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BACKGROUND: 25-hydroxyvitamin D [25(OH)D] assays are characterized by poor between-assay comparability. This result emphasizes the need for reference measurement procedures (RMPs) to establish calibration traceability and assist in method validation. We aimed at developing candidate RMPs on the basis of isotope dilution–liquid chromatography–tandem mass spectrometry (ID-LC-MS/MS) for separate quantification of serum 25(OH)D₂ and 25(OH)D₃.

METHODS: Hexa-deuterated 25(OH)D₃/D₂ was added to serum. This mixture was extracted with n-hexane and fractionated on Sephadex LH-20 before two-dimensional LC-MS/MS. In the first dimension, both procedures used a C4 column; however, in the second dimension, the 25(OH)D₂ procedure used a C18 and the 25(OH)D₃ procedure used a Zorbax SB-CN column. Calibration was traceable to the NIST Standard Reference Material (SRM) 2972. Validation comprised assessment of interference and limit of quantification/detection. Imprecision and trueness were validated by analysis of the SRM 972 against specifications (CV <5% and bias <1.7%). The expanded uncertainty for quadruplicate measurements was estimated.

RESULTS: Testing of potentially interfering substances was negative. Interference by 3-epi-25(OH)D₃ was resolved by sufficient chromatographic resolution. The limits of quantification/detection were 1.1 nmol/L and 0.09 pmol/L for 25(OH)D₃ and 1.2 nmol/L and 0.05 pmol/L for 25(OH)D₂. Mean total CVs and differences from the SRM 972 target (± one-sided 95% CI) were 2.1% and 1.1% ± 1.5% [25(OH)D₃] and 3% and 1.3% ± 0.6% [25(OH)D₂], respectively. The expanded respective uncertainties were 3.4% and 3.9%.

CONCLUSIONS: From the validation data, we conclude that we achieved our objective of 2 state-of-the-art candidate RMPs for serum 25(OH)D₃ and 25(OH)D₂.

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olites and sum up the results to the total 25(OH)D concentration. However, Singh et al. (8) showed that commonly used LC-MS/MS procedures may overestimate the 25(OH)D concentration when the C-3 epimer accounts for a substantial proportion of the circulating 25(OH)D$_3$ concentration. This occurrence results from insufficient chromatographic resolution to separate the 3-epi metabolite. Another major limitation that applies for all current 25(OH)D methods is the poor comparability of results, as demonstrated in dedicated proficiency testing schemes (9, 10). This is confounding for diagnosis against expert recommendations to maintain circulating 25(OH)D concentrations above a certain concentration for optimal health (11–14). This concern increasingly calls for standardization (15–17). There is general consensus that this should be done by using a higher-order reference measurement system to establish traceability to the Système International d’Unités (SI) (18). SI traceability or trueness is indeed the most valid basis for generation of laboratory data that serve the establishment of guidelines and clinical diagnosis, long-term epidemiologic studies, and programs to evaluate the effect and safety of dietary supplements (19–21). It was in this regard that the Office of Dietary Supplements from the National Institutes of Health worked together with the NIST to develop Standard Reference Materials (SRMs) for 25(OH)D$_3$/D$_2$, i.e., the serum-based SRM 972 (4 levels) and the SRM 2972 calibration solutions (22–24). The latter was helpful in decreasing interlaboratory variation of chromatographic procedures (25). The SRM 972, certified with isotope dilution (ID)-LC-MS/MS reference measurement procedures (RMPs) (26), was intended for validation of the trueness of hierarchically lower methods (18). However, some levels of the SRM were deemed noncommutable when measured with immunoassays and therefore not fit for trueness assessment (27). This problem is not expected for routine MS/MS procedures because they are based on the same measurement principle as the certification procedure. Because of the issue of noncommutability of reference materials, the approach of directly using deuterated analogs [d$_6$-25(OH)D$_3$/D$_2$] was obtained from Medical Isotopes. SRM 972 and SRM 2972 were obtained from NIST, Sephadex LH-20 from GE Healthcare, and horse serum from Invitrogen. The components tested for interference were a gift from Immunotech and H. DeLuca (University of Wisconsin, Madison). Chemicals and solvents were analytical grade, with the exception of methanol and water of ultra-performance liquid chromatography (UPLC) grade (Biosolve). For details on the measured human serum samples, see File 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue3.

