[APPLICATION NOTE]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Quantification of 1,25-Dihydroxyvitamin D_2 and D_3 From Human Serum Using Immunopurification, ACQUITY UPLC, and Xevo TQ-S micro

Nikunj Tanna, Mark Wrona, and Kelly Doering
Waters Corporation, Milford, MA

APPLICATION BENEFITS

- Highly sensitive and robust method
- Ability to detect 5 pg/mL using Waters® ACQUITY UPLC® and Xevo® TQ-S micro systems
- Accuracy and precision of <15% across the calibration range of 5–500 pg/mL

WATERS SOLUTIONS

ACQUITY UPLC System

ACQUITY UPLC BEH Column

Xevo TQ-S micro

MassLynx® Software

KEY WORDS

Small molecule bioanalysis, high sensitivity, accuracy and precision, reproducibility, 1,25 $(OH)_2$ vitamin D, DMPK, ADME

INTRODUCTION

Vitamin D is an important fat-soluble vitamin, which helps maintain bone health. Vitamin D exists in two primary forms: Vitamin D_3 (cholcalciferol) synthesized from 7-dehydrocholesterol when the skin is exposed to UV radiation from sunlight, and Vitamin D_2 (ergocalciferol) produced by plants and fungi through solar irradiation of ergosterol. Vitamin D is converted first to 25-hydroxyl (OH) Vitamin D by the liver via the CYP family of enzymes (Figure 1). 25 (OH) 25 (OH) Vitamin D is then hydroxylated into its biologically-active form: 1,25-dihydroxy $(OH)_2$ Vitamin D in the kidneys.

This conversion is tightly controlled through a cascade pathway which involves calcium, phosphorous, parathyroid hormone, and Vitamin D receptors. Regulated by a feedback mechanism process, 1,25 $(OH)_2$ Vitamin D circulates in the pg/mL-level range in serum. Main application areas for the measurement of Vitamin D are in nutrition, pharmacokinetic studies, clinical studies, and quality control for foods and supplements.[1]

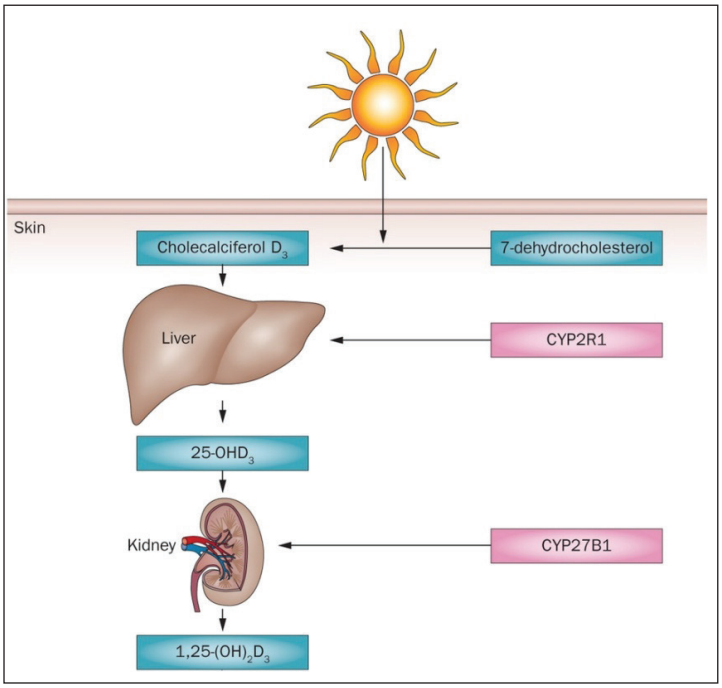


Figure 1. Process for conversion of 7-dehydrocholesterol to 1,25 $(OH)_2$ Vitamin D_3.[2]

In recent years, LC-MS/MS assays – compared to immunoassays – have gained popularity as the method of choice for quantification of 1,25 $(OH)_2$ Vitamin D. LC-MS/MS assays provide orthogonal selectivity. The identity of every compound is based upon its retention time as well as its unique MRM transition. The addition of immunoaffinity using specific antibodies in sample cleanup adds an extra layer of selectivity to the assay.

