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High-throughput measurement of 25-hydroxyvitamin D by LC–MS/MS with separation of the C3-epimer interference for pediatric populations

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ABSTRACT

Background: Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is widely used for the measurement of 25-hydroxyvitamin D_3 ($25OHD_3$) and 25-hydroxyvitamin D_2 ($25OHD_2$) in blood. However, the presence of $25OHD_3$ C3-epimer (3-epi-$25OHD_3$) may cause interference and overestimation of the $25OHD_3$ level. We developed a rapid and simple assay for measurement of 25OHD that separates this interference and to investigate its impact on $25OHD_3$ measurement in adult and pediatric populations.
Methods: Sample preparation consisted of protein precipitation followed by solid-phase extraction with an LC run time of 4.8 min. Method comparison with another LC–MS/MS method of a major reference laboratory that does not separate the C3-epimer interference was performed using adult ($n = 52$) and pediatric ($n = 40$) samples.
Results: This method is free from significant ion suppression, carryover and interference. The assay can separate $25OHD_3$ from the 3-epi-$25OHD_3$, and can measure $25OHD_2$ and $25OHD_3$ from 4.2 to 310.7 ng/ml and 5.2 to 311.1 ng/ml, respectively. Method comparison with a LC–MS method that does not separate the interference revealed biases of −0.15 and 4.54 for $25OHD_3$ measurement in adult and pediatric samples, respectively.
Conclusion: A fast and simple LC–MS/MS method for quantification of $25OHD_3$ and $25OHD_2$ without 3-epi-$25OHD_3$ interference was developed. This assay is required for accurate quantitation of $25OHD_3$ in pediatric samples and suitable for routine use in a high volume clinical laboratory.

1. Introduction

Various forms of vitamin D and their metabolites play important roles in calcium regulation and homeostasis. Additionally, vitamin D deficiency has been implicated in other disease areas, such as insulin resistance, cardiovascular diseases, and chronic kidney diseases [1,2]. Vitamin D levels in the human body are maintained through sun exposure, dietary uptake, and nutrition supplements. Through sun exposure, 7-dehydrocholesterol in the skin can be converted into vitamin D_3, while dietary uptake and nutritional supplements contain both vitamin D_3 and vitamin D_2. Both forms of vitamin D go through hydroxylation by 25-hydroxylase in the liver, subsequently forming 25-hydroxyvitamin D_3 ($25OHD_3$) and 25-hydroxyvitamin D_2 ($25OHD_2$) [3]. Although the $25OHD_3$ and D_2 are the main circulating forms of vitamin D metabolites in blood, they are not the active forms. $25OHD_3$ and D_2 can be further hydroxylated by a second C-1α hydroxylase to form 1α, 25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ D_3) and D_2, which are the strongest agonists for the vitamin D receptor. Compared to 1,25-$(OH)_2$ D_2 and D_3, $25OHD_2$ and D_3 have a much longer half-life in blood and their levels are independent of calcium, phosphorus or PTH levels. Therefore, blood concentrations of $25OHD_2$ and D_3 are the best biomarkers for the evaluation of vitamin D sufficiency status in the human body. In addition to the known vitamin D metabolites above, recent studies have also revealed that there are significant levels of circulating 3-epi-$25OHD_3$ in serum/plasma of both infants and adults [4,5]. The epimers are generated through the C3-epimerization pathway and epimers exist for other vitamin D_3 intermediate metabolites [6]. The C3-epimers are shown to have a significantly lower level of regulatory effects on calcium homeostasis [7].

For clinical applications, the quantification of plasma/serum $25OHD_3$ and D_2 levels is most commonly performed by immunoassays and liquid chromatography–tandem mass spectrometry (LC–MS/MS). The antibody-based immunoassay suffers from poor antibody specificity, cross-reactivity with other metabolites, and insufficient extraction from vitamin D-binding protein [8,9]. The LC–MS/MS based quantification assay can distinguish different forms of vitamin D metabolites based on their different molecular mass and chromatographic properties and is regarded as the gold standard method for quantification of 25OHD [10]. However, because both 3-epi-$25OHD_3$ and the native form $25OHD_3$ have the same molecular weight and fragmentation pattern, the presence of 3-epi-$25OHD_3$ in blood would lead to an overestimation of $25OHD_3$ in LC–MS/MS methods that do not separate this

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isobaric interference [11]. Existing LC–MS/MS methods that were developed for C3-epimer separation require long chromatography separation time, extensive sample preparation, or high-resolution mass spectrometry [7,11–13], which are not compatible with high-volume clinical applications.

