National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2972

25-Hydroxyvitamin D_2 and D_3 Calibration Solutions

This Standard Reference Material (SRM) consists of two separate solutions of the vitamin D metabolites 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in ethanol. SRM 2972 is intended primarily for use in calibration of instruments and techniques used for the determination of these metabolites. A unit consists of ten, two-milliliter ampoules, five of which contain approximately 1.2 mL of the 25-hydroxyvitamin D_2 solution and five of which contain approximately 1.2 mL of the 25-hydroxyvitamin D_3 solution.

Development of SRM 2972 was through collaboration between the National Institute of Standards and Technology (NIST) and the National Institutes of Health (NIH), Office of Dietary Supplements (ODS).

Certified Mass Fraction Values: The certified values for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 presented in Table 1 are based on the analytical results determined using isotope dilution liquid chromatography with mass spectrometric detection (ID-LC-MS) and LC with absorbance detection (LC-absorbance). A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. The measurand is the total concentration of each analyte, expressed with either mass fraction (ng/g) or amount-of-substance units (nmol/L), in Table 1 [2]. Metrological traceability is to the SI unit of mass. The amount-of-substance concentrations were calculated from the mass fraction values using the density of ethanol at 22 °C (0.78775 g/mL) and the relative molecular masses of 412.65 g/mol for 25-hydroxyvitamin D_2 and 400.64 g/mol for 25-hydroxyvitamin D_3 and 3-epi-25-hydroxyvitamin D_3. An allowance for the change in density over the range from 16 °C to 30 °C is included in the uncertainty.

Expiration of Certification: The certification of **SRM 2972** is valid, within the measurement uncertainty specified, until **31 August 2017**, provided the SRM is handled in accordance with the instructions given in this certificate (see "Instructions for Storage and Use"). The certification will be nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Coordination of the technical measurements leading to the certification of this SRM were performed by M. Bedner and L.C. Sander of the NIST Chemical Sciences Division and K.W. Phinney, presently of the NIST Biomolecular Measurement Division. Analytical measurements at NIST were performed by M. Bedner, K.A. Lippa, and M.A. Nelson of the Chemical Sciences Division and B.E. Lang of the Biosystems and Biomaterials Division.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

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Certificate Issue Date: 21 October 2014

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Table 1. Certified Values for 25-Hydroxyvitamin D_2 and 25-Hydroxyvitamin D_3 in SRM 2972

	Mass Fraction[(a)] (ng/g)	Concentration[(b)] (nmol/L)
25-Hydroxyvitamin D_2	293.6 ± 9.1	560.4 ± 19.9
25-Hydroxyvitamin D_3	410.0 ± 14.9	806.2 ± 32.4

[(a)] The uncertainty provided with each value is an expanded uncertainty about the weighted mean to cover the measurand with approximately 95 % confidence. The expanded uncertainty is calculated as $U = k\, u_c$, where u_c incorporates the observed difference between the results from the methods and their respective uncertainties, as well as uncertainties related to purity estimation of the standards and possible degradation over time, consistent with the ISO/JCGM Guide and its Supplement 1, and k is a coverage factor ($k = 2$) corresponding to approximately 95 % confidence [3-7].

[(b)] The amount-of-substance concentrations (nmol/L) were obtained by multiplying the certified values in mass fraction units by the density of ethanol at 22 °C and dividing by the relative molecular masses of each of the compounds. These concentrations are for use in the temperature range of 16 °C to 30 °C, and an allowance for the change in density over this temperature range is included in the uncertainties.

METROLOGICAL TRACEABILITY

Metrological traceability of measurement results to a given reference must be established through an unbroken chain of calibrations and/or comparisons, each having stated uncertainties [8], using measurement standards that are appropriate for the property measured. The certified values in this calibration SRM are traceable to the International System of Units (SI) through such chains. These chains include: confirmation of the chemical identity and determination of the purity of primary standards, fitness evaluation of the solvent used to prepare each SRM solution, calibration of the devices used to determine mass, control of known influence factors such as temperature and ultraviolet (UV) radiation, and evaluation of the homogeneity and stability of each certified property as delivered in the SRM units.

