Rapid Quantitation of 25-Hydroxyvitamin D2 and D3 in Human Serum Using Liquid Chromatography/drift Tube Ion Mobility-Mass Spectrometry

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Rapid quantitation of 25-hydroxyvitamin D2 and D3 in human serum using liquid chromatography/drift tube ion mobility-mass spectrometry

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Abstract

Ion mobility was integrated with liquid chromatography/high resolution mass spectrometry (LC/IM-HRMS) to quantify 25-hydroxyvitamin D (25OHD) in human serum. It has previously been shown that 25OHD adopts two gas-phase conformations which are resolved using ion mobility; in contrast, the inactive epimer, 3-epi-25-hydroxyvitamin D (epi25OHD) only adopts one. Interference from epi25OHD was eliminated by filtering the chromatogram to retain the drift time that corresponds to the unique gas-phase conformation of 25OHD. Although ion mobility separates the epimers, some chromatography is required to separate compounds which interfere with ionization or fall at the same nominal m/z . Standards were prepared in 4% albumin solutions and compared against commercial serum quality controls. Standards and quality controls were analyzed and validated using a two-minute LC/IM-MS method. 25-hydroxyvitamin D3 and D2 were quantified over the range between 2 and 500 ng/mL with bias and precision within 15%. When epi25OHD was spiked into quality control samples, no significant bias was introduced, and analysis of 30 patient samples shows good agreement between this LC/IM-MS and traditional LC/MS/MS methods. This work shows that ion mobility can be incorporated with liquid chromatography and mass spectrometry for rapid quantitation of 25OHD in human serum.

Introduction

Vitamin D, a class of secosteroids, is responsible for regulating calcium absorption, bone development, and serum calcium homeostasis¹. Evidence is growing which links vitamin D deficiency to a myriad of diseases, including osteomalacia, cardiovascular disease, diabetes, and cancer^{2,3}, while during pregnancy, vitamin D deficiency has been linked to low birth weight and preterm birth⁴⁻⁶. Vitamin D is obtained either by consumption from dietary sources or by supplementation (vitamin D2 from plant and D3 from animal sources) or biosynthesized from 7-hydrocholesterol upon exposure to UV radiation (vitamin D3). In the liver, vitamin D is converted to 25-hydroxyvitamin D (25OHD), the main circulating metabolite, and further metabolism in the kidneys produces 1,25-dihydroxyvitamin D, which is responsible for the genomic and rapid response functions⁷.

Although inactive, 25OHD circulates at the highest serum concentrations and is unaffected by homeostatic control; therefore, it is the most common target of analytical assays⁸. Immunoassay accounts for most testing due to its high throughput and ease of automation⁹; however, it suffers from poor accuracy, especially at low concentrations¹⁰. Unequal antibody affinity for 25OHD2 and 25OHD3 results in a negative bias, and cross reactions with other vitamin D metabolites such as 24,25-dihydroxyvitamin D result in a positive bias^{10,11}. Using liquid chromatography/mass spectrometry (LC/MS), these biases can be avoided, since LC/MS can differentiate the vitamin D metabolites, but these methods require extensive chromatography times to separate interference from the stereoisomer 3-epi-25-hydroxyvitamin D (epi25OHD)¹². The epimer differs from 25OHD by the chirality of the hydroxyl group on the A-ring, which converts from the β to

α position. No definitive biological role has been attributed to the epimer¹³, and, while circulating concentrations of epi25OHD are low in normal adult populations⁸, epimer concentrations can be elevated in maternal populations (up to 20% of total vitamin D⁴) and infants (up to 60% of total vitamin D¹⁴). Failure to separate interfering epimers risks over reporting total 25OHD concentrations by including contributions from the epimer, which can affect the clinical diagnosis when apparent concentrations are close to the cut-off for vitamin D deficiency^{14,15}, typically 15-20 ng/mL^{16,17}. Because of long chromatography times required to separate the epimer pair (typically 7-14 minutes¹²), analysis times can easily exceed ten minutes per sample, which is undesirable in a routine testing lab prioritizing high-throughput, leading many laboratories to forgo separating the epimer¹⁸.

Ion mobility spectrometry (IMS) can resolve ions based on differences in their gas-phase mobility, a property which describes the velocity of an ion travelling through a buffer gas under the influence of an electric field¹⁹. The mobility depends on an ion's mass, charge, and size or collision cross section (CCS)²⁰. Larger ions encounter more collisions with the buffer gas and are detected at a later drift time. Coupling IMS with mass spectrometry (IM-MS) measures m/z which can be combined with drift time to calculate the CCS based on the Mason-Schamp equation¹⁹. This measurement is useful for the separation of isomers which have the same m/z but different CCS. One such example of isomer separation is that of 25OHD. Recent work in our lab has shown that in the gas phase, sodium adducts of 25OHD2 and 25OHD3 can adopt either a compact (closed) or extended (open) conformation, whereas epi25OHD2 and epi25OHD3 can only adopt the compact conformer²¹. The extended conformer of

25OHD is detected as a drift peak that is completely resolved from the compact conformer and free from interference from epi25OHD, achieving separation of the epimers in 30 ms²¹. The millisecond timescale of IMS is faster than that of a liquid chromatography (LC) peak, which individually lasts several seconds. Across a single chromatographic peak, hundreds of mobility scans can be completed and summed to provide detailed ion mobility information, making IM-MS well-suited for coupling with LC during routine quantitation assays.

Often, IM-MS is coupled with liquid chromatography for quantitation to either reduce background noise^{22,23} or increase peak capacity²⁴. In this work, one of two possible conformers of the same ion is quantified under the assumption that the unique conformer is representative of the solution concentration. Work in our lab has shown that the ratio of the conformers depends on the instrument settings and ion flux within the mass spectrometer where the unique open conformer converts to the closed conformer, but potential bias from interconversion can be avoided with the use of an isotopically labeled internal standard, as shown in our previous work²⁵.

A two-minute LC/IM-MS method was developed and validated for the quantitation of 25OHD2 and 25OHD3 in human serum. Bias, precision, linear range and method efficiency measurements were evaluated for both analytes, and the method was shown to be effective at removing interference from epi25OHD. Patient samples, previously analyzed for 25OHD using a validated LC/MS/MS method, were analyzed by the new method and the results were shown to be in good agreement. This method represents a significant reduction in sample analysis time for vitamin D quantitation and illustrates the

utility of combining IMS with traditional LC/MS to improve the speed and accuracy of clinical quantitation.

Methods

Chemicals

25-hydroxyvitamin D3, 3-epi-25-hydroxyvitamin D3, 25-hydroxyvitamin D3-[2H6] (d6-25OHD3), 25-hydroxyvitamin D2, 3-epi-25-hydroxyvitamin D2, 25-hydroxyvitamin D2-[2H3] (d3-25OHD2) solutions (100 µg/mL in ethanol, Certimass solutions) were purchased from Isosciences (Ambler, PA). Methanol, water, isopropanol (LC/MS-grade), ethyl acetate, hexane (reagent-grade) and formic acid (99%) were purchased from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin (10% in phosphate buffered saline) and 0.1 M phosphate buffered saline (PBS) were purchased from MilliporeSigma (St. Louis, MO). Quality control references standards and normal human serum were purchased from UTAK labs (Valencia, CA). Three quality control levels were purchased (low, level 1, level 2) with reference values of 11.0, 26.3, 75 ng/mL (26.7, 63.7, 181.7 nmol/L) for 25OHD2 and 9.9, 27.1, 74 ng/mL (24.7, 67.6, 184.7 nmol/L) for 25OHD3. The NIST SRM 972a vitamin D metabolites set was purchased from NIST.

Sample Preparation

Calibrator working solutions containing both 25OHD3 and 25OHD2 were prepared by serial dilution to concentrations of 10000, 4000, 2000, 1000, 400, 200, 100, 40 ng/mL in methanol. Internal standard working solution containing d6-25OHD3 and d3-25OHD2 was prepared at 1000 ng/mL in methanol. Calibrators were prepared by spiking 4% bovine serum albumin in 0.1 M PBS (BSA/PBS) with working solution to final

concentrations of 250, 100, 50, 25, 10, 5, 2.5, 1 ng/mL (600, 240, 120, 60, 24, 12, 6, 2.4 nmol/L for 25OHD₂ and 625, 250, 125, 62.5, 25, 12.5, 6.25, 2.5 nmol/L for 25OHD₃). Quality control material was prepared according to the manufacturer's directions by adding 5 mL of LC/MS-grade water and allowing to sit for 15 minutes.

