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Development and Certification of a Standard Reference Material for Vitamin D Metabolites in Human Serum[a]

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Abstract

The National Institute of Standards and Technology (NIST), in collaboration with the National Institutes of Health's Office of Dietary Supplements (NIH-ODS), has developed a Standard Reference Material (SRM) for the determination of 25-hydroxyvitamin D [25(OH)D] in serum. SRM 972 Vitamin D in Human Serum consists of four serum pools with different levels of vitamin D metabolites and has certified and reference values for 25(OH)D_2, 25(OH)D_3, and 3-epi-25(OH)D_3. Value assignment of this SRM was accomplished using a combination of three isotope-dilution mass spectrometry approaches, with measurements performed at NIST and at the Centers for Disease Control and Prevention (CDC). Chromatographic resolution of the 3-epimer of 25(OH)D_3 proved to be essential for accurate determination of the metabolites.

Introduction

The essential role of vitamin D in bone metabolism and calcium homeostasis has been known for many years.[1] Vitamin D deficiency can lead to rickets in children and osteomalacia in adults. Recently, however, vitamin D has been the subject of renewed interest because of its function in regulation of the cell cycle, including apoptosis and differentiation.[2] Both epidemiological data and animal model studies have pointed toward a link between vitamin D status and cancer risk;[3,4] many of these studies have focused on cancers of the colon, prostate, and breast.[5-7] At the same time, a number of surveys have suggested that vitamin D deficiency or insufficiency may be more common than previously believed.[8,9] Because of the implications of these observations for public health, considerable effort is now being given to examining the vitamin D exposure of various populations and to

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[c]This work is dedicated to the memory of Dr. Mary Frances Picciano, who led the NIH-ODS Vitamin D Initiative and championed the development of this reference material

determining the levels of vitamin D that are associated with reduced cancer risk and optimal bone health.

Although known as a vitamin, vitamin D is actually a prohormone and is part of the structural family of secosteroids. In humans, vitamin D can arise from various sources. Exposure to sunlight results in production of vitamin D_3 (cholecalciferol) in the skin from 7-dehydrocholesterol. Vitamin D_2 (ergocalciferol) is formed in plants through the irradiation of sterols; consumption of plants generally represents a very minor source of vitamin D in humans. With the exception of some types of fatty fish and egg yolks, food is a poor source of vitamin D, although certain foods, including milk, have been fortified with vitamin D. Dietary supplements, including multivitamins, are also a potential source of either vitamin D_2 or vitamin D_3.[10]

Vitamin D, whether formed in the skin or obtained through the diet, is rapidly hydroxylated in the liver to 25-hydroxyvitamin D [25(OH)D], which is also known as calcifediol. The process of hydroxylation does not appear to be tightly regulated and is limited only by availability of the substrate.[10] This metabolite is further hydroxylated, primarily in the kidney, to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D, calcitriol], which is the biologically active metabolite. Like steroid hormones such as estrogen and testosterone, calcitriol exerts its influence through binding to a nuclear vitamin D receptor (VDR) found in the nuclei of cells of target organs, including kidneys, bone, and intestinal tissues. Binding of calcitriol to the VDR influences expression of genes involved in calcium uptake. Expression of the VDR has also been reported in elements of the immune system, indicating a possible role for vitamin D in modulating immunological response.[1]

Although 1,25$(OH)_2$D is the physiologically active metabolite of vitamin D, it has limited utility as an indicator of vitamin D status because its half-life is relatively short (≈15 hr).[11] In addition, its levels are influenced by concentrations of both parathyroid hormone (PTH) and calcium. Similarly, vitamin D is also a poor marker of vitamin D status because it is rapidly hydroxylated to form 25(OH)D. In contrast, the half-life of circulating 25(OH)D is 2-3 weeks, and therefore measurement of 25(OH)D has become the preferred indicator of vitamin D nutritional status. Because 25(OH)D can arise from hydroxylation of either vitamin D_2 or D_3, both resulting metabolites, 25$(OH)D_2$ and 25$(OH)D_3$, should be considered in order to achieve an accurate assessment of vitamin D status.

