Published in final edited form as:

Anal Chem. 2017 May 02; 89(9): 4907–4913. doi:10.1021/acs.analchem.6b05168.

Development of an Improved Standard Reference Material for Vitamin D Metabolites in Human Serum^a

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Supporting information Available: Calibration and analysis procedures for the NIST LC-MS, NIST LC-MS/MS, and CDC LC-MS/MS methods used in the value assignment of Standard Reference Material (SRM) 972a Vitamin D Metabolites in Human Serum; specific information on chromatographic columns and internal standards used for each of the three methods (Table S-1); description of the procedures used in the commutability assessment.

The Supporting Information is available free of charge via the Internet at <http://pubs.acs.org>

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Abstract

The National Institute of Standards and Technology (NIST) has developed Standard Reference Material (SRM) 972a Vitamin D Metabolites in Frozen Human Serum as a replacement for SRM 972, which is no longer available. SRM 972a was developed in collaboration with the National Institutes of Health's Office of Dietary Supplements. In contrast to the previous reference material, three of the four levels of SRM 972a are comprised of unmodified human serum. This SRM has certified and reference values for the following 25-hydroxyvitamin D [25(OH)D] species: 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃. The value assignment and certification process included three isotope-dilution mass spectrometry approaches, with measurements performed at NIST and at the Centers for Disease Control and Prevention (CDC). The value assignment methods employed have been modified from those utilized for the previous SRM, and all three

approaches now incorporate chromatographic resolution of the stereoisomers, 25(OH)D₃ and 3-epi-25(OH)D₃.

Graphical Abstract

INTRODUCTION

Vitamin D has a well-established role in bone health, and vitamin D deficiency leads to conditions such as rickets and osteomalacia.¹ Although the Institute of Medicine (IOM) recently issued new guidance on recommended dietary intakes of vitamin D,² the debate over optimal dietary intake or sun exposure has continued.^{3, 4} This is, in part, because the vitamin D exposure required for bone health may differ from that needed for other non-skeletal health outcomes.⁵ Vitamin D receptors have been identified in many tissues throughout the body, suggesting that vitamin D may play a much broader role in overall health.⁶

Vitamin D, as either vitamin D₂ or vitamin D₃, is considered a pro-hormone because it must be metabolized to become biologically active.⁷ The initial metabolic step for both vitamin D₂ and D₃ is hydroxylation, primarily in the liver, to form the corresponding metabolites, 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ [25(OH)D₂ and 25(OH)D₃]. Although these metabolites may have some effect on calcium absorption,⁸ the primary active metabolite of vitamin D is 1,25(OH)₂D, which results from an additional hydroxylation step that occurs primarily in the kidneys. Measurement of circulating 25(OH)D is considered the best indicator of vitamin D exposure because it takes into account both dietary sources of vitamin D as well as that arising from sun exposure.^{9–10} In addition, the half-life of this metabolite is sufficiently long (≈15 days) for meaningful measurement, whereas the half-life of 1,25(OH)₂D is typically less than 24 h.¹¹ Because both vitamin D₂ and D₃ undergo similar metabolism, measurement of 25(OH)D should incorporate both 25(OH)D₂ and 25(OH)D₃.

Quantitative assays for 25(OH)D can generally be divided into chromatographic methods, including those with mass spectrometric (MS) detection, and techniques based upon competitive immunoassays. Several recent reviews provide details on the performance characteristics of these methods.^{12–15} Concerns about the prevalence of vitamin D deficiency as well as widespread media attention have fueled dramatic increases in the

demand for 25(OH)D testing. There have been ongoing concerns, however, about the accuracy and comparability of 25(OH)D assays.¹⁶ Such potential inconsistencies can limit the value of 25(OH)D assays in identifying those who are vitamin D deficient as well as trigger treatment for individuals who do not need it.¹⁷ Discrepancies between assay methods or changes in the performance of a single assay (assay drift) over time are also problematic for long-term research studies into the potential benefits of vitamin D supplementation and for population survey data used in public health policy decisions.^{18–20}