Samples were extracted by solid-phase extraction using the Cerex Trace-N 1 mL SPE tube (Tecan, Baldwin Park, CA) using a positive pressure SPE manifold (Tecan). In 1.5 mL polypropylene centrifuge tube, 100 μ L of sample was spiked with 5 μ L internal standard solution and vortexed. To each centrifuge tube, 800 μ L of 72:28 water:isopropanol was added, vortexed, and allowed to rest for 5 minutes. Samples were centrifuged at 10,000 rpm for 10 minutes. SPE cartridges were conditioned sequentially with 800 μ L methanol and 500 μ L water; 800 μ L of sample supernatant was added to the column; the column was washed with 300 μ L 72:28 water:isopropanol and dried with nitrogen for 8 minutes. Samples were eluted with 600 μ L of 80:20 hexane:ethyl acetate into autosampler vials, dried under nitrogen, and reconstituted in 50 μ L of 90% methanol in water.

Instrumentation

All experiments were performed on the Agilent 6560 ion mobility-quadrupole time-of-flight mass spectrometer (Santa Clara, CA) with dual Jetstream electrospray ionization source operated in positive ion mode. Ion source voltages were set to the following: capillary voltage = 3000 V, nozzle voltage = 500 V, fragmentor = 400 V and octopole RF = 750 V. The drying gas was 350 °C at 12 L/min, nebulizer gas pressure was 30 psig, and the sheath gas was 275 °C at 9 L/min. Ion mobility accumulation time was 25 ms, release time was 250 μ s, and ion mobility transient time was 32 ms. The

instrument was calibrated daily using the Agilent autotune option and mass accuracy and pressure calibration factor were measured daily using the Agilent tune mix and stepped-field calibration measurement^{26,27}. Liquid chromatography separations were performed using the Agilent Infinity II 1290 UHPLC with quaternary pump, column heater and sample chiller. Column heater was set to 40°C and sample cooler was set to 4°C during experiments.

Separations were performed using an Agilent Eclipse C18 column (50 x 2.1 mm, 1.7 micron). Mobile phase A consisted of LC/MS-grade water, and mobile phase B consisted of LC/MS-grade methanol. A 30-second gradient from 90% to 100% B was followed by a 36-second isocratic portion at 100% B and a 54-second re-equilibration at 90% B. At a flow rate of 700 µL/min, 25OHD3 and d6-25OHD3 were detected at 0.64 min, and 25OHD2 and d3-25OHD2 were detected at 0.68 min. Fragmentation of sodiated adducts was poor (0% fragmentation efficiency), so only the accurate mass of the mobility selected conformers was used for quantitation.

Data Processing

Data were acquired using Agilent MassHunter version B.08.00, and analytes were quantified using Skyline version 4.2.0²⁸. Peak areas were calculated for the sodiated adducts of 25OHD3 (m/z 423.3239), d6-25OHD3 (m/z 429.3600), 25OHD2 (m/z 435.3239) and d3-25OHD2 (m/z 438.3424) using a mass window of ± 0.01 Da and IMS resolving power of 50. Matrix effects and technical variation were corrected by dividing analyte peak area by their respective isotopically labelled internal standards.

Method Validation

Acceptance criteria for the method validation are based on recommendations from the FDA industry guidance for bioanalytical method validation²⁹. Bias and imprecision must be below 15% except at the lowest level where 20% is acceptable. Validation consists of bias measurements, imprecision (within-run and between-run), autosampler stability, linear quantitative range, limit of detection (LOD), limit of quantitation (LOQ), carryover, epimer interference, ionization efficiency, extraction recovery. Except for epimer interference, three concentration levels were evaluated.

Ionization efficiency, extraction efficiency and overall efficiency using the spike-order method³⁰ are evaluated at 75, 30 and 10 ng/mL (180, 72, 24 nmol/L for 25OHD2 and 188, 75, 25 nmol/L for 25OHD3). Pooled human serum is fortified with d3-25OHD2 and d6-25OHD3 and extracted in triplicate. Blank extracts and methanol samples are fortified to the equivalent concentrations of the previous spiked samples.

The limit of detection (LOD) is evaluated by extracting 10 BSA/PBS blanks and calculating the peak area across the expected analyte retention time window. The LOD is defined as the average blank response plus 3.3 standard deviations. The limit of quantitation (LOQ) is defined as the lowest calibrator whose peak area exceeded the average blank response plus 10 standard deviations. Linearity is assessed with an eight-level calibration curve extracted with five replicates per level. Carryover is measured by injecting a blank following each replicate of the highest calibrator. The ability to distinguish interference from epi25OHD2 and epi25OHD3 is measured by fortifying the low and high QC to 100 ng/mL of each epimer and quantifying against the calibration curve.

Bias and imprecision are evaluated using the UTAK low, level 1 and level 2 controls, $n = 5$ / level / day over four days. Bias and precision are calculated using the one-way ANOVA method from the Scientific Working Group for Forensic Toxicology protocol for method validation³¹. Autosampler stability is assessed by reinjecting the day 1 quality control samples after 4 days using freshly prepared and extracted calibrators. The NIST SRM 972a vitamin D metabolite Standard Reference Material was analyzed in triplicate to evaluate the trueness of the quantitation.

Method Comparison

Thirty deidentified patient samples were obtained from LabCorp (Burlington, NC). No specific patient criteria we used. Samples are analyzed and 25OHD3 values compared to the values obtained by the clinical lab, which used a 10-minute LC/MS/MS method that has been validated to separate the epimer and certified by the Centers for Disease Control's Vitamin D Standardization Certification Program. Correlation was assessed using a Bland-Altman plot to compare agreement between the two methods.

Results

Both sodiated adducts of 25OHD2 and 25OHD3 are identified by retention time, accurate mass, and drift time using the Skyline software. Figure 1 shows the 2-dimensional drift-time versus m/z plot of the unique open conformers for each analyte and their respective internal standards extracted from the total ion chromatogram of a high serum QC sample. The linear range of the analyte-to-internal standard ratio is validated between 2 and 500 ng/mL for 25OHD3 and 25OHD2 (Figure S1). A 1/x weighted linear least-squares regression is applied to three replicates at each concentration level with an $R^2 = 0.996$ (25OHD3) and $R^2 = 0.994$ (25OHD2). Limits of

detection are measured to be 367 counts (25OHD3) and 72 counts (25OHD2), the mean plus 3.3 standard deviations of the peak area from ten blank BSA/PBS extracts. The limit of quantitation is calculated as the calibrator level above the mean plus ten standard deviations of the peak area from ten blank BSA/PBS extracts, calculated as 2 ng/mL for both analytes, which exceeds the limit for vitamin D deficiency (15 ng/mL, 36 nmol/L)^{16,17} and for the CDC vitamin D standardization certification program (9 ng/mL, 22 nmol/L)³².

Table 1 contains a summary of the validation results for bias, precision, and method efficiency. Using three levels of reference materials (11.0, 26.3, 75 ng/mL for 25OHD2 and 9.9, 27.1, 74 ng/mL for 25OHD3) as quality control samples, bias and imprecision are measured over four days. Both within-run and between-run precision are below 15% for all concentration levels and each analyte. Bias, deviation from the target concentration, was under $\pm 15\%$ of target for both analytes. Ionization efficiency for 25OHD3 was measured between 77% and 115% and overall efficiency between 65% and 92%; for 25OHD2 ionization efficiency was measured between 77% and 121% and overall efficiency between 59% and 85%.

Potential interference of epi25OHD2 and epi25OHD3 is measured by fortifying the high and low quality controls with an equal concentration of epi25OHD2 and epi25OHD3. For each analyte, the measured concentrations are within $\pm 15\%$ of the target, and within-run coefficient of variance is below 15% (Table 1). The low bias and imprecision measured in the epimer-spiked samples show that ion mobility effectively removed the interference of epi25OHD by separating the shared closed conformer and unique open conformer of sodiated 25OHD without additional chromatography time.

Carryover is measured with a blank following the highest calibrator; signal was below the limit of detection for 25OHD2 and 25OHD3. Quality control samples are left in the autosampler at 4 °C for 4 days and reinjected with freshly prepared calibrators. Both analytes show bias below $\pm 15\%$ of target and precision under 15%, as summarized in Table 1. The average bias and precision for 25OHD3 in the four levels of SRM972a, analyzed in triplicate, are 1.5% and 5% respectively.

Discussion

Using ion mobility after ionization, interference from epi25OHD is removed without the need for chromatographic separation from 25OHD. Figure 2 shows the extracted ion chromatogram (EIC) of m/z 423.3234, corresponding to the sodiated adduct ($[M+Na]^+$) of 25OHD3 and epi25OHD3 for a calibrator fortified with 75 ng/mL 25OHD3 and the same standard fortified with 75 ng/mL of 25OHD3 and 100 ng/mL of epi25OHD3. Using a two-minute gradient, both compounds coelute at 0.6 minutes, resulting in a peak area greater in the calibrator containing epimer. By summing the mass spectrometer scans across the chromatographic peak, drift time information can be extracted for m/z 423.32 in Figure 3. The peak area at 25 ms, corresponding to the closed conformer, is greater in the epimer sample, consistent with a contribution from the epimer which only adopts the closed conformation. The calibrator without the epimer also contributes to the peak area of the closed conformer since 25OHD3 can adopt either conformation; therefore, the closed conformer is less useful for quantitation. While both isomers contribute to the early drift peak, only 25OHD3 adopts the later open conformation; thus, the peak area at 29 ms, corresponding to the open conformer, is the same for both samples absent slight variations from instrument or sample

preparation variation (Figure S2). The presence of the epimer does not interfere with the response of the open conformation drift peak, and the unique mobility separated peak can be selectively monitored for quantitation of 25OHD without interference from epi25OHD. It should be noted that, although this description focuses on 25OHD3, the same behavior is observed for 25OHD2, epi25OHD2 and their respective isotopically labeled internal standards.

