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Development of a Candidate Reference Measurement Procedure for the Determination of 25-Hydroxyvitamin D_3 and 25-Hydroxyvitamin D_2 in Human Serum Using Isotope-Dilution Liquid Chromatography−Tandem Mass Spectrometry

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Vitamin D exists in two major forms, vitamin D_3 and vitamin D_2. Vitamin D helps the body absorb calcium and promote optimal bone health. Both forms of vitamin D are metabolized to 25-hydroxyvitamin D in the body, and the levels of 25-hydroxyvitamin D_3 [25(OH)D_3] and 25-hydroxyvitamin D_2 [25(OH)D_2] in serum are considered the best indicators of vitamin D status. A candidate reference measurement procedure for serum 25(OH)D_3 and 25(OH)D_2 has been developed and critically evaluated. The deuterated compounds 25(OH)D_3-d_3 and 25(OH)D_2-d_3 are used as internal standards for 25(OH)D_3 and 25(OH)D_2, respectively. The 25(OH)D_3 and 25(OH)D_2 and their respective labeled internal standards are simultaneously extracted from serum using liquid−liquid extraction prior to reversed-phase liquid chromatography−tandem mass spectrometry (LC−MS/MS). Chromatographic separation was performed using a cyano (CN) column for both 25(OH)D_3 and 25(OH)D_2. Atmospheric pressure chemical ionization (APCI) in the positive ion mode and multiple reaction monitoring (MRM) were used for LC−MS/MS. The accuracy of the method was evaluated by recovery studies of measuring 25-hydroxyvitamin D [25(OH)D] in spiked samples with known 25(OH)D levels. The recoveries of the added 25(OH)D_3 and 25(OH)D_2 ranged from 99.0% to 101.0%. The absolute recoveries with this method were 97% and 92% for 25(OH)D_3 and 25(OH)D_2, respectively. Excellent precision was obtained with between-set coefficients of variation (CVs) of 0.2−0.6% for 25(OH)D levels >1 ng/g and within 2% for the level of <1 ng/g. Chromatographic separation of 25(OH)D_3 and 25(OH)D_2 from their respective isomers 3-epi-25(OH)D_3 and 3-epi-25(OH)D_2 was achieved. The limit of detection at a signal-to-noise ratio of ~3 was 40 pg of 25(OH)D on column (or ~0.15 ng/g as expressed as a concentration). This candidate reference measurement procedure for serum 25(OH)D_3 and 25(OH)D_2 demonstrates good accuracy and precision and low susceptibility to interferences. It can be used to provide an accuracy base to which clinical methods for 25(OH)D_3 and 25(OH)D_2 can be compared and that will serve as a standard of higher order for measurement traceability.

Vitamin D is a fat-soluble vitamin that plays an important role in maintaining calcium homeostasis in the body. Vitamin D, together with calcium, promotes bone formation in children and helps maintain bone strength in adults. In children, vitamin D deficiency can cause skeletal deformities known as rickets. In adults, vitamin D deficiency can lead to osteoporosis.[1] Recent studies suggest that higher serum levels of the main circulating form of vitamin D are associated with substantially lower incidence of breast, colorectal, and prostate cancers.[2] Studies also indicate that vitamin D deficiency may be a potential risk factor for cardiovascular diseases.[3]

Vitamin D exists in two major forms, vitamin D_3 and vitamin D_2. Exposure to sunlight, which triggers vitamin D production in the skin, is the primary source of vitamin D_3 for most individuals. Vitamin D_2 is found in dietary supplements, fortified foods, and certain types of plants. Both forms of vitamin D are metabolized in the liver to 25-hydroxyvitamin D [25(OH)D] and then further metabolized in the kidney to 1,25-dihydroxyvitamin D [1,25(OH)$_2$D].[1]

Vitamin D and its metabolites are primarily bound to the vitamin D binding protein (DBP) in blood, and only 0.03% of the 25(OH)D is in the free (unbound) form.[1] Because vitamin D and 1,25(OH)$_2$D have relatively short half-lives (<2 days), levels of circulating 25(OH)D have been the recommended indicator of vitamin D status.[1,4] Typical concentrations of 25(OH)D in serum are approximately 10−50 ng/mL. Very high levels of 25(OH)D (>200 ng/mL) may be associated with toxicity and adverse health effects.[5]

Measurement of 25(OH)D has been performed using a number of different analytical techniques. Historically, immunoassays have

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been the primary method used for measuring serum 25(OH)D, and they remain the most widely used technique.[6,7] These methods incorporate antibodies that are designed to be specific for the 25(OH)D_2 and/or 25(OH)D_3 species.[8] Liquid chromatographic methods incorporating UV or electrochemical detection have also been reported.[9] More recently, tandem mass spectrometric methods (LC−MS/MS) with high selectivity and specificity have been developed.[10−16] and are increasingly used in clinical laboratories. These methods generally utilize one or more labeled internal standards for quantification of 25(OH)D.

