Application Note

Clinical Research

Sensitive and Robust Measurement of Vitamin D Hydroxy Metabolites and Epimers in Serum Using the Agilent Ultivo Triple Quadrupole LC/MS

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Abstract

This application note presents a sensitive and robust analytical method for the simultaneous quantitation of 25-hydroxy vitamin D [25(OH)D] metabolites and their epimers in serum using the Agilent Ultivo triple quadrupole LC/MS system. Specific MRM transitions helped to differentiate between 25(OH)D2 and 25(OH)D3 metabolites, while a 10-minute chromatographic method using Agilent InfinityLab Poroshell 120 PFP column offered baseline separation of 25(OH)D from their epimer forms. Deuterated internal standards were used for the quantification of targets. Significant signal response was observed with serum spiked at 1 ng/mL concentration, confirming the method's analytical sensitivity. Calibration curves were plotted from the LOQ to 200 ng/mL with a coefficient of correlation (R^2) ≥0.999. Target peak area response showed RSD < 5%, retention time RSD was <0.5%, and method accuracy values were within 95 to 115% across the linearity range. The average recovery of targets for three QC levels was within 92 to 109%, with a repeatability RSD of ≤2% (n = 4). Injection-to-injection reproducibility of the response ratio resulted in excellent RSD of less than 5% across 500 continuous injections, confirming the method's robustness. The method performance was tested using ChromSystems certified reference standards and results were within the acceptable limits.

Introduction

Vitamin D is a group of lipophilic secosteroids that have been associated with several pathologies. Vitamin D status is assessed by measuring two main circulating metabolites: 25-hydroxyvitamin D2 [25(OH)D2] and 25-hydroxyvitamin D3 [25(OH)D3]. LC/MS/MS analysis typically offers a specific estimation of 25(OH)D2 and 25(OH)D3. However, the presence of epimeric forms of 25(OH) can interfere with identification and quantitation, resulting in potential overestimation of 25(OH)D2 and/or 25(OH)D3. Epimers differ only by the spatial arrangement of the C3-hydroxyl, which makes them isobaric (therefore indistinguishable by MS) and difficult to separate chromatographically. This study developed a 10-minute chromatographic method offering baseline separation of 25(OH)D and epimers for both D2 and D3 using InfinityLab Poroshell 120 PFP column. The LC/MS/MS method described here allows simultaneous and sensitive quantification of all four 25(OH)D analogs with baseline separation of the epimers.

Experimental

Chemicals and reagents

DC Mass Spect Gold Serum was purchased from Sigma-Aldrich (St. Louis, MO, USA) to prepare matrix-spiked calibrators and quality control (QC) samples. LC/MS-grade acetonitrile, methanol, and formic acid were purchased from Sigma-Aldrich. Ultrapure Milli Q water was produced using an in-house water purification system (Merck Millipore, MA, USA). All other LC/MS-grade solvents and reagents were purchased from Sigma-Aldrich.

Standard and internal standard mix

Labeled and unlabeled standards of 25(OH)D2, 25(OH)D3, and 25(OH)D3-epi at 100 µg/mL concentration were purchased from Isosciences (PA, USA). When not in use, all standards were stored at –20 °C. Deuterated internal standards (25(OH)D2-d3, 25(OH)D3-d3, and 25(OH)D3-epi-d3) were used for accurate quantitation. Standard solutions at various concentrations were prepared using 50/50 acetonitrile/water as a diluent, as follows:

1. **Standard stock solution-A (2,000 ng/mL):** add appropriate amounts of individual standard solutions (100 µg/mL) and diluent, mix well, and store the solution at –20 °C to ensure stock stability.
2. **Diluted stock solution-B (200 ng/mL):** in a clean container, add 100 µL of stock solution A and 900 µL of diluent. Mix well and store at –20 °C.
3. **Diluted stock solution-C (20 ng/mL):** in a clean container, add 100 µL of stock solution B and 900 µL of diluent. Mix well and store at –20 °C.
4. **Diluted stock solution-D (400 ng/mL) for QC samples:** in a clean container, add 200 µL of stock solution A and 800 µL of diluent. Mix well and store at –20 °C.
5. **Internal standard stock solution-E (4,000 ng/mL):** add appropriate amounts of individual internal standard solutions and diluent, mix well, and store the solution at –20 °C to ensure stock stability.
6. **Extraction solvent (acetonitrile with 1% formic acid and 8 ng/mL internal standards):** in a suitable container, add 15.824 mL of acetonitrile, 160 µL of formic acid, and 16 µL of 4,000 ng/mL internal standard stock solution E. Mix well and refrigerate at –20 °C for at least for 30 minutes before use.