LC-MS/MS is accepted by the FDA as the gold standard analytical technique for pharmacokinetic studies of small molecules. The FDA has set specific guidelines to be followed while developing and validating LC-MS/MS assays. These guidelines involve intra-day (within the same day) and inter-day (across multiple days) accuracy and precision studies, linearity, and reproducibility. The method presented here followed the Bioanalytical Method Validation guidelines set out by the FDA. Accuracy, precision, linear range, and reproducibility of the method was evaluated, and the method met the criteria detailed in the FDA guidelines.[3]

The data presented in this application note was generated using a Waters ACQUITY UPLC System and Xevo TQ-S micro Mass Spectrometer.

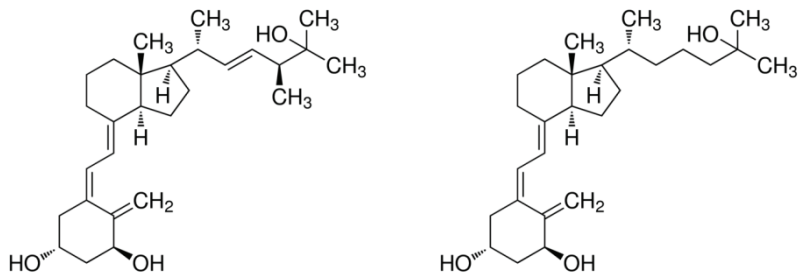


Figure 2a Figure 2b

Figure 2a. 1,25 di-hydroxy Vitamin D_2. Figure 2b. 1,25 di-hydroxy Vitamin D_3.

EXPERIMENTAL

Sample description

Commercially-available immunopurification kits were purchased from ALPCO Diagnostics and used to extract 1,25 $(OH)_2$ Vitamin D from 500 µL of human serum. The eluate from the last step was evaporated to dryness. Samples were derivatized with PTAD (4-phenyl-1,2,4-triazoline-3,5-dione) using 100 µL of 0.75 mg/mL PTAD in acetonitrile, which was added to each tube and allowed to incubate in the dark – at room temperature – for one hour. PTAD was then evaporated using a CryoVac system. The contents of the tubes were reconstituted using 50 µL of 50:50 water–methanol mix. This solution was then transferred to LC-MS/MS vials, and 20 µL were injected into a column.

Method conditions

LC conditions

Instrument:	Waters ACQUITY UPLC
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 mm x 50 mm (P/N 1860002350)
Column temp.:	60 °C
Sample temp.:	4 °C
Injection volume:	20 µL
Flow rate:	0.500 mL
Mobile phase A:	100% water, 0.1% formic acid, 2 mM methylamine
Mobile phase B:	100% methanol, 0.1% formic acid, 2 mM methylamine
Gradient:	Start with 50% A and hold for two minutes. Change to 80% B between 2–4 minutes. Followed by one minute of flushing and one minute of equilibration.

MS conditions

Instrument:	Xevo TQ-S micro
Ionization mode:	ESI+
Transitions:	1,25 $(OH)_2$ Vit D_3 – 574.2>314.1 1,25 $(OH)_2$ Vit D_2 – 635.3>314.1 1,25 $(OH)_2$ Vit D_3 Int std – 580.3>314.1 1,25 $(OH)_2$ Vit D_2 Int std – 641.3>314.1
Capillary voltage:	2.5 kV
Cone voltage:	25 V
Desolvation temp.:	500 °C
Desolvation gas:	1000 L/Hr
Cone gas:	25 L/Hr

