Ultra low level determination of 25-mono-hydroxy-vitamin D2 and D3 in human plasma by acetonitrile- free XLC-MS/MS using the Symbiosis System

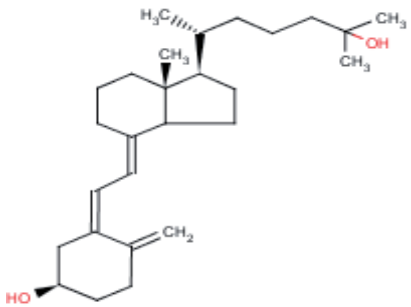
0053-080-01

Summary

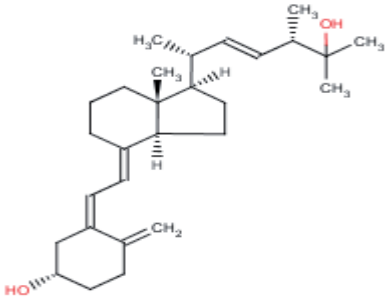
A fast, sensitive and simple method has been developed for the analysis of 25-mono hydroxy vitamin D2 (MHVD2) and 25-mono hydroxy vitamin D3 (MHVD3) in human plasma. The sample is only diluted with a protein disrupting buffer before direct injection into the Symbiosis system. A detection limit of 1 ng/mL per compound is achieved with a total runtime of 4 minutes, including on-line spe.

Introduction

Vitamin D is a group of fat-soluble pro-hormones that helps the body to absorb calcium and maintain proper levels of calcium and phosphorus in the blood. Vitamin D deficiency results in impaired bone mineralization and leads to bone softening diseases. A vitamin D overdose can raise blood levels of calcium and cause gastrointestinal symptoms and kidney disease.



25-Mono-Hydroxy-Vitamin D3 (MHVD3)



25-Mono-Hydroxy-Vitamin D2 (MHVD2)

Symbiosis is Spark Holland's solution for integrated online SPE-LC-MS (XLC-MS).

Instrumental conditions

Autosampler Conditions

250 µL of sample is injected using the standard autosampler configuration.
Washing is performed with two wash solvents.

Wash solvent 1:	40% methanol with 0.2% formic acid (fa)
Wash solvent 2:	80% methanol, 10% 2-propanol and 10% water.

Autosampler wash routine.	
Wash solvent	Wash volume
1	700 µL
2	700 µL
1	1000 µL

SPE conditions

Automated SPE sorbent screening has been used to find the best performing SPE cartridge and optimal wash conditions for clean-up. HySphere C8EC-SE gave the highest signal and also the best peak shape. Recovery compared to a LC injection area is higher then 90%.

SPE Cartridge:	10 x 2 mm HySphere C8EC-SE (Spark P/N:0822.611)	
Solvation:	1 mL methanol	5 mL/min
Equilibration:	1 mL 40% methanol in water, 0.2 % fa	5 mL/min
Sample Loading:	1 mL 40% methanol in water, 0.2 % fa	2 mL/min
Wash 1:	1 mL 40% methanol in water, 0.2 % fa	5 mL/min
Wash 2:	1 mL 60% methanol in water, 0.2 % fa	5 mL/min
Elution	1 min. 30 sec (with LC gradient at 1 mL/min)	

LC conditions

Column:	Merck KgaA Chromolith SpeedROD C18e 50X4.6 mm (P/N:1.51450.0001)
Mobile phase A:	Water, 0.2% fa
Mobile phase B:	Methanol, 0.2% fa

LC gradient

Time (mm:ss)	Flow (mL/min.)	A (%)	B (%)
00:00:01	1.00	20	80
00:00:05	1.00	20	80
00:03:00	1.00	10	90
00:03:10	1.00	10	90
00:03:15	1.00	20	80
00:03:45	1.00	20	80

MS Conditions

A Sciex API 4000 MS/MS (Applied Biosystems/MDS Sciex) is used with a Heated Nebulizer interface in positive mode.

