〈Review Article〉

National Institute of Standards and Technology SRM 972 as a reference material for serum total 25-hydroxyvitamin D measurements

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on behalf of the Committee on Nutrition of the Japan Society of Clinical Chemistry and the Japan Committee for Vitamin Laboratory Standards

Summary The metabolic functions of vitamin D are accepted to maintain calcium homeostasis. Vitamin D is converted to 25-hydroxyvitamin D in the liver and further hydroxylated into the active metabolites, 1,25-dihydroxyvitamin D in the kidney. 1,25-Dihydroxyvitamin D is transported to the target organ, where binding to nuclear vitamin D receptor occurs. Nowadays, 1,25-dihydroxyvitamin D is measured for the evaluation of calcium metabolism, and 25-hydroxyvitamin D is measured for the evaluation of vitamin D nutritional status. However, the assay variation present in measurements

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of serum total 25-hydroxyvitamin D (i.e., the sum of the molar concentration of 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and 3-epi-25-hydroxyvitamin D_3 expressed as ng/mL in terms of 25-hydroxyvitamin D_3 equivalents) confounds the diagnosis of hypovitaminosis D, and requires standardization from clinical and nutritional standpoints. Because the National Institute of Standards and Technology (NIST) developed Standard Reference Material 972 (SRM 972), in this review, we introduce a standardized procedure for serum total 25-hydroxyvitamin D measurements using SRM 972.

Key words: Vitamin D, Liquid chromatography-tandem mass spectrometry (LC-MS/MS), National Institute of Standards and Technology (NIST), Nutritional assessment, Standardization

Vitamin D, a fat-soluble vitamin, has a role in regulating calcium absorption in the gut and calcium excretion into the urine through the kidney, and maintains levels of serum calcium and phosphate in the circulation of blood. Vitamin D also stimulates bone cells to form new bone, but at lower serum calcium levels, it promotes calcium mobilization from old bone. A lack of vitamin D causes rickets in infants and children, and osteomalacia and osteoporosis in the elderly[1].

We obtain vitamin D from diet, i.e., ergocalciferol (vitamin D_2) from plant foods and cholecalciferol (vitamin D_3) from animal products. We receive additional vitamin D_3 through exposure to sunlight. Once absorbed from diets or generated in the skin, vitamin D_2 and D_3 are converted to their metabolites, 25-hydroxyvitamin D_2 and D_3, respectively, in the liver (Fig. 1). These metabolites are physiologically inactive, but circulating 25-hydroxyvitamin D_2 and D_3 are converted to their active metabolites, 1,25-dihydroxyvitamin D_2 and D_3, respectively, by a mitochondrial enzyme 25-hydroxyvitamin D-1-alpha-hydroxylase (encoded by the gene CYP27B1) in the kidney[2]. Parathyroid hormone (PTH) not only induces gene expression of 25-hydroxyvitamin D-1-alpha-hydroxylase, but also affects bone and kidney via a common G protein-coupled, seven-transmembrane helix receptor, so as to regulate serum calcium levels. 1,25-Dihydroxyvitamin D_2 and D_3 bind to the nuclear vitamin D receptor (VDR) in cells, and regulate gene expression of functional proteins (i.e., a calcium binding protein, calbindin and a calcium channel protein, TRPV6)[3] (Fig. 2).