In order to address the recognized need for standardization of 25(OH)D measurements,^{21–22} NIST introduced Standard Reference Material (SRM) 972 Vitamin D in Frozen Human Serum in 2009.²³ This material represented the first available certified reference material (CRM) for assessment of the accuracy of methods for the determination of 25(OH)D and was developed in collaboration with the National Institutes of Health's Office of Dietary Supplements (NIH-ODS). This reference material consisted of four levels, each having different concentrations of vitamin D metabolites. Although this material represented an initial step toward standardization of 25(OH)D measurements, it did have some limitations. Only one of the four levels of SRM 972 was comprised of unmodified human serum. Two of the levels were fortified with exogenous compounds [25(OH)D₂ or 3-epi-25(OH)D₃] and one level was diluted with horse serum to obtain a lower concentration of 25OHD₃. These techniques were successful in achieving the desired metabolite concentrations, but the presence of non-human serum and the addition of exogenous metabolites seemed to pose problems for certain 25(OH)D assays.^{17, 24–25}

Despite the limitations of SRM 972, the inventory of this SRM was rapidly depleted, and therefore design of a replacement material was initiated. Design of the new material, SRM 972a Vitamin D Metabolites in Frozen Human Serum, was guided by lessons learned from the first material. In addition, further work was done at NIST and at the Centers for Disease Control and Prevention (CDC, Atlanta, GA) to optimize the analytical methods used in measurement of 25(OH)D in serum. This new reference material represents a continued collaboration with NIH-ODS through their Vitamin D Initiative. Details of the preparation of the new material and approaches to value assignment are described in this manuscript, with an emphasis on improvements made since the initial reference material was issued.

EXPERIMENTAL SECTION

Disclaimer—The authors declare no competing financial interests. 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. The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the official views or positions of the Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry, the National Institutes of Health, or the Department of Health and Human Services

Safety Considerations—The human sera used in the preparation of SRM 972a Vitamin D Metabolites in Frozen Human Serum were screened and found to be nonreactive 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.²⁶

NIST LC-MS and LC-MS/MS Methods

Materials—Standards for 25(OH)D₂ and 3-epi-25(OH)D₃ were obtained from IsoSciences (King of Prussia, PA). 25-Hydroxyvitamin D₃ was obtained from the U.S. Pharmacopeia (USP, Rockville, MD). The purity of each of these materials was evaluated at NIST,²⁷ and analytical results were corrected for the purity of the standards. Stable isotope-labeled internal standards, 25(OH)D₂-d₃, 25(OH)D₃-d₆, and 3-epi-25(OH)D₃-d₃, were obtained from IsoSciences, Medical Isotopes, Inc. (Pelham, NH), and Cerilliant (Round Rock, TX).

Sample Preparation for LC-MS—Samples of SRM 972a, SRM 972 (used as a quality control material with each set of samples), and the internal standard solution were removed from the freezer, allowed to reach room temperature, and swirled gently to mix. Preparation of the internal standard and calibration solutions is described in the Supporting Information. For each sample, approximately 110 mg (150 µL) of internal standard solution was accurately weighed into an 8 mL glass tube with a screw cap, and an additional 250 µL of 2-propanol:methanol (20:80, volume fractions) was added to the tube. Approximately 450 mg of serum (exact mass known) was added to the tube, and the tube was vortex-mixed and allowed to stand for 5 min. The sample was extracted twice with hexane (2 mL), followed by combining the extracts and evaporation to dryness at 40 °C under nitrogen.²⁸ The residue was reconstituted with 200 µL methanol and vortex-mixed.

Analysis by LC-MS—Measurements of vitamin D metabolites were performed on an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC coupled to an SL series mass spectrometric detector. Atmospheric pressure chemical ionization (APCI) was employed in the positive ion mode with the following parameters: drying gas flow, 5.0 L/min; nebulizer pressure, 0.345 MPa (50 psi); drying gas temperature, 350 °C; capillary voltage, +3600 V; corona current, 4 µA; fragmentor voltage, 150 V; and gain, 2. The vaporizer temperature was either 350 °C or 400 °C, depending upon the LC flow rate used. The ions monitored for quantification included *m/z* 383 for 25(OH)D₃ and 3-epi-25(OH)D₃; *m/z* 386 for 3-epi-25(OH)D₃-d₃; *m/z* 389 for 25(OH)D₃-d₆; *m/z* 395 for 25(OH)D₂; and *m/z* 398 for 25(OH)D₂-d₃. All samples and calibrants were analyzed with two sets of chromatographic conditions that resolved the diastereomers 25(OH)D₃ and 3-epi-25(OH)D₃. The first method utilized an Ascentis Express F5 column from Supelco (Bellefonte, PA) with dimensions of 150 mm × 4.6 mm ID and 2.7 µm particles. The column was maintained at a temperature of 15 °C, and analyses were performed under isocratic conditions with a water:methanol (26:74, volume fractions) mobile phase at a flow rate of 0.8 mL/min. The second method utilized a Zorbax SB-CN column (Agilent Technologies) that was 250 mm × 4.6 mm ID with 5 µm particles. The column temperature was maintained at 45 °C, and analyte elution

