



Quantification of 25-hydroxyvitamin D2 and D3 with chromatographic resolution of the C3-epimer in plasma or serum by liquid chromatography-tandem mass spectrometry for research use

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Keywords

25-hydroxyvitamin D2,
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Application benefits

- Separation of the C3-epimer of 25-hydroxyvitamin D3
- Offline sample preparation: simple protein precipitation followed by online SPE

Goal

Development and implementation of an analytical method for the quantification of 25-hydroxyvitamin D2 and D3 in human plasma or serum with chromatographic separation of the C3-epimer of 25-hydroxyvitamin D3 using a Thermo Scientific™ Transcend™ II TLX-1 system coupled to a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer.

Introduction

An analytical method for clinical research for the quantification of 25-hydroxyvitamin D2 and D3 in human donor plasma or serum is reported. The method, based on the MS7000 ClinMass® LC-MS/MS Complete Kit for 25-OH-Vitamin D2/D3 in Plasma and Serum – On-Line Analysis from RECIPE® Chemicals + Instruments GmbH, allows for the chromatographic separation of the C-3 epimeric form of 25-hydroxyvitamin D3 (3-epi-25-OH-vitamin D3). Sample extraction was performed by protein precipitation followed by online solid phase extraction (SPE) using a Transcend II TLX-1 system. A TSQ Endura triple quadrupole mass spectrometer with

atmospheric pressure chemical ionization (APCI) was used for detection by single reaction monitoring (SRM) using 25-hydroxyvitamin D3-d6 as the internal standard. Implementation of the kit included the evaluation of the limits of quantification, linearity ranges, accuracy, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

- 25-hydroxyvitamin D2
- 25-hydroxyvitamin D3
- 25-hydroxyvitamin D3-d6 (internal standard)

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE covering a concentration range between 8.2 and 68.5 µg/L for 25-hydroxyvitamin D2 and between 9.4 and 77.3 µg/L for 25-hydroxyvitamin D3. For the quantification of both analytes, 25-hydroxyvitamin D3-d6 was used as the internal standard. Sample cleanup was performed by a preliminary protein precipitation with internal standard addition followed by online SPE on a Transcend II TLX-1 system using mobile phases, SPE cartridge, and analytical column provided by RECIPE.

Liquid chromatography

The LC method used for online SPE and chromatographic separation is reported in Table 1. A schematic representation of the LC configuration is reported in Figure 1.

Mass spectrometry

Detection was performed by SRM on a TSQ Endura triple quadrupole mass spectrometer with atmospheric pressure chemical ionization operated in positive mode. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively. The following MS conditions were used:

| | |
|-------------------------------|-----------------------|
| Source type | APCI in positive mode |
| Vaporizer temperature | 350 °C |
| Ion transfer tube temperature | 275 °C |
| Discharge current | 4.5 µA |
| Sheath gas | 50 AU |
| Sweep gas | 0 AU |
| Auxiliary gas | 5 AU |
| Data acquisition mode | SRM |
| Chrom filter peak width | 3.0 s |
| Collision gas pressure | 1.5 mTorr |
| Cycle time | 0.4 s |
| Q1 (FWHM) | 0.7 |
| Q3 (FWHM) | 0.7 |

Table 1. LC method description.

| Time (min) | ASV Position | Pump P1 (SPE Buffer) | | Pump P2 (Mobile Phase) | |
|------------|--------------|----------------------|------------------|------------------------|-------------------------|
| | | Flow (mL/min) | Event SPE Column | Flow (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 | | | |