Concentrations of 25(OH)D in serum less than 50 nmol/L are generally considered deficient,[12] and concentrations greater than 250 nmol/L can lead to toxicity.[13] The first methods for determination of 25(OH)D in serum were based upon competitive protein binding and utilized vitamin D binding protein as the binding agent. These approaches have largely been replaced because they require labor-intensive sample preparation, as noted in recent reviews on the measurement of 25(OH)D.[14] Current assays can generally be divided into either immunoassay-based methods or chromatographic approaches with either UV absorbance or mass spectrometric (MS) detection. Efforts to develop a gas chromatography – mass spectrometry (GC-MS) reference method are also underway.[15]

No matter what technique is used, there are several important factors that must be considered in the measurement of 25(OH)D. The analyte of interest is highly protein bound and generally must be liberated from vitamin D binding protein prior to its measurement. Because vitamin D_3 and/or vitamin D_2 may contribute to an individual's vitamin D exposure, the assay method should respond equally to metabolites of both forms of vitamin D, even if concentrations of 25$(OH)D_2$ and 25$(OH)D_3$ are not reported separately. A structural isomer of 25(OH)D, known as 3-epi-25(OH)D has been reported in certain serum

samples, particularly those from infants. Although its biological activity in humans has not yet been determined, it can be a potential source of measurement bias.[16]

Despite the analytical challenges noted above that are associated with measurement of 25(OH)D, the demand for 25(OH)D testing has grown dramatically in many settings, and an increasing number of laboratories are either implementing commercial assay products or developing their own assay methodologies for 25(OH)D. As new methods have been developed, publications have typically compared the candidate method with an established one, including Food and Drug Administration- (FDA-) cleared assays for 25(OH)D. Such approaches are useful for assessing the comparability of results from different assay techniques and can lend insight into methodological differences. However, there are numerous literature reports describing discrepancies between the results of different assays for 25(OH)D, and a number of editorials have also appeared on the subject.[17-19] In the absence of certified reference materials (CRMs) for 25(OH)D, establishing the accuracy of 25(OH)D assays has remained elusive, and comparisons of one technique to another have done little to foster confidence in 25(OH)D measurements. As a result, serious questions remain about the value of 25(OH)D testing and its ability to identify accurately individuals with sub-optimal 25(OH)D levels.[20-22]

The National Institute of Standards and Technology (NIST) has developed a Standard Reference Material (SRM) to support accuracy in the measurement of 25(OH)D in serum or plasma. SRM 972 Vitamin D in Human Serum was developed in collaboration with the National Institutes of Health's Office of Dietary Supplements (NIH-ODS) and is the first CRM for determination of 25(OH)D in serum. The SRM consists of four pools of serum, each with different levels of vitamin D metabolites. Value assignment of this SRM involved measurements using three different isotope-dilution (ID) mass spectrometry approaches, with measurements performed at NIST and at the Centers for Disease Control and Prevention (CDC, Atlanta, GA). This work also represents the first application of ID liquid chromatography-mass spectrometry (LC-MS) methodology for the determination of 25(OH)D in serum. Chromatographic resolution of the 3-epimer of 25(OH)D_3 proved to be essential for accurate measurement of the vitamin D metabolites present.

EXPERIMENTAL SECTION*

Safety Considerations

The human sera used in the preparation of SRM 972 Vitamin D in Human Serum were screened and found to be non-reactive for hepatitis B surface antigen (HBsAg), human immunodeficiency virus (HIV), hepatitis C virus (HCV), and human immunodeficiency virus 1 antigen (HIV-1Ag) by Food and Drug Administration (FDA)-licensed tests. However, because no test method can guarantee that these infectious agents are absent, appropriate safety precautions should be taken when handling these, or any other potentially infectious human serum or blood specimens.[23]

NIST LC-MS and LC-MS/MS Methods

Materials

Standards for 25(OH)D_2 and 25(OH)D_3 were obtained from IsoSciences (King of Prussia, PA) and the United States Pharmacopeia (USP, Rockville, MD), respectively. Purity of these compounds was assessed by NIST using multiple techniques, and analytical results

*__Disclaimer:__ 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.

were corrected for the purity of the standards.[24] Stable isotope-labeled internal standards, 25(OH)D_2-d_3 and 25(OH)D_3-d_3 were obtained from IsoSciences. Both of these compounds had isotopic purities > 99%. An additional stable isotope-labeled internal standard, 25(OH)D_3-d_6, with an isotopic purity of 99% was obtained from Medical Isotopes, Inc. (Pelham, NH). The 3-epimer of 25(OH)D_3, 3-epi-25(OH)D_3, was obtained from IsoSciences. Structures of 25(OH)D_2, 25(OH)D_3, and 3-epi-25(OH)D_3 are shown in Figure S1. The 3- and 25-positions of these compounds are noted on the structures.