was achieved under isocratic conditions with a water:methanol (33:67, volume fractions) mobile phase and a flow rate of 1.0 mL/min. Additional details on the chromatographic conditions are provided in the Supporting Information. Quantitation was performed using averaged response factors for each analyte. Although limits of quantitation (LOQs) were not explicitly determined for this method, results observed during use of the method indicated that LOQs for 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃ were consistent with those of the other two methods (0.6 ng/mL).

Sample Preparation for LC-MS/MS—Serum samples (1 g to 2 g) were weighed into a 50 mL glass centrifuge tube. At least one level of SRM 972 was used as a quality control material with each set of samples. Each sample was spiked (exact mass known) with an appropriate amount of the internal standard solution (see Supporting Information) to achieve an approximately 1:1 ratio of analyte to internal standard. After equilibration 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). Analytes were isolated from the serum matrix by liquid-liquid extraction with 8 mL hexane:ethyl acetate (50:50, volume fractions).²⁹ Samples were extracted twice, and the extracts were combined and dried under nitrogen at 45 °C. The residue was reconstituted with methanol (200 µL to 250 µL).

Analysis by LC-MS/MS—Measurements were performed on an Applied Biosystems (Framingham, MA) API 4000 LC-MS/MS system coupled to an Agilent 1100 Series LC. Atmospheric pressure chemical ionization (APCI) in the positive ion mode and multiple reaction monitoring (MRM) mode were used, and the following transitions were monitored for quantification: m/z 401 → m/z 383 for 25(OH)D₃ and 3-epi-25(OH)D₃; m/z 407 → m/z 389 for 25(OH)D₃-d₆; m/z 404 → m/z 386 for 3-epi-25(OH)D₃-d₃; m/z 413 → m/z 395 for 25(OH)D₂; and m/z 416 → m/z 398 for 25(OH)D₂-d₃. Additional instrument parameters and a description of the procedures used in preparing calibration solutions are provided in the Supporting Information. Samples and calibrants were analyzed using either an Ascentis Express F5 (15 cm × 4.6 mm, 2.7 µm particle diameter) or a Zorbax SB-CN column (25 cm × 4.6 mm, 5 µm particle diameter) under isocratic conditions with water:methanol mobile phases. A shorter cyano column (15 cm × 4.6 mm, 3.5 µm particle diameter) was used for measurement of 3-epi-25(OH)D₃ in SRM 972a and for 25(OH)D₃ in Level 3 of the SRM. The LOQs for 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃ were all 0.6 ng/mL. Further details on the chromatographic conditions are provided in the Supporting Information.

CDC LC-MS/MS Method

Materials—Bovine serum albumin (96%), phosphate-buffered saline (PBS), 25(OH)D₂ and 25(OH)D₃ were obtained from Sigma (St. Louis, MO). 3-Epi-25(OH)D₃ was obtained from IsoSciences. Stable isotope-labeled internal standards, 25(OH)D₃-d₆ and 3-epi-25(OH)D₃-d₃ were obtained from Medical Isotopes, while 25(OH)D₂-d₃ was obtained from IsoSciences.

Sample Preparation for LC-MS/MS—A Hamilton Microlab Starlet (Reno, NV) workstation was used for sample preparation. Preparation of the internal standard solution is described in the Supporting Information. Serum samples (100 µL) were added to 13 × 100

mm disposable borosilicate glass culture tubes, the internal standard solution (75 μ L) was added, and the tubes were mixed for 30 s. A portion (100 μ L) of methanol:water (69:31, volume fractions) was added to the tube, and the contents were mixed again for 30 s. Liquid-liquid extraction was performed by adding hexane (1.5 mL) to each tube, covering the tube with Parafilm, vortexing for 3 min, and allowing the contents to rest for 1 min. Three cycles of the mixing and rest periods were performed, for a total of 9 min mixing. The tubes were centrifuged at 1,000 $\times$ g for 5 min, and 1 mL of the supernatant in each tube was aspirated and transferred to a 96-well plate. The plate was dried under nitrogen at 25 $^{\circ}$ C, followed by reconstitution of each well with 300 μ L methanol:water (69:31, volume fractions). The reconstituted plate was sealed with a silicone cover, shaken gently on a plate shaker (10 min), and transferred to the thermostatted (7 $^{\circ}$ C) chromatographic autosampler.