Approaches to establishing the traceability of other measurement results of the certified property include calibration of a measurement process using the SRM as-is or by preparing in-house solutions though dilution of this SRM. The property values of in-house solutions can be made traceable to the SRM's certified value and through it to the SI by properly evaluating the uncertainties involved in procedures used in their preparation. Gravimetric and volumetric methods for preparing such in-house solutions are described in Appendix A: Guidance for Diluting SRM 2972 25-Hydroxyvitamin D_2 and D_3 Calibration Solutions. The property value uncertainties assigned to the in-house solutions must include the uncertainty of the SRM's certified value appropriately combined with the uncertainties of the preparative process.

The SI traceability of measurement results made using a measurement process calibrated to the SRM directly or to in-house solutions prepared from this SRM can then be established by properly evaluating the uncertainty of the calibration function resulting from the calibration process. The property value uncertainties assigned to measurement results from the calibrated process must properly combine the calibration function uncertainty with the measurement process imprecision appropriate to the sample analyzed.

Guidance on evaluating and combining uncertainties is provided in reference 9.

NOTICE AND WARNING TO USER

These solutions contain primarily ethanol, which is a flammable solvent. Open flames and sources of spark should be avoided while using this SRM. Use proper methods for disposal of flammable, potentially hazardous waste. Consult the Safety Data Sheet (SDS), enclosed with the SRM shipment, for health and safety information.

INSTRUCTIONS FOR STORAGE AND USE

Storage: Sealed ampoules, as received, should be stored immediately in the dark at temperatures below –20 °C because of analyte instability at higher temperatures.

Use: Ampoules should be removed from the freezer and allowed to equilibrate to room temperature before weighing or volumetrically transferring. Due to the instability of the analytes at temperatures greater than –20 °C, the total amount of time at room temperature for equilibrating and processing should be minimized to less than 3 h. Precautions should be taken to avoid exposure of ampoules and test portions to strong UV light and direct sunlight.

Test portions for use should be withdrawn immediately after opening the ampoules, and should be processed or diluted without delay for the certified concentration to be valid within the stated uncertainty. Because of the volatility of ethanol, the certified concentration value is NOT applicable to material stored in ampoules that have been opened for more than 2 min, even if they are resealed. The certified concentration values listed in Table 1 apply only to aliquots removed at 16 °C to 30 °C. If possible, samples should be placed in thermostatted compartments at 4 °C or colder during analysis.

Guidance for diluting and storing diluted SRM 2972 solutions is provided in Appendix A.

PREPARATION AND ANALYSIS[(1)]

The solutions were prepared gravimetrically at NIST from anhydrous ethanol and primary standards for 25-hydroxyvitamin D_2 obtained from IsoSciences (King of Prussia, PA) and 25-hydroxyvitamin D_3 obtained from the United States Pharmacopeia (Rockville, MD). The solutions were mixed overnight (a minimum of 16 h). On the morning following preparation, the solutions were aliquoted into 2 mL amber glass ampoules that had been purged with argon prior to addition of the solution. The ampoules were then flame-sealed.

NIST Analysis of Vitamin D Metabolites Using Isotope Dilution Liquid Chromatography with Mass Spectrometric Detection: Mass fractions of the vitamin D metabolites were measured at NIST using ID-LC-MS. Separate calibrants were prepared gravimetrically for each of the analytes at concentrations intended to approximate the levels of the vitamin D metabolites in each solution of the SRM. Stable-isotope labeled internal standard solutions were used for each analyte and were added to the calibrants and the SRM 2972a samples. The internal standards consisted of separate solutions containing $^2H_6$-25-hydroxyvitamin D_3 and $^2H_3$-25-hydroxyvitamin D_2. Test portions were approximately 450 mg (approximately 600 µL). For both SRM solutions, duplicate test portions from each of 10 ampoules selected using a stratified random sampling scheme were accurately weighed into 2 mL amber autosampler vials. An aliquot of the internal standard solution corresponding to the metabolite being measured was added, followed by mixing and injection. Details of the separation and a typical chromatogram for each of the metabolites are provided in Figure 1. MS detection with selected ion monitoring was used for quantitation. 25-Hydroxyvitamin D_3 and $^2H_6$-25-hydroxyvitamin D_3 were monitored at *m/z* 383 and *m/z* 389, respectively. 25-Hydroxyvitamin D_2 and $^2H_3$-25-hydroxyvitamin D_2 were monitored at *m/z* 395 and *m/z* 398, respectively.