Quantitation software comparison

The Agilent MassHunter Quantitation software is unable to directly process the extra ion mobility dimension to automatically filter the drift time region specific to the open conformer of 25-hydroxyvitamin D (25OHD). Preprocessing of the data files is required prior to importing into the MassHunter quantitation software. The open source Skyline platform²⁸ supports nested LC/IM-HRMS data files and does not require the preprocessing step, making it a potential alternative. Because each software package handles ion mobility data differently, it is useful, to compare the relative agreement between the two software platforms. Hoofnagle and coworkers showed equivalent results between vendor and Skyline software for the quantitation 25OHD without ion mobility using traditional LC/MS/MS³³. MacLean et al. used Skyline to analyze LC/IM-HRMS data files from BSA tryptic digests, finding that IMS lowered limits of detection by eliminating noise, although they did not validate a quantitative method³⁴. In these datasets, Skyline was able to automatically analyze each file, in contrast to vendor software which required manual analysis. In this work, Skyline is applied to small-molecule datasets to compare the accuracy and precision of a validated LC/IM-HRMS quantitation method.

For the vendor software, the Agilent Quantitative Analysis program version B.08.00 was used for quantitation after drift time information is extracted using Agilent IM browser software version B.08.00. In the IM Browser program, the drift-time region from 28.51 to 29.85 ms was selected and filtered to retain retention time, mass-to-charge and intensity information. Two possible preprocessing approaches could be used for the vendor software. The drift time spectrum (e.g. Figure 3) could be used directly by summing the scans across the chromatographic peak, converting the drift time to retention time, and importing in to the quantitation software, or the chromatogram could be rebuilt by filtering only the relevant drift time window (e.g. Figure S2). Of the two, the latter approach was chosen to preserve the traditional LC/MS workflow as much as possible and because drift time is more stable than retention time from sample to sample; less variation due to time shifts is expected when a fixed drift time window is used for filtering rather than retention time window. The relative standard deviation measured across a 46-sample experiments was 0.4% for drift time and 1.6% for retention time. The rebuilt chromatograms have lower noise than the drift-time files; by selecting a larger retention time window to capture both 25OHD2 and 25OHD3, a larger window is summed together than is necessary than if each analyte were selected separately. When using the chromatogram rebuilding approach, only the drift time region consistent with the open conformers is saved. Since the drift times for 25OHD2 and 25OHD3 are effectively the same, a smaller window is required than with the drift-time conversion. An “extracted drift time chromatogram” is created where only data from 28.51 – 29.85 ms is saved, creating an LC/MS datafile that contains only the retention time, m/z , and intensity information within that drift time

region, which is compatible with the respective quantitation software package. Since drift time, as opposed to CCS, is an experimental value, dependent on the electric field, instrument geometry, gas pressure, and temperature, the drift time range is selected manually to include the two analytes and their internal standards. By quantifying using the internal standard ratio, variation in the selection window is corrected.

The thirty patient sample data set is analyzed using both software platforms. The chromatographic peaks are manually integrated in the vendor software to ensure that the integration range is the same, so that the only observed differences are due to IMS-MS peak picking algorithm. Figure S3 shows a correlation plot of the two software platforms fit with an orthogonal, equal variance regression with slope of 1.006 and correlation of 0.9989, showing almost perfect agreement between the two data sets. This correlation suggests that the preprocessing step does not affect the data quality, and either software is suitable for LC/IM-HRMS quantitation.

In the above analysis, the vendor chromatographic peaks are manually integrated to match those of Skyline, but the need to manually integrate highlights an important difference in the two software; during automatic integration, the two programs use a different approach. In MassHunter, the analyte and internal standard are integrated separately so that the retention time range for each peak is different, according to the time it took for the compound to elute. At lower concentrations, the peak base width appears narrower than at higher concentrations because of the intensity threshold of the instrument. With Skyline, both the analyte and labeled internal standard are integrated over the same retention time range. When the concentration is low and the peaks appear narrow relative to the higher-concentration internal standard,

more noise is captured in the integration over this retention time region. Table S1 shows the results for accuracy and precision for both programs using the automatic peak integration methods. At the low QC level, an average 2% increase in the imprecision was observed using Skyline, representing the noise added to the quantitation. At the high QC level, an average 1.6% decrease in the imprecision is observed. Skyline uses resolving power and drift time to select the drift-time window it extracts, whereas MassHunter is based on a defined drift-time window. This fixed window likely leads to saturation effects where the edges of the peaks are truncated during manual filtering, resulting in lower precision.

This analysis is not to say which approach is better; however, to decide which platform to use for this specific method, the data sets can be compared to the reference LC/MS/MS method by analyzing the patient samples with each platform using the auto-integration method. Figure S4 shows a relative difference plot comparing MassHunter (blue circle) and Skyline (red box) with the reference method. One important assumption is required, that the reference data set is accurate. As the LabCorp method participates in the CDC vitamin D standardization certification based on the vitamin D standardization program³⁵, high confidence in the results is expected; still, some error may be assumed. A shift in the mean difference from 8% to 4% is seen between MassHunter (solid blue line) and Skyline (dotted red line); in addition, Skyline provides quantities more closely matched to the reference method. Multiple runs of the same patient samples by both the LC/IM-HRMS and LC/MS/MS would be required to account for run-to-run variability.

Relative informing power of LC/IM-MS

Although epimer separation occurs in 30 ms using IMS, formation of the sodiated adduct, $[M+Na]^+$ is still impacted by ion suppression from coeluting compounds since the IMS separation occurs post-ionization; therefore, liquid chromatography is still required to resolve suppressing compounds such as phospholipids and salts, and to resolve potential interferents at the same nominal m/z . In addition to epi25OHD interference, isobars have been identified as potentially interfering species³⁶. In contrast to traditional tandem LC/MS methods that feature ion fragment transitions for quantitation and qualification, this method only quantified the parent ion, since fragmentation of the $[M+Na]^+$ ion proceeds at 0% fragmentation efficiency. No quantitatively appropriate MS/MS transitions are detected, likely due to dissociation to form sodium ion and neutral 25OHD. Alternatively, atmospheric pressure chemical ionization (APCI) favors the formation of protonated ions, which generate specific fragment ions during collision-induced dissociation. Using APCI, 25OHD forms the $[M+H]^+$ ion; however, when analyzed by IMS, the $[M+H]^+$ ion is found to form only one conformer that is not resolved from the epimer. Therefore, IMS had little value for 25OHD quantitation using $[M+H]^+$ ions formed by APCI, since chromatographic resolution of the epimers would be required. Traditional LC-MS/MS methods that utilize a triple-quadrupole mass spectrometer, operate at unit mass resolution (resolving power = ion m/z), whereas the LC/IM-HRMS method utilizes a high-resolution TOF mass analyzer with a resolving power over 10,000. A single stage of mass analysis affords much higher resolution of isobaric ions in addition to the separation power of IMS. The lack of fragment ions with which to improve specificity may be troublesome to a method

developer, so it is useful to consider the differences in the informing power of each approach.

Efforts to quantify the informing power of tandem mass spectrometric methods include information theory as described by Fetterolf³⁷. Peak capacity (the total number of peaks that can be observed, as used in chromatography) has been used to approximate the potential information from an IM-HRMS measurement³⁸. For a tandem separation device, the peak capacity is the product of the resolving power of each stage and fraction of orthogonality, a measure of the difference in the properties measured. For example, LC, which separates based on affinity between a stationary and mobile phase, is orthogonal to MS, which separates based on m/z . On the other hand, CCS generally increases with m/z , reducing the fraction of orthogonality. A triple-quadrupole mass spectrometer with unit resolution in each stage, has a resolving power of 423 for sodiated 25OHD3 (m/z 423.3) for the first quadrupole and 159 for the final quadrupole (based on the most common fragment ion used, m/z 159.1). Assuming the fragment ion m/z measured with the final quadrupole is 100% orthogonal to the fragment ion measured with the first, by virtue of the collision-induced dissociation, the total peak capacity is $423 \times 159 \times 1 = 67,000$. The IM-QTOF has a resolving power of 13,000 (m/z 423.3239) in the TOF and 52 in the IMS drift tube. Dwivedi estimates a fraction of orthogonality of 14% for drift-tube IM-HRMS³⁹; therefore, the total peak capacity is 96,000, slightly higher than that of the triple-quadrupole method. This is not to say that IM-MS provides better specificity than tandem MS, but that the informing power is on the same order for the two methods; i.e. nothing is lost by using IMS and high-resolution

mass spectrometry. Ultimately, the test of specificity is low bias and imprecision during method validation for which the LC/IM-HRMS method is similar to that of LC/MS/MS.

