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Quantitation of 25-OH Vit D in human plasma and serum for clinical research by LC-MS/MS

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Application benefits

- Simple offline sample preparation by protein precipitation followed by online SPE
- Robustness and sensitivity with entry level triple quadrupole MS

Goal

Implementation of an analytical method for quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma and serum on a Thermo Scientific™ TSQ Fortis™ triple-stage quadrupole mass spectrometer for clinical research.

Introduction

A robust LC-MS/MS analytical method for the quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma and serum for clinical research is reported. Samples were extracted offline by protein precipitation with concomitant addition of the internal standard. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Flex UHPLC system configured for online solid-phase extraction (SPE). A TSQ Fortis triple-stage quadrupole mass spectrometer

with atmospheric pressure chemical ionization operated in positive ionization mode was used for the detection of all analytes. Data were acquired by selected reaction monitoring (SRM) using d_6-25-hydroxyvitamin D_3 as the internal standard for both analytes. Method performance was evaluated using the ClinMass® LC-MS/MS Complete Kit 25-OH-Vitamin D_2/D_3 in Plasma and Serum from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, carryover, accuracy, and intra- and inter-assay precision for both analytes.

Experimental

Target analytes

- 25-OH-vitamin D_2
- 25-OH-vitamin D_3
- d_6-25-OH-vitamin D_3 (internal standard)

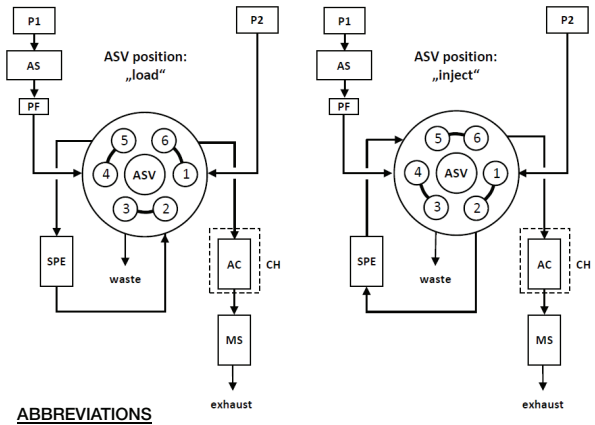
The concentration ranges covered by the calibrators are reported in Table 1.

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE, as well as d_6-25-hydroxyvitamin D_3, which is used as the internal standard for the quantification of both analytes. Samples of 50 µL of serum were protein precipitated using 150 µL of precipitant containing the internal standard. Precipitated samples were vortex-mixed, incubated for 10 min at 4 °C, vortex-mixed again, and then centrifuged. The supernatant was transferred to a clean vial for LC-MS/MS analysis.

Liquid chromatography

Online SPE and chromatographic separation of analytes were achieved on Vanquish Flex UHPLC system comprising a binary and a quaternary pump together with a 2-position/6-port column switching valve (P/N 6036.1560) for online SPE. Sample extraction and LC separation were achieved using an SPE cartridge, analytical column, and mobile phases from RECIPE. A schematic representation of the LC configuration is reported in Figure 1. Details of the analytical method are reported in Table 2. Total runtime was 3.0 minutes.



ABBREVIATIONS

P1 – SPE pump
P2 – HPLC pump
AS – Autosampler
PF – Inline filter
ASV – Automatic switching valve
SPE – SPE column
AC – Analytical column
CH – Column heater
MS – Tandem mass spectrometer

Figure 1. Schematic representation of the LC configuration used for online SPE

Table 1. Concentration ranges covered by calibrators

Analyte	Concentration range (ng/mL)
25-hydroxyvitamin D_2	9.84–81.0
25-hydroxyvitamin D_3	9.04–78.9

Table 2. LC method description

Time (min)	ASV position	Pump P1 (SPE buffer)		Pump 2 (mobile phase)	
		Flow rate (mL/min)	Event SPE column	Flow rate (mL/min)	Event analytical column
0.00	Load	0.1	Loading	0.5	Equilibration
0.01		5.0			
0.75	Inject	5.0	Elution	0.5	Loading
0.85		0.1			Separation
2.15		0.1			
2.20	Load	2.0	Equilibration	0.5	Equilibration
2.85		2.0			
2.90		0.1			
3.00		0.1			

Mass spectrometry

Analytes and internal standard were detected by SRM on a TSQ Fortis triple-stage quadrupole mass spectrometer with atmospheric pressure chemical ionization operated in positive ionization mode. A summary of the MS conditions is reported in Table 3. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively.