2. Materials and methods

2.1. Chemicals, reagents and solutions

LC–MS/MS grade and ACS grade solvents and reagents were used. Methanol and formic acid from Thermo Fisher Scientific, and ammonium acetate from Sigma-Aldrich were used for preparation of chromatographic mobile phases and Solid Phase Extraction (SPE) solutions. The Oasis HLB μElution 96-well plates from Waters were used for SPE in sample preparation.

Stable isotope standards 25-Hydroxyvitamin D_3 (6, 19,19-D_3, 97%) 50 μg/ml, 25-Hydroxyvitamin D_2 (6, 19, 19-D_3, 97%) 50 μg/ml, and unlabeled 3-epi-25-Hydroxyvitamin D_3 50 μg/ml were from Cambridge Isotope Laboratories. Analyte standards 25-Hydroxyvitamin D_3, 100 μg/ml and 25-Hydroxyvitamin D_2, 50 μg/ml were from Cerilliant. The calibrator materials of $25OHD_2$ and D_3 (4 levels, lyophilized serum) and the control materials (2 levels, lyophilized serum) for daily clinical quality control were both purchased from Recipe chemicals. Certified standard reference material SRM 972a (4 levels, serum-based) was from the National Institute of Standards and Technology.

The working internal standard mix (IS mix) contained 250 ng/ml d_3-$25OHD_3$ and 250 ng/ml d_3-$25OHD_2$ in 80/20 v/v methanol/isopropyl alcohol mixture. The analytical measurement range (AMR) samples were prepared by adding known amount of $25OHD_3$ and $25OHD_2$ to the vitamin D-free serum matrix (Golden Western Biologicals). The highest level AMR level 9 contains 300 ng/ml of both $25OHD_3$ and $25OHD_2$. The intermediate levels (level 1 to level 9) of AMR samples were prepared by serial dilution of AMR level 9 with vitamin D-free serum.

2.2. Sample preparation

During sample preparation, 20 μl of the IS mix was first added to 150 μl of heparinized plasma (with gel separator), standards, or quality control samples in microcentrifuge tubes. After vortex mixing, 150 μl of 0.2 mol/l zinc sulfate solution, and 600 μl of methanol were added consecutively to each tube. The mixture was then centrifuged for 2 min at 13,000 × *g*. After centrifugation, 600 μl of the supernatant from each tube was added to the pre-conditioned Waters Oasis HLB μElution 96-well plate. Pre-conditioning of the Waters Oasis HLB μElution 96-well plate involved washing each well with methanol (200 μl) then 60% methanol (200 μl). After sample loading, each well was then consecutively washed with 5% methanol (200 μl) and 60% methanol (200 μl) then eluted with methanol/IPA (80 μl) and water (50 μl). From each eluate, 20 μl was injected onto the LC–MS/MS system for analysis.

