Kerstin Kandler, Neeraj Singh, Friederike Bauland, Elie Fux, Andrea Geistanger, Christian Geletneky and Judith Taibon*

An isotope dilution-liquid chromatography-tandem mass spectrometry based candidate reference measurement procedure for the simultaneous quantification of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum and plasma

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Abstract

Objectives: An isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)-based candidate reference measurement procedure (RMP) for the quantification of 25-hydroxyvitamin D2 (25OHD2) and 25-hydroxyvitamin D3 (25OHD3) in human serum and plasma is presented.

Methods: Quantitative Nuclear Magnetic Resonance (qNMR) spectroscopic methodology has been utilized to assign absolute content (g/g) and International System of Units (SI)-traceability to the reference materials used as primary calibrators. This RMP was developed for the simultaneous quantification of 25OHD2 and 25OHD3 in human samples, utilizing supported liquid extraction (SLE) clean-up and a two-dimensional heart-cut ID-LC-MS/MS method to minimize matrix effects and prevent the co-elution of 3-Epi-25OHD3 and 3-Epi-25OHD2. The method underwent validation in accordance with current guidelines. Selectivity was assessed using spiked samples. To evaluate potential matrix effects, a post-column infusion experiment and a comparison of standard line slopes were performed. A 5-day validation study was conducted to determine precision, accuracy and trueness of the method. Measurement uncertainty for reference value assignment was evaluated in line with the Guide to the Expression of Uncertainty in Measurement (GUM). Equivalence to Joint Committee on Traceability in Laboratory Medicine (JCTLM) listed RMPs was demonstrated through the participation in the CDC Vitamin D Standardization-Certification Program (VDSCP) as well as the RELA scheme.

Results: The RMP enabled the quantification of 25OHD2 and 25OHD3 within the range of 1.50 ng/mL–180 ng/mL (3.64–436 nmol/L for 25OHD2 and 3.74–449 nmol/L for 25OHD3), without interference from their respective epimer and no evidence of matrix effects. Intermediate precision was determined to be ≤4.0 % for 25OHD2 and ≤3.6 % for 25OHD3, while repeatability was ≤3.3 % for 25OHD2 and ≤2.9 % for 25OHD3 across all concentration levels. The relative mean bias for the secondary reference materials varied from −1.0 to 1.1 %, regardless of the analyte. For the spiked samples, the relative mean bias ranged from −4.2 to 1.0 % for 25OHD2 and from −3.9 to 0.9 % for 25OHD3, irrespective of all levels and matrices. Expanded measurement uncertainties (k=2) for target value assignment (n=6) were ≤3.9 % for 25OHD2 and ≤3.2 % for 25OHD3. Participation in the VDSCP and the RELA scheme showed a good agreement with results from the JCTLM listed RMPs and laboratories.

Conclusions: The RMP enables the accurate, precise and consistent determination of 25OHD3 and 25OHD2. The robust performance of this method supports standardization of routine assays and guarantees traceability in the measurement of individual patient samples.

Keywords: 25-hydroxyvitamin D2; 25-hydroxyvitamin D3; isotope dilution-liquid chromatography-tandem mass spectrometry; reference measurement procedure; qNMR characterization; traceability

***Corresponding author: Judith Taibon**, PhD, Roche Diagnostics GmbH, Nonnenwald 2, 82377, Penzberg, Germany,
E-mail: judith.taibon@roche.com
Kerstin Kandler, Neeraj Singh, Elie Fux, Andrea Geistanger and Christian Geletneky, Roche Diagnostics GmbH, Penzberg, Germany
Friederike Bauland, Chrestos Concept GmbH & Co. KG, Essen, Germany

Introduction

The measurement of 25-hydroxyvitamin D2 (25OHD2) and 25-hydroxyvitamin D3 (25OHD3) in clinical chemistry

laboratories is essential to assess overall vitamin D status, diagnose and manage deficiencies, and monitor treatment efficacy. These measurements are crucial for differentiating sources of vitamin D (vitamin D2 from plant-based foods and supplements, and vitamin D3 from animal-based foods and sunlight), investigating hypercalcemia, assessing risk of chronic diseases, and evaluating parathyroid function [1].