**CALIBRATION**

Three working solutions of approximately 60/21 nmol/L 25(OH)D$_3$/D$_2$ in ethanol were prepared. They were assigned with exact values from a comparison protocol with the SRM 2972, further referred to as “calibration transfer protocol.” The solutions of d$_6$-25(OH)D$_3$/D$_2$ were prepared similarly. For quantification, a one-point calibration procedure at the 1:1 isotope ratio (± 25%) was used (30) (see Supplemental Data File 1).

**SAMPLE PREPARATION**

Sample preparation is fully described in Supplemental Data File 1. Typically, 250 μL serum (maximum 500 μL) was sampled and diluted to 1 mL with 0.9% (g/g) sodium chloride solution. Subsequently, equal absolute amounts of either d$_6$-25(OH)D$_3$ or d$_6$-25(OH)D$_2$ were added. After equilibration, the serum was alkalinized, extracted with n-hexane, and fractionated by Sephadex LH-20 chromatography.

**LC-MS/MS ANALYSIS**

A two-dimensional Acquity® UPLC system connected to an Acquity® tandem quadrupole mass detector (both from Waters), operating in the positive electrospray ionization mode, was used. The first dimension used an Acquity® BEH300 C4 column (2.1 × 50 mm, 1.7 μm, 300 Å). The second dimension columns were method specific: the 25(OH)D$_2$ candidate RMP used an Acquity® BEH C18 column (2.1 × 50 mm, 1.7 μm, 130 Å), while the 25(OH)D$_3$ a Zorbax SB-CN column (2.1 × 250 mm, 5 μm, 80 Å; held at 30 °C, Agilent Technologies). The selected reaction monitoring tran-
25-Hydroxyvitamin D Candidate Reference Measurement Procedures

METHOD VALIDATION

Calibration. We estimated the uncertainty of the calibration transfer protocol from the imprecision of the experimental design used for value assignment. Furthermore, we assessed whether it was justified to do the calibration from direct analysis of the calibrators, i.e., without submission to the sample pretreatment procedure. The experimental protocol is described in Supplemental Data File 1.

Recovery and matrix effect on ionization. The absolute recovery of the sample preparation procedure for 25(OH)D₃/D₂ was investigated by comparison of the isotope ratios obtained for 2 sets of 6 aliquots from the same serum pool. To the first set, the isotopically labeled analog was added before sample preparation; to the second set, it was added only after extraction and fractionation (see the Supplemental Data File 1). Matrix effect on ionization was investigated by comparison of the area under the peak obtained from direct injection of the labeled analog vs injection after addition to a processed serum matrix (n = 6).

Interference. Structural analogs of 25(OH)D₃/D₂ were tested for interference (for the amounts injected, see Supplemental Data File 1). The relative molecular mass of the [M+H]⁺ and [M+Na]⁺ ions was a first criterion for potential interference at the m/z values monitored for 25(OH)D₃/D₂ and the d₆ analogs. For the substances with a higher relative molecular mass, a full scan spectrum was registered (m/z range of 350–450) to verify the presence of an interfering MS signal. The substances testing positive were evaluated for their retention time relative to 25(OH)D₃/D₂ in the first dimension of the UPLC procedure only. In addition, 87 native serum samples, not supplemented with d₆-25(OH)D₃/D₂, were analyzed to assess interference.

Limit of quantification (LoQ) and limit of detection (LoD). The LoQ for 25(OH)D₂ was evaluated from analysis of SRM 972 level 1 (certified concentration: 1.46 ± 0.49 nmol/L [expanded uncertainty]) and from an analytical recovery experiment with a human serum pool supplemented to a total concentration of 2.98 nmol/L. Note that the endogenous concentration was 1.49 (0.03) nmol/L (two-sided 95% CI). For 25(OH)D₃, the LoQ was estimated from analysis of horse serum supplemented with 1.02 nmol/L. All samples were quantified in duplicate on 5 independent days (n = 10), which allowed the estimation of the total CV and the mean signal-to-noise (S/N) ratio. Note that the same protocol was used to determine the endogenous concentration in the sera of the recovery experiment. Our specifications for LoQ were a total CV <7% and a maximum absolute deviation <0.13 nmol/L (see Discussion). The estimation of the LoD at an S/N ratio of 3 was done based on the LoQ samples.