MS parameters

CUR:	10.00
GS1:	50.00
GS2:	0.00
NC:	5.00
TEM:	500.00
ihe:	ON
CAD:	4.00
IQ2	-18.00

Compound dependable MS settings			
	MHVD 2	MHVD 3	MHVD3 D6
Mass	412	400	406
Q1 mass	395.1	383.1	389.1
Q3 mass	209.1	211.1	211.1
Dwell time	100	100	100
DP	50.00	50.00	50.00
EP	10.00	10.00	10.00
CE	35.00	35.00	35.00
CXP	15.00	15.00	15.00

Sample preparation

Reagents

1. Protein Disrupting Buffer (PDB):
   This buffer has been especially developed by Spark Holland for rapid and efficient reduction of protein binding. For information about this buffer please contact us at Application@sparkholland.com.
2. Standards working solutions:
   2500, 250 and 25 ng/mL MHVD2 and MHVD 3 in PDB.
3. Internal standard for Patient samples (ISp):
   100 ng/mL MHVD3-D6 in PDB.
4. Internal standard for Calibration samples (ISc):
   1000 ng/mL MHVD3-D6 in PDB.

Samples prepared as described below are stable for at least 24 hours at 25° C, in the dark. When stored at 4° C the samples are stable for one week.

Patient samples:

Pipette 250 µL of plasma sample into an amber autosampler vial.
Add 250 µL of ISp to the vial.
Vortex sample for a few seconds before placing the vial into the autosampler.

Calibration samples

Calibration samples are prepared in Human Sodium Heparin Plasma at 1; 5; 10; 25; 50; 75; 100; 250; 500 ng/mL as follows:

Transfer 500 µL of plasma sample into an amber autosampler vial.
Add 50 µL of ISc to the vial.
Add volumes of PDB and standard solutions (MHVD2 and MHVD3) for various calibration points as shown in table below.
Vortex sample for a few seconds before placing the vial into the autosampler.

Calibration curve sample preparation

Calibration point (plasma concentration in ng/mL)	Volumes mixed (µl)			
	PDB	2500 ng/mL in PDB	250 ng/mL in PDB	25 ng/mL in PDB
1	430			20
5	350			100
10	430		20	
25	400		50	
50	350		100	
75	300		150	
100	250		200	
250	400	50		
500	350	100		

Quality control samples

Calibration standards are prepared in Human Sodium Heparin Plasma at 5; 75 and 400 ng/mL as follows:

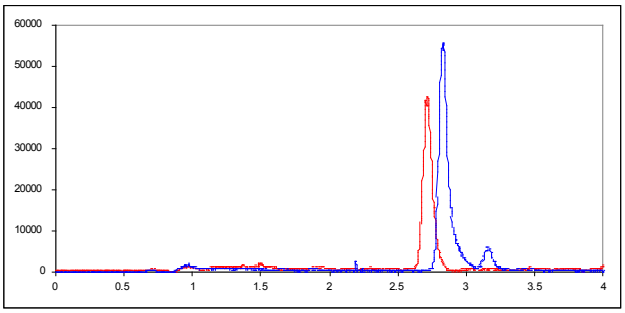
Transfer 500 µL of plasma sample into an amber autosampler vial.
Add 50 µL of ISc to the vial.
Add volumes of protein disrupting buffer and standard solutions (MHVD2 and MHVD3) for various quality control points as shown in table below.
Vortex sample for a few seconds before placing the vial into the autosampler.