Method validation figures of merit

Adequate separation of 25OHD epimers is achieved using a two-minute chromatography run coupled to the IM-MS to validate quantitation of 25OHD3 and D2 in human serum. Both bias and precision are within a strict 15% criterion, and the linear range (2-500 ng/mL) exceeds the cut-off points for toxicity and deficiency, which vary among labs between 15 and 20 ng/mL of total 25OHD for vitamin D deficiency and 150 ng/mL total 25OHD for toxicity^{16,17}. To assess the ability of the method to accurately measure patient samples, 30 samples previously analyzed by a clinical laboratory using a traditional LC/MS/MS assay are reanalyzed for 25OHD3 using this LC/IM-HRMS approach. A Bland-Altman plot for 25OHD3 (Figure 4) shows limits of agreement of $\pm 13\%$ and average bias of 4%, biased toward the LC/IM-HRMS method. Only seven samples have 25OHD2 levels above the limit of quantitation, and of these samples, the average bias is 1.4 ng/mL $\pm 10\%$. A low mean bias and limits of agreement below 15% suggest that the LC/IM-HRMS is similar to the traditional LC/MS/MS method but requires only 2 minutes of chromatography time instead of 10 minutes.

Simultaneous quantitation of 25OHD and epi25OHD is not straightforward using this LC/IM-HRMS approach. Because 25OHD forms the unique open conformer, interference from the epimer is removed; however, 25OHD also forms the closed conformer which is shared with epi25OHD. As seen in Figure 3, under ideal conditions, approximately 10% of 25OHD3 remains in the closed conformer. Additionally, it has been shown that the open conformer readily converts to the closed conformer under

high-energy conditions²⁵. Strategies that reduce the abundance of the closed conformer of 25OHD such as alternative cation adducts⁴⁰, could be implemented to improve simultaneous quantitation of the epimers, but introduction of alternative cations into the LC/IM-HRMS method is not straightforward and the utility of rapid quantitation of the epimers is low. Because the role of epi25OHD is poorly understood, there is little demand for its measurement in clinical testing¹², and although interest in epi25OHD quantitation may come from cases involving infants^{12,14} or in research settings, the number of samples would likely be too low to necessitate a high-throughput method. In these cases, a traditional quantitation method with a longer LC separation to resolve the epimer would be appropriate.

Impact of IMS on internal standard response

Previous work in our lab proposed that sample-to-sample variations in total ion intensity could drive conversion of the open conformer into the closed conformer leading to suppression of the analyte, but could be compensated by similar interconversion of the isotopically-labeled internal standard²⁵; therefore, it is important to consider the degree to which the ion mobility separation affects variation in the internal standard response. In this study, variation in the internal standard response is observed across a 46-sample run including 30 patient samples, 6 commercial QC samples, 2 blanks, and 8 calibrators. Figure 5 shows the normalized internal standard intensity, total ion counts (TIC or ion density), and percentage of open conformers (relative to the total open and closed conformers) for d3-25OHD2 during the validation experiment; the internal standard peak area response varies between 0.56 and 0.84. As the ion density (TIC) increases, the probability increases for ion heating to convert the open conformer to the

closed conformer, suppressing the signal of the analyte and internal standard. The total ion counts, measured at the retention time for d3-25OHD2 varies between 0.56 and 0.68; however, only a $\pm 3\%$ change is observed from the median conformer ratio of 91%; therefore, ion mobility does not contribute significantly to ion suppression, and most variation can be attributed to non-IMS instrument performance and sample-preparation.

Resolution and transmission of the unique open conformer is critical for quantitation. In this application, we validate the method using drift-tube ion mobility, which is one of many ion mobility implementations; therefore, it is appropriate to consider its performance on other mobility platforms⁴¹. We've shown that the unique open conformer remains stable and resolved on several low-field ion mobility instruments using neat standards, suggesting the flexibility of ion mobility for quantitation; however, as with any method transfer to a new instrument, validation of method performance would be required on each platform.

Conclusion

This work develops and validates a rapid LC/IM-HRMS method to quantify 25OHD2 and 25OHD3 in human serum. It demonstrates how ion mobility can be used to reduce liquid chromatography time by separating 25OHD from epi25OHD on a millisecond timescale. It also demonstrates how to integrate the multidimensional data into existing quantitation software platforms. Bias and imprecision are low, and the linear range exceeds cut-off points for toxicity and deficiency, while good agreement is shown between patient samples analyzed by traditional LC/MS/MS and LC/IM-HRMS. The utility of ion mobility is clear in this application by reducing chromatography time from 10 minutes to 2 minutes per sample, enabling high-throughput analysis of 25OHD.

The analysis of 25OHD is a high-demand clinical assay that benefits from the use of ion mobility to improve sample throughput while maintaining the specificity and accuracy inherent in mass spectrometry. As more high-demand and novel implementations of ion mobility in clinical quantitation are developed, ion mobility will become a routine tool within the clinical laboratory.

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Table 1. Results for bias, precision and method efficiency for 25OHD2 and 25OHD3

parameter	25OHD2			25OHD3		
concentration level	low	mid	high	low	mid	high
bias (n = 20, % target)	-4	-8	-2	-6	-10	-4
within run precision (n = 20, %CV)	9	5	5	7	6	4
among run precision (n = 20, %CV)	9	8	7	7	8	6
interference (bias, n = 3, % target)	0	N/A	4	-5	N/A	0
interference (precision, n = 3, %CV)	8	N/A	3	7	N/A	4
autosampler (bias, n = 3, % target)	-5	-3	2	0	-7	4
autosampler (precision, n = 5, %CV)	8	4	5	8	2	5
ionization efficiency (n = 3, % target)	121	91	77	115	90	77
recovery (n = 3, % target)	70	77	76	80	80	85
overall efficiency (n = 3, % target)	85	70	59	92	72	65
NIST SRM 972a (25OHD3)	level 1	level 2		level 3	level 4	
bias (n = 3, % target)	7	-1		0	0	
precision (n = 3, %CV)	9	3		4	3

Figure 1. Total ion chromatogram for high-level serum quality control sample. Combining scans across the chromatographic peak builds a 2-dimensional plot of the drift time and m/z for the target analytes. The unique open conformers at 29 ms are resolved from the noise. The feature around drift time 29 ms can be filtered to create a chromatogram containing only the unique conformer.

Figure 2. Extracted ion chromatogram (m/z 423.3239) of 75 ng/mL quality control with epi25OHD3 (blue) and without (red). Both 25OHD3 and epi25OHD3 coelute, contributing to the higher peak intensity of the sample containing the epimer.

Figure 3. Extracted ion drift time spectrum (m/z 423.3239 and RT 0.75-0.85 min) of the chromatographic peak containing 25OHD3 and epi25OHD3. The drift peak at 25 ms, representing the closed conformer, contains signal from both compounds; however, the peak at 29 ms is unique to 25OHD3 and is the same intensity for both samples.

Figure 4. Bland-Altman means plot for 30 patient samples analyzed by LC/IM-MS and LC/MS/MS. An average difference of 4% biased toward the LC/IM-MS method and limits of agreement of $\pm 13\%$ was measured between the two methods. The LC/MS/MS method separates 3-epi-25-hydroxyvitamin D and requires 10 minutes of chromatography time versus 2 minutes for the LC/IM-MS method.

Figure 5. Box and whisker plot of the internal standard peak area response, total ion counts (TIC) and percent of d3-25OHD2 in the open conformation relative to total open and closed conformers. While ion heating during IMS analysis at high ion density can drive conversion to the closed conformer, little variation is seen across this sample run.

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Rapid quantitation of 25-hydroxyvitamin D2 and D3 in human serum using liquid chromatography/drift tube ion mobility-mass spectrometry

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Abstract

Ion mobility was integrated with liquid chromatography/high resolution mass spectrometry (LC/IM-HRMS) to quantify 25-hydroxyvitamin D (25OHD) in human serum. It has previously been shown that 25OHD adopts two gas-phase conformations which are resolved using ion mobility; in contrast, the inactive epimer, 3-epi-25-hydroxyvitamin D (epi25OHD) only adopts one. Interference from epi25OHD was eliminated by filtering the chromatogram to retain the drift time that corresponds to the unique gas-phase conformation of 25OHD. Although ion mobility separates the epimers, some chromatography is required to separate compounds which interfere with ionization or fall at the same nominal m/z . Standards were prepared in 4% albumin solutions and compared against commercial serum quality controls. Standards and quality controls were analyzed and validated using a two-minute LC/IM-MS method. 25-hydroxyvitamin D3 and D2 were quantified over the range between 2 and 500 ng/mL with bias and precision within 15%. When epi25OHD was spiked into quality control samples, no significant bias was introduced, and analysis of 30 patient samples shows good agreement between this LC/IM-MS and traditional LC/MS/MS methods. This work shows that ion mobility can be incorporated with liquid chromatography and mass spectrometry for rapid quantitation of 25OHD in human serum.

