Development and comparison of three liquid chromatography–atmospheric pressure chemical ionization/mass spectrometry methods for determining vitamin D metabolites in human serum

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ABSTRACT

Liquid chromatographic methods with atmospheric pressure chemical ionization mass spectrometry were developed for the determination of the vitamin D metabolites 25-hydroxyvitamin D_2 (25(OH)D_2), 25-hydroxyvitamin D_3 (25(OH)D_3), and 3-epi-25-hydroxyvitamin-D_3 (3-epi-25(OH)D_3) in the four Levels of SRM 972, Vitamin D in Human Serum. One method utilized a C18 column, which separates 25(OH)D_2 and 25(OH)D_3, and one method utilized a CN column that also resolves the diastereomers 25(OH)D_3 and 3-epi-25(OH)D_3. Both methods utilized stable isotope labeled internal standards for quantitation of 25(OH)D_2 and 25(OH)D_3. These methods were subsequently used to evaluate SRM 909c Human Serum, and 25(OH)D_3 was the only vitamin D metabolite detected in this material. However, SRM 909c samples contained matrix peaks that interfered with the determination of the [$^2H_6$]-25(OH)D_3 peak area. The chromatographic conditions for the C18 column were modified to remove this interference, but conditions that separated the matrix peaks from [$^2H_6$]-25(OH)D_3 on the CN column could not be identified. The alternate internal standard [$^2H_3$]-25(OH)D_3 did not suffer from matrix interferences and was used for quantitation of 25(OH)D_3 in SRM 909c. During the evaluation of SRM 909c samples, a third method was developed using a pentafluorophenylpropyl column that also separates the diastereomers 25(OH)D_3 and 3-epi-25(OH)D_3. The 25(OH)D_3 was measured in SRM 909c using all three methods, and the results were compared.

Published by Elsevier B.V.

1. Introduction

Vitamin D is a prohormone that helps the body regulate calcium and phosphate metabolism and is important for bone health. Vitamin D exists primarily in two forms, as vitamin D_3, which is native to animals and can be formed in the skin by reaction of UV light with 7-dehydrocholesterol, and as vitamin D_2, which is formed in some plant species. Dietary supplements are available that contain either form of vitamin D and are being increasingly used, especially for individuals who have been diagnosed with vitamin D deficiency. Vitamin D_2 and vitamin D_3 are hydroxylated by the liver to form the metabolites 25-hydroxyvitamin D_2 (25(OH)D_2) and 25-hydroxyvitamin D_3 (25(OH)D_3), respectively. The sum of these two species is referred to as 25-hydroxyvitamin D_{Total} (25(OH)D), and the 25(OH)D concentration in human serum is used clinically to assess vitamin D status.

There are several different analytical techniques that can be used to measure 25(OH)D. Radioimmunoassay, enzyme immunoassay,

and chemiluminescent immunoassay platform techniques are commonly used, but these assays do not differentiate between the 25(OH)D_2 and 25(OH)D_3 forms and may not provide equal responses for these two metabolites (see review of immunoassay methods in [1]). Therefore, many clinical labs are using liquid chromatography (LC) with tandem mass spectrometric detection (MS/MS), which can provide quantitation of 25(OH)D_2 and 25(OH)D_3 through the proper selection of column and mobile phase conditions and/or by monitoring different mass transitions for each of the two metabolites. To date, there are several published methods for determining the vitamin D metabolites using LC–MS/MS, most of which use stable isotopically labeled standards such as [$^2H_3$]-25(OH)D_3 or [$^2H_6$]-25(OH)D_3 for quantitation of 25(OH)D_3 but do not use analogous labeled standards for quantitation of 25(OH)D_2. Exceptions are the recently published LC–MS/MS methods by Tai et al. [2] and Hoofnagle et al. [3], which used labeled standards for both metabolites. Specific details of many published immunoassay and LC–MS/MS methods may be found in recent review articles [1,4–6], but several LC–MS/MS methods have subsequently been reported [7–14].

Liquid chromatography with single quadrupole mass spectrometric detection (LC–MS) has been overlooked as a useful

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technique for determining the vitamin D metabolites in human serum. Of the three reported literature methods [15–17], two measured the metabolites directly [15,17], while the other used derivatization with an ion trap MS [16]. LC–MS has significant advantages over LC–MS/MS methods including ease of use and affordability. While potential disadvantages exist such as increased susceptibility to matrix interferences and decreased sensitivity, these obstacles can often be overcome by optimization of the analytical separation conditions and sample clean-up procedures.

In addition to the traditional vitamin D metabolites 25(OH)D$_2$ and 25(OH)D$_3$, there has been increasing interest in the epimer form of 25(OH)D$_3$, 3-epi-25-hydroxyvitamin D$_3$ (3-epi-25(OH)D$_3$). Kamao et al. first isolated and identified 3-epi-25(OH)D$_3$ as a major metabolite of 25(OH)D$_3$ [18]. To determine if 3-epi-25(OH)D$_3$ is present as a metabolite in human serum, this diastereomer must be chromatographically resolved from 25(OH)D$_3$ because these compounds produce the same ions and cannot be distinguished solely with MS or MS/MS detection. Singh et al. used LC–MS/MS with a chiral column that separated the diastereomers and identified 3-epi-25(OH)D$_3$ as a major component in the human serum of infants [19]. Most LC–MS/MS methods utilize C18 columns, which do not resolve these compounds and create a potential bias if significant 3-epi-25(OH)D$_3$ is present in the sample. For the few reported methods that use columns and mobile phase conditions that resolve these diastereomers, the 3-epi-25(OH)D$_3$ is frequently detected in serum collected from adults [2,9,20–24]. In a recent study that evaluated the prevalence of 3-epi-25(OH)D$_3$ in serum samples collected from 501 patients aged 1–94 years, the percentage of 3-epi-25(OH)D$_3$ (relative to the 25(OH)D$_3$) ranged from 0% to 61% [24].

