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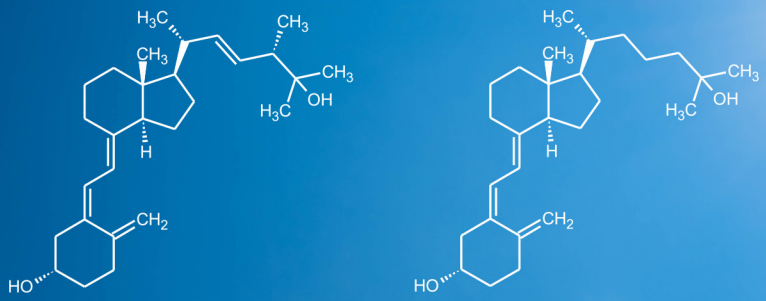

TECHNICAL NOTE 65471

Quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma by liquid chromatography-tandem mass spectrometry for clinical research

Authors

Claudio De Nardi, Thermo Fisher Scientific GmbH, Dreieich, Germany

Paolo Brambilla, Ospedale di Desio, Desio, Italy

Maura Brambilla, Ospedale di Desio, Desio, Italy

Chiara Fania, Università degli Studi Milano-Bicocca, Milano, Italy

Keywords

25-hydroxyvitamin D, offline sample preparation, blood, mass spectrometry, TSQ Quantis

Application benefits

- Increased accuracy of method by implementation of a comprehensive ClinMass® kit for sample preparation
- Fast acquisition time allows for increased productivity of the assay
- 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™ TSQ Quantis™ triple quadrupole 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 (e.g., rickets, osteomalacia), along with several other disorders (nephritic syndrome, granulomatous diseases, hypocalcemia, etc.) In addition, several other diseases, including cardiovascular disease, cancer, and autoimmune disorders, have recently been found to be influenced by vitamin D deficiencies.

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Vitamin D is metabolized to 25-hydroxyvitamin D in the liver, and total vitamin D is best determined by measuring total 25-hydroxyvitamin D (D_2 and D_3) in serum because the half-life of 25-hydroxyvitamin D is about three weeks with serum concentrations of 10–50 ng/mL. Due to its importance across all age groups, there is an increased focus on the analysis and quantitation of vitamin D in human plasma/serum. However, vitamin D is typically not found free in serum samples. This poses a challenge for sensitive and reproducible assays, generally developed using liquid chromatography (LC) coupled to mass spectrometry (MS).

Various sample matrix constituents can cause ion suppression, thus reducing accuracy and reproducibility of vitamin D assays. In this report, an analytical method for clinical research for the quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma is reported. Plasma samples are extracted by offline internal standard addition and protein precipitation. Extracted samples are injected onto a Thermo Scientific™ Transcend™ II TLX-2 system for online SPE and LC separation. Detection is performed on a TSQ Quantis triple quadrupole mass spectrometer with atmospheric pressure chemical ionization by selected reaction monitoring (SRM) using d_6-25-hydroxyvitamin D_3 as the internal standard for both analytes. 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 using the ClinMass LC-MS/MS Complete Kit for 25-hydroxyvitamin D_2/D_3 in Plasma and Serum – on-line Analysis from RECIPE® Chemicals + Instruments GmbH (Munich, Germany).

Experimental

Target analytes

The concentration ranges covered by the calibrators 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 using mobile phases, SPE cartridge, and analytical column 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.