Preparation of calibrators and QC samples

Nine calibrators were prepared by adding appropriate volumes of standard stock solutions (A, B, or C) into the serum matrix. Three QC samples were prepared by spiking the diluted stock solution-D into the serum, 8 ng/mL for low-range QC (LQC), 20 ng/mL for mid-range QC (MQC), and 40 ng/mL for high-range QC (HQC), respectively. Each QC level was prepared in four technical replicates. The preparation calibrators and QCs are summarized in Table 1. The MQC samples were used for 500 continuous injections to assess method robustness. Commercially available calibrators and QC samples from ChromSystems (Gräfelfing, Germany) were purchased to test linearity and recovery analysis, thus evaluating the efficiency of the newly developed method.

Sample preparation

Protein crash using acidified cold acetonitrile followed by centrifugation was adopted for sample cleanup. Each 200 µL serum calibrator, QCs, and the blank sample were protein precipitated using three volumes of cold extraction solvent. Samples were vortexed for 1 minute and allowed to incubate for 15 to 20 minutes at –20 °C. Samples were then vortexed once again for 30 seconds, centrifuged for 10 minutes at 10,000 rpm, and 200 µL of the supernatants were transferred to an Agilent amber vial with inserts (part number 5188-6592) ready for LC/MS/MS analysis.

Instrumentation

An Agilent 1260 Infinity II LC system was used for the analysis. The system consisted of:

- Agilent 1260 Infinity II flexible pump (G7104C)
- Agilent 1260 Infinity II multisampler (G7167A)
- Agilent 1260 Infinity II multicolumn compartment (G7116A)

A 0.3 µm inline filter (part number 5067-6189) was installed between the autosampler injector valve (port 6) and the multicolumn compartment to prevent contaminants from accumulating on the analytical column. The LC conditions are listed in Table 2. An Agilent Ultivo LC/TQ was equipped with electrospray ionization (ESI). Ultivo LC/TQ source parameters were optimized using Agilent MassHunter source optimizer software (Table 3).

Table 1. Preparation of calibration levels.

Standard Mix Used	Level	Volume of Standard Mix Used for Spiking (µL)	Volume of Blank Serum Taken (µL)	Volume of Diluent (µL)	Volume After Spiking (µL)	Concentration (ng/mL)
Standard Stock Solution A	L9	20	180	0	200	200
	L8	10	180	10	200	100
	L7	4	180	16	200	40
Diluted Stock Solution B	L6	20	180	0	200	20
	L5	10	180	10	200	10
	L4	4	180	16	200	4
Diluted Stock Solution C	L3	20	180	0	200	2
	L2	10	180	10	200	1
	L1	4	180	16	200	0.4
NA	Blank	0	180	20	200	0
Diluted Stock Solution D	HQC	20	180	0	200	40
	MQC	10	180	10	200	20
	LQC	4	180	16	200	8

Table 2. Agilent 1260 Infinity II LC parameters.

Parameter	Value
Needle Wash	Standard wash, flush port, 15 s; 60/40 acetonitrile/water
Autosampler Temperature	6 °C
Injection Volume	7 µL
Analytical Column	Agilent InfinityLab Poroshell 120 PFP, 3.0 × 100 mm, 2.7 µm (p/n 695975-308)
Column Temperature	20 °C
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Methanol with 0.1% formic acid
Flow Rate	0.550 mL/min
Gradient	Time (min) %B 0.00 65 1.00 75 6.00 80 6.50 95 8.40 95 8.50 65 10.00 65
Stop Time	10.00 min

Table 3. Agilent Ultivo LC/TQ mass spectrometer configuration and parameters.