To address the lack of LC–MS methods for determining 25(OH)D, two methods using atmospheric pressure chemical ionization (APCI) and quantitation with stable isotope labeled standards for both 25(OH)D$_2$ and 25(OH)D$_3$ were developed. One method uses a C18 column, which separates 25(OH)D$_2$ and 25(OH)D$_3$, and one method uses a CN column that also resolves the diastereomers 25(OH)D$_3$ and 3-epi-25(OH)D$_3$ [20]. Both methods were used for the certification measurements of Standard Reference Material (SRM) 972 Vitamin D in Human Serum, which was developed by the National Institute of Standards and Technology (NIST) to support accurate measurements of vitamin D metabolites in the clinical community. SRM 972 consists of four Levels with different concentrations of 25(OH)D$_3$, 25(OH)D$_2$, and 3-epi-25(OH)D$_3$; details about the preparation and certification measurements of SRM 972 are reported elsewhere [22]. NIST is also providing certified values for the vitamin D metabolites in SRM 909c Human Serum, which has been characterized for other important clinical analytes such as cholesterol, creatinine, and glucose. When SRM 909c was subsequently evaluated using the C18 and CN methods, several matrix interferences were observed, and the methods required modification. This manuscript details the development of the C18 and CN LC–MS methods used for the certification measurements of SRM 972, the modification of these methods for SRM 909c samples, and the development of a third method using a pentafluorophenyl-propyl (PFPP) analytical column that also separates 25(OH)D$_3$ and 3-epi-25(OH)D$_3$. The 25(OH)D$_3$ in both SRM 909c and SRM 972 Level 1 (measurement control) was successfully quantitated using all three methods. The use of multiple chromatographic separation methods was important for identifying and quantitating the vitamin D metabolites in complex matrix samples like SRM 909c.

2. Materials and methods[1]

2.1. Materials

The reference standard for 25-hydroxyvitamin D$_3$ was obtained from United States Pharmacopeia (USP, Rockville, MD, USA). Standards for 25-hydroxyvitamin D$_2$, 25-hydroxyvitamin D$_3$–[^{2}H$_3$] ([^{2}H$_3$]-25(OH)D$_3$), 25-hydroxyvitamin D$_2$–[^{2}H$_3$] ([^{2}H$_3$]-25(OH)D$_2$), and 3-epi-25-hydroxyvitamin D$_3$ were obtained from IsoSciences (King of Prussia, PA, USA). Isotopically labeled 25-hydroxyvitamin D$_3$–[^{2}H$_6$] ([^{2}H$_6$]-25(OH)D$_3$) was obtained from Medical Isotopes, Inc. (Pelham, NH, USA). Samples of SRM 972 and SRM 909c were obtained from the Measurement Services Division (NIST). Optima LC–MS grade methanol and water were used for the mobile phase (Fisher Scientific, Pittsburgh, PA, USA). All other solvents were HPLC-grade.

2.2. Instrumentation

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC with an SL series MS detector and an APCI source was used to determine vitamin D metabolites. The following three optimized chromatographic methods were used to determine 25(OH)D in SRM 909c.

The first method used a Luna C18(2) column with dimensions 250 mm × 4.6 mm ID and 5 μm particles and a 3.0 mm × 4 mm C18 Security Guard column (Phenomenex, Torrance, CA, USA). The column temperature was maintained at 15 °C, and the mobile phase composition used to separate 25(OH)D$_2$ and 25(OH)D$_3$ was 7% water, 93% methanol (volume fractions) at 1.0 mL/min for 11 min. When serum samples were injected, a step gradient was incorporated into the method at the end of the run to elute retained matrix constituents. The step gradient was from 93% methanol to 100% methanol from 11 min to 14 min, followed by a hold at 100% methanol until 26 min. The MS was turned off at 12 min into the run to avoid contamination of the MS with the long-retained components. After returning to the initial conditions, a post-run time of 7 min was used to allow equilibration prior to injection of the next sample. Injection volumes of 15 μL were used.

The second method utilized a Zorbax SB-CN column that was 250 mm × 4.6 mm ID with 5 μm particles and a corresponding guard column that was 12.5 mm × 4.6 mm ID (Agilent). The column temperature was maintained at 45 °C, and an isocratic mobile phase of 32% water, 68% methanol at 1.0 mL/min for 27 min was used to provide separation of 25(OH)D$_3$ and 3-epi-25(OH)D$_3$. As with the C18 column, a step gradient was incorporated into the method at the end of the run to elute retained matrix constituents. The step gradient was from 69% methanol to 100% methanol from 27 min to 29 min followed by a hold at 100% methanol from 29 to 39 min. The MS was turned off at 27 min into the run to avoid contamination of the MS with the long-retained components. After returning to the initial conditions, a post-run time of 7 min was used to allow equilibration prior to injection of the next sample. An injection volume of 15 μL was used.

An Ascentis Express F5 (PFPP) column from Supelco (Bellefonte, PA, USA) with dimensions of 150 mm × 4.6 mm ID and 2.7 μm particles and a corresponding guard column that was 5 mm × 4.6 mm ID was used for the third method. The column was maintained at a temperature of 15 °C, and an isocratic mobile phase of 26% water, 74% methanol at 0.8 mL/min for 25 min was used to provide separation of 25(OH)D$_3$ and 3-epi-25(OH)D$_3$. A step gradient was employed when serum extracts were injected as a flush to remove long-retained compounds from the column. The step gradient was from 74% methanol to 100% methanol from 25 min to 27 min, followed by a hold at 100% methanol from 27 min to 37 min. The MS was turned off at 28 min into the run to avoid contamination of

the MS with the long-retained components. After returning to the initial conditions, a post-run time of 10 min was used to allow equilibration prior to injection of the next sample. An injection volume of 7 μL was used.

All three methods utilized APCI-MS detection with positive polarity at the $[M-H_2O+H]^+$ ion for all species (M is the molecular mass). The ions monitored included m/z 383 for 25(OH)D$_3$ and for 3-epi-25(OH)D$_3$; m/z 386 for [^{2}H$_3$]-25(OH)D$_3$; m/z 389 for [^{2}H$_6$]-25(OH)D$_3$; m/z 395 for 25(OH)D$_2$; and m/z 398 for [^{2}H$_3$]-25(OH)D$_2$. The following optimized APCI-MS parameters were used for detection: drying gas flow, 5.0 L/min; nebulizer pressure, 0.34 kPa (50 psi); drying gas temperature, 350 °C; vaporizer temperature, 350 °C; capillary voltage, +3600 V; corona current, 4 μA; fragmentor, 150 V; and gain, 2.