Sample Preparation for LC-MS

Samples for analysis were selected from nine or ten different trays of each of the four levels of SRM 972. The SRM vials were allowed to reach room temperature prior to analysis. Approximately 110 mg (150 μL, exact mass known) of internal standard solution was weighed into an 8 mL glass tube with a screw cap. An additional 600 μL of 2-propanol:methanol (20:80, volume fractions) was added to the tube. An aliquot of serum (450 mg, exact mass known) was weighed into the tube, and the tube was vortex-mixed and allowed to stand for 5 min. Hexane (2 mL) was added to the tube, and the tube was vortex-mixed for 30 s. The samples were centrifuged at 3000 rpm for 20 min, and 1.5 mL of the hexane layer was removed and transferred to a separate glass tube with a cap. A second extraction with hexane (2 mL) was performed in the same manner, and 2 mL of the hexane layer was removed and combined with the first extract. The extracts were evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted with methanol (300 μL) and vortex-mixed. The samples were transferred to disposable centrifuge filters with a cellulose acetate membrane (1.0 mL, 0.45 μm pores) and centrifuged at 4000 rpm for 5 min. Approximately 200 μL of the filtrate was transferred to an autosampler vial with a 300 μL insert for analysis.

Analysis by LC-MS

Measurements were performed using atmospheric pressure chemical ionization (APCI) in the positive ion mode using an Agilent 1100 series LC coupled to an Agilent SL series mass spectrometric detector. The relevant instrumental parameters include the nebulizer pressure (345 kPa, 50 psig), drying gas temperature (325 °C), drying gas flow (5 L/min), capillary voltage (+3600 V), vaporizer temperature (300 °C), corona current (4 μA), and fragmentor voltage (150 V). Multiple injections of the metabolites of interest and their respective labeled internal standards were used to identify appropriate ions for monitoring and quantification. The ions selected for quantification were *m/z* 383 and *m/z* 389 for 25(OH)D_3 and 25(OH)D_3-d_6, respectively; and *m/z* 395 and *m/z* 398 for 25(OH)D_2 and 25(OH)D_2-d_3, respectively. Chromatographic separation was achieved using either a Luna C18(2) stationary phase (4.6 mm × 250 mm, 5 μm particles, Phenomenex, Torrance, CA) or a Zorbax SB-CN (cyanopropyl) stationary phase (4.6 mm × 250 mm, 5 μm particles, Agilent Technologies, Wilmington, DE). For the C_{18} column, the analytes were eluted under isocratic conditions using a mobile phase comprised of water:methanol (90:10, volume fractions) and a flow rate of 1.0 mL/min. The column was thermostatted at 45 °C. For the cyano column, the analytes were eluted under isocratic conditions using a water:methanol (32:68, volume fractions) mobile phase and a flow rate of 1.0 mL/min. The column was maintained at 45 °C. Injection volumes for both methods were 15 μL and 10 μL for samples and calibrants, respectively. Preparation of the calibration solutions is described in the Supporting Information.

Sample Preparation for LC-MS/MS

Samples for analysis were selected from six different trays of each of the four levels of SRM 972. The SRM vials were allowed to reach room temperature prior to analysis. Serum samples (2 g) were weighed into 50 mL glass centrifuge tubes. In order to obtain this mass

of sample, the contents of two vials of SRM 972 were combined for Levels 1, 3, and 4, while the contents of three vials were combined for Level 2. For Levels 1, 2, and 3, water (1 mL) was added to each sample to prevent protein precipitation upon addition of the internal standard solution. For Level 4, a slightly larger volume of water (1.4 mL) was added to the samples. Each sample was spiked with an appropriate amount of the internal standard solutions to achieve approximately a 1:1 ratio of analyte to internal standard. After addition of the internal standard solutions, the samples were allowed to equilibrate for 1 h at room temperature. The pH of each sample was adjusted to pH 9.8 ± 0.2 with carbonate buffer (0.1 g/mL, pH 9.8). The analytes were extracted from the serum matrix with 8 mL hexane:ethyl acetate (50:50, volume fractions). Samples were mixed vigorously by a mechanical shaker for 10 min. The hexane:ethyl acetate layer was removed, transferred to a 50 mL centrifuge tube, and the extraction process was repeated with an additional 8 mL solvent. The extracts were combined, dried under nitrogen at 45 °C, and reconstituted with 120 μL methanol prior to analysis.

Analysis by LC-MS/MS

Preparation of the calibration solutions is described in the Supporting Information. Measurements were performed using APCI in the positive ion mode on an Applied Biosystems API 4000 LC-MS/MS system coupled to an Agilent 1100 series LC system. The specific transitions monitored were *m/z* 401 → *m/z* 383 for 25(OH)D_3 and 3-epi-25(OH)D_3, *m/z* 404 → *m/z* 386 for 25(OH)D_3-d_3, *m/z* 413 → *m/z* 395 for 25(OH)D_2, and *m/z* 416 → *m/z* 398 for 25(OH)D_2-d_3. The relevant MS parameters for the measurement of 25(OH)D_3 and 3-epi-25(OH)D_3 included dwell time (0.25 s), curtain gas and collision gas (nitrogen at settings of 276 kPa and 21 kPa, respectively), and ion source gas 1 and ion source gas 2 (air, 483 kPa and 241 kPa, respectively). The needle current was set at 5 μA and the temperature was maintained at 350 °C. The declustering potential, entrance potential, collision energy, and collision exit potential were set at 86 V, 10 V, 15 V, and 10 V, respectively.

