Accurate and reliable quantification of 25-hydroxy-vitamin D species by liquid chromatography high-resolution tandem mass spectrometry

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Short Title: 25-OH-vitamin D quantification by LC-MS/HRMS

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Abbreviations

25(OH)D  25-hydroxyvitamin D  
BHT  butylated hydroxytoluene  
HR-MS  high resolution tandem mass spectrometry  
IS  internal standard  
LC-MS/HR-MS  liquid chromatography-high resolution tandem mass spectrometry  
LoD  Limit of detection  
LoQ  Limit of quantification  
MF  matrix factor  
QQQ  triple quadrupole mass spectrometers  
SIM  selected ion monitoring
Abstract

In general, mass spectrometric quantification of small molecules in routine laboratory testing utilizes liquid chromatography coupled to low mass resolution triple quadrupole mass spectrometers (QQQ). Here we introduce high resolution tandem mass spectrometry (quadrupole-Orbitrap) for the quantification of 25-hydroxy-vitamin D [25(OH)D], a marker of the vitamin D status, since the specificity of 25(OH)D-immunoassays is still questionable and mass spectrometric quantification is becoming increasingly important. Liquid chromatography coupled to high resolution tandem mass spectrometry (LC-MS/HR-MS) was used to quantify 25-hydroxycholecalciferol [25(OH)D₃], 25-hydroxy-ergocalciferol [25(OH)D₂] and their C3-epimers 3-epi-25(OH)D₃, 3-epi-25(OH)D₂. The method has a run time of 5min and was validated according to the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines. High mass resolution was advantageously applied to separate a quasi-isobaric interference of the internal standard D₆-25(OH)D₂ with 3-epi-25(OH)D₃. All analytes showed an imprecision of below 10% CV, trueness between 90 and 110% as well as LoQs below 10nmol/l. Concentrations measured by LC-MS/HR-MS are in good agreement with those of the National Institute of Standards and Technology (NIST) reference methods using LC-MS/MS (QQQ). In conclusion, quantification of 25(OH)D by LC-MS/HR-MS is applicable for routine testing and holds promise also for highly specific quantification of other small molecules.
Introduction

It is increasingly recognized that an adequate vitamin D status beside the regulation of bone and calcium-phosphate metabolism seems to be protective against a number of diseases including diabetes, cancer, musculoskeletal disorders, cardiovascular, infectious, autoimmune diseases and dementia (1). 25-hydroxyvitamin D [25(OH)D] is widely accepted as a reliable indicator of the vitamin D status. There is an ongoing debate about the standardization and specificity of 25(OH)D immunoassays (2-4). Thus, sources of inaccuracy may be related to variations in the levels of vitamin D binding protein (5) and cross-reactivity to 24,25-dihydroxyvitamin D (6). Therefore, a number of laboratories introduced liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the quantification of 25(OH)D (reviewed in (7)). Moreover, global standardization is advanced with LC-MS/MS reference methods (8;9) and the availability of NIST reference material (10).

Application of LC-MS/MS allows the separation of different 25(OH)D species i.e. 25-hydroxy-cholecalciferol [25(OH)D\(_3\)], 25-hydroxy-ergocalciferol [25(OH)D\(_2\)] and their C3-epimers 3-epi-25(OH)D\(_3\), 3-epi-25(OH)D\(_2\). Meanwhile it is recognized that accurate quantification of 25(OH)D by LC-MS/MS requires LC separation of 25(OH)D-epimers due to an increased analytical response of the epimers (11;12) and significant concentrations of epimers not only in infants but also adults (13;14).