Introduction

Vitamin D, a class of secosteroids, is responsible for regulating calcium absorption, bone development, and serum calcium homeostasis¹. Evidence is growing which links vitamin D deficiency to a myriad of diseases, including osteomalacia, cardiovascular disease, diabetes, and cancer^{2,3}, while during pregnancy, vitamin D deficiency has been linked to low birth weight and preterm birth⁴⁻⁶. Vitamin D is obtained either by consumption from dietary sources or by supplementation (vitamin D2 from plant and D3 from animal sources) or biosynthesized from 7-hydrocholesterol upon exposure to UV radiation (vitamin D3). In the liver, vitamin D is converted to 25-hydroxyvitamin D (25OHD), the main circulating metabolite, and further metabolism in the kidneys produces 1,25-dihydroxyvitamin D, which is responsible for the genomic and rapid response functions⁷.

Although inactive, 25OHD circulates at the highest serum concentrations and is unaffected by homeostatic control; therefore, it is the most common target of analytical assays⁸. Immunoassay accounts for most testing due to its high throughput and ease of automation⁹; however, it suffers from poor accuracy, especially at low concentrations¹⁰. Unequal antibody affinity for 25OHD2 and 25OHD3 results in a negative bias, and cross reactions with other vitamin D metabolites such as 24,25-dihydroxyvitamin D result in a positive bias^{10,11}. Using liquid chromatography/mass spectrometry (LC/MS), these biases can be avoided, since LC/MS can differentiate the vitamin D metabolites, but these methods require extensive chromatography times to separate interference from the stereoisomer 3-epi-25-hydroxyvitamin D (epi25OHD)¹². The epimer differs from 25OHD by the chirality of the hydroxyl group on the A-ring,

which converts from the β to α position. No definitive biological role has been attributed to the epimer¹³, and, while circulating concentrations of epi25OHD are low in normal adult populations⁸, epimer concentrations can be elevated in maternal populations (up to 20% of total vitamin D⁴) and infants (up to 60% of total vitamin D¹⁴). Failure to separate interfering epimers risks over reporting total 25OHD concentrations by including contributions from the epimer, which can affect the clinical diagnosis when apparent concentrations are close to the cut-off for vitamin D deficiency^{14,15}, typically 15-20 ng/mL^{16,17}. Because of long chromatography times required to separate the epimer pair (typically 7-14 minutes¹²), analysis times can easily exceed ten minutes per sample, which is undesirable in a routine testing lab prioritizing high-throughput, leading many laboratories to forgo separating the epimer¹⁸.

Ion mobility spectrometry (IMS) can resolve ions based on differences in their gas-phase mobility, a property which describes the velocity of an ion travelling through a buffer gas under the influence of an electric field¹⁹. The mobility depends on an ion's mass, charge, and size or collision cross section (CCS)²⁰. Larger ions encounter more collisions with the buffer gas and are detected at a later drift time. Coupling IMS with mass spectrometry (IM-MS) measures m/z which can be combined with drift time to calculate the CCS based on the Mason-Schamp equation¹⁹. This measurement is useful for the separation of isomers which have the same m/z but different CCS. One such example of isomer separation is that of 25OHD. Recent work in our lab has shown that in the gas phase, sodium adducts of 25OHD2 and 25OHD3 can adopt either a compact (closed) or extended (open) conformation, whereas epi25OHD2 and epi25OHD3 can only adopt the compact conformer²¹. The extended conformer of

25OHD is detected as a drift peak that is completely resolved from the compact conformer and free from interference from epi25OHD, achieving separation of the epimers in 30 ms²¹. The millisecond timescale of IMS is faster than that of a liquid chromatography (LC) peak, which individually lasts several seconds. Across a single chromatographic peak, hundreds of mobility scans can be completed and summed to provide detailed ion mobility information, making IM-MS well-suited for coupling with LC during routine quantitation assays.

Often, IM-MS is coupled with liquid chromatography for quantitation to either reduce background noise^{22,23} or increase peak capacity²⁴. In this work, one of two possible conformers of the same ion is quantified under the assumption that the unique conformer is representative of the solution concentration. Work in our lab has shown that the ratio of the conformers depends on the instrument settings and ion flux within the mass spectrometer where the unique open conformer converts to the closed conformer, but potential bias from interconversion can be avoided with the use of an isotopically labeled internal standard, as shown in our previous work²⁵.

A two-minute LC/IM-MS method was developed and validated for the quantitation of 25OHD2 and 25OHD3 in human serum. Bias, precision, linear range and method efficiency measurements were evaluated for both analytes, and the method was shown to be effective at removing interference from epi25OHD. Patient samples, previously analyzed for 25OHD using a validated LC/MS/MS method, were analyzed by the new method and the results were shown to be in good agreement. This method represents a significant reduction in sample analysis time for vitamin D quantitation and illustrates the

utility of combining IMS with traditional LC/MS to improve the speed and accuracy of clinical quantitation.

Methods

Chemicals

25-hydroxyvitamin D3, 3-epi-25-hydroxyvitamin D3, 25-hydroxyvitamin D3-[2H6] (d6-25OHD3), 25-hydroxyvitamin D2, 3-epi-25-hydroxyvitamin D2, 25-hydroxyvitamin D2-[2H3] (d3-25OHD2) solutions (100 µg/mL in ethanol, Certimass solutions) were purchased from Isosciences (Ambler, PA). Methanol, water, isopropanol (LC/MS-grade), ethyl acetate, hexane (reagent-grade) and formic acid (99%) were purchased from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin (10% in phosphate buffered saline) and 0.1 M phosphate buffered saline (PBS) were purchased from MilliporeSigma (St. Louis, MO). Quality control references standards and normal human serum were purchased from UTAK labs (Valencia, CA). Three quality control levels were purchased (low, level 1, level 2) with reference values of 11.0, 26.3, 75 ng/mL (26.7, 63.7, 181.7 nmol/L) for 25OHD2 and 9.9, 27.1, 74 ng/mL (24.7, 67.6, 184.7 nmol/L) for 25OHD3. The NIST SRM 972a vitamin D metabolites set was purchased from NIST.

Sample Preparation

Calibrator working solutions containing both 25OHD3 and 25OHD2 were prepared by serial dilution to concentrations of 10000, 4000, 2000, 1000, 400, 200, 100, 40 ng/mL in methanol. Internal standard working solution containing d6-25OHD3 and d3-25OHD2 was prepared at 1000 ng/mL in methanol. Calibrators were prepared by spiking 4% bovine serum albumin in 0.1 M PBS (BSA/PBS) with working solution to final

concentrations of 250, 100, 50, 25, 10, 5, 2.5, 1 ng/mL (600, 240, 120, 60, 24, 12, 6, 2.4 nmol/L for 25OHD₂ and 625, 250, 125, 62.5, 25, 12.5, 6.25, 2.5 nmol/L for 25OHD₃). Quality control material was prepared according to the manufacturer's directions by adding 5 mL of LC/MS-grade water and allowing to sit for 15 minutes.

Samples were extracted by solid-phase extraction using the Cerex Trace-N 1 mL SPE tube (Tecan, Baldwin Park, CA) using a positive pressure SPE manifold (Tecan). In 1.5 mL polypropylene centrifuge tube, 100 μ L of sample was spiked with 5 μ L internal standard solution and vortexed. To each centrifuge tube, 800 μ L of 72:28 water:isopropanol was added, vortexed, and allowed to rest for 5 minutes. Samples were centrifuged at 10,000 rpm for 10 minutes. SPE cartridges were conditioned sequentially with 800 μ L methanol and 500 μ L water; 800 μ L of sample supernatant was added to the column; the column was washed with 300 μ L 72:28 water:isopropanol and dried with nitrogen for 8 minutes. Samples were eluted with 600 μ L of 80:20 hexane:ethyl acetate into autosampler vials, dried under nitrogen, and reconstituted in 50 μ L of 90% methanol in water.

Instrumentation

All experiments were performed on the Agilent 6560 ion mobility-quadrupole time-of-flight mass spectrometer (Santa Clara, CA) with dual Jetstream electrospray ionization source operated in positive ion mode. Ion source voltages were set to the following: capillary voltage = 3000 V, nozzle voltage = 500 V, fragmentor = 400 V and octopole RF = 750 V. The drying gas was 350 °C at 12 L/min, nebulizer gas pressure was 30 psig, and the sheath gas was 275 °C at 9 L/min. Ion mobility accumulation time was 25 ms, release time was 250 μ s, and ion mobility transient time was 32 ms. The

instrument was calibrated daily using the Agilent autotune option and mass accuracy and pressure calibration factor were measured daily using the Agilent tune mix and stepped-field calibration measurement^{26,27}. Liquid chromatography separations were performed using the Agilent Infinity II 1290 UHPLC with quaternary pump, column heater and sample chiller. Column heater was set to 40°C and sample cooler was set to 4°C during experiments.