The relevant MS parameters for the measurement of 25(OH)D_2 included dwell time (0.25 s), curtain gas and collision gas (nitrogen, 276 kPa and 21 kPa, respectively), and ion source gas 1 and ion source gas 2 (air, 483 kPa and 241 kPa, respectively). The needle current was set at 5 A and the temperature was maintained at 350 °C. The declustering potential, entrance potential, collision energy, and collision exit potential were set at 91 V, 10 V, 13 V, and 12 V, respectively.

Chromatographic separation of the analytes of interest was performed on either a Zorbax Eclipse XDB-C_{18} stationary phase (4.6 mm × 250 mm, 5 μm particles, Agilent Technologies, Palo Alto, CA) or a Zorbax SB CN (cyano) column (4.6 mm × 250 mm, 5 μm particles, Agilent Technologies). For the measurement of 25(OH)D_2 on the C_{18} column, a water:methanol mobile phase was used under isocratic conditions. For Levels 1, 3, and 4, the water:methanol composition was 14:86 (volume fractions), and for Level 2, the composition was 15:85 (volume fractions). For the cyanopropyl stationary phase, the mobile phase was water:methanol (34:66, volume fractions), and isocratic elution conditions were employed. The chromatographic column was thermostatted at 30 °C and the flow rate was 1.0 mL/min.

CDC LC-MS/MS Method

Materials

Bovine serum albumin, 25(OH)D_3 (≥98% pure), 25(OH)D_2 (≥98% pure), and phosphate-buffered saline were obtained from Sigma (St. Louis, MO). The internal standard, 25(OH)D_3-d_6 (95% chemically pure and 98% isotopically pure), was from Synthetica (Oslo,

Norway). Protein precipitation plates (Sirocco) and 96-well plates were obtained from Waters Corporation (Milford, MA). Hydrophobic durapore membrane filters were from Millipore (Bedford, MA).

Sample Preparation for LC-MS/MS

A Hamilton Starlet (Reno, NV) robotic liquid handler was used to process samples including mixing steps (aspirate-dispense). Patient serum, quality controls and calibrator samples (100 μL), thawed to room temperature, were added to a 96-well plate containing 75 μL deionized water and 75 μL of internal standard solution. The wells were mixed, a 200 μL aliquot of each was transferred to a protein precipitation plate where each well contained 300 μL acetonitrile, and the wells were mixed again. To ensure complete protein precipitation, the plate was shaken gently on an orbital shaker for 3 minutes then allowed to rest for 20 minutes. The protein precipitation plate was placed over a fresh 96-well plate then centrifuged at 2,000 relative centrifugal force (RCF) for 6 min. The receiving plate containing the resulting extract was ready for analysis.

Analysis by LC-MS/MS

Preparation of the calibration solutions is described in the Supporting Information. Aliquots (60 μL) of the sample extracts were measured using APCI in the positive ion mode on a Thermo Quantum TSQ coupled to a Thermo Surveyor HPLC system. A 5 μm 2 × 30 mm C_8 column (Phenomenex Torrence, CA) was employed for online solid phase extraction (SPE) under gradient conditions with methanol:water (volume fractions) as the mobile phase (50:50 to 87:13, followed by a 100:0 cleanout step). Online solid-phase extraction (SPE) was followed by chromatographic separation on a 3 μm 2 × 50 mm C18(2) column (Phenomenex Torrence, CA) for quantification of 25(OH)D_2 and 25(OH)D_3 under gradient conditions with methanol:water (volume fractions) as the mobile phase. The elution conditions employed were a gradient applied from 86:14 to 92:8. The flow rate on both columns was 0.4 mL/min at elution and both the columns were operated at room temperature. The relevant instrument parameters include corona current (10.0 μA), collision energy (10 V), skimmer offset (−7 V), tube lens offset (100 V), capillary temperature (150 °C), vaporizer temperature (275 °C), collision pressure (0.107 Pa, 0.8 mTorr), sheath gas pressure (40 psi), ion sweep gas pressure (0 psi), and auxiliary gas pressure (8 psi). The specific transitions monitored for quantification were *m/z* 401 → *m/z* 383, *m/z* 413 → *m/z* 395, *m/z* 407 → *m/z* 389, for 25(OH)D_3, 25(OH)D_2, and 25(OH)D_3-d_6, respectively. Tandem mass spectrometry parameters and transitions were selected based upon direct infusions of each analyte and the labeled internal standard.