Analysis by LC-MS/MS—Measurements were performed on an Accela UHPLC system coupled to a TSQ Vantage mass spectrometer (ThermoElectron Corp., West Palm Beach, FL). Analytes were eluted under isocratic conditions with a methanol:water (69:31, volume fractions) mobile phase and a flow rate of 0.4 mL/min. The chromatographic column was a Thermo Hypersil GOLD pentafluorophenyl (PFP) column maintained at 28 $^{\circ}$ C. Atmospheric pressure chemical ionization (APCI) was employed in the positive ion mode using a published method³⁰ with minor modifications to accommodate a more specific internal standard, namely, 3-epi-25(OH)D₃-d₃. Two transitions per vitamin D metabolite along with one transition per internal standard were monitored: 25(OH)D₃: m/z 383 $\rightarrow$ m/z 365 and m/z 383 $\rightarrow$ m/z 105; 25(OH)D₃-d₆: m/z 389 $\rightarrow$ m/z 371; 3-epi-25(OH)D₃: m/z 383 $\rightarrow$ m/z 365 and m/z 383 $\rightarrow$ m/z 105; 3-epi-25(OH)D₃-d₃: m/z 386 $\rightarrow$ m/z 368; 25(OH)D₂: m/z 395 $\rightarrow$ m/z 377 and m/z 395 $\rightarrow$ m/z 209; 25(OH)D₂-d₃: m/z 398 $\rightarrow$ m/z 380. Analytes were quantitated using six-point linear calibration curves, which were traceable to SRM 2972 for 25(OH)D₂ and 25(OH)D₃, and internal standards were used to correct for recovery. Preparation of the calibration solutions is described in the Supporting Information. The LOQs for 25(OH)D₂ and 25(OH)D₃ were 0.6 ng/mL. The LOQ for 3-epi-25(OH)D₃ was not determined.

RESULTS AND DISCUSSION

Preparation of SRM 972a

A primary objective of developing a reference material for vitamin D metabolites is to improve the accuracy and comparability of routine measurement methods for these analytes in serum. As noted earlier, some aspects of the design of the initial reference material, SRM 972, proved to be problematic and limited the potential impact of the SRM. The use of non-human serum as a diluent to achieve lower concentrations of vitamin D metabolites and employing fortification (spiking) to achieve higher concentrations reduced the utility of some of the levels of SRM 972 for immunoassay users.¹⁷ Studies done through the Vitamin D External Quality Assessment Scheme (DEQAS) and the NIST/NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP) have indicated that exogenous 25(OH)D₂ is not recovered to the same extent by immunoassays as endogenous 25(OH)D₂.^{25, 31} One possible explanation for this behavior is that exogenous 25(OH)D is incorporated into the serum matrix by binding to various components including albumin and

vitamin D binding protein (DBP). In contrast, endogenous 25(OH)D is primarily bound to the DBP. Assays that rely upon the dissociation of metabolites from the DBP may therefore yield very different results for exogenous 25(OH)D than chromatographic methods which incorporate an extraction step and denature serum proteins, thereby liberating any protein-bound 25(OH)D.

The new SRM was designed with these limitations in mind, and three of the four levels of SRM 972a contain naturally occurring concentrations of all vitamin D metabolites. SRM 972a was prepared by Solomon Park Research Laboratories (Kirkland, WA) according to specifications provided by NIST. Level 1 and Level 2 contain primarily 25(OH)D₃ but the concentration is lower in Level 2. The total 25(OH)D concentration [25(OH)D₂ + 25(OH)D₃] was intended to fall within the “adequate” health status range (≥ 20 ng/mL) for Level 1 and within the “inadequate” range (12 ng/mL to 20 ng/mL) for Level 2, as defined by the IOM guidelines. Level 3 contains both 25(OH)D₂ and 25(OH)D₃ at concentrations greater than 10 ng/mL. This level possesses the highest total 25(OH)D concentration.