NIST Analysis of Vitamin D Metabolites Using Liquid Chromatography with Absorbance Detection: Mass fractions of the vitamin D metabolites were measured at NIST using LC-absorbance at 265 nm. Separate calibrants were prepared gravimetrically for each of the analytes at concentrations intended to approximate the levels of the vitamin D metabolites in each solution of the SRM. The LC-absorbance measurements were calibrated using an external standard approach and hence no internal standard was used. Aliquots from 10 ampoules, selected using a stratified random sampling scheme, were analyzed with LC-absorbance using both a C_{18} and a pentafluorophenylpropyl (PFP) column. Representative chromatograms and the separation conditions for each of the metabolites are presented in Figure 2.

Purity Assessment: The concentrations determined by ID-LC-MS and LC-absorbance were adjusted for the purity estimates of the primary standards, which were determined using LC-absorbance and three different stationary phases, LC-MS, thermogravimetric analysis, Karl Fischer titration, and quantitative nuclear magnetic resonance spectroscopy with an internal standard.

Homogeneity Assessment: The homogeneity of vitamin D metabolites in this SRM was assessed at NIST using the methods and test portion sizes described above, graphical analyses, and analyses of variance at the 5 % significance level. No significant inhomogeneity was observed for any of the vitamin D metabolites.

Value Assignment: The weighted mean of NIST results provided by ID-LC-MS and LC-absorbance were used to calculate assigned values for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2.

[(1)] Certain commercial equipment, instruments or materials are identified in this certificate to adequately specify 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.