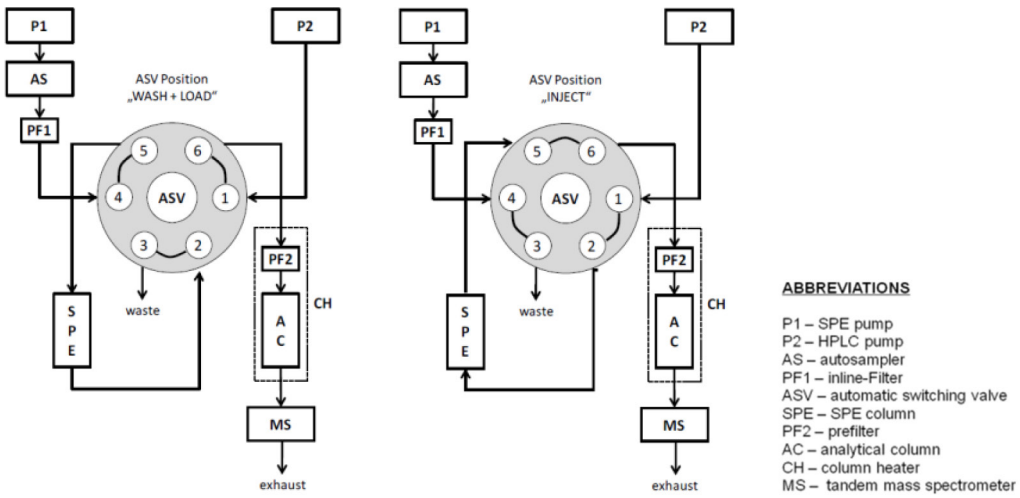

Figure 1. Schematic representation of the Transcend II system configuration used for onine SPE

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			
0.75	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			
Other Parameters					
Injection volume (µL)		30			
Column temperature (°C)		40			

Mass spectrometry

Analytes and internal standard were detected by SRM on a TSQ Quantis triple quadrupole mass spectrometer with atmospheric pressure chemical ionization operated in positive 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.

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 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).

Table 3. MS settings

Source type:	Atmospheric pressure chemical ionization (APCI)
Vaporizer temperature:	400 °C
Capillary temperature:	300 °C
Spray current (positive mode):	4 µA
Sheath gas:	40 AU
Sweep gas:	2 AU
Auxiliary gas:	2 AU
Data acquisition mode:	Selected-reaction monitoring (SRM)
Collision gas pressure:	1.5 mTorr
Cycle time:	0.300 s
Q1 mass resolution (FWMH):	0.7
Q3 mass resolution (FWMH):	0.7

Data analysis

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

Results and discussion

A linear interpolation with 1/x weighting was used for all the analytes. The percentage bias between nominal and back-calculated concentration was always within ±10% for all the calibrators in all the runs. Representative chromatograms for the lowest calibrator for all the analytes and their internal standards 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 just after the highest calibrator.

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 -5.5% and -1.7% (Table 4). The %CV for intra-assay precision was always below 2.4% for all the analytes. The maximum %CV for inter-assay precision including all the analytes was 2.8%. Results for intra- and inter-assay precision are reported in Table 5.

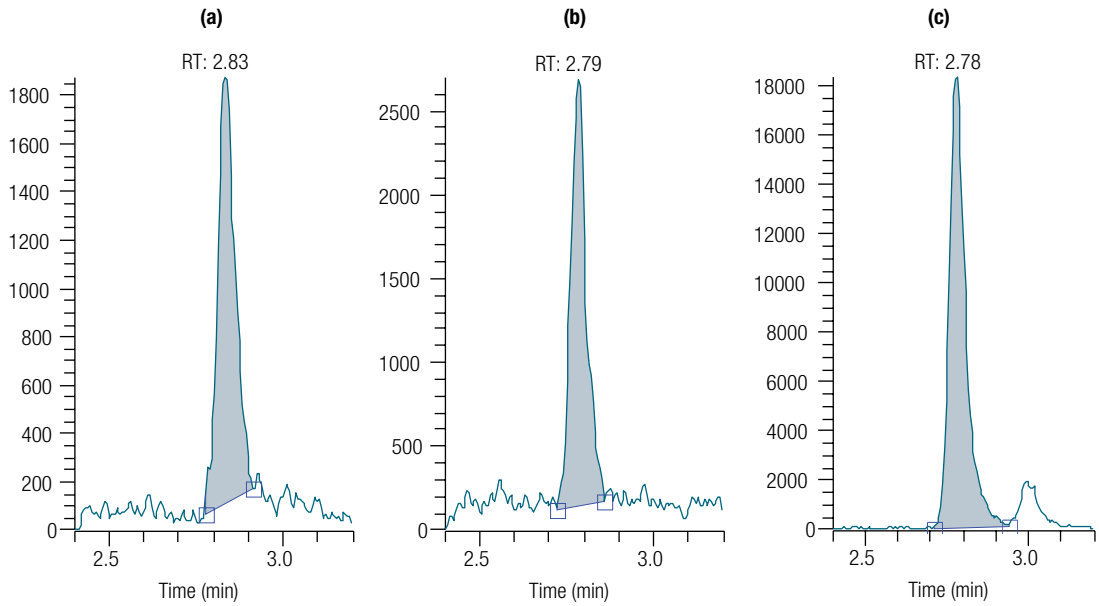

Figure 2. Representative chromatograms of the lowest calibrator for (a) 25-hydroxyvitamin D_2, (b) 25-hydroxyvitamin D_3, and (c) d_6-25-hydroxyvitamin D_3

