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# Confident quantitation of 25-hydroxyvitamin $D_2$ and $D_3$ in human plasma for clinical research by liquid chromatography-tandem mass spectrometry

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## ABSTRACT

**Purpose:** 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 analysis and quantitation of Vitamin D in biological matrices. An analytical method for high throughput quantitation of 25 hydroxyvitamin $D_2$ and $D_3$ in human plasma with simple sample preparation is reported for clinical research.

**Methods:** Samples were prepared by protein precipitation. Chromatographic separation was carried out on a Thermo Scientific™ Transcend™ LX-2 system coupled to a Thermo Scientific™ TSQ Quantis™ triple-stage quadrupole mass spectrometer. Precision was evaluated by analyzing replicate concentrations over three days.

**Results:** A method for analysis of 25-hydroxivitamin $D_2$ and D3 was developed. Inter-assay precision of replicate injections of the controls over three days were below 3.6% for both analytes. Accuracy of analysis was demonstrated, the calculated concentrations for the controls re within 3% of reference values.

## INTRODUCTION

Vitamin D is hydrolized to its prohormone 25-hydroxyvitamin D in the liver, and being a predominant metabolite, circulating 25-hydroxyvitamin D serves as the preferred analyte that is monitored to determine nutritional status of Vitamin D.

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.

## MATERIALS AND METHODS

### Sample Preparation

Reagents included four calibrators (including blank) and two controls from RECIPE® Chemicals + Instruments GmbH (Munich, Germany), as well as $d_6$-25-hydroxyvitamin $D_3$ as the internal standard for quantification. Concentration ranges are reported in Table 1.

Samples were prepared by precipitating 50 µL of plasma with 150 µL of Acetonitrile containing the internal standards. Precipitated samples were vortex-mixed, then centrifuged. The supernatant was transferred to a clean plate or vial.

**Table 1. Concentrations covered by the calibrators.**

| | Concentration Range (ng/mL) |
|---|---|
| **25-hydroxyvitamin $D_2$** | 9.84-81.0 |
| **25-hydroxyvitamin $D_3$** | 9.04-78.9 |

### Liquid Chromatography

Chromatography was performed on a Transcend LX-2 system used in multichannel LC mode. The system consists of two separate, parallel UHPLC channels connected to a single mass spectrometer (Figure 1). To enhance the productivity of the system, each batch was run by staggering the injections on both channels.

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

LC separation was achieved on a Thermo Scientific™ Hypersil GOLD™ analytical column 50 × 2.1 mm (1.9 µm) kept at 40 ° C. Injection volume was 20 µL.

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 run time was 3.5 minutes.

**Table 2. Description of LC method.**

| Time (min) | Flow Rate (mL/min) | A(%) | B(%) |
|---|---|---|---|
| 0.0 | 0.5 | 20 | 80 |
| 0.25 | 0.5 | 20 | 80 |
| 1.0 | 0.5 | 0 | 100 |
| 2.0 | 0.5 | 0 | 100 |
| 2.01 | 0.5 | 20 | 80 |
| 3.5 | 0.5 | 20 | 80 |

### Mass Spectrometry

Analytes and internal standard were detected by SRM on a TSQ Quantis triple-stage 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.

**Table 3. Mass Spectrometry Settings.**

| | | | |
|---|---|---|---|
| **Source Type** | Atmospheric Pressure Chemical Ionization (APCI) | **Sweep Gas** | 2 AU |
| **Vaporizer Temperature** | 400° C | **Data Acquisition Mode** | Selected Reaction Monitoring (SRM) |
| **Capillary Temperature** | 300° C | **Collision Gas Pressure** | 1.5 mTorr |
| **Spray Current (positive mode)** | 4 µA | **Cycle time** | 0.350 s |
| **Sheath Gas** | 40 AU | **Q1 Mass Resolution (FWHM):** | 0.7 |
| **Auxiliary Gas** | 2 AU | **Q3 Mass Resolution (FWHM):** | 0.7 |

### 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 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).

### Data analysis

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

## RESULTS

### Linearity

A quadratic interpolation with 1/x weighting was used for 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 the analytes and their internal standards are reported in Figure 2. Representative calibration curves are reported in Figure 3.

### Accuracy

No significant carryover was observed for both analytes, 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 -2.5% and 2.6% (Table 4).

### Precision

The %CV for intra-assay precision was always below 3.6% for all the analytes. The maximum % CV for inter-assay precision, including all the analytes, was 4.1%. Results for intra- and inter-assay precision are reported in Table 5.