Clinical laboratory professionals have specific expectations from the IVD industry to ensure the accuracy and reliability of IVD assays. These include developing RMPs tailored to manufacturers' needs, assigning SI-traceable values to calibrators, systematically assessing biases to be included in the uncertainty budget of an assay, and publishing methods in peer-reviewed journals [2].

This publication presents an RMP for the measurement of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 that comply with ISO 15193 guidelines. Our methodology, described in detail, includes technical information and calculation of measurement uncertainty, facilitating the transfer of methods to other laboratories. The validation focused on precision and trueness, with the associated measurement uncertainty calculated according to the Guide to the Expression of Uncertainty in Measurement (GUM) [3].

We have employed quantitative nuclear magnetic resonance spectroscopy (qNMR) to characterize reference materials for 25-hydroxyvitamin D2/D3 for the calibration of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays in order to ensure correct mass fraction value and International System of Units (SI)-traceability [4–6]. qNMR is a potent technique for the determination of analyte mass fraction (g/g) owing to the ease of workflow and non-destructive nature of the test, absolute linearity of detector to analyte amount, and availability of higher-order qNMR internal standards traceable to the kilogram via National Institute of Standards and Technology (NIST) Benzoic acid 350b or NIST PS1 [5, 7]. Additionally, the latest International Union of Pure and Applied Chemistry (IUPAC) Technical Report has included qNMR as a potential primary RMP ideally suited for characterizing primary reference materials [8]. We have already published our qNMR findings with respect to quantitative resonance, impurities, reactivity, solvent profile and kinetics of vitamin D vitamers [5]. In this manuscript, we present our qNMR target value assignments for both hydroxyvitamin D analytes and provide an overview of the probability of homolytic bond-cleavage based on high-level quantum chemical calculations.

Singh et al. [9] demonstrated that widely used LC-MS/MS methods might overestimate 25OHD levels when the C-3 epimer constitutes a significant portion of circulating 25OHD3. This overestimation occurs due to inadequate chromatographic resolution of these diasteromers, which fails to effectively separate the 3-epi metabolite. Therefore, special attention must be dedicated to the establishment of a protocol for the simultaneous quantification of 25OHD2 and 25OHD3 in human samples while minimizing matrix effects and preventing the co-elution of 3-Epi-25OHD3 and 3-Epi-25OHD2.

Additionally, the RMP must meet predetermined acceptance criteria for bias and imprecision which were calculated based on the biological variation outlined by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) database. For 25-hydroxyvitamin D3, the RMP's imprecision (CV_{ref}) should be half that of routine assays (CV_{rou}), and its bias (B_{ref}) should be one-third that of routine assays (B_{rou}). Based on EFLM data indicating CV_{rou} ≤3.4 %, B_{rou} ≤7.7 %, and TE_{rou} ≤13.3 % [10], the acceptance criteria for the RMP are defined as CV_{ref} ≤1.7 %, B_{ref} ≤2.6 % and TE_{ref} ≤5.4 %.

Materials and methods

Details according to the method, including a full list of materials and equipment used, can be found in the Supplementary Material 1.

Chemicals and reagents

25-Hydroxyvitamin D3 (25OHD3, CAS: 19356-17-3) and 25-hydroxyvitamin D2 (25OHD2, CAS: 21343-40-8) from MedChemExpress (MCE®, Monmouth Junction, NJ, USA) purchased via Hoelzel Biotech (Cologne, Germany) were used. The internal standard, 25-hydroxyvitamin D3-[23,24,25,26,27-13C5] in ethanol (25OHD3-13C5, CAS: 19356-17-3), was obtained from Entegris Inc – IsoSciences (Ambler, PA, USA). The internal standard, 25-hydroxyvitamin D2-[252627-13C3] (25OHD2-13C3, CAS: 1325307-59-2), was synthesized by Endotherm – Life Science Molecules (Saarbruecken, Germany). 3-Epi-25-hydroxyvitamin D3 in ethanol (CAS: 73809-05-9) and 3-Epi-25-hydroxyvitamin D2 (CAS: 908126-48-7) in ethanol were both obtained from Entegris Inc – IsoSciences. 25-Hydroxyvitamin D calibration solution (SRM 2972a [11]) and certified secondary reference material (SRM 972a [12], SRM 2970 [13]) were obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