2.3. Quantitation of 25(OH)D$_3$ in SRM 909c

A bulk solution of [^{2}H$_3$]-25(OH)D$_3$ was prepared in a solution of 20% isopropanol in methanol (volume fraction) to be ≈10 μg/g. From this, a diluted solution containing ≈394 ng/g [^{2}H$_3$]-25(OH)D$_3$ in 20% isopropanol in methanol was prepared by mass for use as the internal standard (IS) solution.

Given the very low (ng/mL) levels of 25(OH)D$_3$ in serum, dilutions were required to achieve the target concentrations for the working calibrants. First, three stock solutions of 25(OH)D$_3$ were gravimetrically prepared to be 4.3–5.2 μg/g by weighing 1–2 mg of reference standard in 500 mL ethanol (exact masses and concentrations known). From these stock solutions, three intermediate calibration solutions were gravimetrically prepared by weighing an exact amount of a stock solution and diluting with ethanol to 100 mL or 250 mL ethanol to yield solutions with 25(OH)D$_3$ concentrations ranging from 227 ng/g to 249 ng/g. These intermediate calibration solutions were then used to prepare three working calibrants by weighing fixed volumes of one of the intermediate calibration solutions and the IS solution. The concentration of 25(OH)D$_3$ in the working calibrants was designed to match the concentration in the injected SRM 909c samples (about 60 ng/g). All solutions were prepared in reduced lighting and were stored in amber bottles at −20 °C when not in use.

2.4. Preparation of SRM 909c samples

Ten samples were prepared for the determination of 25(OH)D$_3$ in SRM 909c that included single samples from each of two vials and duplicate samples from each of four vials. Duplicate samples were also prepared from a single vial of SRM 972 Level 1, which was analyzed as a measurement control. Prior to the preparation of samples, vials of SRM 909c and SRM 972 Level 1 were removed from the freezer, thawed at room temperature, and swirled gently to ensure thorough mixing. The sample preparation technique was based on a method for extracting the vitamin D metabolites from serum by Turpeinen et al. [25]. To prepare samples, approximately 110 mg (150 μL) of internal standard was accurately weighed in an 8 mL glass tube with a screw cap. An additional 250 μL of 20% isopropanol in methanol (volume fractions) not containing IS was added to have a sufficient volume for precipitation of serum proteins. Approximately 450 mg of an SRM sample was accurately weighed into the glass tube. The tubes were vortex-mixed and allowed to stand for 5 min. Next, 2 mL of hexane was added to the tube, which was then vortex-mixed for 30 s to extract the vitamin D metabolites. The samples were centrifuged at 314 rad/s (3000 rpm) for 20 min, after which 1.5 mL of the hexane layer was removed and transferred to a separate glass tube with a cap. A second extraction with 2 mL of hexane was performed, but the second time 2.0 mL of the hexane layer was removed and combined with the first extract. The hexane extracts were then evaporated to dryness at 40 °C under

nitrogen. The residues were reconstituted with 200 μL methanol, vortex-mixed, and analyzed using the three LC–MS methods. The autosampler tray was maintained at 15 °C to preserve the samples.

2.5. Evaluation of method performance

Five 1:3 serial dilutions of a 2119 ng/g 25(OH)D$_3$ solution were prepared by mass using ethanol as the diluent, with the final solution having a concentration of 2.168 ng/g. These solutions, in addition to an ethanol blank containing no 25(OH)D$_3$, were used to determine the linearity, limits of detection (LOD), and limits of quantitation (LOQ) for the three reported LC–MS methods. The LOD was calculated using a signal to noise ratio of 3, while the LOQ was calculated as 10 times the signal to noise ratio. All methods were linear over the concentration range investigated. The LOD was determined to be 1 ng/g for the CN and PFPP methods, and 2 ng/g for the C18 method. The LOQ was determined to be 3 ng/g, 5 ng/g, and 6 ng/g for the CN, PFPP, and C18 methods, respectively.
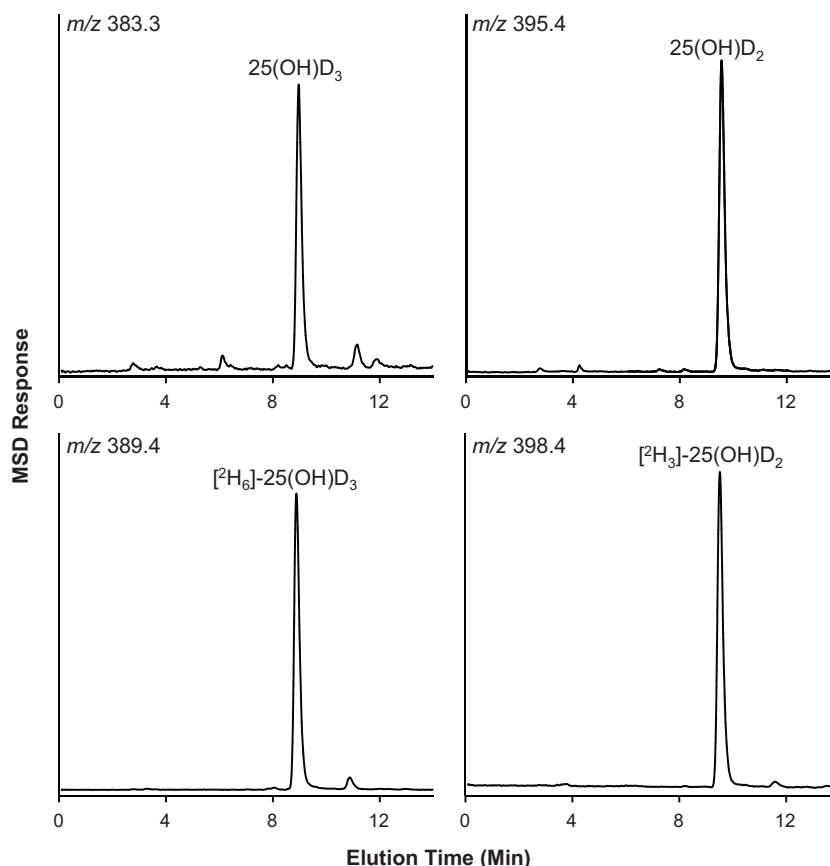
3. Results and discussion

