Ultra Low Level Determination of 25-Mono-Hydroxy-Vitamin D2 and D3 in Plasma by On-line SPE-LC-MS/MS

Martin Sibum and Bert Ooms
Spark Holland B.V., Pieter de Keyserstraat 8, 7825 VE, Emmen, the Netherlands
P +31 591631700, applications@Sparkholland.com, www.sparkholland.com

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. Increasing awareness of these problems and the rapidly aging population in the western world have led to a significant increase in diagnostic analyses. This in turn has stimulated the demand for more automation and to that end, an automated, 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 and injected directly into the analytical system. A detection limit of 1 ng/mL per compound is achieved with a total runtime of 4 minutes, including on-line Solid Phase Extraction. The use of acetonitrile has been avoided to circumvent the current shortage problems.

EXPERIMENTAL

A Symbiosis system (Spark Holland) was used for on-line SPE-LC. The Symbiosis system was coupled to an API 4000 (Applied Biosystems/MDS Sciex) for MS/MS analysis

Autosampler
250 µL of sample is injected using the standard Symbiosis autosampler configuration.
Needle wash in 3 steps:
1.700 µL; 40% methanol in water, 0.2% formic acid (f.a.)
2.700 µL; 80% methanol, 10% 2-propanol and 10% water
3.1000 µL; 40% methanol in water, 0.2% f.a.

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
- Solvation:1 mL methanol
  5 mL/min
- Equilibration:1 mL 40% methanol in water, 0.2 % f.a.
  5 mL/min
- Sample Loading:1 mL 40% methanol in water, 0.2 % f.a.
  2 mL/min
- Wash 1:1 mL 40% methanol in water, 0.2 % f.a.
  5 mL/min
- Wash 2:1 mL 60% methanol in water, 0.2 % f.a.
  5 mL/min
- Elution1 min. 30 sec (with LC gradient at 1 mL/min)

HPLC conditions
Column: Merck KgaA Chromolith SpeedROD C18e 50X4.6 mm
Mobile phase A: Water, 0.2% f.a.
Mobile phase B: Methanol, 0.2% f.a.
Mobile phase flow rate: 1 mL/min

Mobile phase gradient		
Time (mm:ss)	%A	%B
00:01	20	80
00:05	20	80
03:00	10	90
03:10	10	90
03:15	20	80
03:45	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	MHVD-3 D6
Mass	412	400	406
Q1 mass	395.1	383.1	389.1
Q2 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

Symbiosis system

SAMPLE PREPARATION

Reagents
Protein Disrupting Buffer (PDB, Spark Holland): This buffer has been especially developed for rapid and efficient reduction of protein binding.

Standard working solutions:
2500, 250 and 25 ng/mL MHVD2 and MHVD 3 in PDB.

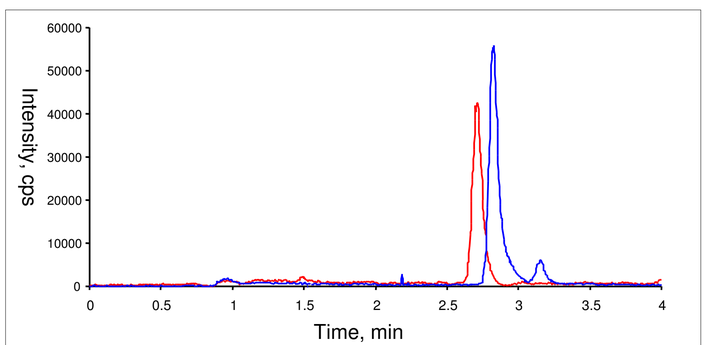
Internal standard for Patient samples (ISp):
100 ng/mL MHVD3 D6 in PDB.

Internal standard for Calibration samples (ISc):
1000 ng/mL MHVD3 D6 in PDB

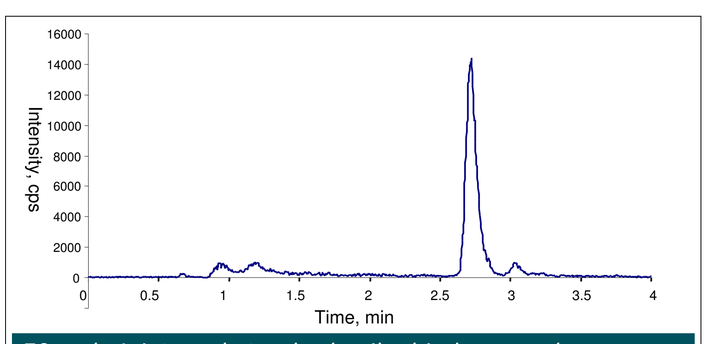
Patient samples:
•Pipette 250 µL of plasma sample into an amber autosampler vial.
•Add 250 µL of ISp to the vial
•Vortex sample for a few seconds before placing the vial into the autosampler.