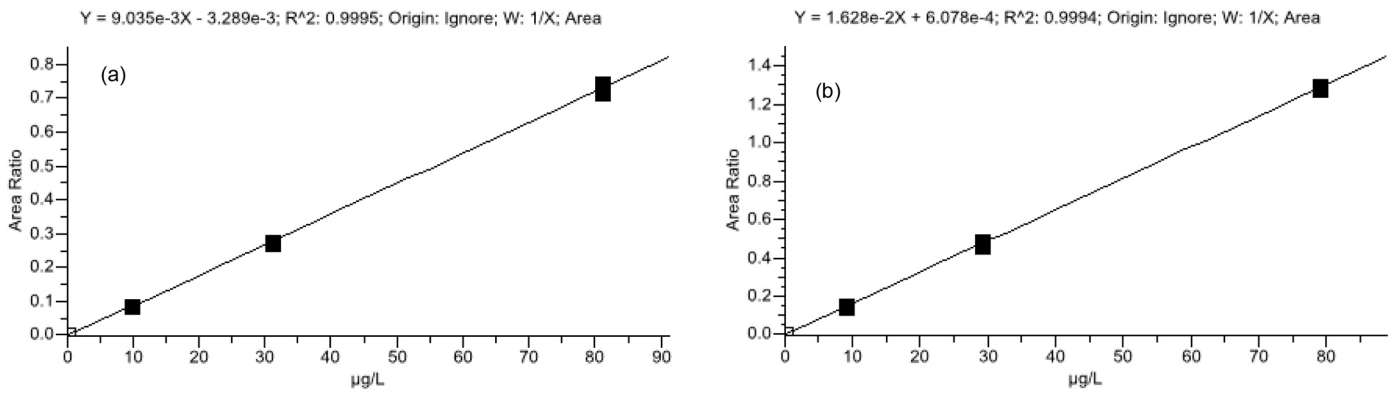

Figure 3. Representative calibration curves for (a) 25-hydroxyvitamin D_2 and (b) 25-hydroxyvitamin D_3

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

Analyte	Control	Nominal Concentration (ng/mL)	Average Calculated Concentration (ng/mL)	Bias (%)
25-hydroxyvitamin D_2	Level I (LOT #1207)	14.7	14.5	-1.7
	Level II (LOT #1207)	42.5	41.1	-3.4
25-hydroxyvitamin D_3	Level I (LOT #1207)	14.9	14.5	-2.8
	Level II (LOT #1207)	42.0	39.7	-5.5

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 Conc. (ng/mL)	CV (%)	Average Calculated Conc. (ng/mL)	CV (%)	Average Calculated Conc. (ng/mL)	CV (%)	Average Calculated Conc. (ng/mL)	CV (%)
25-hydroxyvitamin D_2	Level I (LOT #1207)	14.6	2.3	14.8	1.8	14.0	1.6	14.5	2.8
	Level II (LOT #1207)	40.6	1.7	41.6	1.1	41.0	2.0	41.1	1.9
25-hydroxyvitamin D_3	Level I (LOT #1207)	14.8	1.0	14.3	1.5	14.3	1.1	14.5	1.9
	Level II (LOT #1207)	39.2	2.2	39.8	2.4	40.1	1.7	39.7	2.2

Conclusions

A robust, reproducible, and sensitive liquid chromatography-tandem 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-hydroxyvitamin D_2/D_3 in Plasma and Serum – online Analysis from RECIPE was used. The method was analytically validated on a Transcend II system connected to a TSQ Quantis triple quadrupole mass spectrometer. The method offers quick and simple offline protein precipitation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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