The serum concentration of 1,25-dihydroxyvit-

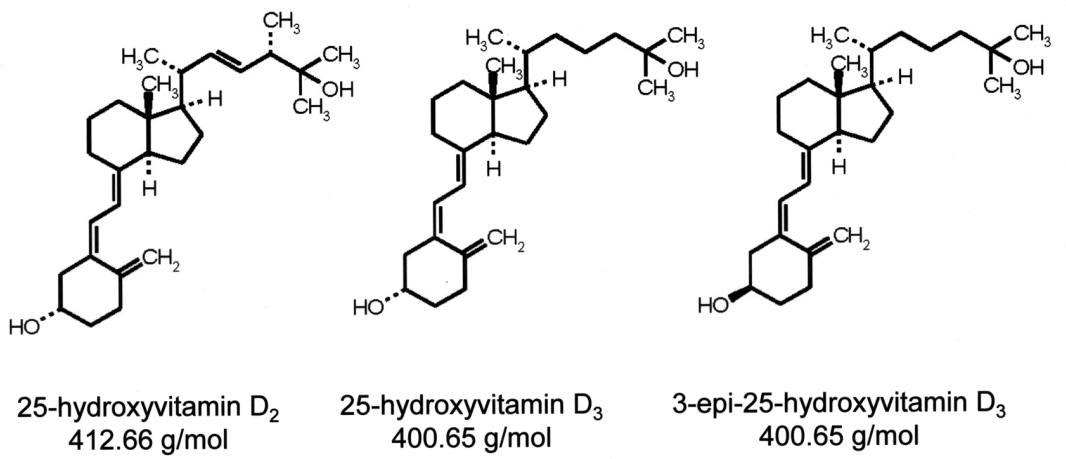

Fig. 1 Structures of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, and 3-epi-25-hydroxyvitamin D_3.

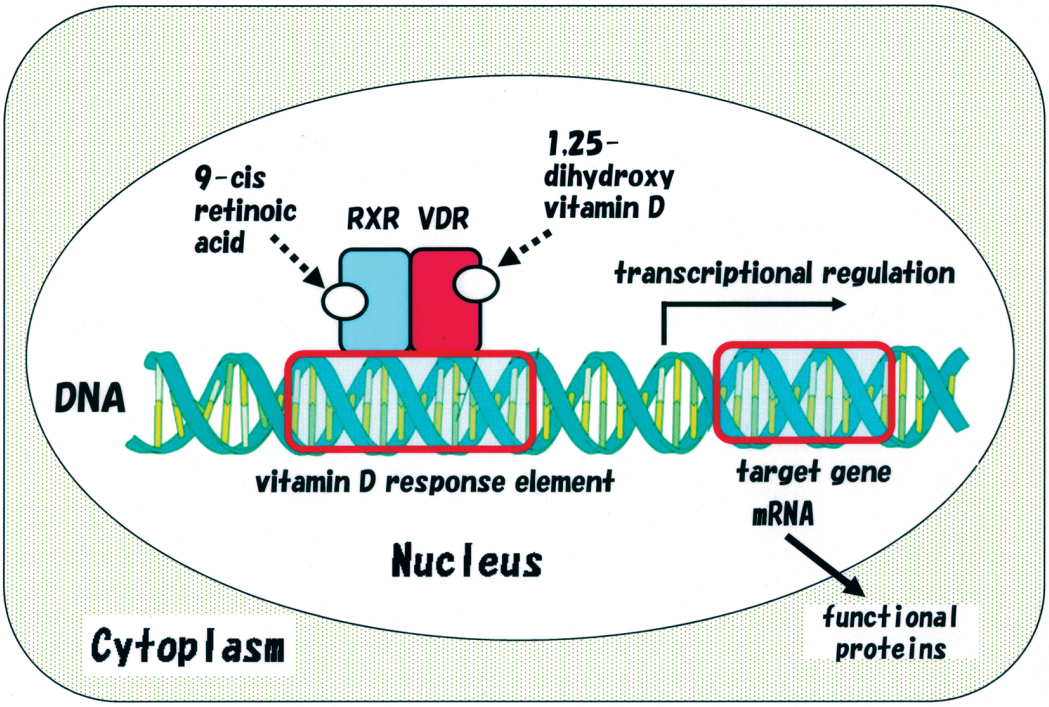

Fig. 2 Schematic model for transcriptional regulation by 1,25-dihydroxyvitamin D.
The vitamin D receptor (VDR) binds as a retinoid X receptor (RXR) heterodimer to target DNA sequences, and facilitates gene expression of functional proteins.

Table 1 Assigned values of SRM 972

	2,5-hydroxyvitamin D_3	2,5-hydroxyvitamin D_2	3-epi-2,5-hydroxyvitamin D_3	total 2,5-hydroxyvitamin D
Level 1	23.9	0.59	1.39	25.9
Level 2	12.3	1.66	0.76	14.7
Level 3	18.5	25.7	1.06	45.3
Level 4	33.0	2.33	37.7	73.0

Total 25-hydroxyvitamin D was the sum of the molar concentration of 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and 3-epi-25-hydroxyvitamin D_3, and all values were expressed as ng/mL in terms of their 25-hydroxyvitamin D_3 equivalens.