Data management

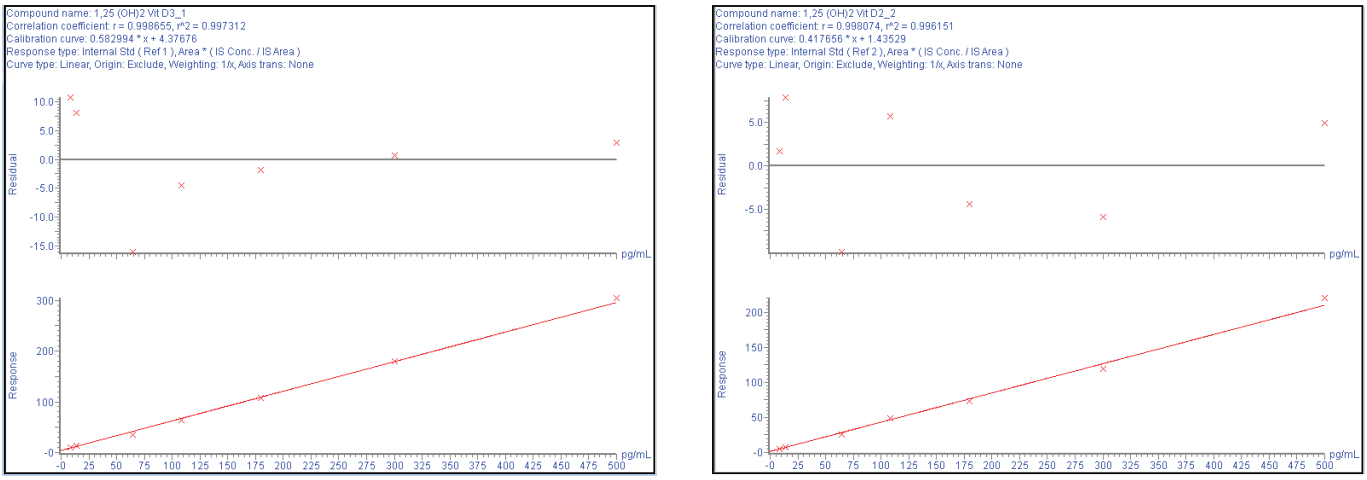
MassLynx 4.1

RESULTS AND DISCUSSION

1,25 $(OH)_2$ Vitamin D is an important biomarker tested routinely in clinical and bioanalytical laboratories. The extremely low circulating levels of this molecule, coupled with lack of ionization in electrospray ionization mode, make this a challenging assay. The method described here combines affinity-based sample preparation combined with derivatization to increase ionization as an elegant solution for this analytically-difficult molecule.

Calibration curve and residual plots

Calibration curve from 5–500 pg/mL is linear and % bias across the range is <15% for both 1,25 $(OH)_2$ Vit D_2 and D_3 as shown in Figure 3a and 3b.



3a 3b

Figure 3a. Residual plot and calibration curve for 1,25 $(OH)_2$ Vit D_3. Figure 3b. Residual plot and calibration curve for 1,25 $(OH)_2$ Vit D_2.

Inter-day accuracy and precision

Six replicates at low (30 pg/mL), mid (150 pg/mL), and high (300 pg/mL) QC levels were extracted and injected across three days. The intra-day and inter-day precision and accuracy were <15% at all levels as shown in Tables 1–4.

1a			Mean	Std dev	% CV	% Bias
Intra-day	30	25.8	29.18	2.89	9.91	-2.72
	30	31.4				
	30	26.4				
	30	28.5				
	30	33.3				
	30	29.7				
	30	34	32.83	4.77	14.51	9.44
	30	26.9				
	30	32.3				
	30	29.3				
	30	40.8				
	30	33.7				
	30	25.6	28.75	3.27	11.37	-4.17
	30	32.1				
	30	30.5				
	30	32.4				
	30	25.6				
	30	26.3				

1b			Mean	Std dev	% CV	% Bias
Intra-day	150	160.7	144.73	14.16	9.78	-3.51
	150	138.9				
	150	160.4				
	150	128.1				
	150	131.5				
	150	148.8				
	150	145.3	148.08	14.44	9.75	-1.28
	150	159.5				
	150	130.2				
	150	135.1				
	150	168.4				
	150	150				
	150	156.4	151.63	15.16	10.00	1.09
	150	134.2				
	150	165.6				
	150	130.9				
	150	159.2				
	150	163.5				

1c			Mean	Std dev	% CV	% Bias
Intra-day	300	270.4	273.82	9.95	3.63	-8.73
	300	280				
	300	260.6				
	300	268.6				
	300	289.4				
	300	273.9				
	300	295.7	303.38	17.24	5.68	1.13
	300	333.1				
	300	296.6				
	300	314.1				
	300	295.2				
	300	285.6				
	300	269.5	294.27	23.47	7.98	-1.91
	300	329.1				
	300	276.2				
	300	304.7				
	300	308.7				
	300	277.4				

Table 1a. Intra-day: 1,25 $(OH)_2 D_2$ – LQC (30 pg/mL). Table 1b. Intra-day: 1,25 $(OH)_2 D_2$ – MQC (150 pg/mL). Table 1c. Intra-day: 1,25 $(OH)_2 D_2$ – HQC (300 pg/mL).