Quality control sample preparation

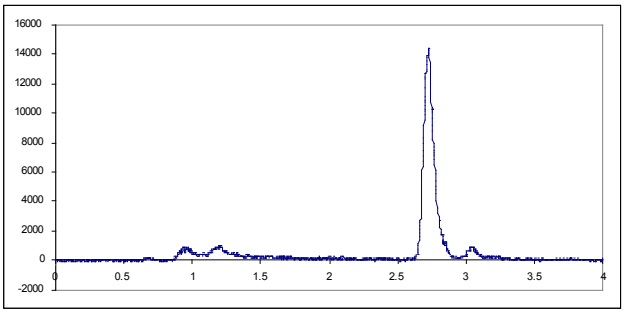
Control (plasma concentration in ng/mL)	Volumes mixed (µl)			
	PDB	2500 ng/mL In PDB	250 ng/mL In PDB	25 ng/mL In PDB
5	350			100
75	300		150	
400	370	80		

Results

Chromatograms



Chromatogram representing 100 ng/mL MHVD3 (red) and MHVD2 (Blue) spiked in human plasma.



50 ng/mL internal standard spiked in human plasma

Linearity

Linearity was determined by injecting the full set of calibration samples ranging from 1 to 500 ng/ml. The resulting calibration curve has a regression coefficient of R = 0.999 with a 1/X weighing for both compounds.

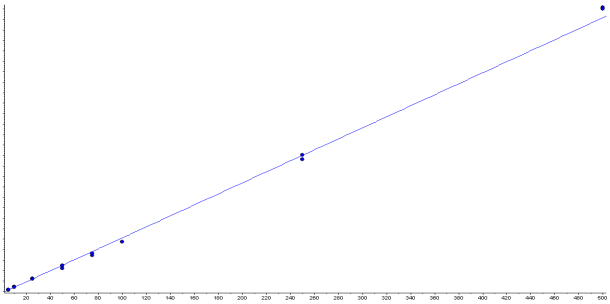


Figure 10: MHVD2 Cal Curve R=0.999 with 1/X weighing

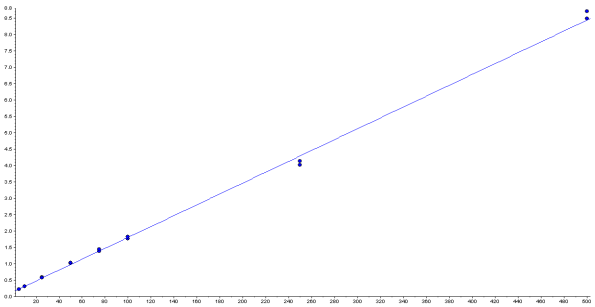


Figure 11: MHVD3 Cal Curve R=0.999 with 1/X weighing

Accuracy and precision

Accuracy calculated by combining 3 sets of data:
2 series of calibration samples were measured with the Symbiosis Pico system en 1 series was measured with the Symbiosis Pharma system.

Calibration Sample (ng/mL)	MHVD 3	MHVD 2
	Accuracy (%)	Accuracy (%)
1	88.8	113
5	92.7	108
10	100	108
25	104	103
50	105	93.5
75	102	93.0
100	99.3	92.5
250	94.6	98.6
500	102	104

Accuracy and precision calculated by combining 3 sets of data:
2 series of QC samples were measured with the Symbiosis Pico system en 1 series was measured with the Symbiosis Pharma system.

