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Quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma by liquid chromatography coupled to high-resolution Orbitrap mass spectrometry for clinical research

Authors: Claudio De Nardi[1], Katharina Kern[2], Steffen Peters[2]

[1]Thermo Fisher Scientific GmbH, Dreieich, Germany;
[2]RECIPE Chemicals + Instruments GmbH, Munich, Germany

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Application benefits

- Increased accuracy of method by implementation of a comprehensive ClinMass® kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- Robust, sensitive hardware enables increased confidence in data

Goal

Implementation of an analytical method for the quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer

Introduction

Vitamin D is an extremely important nutrient that enables intestinal absorption of calcium and phosphate and promotes deposition of these minerals in newly formed bones. Abnormal vitamin D levels can result in several bone-softening diseases (for example, rickets and osteomalacia), along with several other disorders (nephritic syndrome, granulomatous diseases, hypocalcemia, etc.). In addition, several other diseases, including cardiovascular disease, cancer, and autoimmune disorders, are influenced by vitamin D deficiencies.

Vitamin D is metabolized to 25-OH vitamin D in the liver. To determine total vitamin D levels, total 25-hydroxyvitamin D (D_2 and D_3) in serum is measured as the half-life of 25-OH vitamin D is about three weeks with serum concentrations of 10–50 ng/mL. Vitamin D is important across all age groups, and therefore there is an increased focus on the analysis and quantitation of vitamin D in human plasma/serum. Vitamin D is typically not found free in serum samples, which poses a challenge for sensitive and reproducible assays, generally developed using liquid chromatography (LC) coupled to mass spectrometry (MS).

The use of high resolution allows for selectivity and sensitivity even in full scan (FullMS) mode. The additional use of fragmentation in Parallel Reaction Monitoring (PRM) mode provides enhanced specificity to the analytical method.

Various sample matrix constituents can cause ion suppression, thus reducing accuracy and reproducibility of vitamin D assays. In this report, two acquisition approaches, FullMS and PRM, were applied for the quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma. Plasma samples were extracted by offline internal standard addition and protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Duo system for online SPE and LC separation. Detection was performed on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with atmospheric pressure chemical ionization, either by full scan or by PRM 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 for 25-OH-Vitamin D2/D3 in Plasma and Serum – on-line Analysis 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

The concentration ranges covered by the calibrators (MS7013 batch #1108) used are reported in Table 1.

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

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE, as well as d_6-25-hydroxyvitamin D_3 as the internal standard for the quantification. Samples of 50 µL of plasma were protein precipitated using 150 µL of precipitating solution containing the internal standard. Precipitated samples were vortex-mixed, kept at 4 °C for 10 minutes, vortex mixed again, and centrifuged. The supernatant was transferred to a clean plate or vial.

Liquid chromatography

Online SPE and LC separation were achieved on a Vanquish Duo UHPLC system using mobile phases (MS7009 and MS7010), an SPE cartridge (MS7031), and an analytical column (MS7030) provided by 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.

Mass spectrometry

Analytes and internal standard were detected by both FullMS and PRM mode on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with atmospheric pressure chemical ionization operated in positive ion mode. A summary of the MS conditions is reported in Table 3.

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, and intra- and inter-assay precision for both analytes. To determine the LLOQ, the lowest calibrator was diluted down to 20-fold with blank matrix; a full set of calibrators (four levels), diluted calibrators (four levels), and controls (two levels) were extracted in replicates of five (n=5), injected in a single batch and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a CV <20%. Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected just 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), 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).