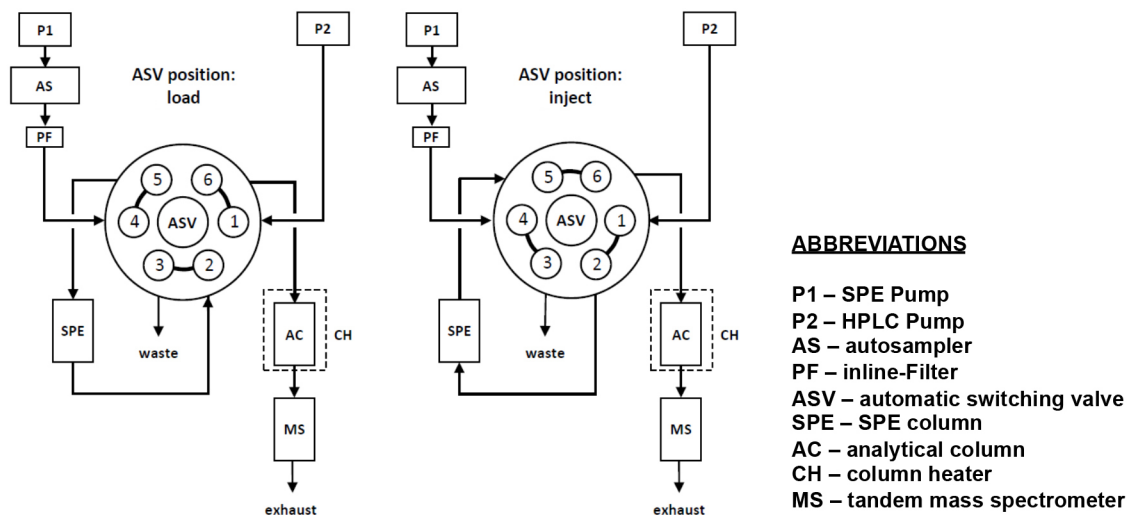


Figure 1. Schematic representation of the Transcend II TLX-1 system configuration used for online SPE.

Method evaluation

The capability of the method was evaluated by achieving limits of quantification, linearity ranges, accuracy, and intra- and inter-assay precision for each analyte. Analytical accuracy was evaluated by using the DEQAS Proficiency Test Samples #466 and #467 prepared and analyzed on five different days in single runs each day. Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using the controls at both levels in replicates of eight (n=8) prepared and analyzed in one batch. Inter-assay precision was evaluated on the same controls in replicates of three (n=3) prepared and analyzed on five different days.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 3.3 software.

Results and discussion

The analytical method was reported to be linear in the calibration range covered by the calibrators and controls with limits of quantification (LOQ) of 2.1 µg/L for both analytes, an upper limit (ULOQ) of 154.7 and 157.0 µg/L for 25-hydroxyvitamin D2 and D3, respectively, and a correlation factor (R^2) always above 0.997. A representative chromatogram of the achievable chromatographic separation of the C-3 epimeric form of 25-hydroxyvitamin D3 is reported in Figure 2. Representative chromatograms at the LOQ are reported in Figure 3 for the analytes and the internal standard. Representative calibration curves for both analytes are reported in Figure 4.

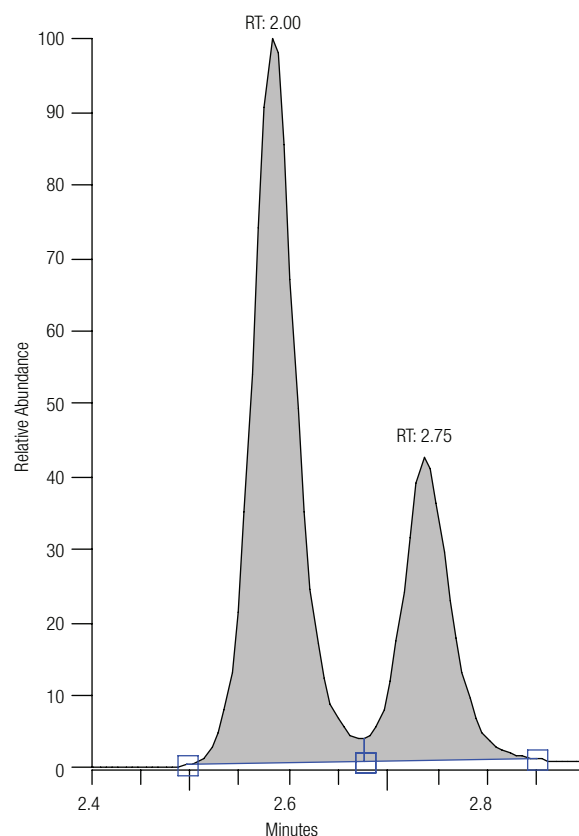


Figure 2. Representative chromatogram of the separation between 25-hydroxyvitamin D3 and the corresponding C3-epimer.