Level 4 of SRM 972a was prepared by fortifying human serum with 3-epi-25(OH)D₃ because of the difficulty in obtaining the required volume of serum with naturally high (> 10 ng/mL) concentrations of this metabolite. The 3-epimers of 25(OH)D are potential sources of measurement bias in mass spectrometry-based methods because they have the same molecular mass as the non-epimeric forms, exhibit similar chromatographic retention, and produce identical mass fragmentation patterns. Because 25(OH)D₃ is generally the dominant form of 25(OH)D present, more interest has been focused on its 3-epimer. Although an initial report suggested that 3-epi-25(OH)D₃ was only present in serum from infants and neonates, subsequent studies have now confirmed that 3-epi-25(OH)D₃ is nearly always present in adult serum.^{32–33} The biological function, if any, of 3-epi-25(OH)D₃ remains unclear; however, failure to account for the potential presence of 3-epi-25(OH)D₃ can result in overestimation of the concentration of 25(OH)D₃, and the extent of this measurement bias is difficult to predict in advance.^{34–35} Given the expanding use of mass spectrometry-based methods for measurement of 25(OH)D, 3-epi-25(OH)D₃ was considered to be an important aspect of the design of SRM 972a.

Value Assignment Measurements

In addition to changes in the material design, the analytical methods used in the value assignment process were also modified from those used in certification of the previous reference material. As in earlier work, analyses were performed at NIST by LC-MS²⁸ and LC-MS/MS²⁹ and at CDC by LC-MS/MS.³⁰ All three approaches incorporated isotopically labeled internal standards for quantification of the vitamin D metabolites of interest (Table S-1, Supporting Information). The NIST LC-MS/MS method is recognized as a reference measurement procedure (RMP) by the Joint Committee for Traceability in Laboratory Medicine (JCTLM), meaning that accuracy, precision, and potential interferences have been thoroughly investigated.

Method modifications included the incorporation of an alternate chromatographic column to resolve 3-epi-25(OH)D₃ from 25(OH)D₃. A cyanopropyl (CN) stationary phase was initially used at NIST in both the LC-MS and LC-MS/MS methods to resolve 3-epi-25(OH)D₃ from

25(OH)D₃. Although this approach was successful, the analysis time was nearly one hour, and baseline resolution was difficult to obtain. An alternative approach utilizing a pentafluorophenyl (PFP) stationary phase was evaluated and found to yield baseline resolution of the two stereoisomers while also reducing the analysis time.²⁸ Figure 1 illustrates the separation of 25(OH)D₃ and 3-epi-25(OH)D₃ on the PFP column for Level 1 of SRM 972a. With the exception of Level 1 of SRM 972a, both the PFP and CN phases were employed in NIST's measurements performed by LC-MS (Table S-1). The use of stationary phases with differing selectivity reduces the likelihood of measurement bias arising from undetected interferences.³⁶ Both PFP and CN columns were also utilized in the NIST LC-MS/MS measurements (see Table S-1, Supporting Information). In particular, a small interference precluded the use of the PFP column in quantification of 25(OH)D₃ in Level 2 of the SRM. Figure 2 illustrates the NIST LC-MS/MS results for the determination of 25(OH)D₃ in Level 3 of SRM 972a. A shorter (15 cm) CN stationary phase with smaller particles (3.5 μm) was employed in these measurements, yielding reduced analysis time while preserving the separation between 25(OH)D₃ and its 3-epimer.

The other change made in the NIST methods was in quantification of 3-epi-25(OH)D₃. During development of SRM 972, no isotope-labeled internal standard was available for this analyte, and therefore it was quantified using the labeled form of 25(OH)D₃ because of its structural similarity and comparable chromatographic retention. In the present work, an isotopically labeled form of 3-epi-25(OH)D₃ was used to quantify it in the NIST LC-MS measurements for all four levels of SRM 972a. For the NIST LC-MS/MS measurements of 3-epi-25(OH)D₃ in Levels 1, 2, and 3, a small interference coeluted with the 3-epi-25(OH)D₃-d₃ internal standard, and therefore the analyte was quantified with 25(OH)D₃-d₆ as the internal standard.