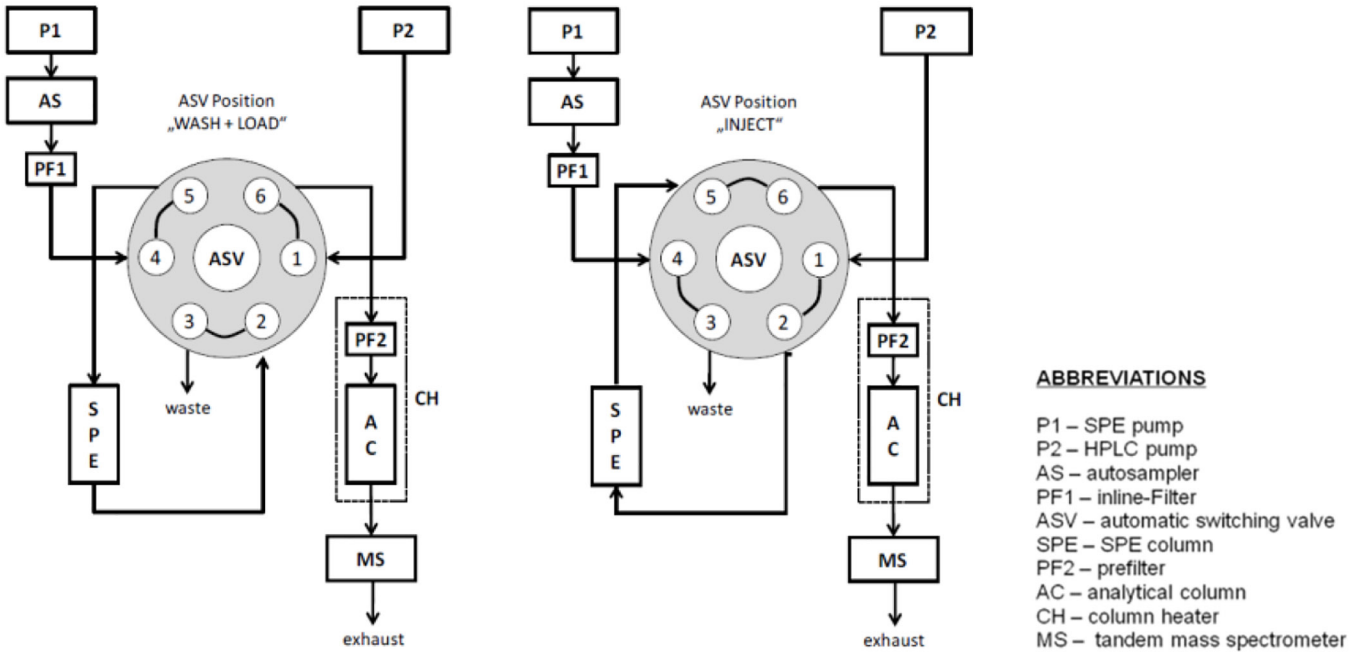

Figure 1. Schematic representation of the Vanquish Duo system configuration used for online SPE

Table 2. LC method description

Gradient profile					
Time (min)	**ASV position**	**Pump P1 flow rate (mL/min)**	**Event SPE column**	**Pump P2 flow rate (mL/min)**	**Event analytical column**
0.00	Load	0.1	Loading	0.5	Equilibration
0.01		5.0		0.5	
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		0.5	
Other parameters					
Injection volume				**Column temperature**	
50 µL				40 °C	

Table 3. MS settings

Property	Setting
Source type	Atmospheric pressure chemical ionization (APCI)
Vaporizer temperature	388 °C
Capillary temperature	244 °C
Spray current (positive mode)	4 µA
Sheath gas	24 AU
Sweep gas	0 AU
Auxiliary gas	5 AU

Data analysis

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

Results and discussion

A linear response with 1/x weighting was obtained for all the analytes not only in the calibration range covered by the calibrators but also down to lower limits of quantification. A summary of the LLOQs obtained using the two different acquisition modes is reported in Table 4. The percentage bias between nominal and back-calculated concentration was always within ±15% for all the calibrators (±20% for the lowest calibrator) in all the runs. Representative chromatograms for the LLOQ for the analytes and their internal standard using both approaches are depicted in Figure 2. Representative calibration curves in the concentration range covered by the kit (three calibrators) are shown in Figure 3.

The maximum reported carryover was 0.4%.

Table 4. Concentrations of the lowest calibrators and the LLOQs

Analyte	Concentration (ng/mL)		
	Lowest calibrator	LLOQ FullMS mode	LLOQ PRM mode
25-hydroxyvitamin D_2	9.84	4.92	4.92
25-hydroxyvitamin D_3	9.04	1.81	4.52