3.1. Optimization of MS parameters for detection of 25(OH)D

Since the levels of the vitamin D metabolites in serum are low (ng/mL), optimization of the MS response was critical to the development of quantitative LC–MS methods. Preliminary MS method development utilized reference standards for 25(OH)D$_2$ and 25(OH)D$_3$. Both electrospray and APCI sources were investigated, but only APCI in positive mode provided sufficient ionization of 25(OH)D$_2$ and 25(OH)D$_3$. Full-scan mass spectra for the vitamin D metabolites and the stable isotope labeled standards revealed predominantly $[M-H_2O+H]^+$ ions, which were used for selected ion monitoring in all further experiments. To obtain the best ionization of the analytes, the APCI-MS detection was optimized for 25(OH)D$_2$ and 25(OH)D$_3$ using flow injection analysis and multiple, successive injections with varying parameters. From these experiments, the optimized conditions listed in Section 2 were determined and were used for all methods. Different organic modifiers and mobile phase additives including methanol, acetonitrile, ammonium acetate, and trifluoroacetic acid were also investigated. As expected, the additives did not improve the ionization of 25(OH)D using APCI, and methanol was found to be a better ionization solvent than acetonitrile. Therefore, methanol/water mobile phases were used to develop the methods for 25(OH)D measurements.

3.2. Development of methods for determining 25(OH)D in SRM 972

The LC–MS methods were originally developed for the determination of the vitamin D metabolites in SRM 972. The initial experiments were focused on separating 25(OH)D$_2$ and 25(OH)D$_3$ because it was not believed that 3-epi-25(OH)D$_3$ would be a metabolite of concern in the adult serum materials that were used to prepare SRM 972. Most literature methods for determining the vitamin D metabolites use C18 columns, so several C18 columns from different vendors and with different dimensions were compared. All C18 columns evaluated readily separated 25(OH)D$_2$ and 25(OH)D$_3$ but were contaminated after multiple injections of serum-based samples, resulting in increased baseline noise and background ionization in the MS, particularly at the monitoring ion for 25(OH)D$_3$ (m/z 383). Changing the guard column more frequently and incorporating a gradient flush (to 100% methanol) at the end of every analysis helped to alleviate the issues with high background ionization, and these practices were followed for all LC–MS methods. Also, the use of a traditional analytical column

Fig. 1. Chromatograms of SRM 972 Level 3 obtained with the C18 column and isocratic elution with 90% methanol, 10% water (volume fractions) and a column temperature of 45 °C.
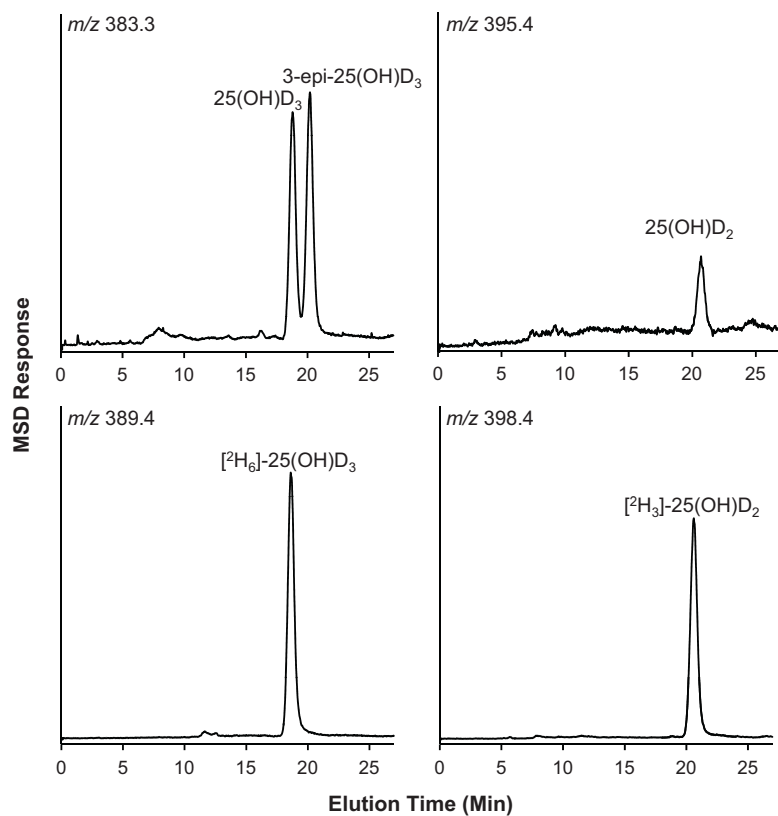
with dimensions of 250 mm × 4.6 mm ID was better for the measurements of 25(OH)D because larger injection volumes could be used as well as higher flow rates, which benefits APCI/MS detection. A Luna C18(2) column was selected for the first method.