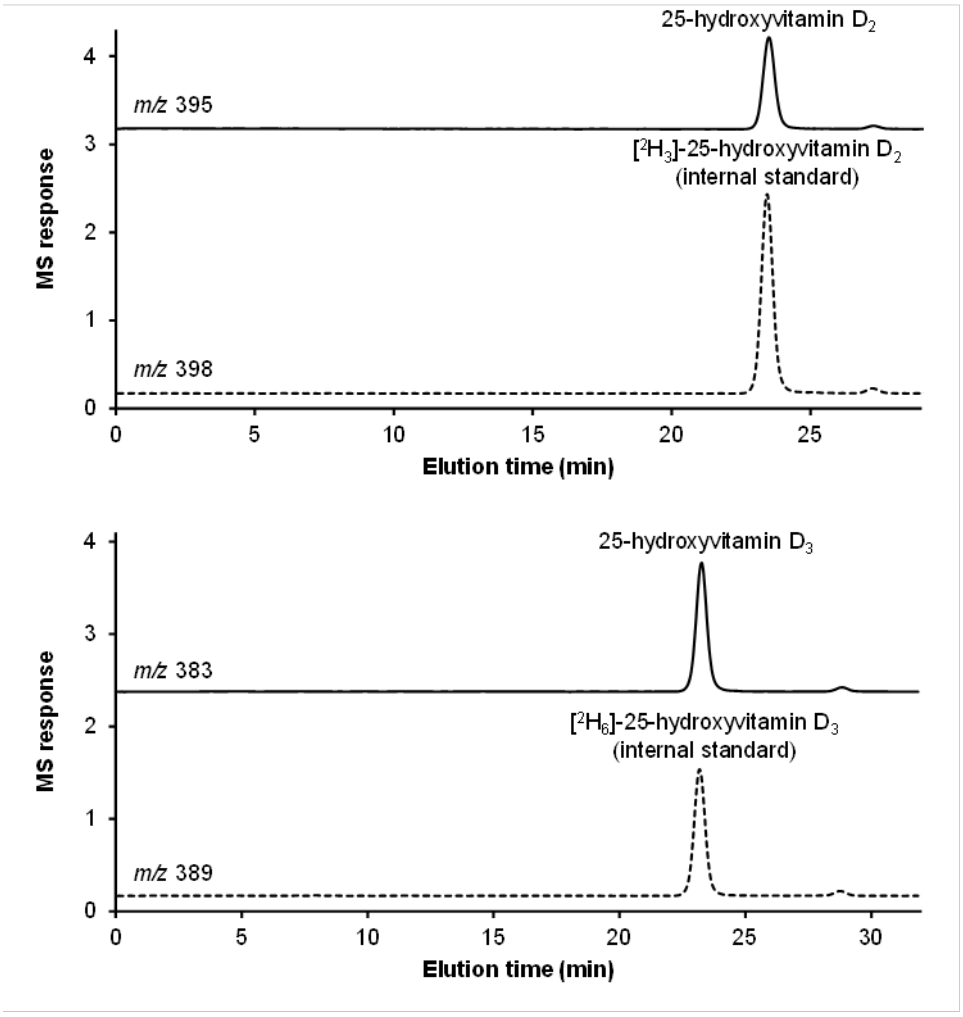

Figure 1. ID-LC-MS chromatograms of SRM 2972 25-Hydroxyvitamin D_2 and D_3 Calibration Solutions. A pentafluorophenylpropyl column with dimensions of 150 mm × 4.6 mm ID and containing 2.7 µm diameter particles and an isocratic mobile phase of 78 % methanol, 22 % water were used with a column temperature of 15 °C. MS detection was achieved using selected ion monitoring at the indicated ions.