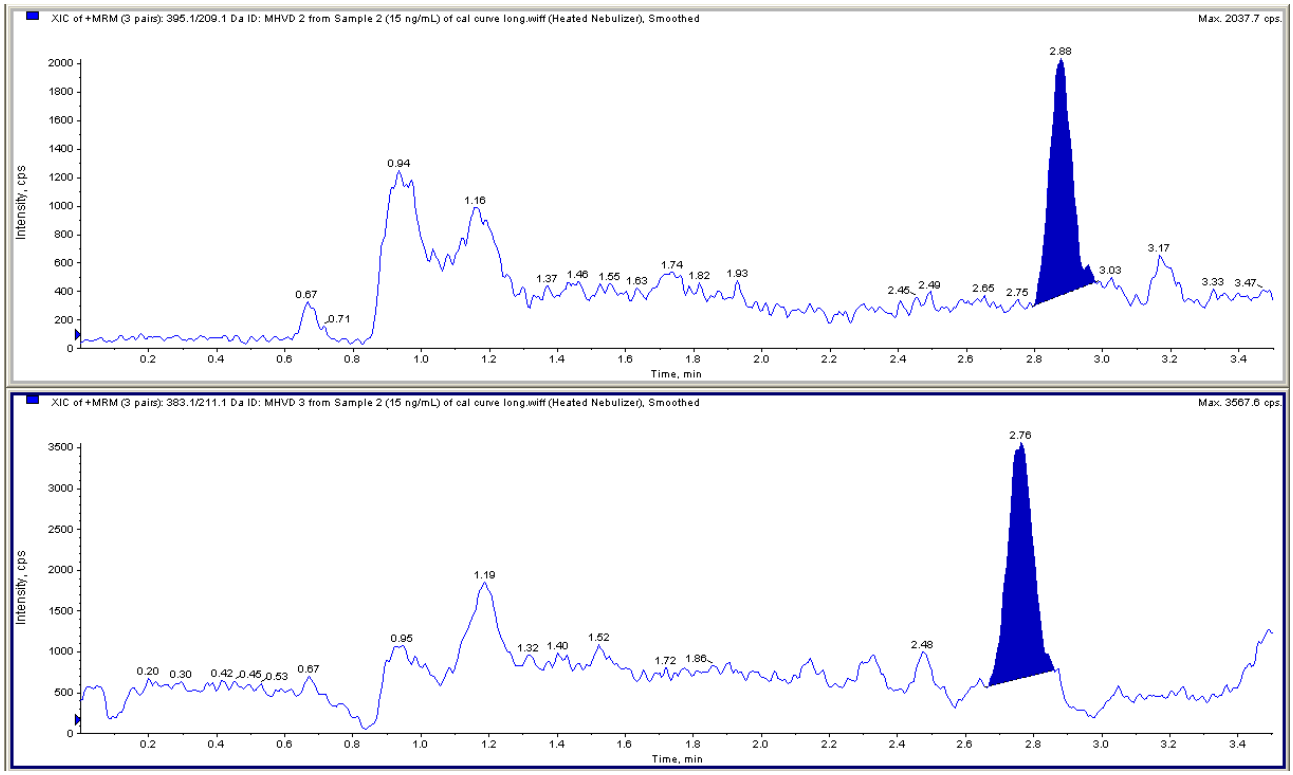
QC Sample (ng/mL)	MHVD 2		MHVD 3	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
5	7.80	103	6.49	106
50	2.61	105	3.04	102
400	2.80	107	3.98	107