Potential limitations exist for all of the current methodologies. Immunoassays may suffer from nonspecific interferences due to cross-reactivity with other metabolites of vitamin D or unequal assay response to 25(OH)D_3 and 25(OH)D_2.[6,7,11,12] Mass spectrometric-based methods may suffer from interferences arising from other species with the same masses as 25(OH)D. In particular, the presence of the 3-epimers of 25(OH)D can pose problems for LC−MS/MS methods. The 3-epimer of 25(OH)D has been identified in samples from neonates.[17] Because the mass and fragmentation patterns are the same as 25(OH)D, failure to account for these metabolites resulted in overestimation of 25(OH)D_3 and 25(OH)D_2.[8,17] There is a need for critically evaluated reference measurement procedures (RMPs) for serum 25(OH)D to assess the accuracy of the test methods used in the clinical laboratories. RMPs can also be used to assign the concentrations of reference materials which can be used as accuracy controls or calibrators for routine test methods. They provide means for demonstrating traceability of routine test methods and materials to higher-order reference materials. The requirements of RMPs for clinical diagnostic markers have been outlined by the International Organization for Standardization (ISO) in ISO 15193 (in vitro diagnostic systems; measurement of quantities in samples of biological origin; presentation of reference measurement procedures).[18] The Joint Committee for Traceability in Laboratory Medicine (JCTLM)[19] reviews potential RMPs and compiles a list of those that meet the requirements of ISO 15193. Currently, there are no RMPs for serum 25(OH)D_3 and 25(OH)D_2 recognized by the JCTLM.

Recently, the National Institute of Standards and Technology (NIST) developed an isotope-dilution (ID) method coupled with LC−MS/MS for the determination of serum 25(OH)D_3 and 25(OH)D_2. This method was critically characterized. All of the requirements for an RMP recognized by the JCTLM have been met except for validation by an interlaboratory study which is currently being planned. The results from this study will complete the requirements for a RMP recognized by the JCTLM.

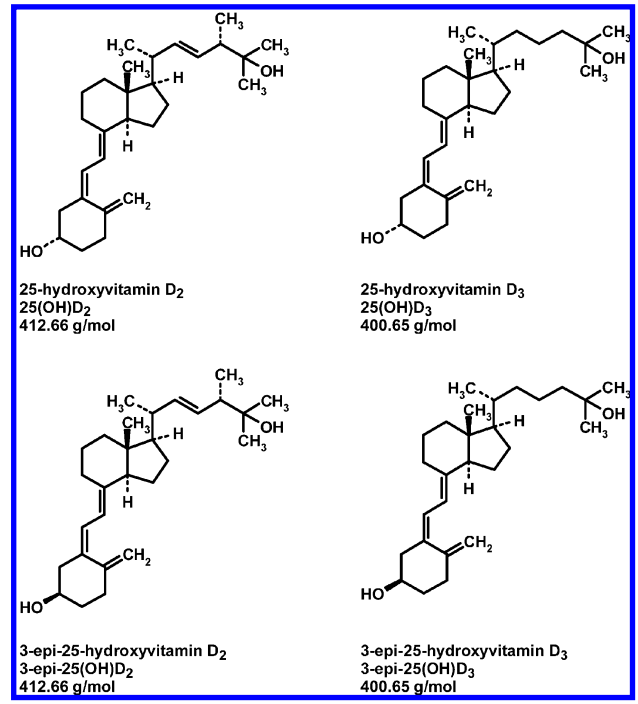

Figure 1. Structures of 25(OH)D_3, 25(OH)D_2, 3-epi-25(OH)D_3, and 3-epi-25(OH)D_2.