Sodium carbonate (CAS: 497-19-8), sodium hydrogen carbonate (CAS: 144-55-8), ethanol absolute for analysis (CAS: 64-17-5), ethyl acetate (CAS: 141-78-6) and 2-propanol (CAS: 67-63-0) were purchased from Merck (Darmstadt, Germany). Methanol (CAS: 67-56-1) and formic acid (CAS: 64-18-6), both LC-MS grade, were obtained from Biosolve (Valkenswaard, The Netherlands).

Vitamin D depleted human serum was obtained from Roche Diagnostics GmbH (Penzberg, Germany) and from Golden West Diagnostics (Temecul, USA). Mixed gender native plasma pools (Li-heparin [CUST-BB-17022020-4C], dipotassium (K2) ethylene diamine tetraacetic acid (EDTA) [CUST-BB-17022020-4D] and tripotassium (K3) EDTA [CUST-BB-17022020-4E] were purchased from Biotrend (Cologne, Germany). The native human serum pool consisted of five samples. According to the Declaration of Helsinki, all native serum and plasma patient samples were anonymized samples.

qNMR for determining the purity of standard materials

qNMR experiments were performed on a Jeol 600 MHz NMR equipped with a Helium-cryoprobe. For both the analytes, single-pulse-$^{1}H\{^{13}C\}$NMR was utilized for the quantitation (alkene proton; δ=6.24 ppm; 1H; tecnazene as qNMR internal standard; $CDCl_3$ as solvent) with an inter-scan delay of 70 s (Supplemental Material 3, Figures 1–4). All the qNMR details about impurities, quantitative resonance, and chemistry in solution have already been published [5]. Further details relevant to this manuscript are available in the Supplemental Material 2.

Additionally, we also performed quantum chemical calculations to determine the most reactive chemical bond towards H-atom transfer (HAT analysis) was also performed owing to the alleged photo-instability of the analytes. Finally, Density Functional Theory (DFT) single-point calculations were performed at the wB97X-V/def2TZVP level of theory and all the information is presented in the Supplemental Material 2. These theoretical analyses contributed in the determination of ideal quantitative resonance for both the analytes along with experimentally available data in the literature.

Preparation of matrix based calibrators and quality control samples

Matrix-based calibrators and quality controls (QCs) containing both 25OHD2 and 25OHD3 were used. Three independently weighed calibrator stock solutions were prepared for 25OHD2 and 25OHD3 using the qNMR characterized material. For each stock solution, 5 mg were weighed using an ultra-microbalance (XP6U/M, Mettler Toledo) and dissolved in 10 mL ethanol in a volumetric flask (0.500 mg/mL; 1.21 mmol/L for 25OHD2 and 1.25 mmol/L for 25OHD3). The purity of each material (25OHD2: 83.7 % ± 0.5 %, 25OHD3: 93.6 % ± 0.4 %, determined by qNMR) was taken into account for the calculation of the final stock solution concentrations. Three working solutions containing both analytes were prepared from these individual stock solutions in 50 % ethanol, all with a concentration of 10 µg/mL for both analytes (24.1 µmol/L for 25OHD2 and 24.7 µmol/L for 25OHD3). These working solutions were used to prepare spike solutions for the eight matrix matched calibrators (ranging from 1.50 to 180 ng/mL; 3.64–436 nmol/L for 25OHD2 and 3.74–449 nmol/L for 25OHD3) and three QC samples (4.50 ng/mL, 80.0 ng/mL and 150 ng/mL; 10.9, 194, 364 nmol/L for 25OHD2 and 11.2, 200, 374 nmol/L for 25OHD3). Both, calibrators and QCs, had the same concentration of 25OHD2 and 25OHD3. Vitamin D depleted human serum was used as surrogate matrix for calibrators and controls. In addition, secondary reference material (NIST SRM 2970, 25OHD2: 23.5 ng/mL, [56.9 nmol/L]; 25OHD3: 9.63 ng/mL, [24.0 nmol/L]) and one native patient sample (25OHD2: <LLMI, 25OHD3: ~35.0 ng/mL [87.4 nmol/L]) were used to monitor the method performance over time.