The original conditions for the C18 column were developed using SRM 972 Level 3 as a test material because it contains significant levels of both $25(OH)D_3$ (native) and $25(OH)D_2$ (spiked). An isocratic mobile phased comprised of 90% methanol, 10% water (volume fractions) and a column temperature of 45 °C were found to separate the analytes from matrix peaks. These conditions were used to determine $25(OH)D_2$ and $25(OH)D_3$ in SRM 972 using $[^2H_3]$-25(OH)D_2 and $[^2H_6]$-25(OH)D_3 as labeled standards for quantitation, respectively. Representative chromatograms of SRM 972 Level 3 obtained using the C18 column and the original conditions are presented in Fig. 1. The y-axis on all chromatograms represents the mass spectrometric detector (MSD) response. These chromatograms demonstrate the sensitivity and selectivity of this LC–MS method for determining $25(OH)D_2$ and $25(OH)D_3$ in SRM 972 Level 3.
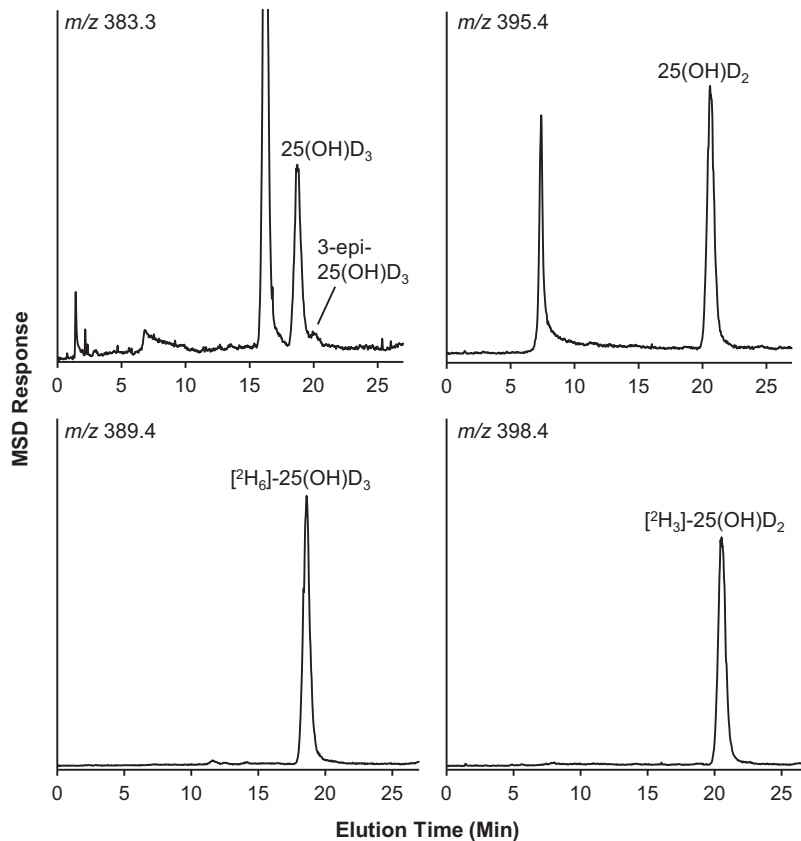
A major limitation of the C18 method is that it is unable to separate $25(OH)D_3$ and 3-epi-25(OH)D_3, which was fortified to be a major component of SRM 972 Level 4. Different stationary phases were investigated for the separation of $25(OH)D_3$ and 3-epi-25(OH)D_3 including phenyl, PFPP, and two CN-based columns. An Agilent SB-CN was the only column investigated at that time that was capable of separating these two species, and it was used to evaluate $25(OH)D_3$, 3-epi-25(OH)D_3, and $25(OH)D_2$ in SRM 972 Level 4. A composition of 68% methanol was found to have the best separation of the $25(OH)D_3$ and 3-epi-25(OH)D_3 while maintaining acceptable signal levels in the MS. The 3-epi-25(OH)D_3 and

$25(OH)D_2$ coelute with this column, but the mass spectrometer can distinguish these compounds based on the different monitoring ions of m/z 383 and m/z 395, respectively. Chromatograms of SRM 972 Level 4 obtained using the CN column and the conditions just described are presented in Fig. 2. These chromatograms reveal near-baseline resolution of $25(OH)D_3$ and 3-epi-25(OH)D_3.

After developing the method with the CN column for SRM 972 Level 4, extracts of SRM 972 Level 1, Level 2, and Level 3 were also evaluated with the CN method. Representative chromatograms of a sample of SRM 972 Level 3 are presented in Fig. 3. A large peak that elutes before the peak for $25(OH)D_3$ is observable in the m/z 383 chromatogram and is displayed off-scale. Due to its large size, the compound is unlikely to be related to $25(OH)D_3$ and was presumed to originate from the matrix. The development of chromatographic conditions that separated this interference from $25(OH)D_3$ were critical because of the limited selectivity of MS detection for complex matrix samples like human serum. There is also a small peak in the m/z 383 chromatogram that elutes as a slight shoulder on the $25(OH)D_3$ peak and has the correct retention time for 3-epi-25(OH)D_3. This peak was also evident in the chromatograms for Level 1 and Level 2, and it was concluded that all Levels of SRM 972 contain detectable amounts of this diastereomer. To avoid bias in the measured values for $25(OH)D_3$, the CN method was used to measure this analyte in SRM 972. However, $25(OH)D_2$ was measured using the C18 column method because of the improved sensitivity of detection and the low levels of this analyte in SRM 972 Levels 1, 2, and 4 (<3 ng/mL). The certified values for SRM 972 [22] were obtained from a combination of the measurements obtained with the LC–MS methods described here as well as the measurements from LC–MS/MS methods developed

Fig. 2. Chromatograms of SRM 972 Level 4 obtained with the CN column and isocratic elution with 68% methanol, 32% water (volume fractions) and a column temperature of 45 °C.

Fig. 3. Chromatograms of SRM 972 Level 3 obtained with the CN column and isocratic elution with 68% methanol, 32% water (volume fractions) and a column temperature of 45 °C.

by both NIST [2] and the US Centers for Disease Control and Prevention [26].