EXPERIMENTAL SECTION

Materials. The 25(OH)D_3 (as monohydrate) reference compound was obtained from the United States Pharmacopeia (USP, Rockville, MD). The 25(OH)D_2 reference compound was obtained from IsoSciences (King of Prussia, PA). The impurities in these materials were evaluated at NIST by liquid chromatography/ultraviolet absorbance (LC/UV), thermogravimetric analysis (TGA), and gas chromatography headspace analysis.[20] Moisture content for 25(OH)D_3 was determined at NIST by Karl Fischer titration. The purities of 25(OH)D_3 and 25(OH)D_2 were determined to be 99.40% ± 0.14% and 95.41% ± 0.15%, respectively. The isotopically labeled compounds 25(OH)D_3-d_3 (isotopic purity of 99%) and 25(OH)D_2-d_3 (isotopic purity of >99%) were obtained from IsoSciences. 3-Epi-25(OH)D_3 and 3-epi-25(OH)D_2 were also obtained from IsoSciences. A Zorbax SB-CN column [4.6 mm (i.d.) × 25 cm, 5 μm particle diameter] was obtained from Agilent Technologies (Palo Alto, CA). Solvents used for LC−MS/MS measurements were HPLC grade, and all other chemicals were reagent grade. Frozen human serum materials from individual donors were obtained from the Interstate Blood Bank, Inc. (Memphis, TN). A 25(OH)D-stripped serum material was a gift from DiaSorin (Stillwater, MN). Structures of 25(OH)D_3, 25(OH)D_2, 3-epi-25(OH)D_3, and 3-epi-25(OH)D_2 are presented in Figure 1.

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Preparation of Calibration Solutions. Three standard stock solutions of 25(OH)D_3 were gravimetrically prepared for calibration. Approximately 2−3 mg of the 25(OH)D_3 reference compound for each stock solution was accurately weighed and dissolved in 100 mL of anhydrous ethanol yielding concentrations that ranged from 25 to 36 μg/g. A working solution was gravimetrically prepared from each stock solution by diluting 1 to 2 mL of the stock solution with 150 mL of anhydrous ethanol. The concentrations of the three 25(OH)D_3 working standard solutions ranged from 270 to 400 ng/g. A solution of the internal standard, 25(OH)D_3-d_3, was prepared to be 256.60 ng/g in anhydrous ethanol.

Three working standard solutions of 25(OH)D_2 were gravimetrically prepared over the range of 240−350 ng/g using the same procedure described for 25(OH)D_3. A solution of 25(OH)D_2-d_3 was prepared to be 252.86 ng/g in anhydrous ethanol [25(OH)D_2-d_3 solution 1 for calibrants and serum samples with 25(OH)D_2 levels >1 ng/g]. A more diluted internal standard solution [25(OH)D_2-d_3 solution 2 for serum samples with 25(OH)D_2 levels < 1 ng/g] was gravimetrically prepared to be 25.212 ng/g by diluting 20.0 mL of 25(OH)D_2-d_3 solution 1 with 200 mL of anhydrous ethanol.

For each set of samples, six calibrants were prepared from the three 25(OH)D_3 and 25(OH)D_2 working standard solutions. Two aliquots [244−419 μL for 25(OH)D_3 and 188−588 μL for 25(OH)D_2] from each of the three working solutions were spiked with the corresponding internal standard solution, yielding six calibrants with mass ratios of unlabeled to labeled compound ranging from 0.65 to 1.40. The mixtures were dried under nitrogen at approximately 45 °C and reconstituted with 150 μL of methanol for LC−MS/MS analysis.

Sample Preparation. Serum materials from three individual donors were used for 25(OH)D_3 studies (6.3, 15.3, and 27.0 ng/g 25(OH)D_3 for sera 1, 2, and 3, respectively), while a serum material from a single donor was used for 25(OH)D_2 studies [0.9 ng/g 25(OH)D_2]. Samples were prepared in three different sets (each on a different day), each set consisting of triplicate aliquots from each of the serum materials. Each serum material was accurately weighed (approximately 2 g) into a 50 mL glass centrifuge tube. Each sample was then spiked with appropriate weighed amounts of 25(OH)D_3-d_3 and 25(OH)D_2-d_3 to get an approximately 1:1 mass ratio of analyte to internal standard. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH 9.8 ± 0.2 with approximately 400 μL of 0.1 g/mL carbonate buffer, pH 9.8 (approximately 200 μL of buffer per mL of liquid). The 25(OH)D_3 and 25(OH)D_2 were simultaneously extracted from the serum matrix with 8 mL of hexane−ethyl acetate (50:50, volume fraction). Each sample was shaken vigorously for 10 min using a mechanical shaker to allow complete mixing. The upper hexane−ethyl acetate layer was transferred to another 50 mL centrifuge tube. Hexane−ethyl acetate extraction was repeated once more with another 8 mL of solvent by shaking for 3 min. The combined extracts were dried under nitrogen at 45 °C, and the residue was reconstituted with 120 μL of methanol for LC−MS/MS analysis.

