National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 972

Vitamin D in Human Serum

Standard Reference Material (SRM) 972 is intended for use as an accuracy control in the critical evaluation of methods for determining the amount of substance concentration of vitamin D metabolites in human serum. This SRM can also be used as a quality assurance tool for assigning values to in-house control materials for these constituents. A unit of SRM 972 consists of four vials (Levels 1 through 4) of frozen serum with different concentration levels of 25-hydroxyvitamin D [25(OH)D]. Measurement of 25(OH)D in serum is generally considered a reliable indicator of vitamin D status. Each vial of SRM 972 contains approximately 1 mL of serum.

Each of the four levels of SRM 972 was prepared with specific target levels of vitamin D metabolites. While some measurement methods might be applicable to each of the four levels of SRM 972, it is recognized that some specific levels may not be applicable to a given method. Individual users will need to assess which level or levels best suit their particular needs. Level 1 of SRM 972 was prepared from "normal" human serum and has not been altered. Level 2 was prepared by diluting Level 1 with horse serum to achieve a lower 25(OH)D concentration. Level 3 contains "normal" human serum that has been fortified with 25-hydroxyvitamin D_2, and Level 4 contains "normal" human serum that has been fortified with 3-epi-25-hydroxyvitamin D_3.

Certified Concentration Values: The certified concentration values for 25-hydroxyvitamin D_3 [25(OH)D_3], 25-hydroxyvitamin D_2 [25(OH)D_2], and 3-epi-25-hydroxyvitamin D_3 [3-epi-25(OH)D_3] are provided in Table 1. Structures of these compounds are provided in Figure 1. 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 certified concentration values for these analytes are based on the agreement of results from isotope dilution liquid chromatography mass spectrometry (ID-LC-MS), and isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) procedures performed at NIST, and from results provided by the Centers for Disease Control and Prevention (CDC), Atlanta, GA.

Reference Concentration Values: Reference concentration values for 25(OH)D_2 and 3-epi-25(OH)D_3 are provided in Table 2. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification, and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [1]. The reference values for 3-epi-25(OH)D_3 are based on LC-MS/MS measurements performed at NIST.

Expiration of Certification: The certification of **SRM 972** is valid, within the measurement uncertainty specified, until **30 September 2015,** provided the SRM is handled in accordance with the instructions given in this certificate (see "Instructions for Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certificate: 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) will facilitate notification.

Support for the development of SRM 972 was provided in part by the National Institutes of Health (NIH) Office of Dietary Supplements (ODS). Technical consultation was provided by J.M. Betz and M.F. Picciano (NIH-ODS).

The overall direction and coordination of the preparation and analytical measurements leading to the certification of this SRM were performed by K.W. Phinney and S.A. Wise of the NIST Analytical Chemistry Division.

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Acquisition of the material was performed by K.E. Sharpless, and analytical measurements were performed by M. Bedner and S.S.-C. Tai of the NIST Analytical Chemistry Division, and V.V. Vamathevan, a guest scientist at NIST from the National Metrology Institute of Australia. Measurements were also performed by L.F. McCoy, H. Chen, M. Chaudhary-Webb, D. LaVoie, C.M. Pfeiffer, and R.L. Schleicher at the CDC.

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 Measurement Services Division.

NOTICE AND WARNING TO USERS

Warning: SRM 972 IS INTENDED FOR IN-VITRO DIAGNOSTIC USE ONLY. THIS IS A HUMAN-SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of the source materials used to prepare this product found the materials to be non-reactive when tested 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 offer complete assurance that HIV, hepatitis viruses, or other infectious agents are absent, this SRM should be handled at the Biosafety Level 2 for any potentially infectious human serum or blood specimen [2].

Storage: Until required for use, SRM 972 should be stored in the dark at a temperature between –20 °C and –80 °C.

Instructions for Use: SRM 972 is provided as a set of four vials of frozen serum. The vial (or vials) to be used should be allowed to thaw at room temperature for at least 30 min under subdued light. The contents of the vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to strong UV light and direct sunlight.

