TECHNICAL NOTE 66025

Quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma by LC-HRAM-MS for clinical research

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Application benefits

- Accurate and confident results, simple sample preparation, and rapid quantitation
- High-resolution mass spectrometry for improved selectivity
- Robust, sensitive LC and MS platforms enable 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™ Orbitrap Exploris™ 120 mass spectrometer

Introduction

Vitamin D is crucial for the regulation of calcium and phosphate homeostasis and has been used as a critical marker for a number of other important biological processes including immune function.[1,2] Vitamin D deficiency can result in metabolic bone diseases, such as rickets, osteoporosis, and osteomalacia.[2,3] In addition, several other diseases, including cardiovascular disease, cancer, and autoimmune disorders, have recently been found to be influenced by vitamin D deficiencies.

Vitamin D exists naturally in two forms, vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). The major source of vitamin D in humans is the photoconversion of 7-dehydrocholesterol to pre-vitamin D_3 in the epidermis, which isomerizes to vitamin D_3.[4] Vitamin D_2 differs from

vitamin D_3 by the presence of a double bond and methyl group on the aliphatic side chain. Both vitamins D_2 and D_3 undergo hydroxylation by 25-hydroxylase enzymes in the liver to produce the most abundant circulating form, 25-hydroxyvitamin D_3 ($25\text{-}OHD_3$) and 25-hydroxyvitamin D_2 ($25\text{-}OHD_2$).[5] Owing to the impact vitamin D has on health and wellbeing, several improvements have been made in relevant analytical techniques. Because of a long serum half-life, measurement of total 25-OHD ($25\text{-}OHD_2$ and $25\text{-}OHD_3$) is routinely monitored for assessing total circulating vitamin D status. Immunoassays have long been the "go to" technique for monitoring vitamin D. However, a measure of total metabolite concentration and equivalent detection of both $25\text{-}OHD_2$ and $25\text{-}OHD_3$ can be challenging, owing to the preferential binding of proteins to $25\text{-}OHD_3$ compared to $25\text{-}OHD_2$.

LC-MS is one of the preferred techniques that can address the above-mentioned challenges because of its inherent selectivity, specificity, sensitivity, and high-resolution capabilities. In this study, an analytical method for clinical research for the quantification of $25\text{-}OHD_2$ and $25\text{-}OHD_3$ in human plasma is reported. Plasma samples are extracted by internal standard addition and protein precipitation.

Method performance was evaluated using calibrators and controls from RECIPE Chemicals + Instruments GmbH (Munich, Germany) 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.

Experimental

Target analytes

The list of analytes and their corresponding internal standard are summarized in Table 1. Concentration ranges covered by the calibrators used are reported in Table 2.

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 quantification. Samples of 50 µL of plasma were protein precipitated using 150 µL of acetonitrile containing the internal standard. Precipitated samples were vortex-mixed and centrifuged. The supernatant was transferred to a clean vial.

Liquid chromatography

LC separation was performed on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system using the following mobile phases:

Mobile phase A:
5 mM ammonium formate + 0.1% formic acid in water

Mobile phase B:
5 mM ammonium formate + 0.1% formic acid in methanol

Table 1. Analytes and internal standard

Analyte	Chemical formula	Expected mass (*m/z*)	tMS²	
			Quantifier ion	Confirming ion
25-hydroxyvitamin D_2	$C_{28}H_{44}O_2$	395.3308	209.1326	269.1902
25-hydroxyvitamin D_3	$C_{27}H_{44}O_2$	383.3308	211.1482	257.2266
d_6-25-hydroxyvitamin D_3	$C_{27}H_{38}D_6O_2$	389.3685	211.1482	---

Table 2. Concentration ranges covered by calibrators

Analyte	Retention time (min)	Concentration (µg/L)		
		L1	L2	L3
25-hydroxyvitamin D_2	1.33	9.84	31.1	81.0
25-hydroxyvitamin D_3	1.28	9.04	29.1	78.9

Chromatographic separation was achieved by gradient elution on a Thermo Scientific™ Hypersil GOLD™ 2.1 × 50 mm (1.9 µm) analytical column (P/N 25002-052130) run at 40° C at a flow rate of 0.5 mL/min. Total run time was 3.5 minutes. The chromatographic conditions are given in Table 3.