Imprecision and trueness. These characteristics were validated from analysis of the 4 levels of the SRM 972 against specifications of a maximum total CV of 5% and a maximum systematic deviation of 1.7% (29). Because of the limited volumes of the NIST SRM materials, measurements were done according to a reduced CLSI EP 5 protocol, i.e., duplicates on 5 independent days (n = 10) (31). For each level, the within-run, between-run, and total CVs were calculated by one-way ANOVA. The mean total CV was calculated as the square root of the mean of the quadratic total CVs. For the different internal quality control samples (see Supplemental Data File 1), the total CV was similarly calculated using one-way ANOVA. The CV for measurement of native serum samples was derived from the difference between the duplicates; however, it was measured as singlicate on 2 independent occasions. The trueness was expressed as % recovery of the NIST-certified values.

Performance of native serum samples. The candidate RMPs were challenged with analysis of 87 native serum samples. After screening of the samples for the presence of 25(OH)D₂ and 3-epi-25(OH)D₃, all 3 metabolites were determined in duplicate with rigorous internal quality control (see Supplemental Data File 1). In the reconstructed ion chromatograms, we determined the typical S/N ratio, verified the presence of nonidentified interfering peaks, and evaluated the resolving power to separate 3-epi-25(OH)D₃ [at 3% of the 25(OH)D₃ peak height]. The 25(OH)D₂ and 25(OH)D₃ concentrations were quantified in absolute terms, whereas for the 3-epi form, the content relative to 25(OH)D₃ (%) was estimated.

Expanded uncertainty of measurement. First, the combined uncertainty was calculated from propagating the uncertainty due to the imprecision, the calibration transfer protocol, the SRM 2972 certification, and unspecific interferences. To obtain a representative imprecision, the total CV estimated from analysis of the different SRM 972 levels was combined with the CV’s calculated for the internal quality control and native serum samples. This step was done by taking the square root of the mean of each quadratic CV value. Finally, the expanded uncertainty was estimated with k = 2 (95%).
**Table 1. Vitamin D analogs tested for potential interference.**

<table>
<thead>
<tr>
<th>Vitamin D metabolite or structural analog</th>
<th>Relative molecular mass</th>
<th>Interfering m/z value</th>
<th>Relative retention time*</th>
<th>Interference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[M+H-H2O]⁺</td>
<td>[M+H]⁺</td>
<td>[M+Na]⁺</td>
<td></td>
</tr>
<tr>
<td>Cholecalciferol</td>
<td>367.7</td>
<td>385.7</td>
<td>407.7</td>
<td>+</td>
</tr>
<tr>
<td>Ergocalciferol</td>
<td>379.7</td>
<td>397.7</td>
<td>419.7</td>
<td>+</td>
</tr>
<tr>
<td>7-Dehydrocholesterol</td>
<td>367.6</td>
<td>385.6</td>
<td>407.6</td>
<td>-</td>
</tr>
<tr>
<td>3-epi-25-Hydroxyvitamin D₃</td>
<td>383.6</td>
<td>401.6</td>
<td>423.6</td>
<td>+</td>
</tr>
<tr>
<td>1α-Hydroxyvitamin D₃</td>
<td>383.6</td>
<td>401.6</td>
<td>423.6</td>
<td>+</td>
</tr>
<tr>
<td>1α,25-Dihydroxyvitamin D₂</td>
<td>411.7</td>
<td>429.7</td>
<td>451.7</td>
<td>-</td>
</tr>
<tr>
<td>1α,25-Dihydroxyvitamin D₃</td>
<td>399.6</td>
<td>417.6</td>
<td>439.6</td>
<td>-</td>
</tr>
<tr>
<td>24,25-Dihydroxyvitamin D₃</td>
<td>399.6</td>
<td>417.6</td>
<td>439.6</td>
<td>-</td>
</tr>
<tr>
<td>25,26-Dihydroxyvitamin D₃</td>
<td>399.6</td>
<td>417.6</td>
<td>439.6</td>
<td>-</td>
</tr>
<tr>
<td>1α,25,26-Trihydroxyvitamin D₃</td>
<td>415.6</td>
<td>433.6</td>
<td>455.6</td>
<td>-</td>
</tr>
</tbody>
</table>

Ø Codes used to describe interfering m/z value: –, no signal, obtained in the scan mode, at the m/z values corresponding to the relative molecular mass of [M+H]⁺: 401.6 [25(OH)D₃], 407.6 [d₂-25 (OH)D₃], 413.7 [25(OH)D₂], and 419.7 [d₂-25(OH)D₂]. +, because the molecule had an interfering m/z value (note: m/z values are in italics), we determined the relative retention time. Only the molecules that eluted from the first dimension column between 5.4 and 6.4 min (corresponding relative retention time: 0.92–1.09 (0.89–1.05) for 25(OH)D₃ [25(OH)D₂] are transferred to the second dimension column).