Preliminary experiments were conducted to evaluate the 25(OH)D concentrations of serum materials. An initial experiment was performed where arbitrary amounts of the internal standard were chosen, and a wider range of mass ratios (0.2−3.0) for the standards was used. Once the approximate 25(OH)D concentration was determined, the quantity of internal standard was calculated to obtain a 1:1 mass ratio.

LC−MS/MS Analysis for 25(OH)D. Samples and calibrants were analyzed with an Applied Biosystems API 4000 LC−MS/MS system equipped with an Agilent 1100 series LC system. An isocratic mobile phase consisting of 34% water, 66% methanol (volume fraction) was used with a Zorbax SB CN column[9] at 30 °C and a flow rate of 1 mL/min. At the completion of each run, the column was flushed with 100% methanol for 12 min and then equilibrated at the initial conditions for 15 min. The injection volume was 10−20 μL. The autosampler tray temperature was set at 10 °C to preserve the samples. Atmospheric pressure chemical ionization (APCI) in the positive ion mode and the multiple reaction monitoring (MRM) mode were used for LC−MS/MS. The respective transitions at m/z 401 → m/z 383 and m/z 404 → m/z 386 for 25(OH)D_3 and 25(OH)D_3-d_3 and at m/z 413 → m/z 395 and m/z 416 → m/z 398 for 25(OH)D_2 and 25(OH)D_2-d_3 were monitored. The dwell times were 0.25 s for MRM. The curtain gas and collision gas were nitrogen at settings of 276 kPa (40 psi) and 21 kPa (3 psi), respectively. The ion source gas 1 and ion source gas 2 were air at settings of 483 kPa (70 psi) and 242 kPa (35 psi), 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, 10, 15, and 10 V, respectively, for 25(OH)D_3, and at 91, 10, 13, and 12 V, respectively, for 25(OH)D_2.

The following measurement protocol was used for LC−MS/MS analysis. The six calibrants were analyzed along with the samples. The calibrants were analyzed first, followed by the samples, and then the samples and calibrants were analyzed in reverse order. Instrumental response was determined from a linear regression fit of the calibration data using a $y = mx + b$ regression model.

Method Validation for 25(OH)D. A 25(OH)D-stripped serum was used for the 25(OH)D_3 recovery study. A commercially available frozen human serum material from a single donor containing a very small amount of 25(OH)D_2 (approximately 0.1 ng/g) was used for the 25(OH)D_2 study. The approaches described in previously published papers for steroid hormones[21,22] were used to determine the absolute recovery of 25(OH)D_3 and 25(OH)D_2 from serum with this extraction method. The recoveries of 25(OH)D were evaluated with the serum samples that were spiked with 25(OH)D at approximately 20 ng/g. For both 25(OH)D_3 and 25(OH)D_2, two groups of samples were prepared. For the first group, serum was spiked with 25(OH)D before extraction and the respective labeled internal standard after extraction. For the second group, both 25(OH)D and the respective labeled internal standard were spiked before extraction. The samples were processed according to the procedure described above in the sample preparation for the LC−MS/MS measurements. The absolute

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recovery of 25(OH)D_3 and 25(OH)D_2 from serum was calculated from the comparison of the results of the two groups.

The conditions needed for equilibration of the internal standard with serum was also studied. Commercially available frozen human serum from a single donor [containing approximately 18 ng/g 25(OH)D_3] was aliquoted into 12 subsamples (2 g). An appropriate amount of 25(OH)D_3-d_3 was added to all subsamples to obtain an approximate 1:1 ratio of analyte to internal standard, The samples were equilibrated with 25(OH)D_3-d_3 at room temperature for periods of 0.5, 1, 2, and 3 h (triplicate subsamples for each time interval) and processed as described above in the sample preparation for LC−MS/MS measurement.