Calibration and QC samples.
Calibration and QC samples are prepared in Human Sodium Heparin Plasma:
•Transfer 500 µL plasma sample into an amber autosampler vial.
•Add 50 µL of ISc to the vial.
•Add appropriate volumes of PDB and standard solutions (MHVD2 and MHVD3) to obtain desired concentration and a total volume of 1000 µL .
•Vortex sample for a few seconds before placing the vial into the autosampler

RESULTS



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



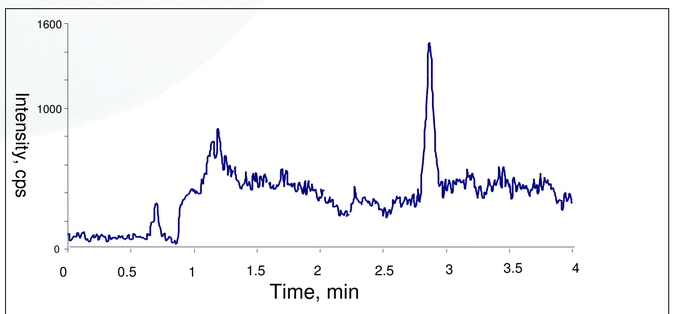
50 ng/mL internal standard spiked in human plasma

Linearity
Linearity was determined by injecting the full set of 9 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 weighting for both compounds.

Accuracy and precision
Accuracy and precision were calculated by combining 2x2 series of QC samples, measured with the Symbiosis Pico system en 2x1 series measured with the Symbiosis Pharma system.

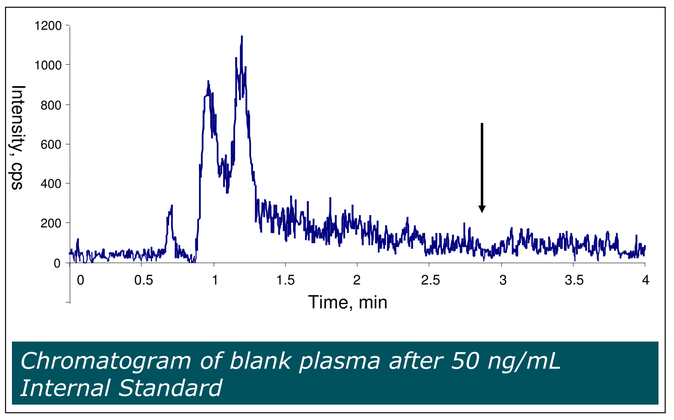
QC sample (ng/ml)	MHVD-2		MHVD-3	
	Precision (% RSD)	Accuracy (%)	Precision (% RSD)	Accuracy (%)
5	7.8	103	6.5	106
50	2.6	105	3.0	102
400	2.8	107	4.0	107

Sensitivity
With 250 µL injection volume (representing 125 µL plasma) a quantitation limit of 5 ng/mL is achieved and 1 ng/mL is still detectable with a signal to noise of > 4. If only 50 µl of plasma is available, resulting in 100 µl injection volume, a quantitation limit of 10 ng/mL is achieved for both compounds.



1 ng/mL MHVD2 in plasma

Carry-over
MHVD2 and MHVD3 are endogenous compounds and always present in human plasma. In order to determine carry-over of the method, the carry-over of the internal standard is measured. No carry-over could be observed as shown below.



Chromatogram of blank plasma after 50 ng/mL Internal Standard

CONCLUSION

A rapid, sensitive and highly automated assay for 25-Mono-Hydroxy-Vitamin D2 and D3 in Plasma by On-line SPE-LC-MS/MS has been developed. The 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. 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 limit of 10 ng/mL is achieved. The assay is acetonitrile-free and runs on both the Symbiosis Pico and Symbiosis Pharma system.

053-871-1
HPLC 2009 Dresden