Separations were performed using an Agilent Eclipse C18 column (50 x 2.1 mm, 1.7 micron). Mobile phase A consisted of LC/MS-grade water, and mobile phase B consisted of LC/MS-grade methanol. A 30-second gradient from 90% to 100% B was followed by a 36-second isocratic portion at 100% B and a 54-second re-equilibration at 90% B. At a flow rate of 700 μ L/min, 25OHD3 and d6-25OHD3 were detected at 0.64 min, and 25OHD2 and d3-25OHD2 were detected at 0.68 min. Fragmentation of sodiated adducts was poor (0% fragmentation efficiency), so only the accurate mass of the mobility selected conformers was used for quantitation.

Data Processing

Data were acquired using Agilent MassHunter version B.08.00, and analytes were quantified using Skyline version 4.2.0²⁸. Peak areas were calculated for the sodiated adducts of 25OHD3 (m/z 423.3239), d6-25OHD3 (m/z 429.3600), 25OHD2 (m/z 435.3239) and d3-25OHD2 (m/z 438.3424) using a mass window of ± 0.01 Da and IMS resolving power of 50. Matrix effects and technical variation were corrected by dividing analyte peak area by their respective isotopically labelled internal standards.

Method Validation

Acceptance criteria for the method validation are based on recommendations from the FDA industry guidance for bioanalytical method validation²⁹. Bias and imprecision must be below 15% except at the lowest level where 20% is acceptable. Validation consists of bias measurements, imprecision (within-run and between-run), autosampler stability, linear quantitative range, limit of detection (LOD), limit of quantitation (LOQ), carryover, epimer interference, ionization efficiency, extraction recovery. Except for epimer interference, three concentration levels were evaluated.

Ionization efficiency, extraction efficiency and overall efficiency using the spike-order method³⁰ are evaluated at 75, 30 and 10 ng/mL (180, 72, 24 nmol/L for 25OHD2 and 188, 75, 25 nmol/L for 25OHD3). Pooled human serum is fortified with d3-25OHD2 and d6-25OHD3 and extracted in triplicate. Blank extracts and methanol samples are fortified to the equivalent concentrations of the previous spiked samples.

The limit of detection (LOD) is evaluated by extracting 10 BSA/PBS blanks and calculating the peak area across the expected analyte retention time window. The LOD is defined as the average blank response plus 3.3 standard deviations. The limit of quantitation (LOQ) is defined as the lowest calibrator whose peak area exceeded the average blank response plus 10 standard deviations. Linearity is assessed with an eight-level calibration curve extracted with five replicates per level. Carryover is measured by injecting a blank following each replicate of the highest calibrator. The ability to distinguish interference from epi25OHD2 and epi25OHD3 is measured by fortifying the low and high QC to 100 ng/mL of each epimer and quantifying against the calibration curve.

Bias and imprecision are evaluated using the UTAK low, level 1 and level 2 controls, $n = 5$ / level / day over four days. Bias and precision are calculated using the one-way ANOVA method from the Scientific Working Group for Forensic Toxicology protocol for method validation ³¹. Autosampler stability is assessed by reinjecting the day 1 quality control samples after 4 days using freshly prepared and extracted calibrators. The NIST SRM 972a vitamin D metabolite Standard Reference Material was analyzed in triplicate to evaluate the trueness of the quantitation.

Method Comparison

Thirty deidentified patient samples were obtained from LabCorp (Burlington, NC). No specific patient criteria we used. Samples are analyzed and 25OHD3 values compared to the values obtained by the clinical lab, which used a 10-minute LC/MS/MS method that has been validated to separate the epimer and certified by the Centers for Disease Control's Vitamin D Standardization Certification Program. Correlation was assessed using a Bland-Altman plot to compare agreement between the two methods.

Results

Both sodiated adducts of 25OHD2 and 25OHD3 are identified by retention time, accurate mass, and drift time using the Skyline software. Figure 1 shows the 2-dimensional drift-time versus m/z plot of the unique open conformers for each analyte and their respective internal standards extracted from the total ion chromatogram of a high serum QC sample. The linear range of the analyte-to-internal standard ratio is validated between 2 and 500 ng/mL for 25OHD3 and 25OHD2 (Figure S1). A $1/x$ weighted linear least-squares regression is applied to three replicates at each concentration level with an $R^2 = 0.996$ (25OHD3) and $R^2 = 0.994$ (25OHD2). Limits of

detection are measured to be 367 counts (25OHD3) and 72 counts (25OHD2), the mean plus 3.3 standard deviations of the peak area from ten blank BSA/PBS extracts. The limit of quantitation is calculated as the calibrator level above the mean plus ten standard deviations of the peak area from ten blank BSA/PBS extracts, calculated as 2 ng/mL for both analytes, which exceeds the limit for vitamin D deficiency (15 ng/mL, 36 nmol/L)^{16,17} and for the CDC vitamin D standardization certification program (9 ng/mL, 22 nmol/L)³².

Table 1 contains a summary of the validation results for bias, precision, and method efficiency. Using three levels of reference materials (11.0, 26.3, 75 ng/mL for 25OHD2 and 9.9, 27.1, 74 ng/mL for 25OHD3) as quality control samples, bias and imprecision are measured over four days. Both within-run and between-run precision are below 15% for all concentration levels and each analyte. Bias, deviation from the target concentration, was under $\pm 15\%$ of target for both analytes. Ionization efficiency for 25OHD3 was measured between 77% and 115% and overall efficiency between 65% and 92%; for 25OHD2 ionization efficiency was measured between 77% and 121% and overall efficiency between 59% and 85%.

Potential interference of epi25OHD2 and epi25OHD3 is measured by fortifying the high and low quality controls with an equal concentration of epi25OHD2 and epi25OHD3. For each analyte, the measured concentrations are within $\pm 15\%$ of the target, and within-run coefficient of variance is below 15% (Table 1). The low bias and imprecision measured in the epimer-spiked samples show that ion mobility effectively removed the interference of epi25OHD by separating the shared closed conformer and unique open conformer of sodiated 25OHD without additional chromatography time.

Carryover is measured with a blank following the highest calibrator; signal was below the limit of detection for 25OHD2 and 25OHD3. Quality control samples are left in the autosampler at 4 °C for 4 days and reinjected with freshly prepared calibrators. Both analytes show bias below $\pm 15\%$ of target and precision under 15%, as summarized in Table 1. The average bias and precision for 25OHD3 in the four levels of SRM972a, analyzed in triplicate, are 1.5% and 5% respectively.

Discussion

Using ion mobility after ionization, interference from epi25OHD is removed without the need for chromatographic separation from 25OHD. Figure 2 shows the extracted ion chromatogram (EIC) of m/z 423.3234, corresponding to the sodiated adduct ($[M+Na]^+$) of 25OHD3 and epi25OHD3 for a calibrator fortified with 75 ng/mL 25OHD3 and the same standard fortified with 75 ng/mL of 25OHD3 and 100 ng/mL of epi25OHD3. Using a two-minute gradient, both compounds coelute at 0.6 minutes, resulting in a peak area greater in the calibrator containing epimer. By summing the mass spectrometer scans across the chromatographic peak, drift time information can be extracted for m/z 423.32 in Figure 3. The peak area at 25 ms, corresponding to the closed conformer, is greater in the epimer sample, consistent with a contribution from the epimer which only adopts the closed conformation. The calibrator without the epimer also contributes to the peak area of the closed conformer since 25OHD3 can adopt either conformation; therefore, the closed conformer is less useful for quantitation. While both isomers contribute to the early drift peak, only 25OHD3 adopts the later open conformation; thus, the peak area at 29 ms, corresponding to the open conformer, is the same for both samples absent slight variations from instrument or sample

preparation variation (Figure S2). The presence of the epimer does not interfere with the response of the open conformation drift peak, and the unique mobility separated peak can be selectively monitored for quantitation of 25OHD without interference from epi25OHD. It should be noted that, although this description focuses on 25OHD3, the same behavior is observed for 25OHD2, epi25OHD2 and their respective isotopically labeled internal standards.