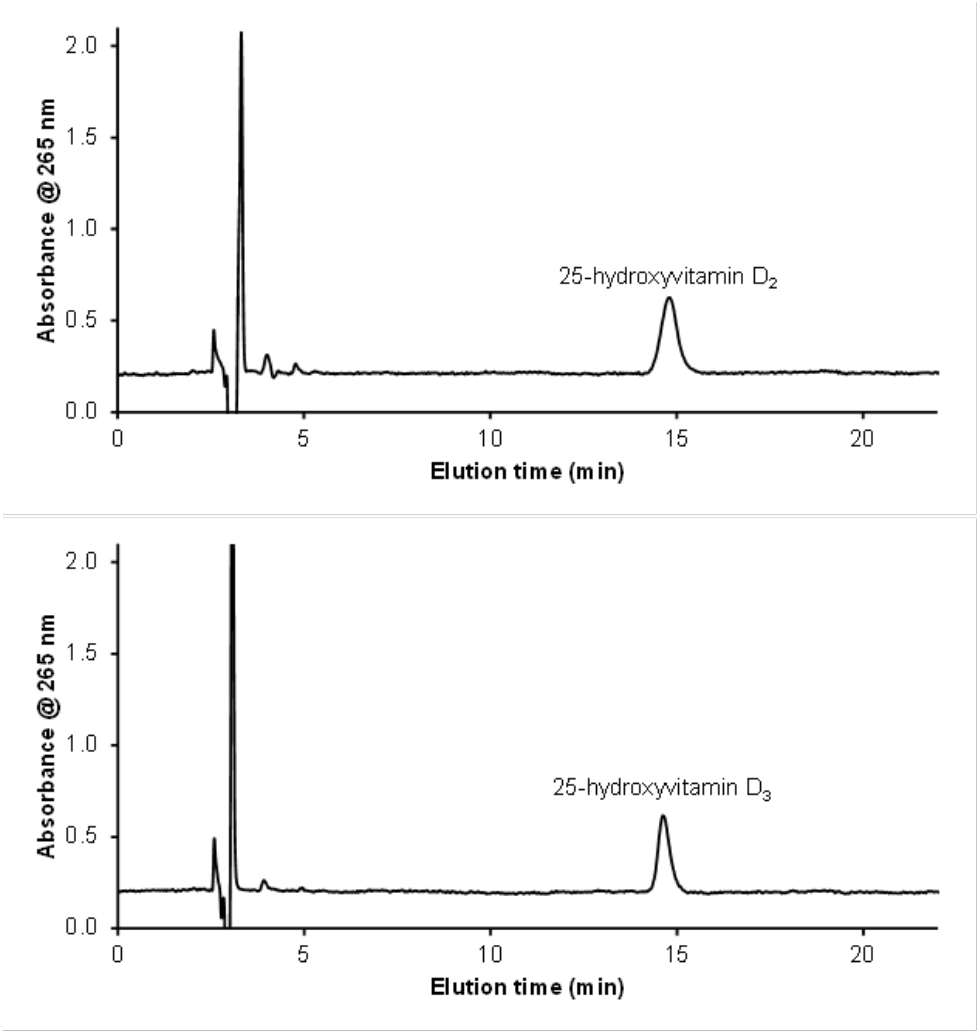

Figure 2. LC-Absorbance chromatograms of SRM 2972 25-Hydroxyvitamin D_2 and D_3 Calibration Solutions. A C_{18} column with dimensions of 250 mm × 4.6 mm ID and containing 5 µm particles and an isocratic mobile phase of 86 % methanol, 14 % water were used with a column temperature of 45 °C. Absorbance detection was achieved using 265 nm.

REFERENCES

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[9] EURACHEM; *EURACHEM/CITAC Guide: Quantifying Uncertainty in Analytical Measurement;* Ellison, S.; Williams, A. Eds.; 3rd ed.; (2012); available at http://www.eurachem.org/index.php/publications/guides (accessed Oct 2014).

Certificate Revision History: 21 October 2014 (Update of certified values, change of the expiration date, addition of description of measurement procedures used at NIST, appendix added, editorial changes); **07 December 2009** (Original certificate date).

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

Appendix A

Guidance for Diluting SRM 2972 25-Hydroxyvitamin D_2 and D_3 Calibration Solutions

The ethanolic calibration solutions of SRM 2972 for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 are of higher concentration than is typically encountered in human serum/plasma samples. Therefore, dilution of the calibration solutions may be required for analysis by many of the common vitamin D metabolite assays. Recommendations for dilution of the calibration solutions are as follows:

1) The solutions should be allowed to reach room temperature and be thoroughly mixed prior to opening the ampoules for dilution. However, due to the instability of the vitamin D metabolites at room temperature, care should be taken to minimize the total time at room temperature for equilibrating, diluting and analyzing to less than 3 h.

2) The most accurate results for dilution will be obtained using gravimetry with a calibrated analytical balance. Both the masses of the SRM 2972 solution and the total amount of solution after dilution are required to calculate the new concentration.

3) For assays that utilize volumetric measurements, use of either a gas-tight syringe or a positive displacement pipette (PDP) is recommended for solution transfer. If using a PDP, ensure all solution is delivered from the capillary (touching the tip of the capillary to the wall of the container may be required to fully deliver the correct volume). The best results for a volume dilution will be obtained if a volumetric flask is used to achieve the desired total volume.

4) If a positive displacement pipette is not available, an air-displacement pipette can be used. However, the errors in the amount of the ethanolic solution dispensed and the concentration of the diluted solution will be greater. Also, in both (3) and (4), use of pipettes that are out of calibration will result in additional error.

5) The choice of diluent does not matter, as long as the ethanol/analytes are soluble. Dilution with organic solvents such as alcohols or acetonitrile is preferable, but water can be used as the diluent to minimize solvent losses due to evaporation. All diluted SRM 2972 solutions should be stored in the dark in a sealed container (e.g., amber threaded bottle with a lined screw cap) to minimize concentration changes that could occur from evaporative losses. Solutions diluted with organic solvents should be stored at –20 °C until ready for analysis (up to 2 months) to minimize metabolite degradation. The viability of solutions that have been diluted with water, stored at –20 °C, and then equilibrated to room temperature for analysis has not been investigated at NIST. Therefore, it is recommended that samples diluted with water be analyzed without delay and discarded after 3 h.