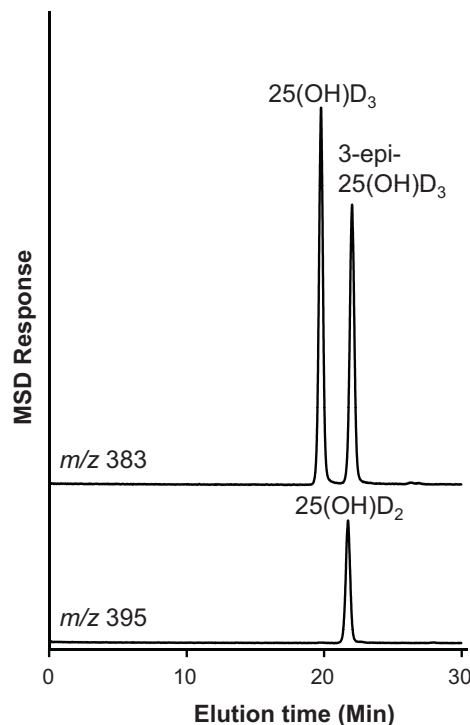### 3.3. Optimization and development of methods for determining 25(OH)D in SRM 909c

Samples of SRM 909c were preliminarily evaluated using the previously described methods for both the C18 and CN columns and $[^2H_6]$-25(OH)D$_3$ as the internal standard. The metabolite 25(OH)D$_2$ was not detected using either method, and the presence of 3-epi-25(OH)D$_3$ in SRM 909c was not confirmed with the CN method. Therefore, 25(OH)D$_3$ was the only vitamin D metabolite detected in SRM 909c. However, interferences at m/z 389 were observed for SRM 909c samples that affected reliable determination of the $[^2H_6]$-25(OH)D$_3$ peak area for both the C18 and CN methods. Interferences at this ion were not previously observed for SRM 972 and were presumed to originate from the SRM 909c matrix. These matrix peaks could possibly represent other unidentified vitamin D metabolites, but identification of these compounds was beyond the scope of the present investigation.

Since $[^2H_6]$-25(OH)D$_3$ is used for quantitation of 25(OH)D$_3$, the existing methods were optimized to achieve better separation of the internal standard and the matrix peaks for SRM 909c samples. First, the mobile phase conditions were varied for the C18 method, but decreasing the methanol content from 90% to 88% did not baseline resolve the matrix interferences and decreased the sensitivity at m/z 383 for detection of 25(OH)D$_3$. The column temperature was then decreased so that the proportion of methanol in the mobile phase could be increased. Conditions of 93% methanol, 7% water and a column temperature of 15 °C were found to separate $[^2H_6]$-25(OH)D$_3$ from the matrix peaks for the C18 method.

The mobile phase compositions were also varied for the CN column in an attempt to resolve the matrix interferences at m/z 389. Mobile phase compositions from 65% to 67% methanol were investigated with a column temperature of 45 °C. A composition of 65% methanol was found to provide resolution of $[^2H_6]$-25(OH)D$_3$ from matrix peaks, but the decreased sensitivity of detection and increased noise for the 25(OH)D$_3$ ion (m/z 383) prohibited use of these conditions for SRM 909c samples. Solvent compositions of 68% methanol to 72% methanol were also investigated with column temperatures of 15 °C and 25 °C, but conditions could not be identified that resolved $[^2H_6]$-25(OH)D$_3$ from matrix interferences. Development of a reliable CN method was critical because it is the only existing method that can resolve 25(OH)D$_3$ from 3-epi-25(OH)D$_3$.

Chromatographic conditions could not be identified that resolved the matrix interferences on the CN column, so modifications in sample preparation were also investigated. Samples of SRM 909c were prepared with and without filtration, with different ratios of the precipitation solvent to serum, and with different protein precipitation solvents including 20% isopropanol in methanol (the original method solvent), ethanol, methanol, and acetonitrile. When the samples were compared with the CN method, none of the procedural changes eliminated the interferences with the $[^2H_6]$-25(OH)D$_3$ peak. These samples were also evaluated at the monitoring ion for $[^2H_3]$-25(OH)D$_3$, m/z 386, which is an alternate internal standard that can be used for quantitation of 25(OH)D$_3$. No interferences were observed near the retention time for $[^2H_3]$-25(OH)D$_3$, which was selected as the internal standard for SRM 909c measurements.

In an attempt to identify alternate approaches to separate the diastereomers 25(OH)D$_3$ and 3-epi-25(OH)D$_3$, a different column chemistry was evaluated. Recently, a number of literature methods reported the use of PFPP columns for resolution of these two isomers [9,24,27]. Of these columns, an Ascentis Express F5 (PFPP)
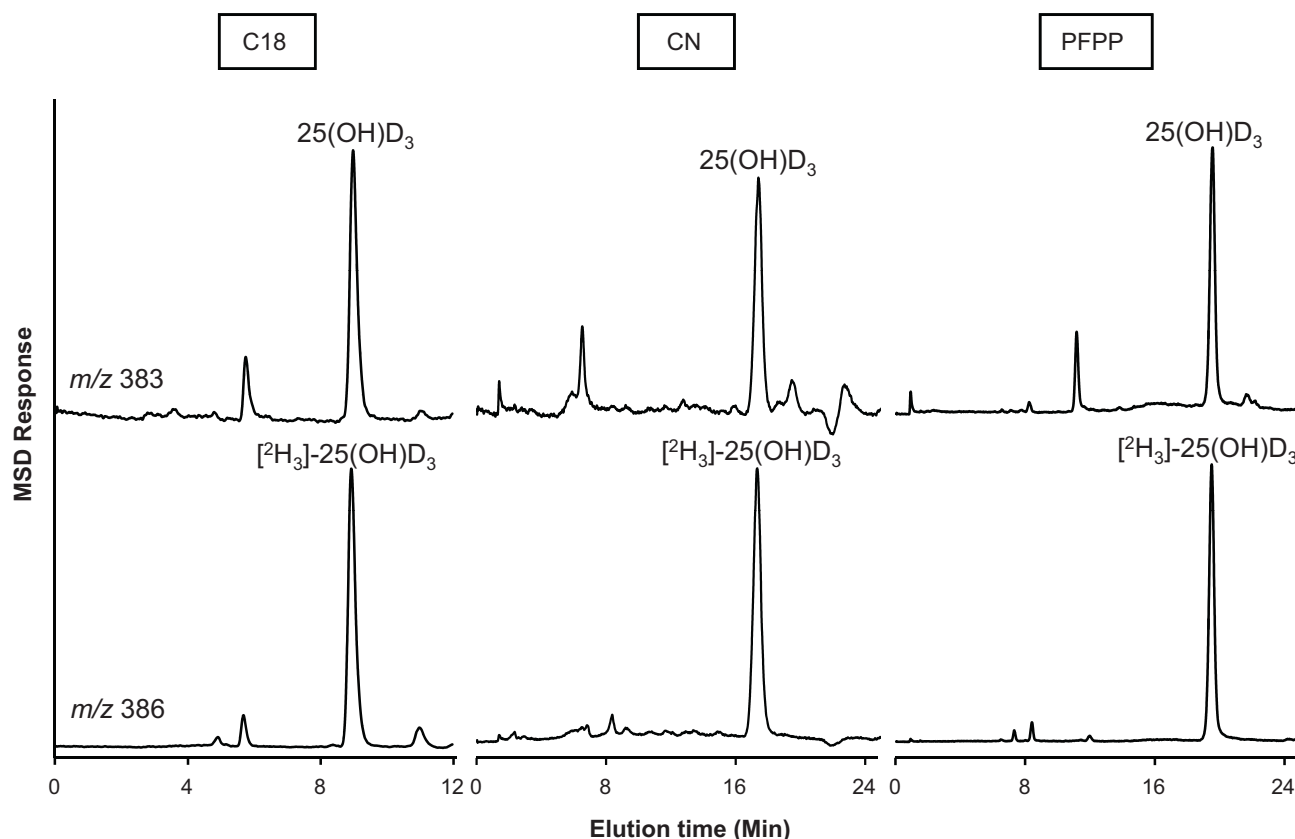