The accuracy of the method was evaluated by measuring 25(OH)D in spiked samples with known 25(OH)D levels. A commercially available frozen human serum material from a single donor containing approximately 0.1 ng/g of 25(OH)D_2 and 3.4 ng/g of 25(OH)D_3 was used for this study. The serum samples were subdivided into 12 aliquots for each of the 25(OH)D_3 and 25(OH)D_2 studies. Triplicate aliquots without addition of unlabeled 25(OH)D were used to determine the endogenous concentration of 25(OH)D in the material. Nine aliquots were spiked with unlabeled 25(OH)D at three different concentrations [triplicate aliquots for each of three concentrations at approximately 8, 16, and 38 ng/g for 25(OH)D_3 and at approximately 8, 16, and 24 ng/g for 25(OH)D_2]. Appropriate amounts of 25(OH)D_3-d_3 and 25(OH)D_2-d_3 were added to all aliquots, and the aliquots were processed using a previously described procedure.

For 25(OH)D_3, this method was applied to frozen serum materials from three individual donors with endogenous 25(OH)D_3 concentrations ranging from approximately 6.3 to 27 ng/g [sera with deficient and normal levels of 25(OH)D]. For 25(OH)D_2, the method was applied to a frozen serum material from a single donor with a naturally low level of 25(OH)D_2 (approximately 0.9 ng/g). Samples were prepared in three different sets (each set on a different day), each set consisting of triplicate aliquots from each serum material. Repeatability (within-set precision) and intermediate precision (between-set precision) were evaluated for the LC−MS/MS method.

Structural analogues of 25(OH)D_3 and 25(OH)D_2 were tested as potential interferences. The 3-epi-isomers of 25(OH)D_3 and 25(OH)D_2, 3-epi-25(OH)D_3, and 3-epi-25(OH)D_2 (structures are shown in Figure 1), having the same molecular masses as 25(OH)D_3 and 25(OH)D_2, were tested using the LC−MS/MS method described above for 25(OH)D_3 and 25(OH)D_2.

Uncertainty Evaluation. Statistical treatment of the data was in accordance with NIST guidelines,[23] which conform to the ISO Guide to the Expression of Uncertainty in Measurement.[24] Potential sources of uncertainty were evaluated, and those factors that could contribute significantly were used to calculate the standard uncertainty. For measurement imprecision uncertainty (type A component), an analysis of variance calculation was performed on the measurement data to determine if set-to-set differences were statistically significant. This analysis determined the number of independent measurements, n, used for calculating the measurement standard deviation of the mean. Other uncertainty components (type B) were based on the uncertainties in the purity of the reference compounds, in the weighing of the reference compounds, and on an allowance for unknown systematic errors in the sample preparation and undetected interferences in the LC−MS/MS analysis. Type A and type B uncertainty components were combined quadratically to determine the standard uncertainty, u_c, which was multiplied by a coverage factor, k, to calculate the expanded uncertainty, U.

RESULTS AND DISCUSSION

Method Validation. To satisfy the requirements of ISO 15193, a reference measurement procedure must be thoroughly tested for sources of bias and uncertainty. Critical parameters that potentially could bias the results were tested and demonstrated below.

The time to achieve equilibration of 25(OH)D in serum with the spiked internal standard was investigated. The serum containing endogenous 25(OH)D_3 at approximately 18 ng/g was used to determine the time required for 25(OH)D to equilibrate with the internal standard, 25(OH)D-d_3. No difference was observed among samples for the four time periods between 0.5 and 3 h. Therefore, it appears that equilibration is complete by 0.5 h. For convenience, a 1 h equilibration time was chosen.