Table 3. MS settings

Source type	Atmospheric pressure chemical ionization (APCI)
Vaporizer temperature	350 °C
Capillary temperature	300 °C
Spray current (positive mode)	2 µA
Sheath gas	50 AU
Sweep gas	1 AU
Auxiliary gas	2 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Collision gas pressure	1.5 mTorr
Cycle time	0.400 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7

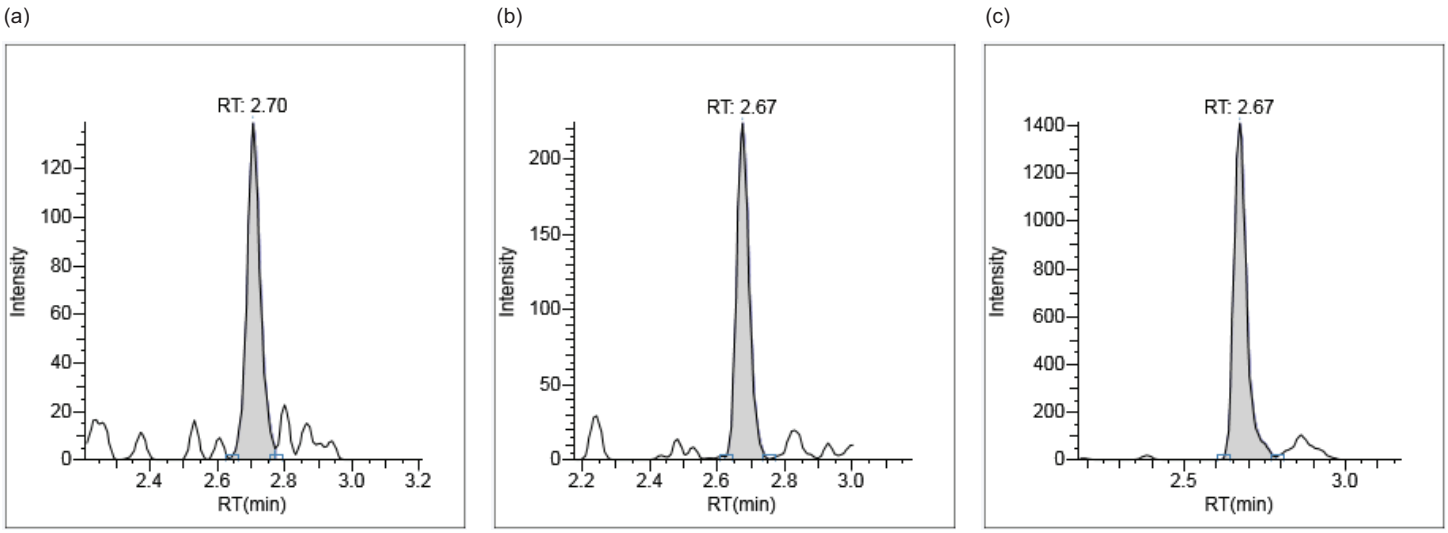


Figure 2. Representative chromatograms of the lowest calibrator for (a) 25-OH-vitamin D_2, (b) 25-OH-vitamin D_3, and (c) their internal standard d_6-25-OH-vitamin D_3

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, carryover, accuracy, and intra- and inter-assay precision for both analytes. Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately after it. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using quality control samples at two different levels provided by RECIPE (MS7082 batch #1207), which were prepared and analyzed in replicates of five on three different days. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

A quadratic interpolation with 1/x weighting was used for both analytes. The percentage bias between nominal and back-calculated concentration was always within ±10% for all calibrators in all runs. Representative chromatograms for the lowest calibrator for both analytes and the internal standard are reported in Figure 2. Representative calibration curves are reported in Figure 3.