The LC-MS/MS method originally developed by CDC for 25(OH)D and incorporated in value assignment of SRM 972 did not resolve 25(OH)D₃ from its 3-epimer.³⁷ The method was developed prior to reports that the presence of the 3-epimer could be anticipated in nearly all samples. Because the CDC method is used in analysis of samples for a national nutrition survey, the occurrence of measurement bias arising from the 3-epimer could affect estimations of the prevalence of vitamin D sufficiency in the U.S. population. Following the recommendations of an expert panel,¹⁸ CDC revised its LC-MS/MS method to include measurement of 3-epi-25(OH)D₃, and this method was employed during value assignment of SRM 972a. The modified CDC method utilizes a PFP stationary phase to achieve chromatographic resolution of 25(OH)D₃ and 3-epi-25(OH)D₃.³⁰ In the measurements performed by CDC, 3-epi-25(OH)D₃ was quantified by using 3-epi-25(OH)D₃-d₃ as the internal standard.

Certification of SRM 972a

Values from the three basic approaches employed (NIST LC-MS, NIST LC-MS/MS, CDC LC-MS/MS) were combined to obtain the certified and reference values for SRM 972a. Results for the determination of 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃ by each method are shown in Table 1. At least one level of the previous reference material, SRM 972, was analyzed as a control material at NIST in each set of LC-MS or LC-MS/MS

analyses of the new reference material. Results from the control material were used to guide selection of appropriate data to be used in statistical analysis of the data and assignment of certified and reference values and expanded uncertainties for the new SRM 972a. If results for the control material were not consistent with the assigned values and uncertainties on the Certificate of Analysis for SRM 972, that set of data was eliminated from further consideration. For Level 1 of the SRM, only the PFP column was used in the LC-MS measurements.

Comparison of the method-specific values in Table 1 reveals the excellent consistency in results among the analytical methods employed. The resulting certified and reference values for SRM 972a are shown in Table 2. 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.^{38–39} A detailed description of the uncertainty determinations is provided in the Certificate of Analysis for SRM 972a. Values for the previous material, SRM 972, are shown in Table 2 for comparison.

During certification of SRM 972, only one method (NIST LC-MS/MS) was available for the determination of 3-epi-25(OH)D₃ in three of the four levels that comprised the SRM.²³ Results from this method were used to adjust the results obtained from methods that did not resolve the 3-epimer. While this approach yielded good agreement among the methods, it also introduced greater uncertainty into the final values. For SRM 972a, data for the 3-epimer was available from all three methods, and certified values are now provided for 3-epi-25(OH)D₃ in three of the four levels. The relative expanded uncertainty for 3-epi-25(OH)D₃ in Level 3 is > 10%, and we elected to make this a reference value. The concentration of 25(OH)D₂ in Levels 1 and 4 is very close to the limit of quantitation for the NIST LC-MS/MS method, and therefore these values are also considered reference values.

Commutability

A reference material prepared from minimally processed human serum should have properties similar to those of serum samples encountered in routine assays for 25(OH)D and can serve as an accuracy or “trueness” control for those assays. However, commutability with these routine methods cannot be assumed because each assay may respond differently to matrix alterations such as pooling of samples, spiking of exogenous components, or other modifications.^{40–41} Commutability of SRM 972a was evaluated as part of the Vitamin D Standardization Program (VDSP), an international effort to standardize the assessment of vitamin D status.²⁰ The four levels of SRM 972a were analyzed by participating laboratories at the same time as 50 single donor patient samples (see Supporting Information). These results were then compared to values obtained by the NIST RMP for the same samples, following the general guidance from the Clinical Laboratory Standards Institute (CLSI) for assessing commutability (C53-A). The complete results of the study will be published elsewhere, but a subset of the data is presented here to illustrate the scope of applicability for this new SRM.

Figure 3 illustrates the results observed for two measurement techniques for total 25(OH)D as compared to the results obtained by the reference method, the NIST RMP. In this study,

the total 25(OH)D concentration as determined by the RMP included only the sum of the 25(OH)D₂ and 25(OH)D₃ concentrations. Level 2 of SRM 972a contains the lowest concentration of total 25(OH)D (see Table 2), and Level 3 has the highest total 25(OH)D concentration. The results in Figure 3A show the data obtained by the Health Canada laboratory with the DiaSorin Liaison automated 25(OH)D immunoassay platform (see Supporting Information), and the results in 3B demonstrate the results obtained by CDC with their LC-MS/MS method. As shown in Figure 3A, the data points for all four levels of SRM 972a lie within the prediction interval (Level 1 and Level 4 are nearly superimposed), suggesting that all four levels of the SRM are commutable with this particular type of assay.