As steroid hormones, 25(OH)D exists mainly in protein-bound forms in serum and is commonly bound with low-affinity noncovalent bonds to serum proteins.[1,21,22,25] Generally, there are two major ways to liberate the analyte from its binding protein, extreme pH (e.g., pH 2 or pH 10).[22,25] or mechanical shaking with extraction solvents.[21] A hexane−ethyl acetate (50:50 by volume) solvent mixture and pH 10 were chosen to liberate 25(OH)D from its binding protein DBP. 25(OH)D was then extracted into the solvent from the serum matrix. This liquid−liquid extraction of serum produced a clean extract with no interferences detected at ions monitored for labeled and unlabeled 25(OH)D by LC−MS/MS. The absolute recovery of 25(OH)D from the serum with this extraction method averaged 97% (0.4% CV, $n = 5$) and 92% (0.9% CV, $n = 5$) for 25(OH)D_3 and 25(OH)D_2, respectively. With isotope dilution, the recovery of 25(OH)D and the labeled internal standards should be equal if equilibration is achieved, and absolute recoveries of less than 100% should not influence the method accuracy because quantitation is based on the ratio of unlabeled to labeled 25(OH)D.

The recoveries of the 25(OH)D_3 and 25(OH)D_2 added to the serum (accuracy test) are listed in Table 1. The average concentrations of endogenous 25(OH)D_3 and 25(OH)D_2 in the serum material used for the accuracy test were determined to be 3.40 and 0.12 ng/g for 25(OH)D_3 and 25(OH)D_2, respectively, using the procedure described in the Experimental Section. This material was spiked with 25(OH)D_3 and 25(OH)D_2 at three different concentrations. For each concentration, the expected result equals the sum of the endogenous 25(OH)D and the amount of 25(OH)D added. For both 25(OH)D_3 and 25(OH)D_2, the amounts recovered and expected were in very good agreement for all three concentrations; the mean recoveries ranged from 100.1% to 101.0% for 25(OH)D_3 and ranged from 99.0% to 99.4% for 25(OH)D_2.

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Table 1. Recovery of 25(OH)D_3[a] and 25(OH)D_2[a] Added to Serum

concn	added ng/g	expected ng/g	detected ng/g	recovery %	mean recovery %	CV[b] $n = 3$
25(OH)D_3						
0	0	3.40	3.40	NA[c]		
1	7.78	11.19	11.19	100.1	100.1	0.1
	8.11	11.51	11.53	100.2		
	8.20	11.60	11.60	100.0		
2	16.97	20.37	20.56	100.9	100.9	0.1
	17.53	20.93	21.12	100.9		
	17.59	20.99	21.20	101.0		
3	38.97	42.38	42.84	101.1	101.0	0.1
	37.85	41.25	41.67	101.0		
	37.69	41.10	41.49	100.9		
25(OH)D_2						
0	0	0.12	0.12	NA[c]		
1	7.78	7.89	7.90	100.1	99.0	1.0
	8.05	8.17	8.01	98.0		
	8.27	8.39	8.30	98.9		
2	16.07	16.19	16.13	99.6	99.3	0.3
	16.01	16.12	15.99	99.2		
	16.03	16.15	15.99	99.0		
3	24.09	24.20	24.09	99.5	99.4	0.2
	23.98	24.10	23.89	99.1		
	24.09	24.21	24.06	99.4		

[a] Based on known additions to a serum sample. [b] CV, coefficient of variation. [c] NA, not applicable.

The 3-epi-isomers of 25(OH)D_3 and 25(OH)D_2 [metabolites of 25(OH)D[26] which have the same relative molecular masses as 25(OH)D_3 and 25(OH)D_2] were evaluated as potential interferences with the measurement of 25(OH)D_3 and 25(OH)D_2. The structures of 25(OH)D and their respective 3-epimers only differ in the OH position on C3; thus, chromatographic separation is difficult. Various columns with different selectivities including chiral, C_{18}, and CN phases were investigated in the preliminary studies. Among the columns studied, only the CN column provided baseline resolution of 25(OH)D_3 and its 3-epi-isomer (see chromatograms in Figure 2). Partial chromatographic separation of 25(OH)D_3 from 3-epi-25(OH)D_3 using a CN column was reported in the literature.[9] 3-epi-25(OH)D_3 was detected in all the adult serum materials that were evaluated in this investigation. Other studies indicated that the 3-epi-isomers of 25(OH)D can account for a significant proportion of the total 25(OH)D (8.7−61.1%) in infants.[17] Thus mass spectrometric-based methods could yield biased high results of 25(OH)D if the separation of 25(OH)D and their 3-epi-isomer is not achieved. The serum materials that were investigated did not contain significant amounts of 25(OH) D_2 and 3-epi-25(OH)D_2. An LC−MS/MS chromatogram of a mixture of 25(OH)D_2 and 3-epi-25(OH)D_2 standards that demonstrates baseline resolution of these two species is presented in Figure 3.