Internal standard (ISTD) solution

An internal standard solution containing both 25OHD2-13C3 and 25OHD3-13C5 was prepared by dissolving 25OHD2-13C3 powder material in ethanol and further dilution to a 100 µg/mL (241 µmol/L) 25OHD2-13C3 working solution. Then, the 25OHD2-13C3 working solution and the commercially available 100 µg/mL (247 µmol/L) 25OHD3-13C5 solution were combined and further diluted with 50 % ethanol, resulting in a 165 ng/mL (397 nmol/L for 25OHD2-13C3 and 407 nmol/L for 25OHD3-13C5) ISTD spike solution.

Sample preparation

Serum and plasma matrices (lithium-heparin plasma, K_2-EDTA and K_3-EDTA) and vitamin D depleted serum can be used as sample matrix.

200 µL of sample (unknown native sample, calibrator or QC) was pipetted into a 0.5 mL brown screw cap vial. 20 µL internal standard spike solution (25OHD2-13C3 and 25OHD3-13C5 in 50 % ethanol) was added and incubated on an overhead shaker (Sarmix M2000; Sarstedt, Nümbrecht, Germany) for 15 min at room temperature. Samples were then treated with 100 µL of carbonate buffer on a thermomixer (Eppendorf 5382 thermomixer C, 10 min, 1,400 rpm), to release analytes from Vitamin D binding protein. Further purification was carried out via supported liquid extraction (Novum SLE 3 cc; Phenomenex, Aschaffenburg, Germany). The obtained extract was evaporated to dryness with an N_2 evaporator (Biotage, Uppsala, Sweden; 30 °C, 1.3 mL/min, 20 min; Biotage, Uppsala, Sweden) and reconstituted in

100 µL 80 % methanol. In a final step, the samples were filtered using centrifuge filter tubes and transferred into 1.5 mL amber sample vials with an insert. Vitamin D metabolites are sensitive to strong UV light, such as direct sunlight, as well as heat, so that precautions must be taken to avoid potential degradation.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The measurement of 25OHD2 and 25OHD3 were performed via a heart-cut two-dimensional LC-MS/MS method on a triple quadrupole Thermo Fisher TSQ Altis coupled to a High Pressure Liquid Chromatography Thermo Fisher Vanquish Horizon with two pumps and a two position 6-port valve. All method details (gradient, source and Multiple Reaction Monitoring [MRM] parameters) are shown in Supplementary Material 1.

10 µL of each sample were injected to a Thermo Fisher Hypersil Gold C4 column (50 × 2.1 mm, 1.9 µm) and transferred onto a Phenomenex Kinetex F5 column (150 × 2.1 mm, 2.6 µm) in the second dimension. Both analytes were transferred simultaneously via a heart-cut of 0.25 min width. Column temperature was kept at 30 °C. The HPLC flow rate was set to 0.3 mL/min in the first dimension and 0.5 mL/min in the second dimension. Mobile phases consisted of ultrapure water (A1), ultrapure water containing 0.1 % formic acid (A2) and methanol (B). Electrospray ionization was performed in positive mode. The MRM transitions for the analytes and their internal standards were: 25OHD2, *m*/*z* 413.3 → 395.3 (quantitation ion (QI)), *m*/*z* 413.3 → 271.2 (confirmation ion (CI)); 25OHD2-13C3: *m*/*z* 416.3 → 398.3 (ISTD QI), *m*/*z* 416.3 → 271.2 (ISTD CI); 25OHD3, *m*/*z* 401.3 → 383.3 (QI), *m*/*z* 401.3 → 365.3 (CI); 25OHD3-13C5, *m*/*z* 406.3 → 388.3 (ISTD QI), *m*/*z* 406.3 → 370.3 (ISTD CI). The total runtime per sample was 11 min.

