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
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$_2$ 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.

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 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. 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) (Fig. 2).

The serum concentration of 1,25-dihydroxyvita...
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. 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). SRM 972 consists of four serum pools with different levels of vitamin D metabolites, and gives certified reference values of 25-hydroxyvitamin D$_3$ and D$_2$, and 3-epi-25-hydroxyvitamin D$_3$ (Table 1).

### Table 1  Assigned values of SRM 972

<table>
<thead>
<tr>
<th>Level</th>
<th>2,5-hydroxyvitamin D$_3$</th>
<th>2,5-hydroxyvitamin D$_2$</th>
<th>3-epi-2,5-hydroxyvitamin D$_3$</th>
<th>total 2,5-hydroxyvitamin D$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>23.9</td>
<td>0.59</td>
<td>1.39</td>
<td>25.9</td>
</tr>
<tr>
<td>Level 2</td>
<td>12.3</td>
<td>1.66</td>
<td>0.76</td>
<td>14.7</td>
</tr>
<tr>
<td>Level 3</td>
<td>18.5</td>
<td>25.7</td>
<td>1.06</td>
<td>45.3</td>
</tr>
<tr>
<td>Level 4</td>
<td>33.0</td>
<td>2.33</td>
<td>37.7</td>
<td>73.0</td>
</tr>
</tbody>
</table>

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$_3$ equivalents.
Table 2  Verification of 25-hydroxyvitamin D₃ values of SRM 972

<table>
<thead>
<tr>
<th>Level</th>
<th>Assigned values (ng/mL)</th>
<th>Observed values by LC-MS/MS (ng/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25-hydroxyvitamin D₃</td>
<td>3-epi-25-hydroxyvitamin D₃</td>
</tr>
<tr>
<td>Level 1</td>
<td>23.9</td>
<td>1.39</td>
</tr>
<tr>
<td>Level 2</td>
<td>12.3</td>
<td>0.76</td>
</tr>
<tr>
<td>Level 3</td>
<td>18.5</td>
<td>1.06</td>
</tr>
<tr>
<td>Level 4</td>
<td>33.0</td>
<td>37.7</td>
</tr>
</tbody>
</table>

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

Table 3  Determination of SRM 972 by two different methods

<table>
<thead>
<tr>
<th>Level</th>
<th>Assigned values (ng/mL)</th>
<th>Observed values (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total 2,5-hydroxyvitamin D including</td>
<td>total 2,5-hydroxyvitamin D excluding</td>
</tr>
<tr>
<td></td>
<td>3-epi-2,5-hydroxyvitamin D₃</td>
<td>3-epi-2,5-hydroxyvitamin D₃</td>
</tr>
<tr>
<td>Level 2</td>
<td>25.0</td>
<td>12.7</td>
</tr>
<tr>
<td>Level 3</td>
<td>45.3</td>
<td>44.2</td>
</tr>
<tr>
<td>Level 4</td>
<td>73.0</td>
<td>35.3</td>
</tr>
</tbody>
</table>

CPBA: Competitive protein binding assay
RIA: 25-Hydroxyvitamin D₁₂⁵-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 D₃ and 3-epi-25-hydroxyvitamin D₃, 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 D₃ were near by a 100% recovery against the expected values (the sum of 25-hydroxyvitamin D₃ and 3-epi-25-hydroxyvitamin D₃: Table 2). They also verified the assigned value of 25-hydroxyvitamin D₃ in Level 3, but found the value to be 33.1 ng/mL and to give a large recovery of 25-hydroxyvitamin D₃ 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 D₃ should be studied more carefully with the use of 25-hydroxyvitamin D₃ naturally present in human serum, since Level 3 was a preparation spiked with 25-hydroxyvitamin D₃.

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 D₁₂⁵-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 D₃. The total 25-hydroxyvitamin D concentration shown in Table 3 was the sum of the molar concentration of 25-hydroxyvitamin D₃, 25-hydroxyvitamin D₃ and 3-epi-25-hydroxyvitamin D₃, and the assay values were expressed as ng/mL in terms of their 25-hydroxyvitamin D₃ 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 D₃, but not with 25-hydroxyvitamin D₃ and 3-epi-25-hydroxyvitamin D₃. 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=...
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$_3$ 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$^9$. 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$^{18}$, 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