Parameter	Value
Configuration	Ultivo LC/TQ (G6465B) equipped with an Agilent Jet Stream (AJS) Electrospray ion source
Ionization Mode	Positive
MS/MS Mode	MRM
Drying Gas Temperature	250 °C
Drying Gas Flow	7 L/min
Nebulizer Pressure	45 psi
Sheath Gas Temperature	325 °C
Sheath Gas Flow	11 L/min
Nozzle Voltage	500 V
Capillary Voltage	4,000 V
Diverter Valve to Waste	At 7 min

MRM optimization

Agilent MassHunter optimizer software (version 1.2) was used to obtain analyte-specific MRM transitions, fragmentor voltage, and collision energies. The MS/MS optimization was performed without chromatographic separation, using 1 µL injections of neat solutions of individual analytes at approximately 2,000 ng/mL. The MS/MS settings used for the analysis are included in Table 4.

Table 4. MRM parameters: MS1 resolution: unit; MS2 resolution: unit; dwell: 100 (ms); polarity: positive.

Analyte	ISTD	Precursor (*m/z*)	Product (*m/z*)	Frag (V)	CE (V)
25(OH)D2 + epi (Quant)	25(OH)D2 d3 + epi d3 (1)	413.3	355.2	105	4
25(OH)D2 + epi (Qual)	–	413.3	395.3	105	4
25(OH)D3 + epi (Quant)	25(OH)D3 d3 + epi d3 (1)	401.3	383.3	107	4
25(OH)D3 + epi (Qual)	–	401.3	365.3	107	8
25(OH)D2 d3 + epi d3 (1)	–	416.4	358.2	96	4
25(OH)D2 d3 + epi d3 (2)	–	416.4	398.4	96	4
25(OH)D3 d3 + epi d3 (1)	–	404.4	386.3	94	4
25(OH)D3 d3 + epi d3 (2)	–	404.4	368.3	94	8

Data acquisition and analysis

Agilent MassHunter LC/MS data acquisition software (version 1.2) and Agilent MassHunter quantitative analysis software (version 10.0) were used to collect and process the data. Each calibration sample, blank, and four technical preparations of each QC level were injected in triplicates. The diluent was injected after the highest calibrator to assess the carryover. Neat standard mix solution at 100 ng/mL concentration was injected to assess matrix effect by comparing the response with L8 calibration level. The pooled MQC samples were injected 500 times to assess method robustness.

Results and discussion

Sensitivity and linearity

Limit of detection (LOD) and limit of quantitation (LOQ) were established using lower levels of serum spiked calibration levels. For each compound, the signal-to-noise ratio (S/N) thresholds were defined as S/N >3 for LOD, and S/N >10 for LOQ. S/N was determined using the peak height and auto-RMS algorithm embedded in MassHunter quantitative analysis software. LOD and LOQ calculations solely based on S/N may be impacted by the presence of endogenous targets in the matrix.

In such cases of matrix contribution to the target signals, LOD was defined as the three-fold peak area of matrix contribution, and LOQ was defined as the five-fold area of matrix contribution.

The overlay of blank, LOD, and LOQ levels of all three analytes are included in Figure 1. Serum blank showed trace amounts of endogenous 25(OH)D2, 25(OH)D3, and 25(OH)D3-epi metabolites. For 25(OH)D2, the L2 (1.0 ng/mL) calibrator met LOD requirements, and L3 (2.0 ng/mL) was assigned as LOQ (Figure 1A). For 25(OH)D3 and 25(OH)D3-epi, the lowest serum spiked level (L1, 0.4 ng/mL) met the LOD requirement and L2 (1.0 ng/mL) was assigned as LOQ (Figure 1B). Linearity curves were plotted for all analytes from the LOQ to the highest spiked calibration level (200 ng/mL) with internal standard correction. All three targets displayed a linear response with R^2 values >0.999 (calibration model type: linear; origin: ignore; weight: 1/x).

Precision and accuracy

Precision was determined by calculating the %RSD of the target response and retention time (RT) using triplicate injections for the serum-spiked calibration levels. Good RT and response precision values for all analytes were obtained, with %RSD values <0.3% and <5.0%, respectively.

The average accuracy value for each serum-spiked calibration level was calculated from triplicate injections (Table 5) and the values for all three analytes across the calibration range were within 95 to 115%.

Table 5. Accuracy values for responses of all three analytes at various linearity levels.