RESULTS AND DISCUSSION

Preparation of SRM 972

One of the goals in designing SRM 972 was to pose similar analytical challenges as those encountered in the analysis of 25(OH)D in routine samples. In addition, the presence of 3-epi-25(OH)D_3 in samples from infants had recently been reported, and the (undetected) presence of this analyte was identified as a potential source of error in mass spectrometry-based measurements of 25(OH)D.[16] In outlining the specifications for SRM 972, NIST worked with NIH-ODS and a panel of experts in the vitamin D field. SRM 972 was prepared by Aalto Scientific, Ltd. (Carlsbad, CA) according to specifications provided by NIST. Additional details on the preparation of SRM 972 can be found in the Certificate of Analysis for SRM 972.[25]

SRM 972 was prepared from three different pools of human serum. Level 1 and Level 2 were prepared from the first pool of serum, while Level 3 and Level 4 were prepared from

the second and third pools, respectively. Level 1 of SRM 972 consists of unaltered human serum and contains a concentration of 25(OH)D_3 that is consistent with "normal" levels of 25(OH)D_3 found in adults. In the absence of supplementation with vitamin D_2, the level of 25(OH)D_2 in Level 1 was expected and was confirmed to be low. To achieve the desired 25(OH)D_3 concentration for Level 2, the human serum used for Level 1 was diluted by approximately 50% with horse serum. Horse serum is naturally low in 25(OH)D_3 and has been used to prepare calibrators for 25(OH)D measurements.[26] The horse serum did contain a small amount of 25(OH)D_2, however, and therefore the concentration of 25(OH)D_2 is slightly higher in level 2 than in Level 1. Level 3 was designed to contain approximately equal concentrations of 25(OH)D_2 and 25(OH)D_3. To achieve this goal, human serum containing a normal level of 25(OH)D_3 was fortified (spiked) with 25(OH)D_2. Level 4 was prepared by fortifying normal human serum with 3-epi-25(OH)D_3.

Measurement of 25(OH)D in SRM 972 by LC-MS (NIST)

Initial value assignment efforts for SRM 972 involved the development of an ID LC-MS approach to measure 25(OH)D. Based on previous literature reports, we did not anticipate measurable concentrations of 3-epi-25(OH)D in Levels 1, 2, or 3 because these pools were based upon human serum collected from adult donors. Work published by Singh et al.[16] suggested that this metabolite was only present in infants and quickly disappeared by age one. Because 25(OH)D_2 and 25(OH)D_3 have different molecular masses, chromatographic separation of the two analytes may not be required for methods employing mass spectrometric detection. However, for methods employed in value assignment of CRMs, every effort should be made to reduce potential sources of measurement bias. Therefore, a chromatographic separation of the two major metabolites of interest, 25(OH)D_2 and 25(OH)D_3, was developed using a C_{18} stationary phase and a methanol:water mobile phase. The two metabolites can readily be resolved from one another, and the mobile phase is compatible with APCI. This method was utilized for the measurement of 25(OH)D_2 and 25(OH)D_3 in Level 1, Level 2, and Level 3 of SRM 972.

The sample preparation scheme for LC-MS measurements employed a mixture of 2-propanol and methanol to precipitate sample proteins and liberate the 25(OH)D species present. This was followed by extraction of the 25(OH)D species into hexane. The isotopically labeled internal standards were added at the beginning of sample processing and were anticipated to compensate for less than 100% extraction efficiency. Labeled internal standards for both 25(OH)D_2 and 25(OH)D_3 were utilized, and the internal standards were added so that their concentrations exceeded those of the unlabeled species to minimize errors in the determination of area ratios.

For Level 4 of SRM 972, which contains 3-epi-25(OH)D_3, a second LC-MS approach was developed to allow the quantification of 25(OH)D_3 and 3-epi-25(OH)D_3. These two structural isomers were not resolved using a C_{18} stationary phase, and therefore we pursued alternative means of achieving the separation of these two species. Lensmeyer et al. previously reported the use of a cyanopropyl stationary phase to achieve the partial chromatographic resolution of 3-epi-25(OH)D_3 from 25(OH)D_3 and 25(OH)D_2 in human serum samples.[27] We modified their mobile phase conditions slightly to improve the resolution between 25(OH)D_3 and 3-epi-25(OH)D_3. We also evaluated several other types of stationary phases but could not identify a column that provided superior resolution to that of the cyano column.