Fig. 4. Separation of mixed standard containing 25(OH)D$_2$, 25(OH)D$_3$, and 3-epi-25(OH)D$_3$ with the PFPP column method using an isocratic mobile phase of 74% methanol, 26% water (volume fractions) and a column temperature of 15 °C.

column was selected and was evaluated for its ability to resolve a standard mixture of 25(OH)D$_3$, 3-epi-25(OH)D$_3$, and 25(OH)D$_2$. Isocratic mobile phase compositions from 72% methanol to 79% methanol in water (volume fractions) were evaluated; 25(OH)D$_3$ and 3-epi-25(OH)D$_3$ were separated under all conditions investigated, but resolution of 25(OH)D$_2$ and 3-epi-25(OH)D$_3$ could not be achieved (this is also true for the CN column). Fortunately, the mass spectrometer can distinguish these compounds based on the different ions of m/z 395 and m/z 383 for 25(OH)D$_2$ and 3-epi-25(OH)D$_3$, respectively. The column was then evaluated with samples of SRM 909c, and the conditions that provided the best separation of the SRM 909c matrix peaks without sacrificing sensitivity included a mobile phase composition of 74% methanol, 26% water and a column temperature of 15 °C. Separation of a mixed standard containing 25(OH)D$_3$, 3-epi-25(OH)D$_3$ and 25(OH)D$_2$ on the PFPP column with these conditions is presented in Fig. 4. The PFPP column offers peak shape and efficiency that are comparable with the C18 column, but it has the advantage of resolving 25(OH)D$_3$ and 3-epi-25(OH)D$_3$.

3.4. Quantitative results for 25(OH)D$_3$ in SRM 909c

Samples of SRM 909c were evaluated with the modified C18 method, the new PFPP method, and the CN method. To prepare samples, the procedure described in Section 2 was used with $[^2H_3]$-25(OH)D$_3$ as the internal standard (25(OH)D$_2$ was not detected in SRM 909c). Example chromatograms of an SRM 909c sample obtained using the three methods are provided in Fig. 5, and all methods demonstrate good sensitivity and selectivity for determining 25(OH)D$_3$ and $[^2H_3]$-25(OH)D$_3$. The m/z 383 chromatogram for the PFPP column reveals a small peak eluting after 25(OH)D$_3$ that corresponds with the retention time of 3-epi-25(OH)D$_3$, but the m/z 383 chromatogram for the CN column does not exhibit a shoulder on the 25(OH)D$_3$ peak, which would be expected if a small amount of 3-epi-25(OH)D$_3$ was present in the sample

Fig. 5. Chromatograms of SRM 909c samples obtained with three methods. The C18 method utilized an isocratic mobile phase of 93% methanol, 7% water, column temperature 15 °C; the CN method utilized an isocratic mobile phase of 68% methanol, 32% water, column temperature 45 °C; the PFPP method used an isocratic mobile phase of 74% methanol, 26% water and a column temperature of 15 °C.

(see chromatogram of SRM 972 in Fig. 3). The other matrix peaks present in each of the chromatograms were not identified.

Since the presence of 3-epi-25(OH)D$_3$ was not positively confirmed, only 25(OH)D$_3$ was measured in SRM 909c. Table 1 presents the quantitative results for 25(OH)D$_3$ in SRM 909c and SRM 972 Level 1, which was used as a control to validate the measurements. The data in Table 1 include the total number of quantitative values measured (N), the average value, the standard deviation (SD), and the percent relative standard deviation (%RSD); the reported concentrations of 25(OH)D$_3$ were corrected for the purity of the reference standard, which was determined at NIST. There is excellent agreement in the results obtained for all three methods for both materials, and the measurement variability was very low (%RSD < 2.2). The equivalence of the results for 25(OH)D$_3$ from all three methods indicates 3-epi-25(OH)D$_3$ is not a significant metabolite in SRM 909c (or SRM 972 Level 1) because the C18 results are not biased high. In addition, the measured values for the 25(OH)D$_3$ in SRM 972 Level 1 are well within the expanded uncertainty range for the certified value of 23.2 ng/g ± 0.8 ng/g,

Table 1
Comparison of 25-hydroxyvitamin D$_3$ concentrations (ng/g) in SRM 972 Level 1 (control) and SRM 909c obtained using the three developed methods.

	SRM 972 Level 1 (control)[a]			SRM 909c		
	C18	CN	PFPP	C18	CN	PFPP
N	2	2	2	10	10	10
Average	23.32	23.43	22.98	19.86	19.86	19.65
SD	0.01	0.31	0.31	0.19	0.29	0.42
%RSD	0.02	1.31	1.35	0.97	1.47	2.12

[a] Certified value for 25(OH)D$_3$: 23.2 ± 0.8 ng/g.

indicating that the measurements were in control during the analysis of SRM 909c.