2.3. LC–MS/MS method

Analysis was performed by reversed-phase LC separation followed by electrospray ionization in positive mode (ESI+) and MS/MS detection using a triple quadrupole. The chromatographic separation was carried out with a Waters ACQUITY UPLC system coupled to a Waters Xevo TQD mass spectrometer. The data were collected and processed using the instrument software TargetLynx. Mobile phase A was 2 mmol/l ammonium acetate in water with 0.1% formic acid. Mobile phase B was 2 mmol/l ammonium acetate in methanol with 0.1% formic acid. Samples were injected on the ACQUITY UPLC HSS PFP column (1.8 μm particle size, 100 × 2.1 mm i.d.) from Waters at a flow rate of 500 μl/min, with an initial mobile phase composition of 32:68 (v/v) A:B for 0.8 min. Then, mobile phase composition was changed linearly to 28:72 (v/v) A:B over 2 min. Last, the column was washed with 2:98 (v/v) A:B for 0.7 min and re-equilibrated for 1.1 min with 32:68 (v/v) A:B before the next injection. The total run time between injections for one channel is 4.8 min, and the column temperature was kept at 50 °C. For the MS/MS detection, the desolvation gas flow was 1200 l/h, and source temperature was 350 °C. The capillary voltage is 1.0 kV, and the cone voltage is 26 V. For each analyte of interest, 2 MS/MS transitions were selected as quantifier and qualifier: $25OHD_3$, *m/z* 401.5 > 159.1 (401.5 > 365.4, qualifier); and $25OHD_2$, *m/z* 413.5 > 83.0 (413.5 > 355.4, qualifier). For the 2 IS, a single transition was monitored: d_3-$25OHD_3$, *m/z* 404.5 > 162.1; d_3-$25OHD_2$, *m/z* 416.5 > 358.4.

2.4. Method validation

2.4.1. Ion suppression evaluation

To evaluate absolute ion suppression, a solution mixture containing 100 ng/ml of d_3-$25OHD_3$ and d_3-$25OHD_2$ internal standards (IS) was infused through a tee connection to combine with the eluates from the LC. While the solution mixture was infused at a constant flow rate of 5 μl/min, 5 solvent blank and 5 patient plasma samples were consecutively injected onto the LC system. The signal intensity of each analyte from the MS/MS measurement was recorded throughout the entire LC separation time. Relative ion suppression was studied to test whether the IS accounted for ion suppression in the matrix for the analytes. It is evaluated by extracting and injecting a candidate matrix solution (vitamin D-free serum), 6 patient samples (3 males and 3 females), and 1:1 mixtures of patient samples with the candidate matrix solution. The criterion for a passing test is that the response ratio (analyte/IS) of each 1:1 mixture was within 20% of the theoretical response calculated from an average of the measured values of the patient and candidate matrix solution.

2.4.2. Interference study

To investigate the interference effects, highly lipemic (L index: 350), hemolyzed (H-index: 524), and icteric (I index: 26) plasma samples were selected. Two different analyte concentrations (low and high) were investigated by mixing each sample 1:1 with vitamin D-free matrix spiked with $25OHD_2$ at 13.8 ng/ml, and $25OHD_3$ at 17.9 ng/ml for low and $25OHD_2$ at 49.8 ng/ml, and $25OHD_3$ at 79.3 ng/ml for high, respectively. It was determined that there was no significant interference if the response ratio of each 1:1 mixture was within 20% of the theoretical response calculated from the average of interference containing sample and the spiked matrix.

2.4.3. Analytical measurement range

The AMR samples at different concentration levels (Blank, Level 1 to Level 9) were examined in triplicate to determine the analytical measurement range. After SPE, the AMR samples were injected onto LC–MS/MS in the order of increasing concentration. The analytical measurement range was determined based on accuracy within 100 ± 20%, a total CV within 20%, and a signal-to-noise ratio > 10.

2.4.4. Carryover evaluation

The AMR samples (Level 3 and Level 9) were used to evaluate the carryover effects. After SPE, the samples were analyzed in 3 independent experiments each consisted of running 2 extractions in the sequence of Level 3_1–Level 9–Level 3_2, where Level 3_2 is a re-injection of Level 3_1. A passing test meant that Level 3_1 is within 20% of Level 3_2, and that Level 3_2 is within 3 standard deviations of the Level 3_1 value. The standard deviation was determined using Level 3_1 values.

2.4.5. Imprecision

Samples containing high, mid, and low levels of $25OHD_2$ and $25OHD_3$ were used for imprecision evaluation. The low samples were vitamin D-free serum spiked with 10 ng/ml of $25OHD_2$ and $25OHD_3$. The high samples were pooled patient samples, spiked with 60 ng/ml of $25OHD_2$ and $25OHD_3$. The mid samples were obtained by mixing