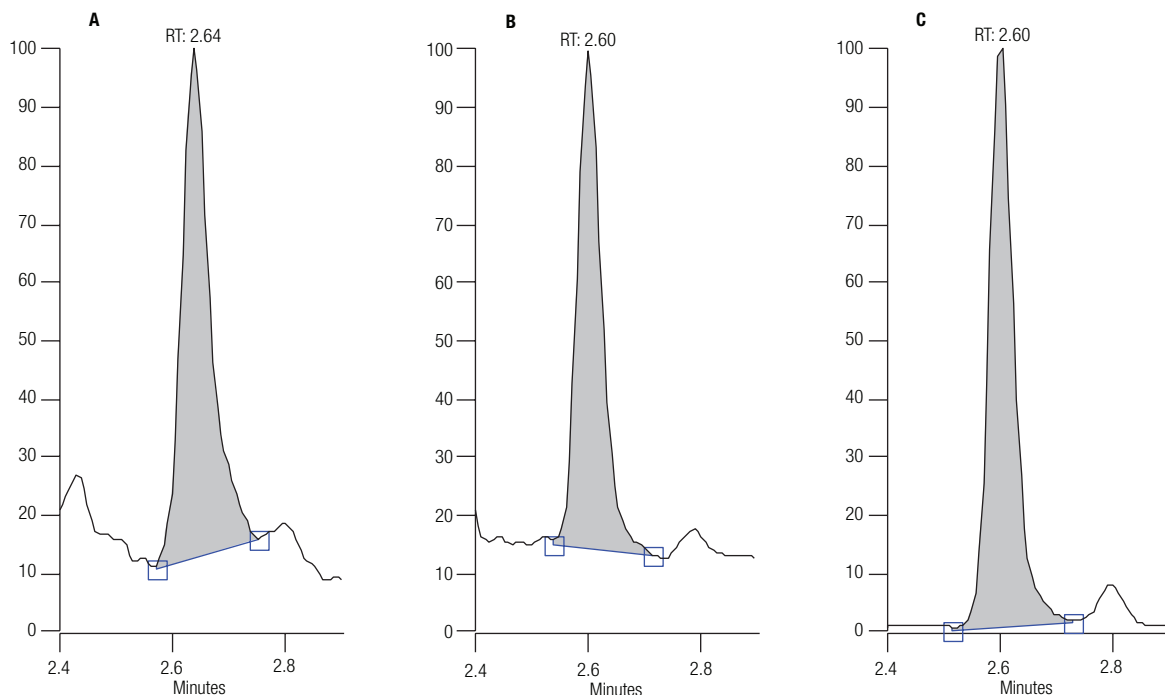


Figure 3. Representative chromatograms at the LOQ and for (A) 25-hydroxyvitamin D2, (B) D3, and (C) their internal standard 25-hydroxyvitamin D3-d6.