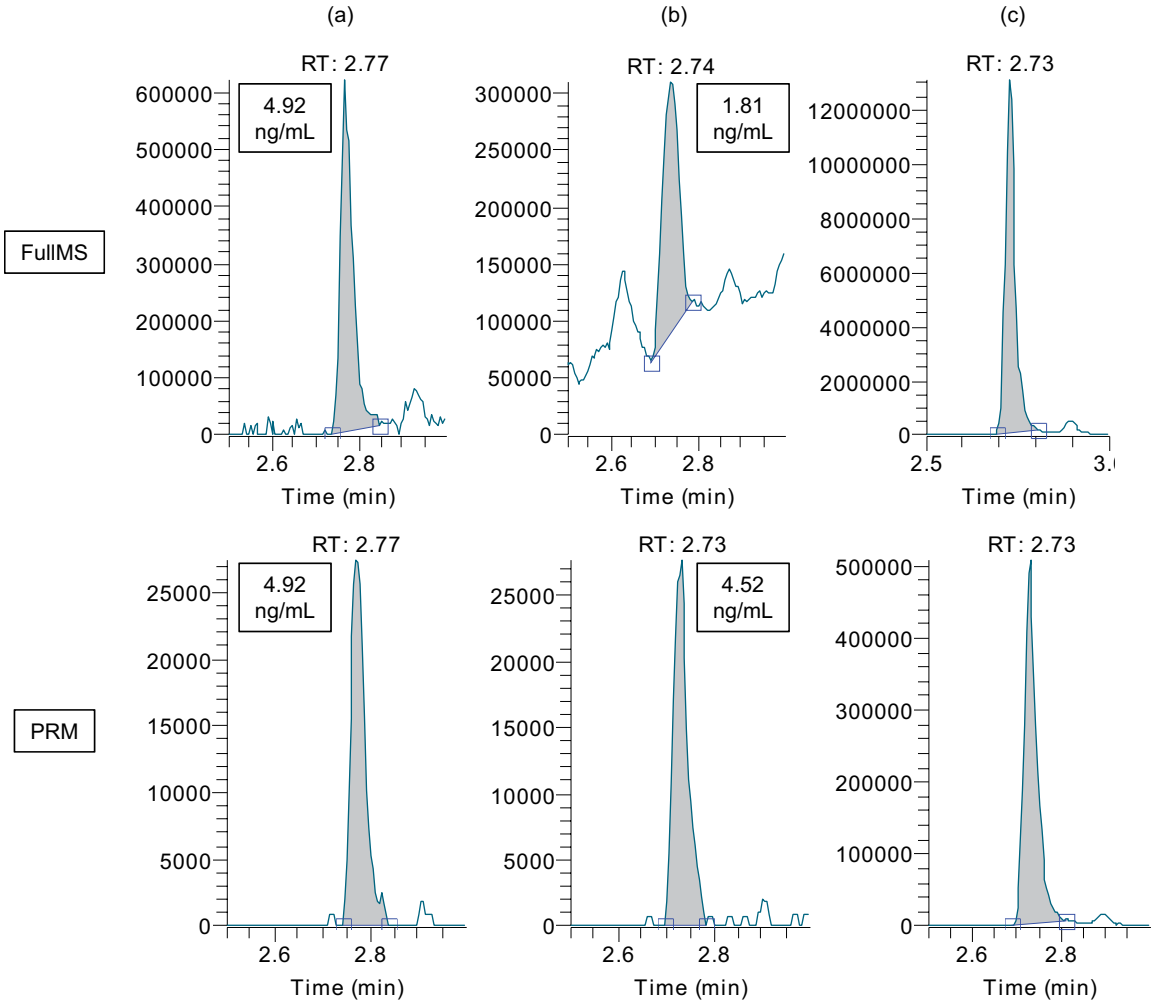

Figure 2. Representative chromatograms of the LLOQ for (a) 25-OH-Vitamin D_2, (b) 25-OH-Vitamin D_3, and (c) d_6-25-OH-Vitamin D_3 using FullMS and PRM acquisition modes

