.9	101.6
381	Sample		41.5		N.D.	
382	Sample		15.4		N.D.	
383	Sample		33.7		N.D.	
384	Sample		12.7		N.D.	
385	Sample		30.5		N.D.	
386	Sample		13.0		N.D.	
387	Sample		23.7		N.D.	
388	Sample		35.8		N.D.	
389	Sample		18.8		N.D.	
390	Sample		27.6		N.D.	
391	Sample		6.7		N.D.	
392	Sample		29.6		N.D.	
393	Sample		12.1		N.D.	
394	Sample		23.7		N.D.	
395	Sample		16.1		N.D.	
396	Sample		11.6		N.D.	
397	Sample		19.2		N.D.	
398	Sample		32.3		N.D.	
399	Sample		14.0		N.D.	
400	Sample		28.3		N.D.	

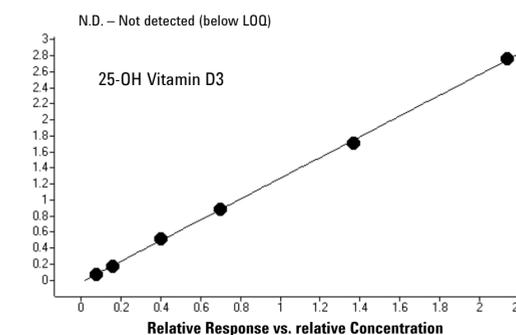


Figure 5. Results table and calibration curves

Sample	DEQAS		LC-MS/MS	
	ng/ml	ng/ml	ng/ml	%Dev
381	40.3	41.5	41.5	-2.8
382	16.0	15.4	15.4	3.9
383	33.7	33.7	33.7	-0.1
384	11.8	12.7	12.7	-6.7
385	26.4	30.5	30.5	-13.4
386	12.2	13.0	13.0	-6.2
387	24.1	23.7	23.7	1.6
388	33.1	35.8	35.8	-7.5
389	19.4	18.8	18.8	3.0
390	28.7	27.6	27.6	4.0
391	6.7	7.5	7.5	-10.7
392	29.6	31.9	31.9	-7.2
393	12.1	14.4	14.4	-14.7
394	23.7	26.2	26.2	-9.5
395	16.1	17.7	17.7	-9.1
396	11.6	13.5	13.5	-14.0
397	19.2	18.6	18.6	3.3
398	32.3	35.1	35.1	-7.9
399	14.0	15.6	15.6	-10.5
400	28.3	29.0	29.0	-2.4

Correlation to DEQAS

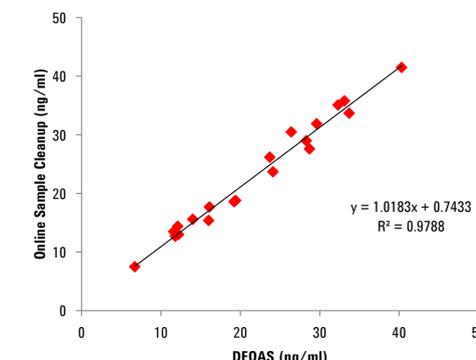


Figure 6. DEQAS correlation

Conclusions

A rapid and accurate quantitative method has been developed for the simultaneous analysis of 25-OH Vitamin D3 and 25-OH Vitamin D2 in plasma using online sample cleanup utilizing a 2-column switching valve system. This approach saves time by decreasing the amount of sample preparation, improves robustness with a cleaner ion source, and compares favorably to DEQAS with good accuracy, linearity, and sensitivity.