Table 3. LC method description

Gradient profile		
Time (min)	**Flow rate (mL/min)**	**%B**
0.00	0.5	80
0.25	0.5	80
1.00	0.5	100
2.00	0.5	100
2.01	0.5	80
3.50	0.5	80
Other parameters		
Column temperature		40 °C
Injection volume		20 µL

Mass spectrometry

Detection was performed on an Orbitrap Exploris 120 mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) ion source operated in positive ionization mode. Data were acquired in both Full Scan mode using a resolution of 60,000 (FWHM) at *m/z* 200 on a scan range of *m/z* 100 to 500 and in targeted MS^2 mode with a resolution of 30,000 (FWHM) at *m/z* 200. The ion source conditions and the mass spectrometer settings are presented in Tables 4 and 5, respectively.

Table 4. Ion source settings

Parameters	Setting
Source type	Atmospheric pressure chemical ionization (APCI)
Vaporizer temperature	400 °C
Ion transfer tube temperature	300 °C
Spray current (positive mode)	4 µA
Sheath gas	40 AU
Sweep gas	2 AU
Auxiliary gas	2 AU

Table 5. MS settings

Full Scan MS properties	
Resolution at *m/z* 200	60,000
Scan range	100–500 *m/z*
AGC target	Standard (1e6)
RF lens	70%
Maximum injection time mode	Auto
Data type	Profile
Polarity	Positive
Source fragmentation	Off
Mild trapping	Off
tMS^2 scan properties	
Resolution @*m/z* 200	30,000
Isolation window (*m/z*)	2
Collision energy mode	Fixed
Collision energy type	Normalized
HCD collision energy (%)	40

Method evaluation

The parameters used to evaluate the performance of the method included linearity of response, LLOQ, intra- and inter-assay accuracy and precision, and carryover 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). The LLOQ was investigated by dilution of the lowest calibrator with blank matrix and was established as the lowest concentration with a mean accuracy and precision better than 20%.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 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 the calibrators in all the runs.

A summary of the LLOQs using both acquisition modes is presented in Table 6.

Representative chromatograms for the LLOQ for the analytes and their internal standards using the different acquisition modes are reported in Figure 1. Representative calibration curves are reported in Figure 2.

No significant carryover was observed for both analytes, with no signal detected in the blank injected just after the highest calibrator.

Table 6. LLOQ values for both acquisition modes

Analyte	Concentration (µg/L)		
	Lowest calibrator	LLOQ Full Scan MS mode	LLOQ tMS^2 mode
25-hydroxyvitamin D_2	9.84	1.97	1.97
25-hydroxyvitamin D_3	9.04	4.52	4.52