Structure of analytical series, calibration and data analysis

A system suitability test (SST) was performed prior to every analytical series to examine sensitivity, retention time, chromatographic separation of the analytes and their corresponding epimer and to evaluate potential analyte carry-over. Two levels (SST1 and SST2) were prepared in methanol/water (8 + 2, v + v) both containing 25OHD2, 25OHD2-13C3, 3-Epi-25OHD2, 25OHD3, 25OHD3-13C5 and 3-Epi-25OHD3 corresponding to the concentrations of calibrator level 1 and level 8. To pass the SST, each signal-to-noise ratio (S/N) had to be ≥10 for SST1 of the respective quantifier transition of both analytes. Both epimers of the analytes are transferred to the second dimension and had to be baseline separated from 25OHD2 or 25OHD3 with a chromatographic resolution of R >1.5. To examine a potential carry-over, SST2 was injected followed by the injection of two solvent blanks. The analyte peak area observed in the first blank needed to be ≤20 % of the analyte peak area of SST1.

For each measurement sequence, a set of calibrator and quality controls were measured at the beginning and the end of the analytical series. Calibrators were measured in increasing concentrations. The calibration functions (using both injections of the 8 calibrators) were obtained by linear regression of the area ratios of the analyte and internal standard (AR=area ratio) against the analyte concentration (c_A) resulting in the function $AR=a*c_A + b$ with $1/c_A$ weighting.

The sequence setup varied due to specific measurement requirements. For reference value assignment, the number of sample preparations (n=x) was dependent on the desired measurement uncertainty and samples were measured on at least two different days. Method comparison or complaint samples were prepared with n=1.

Data processing of the raw data files was done with the Thermo Fisher software Chromeleon™ Version 7.2.10. Please refer to Supplementary Material 1 for more details.

Method validation

The details of the method validation process were previously described in Taibon et al. [14] and set up in accordance to the Clinical & Laboratory Standard Institute (CLSI) Guidelines C62A: “Liquid Chromatography-Mass Spectrometry Methods” [15], the International Conference on Harmonization (ICH) guidance document “Harmonized Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1)” [16] and the Guide to the expression of uncertainty in measurement (GUM) [3].

Selectivity/specificity

Selectivity was evaluated by spiking the analytes 25OHD2, 25OHD3, the corresponding internal standards 25OHD2-13C3, 25OHD3-13C5 and the interferences 3-Epi-25OHD2 and 3-Epi-25OHD3 into a native human serum pool. The chromatographic separation of 25OHD2 and 25OHD3, 3-Epi-25OHD2 and 25OHD2, as well as the separation of 3-Epi-25OHD3 and 25OHD3 was evaluated and analytes and interferences had to show a resolution R ≥1.5 in the second dimension.

Vitamin D depleted serum was spiked with the internal standards 25OHD2-13C3 and 25OHD3-13C5 and measured to determine the amount of residual unlabeled analyte in the internal standard solution. Both, the vitamin D depleted serum and the native human serum pool were checked at the expected retention time to identify possible interfering matrix signals.

Qualitative and quantitative matrix effects

Possible matrix effects were determined by a post-column infusion experiment. Therefore, a neat solution of 150 ng/mL each of 25OHD2 (364 nmol/L), 25OHD2-13C3 (361 nmol/L), 25OHD3 (374 nmol/L) and 25OHD3-13C5 (370 nmol/L) (prepared in methanol/water [1 + 1, v + v]) was infused at a constant flow rate of 10 µL/min. Processed samples (native human serum pool, methanol/water [8 + 2, v + v], vitamin D depleted serum and plasma matrices [K2-EDTA, K3-EDTA, Li-heparin]) were injected to evaluate ion enhancement or suppression effects.