PREPARATION AND ANALYSIS[1]

Preparation of SRM Serum Pools: SRM 972 was prepared by Aalto Scientific, Ltd., Carlsbad, CA. Four serum pools were prepared. Level 1 is a "normal" human serum pool containing 25(OH)D_3; Level 2 is a blend of the "normal" serum used in Level 1 and horse serum to contain approximately half the 25(OH)D_3 concentration of the "normal" pool; Level 3 is a "normal" human serum pool enriched to contain equivalent amounts of 25(OH)D_2 and 25(OH)D_3; and Level 4 is a "normal" human serum pool enriched to contain equivalent amounts of 3-epi-25(OH)D_3 and 25(OH)D_3. Level 3 and Level 4 were prepared from serum pools that were separate from those used in Level 1 and Level 2.

Analytical Approach for Determination of Vitamin D Metabolites: Value assignment of the concentrations of the vitamin D metabolites in SRM 972 was based on the combination of results provided from two analytical methods at NIST (ID-LC-MS and ID-LC-MS/MS using different chromatography), and from ID-LC-MS/MS at CDC.

NIST Analyses for Vitamin D Metabolites: Metabolites of vitamin D were measured at NIST using ID-LC-MS and ID-LC-MS/MS, each with two different types of chromatographic separations. Calibrants were prepared gravimetrically at levels intended to approximate the levels of the metabolites in the SRM. Isotopically labeled internal standards were employed; a single internal standard solution was used for the calibrants and samples.

Measurement of 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 by ID-LC-MS (NIST): Serum (450 mg), and an internal standard solution containing $^2H_6$-25(OH)D_3 and $^2H_3$-25(OH)D_2 were combined in glass tubes, proteins were precipitated, and the metabolites were extracted into hexane twice. The hexane phases were combined and evaporated to dryness at 40 °C under nitrogen. The residues were reconstituted, and were further clarified using centrifuge filters. Extracts were analyzed by using LC-MS with: (1) a deactivated C_{18} stationary phase and (2) a cyanopropyl stationary phase. Chromatograms are provided in Figures 2 and 3, and chromatographic conditions are provided in the figure captions. All solvent compositions represent volume fractions in percent. Atmospheric pressure chemical ionization (APCI-MS) detection with positive polarity was used for both chromatographic methods. The $[M - H_2O + H]^+$ ions were monitored and used for quantification of all species.

[1]Certain commercial products are identified in this certificate to adequately describe 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 products identified are necessarily the best available for this purpose.

The ions monitored included: *m/z* 383, *m/z* 395, *m/z* 389, and *m/z* 398 for $25(OH)D_3$ and 3-epi-$25(OH)D_3$, $25(OH)D_2$, $^2H_6$-$25(OH)D_3$, and $^2H_3$-$25(OH)D_2$ respectively. $25(OH)D_3$ and 3-epi-$25(OH)D_3$ co-eluted using the C_{18} column, and results were corrected by subtracting the 3-epi-$25(OH)D_3$ values obtained using ID-LC-MS/MS as described below.

Measurement of $25(OH)D_3$, $25(OH)D_2$, and 3-epi-$25(OH)D_3$ by ID-LC-MS/MS (NIST): Serum (2.0 g to 2.5 g) was combined with water (to avoid protein precipitation when samples were spiked with internal standard solutions) and an internal standard solution containing $^2H_3$-$25(OH)D_3$ and $^2H_3$-$25(OH)D_2$. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH 9.8 ± 0.2 with carbonate buffer. Analytes were extracted from the serum matrix with a mixture of hexane and ethyl acetate. The combined extracts were dried under nitrogen at 45 °C, and the residues were reconstituted with methanol for LC-MS/MS analysis. Chromatograms are provided in Figure 4, and chromatographic conditions are provided in the caption. APCI in the positive-ion mode and multiple reaction monitoring (MRM) mode were used. The transitions at *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 $^2H_3$-$25(OH)D_3$, *m/z* 413 → *m/z* 395 for $25(OH)D_2$, and *m/z* 416 → *m/z* 398 for $^2H_3$-$25(OH)D_2$ were monitored