During the method development process for Level 4 of SRM 972, several samples of the other three levels of SRM 972 were also analyzed on the cyano column. A small peak eluting after 25(OH)D_3 was observed (Figure 1), and the chromatographic retention and mass (*m/z*) of this species were consistent with that of 3-epi-25(OH)D_3 based upon injection

of a standard solution of 3-epi-25(OH)D_3. Further investigation and consultation with experts in the vitamin D field indicated that 3-epi-25(OH)D_3 could be present in samples from adults. However, the concentration of the putative 3-epi-25(OH)D_3 could not be quantified in the first three levels of SRM 972 with the desired level of precision by LC-MS, and we elected to pursue LC-MS/MS methodology to further investigate this species. Nevertheless, we did choose to employ the LC-MS method with the cyano column for certification measurements, as will be described in more detail in subsequent sections. In addition, this alternative approach was appropriate for determination of 3-epi-25(OH)D_3 in Level 4 of SRM 972, which had been fortified with 3-epi-25(OH)D_3. Because a labeled internal standard was not available for 3-epi-25(OH)D_3, we utilized the labeled 25(OH)D_3 as the internal standard for these measurements.

Measurement of 25(OH)D by LC-MS/MS (NIST)

Development of ID LC-MS/MS was pursued to take advantage of enhanced specificity arising from the use of selected reaction monitoring. In addition, larger serum samples sizes (2 mL) were used in the LC-MS/MS methodology when compared to the LC-MS method to achieve the desired level of sensitivity and measurement precision for the low concentrations of 25(OH)D_2 present in Levels 1, 2, and 4.

Direct infusions of the analytes of interest were performed to optimize the MS parameters and to identify the appropriate transitions for quantification. A detailed description of the optimization process can be found in a recent publication.[24] This method was validated in accordance with guidelines for reference measurement procedures as recognized by the Joint Committee for Traceability in Laboratory Measurement (JCTLM).

Because it appeared from the LC-MS results that 3-epi-25(OH)D_3 was present in Levels 1, 2, and 3 at low concentrations, we elected to include this analyte in the LC-MS/MS method development process. For the determination of 25(OH)D_3 and 3-epi-25(OH)D_3, the chromatographic separation employed in the LC-MS work was further optimized to obtain baseline resolution of these two analytes. Additional stationary phases, including the chiral stationary phase utilized by Singh et al.[16] were also evaluated, but we were unable to further improve the separation of these two structural isomers. Because we did not have a labeled internal standard for 3-epi-25(OH)D_3, we utilized 25(OH)D_3-d_3 for quantification of both 25(OH)D_3 and 3-epi-25(OH)D_3. Results for the LC-MS/MS analysis of Level 4 of SRM 972 are shown in Figure 2A. As shown in the Figure, baseline resolution of 25(OH)D_3 and 3-epi-25(OH)D_3 was achieved on the cyano column. Because these two structural isomers share the same mass and fragmentation patterns, failure to resolve these species chromatographically would result in overestimation of the 25(OH)D_3 concentration.

For the determination of 25(OH)D_2 by LC-MS/MS, a separate analysis employing a C_{18} column was performed.[24] Quantification of this analyte was based upon the labeled internal standard, 25(OH)D_2-d_3. We also investigated the possible presence of 3-epi-25(OH)D_2 in the SRM serum pools using a similar approach to that used for 3-epi-25(OH)D_3, but none was detected. Figure 2B illustrates the measurement of 25(OH)D_2 by LC-MS/MS.

Measurement of 25(OH)D by ID LC-MS/MS (CDC)

The basic methodology utilized by CDC for measurement of 25(OH)D has been described previously,[28] but there were several modifications made to the published method including a change from manual to automated sample processing, use of half as much serum as in the original method, and a switch from solid-phase extraction to on-line gradient C_8 column clean-up which led to improved chromatographic resolution of 25(OH)D_2.[29] It should be

noted that measurement of 3-epi-25(OH)D_3 was not included in the CDC method, and the chromatographic method employed did not resolve 25(OH)D_3 from 3-epi-25(OH)D_3.

Certification of SRM 972 Vitamin D in Human Serum

The multiple methods employed at NIST and CDC yielded a wealth of data to be used for value assignment of SRM 972, and in general the results were in good agreement. The top section of Table 1 provides a summary of the results for measurement of 25(OH)D_3 using the various methods. One fundamental question needed to be resolved, however, before the data could be combined and the expanded measurement uncertainties evaluated. One of the NIST LC-MS methods chromatographically resolved the 3-epi-25(OH)D_3 from 25(OH)D_3 and the other did not. The CDC LC-MS/MS method also did not account for the presence of 3-epi-25(OH)D_3 in any of the four levels of SRM 972. However, the NIST LC-MS/MS method quantified 3-epi-25(OH)D_3 in all four levels of SRM 972, and this value could be used to adjust or correct the other data sets for the presence of 3-epi-25(OH)D_3. While this approach may seem less than ideal, failure to account for this known difference between methods seemed even less justifiable when assigning values to a CRM. Ultimately we elected to use the concentrations of 3-epi-25(OH)D_3 determined by the NIST LC-MS/MS method to adjust the 25(OH)D_3concentrations determined by the NIST LC-MS method employing a C_{18} column and those determined by the CDC LC-MS/MS method. A summary of the approach taken is given in Figure S2. In addition, the CDC results for Level 4 were not used in the value assignment of this level. A summary of the adjusted data for 25(OH)D_3, as well as the certified values for 25(OH)D_3 in each of the four levels of SRM 972, is shown in the lower section of Table 1. As can be seen in the table, there is excellent agreement among the methods after the adjustment for the presence of 3-epi-25(OH)D_3.