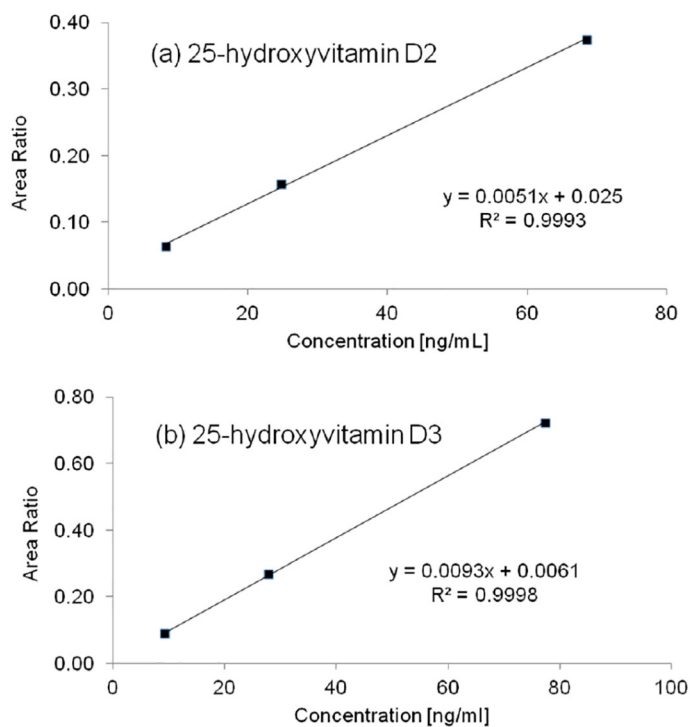


Figure 4. Representative calibration curves for (a) 25-hydroxyvitamin D2 and (b) 25-hydroxyvitamin D3.

The data showed exceptional accuracy with the percentage bias between nominal and average back-calculated concentration for the DEQAS proficiency test samples #466 and #467 being 2.4% and 2.5%, respectively (Table 2).

The %CV for intra-assay precision was 4.5% and 3.1% for the lower and upper control, respectively, for 25-hydroxyvitamin D2, and 2.2% and 2.8% for the lower and upper control, respectively, for 25-hydroxyvitamin D3 (Table 3). For the lower and upper control, respectively, for 25-hydroxyvitamin D2, and 4.3% and 3.0% for the lower and upper control, respectively, for 25-hydroxyvitamin D3 (Table 4).

Table 2. Analytical accuracy for 25-hydroxyvitamin D3.

| Sample | n | Nominal Concentration (ng/mL) | Measured Concentration (ng/mL) | CV (%) | Bias (%) |
|-----------|---|-------------------------------|--------------------------------|--------|----------|
| DEQAS 466 | 5 | 25.8 | 26.4 | 4.3 | 2.4 |
| DEQAS 467 | 5 | 17.8 | 18.2 | 6.5 | 2.5 |

Table 3. Intra-assay precision for (A) 25-hydroxyvitamin D2 and (B) 25-hydroxyvitamin D3 (n = 8).

| | Level I Measured Concentration (ng/mL) | CV (%) | Level II Measured Concentration (ng/mL) | CV (%) |
|---|--|--------|---|--------|
| A | 15.4 | 4.5 | 37.1 | 3.1 |
| B | 19.9 | 2.2 | 45.8 | 2.8 |

Table 4. Inter-assay precision for (a) 25-hydroxyvitamin D2 and (B) 25-hydroxyvitamin D3 (n = 15).

| | Level I Measured Concentration (ng/mL) | CV (%) | Level II Measured Concentration (ng/mL) | CV (%) |
|---|--|--------|---|--------|
| A | 16.4 | 10.0 | 38.7 | 8.3 |
| B | 19.6 | 4.3 | 45.8 | 3.0 |

Conclusions

A robust, reproducible method employing liquid chromatography-tandem mass spectrometry was developed for quantitation of 25-hydroxyvitamin D2 and D3 in human plasma or serum. This analytical method used calibrators and controls from the RECIPE MS7000 ClinMass LC-MS/MS Complete Kit for 25-OH-Vitamin D2/D3 in Plasma and Serum On-Line Analysis was implemented. Separation and detection parameters were analytically validated on a Transcend II system connected

to a TSQ Endura triple quadrupole mass spectrometer. This analytical method allows for the chromatographic resolution between 25-hydroxyvitamin D3 and its C-3 epimeric form, ensuring accurate quantitation of both vitamin D epimers. The method meets research laboratory requirements for analytical sensitivity and linearity of response covering analyte concentration ranges provided by the MS7000 ClinMass LC-MS/MS product.

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