Calibration Level (L)	Concentration (ng/mL)	25(OH)D2	25(OH)D3	25(OH)D3-epi
L1	0.4	–	107 (LOD)	101 (LOD)
L2	1	115 (LOD)	110	103
L3	2	109	114	110
L4	5	95	102	99
L5	10	112	111	111
L6	20	109	110	111
L7	50	98	99	98
L8	100	100	100	100
L9	200	99	98	99

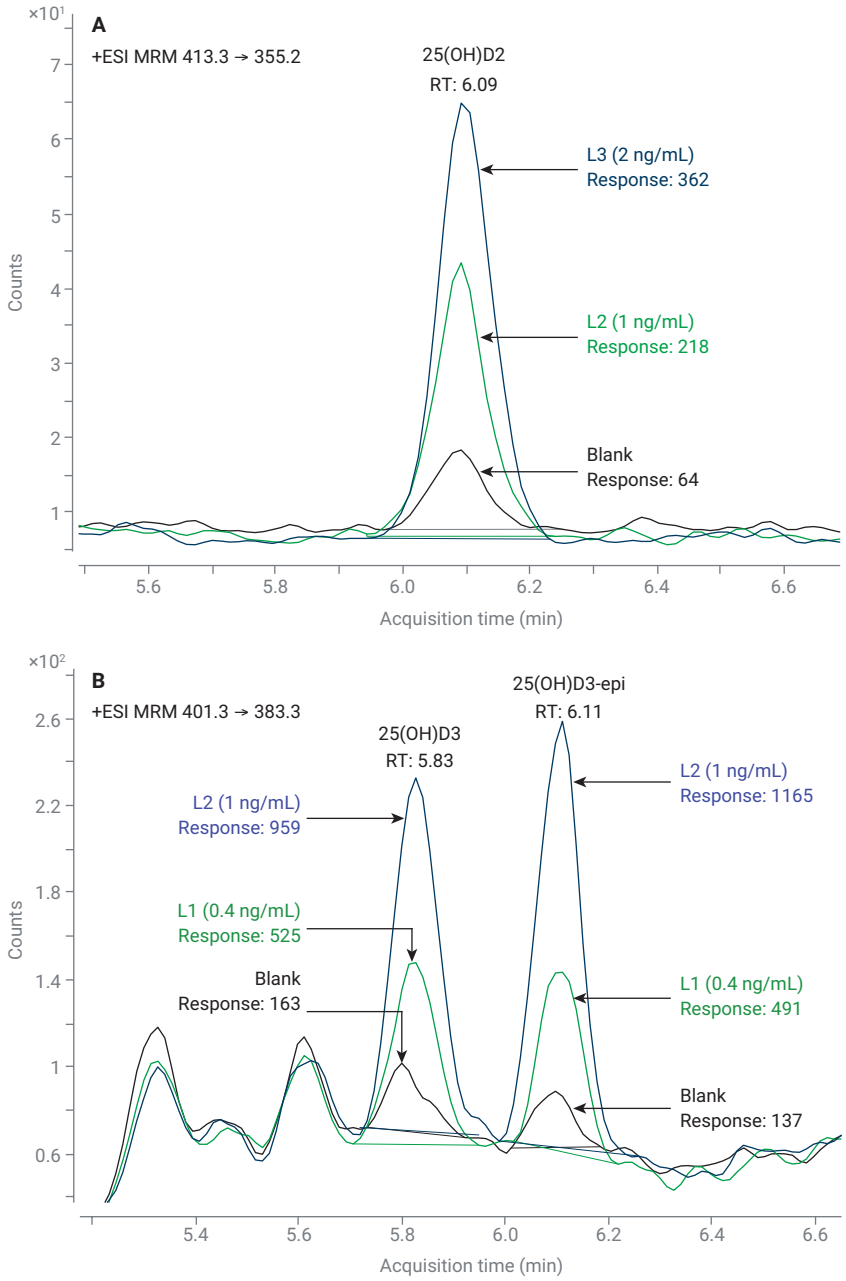

Figure 1. (A) MRM trace of 25(OH)D2 in blank serum (black trace), LOD 1 ng/mL serum spike (green trace), and LOQ 2 ng/mL serum spike (blue trace). (B) MRM trace of 25(OH)D3 and 25(OH)D3-epi in blank serum (black trace), LOD 0.4 ng/mL serum spike (green trace), and LOQ 1 ng/mL serum spike (blue trace).