amin D was determined for the clinical diagnosis of hypocalcemia as well as rickets, osteomalacia, osteoporosis, hypoparathyroidism, and chronic kidney disease. For the nutritional assessment of vitamin D status, serum 25-hydroxyvitamin D concentration was determined, because 25-hydroxyvitamin D is the major circulating form of vitamin D, having a longer half life (2-3 weeks) than 1,25-dihydroxyvitamin D (15 hours). Recently, the serum concentration of 25-hydroxyvitamin D was easily determined by the use of automated immunoassays, which allow many specimens to be assayed. However, inter-laboratory comparison studies revealed that discrepancies between the results obtained using different assay methods, i.e., RIA, automated chemiluminescent immunoassay with a high throughput, and high-performance liquid chromatography, confounded the nutritional evaluation of subjects having low or normal vitamin D status[4,5,6]. Therefore the assay of 25-hydroxyvitamin D must be standardized. For this reason, the National Institute of Standards and Technology (NIST) developed Standard Reference Material 972 (SRM 972)[7,8]. SRM 972 consists of four serum pools with different levels of vitamin D metabolites, and gives certified reference values of 25-hydroxyvitamin D_2 and D_3, and 3-epi-25-hydroxyvitamin D_3 (Table 1).

Table 2 Verification of 25-hydroxyvitamin D_3 values of SRM 972

	Assigned values (ng/mL)		Observed values by LC-MS/MS (ng/mL)*
	2,5-hydroxyvitamin D_3	3-epi-2,5-hydroxyvitamin D_3	2,5-hydroxyvitamin D_3
Level 1	23.9	1.39	25.0
Level 2	12.3	0.76	12.7
Level 3	18.5	1.06	19.0
Level 4	33.0	37.7	67.0

*Verified by Mochizuki et al. (Reference 13).

Table 3 Determination of SRM 972 by two different methods

	Assigned values (ng/mL)		Observed values (ng/mL)	
	total 2,5-hydroxyvitamin D including 3-epi-2,5-hydroxyvitamin D_3	total 2,5-hydroxyvitamin D excluding 3-epi-2,5-hydroxyvitamin D_3	in-house CPBA total 2,5-hydroxyvitamin D	RIA total 2,5-hydroxyvitamin D
Level 1	25.9	24.5	23.0	41
Level 2	14.7	14.0	18.8	22
Level 3	45.3	44.2	29.3	49
Level 4	73.0	35.3	46.7	47

CPBA: Copmetitive protein binding assay

RIA: 25-Hydroxyvitamin D ^{125}I-RIA kit, DiaSorin

Level 1 was prepared from normal human serum and has not been altered. Level 2 was prepared by two-fold diluting of Level 1 with horse serum. Level 3 and Level 4 contained normal human serum fortified with 25-hydroxyvitamin D_2 and 3-epi-25-hydroxyvitamin D_3, respectively. These values were assigned using an isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS), which can be defined as a reference measurement procedure (RMP)[9, 10, 11, 12].

Mochizuki et al.[13] assayed SRM 972 by LC-MS/MS using a triple quadrupole mass spectrometer, and verified these assigned values. Their observed values for 25-hydroxyvitamin D_3 were near by a 100% recovery against the expected values (the sum of 25-hydroxyvitamin D_3 and 3-epi-25-hydroxyvitamin D_3: Table 2). They also verified the assigned value of 25-hydroxyvitamin D_2 in Level 3, but found the value to be 33.1 ng/mL and to give a large recovery of 25-hydroxyvitamin D_2 by 125.5% (data not shown). Although this matter needs further investigation, SRM 972 would be usable as a standard material for LC-MS/MS. The recovery of 25-hydroxyvitamin D_2 should be studied more carefully with the use of 25-hydroxyvitamin D_2 naturally present in human serum, since Level 3 was a preparation spiked with 25-hydroxyvitamin D_2.

We also assayed SRM 972 and 20 sera from healthy subjects (aged 44 to 78 years old, 12 men and 8 women)[14] by in-house competitive protein binding assay (CPBA)[15] and RIA (25-Hydroxyvitamin D ^{125}I-RIA kit, DiaSorin)[16]. Informed consent was obtained from all subjects, and the studies were approved by the guidelines established by the Protection of Human Subjects Committee of Toho University Ohashi Medical Center (IRB number, 22-47). Table 3 shows the assigned values of total 25-hydroxyvitamin D including and excluding 3-epi-25-hydroxyvitamin D_3. The total 25-hydroxyvitamin D concentration shown in Table 3 was the sum of the molar concentration of 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and 3-epi-25-hydroxyvitamin D_3, and the assay values were expressed as ng/mL in terms of their 25-hydroxyvitamin D_3 equivalents. Although a poor correlation ($r= 0.672$, $p= 0.328$) was observed in total 25-hydroxyvitamin D values between CPBA and RIA, both assays seemed to react equally with 25-hydroxyvitamin D_3, but not with 25-hydroxyvitamin D_2 and 3-epi-25-hydroxyvitamin D_3. While for 20 sera from healthy subjects, the measured values of serum total 25-hydroxyvitamin D were significantly correlated between the two assays ($r= 0.832$, $p=$