* Relative retention time in comparison with 25(OH)D₃ or 25(OH)D₂, respectively.

Statistical data analysis. Microsoft Office Excel® (version 2007) was used to perform the Grubbs outlier test, the two-sided F-test, one- or two-sided (depending on the application) Student’s t-tests with equal or unequal variances (based on the F-test), and a one-way ANOVA, all at 95% probability.

**Results**

**Calibration**

The uncertainty of the calibration transfer protocol amounted to 0.7%. The isotope ratios measured in the 2 sets of calibrators (directly analyzed and after submission to sample preparation) were not significantly different [25(OH)D₃]: difference 0.86%, P_{two-sided} = 0.186; 25(OH)D₂: 0.54%, P_{two-sided} = 0.663]. This result together with the evidence for absence of matrix effect on ionization (see below) showed that calibration with directly analyzed calibrators was justified.

**Recovery and Matrix Effect on Ionization**

The absolute recovery ± two-sided 95% CI of the sample preparation procedures was 71% ± 4% [25(OH)D₃] and 70% ± 8% [25(OH)D₂]. The matrix effect on ionization was found to be statistically not significant [25(OH)D₃], observed difference 15%, P_{two-sided} = 0.0516; 25(OH)D₂, 18%, P_{two-sided} = 0.0574].

**Interference**

The results for the interference study are summarized in Table 1 (for the retention time of the examined components, see Supplemental Table S3 in the online Data Supplement). Although the registered full scan spectra identified potential interference by some compounds, the relative retention time allowed to exclude it for all, except 3-epi-25(OH)D₃. As shown in Fig. 1, the 3-epi form is separated at 3% of the 25(OH)D₃ peak height on the Zorbax SB-CN column. Analysis of the 87 samples without added d₆ analogs confirmed absence of interference on the transition m/z values 407.3 → 159.3 and 419.4 → 159.4.

**LoQ and LoD**

The LoQ for 25(OH)D₃, estimated from analysis of SRM 972 level 1, was 1.22 (0.05) nmol/L (two-sided 95% CI). The absolute difference from the target was −0.24 nmol/L, the total CV was 5.3%, and the mean S/N ratio was 76. The LoQ, estimated from the analytical recovery experiment, was 1.43 (0.05) nmol/L, with a deviation of −0.054 nmol/L from the target. The measured total 25(OH)D₃ concentration in the supplemented horse serum was 1.12 (0.05) nmol/L. The LoD, estimated from the above data, was 0.025 pmol (10 pg) and 0.015 pmol (6 pg) on columns for 25(OH)D₃ and 25(OH)D₂, respectively.

**Imprecision and Trueness**

Tables 2 and 3 list the different imprecision and trueness data. The mean within-run and total imprecision (ex-
pressed as % CV) estimated from analysis of the different levels of the SRM 972 were 1.9% and 2.1% for 25(OH)D₃ and 2.9% and 3.0% for 25(OH)D₂. The mean trueness was 101.1% ± 1.5% (one-sided 95% CI) for 25(OH)D₃ and 101.3% ± 0.6% for 25(OH)D₂. None of the obtained % recoveries of the NIST target values exceeded the 1.7% deviation limit, as confirmed by a one-sided t-test against the limits for maximum deviation (i.e., 98.3% or 101.7%) (see Table 3 for the P values).

PERFORMANCE ON NATIVE SERUM SAMPLES
The concentrations of 25(OH)D₃ in 87 samples ranged from 2.4 to 590 nmol/L, with a mean of 73 nmol/L. In all investigated samples, 3-epi-25(OH)D₃ was present. The mean relative amount compared to the 25(OH)D₃ content was 5.3% [<4% 3-epi-25(OH)D₃ in 32% of the samples, between 4% and 8% in 56%, and >8% (with a maximum of 17%) in 12%]. In 6 samples, the 25(OH)D₂ concentration range was from 11 to 281 nmol/L. Fig. 1 shows representative chromatograms of processed serum samples. Typical S/N ratios were approximately 2500 [4.0 pmol (1.6 ng) 25(OH)D₃ on column] and approximately 850 [1.2 pmol (0.5 ng) 25(OH)D₂ on column].

