**Supplemental data to**

**A Candidate Reference Measurement Procedure for Quantifying Serum Concentrations of 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D2 Using Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry**

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**Materials and methods**

*Calibration preparation and control materials*

A master stock solution of 25(OH)D3 was gravimetrically prepared by dissolving between 1-1.5 mg of solid in a 100-mL volumetric flask with absolute ethanol, targeting a concentration of 30-50 μmol/L. On the day of the preparation we used the master stock to prepare 3 independent working solutions (gravimetrically) in ethanol, targeting concentrations of 150-250 nmol/L. The exact concentrations of the working solutions were determined from a parallel analysis with gravimetrically diluted SRM 2972 in 3 independent measurement series (2 sets of 3 or 4 working calibrators prepared from both NIST SRM 2972 and our working solutions). The difference, between the target concentration and the concentration determined by using NIST SRM 2972 (Certificate of Analysis October 21, 2014), among any of the 3 working standard solutions was within 1%. All ethanolic solutions were capped and stored at -20oC and used multiple times.

*LC-MS/MS analysis*

Typical MS parameters: nitrogen sheath gas pressure and auxiliary gas flow were 30 and 8 arbitrary units, respectively. The argon collision gas pressure was 1.2 mTorr. The vaporizer temperature, ion transfer capillary temperature and corona needle current were set at 380 oC, 250 oC and 2 μA, respectively. The tube lens offset and the skimmer voltages were set at 100 V and 6 V, respectively.

*Data analysis and typical measurement series (run) set-up*

The design of a typical measurement series was as follows: a blank sample (spiked with ISTD), a set of four calibrators (increasing concentrations), mobile phase, a second set of four calibrators (increasing concentrations), IQC samples (at least two different materials), SRM (typically one level of either 972 or 972a), unknown samples (prepared in duplicate), SRM (a different level of 972 or 972a level), IQC samples (the same materials, independently prepared). After the initial injection (1→44), the entire run was injected in reverse order (44→1), (Fig. S1). For each calibration level, the response ratios from both calibration sets (including the re-injection, 3 levels from 2 different solutions and duplicate injections = 12 points), were plotted and used for quantitation by Xcalibur software. Within a measurement series we used the mean of all 4 instrument results (2 preparations/2 injections) for every unknown and IQC sample to calculate the mass fraction (ng/g). In Excel, the mean instrument result [mass ratio, calculated off the calibration curve (function of mass ratios of the calibrators versus the response)] was multiplied by the weight of the internal standard solution (g) and by the internal standard mass fraction (ng/g) and divided by the weight of the unknown sample (g) to obtain the mass fraction of 25(OH)D2 and 25(OH)D3 (ng/g) in each unknown sample. To convert mass fraction (ng/g) to commonly used mass concentration (ng/mL) results, we applied the density of the serum samples. We performed serum density measurements on a DMA 35N portable density meter (Anton Paar, Ashland, VA). The final amount of substance, in nanomol per liter (nmol/L), was calculated by multiplying nanograms per milliliter by 2.4233 or 2.4959 for 25(OH)D2 and 25(OH)D3, respectively.

*Ion suppression.* We assessed the effect of matrix on ionization through ion suppression experiments [1]. We used post-column infusion of mixed 25(OH)D3 and 25(OH)D2 stock solutions while injecting matrix samples (QC and unknowns).

*Method validation*

*Direct calibration*

At 2-4 calibration sets (each consisting of 3-6 calibration levels) were prepared in three measurement series. In each series, at least 2 sets were carried through the sample preparation procedure and equal amount of sets were mixed with the ISTD, evaporated (vacuum at 45 oC) and directly injected on the instrument after reconstitution with 73% MeOH/water. Each sample was injected twice, and the mean response was used in the calculations. Mean relative response ratios from all measurements were calculated (directly analyzed and after submission to sample preparation) and used to calculate the % difference between the two sets.

We assessed the effect of matrix on ionization during the sample preparation procedure, as factors that may influence accuracy. Our candidate RMP was free of ion suppression, indicated by the lack of baseline drifts around the elution times of 25(OH)D3 and 25(OH)D2 upon injection of extracted matrix samples.

**References**

1. Annesley T (2003) Ion suppression in mass spectrometry. Clin Chem 49(7):1041-1044

**Table 1S. Estimation of expanded uncertainty for candidate reference measurement procedure measurements of serum concentrations of 25 (OH)D2 and 25 (OH)D3 in internal quality control materials**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Type A factor** | | | | **Type B factor** | | | | |  | **Uncertainty** | | |  | |
| **Mean (nmol/L)** | **SD** | **SD, mean** | | **Purity\*** | **Weighing** | | **Density** | **Other#** | ***uc*a** | | ***U*b** | ***U*c, %** | | |
| **25(OH)D2** | | | | | | | | | | | | | |
| 3.01 | 0.072 | | 0.041 | 0.024 | 2.5e-3 | 8.7e-7 | | 0.030 | 0.057 | | 0.113 | 3.8 | | |
| 3.15 | 0.066 | | 0.038 | 0.025 | 2.5e-3 | 8.7e-7 | | 0.032 | 0.055 | | 0.111 | 3.5 | | |
| 14.0 | 0.212 | | 0.122 | 0.112 | 2.5e-3 | 8.7e-7 | | 0.140 | 0.217 | | 0.434 | 3.1 | | |
| 14.7 | 0.334 | | 0.193 | 0.118 | 2.5e-3 | 8.7e-7 | | 0.147 | 0.270 | | 0.539 | 3.7 | | |
| 17.1 | 0.404 | | 0.233 | 0. 136 | 2.5e-3 | 8.7e-7 | | 0.171 | 0.320 | | 0.639 | 3.7 | | |
| **25(OH)D3** | | | | | | | | | | | | | |
| 16.4 | 0.424 | | 0.245 | 0.131 | 2.5e-3 | 8.7e-7 | | 0.164 | 0.322 | | 0.645 | 3.9 | | |
| 23.1 | 0.478 | | 0.276 | 0.185 | 2.5e-3 | 8.7e-7 | | 0.231 | 0.405 | | 0.809 | 3.5 | | |
| 29.6 | 0.568 | | 0.328 | 0.237 | 2.5e-3 | 8.7e-7 | | 0.296 | 0.501 | | 1.003 | 3.4 | | |
| 39.0 | 0.294 | | 0.170 | 0.312 | 2.5e-3 | 8.7e-7 | | 0.390 | 0.527 | | 1.054 | 2.7 | | |
| 63.5 | 0.377 | | 0.218 | 0.508 | 2.5e-3 | 8.7e-7 | | 0.635 | 0.842 | | 1.684 | 2.7 | | |
| 72.2 | 1.211 | | 0.699 | 0.578 | 2.5e-3 | 8.7e-7 | | 0.722 | 1.159 | | 2.319 | 3.2 | | |

\* 0.8 % uncertainty of purity reference compound

#1% uncertainty of systemic error

a combined standard uncertainty

b expanded uncertainty with coverage factor of 2 (95% confidence interval)

c relative expanded uncertainty

**Figure captions and legends**

**Fig. S1** Typical run set up

**Fig. S2** Analytical imprecision of 25(OH)2 as a function of concentration. NIST SRM materials (n = 8) are depicted by open diamonds and in-house IQC materials (n = 5-8) are represented by closed diamond