Measurement of $25(OH)D_3$, and $25(OH)D_2$ by ID-LC-MS/MS (CDC): Samples of SRM 972 were spiked with an internal standard solution containing $^2H_6$-$25(OH)D_3$. Proteins were precipitated with acetonitrile, and the supernatant mixture was filtered through a protein precipitation plate (Sirocco, Waters Corporation, Milford, MA). The extract was analyzed by using LC-MS/MS in positive-ion mode using a C_8 column for the online solid-phase extraction phase followed by separation on a C_{18} column for quantification. A total of three transitions were monitored, two for the unlabeled analytes and one for the labeled internal standard: *m/z* 401 → *m/z* 383 and *m/z* 413 → *m/z* 395 (unlabeled), and *m/z* 407 → *m/z* 389 (labeled).

Homogeneity Assessment: The homogeneity of SRM 972 was assessed at NIST by using the reversed-phase cyanopropyl LC-MS method described above. An analysis of variance did not show inhomogeneity for the test portions analyzed.

Value Assignment: The equally weighted mean of the NIST method means and the mean of the CDC data were used to calculate each certified concentration value. The reference concentration values are based on the mean of measurements from a single NIST method.

Table 1. Certified Concentration Values for Vitamin D Metabolites in SRM 972[(a)]

	ng/g		ng/mL[(b)]		nmol/L[(c)]	
Level 1						
25-hydroxyvitamin D_3	23.2	± 0.8	23.9	± 0.8	59.6	± 2.1
Level 2						
25-hydroxyvitamin D_2	1.67	± 0.08	1.71	± 0.08	4.14	± 0.19
25-hydroxyvitamin D_3	12.0	± 0.6	12.3	± 0.6	30.8	± 1.5
Level 3						
25-hydroxyvitamin D_2	25.8	± 1.9	26.4	± 2.0	64.1	± 4.8
25-hydroxyvitamin D_3	18.1	± 1.1	18.5	± 1.1	46.2	± 2.8
Level 4						
25-hydroxyvitamin D_2	2.35	± 0.21	2.40	± 0.21	5.81	± 0.52
25-hydroxyvitamin D_3	32.3	± 0.8	33.0	± 0.8	82.3	± 2.0
3-epi-25-hydroxyvitamin D_3	36.9	± 1.1	37.7	± 1.2	94.1	± 2.9

[(a)] Each certified concentration value is an equally weighted mean of the means from the NIST methods and the mean of CDC data corrected for the coelution of 3-epi-$25(OH)D_3$ with $25(OH)D_3$. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [3,4], is expressed as an expanded uncertainty, *U*. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory and within-laboratory components of uncertainty. The coverage factor (*k*) is determined from the Student's *t*-distribution corresponding to the appropriate associated degrees of freedom, and approximately 95 % confidence for each analyte [4-6].

[b]Mass concentration levels were calculated from mass fractions using measured serum densities: Level 1, 1.02900 g/mL; Level 2, 1.02521 g/mL; Level 3, 1.02453 g/mL; and Level 4, 1.01994 g/mL.

[c]Molar concentration levels were calculated from mass concentration levels using the relative molecular masses shown in Figure 1. The equivalent conversion factors are 2.4233 for 25(OH)D_2 and 2.4959 for 25(OH)D_3 and 3-epi-25(OH)D_3.

Table 2. Reference Concentration Values for Vitamin D Metabolites in SRM 972[a]

	ng/g	ng/mL[b]	nmol/L[c]
Level 1			
25-hydroxyvitamin D_2	0.59 ± 0.20	0.60 ± 0.20	1.46 ± 0.49
3-epi-25-hydroxyvitamin D_3	1.35 ± 0.04	1.39 ± 0.04	3.46 ± 0.11
Level 2			
3-epi-25-hydroxyvitamin D_3	0.74 ± 0.02	0.76 ± 0.02	1.9 ± 0.06
Level 3			
3-epi-25-hydroxyvitamin D_3	1.04 ± 0.03	1.06 ± 0.03	2.65 ± 0.06

[a]The reference concentration value for 25(OH)D_2 is an equally weighted mean of the means from the NIST methods and the mean of CDC data. The reference concentration value for 3-epi-25(OH)D_3 is the mean of results from a single NIST LC-MS/MS method. The uncertainty in the reference value, calculated according to the method described in the ISO Guide [3,4], is expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-laboratory and within-laboratory components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom, and approximately 95 % confidence for each analyte [4-6].

