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TECHNICAL NOTE 65559

# Confident quantitation of 25-hydroxyvitamin $D_2$ and $D_3$ in human plasma for clinical research by liquid chromatography-tandem mass spectrometry with simple, robust sample preparation


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## Keywords

25-hydroxyvitamin D, offline sample preparation, human plasma, mass spectrometry, TSQ Quantis, multiplexing, multichannel LC, Transcend LX-2, TraceFinder


## Application benefits

- Simple offline sample preparation via protein precipitation
- Fast acquisition time leveraging multichannel LC ensuring increased productivity
- Best-in-class sensitivity with mid-range triple quadrupole MS

## Goal

Implementation of an analytical method for quantification of 25-hydroxyvitamin $D_2$ and $D_3$ in human plasma with a Thermo Scientific™ Transcend™ LX-2 system and a Thermo Scientific™ TSQ Quantis™ triple-stage quadrupole mass spectrometer.

## Introduction

Vitamin D is an essential nutrient that enables intestinal absorption of calcium and phosphate and promotes deposition of these minerals in newly formed bones. One can acquire the precursors to the bioactive forms, vitamin $D_3$ and vitamin $D_2$, from several sources, including diet, supplements, and sunlight.

Vitamin D is metabolized to 25-hydroxyvitamin D in the liver, and being a predominant metabolite, circulating 25-hydroxyvitamin D serves as the preferred surrogate biomarker of vitamin D nutritional status. 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 demand for the comprehensive analysis and quantitation of

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vitamin D and its metabolites in human plasma/serum. This is a major challenge, as vitamin D is typically not found free in serum samples. Owing to its increased selectivity and specificity while addressing sensitivity, robustness, and reliability requirements, liquid chromatography (LC) coupled to mass spectrometry (MS) has become the platform of choice for identification and quantitation of Vitamin D in biological matrices.

In this study, an analytical method for quantification of 25-hydroxyvitamin $D_2$ and $D_3$ in human plasma is reported for clinical research. Plasma samples were extracted by protein precipitation followed by offline addition of the internal standard. Extracted samples were injected onto a Transcend LX-2 system for LC separation. MS detection was performed using a TSQ Quantis triple-stage quadrupole mass spectrometer with atmospheric pressure chemical ionization operated in positive ionization mode. 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 calibrators and controls 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 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 $D_3$, which was used as the internal standard for the quantification. Samples of 50 µL of plasma were protein precipitated using 150 µL of acetonitrile containing the internal standard. Precipitated samples were vortex-mixed, then centrifuged. The supernatant was transferred to a clean plate or vial.

### Liquid chromatography

A Transcend LX-2 system was used in multichannel LC mode. The system consists of two separate, parallel UHPLC channels connected to a single mass spectrometer (Figure 1). This configuration enhances

**Figure 1. Thermo Scientific Transcend LX-2 system**

productivity by utilizing the mass spectrometer idle time. In our case, each batch was run by staggering the injections on both channels. LC separation was achieved on a Thermo Scientific™ Hypersil GOLD™ analytical column, 50 × 2.1 mm (1.9 µm) kept at 40 °C. Mobile phases A and B consisted of water and methanol, respectively, both containing 10 mM ammonium formate and 0.1% formic acid. Details of the analytical method are reported in Table 2. Total runtime was 3.5 minutes.

**Table 2. LC method description**

| Gradient Profile | | | |
|---|---|---|---|
| **Time (min)** | **Flow Rate (mL/min)** | **A (%)** | **B (%)** |
| 0.00 | 0.5 | 20 | 80 |
| 0.25 | 0.5 | 20 | 80 |
| 1.00 | 0.5 | 0 | 100 |
| 2.00 | 0.5 | 0 | 100 |
| 2.01 | 0.5 | 20 | 80 |
| 3.50 | 0.5 | 20 | 80 |
| **Other Parameters** | | | |
| Injection volume (µL) | | 20 | |
| 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 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), 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: | 0.350 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 quadratic interpolation with 1/x weighting was used for both 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 both the analytes and the internal standard are reported in Figure 2. Representative calibration curves are reported in Figure 3.