EXPANDED UNCERTAINTY OF MEASUREMENT
The combined uncertainty was estimated from propagating the uncertainty components attributed to the imprecision for measurement of the SRM 972, the internal quality control, and patient samples [1.9% for 25(OH)D₃; 2.6% for 25(OH)D₂; the calibration transfer (0.7%); and the NIST SRM 2972 certification (0.8%, (23) and unspecific interferences, estimated at 1%). This result gave an expanded uncertainty (k = 2; 95%) for a single measurement (at a concentration within the normal range) amounting to 4.7% for 25(OH)D₃ and 5.9% for 25(OH)D₂; for measurement in 4 replicates, it was 3.4% and 3.9%, respectively. At the LoQ, the expanded uncertainty for 25(OH)D₃ was 12.9% (n = 1) and 6.9% (n = 4) and for 25(OH)D₂ was 11.0% (n = 1) and 6.0% (n = 4).

Discussion
We developed 2 candidate RMPs for separate quantification of 25(OH)D₃ and 25(OH)D₂. These RMPs were developed to enable unequivocal validation of immunoassays that claim equimolar measurement, which is indeed a prerequisite for correct assessment of the vitamin D status when dietary supplementation with vitamin D₂ is done. It is expected that vitamin D₂ supplementation will be continued as long as the controversy about the effectiveness of the 2 vitamin forms in maintaining circulating 25(OH)D concentrations is unresolved (32–34). We further ensured traceability of the
calibration to the hierarchically highest primary calibrators currently available, i.e., the SRM 2972 (23). For the same reason, we validated the trueness and imprecision in a combined measurement protocol of the 4 levels of the SRM 972 (24). We performed this validation against specifications previously proposed for an RMP. These specifications are on the basis of the biological variation diagnosis model, i.e., a total CV/≤5% and a systematic deviation/≤1.7% (29). The strength of these quality goals is that they represent a good balance between state-of-the-art performance of typical ID-LC-MS/MS procedures and the imprecision and trueness needed for an RMP fit for the purpose of establishing/validating the SI traceability of routine diagnostic methods. We realized, however, that because the aforementioned goals are limits, our stable performance should be better. The values in Tables 2 and 3 demonstrate that we achieved this for our 2 candidate RMPs. Note that the imprecision data generated from applying the reduced CLSI EP5 protocol for analysis of the SRM 972 were confirmed by the internal quality control CVs at higher n values (Table 2). In addition, the estimated expanded uncertainty of measurement supports the fitness of the candidate RMPs, in particular, with a typical measurement protocol of 4 replicates.

We further paid attention to typical points of concern for a candidate RMP on the basis of ID-LC-MS/MS. These points comprised calibration at the 1:1 isotope ratio, while accounting for the influence of the spectral overlap (30), assessment of absence of matrix effect on ionization and sufficient recovery to not negatively influence the accuracy and imprecision, and assessment that it was justified to perform matrix-free calibration. The measures taken to minimize matrix effect on ionization and to justify matrix-free calibration comprised (a) use of an extensive sample pretreatment strategy, (b) collection of only a narrow Sephadex LH-20 window, and (c) incorporation of a two-dimensional UPLC approach. The observed differences in isotope ratios between extracted and nonextracted calibrators were not statistically significant. Another point of concern was thorough assessment of absence of interference. This was done from general

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**Table 2. Imprecision data obtained from analysis of 25(OH)D₃ and 25(OH)D₂ in the SRM 972, at the LoQ, and in internal quality control samples and native serum samples.**

<table>
<thead>
<tr>
<th>Target (nmol/L)</th>
<th>25(OH)D₃</th>
<th>25(OH)D₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRM 972</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1</td>
<td>59.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Level 2</td>
<td>30.8</td>
<td>6.2</td>
</tr>
<tr>
<td>Level 3</td>
<td>46.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Level 4</td>
<td>82.3</td>
<td>4.6</td>
</tr>
<tr>
<td>IQC</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Pool 1</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Pool 2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Native serum samples (n = 87)</td>
<td>1.6</td>
<td>2</td>
</tr>
<tr>
<td>25(OH)D₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRM 972</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 2</td>
<td>4.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Level 3</td>
<td>64.1</td>
<td>2.0</td>
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<tr>
<td>Level 4</td>
<td>5.8</td>
<td>3.0</td>
</tr>
<tr>
<td>IQC</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Pool 3</td>
<td>15.4</td>
<td>6.2</td>
</tr>
<tr>
<td>Native serum samples (n = 6)</td>
<td>1.2</td>
<td>2</td>
</tr>
<tr>
<td>LoQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D₃ (SRM 972)</td>
<td>1.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* Always from duplicate analysis.

b IQC, internal quality control.