		LQC	MQC	HQC
Intra-day	Mean	30.26	148.15	290.49
	Std dev	3.98	14.01	20.99
	% CV	13.15	9.46	7.22
	% Bias	0.85	-1.23	-3.17

Table 2. Inter-day precision data: 1,25 $(OH)_2$ Vitamin D_2.

3a			Mean	Std dev	% CV	% Bias
Intra-day	30	32	31.47	3.21	10.20	4.89
	30	27.2				
	30	27.7				
	30	34.2				
	30	33.8				
	30	33.9				
	30	26.5	29.75	3.92	13.19	-0.83
	30	31.7				
	30	33.6				
	30	34.4				
	30	25.8				
	30	26.5				
	30	26.5	28.72	2.62	9.13	-4.28
	30	25.8				
	30	29.5				
	30	29				
	30	28.3				
	30	33.2				

3b			Mean	Std dev	% CV	% Bias
Intra-day	150	168.4	166.90	3.48	2.09	-11.27
	150	170.8				
	150	167.8				
	150	161.8				
	150	169.1				
	150	163.5				
	150	162.3	153.17	10.86	7.09	-2.11
	150	166.3				
	150	144.6				
	150	145.2				
	150	140.8				
	150	159.8				
	150	157.4	156.10	3.85	2.47	-4.07
	150	154				
	150	154.8				
	150	151				
	150	157				
	150	162.4				

3c			Mean	Std dev	% CV	% Bias
Intra-day	300	334.3	332.25	12.83	3.86	-10.75
	300	336.3				
	300	342.5				
	300	332.2				
	300	307.3				
	300	340.9				
	300	314.9	316.30	22.89	7.24	-5.43
	300	313.5				
	300	321.5				
	300	274.9				
	300	341.6				
	300	331.4				
	300	260.1	293.32	27.33	9.32	2.23
	300	316.8				
	300	307.5				
	300	256.5				
	300	309.1				
	300	309.9				

Table 3a. Intra-day: 1,25 $(OH)_2 D_3$ – LQC (30 pg/mL). Table 3b. Intra-day: 1,25 $(OH)_2 D_3$ – MQC (150 pg/mL). Table 3c. Intra-day: 1,25 $(OH)_2 D_3$ – HQC (300 pg/mL).

		LQC	MQC	HQC
Intra-day	Mean	29.98	158.72	313.96
	Std dev	3.31	8.92	26.32
	%CV	11.03	5.62	8.38
	% Bias	-0.07	-5.81	-4.65

Table 4. Inter-day precision data: 1,25 $(OH)_2$ Vitamin D_3.

Injection reproducibility

The Waters ACQUITY UPLC and Xevo TQ-S micro displayed robust injection reproducibility as shown below. The % CV for the retention times was 0.32% (Figure 4a) and the % CV for analyte area counts was 5.21% (Figure 4b) – both of which are well within the acceptable criteria.

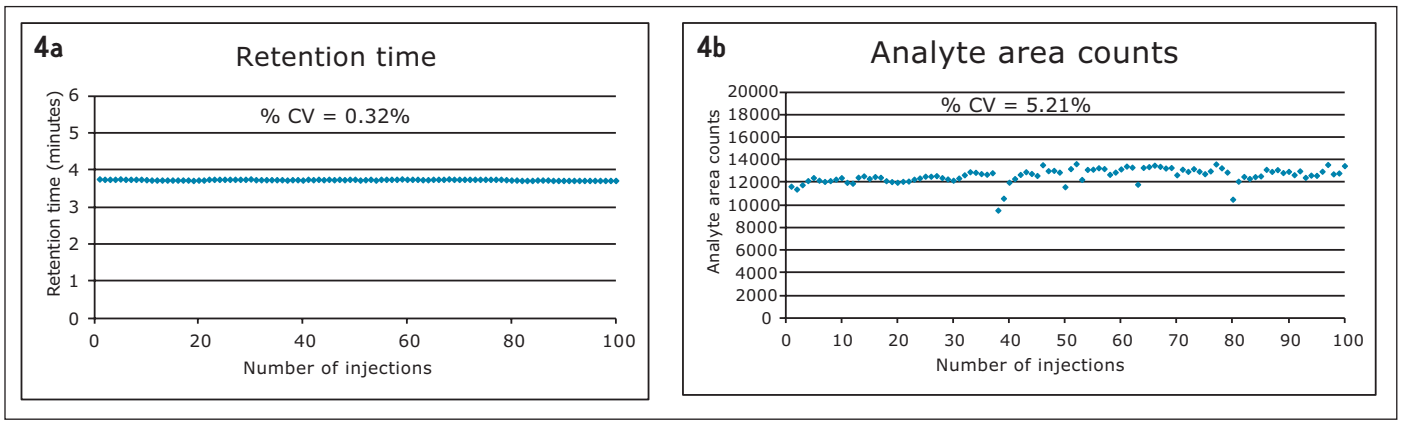


Figure 4a. % CV for retention time over 100 injections. Figure 4b. % CV for analyte area counts over 100 injections.