Recovery

In this experiment, the impact of sample preparation on target recovery was assessed using four technical preparations of three levels of QC samples (LQC, MQC, and HQC). Each technical preparation was injected into the mass spectrometer in triplicates and recovery (%) was calculated using respective calibration curve equations (Figure 2). The recovery repeatability was measured as %RSD of recovery values calculated using four technical preparations. Recoveries for overall analytes were within 91 to 111% with intra-batch RSD ≤2%.

Carryover analysis

Carryover was measured by injecting 200 ng/mL of serum-spiked calibrator, followed by injection of diluent. The % carryover observed in the diluent injection was negligible at less than 0.004%.

Matrix effect assessment

The matrix effect (ME) is an important parameter for method sensitivity and reliability assessments. ME is defined as the ratio of analyte response (I) in serum-spiked samples with those in the corresponding neat standards (Equation 1). The closer the ME value is to 100%, the lower the ME.

Equation 1.

$$ME\% = \frac{I_{matrix}}{I_{solvent}} \times 100$$

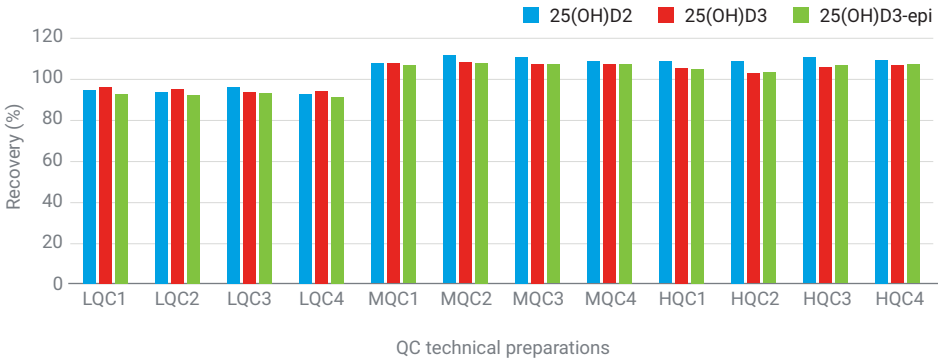

Figure 2. Average recovery values of targets using four technical preparations of three QC levels.

To assess the ME, the response of serum-spiked L8 calibrator level (100 ng/mL) was compared with that from the corresponding neat standard. All three analytes showed ME of >87%, indicating minor matrix suppression.

Robustness

The method robustness was assessed from 500 continuous injections of the MQC (20 ng/mL) sample. The reproducibility of calculated concentration and RT were monitored for all three targets over time and %RSD values were calculated to assess the robustness. The data acquisition was continuous, and the Ultivo LC/TQ was operated without readjusting any tune parameters. The entire run lasted 3.5 days.

The elution profile using the InfinityLab Poroshell 120 PFP column was extremely consistent over 500 injections. Good reproducibility of calculated concentration with RSD <5.0% and RT RSD <0.2% were observed for all three targets. The reproducibility of calculated concentrations for three analytes over 500 continuous injections is summarized in Figure 3. The innovative ion transfer optics design of the Ultivo LC/TQ minimizes the source contamination from the serum matrix, thus providing a robust analytical platform for the confident routine analysis of trace analytes (Figure 4). Instrumentation innovations such as ion guide, collision cell, and small hyperbolic quadrupoles were designed to maximize quantitative performance within a smaller footprint. The sample preparation procedure discussed earlier provided efficient sample matrix cleanup, greatly reduced the matrix residue accumulation on the ion source interface, and provided extended column lifetime and detection consistency. The method robustness, calculated from 500 continuous data acquisitions, confirmed the sustainable performance of the Ultivo LC/TQ for day-to-day operations.