Repeatability (within-set precision) and intermediate precision (between-set precision) were evaluated for this LC−MS/MS method. For 25(OH)D_3, the method was applied to frozen serum materials from three individual donors with endogenous 25(OH)D_3 concentrations ranging from approximately 6.3 to 27.0 ng/g. For 25(OH)D_2, the levels of 25(OH)D_2 found in the serum materials that were investigated were very low (<1 ng/g) and one of these serum materials with an endogenous 25(OH)D_2 concentration of approximately 0.9 ng/g was used for this study. Three sets of samples were analyzed, each set consisting of three samples from each serum material. The

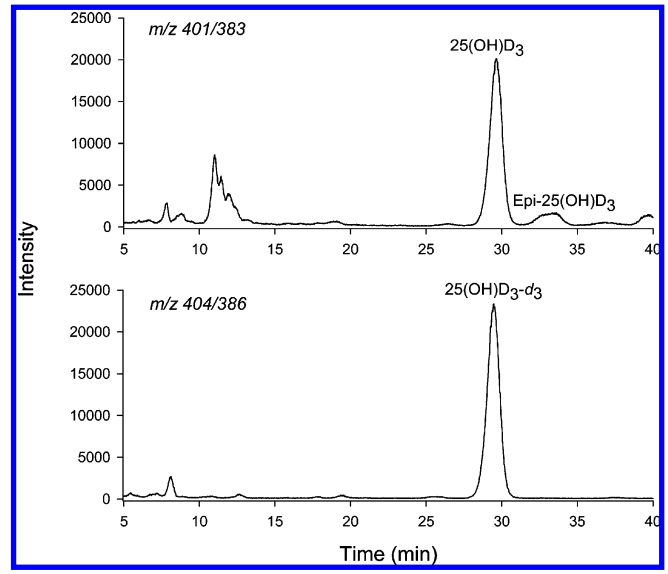

Figure 2. Selected ion chromatograms for 25(OH)D_3 and 25(OH)D_3-d_3 in serum at a concentration of 6.3 ng/g obtained by LC−MS/MS.

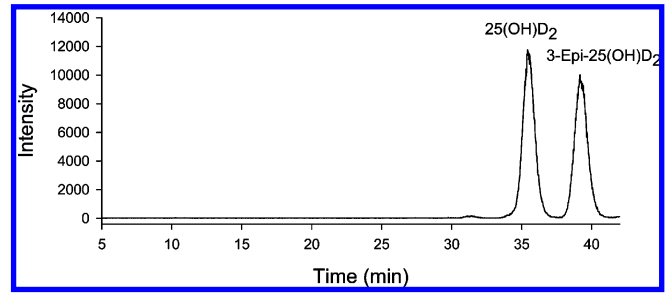

Figure 3. Selected ion chromatograms for a mixture of 25(OH)D_2 and 3-epi-25(OH)D_2 standards obtained by LC−MS/MS.

(26) Kamao, M.; Tatematsu, S.; Hatakeyama, S.; Sakaki, T.; Sawada, N.; Inouye, K.; Ozono, K.; Kubodera, N.; Reddy, G. S.; Okano, T. *J. Biol. Chem.* **2004**, *279*, 15897–15907.

Table 2. Repeatability of LC–MS/MS Measurements of Serum 25(OH)D_3 and 25(OH)D_2

serum	set	mean ng/g	within-set CV, %	overall mean, ng/g	between-set CV, %
25(OH)D_3					
	1	6.31	0.4	6.31	0.6
1	2	6.27	0.2		
	3	6.35	0.4		
	1	15.30	0.4	15.29	0.3
2	2	15.28	0.5		
	3	15.28	0.2		
	1	26.97	0.1	27.00	0.2
3	2	27.01	0.3		
	3	26.95	0.3		
25(OH)D_2					
	1	0.86	0.9	0.86	2.0
1	2	0.85	2.9		
	3	0.86	2.0		

Table 3. (A) Estimation of Expanded Uncertainties for LC–MS/MS Measurements of Serum 25(OH)D_3 and (B) Estimation of Expanded Uncertainties for LC–MS/MS Measurements of Serum 25(OH)D_2