Quantitation software comparison

The Agilent MassHunter Quantitation software is unable to directly process the extra ion mobility dimension to automatically filter the drift time region specific to the open conformer of 25-hydroxyvitamin D (25OHD). Preprocessing of the data files is required prior to importing into the MassHunter quantitation software. The open source Skyline platform²⁸ supports nested LC/IM-HRMS data files and does not require the preprocessing step, making it a potential alternative. Because each software package handles ion mobility data differently, it is useful, to compare the relative agreement between the two software platforms. Hoofnagle and coworkers showed equivalent results between vendor and Skyline software for the quantitation 25OHD without ion mobility using traditional LC/MS/MS³³. MacLean et al. used Skyline to analyze LC/IM-HRMS data files from BSA tryptic digests, finding that IMS lowered limits of detection by eliminating noise, although they did not validate a quantitative method³⁴. In these datasets, Skyline was able to automatically analyze each file, in contrast to vendor software which required manual analysis. In this work, Skyline is applied to small-molecule datasets to compare the accuracy and precision of a validated LC/IM-HRMS quantitation method.

For the vendor software, the Agilent Quantitative Analysis program version B.08.00 was used for quantitation after drift time information is extracted using Agilent IM browser software version B.08.00. In the IM Browser program, the drift-time region from 28.51 to 29.85 ms was selected and filtered to retain retention time, mass-to-charge and intensity information. Two possible preprocessing approaches could be used for the vendor software. The drift time spectrum (e.g. Figure 3) could be used directly by summing the scans across the chromatographic peak, converting the drift time to retention time, and importing in to the quantitation software, or the chromatogram could be rebuilt by filtering only the relevant drift time window (e.g. Figure S2). Of the two, the latter approach was chosen to preserve the traditional LC/MS workflow as much as possible and because drift time is more stable than retention time from sample to sample; less variation due to time shifts is expected when a fixed drift time window is used for filtering rather than retention time window. The relative standard deviation measured across a 46-sample experiments was 0.4% for drift time and 1.6% for retention time. The rebuilt chromatograms have lower noise than the drift-time files; by selecting a larger retention time window to capture both 25OHD2 and 25OHD3, a larger window is summed together than is necessary than if each analyte were selected separately. When using the chromatogram rebuilding approach, only the drift time region consistent with the open conformers is saved. Since the drift times for 25OHD2 and 25OHD3 are effectively the same, a smaller window is required than with the drift-time conversion. An “extracted drift time chromatogram” is created where only data from 28.51 – 29.85 ms is saved, creating an LC/MS datafile that contains only the retention time, m/z , and intensity information within that drift time

region, which is compatible with the respective quantitation software package. Since drift time, as opposed to CCS, is an experimental value, dependent on the electric field, instrument geometry, gas pressure, and temperature, the drift time range is selected manually to include the two analytes and their internal standards. By quantifying using the internal standard ratio, variation in the selection window is corrected.

The thirty patient sample data set is analyzed using both software platforms. The chromatographic peaks are manually integrated in the vendor software to ensure that the integration range is the same, so that the only observed differences are due to IMS-MS peak picking algorithm. Figure S3 shows a correlation plot of the two software platforms fit with an orthogonal, equal variance regression with slope of 1.006 and correlation of 0.9989, showing almost perfect agreement between the two data sets. This correlation suggests that the preprocessing step does not affect the data quality, and either software is suitable for LC/IM-HRMS quantitation.

In the above analysis, the vendor chromatographic peaks are manually integrated to match those of Skyline, but the need to manually integrate highlights an important difference in the two software; during automatic integration, the two programs use a different approach. In MassHunter, the analyte and internal standard are integrated separately so that the retention time range for each peak is different, according to the time it took for the compound to elute. At lower concentrations, the peak base width appears narrower than at higher concentrations because of the intensity threshold of the instrument. With Skyline, both the analyte and labeled internal standard are integrated over the same retention time range. When the concentration is low and the peaks appear narrow relative to the higher-concentration internal standard,

more noise is captured in the integration over this retention time region. Table S1 shows the results for accuracy and precision for both programs using the automatic peak integration methods. At the low QC level, an average 2% increase in the imprecision was observed using Skyline, representing the noise added to the quantitation. At the high QC level, an average 1.6% decrease in the imprecision is observed. Skyline uses resolving power and drift time to select the drift-time window it extracts, whereas MassHunter is based on a defined drift-time window. This fixed window likely leads to saturation effects where the edges of the peaks are truncated during manual filtering, resulting in lower precision.

This analysis is not to say which approach is better; however, to decide which platform to use for this specific method, the data sets can be compared to the reference LC/MS/MS method by analyzing the patient samples with each platform using the auto-integration method. Figure S4 shows a relative difference plot comparing MassHunter (blue circle) and Skyline (red box) with the reference method. One important assumption is required, that the reference data set is accurate. As the LabCorp method participates in the CDC vitamin D standardization certification based on the vitamin D standardization program³⁵, high confidence in the results is expected; still, some error may be assumed. A shift in the mean difference from 8% to 4% is seen between MassHunter (solid blue line) and Skyline (dotted red line); in addition, Skyline provides quantities more closely matched to the reference method. Multiple runs of the same patient samples by both the LC/IM-HRMS and LC/MS/MS would be required to account for run-to-run variability.

Relative informing power of LC/IM-MS

Although epimer separation occurs in 30 ms using IMS, formation of the sodiated adduct, $[M+Na]^+$ is still impacted by ion suppression from coeluting compounds since the IMS separation occurs post-ionization; therefore, liquid chromatography is still required to resolve suppressing compounds such as phospholipids and salts, and to resolve potential interferents at the same nominal m/z . In addition to epi25OHD interference, isobars have been identified as potentially interfering species³⁶. In contrast to traditional tandem LC/MS methods that feature ion fragment transitions for quantitation and qualification, this method only quantified the parent ion, since fragmentation of the $[M+Na]^+$ ion proceeds at 0% fragmentation efficiency. No quantitatively appropriate MS/MS transitions are detected, likely due to dissociation to form sodium ion and neutral 25OHD. Alternatively, atmospheric pressure chemical ionization (APCI) favors the formation of protonated ions, which generate specific fragment ions during collision-induced dissociation. Using APCI, 25OHD forms the $[M+H]^+$ ion; however, when analyzed by IMS, the $[M+H]^+$ ion is found to form only one conformer that is not resolved from the epimer. Therefore, IMS had little value for 25OHD quantitation using $[M+H]^+$ ions formed by APCI, since chromatographic resolution of the epimers would be required. Traditional LC-MS/MS methods that utilize a triple-quadrupole mass spectrometer, operate at unit mass resolution (resolving power = ion m/z), whereas the LC/IM-HRMS method utilizes a high-resolution TOF mass analyzer with a resolving power over 10,000. A single stage of mass analysis affords much higher resolution of isobaric ions in addition to the separation power of IMS. The lack of fragment ions with which to improve specificity may be troublesome to a method

developer, so it is useful to consider the differences in the informing power of each approach.

Efforts to quantify the informing power of tandem mass spectrometric methods include information theory as described by Fetterolf³⁷. Peak capacity (the total number of peaks that can be observed, as used in chromatography) has been used to approximate the potential information from an IM-HRMS measurement³⁸. For a tandem separation device, the peak capacity is the product of the resolving power of each stage and fraction of orthogonality, a measure of the difference in the properties measured. For example, LC, which separates based on affinity between a stationary and mobile phase, is orthogonal to MS, which separates based on m/z . On the other hand, CCS generally increases with m/z , reducing the fraction of orthogonality. A triple-quadrupole mass spectrometer with unit resolution in each stage, has a resolving power of 423 for sodiated 25OHD3 (m/z 423.3) for the first quadrupole and 159 for the final quadrupole (based on the most common fragment ion used, m/z 159.1). Assuming the fragment ion m/z measured with the final quadrupole is 100% orthogonal to the fragment ion measured with the first, by virtue of the collision-induced dissociation, the total peak capacity is $423 \times 159 \times 1 = 67,000$. The IM-QTOF has a resolving power of 13,000 (m/z 423.3239) in the TOF and 52 in the IMS drift tube. Dwivedi estimates a fraction of orthogonality of 14% for drift-tube IM-HRMS³⁹; therefore, the total peak capacity is 96,000, slightly higher than that of the triple-quadrupole method. This is not to say that IM-MS provides better specificity than tandem MS, but that the informing power is on the same order for the two methods; i.e. nothing is lost by using IMS and high-resolution

mass spectrometry. Ultimately, the test of specificity is low bias and imprecision during method validation for which the LC/IM-HRMS method is similar to that of LC/MS/MS.

Method validation figures of merit

Adequate separation of 25OHD epimers is achieved using a two-minute chromatography run coupled to the IM-MS to validate quantitation of 25OHD3 and D2 in human serum. Both bias and precision are within a strict 15% criterion, and the linear range (2-500 ng/mL) exceeds the cut-off points for toxicity and deficiency, which vary among labs between 15 and 20 ng/mL of total 25OHD for vitamin D deficiency and 150 ng/mL total 25OHD for toxicity^{16,17}. To assess the ability of the method to accurately measure patient samples, 30 samples previously analyzed by a clinical laboratory using a traditional LC/MS/MS assay are reanalyzed for 25OHD3 using this LC/IM-HRMS approach. A Bland-Altman plot for 25OHD3 (Figure 4) shows limits of agreement of $\pm 13\%$ and average bias of 4%, biased toward the LC/IM-HRMS method. Only seven samples have 25OHD2 levels above the limit of quantitation, and of these samples, the average bias is 1.4 ng/mL $\pm 10\%$. A low mean bias and limits of agreement below 15% suggest that the LC/IM-HRMS is similar to the traditional LC/MS/MS method but requires only 2 minutes of chromatography time instead of 10 minutes.