In general, quantification of small molecules involves triple quadrupole instruments (QQQ) operated at unit mass resolution. Until now there have been only two methods published for 25(OH)D quantification by high resolution mass spectrometry (HR-MS). One applied LC-HR-MS (15) and the second performed LC-HR-MS\(^3\) of derivatized 25(OH)D\(_3\) species with an ion trap-Orbitrap mass spectrometer (16). Here we present a novel method for the fast and accurate quantification of 25(OH)D\(_3\), 25(OH)D\(_2\) and...
their C3-epimers by liquid chromatography-high resolution tandem mass spectrometry (LC-MS/HR-MS) using a quadrupole-Orbitrap instrument.
Material and Methods

Reagents
Ammonium acetate analytical grade, formic acid analytical grade, ethanol absolute EMSURE and isopropanol LiChrosolv were purchased from Merck (Darmstadt, Germany). Chloroform ROTISOLV® was from Carl Roth GmbH (Karlsruhe, Germany) and methanol LC-MS Chromasolv from Fluka (Buchs, Switzerland). 25(OH)D₃, 25(OH)D₂, D₆-25(OH)D₃, D₆-25(OH)D₂ and 3-epi-25(OH)D₃ were purchased from Toronto research chemicals (Toronto, Canada). Butylated hydroxytoluene (BHT), 3-epi-25(OH)D₂, iso-octane ACS reagent and Zone-Free Films were from Sigma Aldrich (München, Germany).

Calibrators and quality controls
Assay validation was performed with serum controls MassCheck® 3-epi-25-OH-D₃/D₂ and 25-OH-D₃/D₂ Level I (medium) and II (high) (Table 2), purchased from Chromsystems (München, Germany). A low level control was prepared by 5-fold dilution of Level I with physiological human albumin solution ALBUNORM 5% (Octapharma, Langenfeld, Germany). Additionally, pooled serum was used as an in-house quality control. Calibrators were prepared by standard addition from methanolic solutions of authentic standards to pooled human serum. ALBUNORM was used as analyte free level and did not contain any detectable 25(OH)D species (please see Figures S4 to 7, Cal 0). The level I calibrator was prepared by 4-fold dilution of a serum pool with ALBUNORM. The in-house calibrators (blank + 5 levels, see Supplemental Figures 4-7) were calibrated by repeated quantification (n=6) using NIST traceable serum calibrators 3PLUS1® Multilevel Serum Calibrator Set 3-epi-25-
OH-D3/D2 and 25-OH-D3/D2 (four calibration levels including a low level) obtained from Chromsystems (München, Germany).

Sample preparation

Liquid-liquid extraction was used as described by Midttun et al. (17). In brief, 100µl serum/control/calibrator were placed into 96-well deep-wells (2ml Costar Assay Block; Corning, Amsterdam, The Netherlands) and deproteinized by 200µl of an ethanolic solution containing 50ng/ml each D\textsubscript{6}-25(OH)D\textsubscript{3}, D\textsubscript{6}-25(OH)D\textsubscript{2} and 1g/l BHT. Extraction was performed with 600µl iso-octane/chloroform = 3/1 (v/v). 400µl of the upper phase was recovered using a Tecan Genesis (Männedorf, Switzerland) and transferred to another 96-well deep-well plate. Solvent was removed by vacuum-centrifugation. The samples were re-dissolved in 50µl methanol containing 1g/l BHT and sealed with Zone-Free Film.

LC-MS/HR-MS:

25(OH)D analysis was performed by liquid chromatography-high resolution tandem mass spectrometry (LC-MS/HR-MS). The LC consisted of an UltiMate 3000 XRS quaternary UHPLC pump, an UltiMate 3000 RS column oven and an UltiMate 3000 isocratic pump (Thermo Fisher Scientific Waltham, MA USA) connected to a PAL HTS-xt autosampler (CTC Analytics, Zwingen, CH) and a hybrid quadrupole-orbitrap mass spectrometer QExactive (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source. 5µl of the re-dissolved samples were injected and separated on a Kinetex™ 2.6 µm PFP (50 x 2.1 mm, Phenomenex, Aschaffenburg, Germany) equipped with a 0.5µm inline filter (Vici Valco, Schenkon, Switzerland) at a column temperature of 40°C. Mobile phase A consisted of methanol/water (5/95; v/v), mobile phase B was 100% methanol, both
containing 0.1% formic acid and 2mmol/L ammonium acetate. Gradient elution started at 100% A with a flow rate of 500µl/min, a linear increase to 68% B in 0.1 min, followed by an increase to 73% B until 4 min. For column cleaning the methanol percentage and flow were increased to 100% and 800µl/min within 0.1 min, respectively. After flushing for 0.5 min, the solvent composition was changed to 100% A within 0.1 min and hold until 5 min at a flow rate of 800µL/min. To minimize contamination of the mass spectrometer, the column flow was directed only from 3.0 to 4.0 min into the mass spectrometer using a divert valve. Otherwise methanol with a flow rate of 200µL/min was delivered into the mass spectrometer. The ion source was operated in the positive ion-mode using the following settings: Ion spray 3500V, sheath gas 53, aux gas 14, sweep gas 3 and aux gas heater temperature of 250°C. Capillary temperature was set to 269°C and the S-lens RF level to 55. Data were collected from 3.0 to 4.0 min in the targeted MS2 mode with the following settings: Resolution 35,000, AGC target: 5e5, maximum IT 100 ms with a multiplex of 2 and quadrupole isolation window of 0.8 m/z. Data analysis was performed with TraceFinder 3.1 Clinical (Thermo Fisher Scientific), a software module which extracts target ions (Table 1) within ±5 ppm mass window, generates calibration lines (Figures S-4 to S-7), checks quality controls and ion ratios of quantifier to qualifier ions (Table 1).

Method validation

Method validation was performed on the basis of the FDA (18) and EMA (19) guidelines on bioanalytical method validation (for details see Supplemental Data).

Results
Aim of the current study was to develop an accurate and fast method for the quantification of 25(OH)D species by LC-MS/HR-MS using a quadrupole-Orbitrap hybrid mass spectrometer. A core-shell pentafluorophenyl column with polar and aromatic selectivity was selected to separate the isobaric 3β-25(OH)D and 3α-25(OH)D (= epi) isomers as described in previous studies (7). We could achieve almost base-line separation of 25(OH)D₃ and 3-epi-25(OH)D₃ as well as 25(OH)D₂ and 3-epi-25(OH)D₂ within a run-time of 5min (Figure 1A). Due to the fact that HR-MS offers increased specificity compared to unit resolution usually provided by quadrupoles, [M+H-H₂O]⁺ ions were selected as sensitive fragment ions for all analytes (Table 1). During first tests including the internal standards (IS) D₆-25(OH)D₃ and D₆-25(OH)D₂ an immediate drop of the signal was recorded for 3-epi-25(OH)D₃ (Figure S-1). Product ion spectra displayed a mass shift for the m/z 383 product ion beyond the 5ppm mass window of the [25(OH)D₃+H-H₂O]⁺ from 3.68min to 3.70min (Figures S-1 and S-2). An increase of the mass resolution settings from 17,500 to 35,000 separated two ions m/z 383.3314 [M+H-H₂O]⁺ of 25(OH)D₃ and m/z 383.3585 [M+H-2H₂O]⁺ of D₆-25(OH)D₂ resulting from an in-source fragmentation (Table S-1 and Figure S-3). With mass resolution setting 35,000 no signal drop could be observed for 3-epi-25(OH)D₃ (Figure S-3).

Specificity and matrix effects

The specificity of the method was investigated using qualifier ions (Table 1) in six different patient samples including potential interferences from hemolysis, icterus and lipemia. 25(OH)D₂, 3-epi-25(OH)D₂ were not present in the tested patient samples above LoQ and 3-epi-25(OH)D₃ was present below or close to LoQ. Therefore, these analytes were evaluated only in spiked samples while 25(OH)D₃ specificity was also checked in unspiked samples. The ion ratios quantifier/qualifier correspond to those
of authentic standards with a maximum deviation of ±15% for all analytes (except for one sample with a low concentration of 25(OH)D₃ and the low spike of 25(OH)D₂; data not shown). Moreover, ion ratios are evaluated for all patient samples during routine diagnostic with an acceptance criterion of ±20% as the maximum relative deviation. We only observed a failure of the ion ratio criterion for concentrations close to LoQ due to qualifier intensities at or below LoD (signal dropout of qualifier ions was partly due to a mass shift outside of the 5ppm mass window).