(A) 25(OH)D_3

	concn 1 (ng/g)	concn 2 (ng/g)	concn 3 (ng/g)
mean	6.311	15.287	26.972
type A			
standard deviation	0.037	0.052	0.062
standard deviation of mean	0.012	0.017	0.021
type B			
1% uncertainty of systemic error	0.063	0.153	0.270
0.14% uncertainty of purity of reference compound	0.009	0.021	0.038
0.1% uncertainty of weighing	0.006	0.015	0.027
combined standard uncertainty (u_c)	0.065	0.156	0.274
coverage factor (k)	2	2	2
expanded uncertainty (U)[a]	0.130	0.312	0.549
relative expanded uncertainty, %	2.1	2.0	2.0

(B) 25(OH)D_2

	concn (ng/g)
mean	0.857
type A	
standard deviation	0.017
standard deviation of mean	0.006
type B	
1% uncertainty of systemic error	0.009
0.15% uncertainty of purity of reference compound	0.001
0.1% uncertainty of weighing	0.001
combined standard uncertainty (u_c)	0.010
coverage factor (k)	2
expanded uncertainty (U)[a]	0.021
relative expanded uncertainty, %	2.4

[a] 95% confidence interval.

results for 25(OH)D_3 and 25(OH)D_2 are shown in Table 2. For 25(OH)D levels > 1 ng/g, within-set coefficients of variations (CVs) of 0.1–0.5% and between-set CVs of 0.2–0.6% were obtained. For levels < 1 ng/g, within-set CVs of 0.9–2.9% and a between-set CV of 2.0% were demonstrated.

Excellent linearity was obtained for all calibration curves for both 25(OH)D_3 and 25(OH)D_2. A typical regression line was $y = 0.98617x + 0.00580$ ($R = 0.9997$; standard error = 0.006 23; $n = 12$). The detection limit at a signal-to-noise ratio of ~3 for 25(OH)D in serum was 40 pg on column (or ~0.15 ng/g as expressed as a concentration). Selected ion chromatograms for 25(OH)D_3 at a concentration of 6.3 ng/g in serum are shown in Figure 2.

Statistical Analysis of Results. The summaries of the statistical analyses for the results are shown in parts A and B of Table 3 for 25(OH)D_3 and 25(OH)D_2, respectively. For each level, three sets of three samples were analyzed. To determine if set-to-set differences were significant, the duplicate measurements of each sample were averaged to obtain a sample mean, which was then normalized by dividing by the overall mean of

that level. An analysis of variance determined that the *p*-values were greater than 0.05 for all the normalized sample means, indicating that set-to-set differences were not statistically significant. Thus, the standard deviation of the mean for each level was calculated by dividing the standard deviation of the sample means for that level by the square root of n, where $n = 9$. To calculate the standard uncertainty, u_c, the standard deviation of the mean for the measurements was combined quadratically with the type B factors, which include uncertainties related to the purity of the unlabeled 25(OH)D reference compounds, the weighing of the reference compounds, and unknown systematic errors in the sample preparation and in the LC−MS/MS system (undetected interferences from other metabolites of vitamin D). The uncertainties in the purity of the reference compound were estimated to correspond to a relative standard deviation of 0.14% and 0.15% for 25(OH)D_3 and 25(OH)D_2, respectively. The uncertainty in the weighing was estimated to be 0.1%. Finally, the uncertainty of other unknown sources in the sample preparation was estimated to be 1%. Because the type B components contribute most of the uncertainty and have very large degrees of freedom, the effective degrees of freedom for each level are large, resulting in a coverage factor, k, near 2. A value of 2 was used for multiplying the standard uncertainty for each level to calculate the expanded uncertainty, which is intended to represent a 95% confidence interval. The relative expanded uncertainties for all levels ranged from 2.0% to 2.4% for both 25(OH)D_3 and 25(OH)D_2.

CONCLUSIONS

An LC−MS/MS reference measurement procedure for serum 25(OH)D_3 and 25(OH)D_2 has been developed. This method demonstrates good accuracy and low susceptibility to interferences and produces results with relatively small uncertainties for serum 25(OH)D. NIST has used this candidate reference measurement procedure to certify the concentrations of 25(OH)D_3 and 25(OH)D_2 in a new standard reference method (SRM) for vitamin D in human serum which can be used to validate the accuracy for the test methods used in clinical laboratories.

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