Instrument dynamic range

The analytical range for this method is from 5–500 pg/mL and covers the relevant concentrations typically found in serum.

1,25 $(OH)_2$ Vitamin D showed linearity from 50 pg/mL–1 µg/mL using the ACQUITY UPLC and TQ-S micro for the method described here.

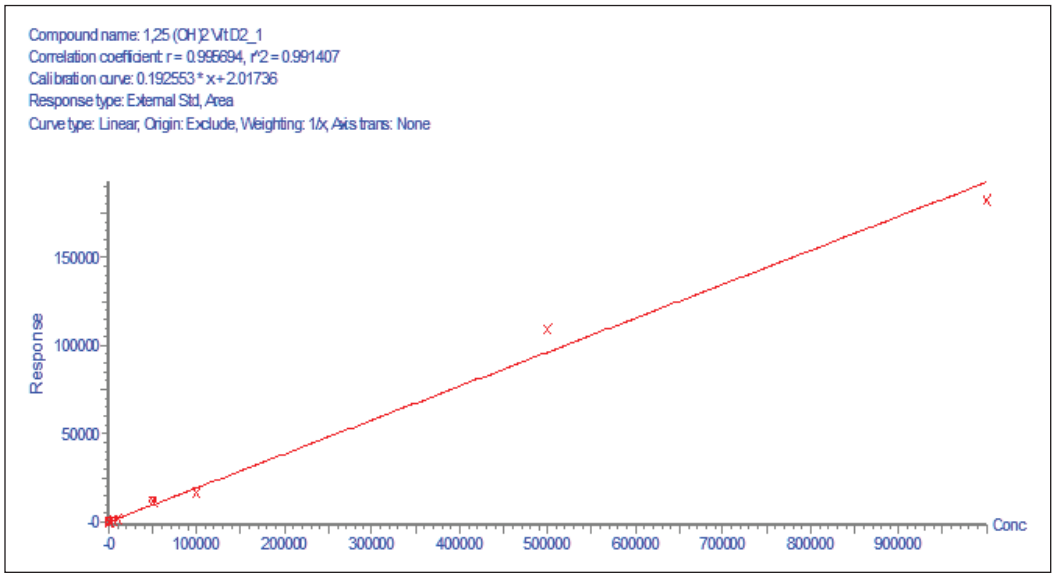


Figure 5. Linear dynamic range >4x.

CONCLUSIONS

A robust, selective, and sensitive analytical method for the quantification of 1,25 $(OH)_2$ Vitamin D_2 and D_3 from human serum was developed using the Waters ACQUITY UPLC System and Xevo TQ-S micro Mass Spectrometer. A limit of quantification of 5 pg/mL was readily achieved while maintaining excellent linearity. Calibration curves for both 1,25 $(OH)_2$ D_2 and D_3 were linear over the range of 5–500 pg/mL with r^2>0.99. Across three days, the intra- and inter-day CV as well as the % bias were <15% for both 1,25 $(OH)_2$ Vitamin D_2 and D_3. Injection reproducibility was excellent with % CV <0.32% for retention time and <5.5% for area counts.

Today's analytical laboratories are becoming more diverse and multi-functional. Lab managers are expected to diversify their analytical platforms within limited lab spaces. The Xevo TQ-S micro has the ideal combination of sensitivity, reproducibility, and versatility to provide an excellent option for all types of bioanalytical labs.

References

1. van den Ouweland, M. W. J.; Vogeser, M.; Bächer, S. Vitamin D and metabolites measurement by tandem mass spectrometry. *Rev Endocr Metab Disord*. (2013): 159–184.
2. Jensen, M. J. Vitamin D and male reproduction. *Nature Reviews Endocrinology*. (2014): 175–186.
3. Guidance for Industry, Bioanalytical Method Validation. *Food and Drug Administration* (2001).

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com