[b]Mass concentration levels were calculated from mass fractions using measured serum densities provided in Table 1, footnote b.

[c]Molar concentration levels were calculated from mass concentration levels using the relative molecular masses shown in Figure 1. The equivalent conversion factors are 2.4233 for 25(OH)D_2 and 2.4959 for 25(OH)D_3 and 3-epi-25(OH)D_3.

REFERENCES

[1] May, W.; Parris, R.; Beck, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements;* NIST Special Publication 260-136, U.S. Government Printing Office, Gaithersburg, MD (2000); available at http://www.cstl.nist.gov/nist839/NIST_special_publications.htm

[2] CDC/NIH; *Biosafety in Microbiological and Biomedical Laboratories, 5th ed*.; Richardson, J.; Barkley, W.E.; Richmond, J.; McKinney, R.W., Eds.; U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health; US Government Printing Office: Washington, D.C. (2007); available at http://www.cdc.gov/OD/OHS/biosfty/bmbl5/BMBL_5th_Edition.pdf.

[3] *Evaluation of Measurement Data – Guide to the Expression of Uncertainty in Measurement*, BIPM – JCGM 100: 2008, GUM 1995 with minor corrections (2008); available at http://www.bipm.org/en/publications/guides/gum.html.

[4] Levenson, M.S., Banks, D.L., Eberhardt, K.R., Gill, L.M., Guthrie, W.F., Liu, H.K., Vangel, M.G., Yen, J.H., Zhang, N.F.; *An Approach to Combining Results From Multiple Methods Motivated by the ISO GUM;* J. Res. Natl. Inst. Stand. Technol., Vol. 105, pp. 571-579 (2000).

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[6] Schiller, S.; Eberhardt, K.; *Combining Data from Independent Chemical Analysis Methods*; Spectrochim. Acta, Vol. 46B, pp. 1607–1613 (1991).

Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-2200; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