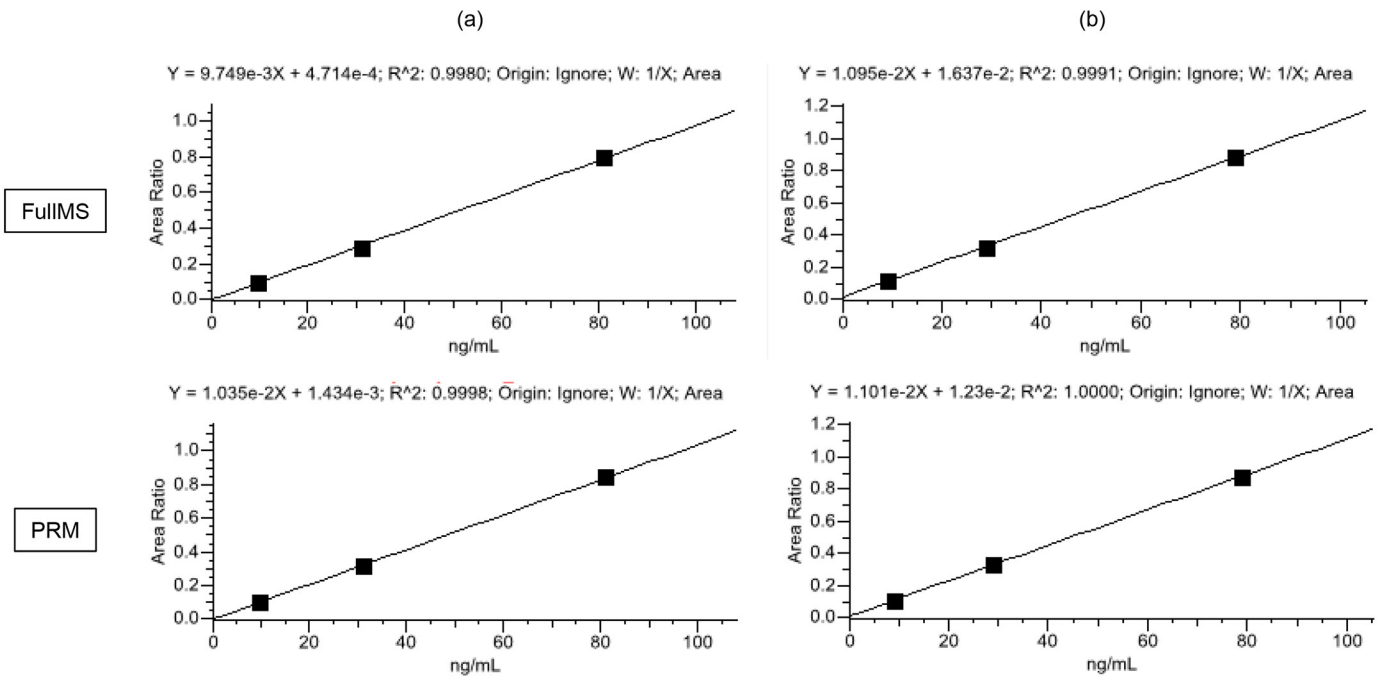

Figure 3. Representative calibration curves for (a) 25-OH-Vitamin D_2 and (b) 25-OH-Vitamin D_3 using FullMS and PRM acquisition modes

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -4.2% and 1.2% in FullMS mode and between 0.2% and 6.3% in PRM mode (Table 5). The %CV for intra-assay precision was always below 11.3% for both acquisition modes. The maximum %CV for inter-assay precision including both acquisition modes was 9.3%. Results for intra- and inter-assay precision are reported in Table 6 and Table 7 for FullMS and PRM mode, respectively.

Table 5. Analytical accuracy results for control MS7082 batch #1207

			FullMS mode		PRM mode	
Analyte	Control	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)	Average calculated concentration (ng/mL)	Bias (%)
25-OH-Vitamin-D_2	Level I	14.7	14.7	-0.1	14.8	0.7
	Level II	42.5	42.8	0.7	43.5	2.4
25-OH-Vitamin-D_3	Level I	14.9	14.3	-4.2	14.9	0.2
	Level II	42.0	42.5	1.2	44.6	6.3

Table 6. Analytical intra- and inter-assay precision results for control MS7082 batch #1207 – FullMS mode

		Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
Analyte	Control	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	14.8	6.2	14.7	11.3	14.6	10.3	14.7	8.8
	Level II	40.8	6.5	43.5	5.0	44.1	4.5	42.8	6.1
25-OH-Vitamin-D_3	Level I	13.9	7.0	14.5	2.9	14.4	2.5	14.3	4.7
	Level II	41.4	2.2	43.5	2.0	42.6	1.8	42.5	2.8

Table 7. Analytical intra- and inter-assay precision results for control MS7082 batch #1207 – PRM mode

		Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
Analyte	Control	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	14.6	3.5	15.3	7.3	14.6	7.5	14.8	6.4
	Level II	46.4	6.8	44.4	4.9	39.7	9.0	43.5	9.3
25-OH-Vitamin-D_3	Level I	15.1	10.4	15.4	5.1	14.4	6.0	14.9	7.6
	Level II	45.6	6.4	44.4	3.9	43.9	8.6	44.6	6.3

Conclusions

A robust, reproducible, and sensitive liquid chromatography-high-resolution Orbitrap mass spectrometry method for clinical research for quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma was developed and implemented. The ClinMass LC-MS/MS Complete Kit for 25-OH-Vitamin D2/D3 in Plasma and Serum – on-line Analysis from RECIPE was used. The method was analytically validated on a Vanquish Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with atmospheric pressure chemical ionization. FullMS and PRM experiments were used for data acquisition. The method described here offers quick and simple offline protein precipitation with concomitant internal standard addition. Both FullMS and PRM approaches meet research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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