In Figure 3B, the prediction interval is much narrower than in Figure 3A, which likely reflects the improved specificity of LC-MS/MS methods when compared to immunoassays for 25(OH)D. The CDC LC-MS/MS method also shares characteristics with the NIST RMP because it includes chromatographic separation of 25(OH)D₃ from its 3-epimer. As shown in Figure 3B, the data points for three of the four levels of SRM 972a are clearly within the prediction interval (Level 1 and Level 4 are nearly superimposed), and the data point for Level 3 is just at the edge of the prediction interval. These results indicate that SRM 972a is also commutable with this LC-MS/MS method.

CONCLUSIONS

Research into the link between vitamin D and health is continuing, as are studies to identify optimal vitamin D exposure and inform public health policies. SRM 972a serves as a foundation for these studies, by helping to ensure the accuracy of clinical and research laboratory results and serving as a key element in international standardization efforts. At the same time, reliable 25(OH)D measurements are essential for identifying individuals affected by vitamin D deficiency. SRM 972a represents a significant improvement over its predecessor in terms of its design and applicability, and it can serve as an anchor point for new assay methodologies as they are developed and implemented.

Supplementary Material

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Figure 1. Analysis of SRM 972a Level 1 by the NIST LC-MS method with a pentafluorophenyl (PFP) stationary phase.

Figure 2.
Analysis of Level 3 of SRM 972a by the NIST LC-MS/MS method with a 15 cm cyano column.

3A)

3B)

Figure 3.

Results from the DiaSorin Liaison immunoassay in the Health Canada laboratory (3A) and the CDC LC-MS/MS method (3B) as compared to values determined by the NIST reference measurement procedure. All values in nmol/L (values can be converted to ng/mL by dividing by ≈ 2.5). Results for the 50 single donor patient samples are represented by open circles.

Table 1

Method specific results for vitamin D metabolites in SRM 972a (ng/g). Relative standard deviations (% RSD) are given in parentheses.

25(OH)D₂	CDC	LC-MS PFP	LC-MS CN	LC-MS/MS
Level 1	0.58 (20)	0.53 (7.3)	-	0.47 (4.7)
Level 2	0.93 (14)	0.80 (5.4)	0.77 (3.1)	0.76 (1.1)
Level 3	13.1 (5.6)	13.22 (3.8)	12.72 (1.2)	13.05 (0.4)
Level 4	0.66 (16)	0.57 (8.3)	0.47 (5.5)	0.48 (5.2)
25(OH)D₃	CDC	LC-MS PFP	LC-MS CN	LC-MS/MS
Level 1	28.0 (3.9)	27.46 (1.4)	-	28.88 (0.5)
Level 2	17.5 (3.6)	-	17.60 (1.3)	17.76 (0.9)
Level 3	19.4 (1.9)	18.68 (4.6)	19.66 (0.7)	19.39 (0.6)
Level 4	28.0 (5.2)	28.70 (1.6)	28.52 (1.2)	29.28 (0.5)
3-epi-25(OH)D₃	CDC	LC-MS PFP	LC-MS CN	LC-MS/MS
Level 1	1.84 (13)	1.74 (5.2)	-	1.83 (1.2)
Level 2	1.39 (20)	1.02 (4.1)	1.46	1.24 (0.9)
Level 3	1.25 (17)	0.77 (9.8)	1.34 (6.9)	1.04 (1.4)
Level 4	25.3 (11)	25.33 (3.9)	26.55 (3.6)	25.68 (3.4)

Table 2

Certified (in bold) and reference values for vitamin D metabolites in SRM 972a. Values for the previous material, SRM 972, are shown for comparison. All results are given in ng/mL.

Analyte	SRM	Level 1	Level 2	Level 3	Level 4
25(OH)D ₃	972a	28.8 ± 1.1	18.1 ± 0.4	19.8 ± 0.4	29.4 ± 0.9
	972	23.9 ± 0.8	12.3 ± 0.6	18.5 ± 1.1	33.0 ± 0.8
25(OH)D ₂	972a	0.54 ± 0.06	0.81 ± 0.06	13.3 ± 0.3	0.55 ± 0.10
	972	0.60 ± 0.20	1.71 ± 0.08	26.4 ± 2.0	2.40 ± 0.21
3- <i>epi</i> -25(OH)D ₃	972a	1.81 ± 0.10	1.28 ± 0.09	1.17 ± 0.14	26.0 ± 2.2
	972	1.39 ± 0.04	0.76 ± 0.02	1.06 ± 0.03	37.7 ± 1.2