4. Conclusions

LC-APCI/MS methods using C18 and CN columns were developed to determine the vitamin D metabolites in SRM 972. The methods utilized optimized detection parameters, sample preparation, and separation conditions for resolution of matrix constituents from the analytes and labeled standards. When these methods were used to evaluate SRM 909c, matrix peaks were observed that interfered with the measurement of [^{2}H$_6$]-25(OH)D$_3$ and hence 25(OH)D$_3$. The conditions for the C18 method were modified to separate the matrix peaks, but only selection of [^{2}H$_3$]-25(OH)D$_3$ as an internal standard eliminated the interference for the CN method. Since SRM 909c samples were found to have matrix peaks and interferences that were not present in the four Levels of SRM 972, multiple robust methods to accurately assess both the identity and concentrations of the vitamin D metabolites were necessary. Therefore, a third method that utilizes a PFPP column was also developed for evaluating SRM 909c samples. Of the two methods that resolve 25(OH)D$_3$ and 3-epi-25(OH)D$_3$, the PFPP method offered better resolution over the CN method for the SRM 909c samples. However, since 3-epi-25(OH)D$_3$ was not detected in SRM 909c, the results for 25(OH)D$_3$ obtained with the C18 method were unbiased and found to be equivalent to the results obtained for the CN and PFPP methods.

Acknowledgment

References

[1] A.M. Wallace, S. Gibson, A. de la Hunty, C. Lamberg-Allardt, M. Ashwell, Steroids 75 (2010) 477.
[2] S.S.C. Tai, M. Bedner, K.W. Phinney, Anal. Chem. 82 (2010) 1942.
[3] A.N. Hoofnagle, T.J. Laha, T.F. Donaldson, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 1639.
[4] J.E. Zerwekh, Ann. Clin. Biochem. 41 (2004) 272.
[5] M. Vogeser, J. Steroid Biochem. 121 (2010) 565.
[6] J.M. El-Khoury, E.Z. Reineks, S.H. Wang, Clin. Biochem. 44 (2011) 66.
[7] J. Adamec, A. Jannasch, J.J. Huang, E. Hohman, J.C. Fleet, M. Peacock, M.G. Ferruzzi, B. Martin, C.M. Weaver, J. Sep. Sci. 34 (2011) 11.
[8] M.M. Kushnir, J.A. Ray, A.L. Rockwood, W.L. Roberts, S.L. La'ulu, J.E. Whittington, A.W. Meikle, Am. J. Clin. Nutr. 134 (2010) 148.
[9] R.L. Schleicher, S.E. Encisco, M. Chaudhary-Webb, E. Paliakov, L.F. Mccoy, C.M. Pfeiffer, Clin. Chim. Acta 412 (2011) 1594.
[10] H.C.M. Stepman, A. Vanderroost, K. Van Uytfanghe, L.M. Thienpont, Clin. Chem. 57 (2011) 441.
[11] W. Xie, C.M. Chavez-Eng, W. Fang, M.L. Constanzer, B.K. Matuszewski, W.M. Mullett, J. Pawliszyn, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 879 (2011) 1457.
[12] B.C. Netzel, K.W. Cradic, E.T. Bro, A.B. Girtman, R.C. Cyr, R.J. Singh, S.K.G. Grebe, Clin. Chem. 57 (2011) 431.
[13] T. Higashi, M. Suzuki, J. Hanai, S. Inagaki, J.Z. Min, K. Shimada, T. Toyo'oka, J. Sep. Sci. 34 (2011) 725.
[14] P. Koerner, M. McGinley, LCGC North Am. (2011) 8.
[15] M. Blum, G. Dolnikowski, E. Seyoum, S.S. Harris, S.L. Booth, J. Peterson, E. Saltzman, B. wson-Hughes, Endocrine 33 (2008) 90.
[16] T. Higashi, A. Yamauchi, K. Shimada, Anal. Sci. 19 (2003) 941.
[17] T. Holmoy, S.M. Moen, T.A. Gunderson, M.F. Holick, E. Fainardi, M. Castellazzi, I. Casetta, Mult. Scler. 15 (2009) 1280.
[18] M. Kamao, S. Tatematsu, S. Hatakeyama, T. Sakaki, N. Sawada, K. Inouye, K. Ozono, N. Kubodera, G.S. Reddy, T. Okano, J. Biol. Chem. 279 (2004) 15897.
[19] R.J. Singh, R.L. Taylor, G.S. Reddy, S.K.G. Grebe, J. Clin. Endocrinol. 91 (2006) 3055.
[20] G.L. Lensmeyer, D.A. Wiebe, N. Binkley, M.K. Drezner, Clin. Chem. 52 (2006) 1120.
[21] G.L. Lensmeyer, D.A. Wiebe, N. Binkley, M.K. Drezner, Clin. Chem. 52 (2006) 2305.
[22] K.W. Phinney, M. Bedner, S.S.C. Tai, V.V. Vamathevan, L.C. Sander, K.E. Sharpless, S.A. Wise, J.H. Yen, R.L. Schleicher, M. Chaudhary-Webb, C.M. Pfeiffer, J.M. Betz, P.M. Coates, M.F. Picciano, Anal. Chem. 84 (2012) 956.
[23] H.C.M. Stepman, A. Vanderroost, D. Stockl, L.M. Thienpont, Clin. Chem. Lab. Med. 49 (2011) 253.
[24] F.G. Strathmann, K. Sadilkova, T.J. Laha, S.E. LeSourd, J.A. Bornhorst, A.N. Hoofnagle, R. Jack, Clin. Chim. Acta 413 (2012) 203.
[25] U. Turpeinen, U. Hohenthal, U.H. Stenman, Clin. Chem. 49 (2003) 1521.
[26] H.P. Chen, L.F. Mccoy, R.L. Schleicher, C.M. Pfeiffer, Clin. Chim. Acta 391 (2008) 6.
[27] C.R. Aurand, D.S. Bell, Reporter 29.2 (2011) 3.