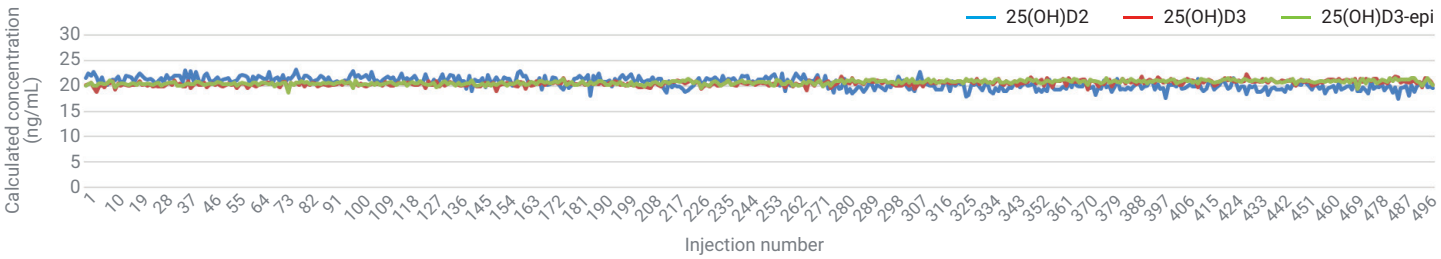

Figure 3. The reproducibility of calculated concentration of all three targets over 500 injections using MQC sample.

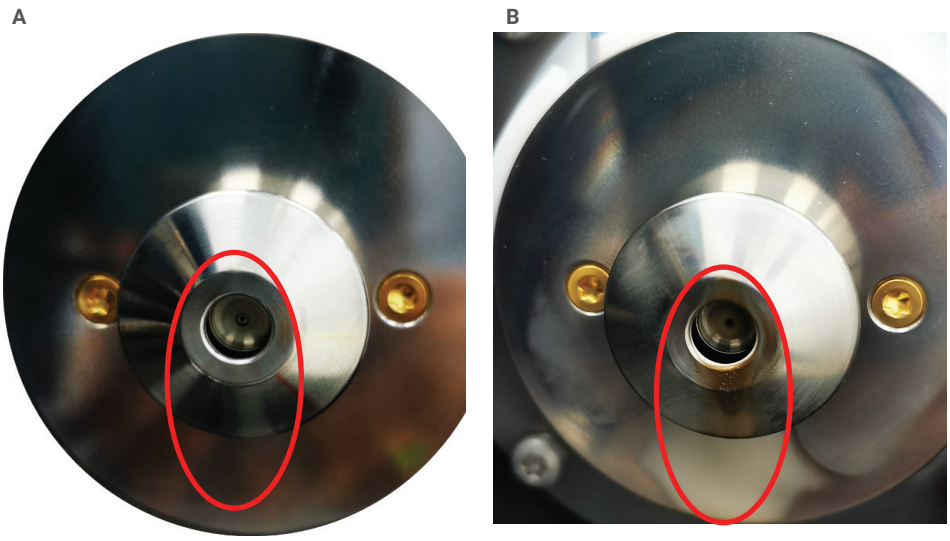

Figure 4. The Agilent Jet Stream (AJS) technology ion source of the Agilent Ultivo LC/TQ before (A) and after (B) >500 continuous injections of serum sample.

Performance testing using ChromSystems reference standards

ChromSystems sample set includes lyophilized blank, three level calibrators, and two QC levels. In addition to the three studied analytes 25(OH) D2, 25(OH)D3, and 25(OH)D3-epi, ChromSystems lyophilized standard set also includes 25(OH)D2-epi. The lyophilized samples were reconstituted as per the manufacturer's protocol. The blank, calibrators, and four technical preparations of each QC level were then processed using the in-house developed sample preparation protocol and acquired using the developed Ultivo LC/TQ method in triplicates. Four technical preparations of each QC level were used to calculate the recovery (%) and repeatability (%RSD) values.

Good separation was observed between 25(OH)D3 and 25(OH)D3-epi in the reference standards (Figure 5A). In addition, although 25(OH)D2-epi was not included in the initial method development, its presence in the reference standards demonstrated that the method also achieves baseline separation of 25(OH)D2 and 25(OH)D2-epi (Figure 5B). The linearity curves were plotted using the three-level calibrators and all four targets displayed a linear response with R^2 values >0.999 (Figure 6). The average recovery of all four targets using both QC levels was within 97 to 109%, with a repeatability RSD of ≤4%. The recovery results using four technical reparations of each Chromsystems QC sample were well within the certified allowed deviation range (Table 6).