Simultaneous quantitation of 25OHD and epi25OHD is not straightforward using this LC/IM-HRMS approach. Because 25OHD forms the unique open conformer, interference from the epimer is removed; however, 25OHD also forms the closed conformer which is shared with epi25OHD. As seen in Figure 3, under ideal conditions, approximately 10% of 25OHD3 remains in the closed conformer. Additionally, it has been shown that the open conformer readily converts to the closed conformer under

high-energy conditions²⁵. Strategies that reduce the abundance of the closed conformer of 25OHD such as alternative cation adducts⁴⁰, could be implemented to improve simultaneous quantitation of the epimers, but introduction of alternative cations into the LC/IM-HRMS method is not straightforward and the utility of rapid quantitation of the epimers is low. Because the role of epi25OHD is poorly understood, there is little demand for its measurement in clinical testing¹², and although interest in epi25OHD quantitation may come from cases involving infants^{12,14} or in research settings, the number of samples would likely be too low to necessitate a high-throughput method. In these cases, a traditional quantitation method with a longer LC separation to resolve the epimer would be appropriate.

Impact of IMS on internal standard response

Previous work in our lab proposed that sample-to-sample variations in total ion intensity could drive conversion of the open conformer into the closed conformer leading to suppression of the analyte, but could be compensated by similar interconversion of the isotopically-labeled internal standard²⁵; therefore, it is important to consider the degree to which the ion mobility separation affects variation in the internal standard response. In this study, variation in the internal standard response is observed across a 46-sample run including 30 patient samples, 6 commercial QC samples, 2 blanks, and 8 calibrators. Figure 5 shows the normalized internal standard intensity, total ion counts (TIC or ion density), and percentage of open conformers (relative to the total open and closed conformers) for d3-25OHD2 during the validation experiment; the internal standard peak area response varies between 0.56 and 0.84. As the ion density (TIC) increases, the probability increases for ion heating to convert the open conformer to the

closed conformer, suppressing the signal of the analyte and internal standard. The total ion counts, measured at the retention time for d3-25OHD2 varies between 0.56 and 0.68; however, only a $\pm 3\%$ change is observed from the median conformer ratio of 91%; therefore, ion mobility does not contribute significantly to ion suppression, and most variation can be attributed to non-IMS instrument performance and sample-preparation.

Resolution and transmission of the unique open conformer is critical for quantitation. In this application, we validate the method using drift-tube ion mobility, which is one of many ion mobility implementations; therefore, it is appropriate to consider its performance on other mobility platforms⁴¹. We've shown that the unique open conformer remains stable and resolved on several low-field ion mobility instruments using neat standards, suggesting the flexibility of ion mobility for quantitation; however, as with any method transfer to a new instrument, validation of method performance would be required on each platform.

Conclusion

This work develops and validates a rapid LC/IM-HRMS method to quantify 25OHD2 and 25OHD3 in human serum. It demonstrates how ion mobility can be used to reduce liquid chromatography time by separating 25OHD from epi25OHD on a millisecond timescale. It also demonstrates how to integrate the multidimensional data into existing quantitation software platforms. Bias and imprecision are low, and the linear range exceeds cut-off points for toxicity and deficiency, while good agreement is shown between patient samples analyzed by traditional LC/MS/MS and LC/IM-HRMS. The utility of ion mobility is clear in this application by reducing chromatography time from 10 minutes to 2 minutes per sample, enabling high-throughput analysis of 25OHD.

The analysis of 25OHD is a high-demand clinical assay that benefits from the use of ion mobility to improve sample throughput while maintaining the specificity and accuracy inherent in mass spectrometry. As more high-demand and novel implementations of ion mobility in clinical quantitation are developed, ion mobility will become a routine tool within the clinical laboratory.

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Table 1. Results for bias, precision and method efficiency for 25OHD2 and 25OHD3

parameter	25OHD2			25OHD3		
	low	mid	high	low	mid	high
concentration level						
bias (n = 20, % target)	-4	-8	-2	-6	-10	-4
within run precision (n = 20, %CV)	9	5	5	7	6	4
among run precision (n = 20, %CV)	9	8	7	7	8	6
interference (bias, n = 3, % target)	0	N/A	4	-5	N/A	0
interference (precision, n = 3, %CV)	8	N/A	3	7	N/A	4
autosampler (bias, n = 3, % target)	-5	-3	2	0	-7	4
autosampler (precision, n = 5, %CV)	8	4	5	8	2	5
ionization efficiency (n = 3, % target)	121	91	77	115	90	77
recovery (n = 3, % target)	70	77	76	80	80	85
overall efficiency (n = 3, % target)	85	70	59	92	72	65
NIST SRM 972a (25OHD3)	level 1	level 2		level 3	level 4	
bias (n = 3, % target)	7	-1		0	0	
precision (n = 3, %CV)	9	3		4	3

Figure 1. Total ion chromatogram for high-level serum quality control sample. Combining scans across the chromatographic peak builds a 2-dimensional plot of the drift time and m/z for the target analytes. The unique open conformers at 29 ms are resolved from the noise. The feature around drift time 29 ms can be filtered to create a chromatogram containing only the unique conformer.

Figure 2. Extracted ion chromatogram (m/z 423.3239) of 75 ng/mL quality control with epi25OHD3 (blue) and without (red). Both 25OHD3 and epi25OHD3 coelute, contributing to the higher peak intensity of the sample containing the epimer.

Figure 3. Extracted ion drift time spectrum (m/z 423.3239 and RT 0.75-0.85 min) of the chromatographic peak containing 25OHD3 and epi25OHD3. The drift peak at 25 ms, representing the closed conformer, contains signal from both compounds; however, the peak at 29 ms is unique to 25OHD3 and is the same intensity for both samples.

Figure 4. Bland-Altman means plot for 30 patient samples analyzed by LC/IM-MS and LC/MS/MS. An average difference of 4% biased toward the LC/IM-MS method and limits of agreement of $\pm 13\%$ was measured between the two methods. The LC/MS/MS method separates 3-epi-25-hydroxyvitamin D and requires 10 minutes of chromatography time versus 2 minutes for the LC/IM-MS method.

Figure 5. Box and whisker plot of the internal standard peak area response, total ion counts (TIC) and percent of d3-25OHD2 in the open conformation relative to total open and closed conformers. While ion heating during IMS analysis at high ion density can drive conversion to the closed conformer, little variation is seen across this sample run.

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Rapid quantitation of 25-hydroxyvitamin D2 and D3 in human serum using liquid chromatography/drift tube ion mobility-mass spectrometry

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Contents:

S-2. calibration curves for 25-hydroxyvitamin D2 and D3

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Figure S1. Eight-point calibration curves ($n=5/\text{level}$) showing linearity between 2 and 500 ng/mL for 25OHD2 and 25OHD3

Figure S2. Extracted drift time and ion chromatogram for 75 ng/mL quality control with and without 50 ng/mL epi25OHD3. Drift time filtering removed interference from the epimer.

Table S1. Comparison of results from the four-day bias and precision study using MassHunter (left of slash) and Skyline (right of slash). Results where Mass hunter performed better are in italics and results where Skyline perform better are in bold. Overall bias is similar for the two software, but precision is better at low concentrations using MassHunter and better at high concentrations using Skyline. Values that improved using Skyline are bolded; values that worsen with Skyline are italicized

parameter	Mass Hunter / Skyline		
	low	mid	high
concentration level			
bias (n = 20, % target)	-2 / -6	-10 / -10	-4 / -4
within run precision (n = 20, %CV)	<i>5 / 7</i>	6 / 6	8 / 4
among run precision (n = 20, %CV)	<i>6 / 7</i>	8 / 8	8 / 6
interference (bias, n = 3, % target)	-7 / -5	N/A	6 / 0
interference (precision, n = 3, %CV)	<i>2 / 7</i>	N/A	<i>2 / 4</i>
autosampler (bias, n = 3, % target)	4 / 0	-5 / -7	6 / 4
autosampler (precision, n = 5, %CV)	<i>6 / 8</i>	<i>3 / 2</i>	<i>5 / 5</i>

Figure S3. Correlation plot of patient samples between MassHunter and Skyline with orthogonal, equal variance regression. Good correlation is seen between the two methods.

Figure S4. Difference plots for the patient sample validation run compared to the LC/MS/MS reference laboratory results using both software packages. The mean difference using skyline (red dotted line) is lower than using MassHunter (solid blue line)

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