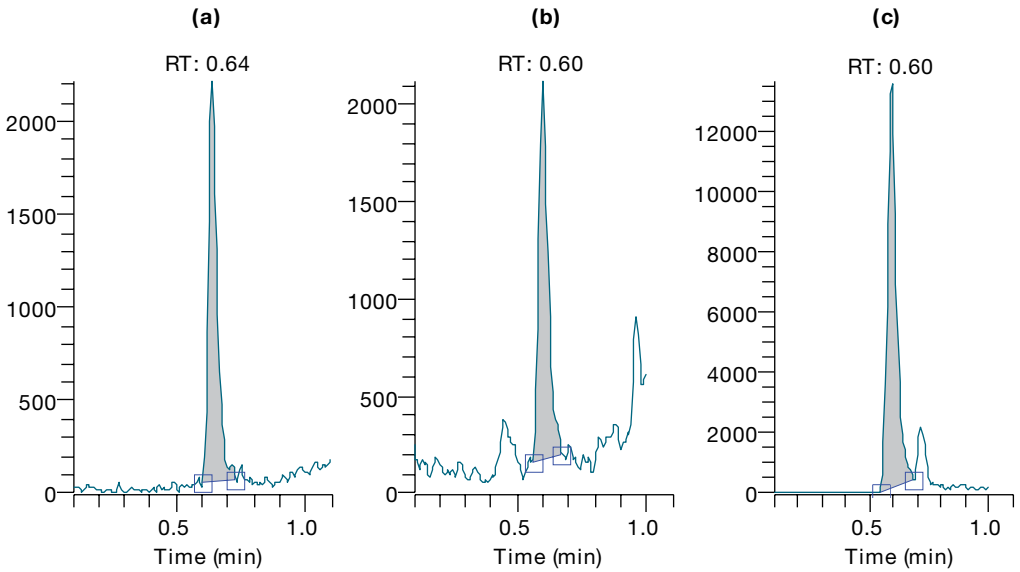


**Figure 2. Representative chromatograms of the lowest calibrator for (a) 25-hydroxyvitamin $D_2$, (b) 25-hydroxyvitamin $D_3$, and (c) $d_6$-25-hydroxyvitamin $D_3$**

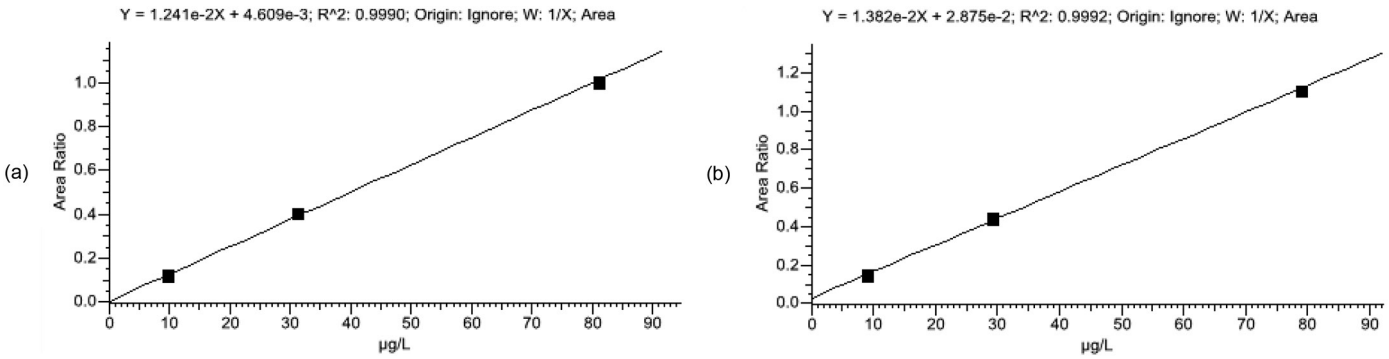


**Figure 3. Representative calibration curves for (a) 25-hydroxyvitamin $D_2$, and (b) 25-hydroxyvitamin $D_3$ – day 2**

No significant carryover was observed for either analyte, with no signal detected in the blank injected immediately 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 control samples ranging between -2.5% and 2.6% (Table 4). The %CV for intra-assay precision was always below 3.6% for both the analytes. The maximum %CV for inter-assay precision including both the analytes was 4.1%. 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-hydroxyvitamin $D_2$ | Level I (LOT #1207) | 14.7 | 14.8 | 0.6 |
| | Level II (LOT #1207) | 42.5 | 43.6 | 2.6 |
| 25-hydroxyvitamin $D_3$ | Level I (LOT #1207) | 14.9 | 14.5 | -2.5 |
| | Level II (LOT #1207) | 42.0 | 41.9 | -0.3 |

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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) | 14.3 | 3.2 | 15.2 | 2.7 | 14.9 | 1.3 | 14.8 | 3.7 |
| | Level II (LOT #1207) | 41.7 | 3.1 | 45.3 | 0.9 | 43.8 | 2.0 | 43.6 | 4.1 |
| 25-hydroxyvitamin $D_3$ | Level I (LOT #1207) | 14.6 | 1.4 | 14.7 | 3.6 | 14.3 | 0.9 | 14.5 | 2.4 |
| | Level II (LOT #1207) | 40.3 | 1.9 | 43.4 | 2.0 | 41.9 | 2.8 | 41.9 | 3.8 |

## Conclusions

A robust, reproducible, and reliable analytical method was developed using liquid chromatography-tandem mass spectrometry for clinical research for quantification of 25-hydroxyvitamin $D_2$ and $D_3$ in human plasma. The method was analytically validated with a Transcend LX-2 multichannel LC system connected to a TSQ Quantis triple-stage quadrupole mass spectrometer. The Transcend LX-2 system doubled the productivity of the mass spectrometer by achieving the throughput of two separate LC/MS systems with a single MS. Simple and robust protein precipitation of the calibrators and controls obtained from RECIPE was used for sample preparation. 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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