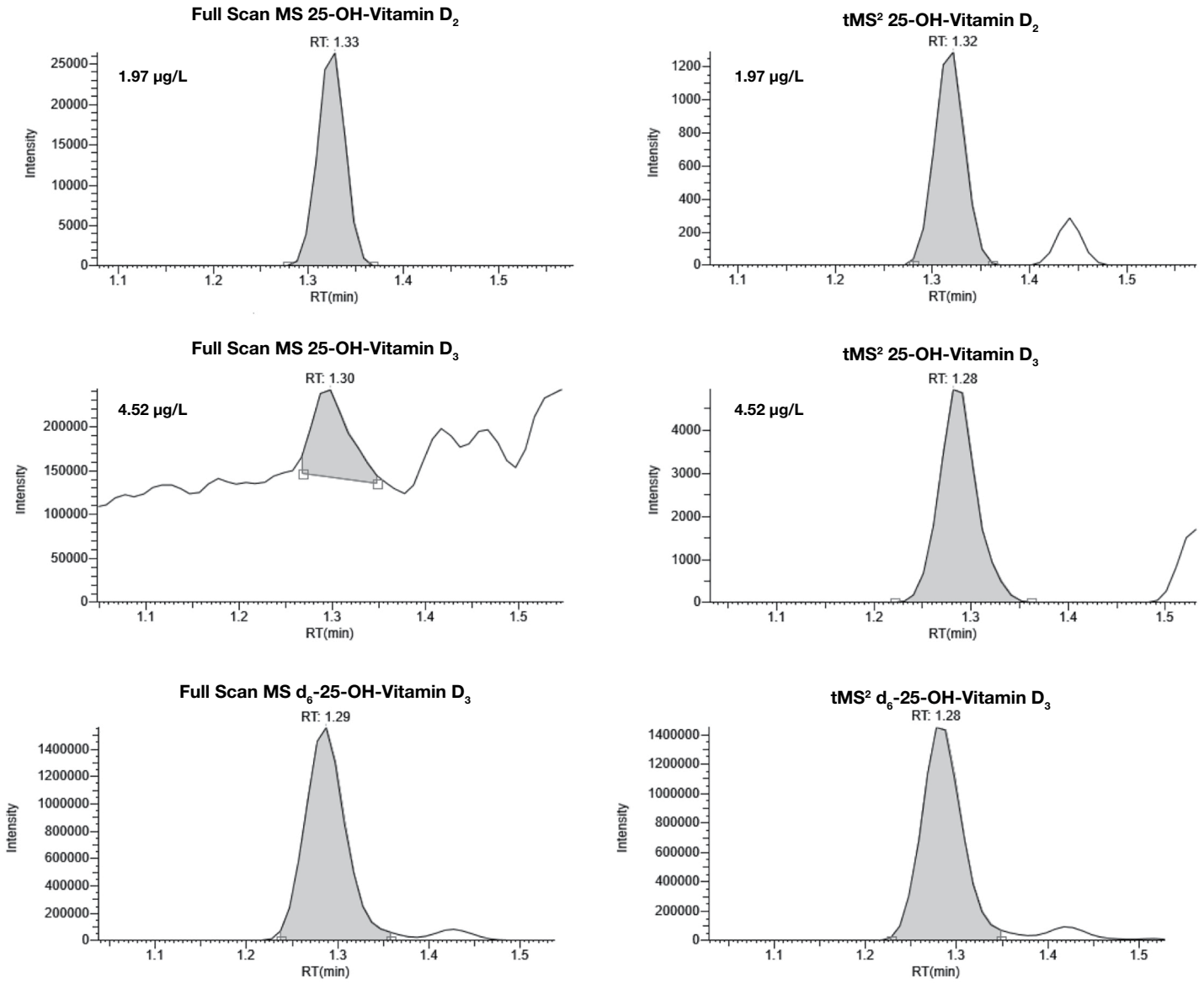

Figure 1. Representative chromatograms of the lowest calibrator for 25-OH-Vitamin D_2, (top) 25-OH-Vitamin D_3 and (middle) and d_6-25-OH-Vitamin D_3 (bottom)