No significant carryover was observed for either analyte, with no signal detected in the blank injected immediately after the highest calibrator.

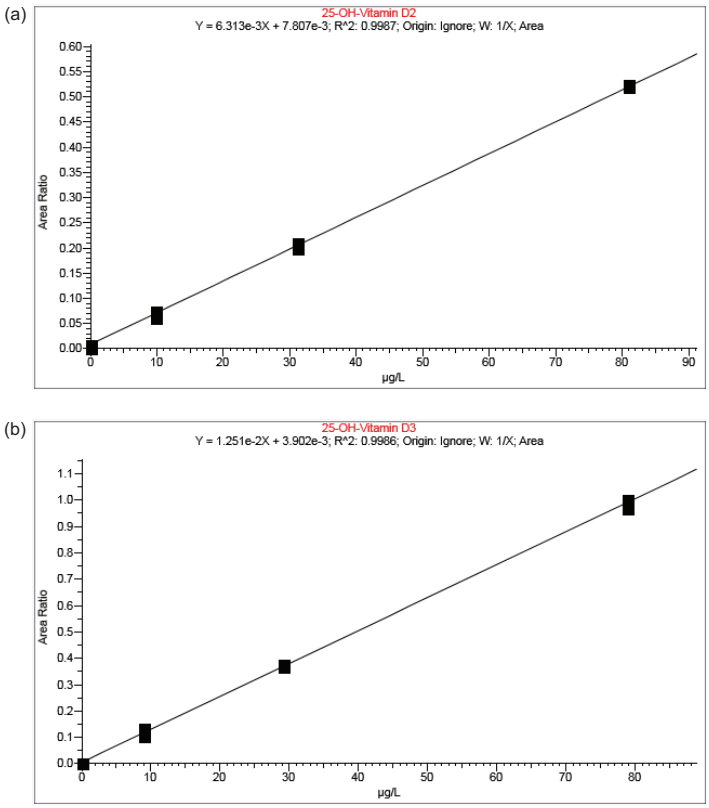


Figure 3. Representative calibration curves for (a) 25-OH-vitamin D_2 and (b) 25-OH-vitamin D_3

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the control samples ranging between -4.3% and 3.8% (Table 4). The %CV for intra-assay precision was always below 9.0% for both analytes. The maximum %CV for inter-assay precision including all the analytes was 7.8%. Results for intra- and inter-assay precision are reported in Table 5.

Table 4. Analytical accuracy results for control MS7082 batch #1207

Analyte	Control	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)
25-OH-vitamin-D_2	Level I (LOT #1207)	14.7	15.1	3.0
	Level II (LOT #1207)	42.5	42.2	-0.7
25-OH-vitamin-D_3	Level I (LOT #1207)	14.9	14.2	-4.3
	Level II (LOT #1207)	42.0	43.6	3.8

Table 5. Analytical intra- and inter-assay precision results for control MS7082 batch #1207

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)
25-OH-vitamin-D_2	Level I (LOT #1207)	15.6	7.5	14.7	7.3	15.1	9.0	15.1	7.8
	Level II (LOT #1207)	43.6	5.1	40.5	6.9	42.4	6.7	42.2	6.6
25-OH-vitamin-D_3	Level I (LOT #1207)	14.1	7.4	14.1	7.3	14.5	4.2	14.2	6.0
	Level II (LOT #1207)	42.7	4.4	43.2	6.5	44.9	7.2	43.6	6.2

Conclusion

A robust, reproducible, and sensitive method for clinical research was reported using liquid chromatography-tandem mass spectrometry for the quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma and serum. The method was analytically validated using a Vanquish Flex UHPLC system configured for online SPE connected to a TSQ Fortis triple-stage quadrupole mass spectrometer. The ClinMass LC-MS/MS Kit 25-OH-Vitamin D_2/D_3 in Plasma and Serum – online Analysis from RECIPE was used. The procedure describes simple, robust protein precipitation prior to online SPE of the samples. The data obtained with the described method successfully met sensitivity, reliability, accuracy, and precision expectations typically demanded by clinical research laboratories.

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