Sensitivity

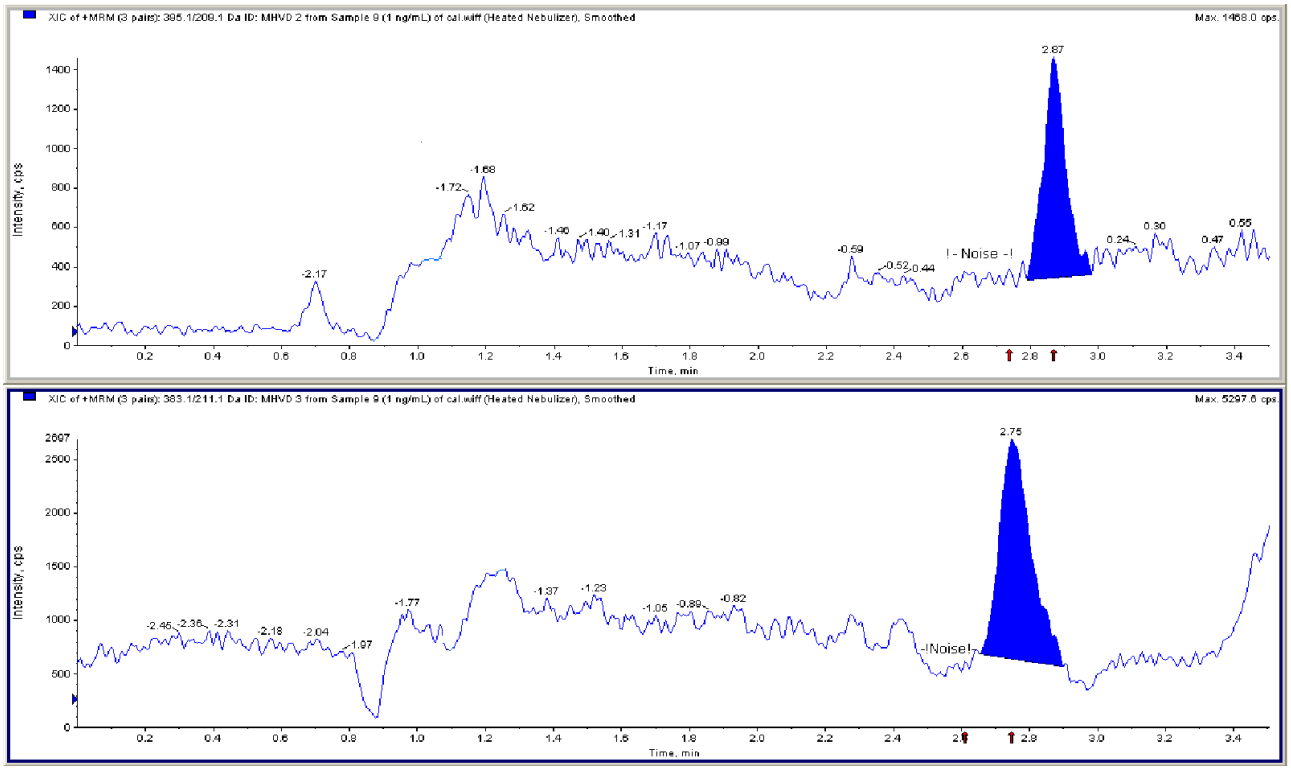
With 250 µL of sample (125 µL plasma) a quantitation limit of 5 ng/mL is achieved. 1 ng/mL is still detectable with a signal to noise of 4 or more:

S/N for 250 µL injections

Concentration (ng/mL)	MHVD2 S/N	MHVD3 S/N
1	4	4
5	16	10
10	40	16



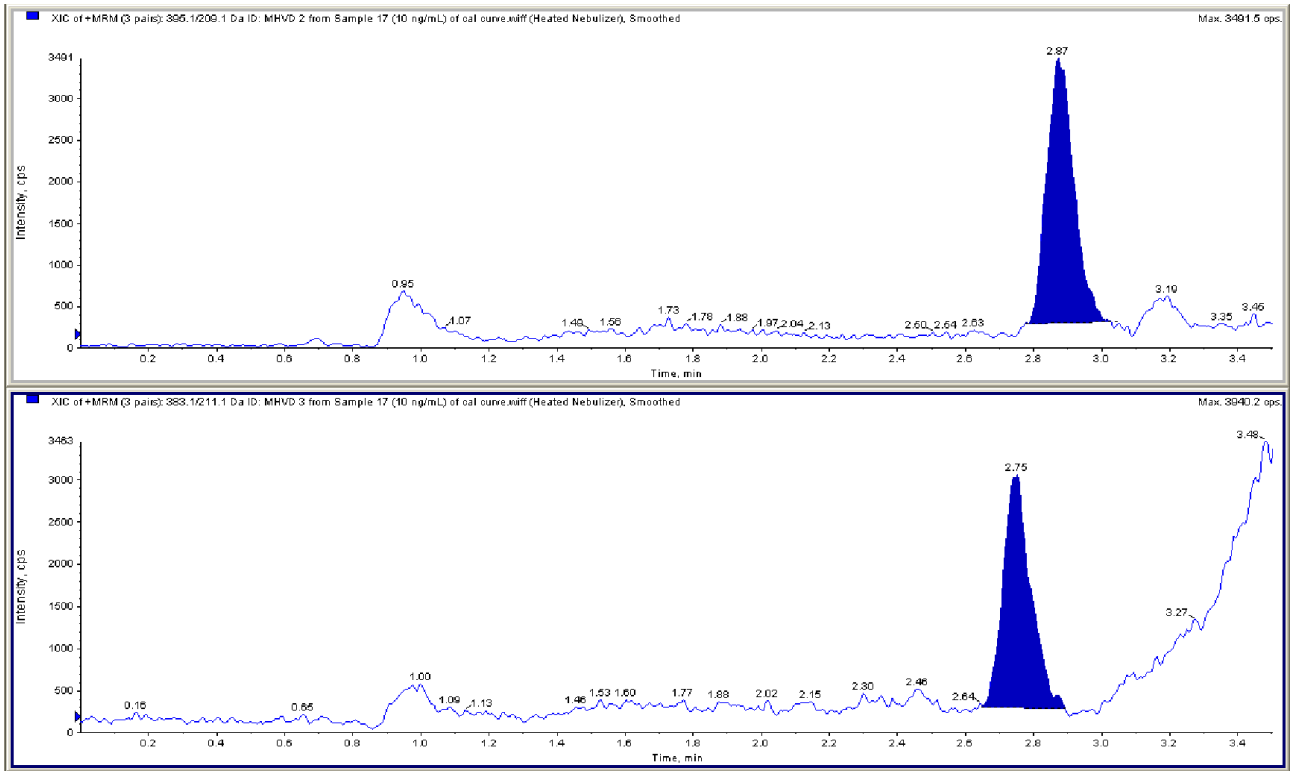
5 ng/ml spiked plasma sample. 250 µL of sample is injected.