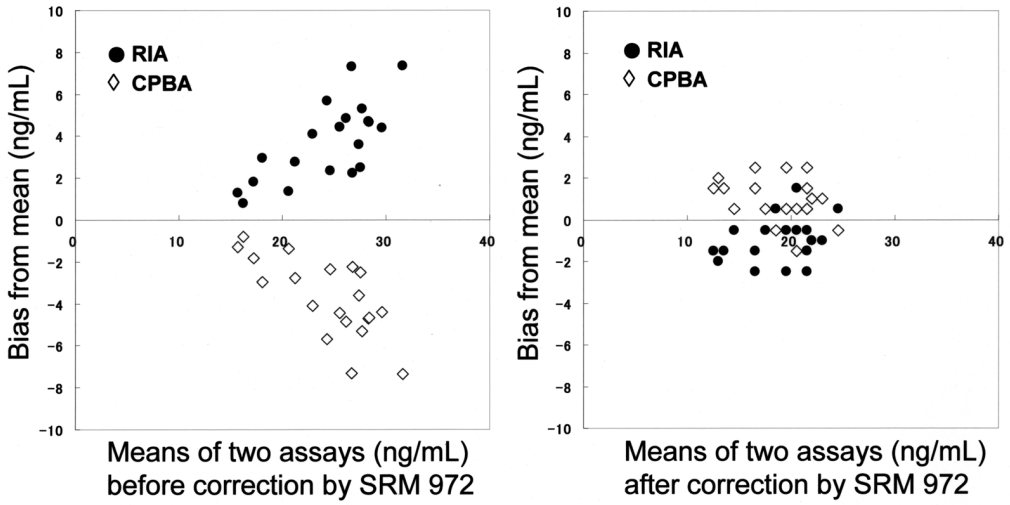

Fig. 3 Comparison of CPBA and RIA for total serum 25-hydroxyvitamin D concentrations with their mean values before and after correction by SRM 972.

0.001). However, the values measured by RIA were markedly higher than those measured by CPBA (Fig. 3). When the values measured by both methods were corrected for using the mean value of SRM 972 Level 1 and Level 2 (20.3 ng/mL), biases from the mean of the two assays were revealed to be reduced to ±3 ng/mL. This led to our conclusion that SRM 972 would be a possible material for standardizing the values of serum total 25-hydroxyvitamin D measured by different methods. However, Level 3 and Level 4 were of no use, because both were fortified with 25-hydroxyvitamin D_2 or 3-epi-25-hydroxyvitamin D_3. Indeed, we failed to reduce biases with the use of Level 3 and Level 4, in addition to Level 1 and Level 2.

Recently, a considerable amount of 3-epi-25-hydroxyvitamin D_3 was reported to be present (27.8% of total 25-hydroxyvitamin D: 8.7-61.1%) in sera from infants less than 1 year old[17]. It was also reported that the amount of 3-epi-25-hydroxyvitamin D_3 present in adult sera was 0-61% of total 25-hydroxyvitamin D, although the most of the amount of serum 3-epi-25-hydroxyvitamin D_3 was less than 10%[18]. At present, although the physiological importance of 3-epi-25-hydroxyvitamin D_3 is uncertain[19], it is known to be converted to 3-epi-1,25-dihydroxyvitamin D_3 by 25-hydroxyvitamin D-1-alpha-hydroxylase. Although 3-epi-1,25-dihydroxyvitamin D_3 can bind to VDR, it regulates gene expression of functional proteins poorly[20]. This suggests that we should standardize the measured values of serum total 25-hydroxyvitamin D excluding 3-epi-25-hydroxyvitamin D_3, and that all assay methods are undesirable for measuring this epimer. On the contrary, 3-epi-1,25-dihydroxyvitamin D_3 was reported to negatively regulate PTH gene transcription more effectively than 1,25-dihydroxyvitamin D_3[21]. If this is the case, 3-epi-25-hydroxyvitamin D_3 should be considered for future development of the standardization of serum total 25-hydroxyvitamin D measurement.