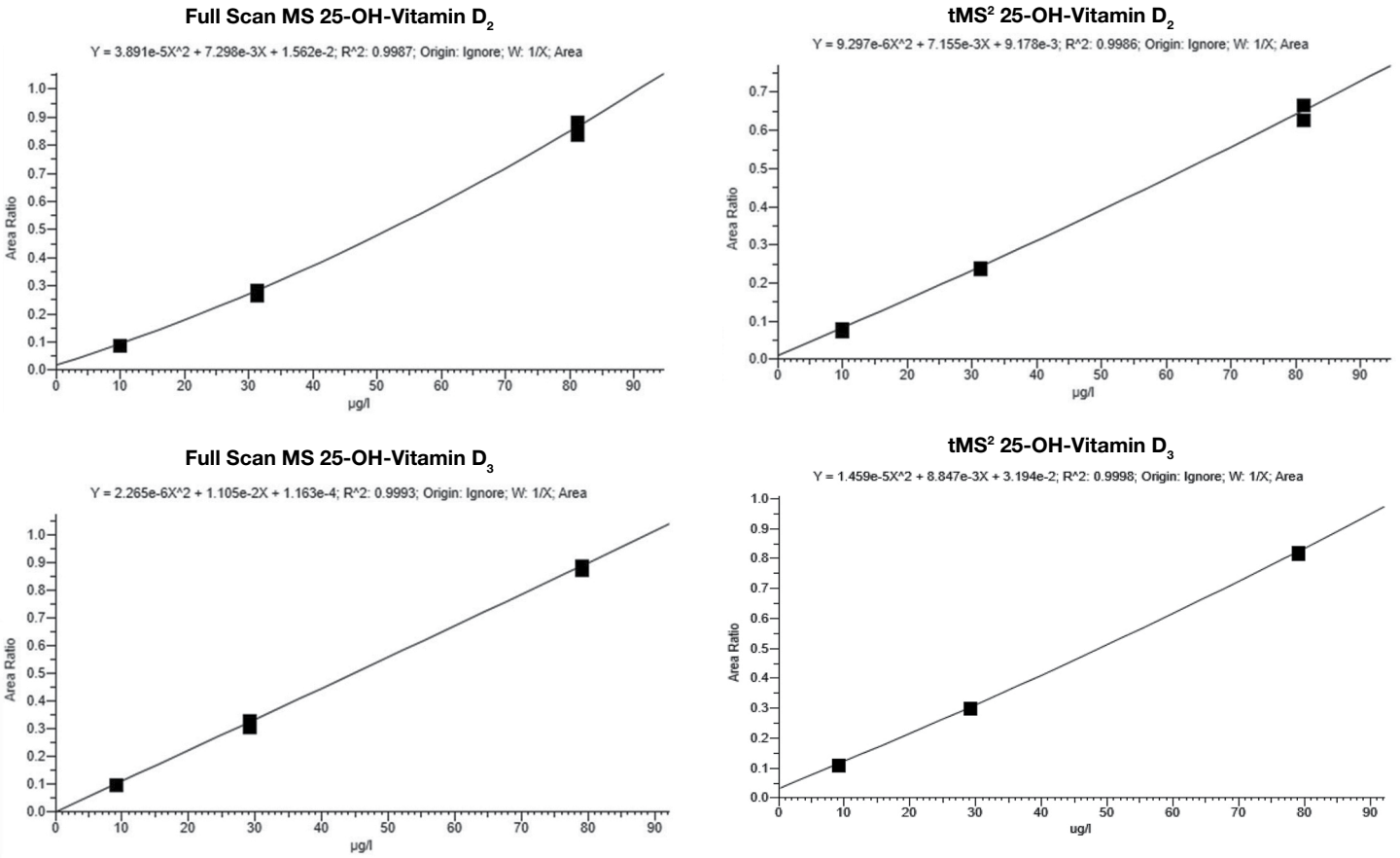

Figure 2. Representative calibration curves for 25-OH-Vitamin D_2 and 25-OH-Vitamin D_3 using Full Scan MS mode (left) and tMS^2 mode (right)

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 1.9% and 5.8% in Full Scan mode and between 1.8% and 6.7% in tMS^2 mode (Table 7). The %CV for intra-assay precision in Full Scan mode was always below 7.5% for all the analytes and below 8.4% for the tMS^2 mode. The maximum %CV for inter-assay precision including all the analytes was 5.0% for Full Scan mode and 5.9% for tMS^2 mode. Results for intra- and inter-assay precision are reported in Tables 8 and 9 for Full Scan and tMS^2, respectively.

Table 7. Analytical accuracy results for control MS7082 batch #1207

			Full Scan MS		tMS^2	
Analyte	**Control**	**Nominal concentration (µg/mL)**	**Average calculated concentration (µg/mL)**	**Bias (%)**	**Average calculated concentration (µg/mL)**	**Bias (%)**
25-OH-Vitamin-D_2	Level I	14.7	15.5	5.8	15.5	5.2
	Level II	42.5	44.9	5.6	45.3	6.7
25-OH-Vitamin-D_3	Level I	14.9	15.3	2.7	15.2	1.8
	Level II	42.0	42.8	1.9	43.2	2.8

Table 8. Analytical intra- and inter-assay precision results for control MS7082 batch #1207 - Full Scan MS

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)
25-OH-Vitamin-D_2	Level I	15.7	2.8	15.5	4.9	15.5	7.5	15.5	5.0
	Level II	45.4	5.1	43.7	4.2	45.3	4.1	44.9	3.9
25-OH-Vitamin-D_3	Level I	15.4	4.9	15.3	2.7	15.2	5.3	15.3	4.3
	Level II	42.1	3.3	42.2	3.6	44.1	6.2	42.8	4.8

Table 9. Analytical intra- and inter-assay precision results for control MS7082 batch #1207 - tMS^2

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)
25-OH-Vitamin-D_2	Level I	15.2	6.0	15.4	7.2	15.7	4.3	15.5	5.7
	Level II	45.4	3.5	44.1	6.1	46.1	2.5	45.3	3.5
25-OH-Vitamin-D_3	Level I	15.5	4.1	14.8	2.7	15.2	8.4	15.2	5.9
	Level II	42.7	1.7	42.4	1.7	44.4	5.4	43.2	3.9

Conclusions

A reproducible and sensitive liquid chromatography–high-resolution mass spectrometry method for clinical research for quantification of 25-hydroxyvitamin D_2 and D_3 in human plasma was developed, implemented, and analytically validated. Method performance was evaluated using calibrators and controls from RECIPE Chemicals + Instruments GmbH. Sample preparation is based on a rapid 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.

References

1. Mora, J.R.; Iwata, M.; von Andrian, U.H. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol.* **2008,** *8,* 685-698.
2. Plum, L.A.; DeLuca, H.F. Vitamin D, disease and therapeutic opportunities. *Nat Rev Drug Discov.* **2010,** *9,* 941–955.
3. DeLuca, H.F. The vitamin D story: a collaborative effort of basic science and clinical medicine. *The FASEB Journal.* **1988,** *2,* 224–236.
4. Holick, M.F. Vitamin D *Deficiency. N Engl J Med.* **2007,** *357,* 266–281.
5. Ohyama, Y.; Yamasaki, T. Eight cytochrome P450s catalyze vitamin D metabolism. *Front Biosci.* **2004,** *9,* 3007–3018.

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