Similarly, matrix effects were evaluated in patient samples with potential interferences at low and high spike concentrations. For all patient samples the IS-normalized matrix factor was within a window 100±15% (data not shown).

**Calibration**

D₆-25(OH)D₃ was used as IS for 25(OH)D₃ and D₆-25(OH)D₂ was used for 25(OH)D₂, 3-epi-25(OH)D₃ and 3-epi-25(OH)D₂. Linear calibration lines were found for all analytes within the analyzed range (Table 1). Calibration lines were weighted 1/x and back calculated concentrations were found within ±15% of the nominal values (Figures S-4 to S-7).

**Limit of quantification (LoQ)**

Determination of LoQ for LC-MS/MS usually involves calculation of signal to noise ratios. Due to its high specificity we did not observe baseline noise in LC-MS/HR-MS for 25(OH)D species. Therefore, LoQ was determined by functional testing with serial dilutions of control and calibrator samples (Figure S-8). LoQ for all analytes were found below 10nmol/l (Table 1). Typically, signals below the LoQ showed signal dropouts due to reduced mass precision and target masses outside the 5ppm mass window as shown for 25(OH)D₂ (Figure 1B).
Imprecision and trueness

Commercial serum controls traceable to NIST 972a reference material were used to evaluate imprecision and trueness including a low level prepared by dilution with physiological human albumin solution. For medium and high levels CVs were below 10% and trueness between 90 and 110% for all analytes (Table 2). Imprecision and trueness of the low level control were significantly higher with up to 16% and between 85 and 115%, respectively. The low level of 3-epi-25(OH)D$_2$ was below LoQ and displayed a trueness of about 130%. A serum pool, used as a quality control during routine analysis for 25(OH)D$_3$ (mean 55.5 nmol/l) and 3-epi-25(OH)D$_3$ (mean 3.33 nmol/l), showed an impressive between-run precision (n=10) of 3.9% and 3.8% CV, respectively. Results obtained for 10 samples of the Vitamin D External Quality Assessment Scheme (DEQAS) analyzed by NIST reference methods were in good agreement with LC-MS/HR-MS concentrations (Table S-2).

Epimer fractions in patient samples

The fraction of 3-epi-25(OH)D$_3$ related to total 25(OH)D was calculated in patient samples submitted to routine diagnostics at the University Hospital Regensburg (Figure 2). As expected children below 1 year displayed the highest fraction with a median of 14% and a maximum over 30% (Figure 2A). An age-dependent decrease was observed with a median of 4.3% in adult patients. 3-epi-25(OH)D$_3$ concentrations above LoQ were detected in about 60% of the samples of adult patients and more the 10% of these samples contained a 3-epi-25(OH)D$_3$ fraction above 10% of total 25(OH)D (Figure 2B).
Discussion

Here we present a fast and accurate method for the quantification of 25(OH)D species using LC-MS/HR-MS. Two studies showed an increased analytical response of 3-epi-25(OH)D$_3$ compared to 25(OH)D$_3$ (11;12). Consequently, only chromatographic separation of these isobaric analytes avoids overestimation of 25(OH)D$_3$ concentrations in samples with significant 3-epi-25(OH)D$_3$ fractions. Our method has a run-time of 5min which is acceptable for routine analysis and comparable to the fastest published methods by van den Ouweland et al. with 6.5min (20) and 5min (11). Moreover, since only a 1min-window of the LC-run was introduced into the mass spectrometer, a multiplexing of LC-channels may increase the throughput up to 5-fold.