Declaration of Interest

The authors declare no potential conflicts of interest.

References

1. Sauberlich HE: Vitamin D. Laboratory Tests for the Assessment of Nutritional Status, 2nd ed., 233-248, CRC Press, Washington, D.C., 1999.
2. Eitenmiller RR, Landen WO Jr: Vitamin D. Vitamin Analysis for the Health and Food Sciences. 77-107, CRC Press, Washington, D.C., 1999.
3. Edited by De Leenheer AP, Lambert WE, Van Bocxlaer JF: Jones G, Makin HLJ: Vitamin Ds: Metabolites and Analogs. Modern Chromatographic Analysis of Vitamins, 3rd ed., Revised and Expanded. 75-141, Marcel Dekker Inc., New York, 2000.
4. Binkley N, Krueger D, Cowgill CS, et al.: Assay

variation confounds the diagnosis of hypovitaminosis D: a call for standardization. J Clin Endocrinol Metab, 89: 3152-3157, 2004.

5. Singh RJ: Are clinical laboratories prepared for accurate testing of 25-hydroxy vitamin D? Clin Chem, 54: 221-223, 2008.

6. Carter GD, Carter CR, Gunter E, et al: Measurement of Vitamin D metabolites: an international perspective on methodology and clinical interpretation. J Steroid Biochem Mol Biol, 89-90: 467-471, 2004.

7. Phinney KW: Development of a standard reference material for vitamin D in serum. Am J Clin Nutr, 88: 511S-512S, 2008.

8. Phinney KW, Bedner M, Tai SS, et al.: Development and certification of a standard reference material for vitamin D metabolites in human serum. Anal Chem, 84: 956-962, 2012.

9. Vogeser M, Kyriatsoulis A, Huber E, et al: Candidate reference method for the quantification of circulating 25-hydroxyvitamin D_3 by liquid chromatography-tandem mass spectrometry. Clin Chem, 50: 1415-1417, 2004.

10. Tai SS, Bedner M, Phinney KW: 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. Anal Chem, 82: 1942-1948, 2010.

11. Committee on Nutrition, Japan Society of Clinical Chemistry: Comment on standardization of serum 25-hydroxyyitamin D measurement in Japan[Jpn]. Jpn J Clin Chem, 38: 140-151, 2009.

12. Tsugawa N, Suhara Y, Kamao M, Okano T: Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography-tandem mass spectrometry. Anal Chem, 77: 3001-3007, 2005.

13. Mochizuki A, Kodera Y, Saito T, et al.: Preanalytical evaluation of serum 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 measurements using LC-MS/MS. Clin Chim Acta, 420: 114-120, 2013.

14. Ihara H: Recent progress in serum vitamin D analytical method [Jpn]. Rinsho Kensa (Journal of Medical Technology), 55: 967-972, 2011.

15. Kao PC, Heser DW: Simultaneous determination of 25-hydroxy- and 1,25-dihydroxyvitamin D from a single sample by dual-cartridge extraction. Clin Chem, 30: 56-61, 1984.

16. 25-Hydroxyvitamin D ^{125}I-RIA kit instruction manual. DiaSorin, Stillwater, Minnesota 55082-0285, U.S.A.

17. Singh RJ, Taylor RL, Reddy GS, et al.: C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. J Clin Endocrinol Metab, 91: 3055-3061, 2006.

18. Strathmann FG, Sadilkova K, Laha TJ, et al.: 3-epi-25 hydroxyvitamin D concentrations are not correlated with age in a cohort of infants and adults. Clin Chim Acta, 413: 203-206, 2012.

19. Lensmeyer G, Poquette M, Wiebe D, Binkley N: The C-3 epimer of 25-hydroxyvitamin D_3 is present in adult serum. J Clin Endocrinol Metab, 97: 163-168, 2012.

20. Brown AJ, Ritter CS, Weiskopf AS, et al.: Isolation and identification of 1 α -hydroxy-3-epi-vitamin D_3, a potent suppressor of parathyroid hormone secretion. J Cell Biochem, 96: 569-578. 2005.

21. Brown AJ, Ritter C, Slatopolsky E, Muralidharan KR, Okamura WH, Reddy GS: 1α,25-dihydroxy-3-epi-vitamin D_3, a natural metabolite of 1α,25-dihydroxyvitamin D_3, is a potent suppressor of parathyroid hormone secretion. J Cell Biochem, 73: 106-113, 1999.