1 ng/ml spiked plasma sample. 250 µL of sample is injected.

When only 50 µl of plasma is available, resulting in 100 µl sample, a quantitation limit of 10 ng/mL is achieved for both compounds:

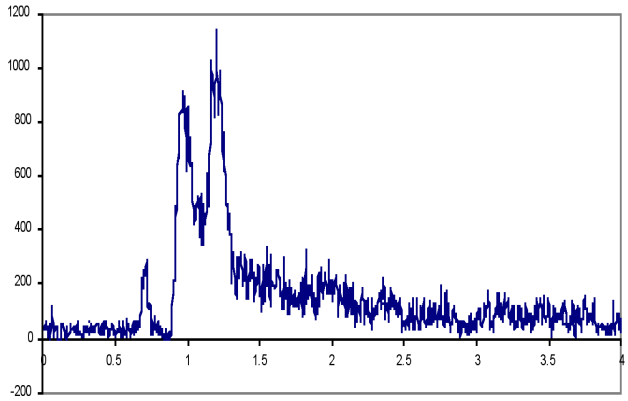
S/N for 100µL injections		
Concentration (ng/mL)	MHVD2 S/N	MHVD3 S/N
10	18	8



10 ng/mL spiked plasma. 100 µL injected

Carry-over

MHVD2 and MHVD3 are endogenous compounds and therefore always present in human plasma, which means that there will be no zero blank. Therefore, in order to determine carry-over of the method, the carry-over of the internal standard is measured. No measurable carry-over was observed as shown in the chromatogram below.



Chromatogram representing blank after 50 ng/mL Internal Standard.

Conclusion

This method was evaluated using spiked human sodium heparin plasma samples. The developed XLC-MS/MS method has an absolute recovery of more then 90% and a linear range from 1-500 ng/ml (R=0.999) for both compounds. Accuracy is 89-117% and precision 0.1-7.9 %CV. The estimated quantitation limits for both analytes are more than sufficient to allow the method to be used for therapeutic drug monitoring. For 250 µl injections, containing 125 µL of plasma, a detection limit of 1 ng/mL and a quantitation limit of 5 ng/ml is achieved. When only 50 µl of plasma is available, still a quantitation and detection limit of 10 ng/mL is achieved. The assay runs on both the Symbiosis Pico and Symbiosis Pharma system.