The method-specific results for 25(OH)D_2 are shown in Table 2. The concentrations of 25(OH)D_2 were too low in Levels 1, 2, and 4 to be quantified reliably by LC-MS using the cyano column. Therefore this data was not included in the assignment of the certified and reference values for this analyte. Because the corresponding 3-epimer, 3-epi-25(OH)D_2, was not detected in any of the four levels of SRM 972, the combination of measurements to obtain the certified or reference values for 25(OH)D_2 was relatively straightforward, and no correction of the data was necessary.

The results from the method-specific sets of measurements were combined using equal weighting for the method means. Because the measurements from the two NIST LC-MS methods (C_{18} and cyano columns) were made using the same sample preparation, these were not completely independent methods. Therefore an average value was calculated based upon the adjusted C_{18} results (corrected for the 3-epimer) and the measurements using the cyano column. Hence, for the majority of the certified values for 25(OH)D_3, three data sets (NIST LC-MS, NIST LC-MS/MS, and CDC LC-MS/MS) were combined in the value assignment. This process is summarized in Figure S2. Calculation of the expanded uncertainties for each of the certified and reference values also included consideration of Type B contributions to the overall uncertainty, and were calculated according to the method described in the ISO Guide.[30,31]

Table 3 provides a summary of the certified and reference values for 25(OH)D_2, 25(OH)D_3, and 3-epi-25(OH)D_3 in SRM 972. Certified values were assigned for 25(OH)D_3 in all four levels of SRM 972. Because of the somewhat higher uncertainty associated with the determination of 25(OH)D_2 in Level 1, this concentration is a reference value. The concentration of 3-epi-25(OH)D_3 in Levels 1, 2, and 3 was determined using a single method (NIST LC-MS/MS), and these concentrations are also reported as reference values. As shown in Table 3, it was possible to assign values to these analytes at concentrations

near, or below 1 ng/g. The certified value for 3-epi-25(OH)D_3 in Level 4 was assigned using data from the NIST LC-MS (cyano column) and NIST LC-MS/MS methods.

CONCLUSIONS

SRM 972 represents the first CRM developed for determination of vitamin D metabolites in human serum. This reference material provides a mechanism to ensure measurement accuracy and comparability and represents a first step toward standardization of 25(OH)D measurements. In addition, the agreement among the analytical methods, which included different sample preparation schemes, chromatographic separations, and detection methods, provides a high level of confidence in the assigned values for vitamin D metabolites in this SRM. Although a commutability study has not been performed for this SRM, the comparable results for the methods employed suggest that this SRM is commutable with higher-order methods. The results of this work also provided evidence that the potential presence of the 3-epimers of 25(OH)D should be considered for both adult and pediatric samples in order to avoid measurement bias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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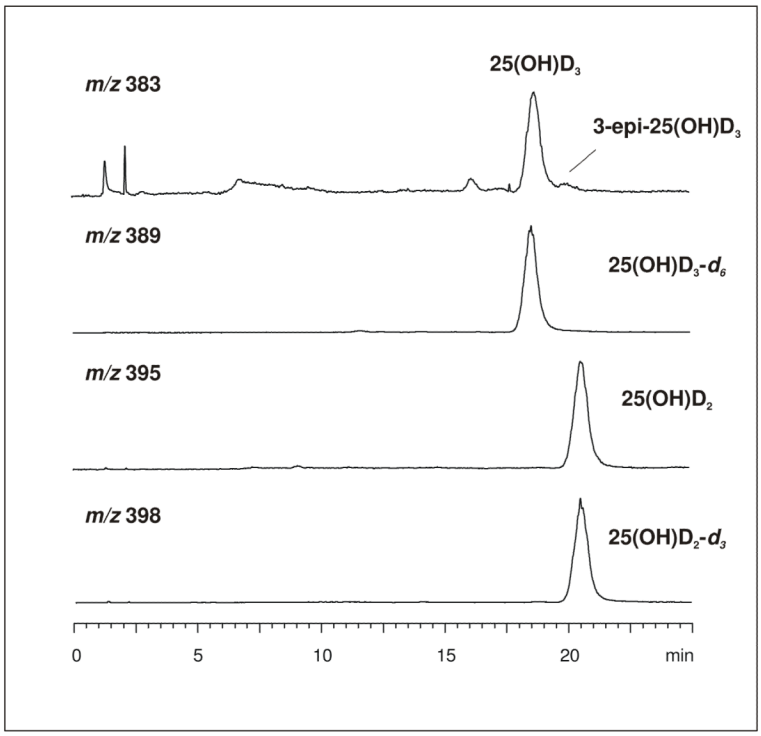