Appendix

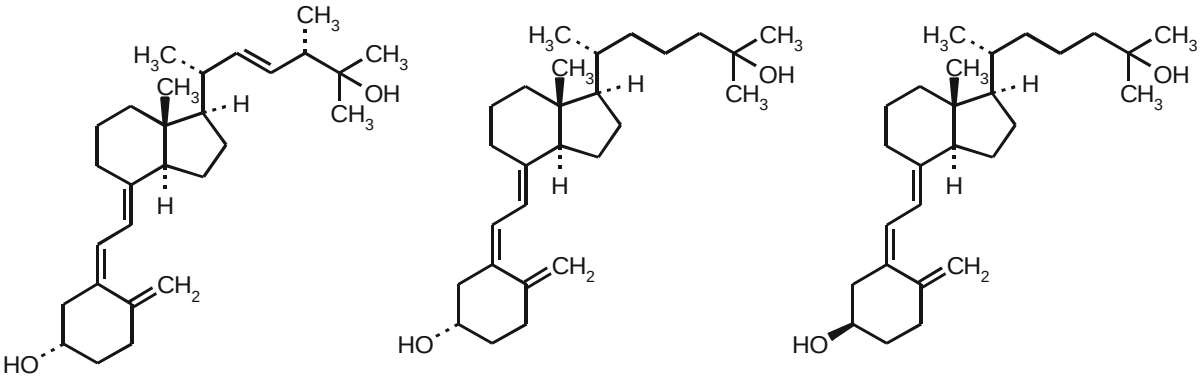

Figure l. Structures of $25(OH)D_2$, $25(OH)D_3$, and 3-epi-$25(OH)D_3$

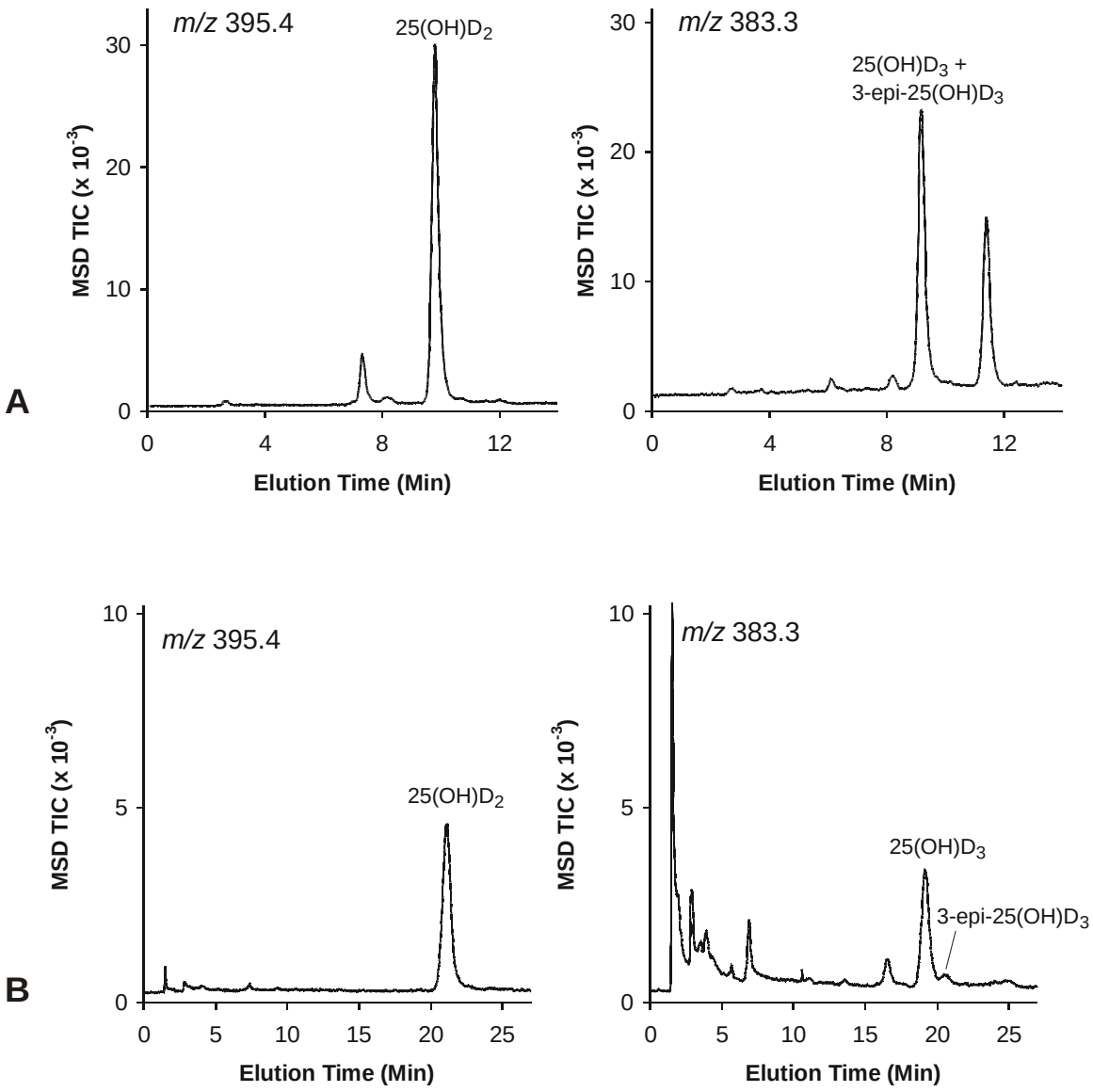

Figure 2. LC-MS selected ion monitoring chromatograms for SRM 972 Level 3.