**Figure 2. Representative chromatograms of the lowest calibrator for (a) 25-OH-Vitamin D2, (b) 25-OH-Vitamin D3 and (c) d6-25-OH-Vitamin D3**

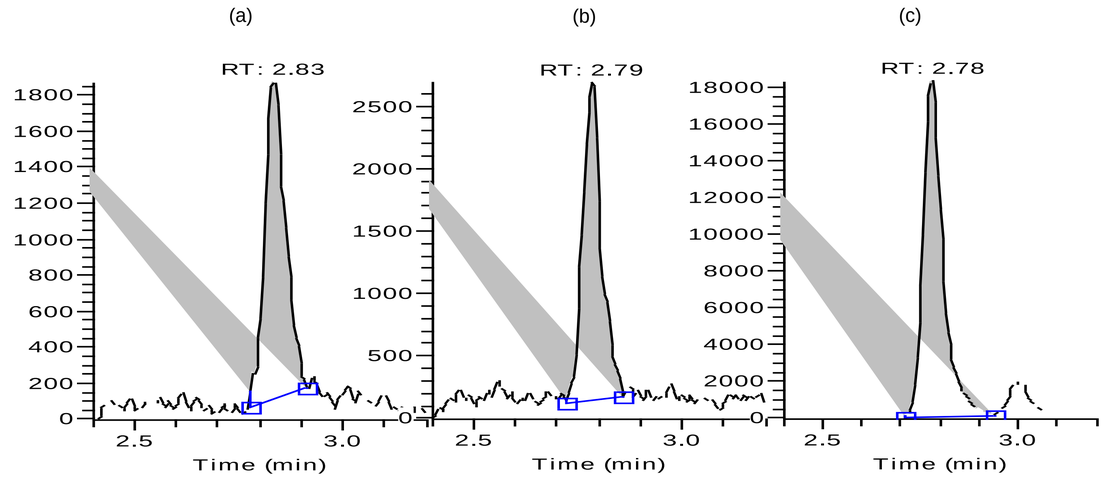


**Figure 3. Representative calibration curves for (a) 25-OH-Vitamin D2 and (b) 25-OH-Vitamin D3**

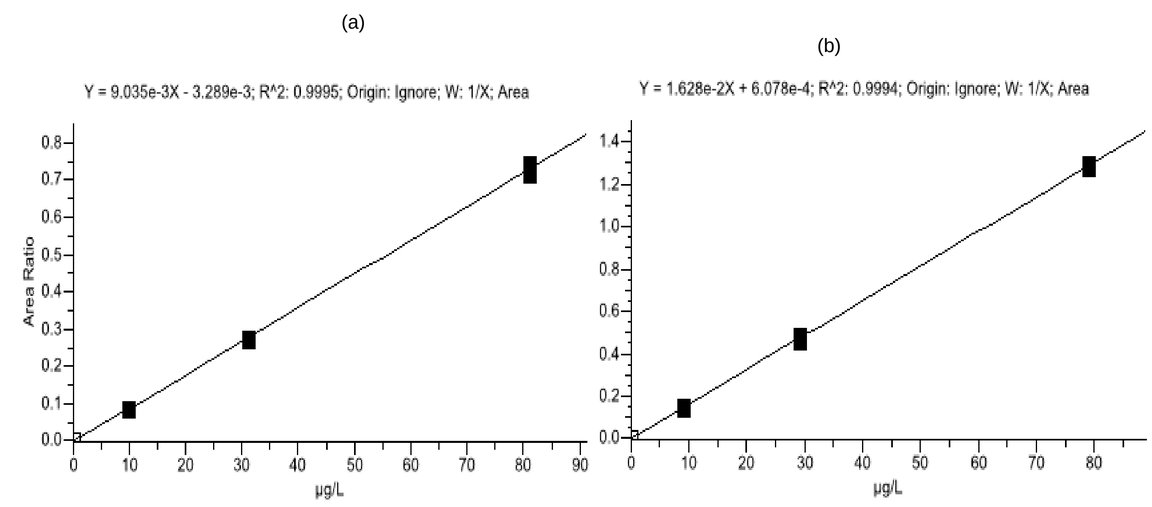


**Table 4. Analytical accuracy results for control MS7082 batch #1207.**

| Analyte | Control | Nominal Concentration (ng/mL) | Average Calculated Concentration (ng/mL) | Bias (%) |
|---|---|---|---|---|
| 25-OH-Vitamin-$D_2$ | Level I (LOT #1207) | 14.7 | 14.5 | -1.7 |
| | Level II (LOT #1207) | 42.5 | 41.1 | -3.4 |
| 25-OH-Vitamin-$D_3$ | 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.**

| | | 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 (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-OH-Vitamin-$D_3$ | 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 D2 and D3 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 Transcend II system connected to a TSQ Quantis triple quadrupole mass spectrometer.
- This 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.

## TRADEMARKS/LICENSING



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