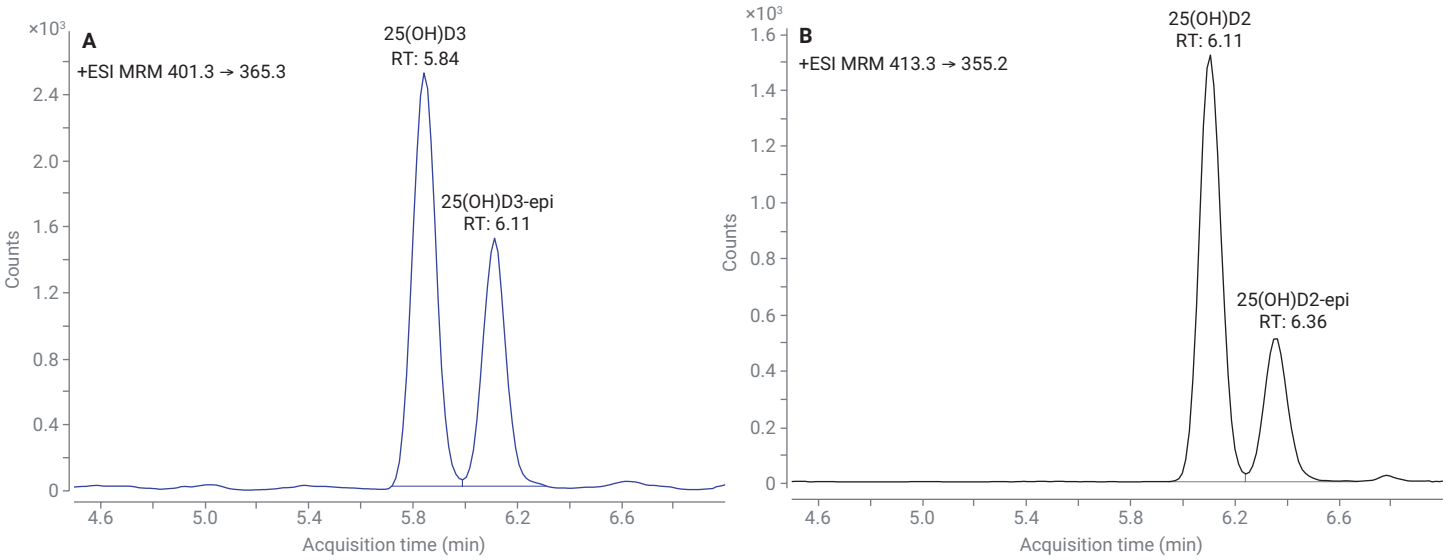

Figure 5. MRM trace of targets indicating baseline chromatographic separation of 25(OH)D and respective epimer. (A) separation between 25(OH)D3 and 25(OH)D3-epi, (B) separation between 25(OH)D2 and 25(OH)D2-epi.