A) A deactivated C_{18} stationary phase (Luna C18(2), Phenomenex, Torrance, CA) was used with an isocratic mobile phase of 10 % water and 90 % methanol at a flow rate of 1.0 mL/min and a column temperature of 45 °C. A step gradient to 100 % methanol was incorporated into the method at the end of the run to elute retained matrix components.

B) A cyanopropyl stationary phase (Zorbax SB-CN, Agilent Technologies, Palo Alto, CA) was used to resolve the C-3 epimers 25(OH)D_3 and 3-epi-25(OH)D_3 in SRM 972. Although 3-epi-25(OH)D_3 was not quantified by this method for Levels 1 through 3, it was chromatographically resolved from 25(OH)D_3 in the determination of this metabolite. An isocratic mobile phase of 32 % water and 68 % methanol, at a flow rate of 1.0 mL/min and a column temperature of 45 °C, were used for the analysis. A step gradient to 100 % methanol was employed to elute retained matrix components.

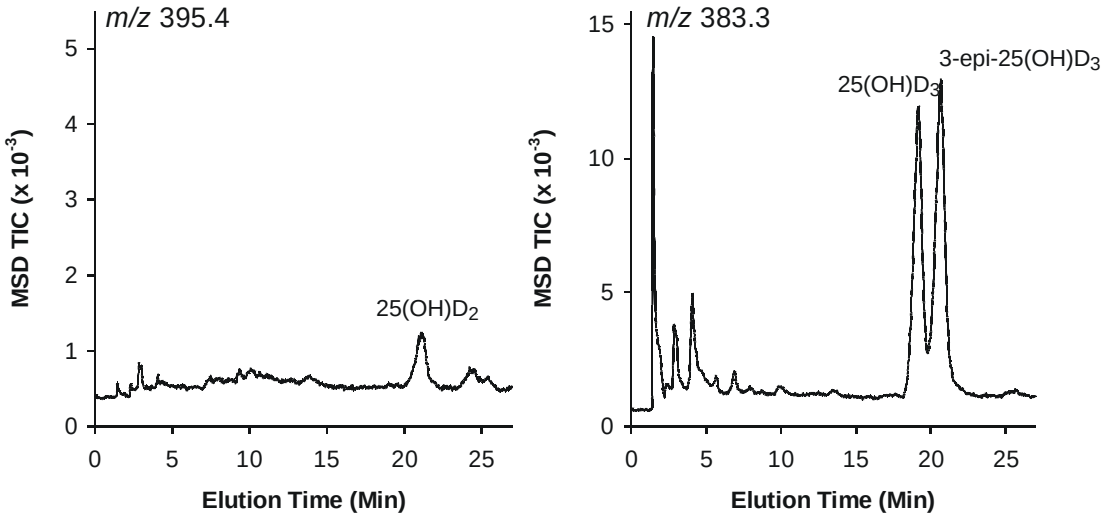

Figure 3. LC-MS selected ion monitoring chromatograms for SRM 972 Level 4, using a cyanopropyl column and chromatographic conditions described in Figure 2.