CV from the differences between duplicates.

Duplicate on 2 different days.
inspection of the presence of unidentified peaks in the reconstructed ion chromatograms obtained for a multitude of patient samples, from analysis of samples without addition of the isotopically labeled analog, and from dedicated investigation of potentially interfering components. We also emphasized having sufficient chromatographic resolution to separate the 3-epi form of 25(OH)D₃, because coelution may lead to overestimation of circulating 25(OH)D₃ concentrations when the 3-epi form is present in a considerable proportion (8). Although there is no current evidence that the 3-epi form is clinically relevant, we found that 12% of the serum samples analyzed in this study had a 3-epi-25(OH)D₃/25(OH)D₃ ratio of 8% relative to their 25(OH)D₃ (with a maximum of 17%). Although we do not claim that we are able to certify the concentration of the 3-epi form with the same quality as for 25(OH)D₃, we estimated the concentration assuming the same MS response factor for the 2 metabolites, which gave for SRM 972 level 4 a result in good agreement with the certified value (1.8% mean systematic deviation and 1.4% total CV, n = 10). Another point of care was the definition of the LoQ with accompanying imprecision and trueness specifications, i.e., a maximum total CV of 7% and systematic deviation of 0.13 nmol/L. These specifications stand into relationship to the aforementioned biologic quality goals (CV <5%; systematic deviation <1.7%) (29). Note that we converted the relative systematic deviation criterion to an absolute value for concentrations <7.7 nmol/L (0.13 nmol/L is 1.7% at 7.7 nmol/L). As shown in Tables 2 and 3, the LoQ for our 25(OH)D₃ RMP met the goals. In contrast, the LoQ for measurement of 25(OH)D₂ only had an acceptable imprecision, whereas the absolute difference from the target exceeded the limit. However, considering that the uncertainty on the 25(OH)D₂ target for level 1 of SRM 972 is quite high (i.e., 34%), we accepted the LoQ. This result was justified by the analytical recovery experiment, demonstrating a similar LoQ with a deviation of only −0.054 nmol/L. The LoQ of 26 fmol (10 pg) 25(OH)D₃ and 15 fmol (6 pg) 25(OH)D₂ on column corresponds to serum concentrations of 89 fmol/L and 48 fmol/L, respectively. Comparison of the performance characteristics of our candidate RMPs with those documented for the previously published procedures is difficult (26, 35). This result occurs because either only the precision is documented (35) or a different experimental design is used (26). For example, for validation of the NIST candidate RMP for 25(OH)D, Tai et al. (26) injected each individually prepared sample twice (resulting in a better within-run CV), while we calculated our data based on a single injection; moreover, they did not report the total CV. For obvious reasons, Tai et al. assessed the trueness from analytical recovery data of supplemented serum, whereas we did the validation from measurement of the SRM 972. Finally, we demonstrated the applicability of our candidate RMPs by quantifying the 25(OH)D₃ concentration in 87 samples and the D₂ metabolite in 6 samples, all covering reasonable concentration ranges from 2.4 to 590 nmol/L [25(OH)D₃] and from 11 to 281 nmol/L [25(OH)D₂]. All samples were analyzed on 2 different occasions with separate sample pretreatment and independent calibration. The difference between the duplicate results never exceeded 3.5%, and the
mean CV was 1.6% and 1.0% for 25(OH)D₃ and 25(OH)D₂, respectively.

In conclusion, we achieved our objective of developing 2 SI-traceable candidate RMPs for serum 25(OH)D₃ and 25(OH)D₂ that are fit to the purpose of establishing/validating the SI traceability of diagnostic routine methods.

**Authors’ Contributions:** All authors contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data; or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**References**

34. Holick MF, Biancuzzo RM, Chen TC, Klein EK, Young A, Bibuld M et al. Vitamin D₃ is as effective as vitamin D₂ in maintaining circulating concentrations of 25-hydroxyvitamin D. J Clin Endocrinol Metab 2008;93:677–81.