Figure 1.
Selected ion chromatograms (LC-MS) for vitamin D metabolites and their labeled internal standards in Level 3 of SRM 972. Experimental details are provided in the text.

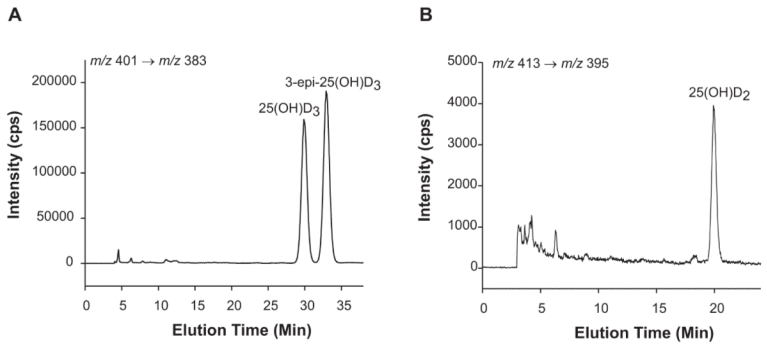

Figure 2.

LC-MS/MS multiple reaction monitoring chromatograms for Level 4 of SRM 972. Measurements of 25(OH)D_3 and 3-epi-25(OH)D_3 were made using a Zorbax CN column (A) and measurements of 25(OH)D_2 were made using a Zorbax XDB-C_{18} column (B). Further experimental details are provided in the text.

Table 1

Method-specific results (ng/g) for measurement of 25-hydroxyvitamin D3 in SRM 972. Relative standard deviations (% RSD) for the measurements are given in parentheses.

Initial results	LC-MS (NIST C_{18})	LC-MS (NIST cyano)	LC-MS/MS (NIST)	LC-MS/MS (CDC)
Level 1	24.26 (2.7)	22.33 (3.0)	23.48 (0.6)	24.90 (4.9)
Level 2	12.25 (2.1)	11.5 (5.0)	12.18 (0.6)	13.15 (4.0)
Level 3	18.45 (1.7)	16.91 (2.3)	18.18 (0.5)	19.91 (4.5)
Level 4		32.00 (1.9)	32.67 (1.2)	

Adjusted data*	LC-MS (NIST C_{18})	LC-MS (NIST cyano)	LC-MS/MS (NIST)	LC-MS/MS (CDC)	Certified value
Level 1	22.95	22.33	23.48	23.55	23.2 ± 0.8
Level 2	11.55	11.50	12.18	12.4	12.0 ± 0.6
Level 3	17.46	16.91	18.18	18.83	18.1 ± 1.1
Level 4		32.00	32.67		32.3 ± 0.8

* Levels were corrected for contributions from unresolved 3-epi-25(OH)D3 based on NIST LC-MS/MS measurements

Table 2

Method specific results (ng/g) for measurement of 25-hydroxyvitamin D2 in SRM 972. Relative standard deviations (% RSD) are given in parentheses.

	LC-MS (NIST C_{18})	LC-MS (NIST cyano)	LC-MS/MS (NIST)	LC-MS/MS (CDC)
Level 1	0.77 (20)	-*	0.49 (3.0)	0.50 (44)
Level 2	1.71 (10)	-	1.63 (2.4)	2.17 (10)
Level 3	24.61 (1.0)	24.97 (3.1)	25.10 (0.4)	27.53 (8.6)
Level 4	2.32 (3.9)	-	2.21 (1.1)	2.52 (8.9)

*Concentrations of 25(OH)D2 in these levels were too low to be quantified reliably

Table 3

Summary of certified and reference values (ng/g) for SRM 972. Certified values are shown in bold.

	25(OH)D_2	**25(OH)D_3**	**3-epi-25(OH)D_3**
Level 1	0.59 ± 0.20	**23.2 ± 0.8**	1.35 ± 0.04
Level 2	**1.67 ± 0.08**	**12.0 ± 0.6**	0.74 ± 0.02
Level 3	**25.8 ± 1.9**	**18.1 ± 1.1**	1.04 ± 0.03
Level 4	**2.35 ± 0.21**	**32.3 ± 0.8**	**36.9 ± 1.1**