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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.

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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 pentafluorophenylpropyl (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–[$^2H_3$] ([$^2H_3$]-25(OH)D_3), 25-hydroxyvitamin D_2–[$^2H_3$] ([$^2H_3$]-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–[$^2H_6$] ([$^2H_6$]-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

[1] Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.