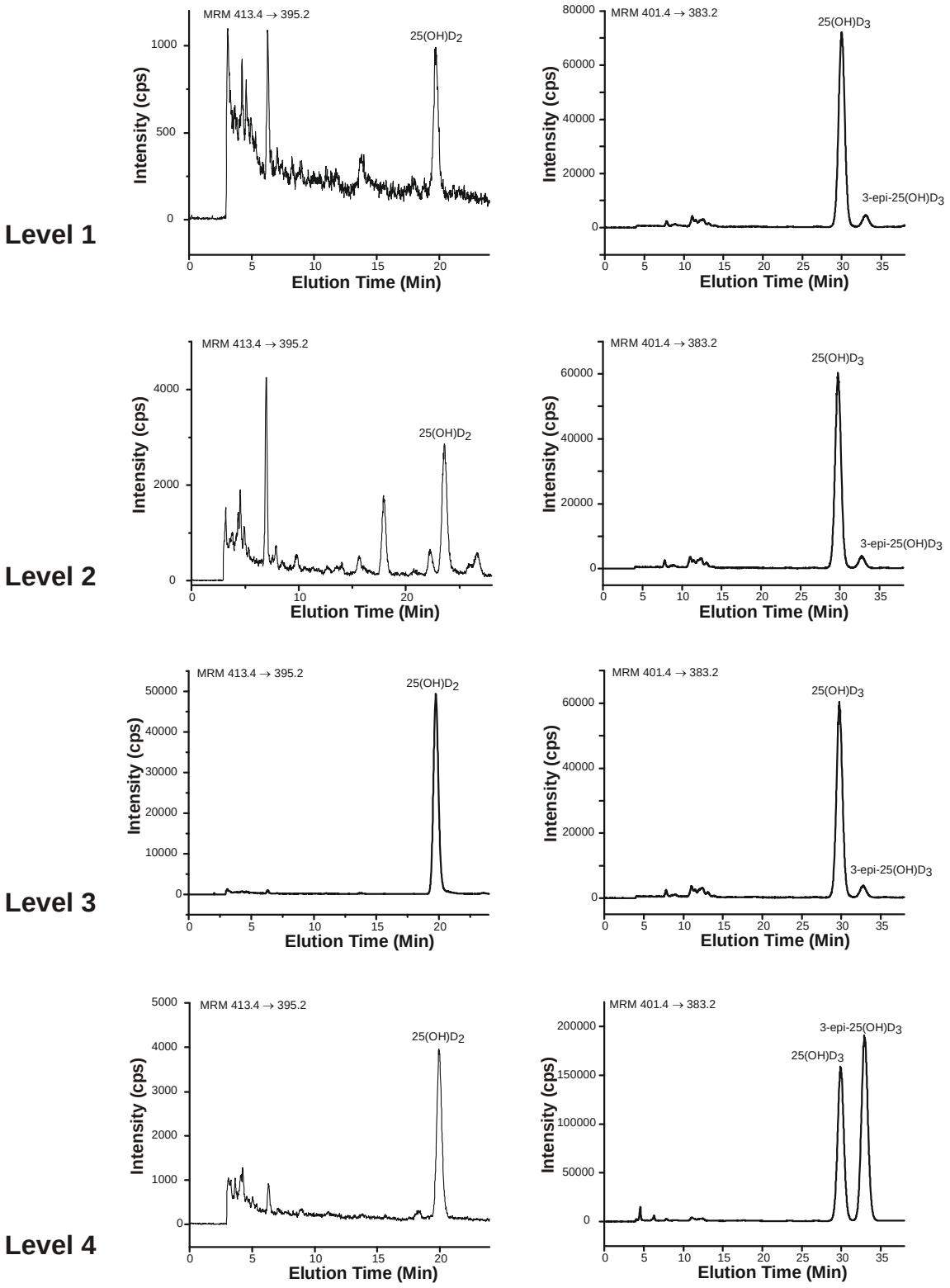

Figure 4. LC-MS/MS MRM chromatograms for vitamin D metabolites in SRM 972. For the measurement of 25(OH)D_3 and 3-epi-25(OH)D_3, an isocratic method was used with a cyanopropyl stationary phase (Zorbax SB CN, Agilent Technologies, Palo Alto, CA) at 30 ºC with 34 % water and 66 % methanol at a flow rate of 1.0 mL/min. The MRM transitions at *m/z* 401 → *m/z* 383 for 25(OH)D_3 and 3-epi-25(OH)D_3, and *m/z* 404 → *m/z* 386 for $^2H_3$-25(OH)D_3 were monitored. For the measurement of 25(OH)D_2, an isocratic method was used with a C_{18} column (Zorbax Eclipse XDB-C_{18}, Agilent Technologies, Palo Alto, CA) at 30 ºC with 14 % water and 86 % methanol at a flow rate of 1.0 mL/min. For Level 2, an isocratic mobile phase of 15 % water and 85 % methanol was used instead to better separate 25(OH)D_2 peak from an interference peak. The MRM transitions at *m/z* 413 → *m/z* 395 and at *m/z* 416 → *m/z* 398 for 25(OH)D_2 and $^2H_3$-25(OH)D_2 respectively, were monitored.