The standard instruments for tandem mass spectrometric quantitation are triple-quadrupole instruments which offer unit mass resolution. In this study, we used a hybrid instrument quadrupole-Orbitrap which offers high mass resolution with up to 140,000 at m/z 200. Based on their high specificity these instruments have also been applied in full scan or targeted selected ion monitoring (SIM) (15). We tested these analysis modes together with MS/HR-MS (data not shown). However, despite high mass resolution, SIM displayed base line noise and unspecific signals comparable to the data by Bruce et al. (15). Therefore, we decided to used MS/HR-MS with a mass resolution setting of 35,000 that permits separation of quasi-isobaric product ions at m/z 383 generated from 25(OH)D$_3$ and D$_6$-25(OH)D$_2$ (Figure S-3). Without high mass resolution D$_6$-25(OH)D$_2$ may interfere with 25(OH)D$_3$ isomers in case of a co-elution (7). Consequently, analysis by low mass resolution instruments requires either an alternative (potentially more expensive) IS for 25(OH)D$_2$ like D$_3$-25(OH)D$_2$, other fragment ions or has to avoid co-elution of D$_6$-25(OH)D$_2$ and 25(OH)D$_3$ isomers. HR-
MS permits the use of $[25\text{(OH)}D+\text{H-H}_2\text{O}]^+$ fragment ions which are prominent and therefore sensitive but generally considered as unspecific. However, in our experiments the extracted ion chromatograms of MS/HR-MS spectra contain no baseline noise which impressively demonstrates the specificity of the method. Thus, LoQ has to be evaluated by functional testing but not by signal to noise measurement (Figure S-8). LoQ for all analytes is below 10nmol/l and sufficient for testing of the vitamin D status. Moreover, the presented LC-MS/HR-MS method exhibits LoQs for 25(OH)D comparable to that reported from triple quadrupole instruments in the lower nmolar range (7;13).

The fraction of epimer detected in patients of the University Hospital Regensburg fits very well to literature data (13;14). Previous studies observed a median of 3 to 6% of the 3-epi-25(OH)D$_3$-fraction in adult patients which is in good agreement to 4.3% found in our patients (Figure 2B).

In conclusion, the presented method shows for the first time the feasibility of LC-MS/HR-MS for small molecule quantification in laboratory routine testing. The high specificity of this technique may be also useful for other analytes as for example steroid hormone quantification.

**Acknowledgement**

We thank Simone Düchtel and Doreen Müller for expert technical assistance.
References


Figure legends

**Figure 1:** Chromatogram of 25-OH-vitamin D isomers

Extracted ion chromatograms (unsmoothed; quantifier ions as displayed in Table 1) are shown for A) a quality control sample (medium level, see Table 2) and for B) a typical patient sample (the following concentrations were calculated: 60.9nmol/l 25(OH)D$_3$, 7.8nmol/l 3-epi-25(OH)D$_3$ and 0.8nmol/l 25(OH)D$_2$, which is below LoQ).

**Figure 2:** Fraction of 3-epi-25(OH)D$_3$ in patient samples

The fraction of 3-epi-25(OH)D$_3$ of 25(OH)D is calculated for 364 patient samples sent for 25(OH)D analysis. A) Age-dependent fraction of 3-epi-25(OH)D$_3$ in patients below one year, from 1 to 5 years, older than 5 to 18 years and adult patients older 18 years. B) Fraction of adult patients with 3-epi-25(OH)D$_3$ fraction below LoQ, up to 5%, 5 to 10%, 10 to 15% and above 15% of total 25(OH)D.
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<th>Mass transitions qualifier [m/z]</th>
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<th>Calibration range [nmol/l]</th>
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**Table 1:** Mass transitions, internal standards (IS) used for quantitation, calibration range and limit of quantification (LoQ) of LC-MS/HR-MS.
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**Table 2: Trueness and imprecision**

Within-run and between-run trueness and imprecision were calculated from each 5 replicates.
Figure 1

(A) 25(OH)D₃, 3-epi-25(OH)D₃, D₂-25(OH)D₃, 25(OH)D₂, 3-epi-25(OH)D₂, D₂-25(OH)D₂

(B) 25(OH)D₃, 3-epi-25(OH)D₃, D₂-25(OH)D₃, 25(OH)D₂, D₂-25(OH)D₂
Figure 2