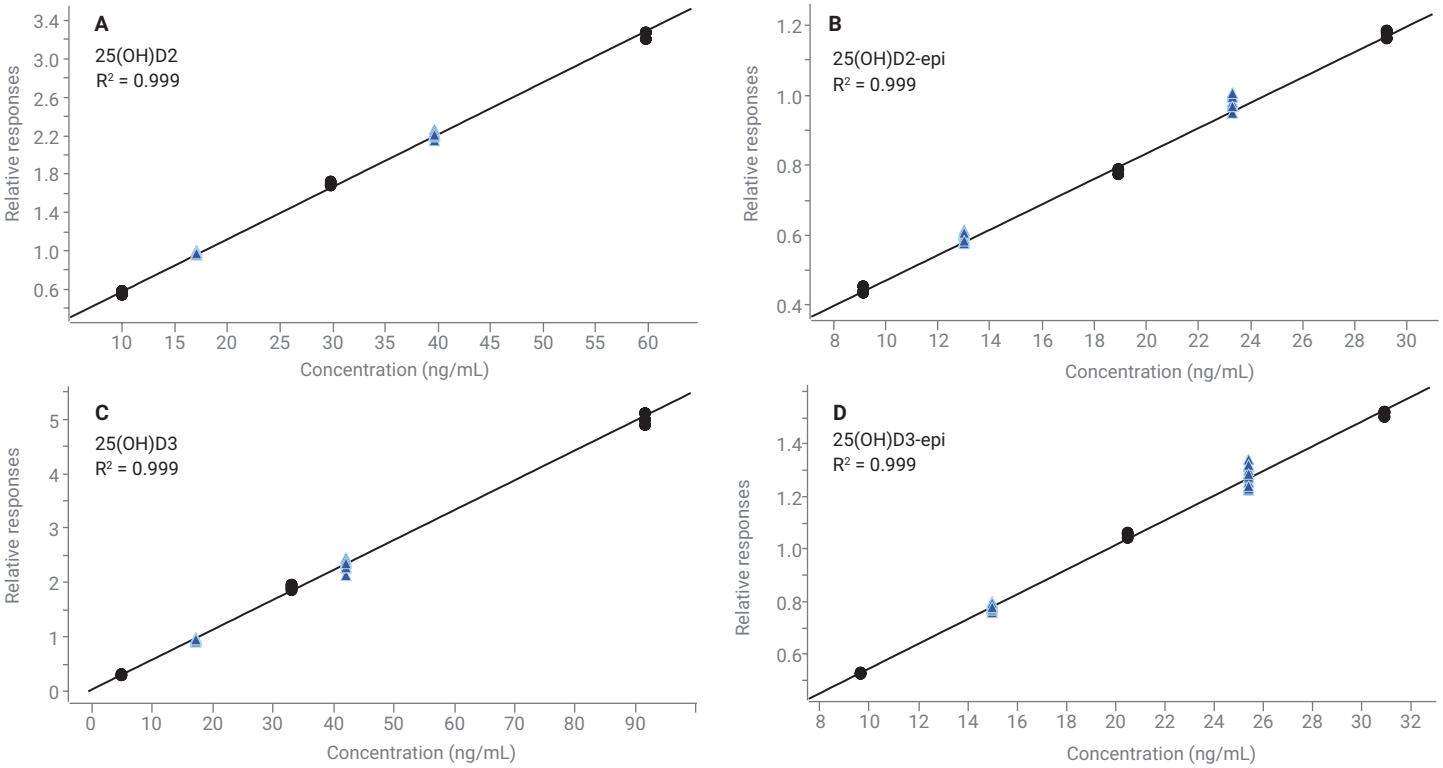

Figure 6. The calibration curves for all four analytes were constructed using Chromsystems three-level calibrators: A: 25(OH)D2; B: 25(OH)D2-epi; C: 25(OH)D3; and D:25(OH)D3-epi. The two QC levels are marked in a blue triangle (calibration model type: linear; origin: ignore; weight: 1/x).

Table 6. Recovery and repeatability results of ChromSystems QC samples. Results are calculated using four technical replicates. The theoretical values and allowed deviations are also included.

QC	Details	25(OH)D2	25(OH)D2-epi	25(OH)D3	25(OH)D3-epi
QC 1	Theoretical Value (ng/mL)	17	13	17.2	15
	Allowed Range (ng/mL)	13.6 to 20.4	10.4 to 15.6	13.7 to 20.6	12 to 18
	Replicate 1	17	14	17	15
	Replicate 2	17	14	17	15
	Replicate 3	18	13	17	15
	Replicate 4	18	13	17	15
	Average Recovery % (n = 4)	103	104	97	100
	SD	0.1	0.2	0.1	0.1
	Recovery Repeatability %RSD (n = 4)	1%	2%	1%	1%
QC 2	Theoretical Value (ng/mL)	39.6	23.3	42	25.8
	Allowed Range (ng/mL)	31.7 to 47.6	18.6 to 27.9	33.6 to 50.4	20.3 to 30.4
	Replicate 1	40	25	42	25
	Replicate 2	40	26	44	27
	Replicate 3	40	26	41	25
	Replicate 4	40	24	41	25
	Average Recovery % (n = 4)	101	109	99	100
	SD	0.2	0.8	1.3	0.9
	Recovery repeatability %RSD (n = 4)	0%	3%	3%	4%

Conclusion

This application note has described a robust method for the simultaneous analysis of vitamin D hydroxy metabolites and their respective epimers. Both 25(OH)D2 and 25(OH)D3 were chromatographically well resolved from their epimeric forms using a 10-minute gradient, thus adding specificity to the confident quantitation of all four targets: 25(OH)D2, 25(OH)D2-epi, 25(OH)D3, and 25(OH)D3-epi. The results confirmed that the Agilent Ultivo triple quadrupole LC/MS system provides excellent linearity, precision, and analytical sensitivity across the range of 1 through 200 ng/mL for 25(OH)D metabolites in serum. The developed method was tested with ChromSystems reference samples and results were in excellent agreement within the allowed range. The robustness assessment using 500 continuous injections further illustrated the method reliability for day-to-day operation.

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