In addition, a quantitative experiment based on the comparison of standard line slopes was performed. For both analytes, an ethanolic calibration solution from NIST (SRM 2972a) is available and was used for the preparation of a calibrator set. Additionally, calibrator sets were prepared in methanol/water (8 + 2, v + v), native human serum pool, vitamin D depleted serum, and lithium-heparin plasma. Although the absolute peak areas of the analytes may vary across different matrices at the same concentration, it is crucial to ensure that the ratios of analyte to internal standard and the resulting slopes do not differ. Each sample was measured once, and a comparison was made between the slopes, correlation coefficients, and confidence intervals of the slopes, which needed to overlap.

Furthermore, the neat calibration was set as standard and the calibrator levels prepared in matrix were examined as controls. Recoveries were reported as the percentage of recovery of the measured concentration relative to the nominal concentration.

Precision, trueness and accuracy

Precision of the method was assessed by performing a 5-day validation experiment, as previously described by Taibon et al. [14]. An analysis of variance (ANOVA)-based variance component analysis (VCA) was used to determine the total variability (type A uncertainty) of the method. An internal statistic program based on the VCA Roche Open Source software package in R was used [17].

Two levels of the certified secondary reference material from NIST (SRM 972a level 3 25OHD2: 13.3 ng/mL, [32.2 nmol/L]; SRM 972a level 3 25OHD3: 19.8 ng/mL, [49.4 nmol/L] and SRM 972a level 4 25OHD3: 29.4 ng/mL, [73.4 nmol/L]), three levels of spiked vitamin D depleted serum covering the measuring range of both analytes (4.50, 80.0 and 150 ng/mL; 10.9, 194, 364 nmol/L for 25OHD2 and 11.2, 200, 374 nmol/L for 25OHD3), and one native serum sample (25OHD2: approx. 48.0 ng/mL, [116 nmol/L]; 25OHD3: approx. 12.3 ng/mL, [30.7 nmol/L]) were used. All samples were prepared in triplicates for two separate measurement parts (part A and part B) and injected twice for each part, resulting in n=12 measurements per day and n=60 measurements over 5 days. For each part, individual calibrator levels were prepared.

The same samples with the exception of the native serum sample were used to determine accuracy and trueness of the method. In addition, to evaluate accuracy and trueness in plasma, lithium heparin plasma was fortified with the same concentration levels as serum samples. For the determination of the nominal concentration the concentration of residual endogenous 25OHD2 and 25OHD3 was taken into account. All samples were prepared in triplicate for each part on a single day resulting in n=6 sample preparations.

For 25-hydroxyvitamin D, the acceptance criteria for the RMP are defined as CV_{ref} ≤1.7 %, B_{ref} ≤2.6 % and TE_{ref} ≤5.4 %.

Lower limit of the measuring interval (LLMI)

The lower limit of the measuring interval (LLMI) was determined using a spiked sample in vitamin D depleted serum at the concentration of the lowest calibrator level (1.50 ng/mL for both analytes; 3.64 nmol/L for 25OHD2 and 3.74 nmol/L for 25OHD3). This sample was prepared fivefold on one day. Recovery, bias and precision were required to be within the range of acceptance criteria defined within the accuracy and precision experiment.

Linearity

To evaluate linearity, calibrator levels with a calibration range extended by 20 % at the lower and upper end of the range (1.20–216 ng/mL, 2.91–523 nmol/L for 25OHD2 and 3.00–539 nmol/L for 25OHD3) were prepared in triplicate in vitamin D depleted serum. Each calibrator set was prepared using individual weighings based on the described test procedure. Calibration curves were evaluated using a linear and quadratic fit, both with 1/x weighting. Suitable mathematical regression must have a correlation of determination R^2 ≥0.99, and the residuals had to be randomly distributed.

In addition, to confirm the linearity of the method for both analytes high concentrated native samples were serially diluted with a low concentrated native sample for 25OHD2 and vitamin D depleted serum for 25OHD3. To achieve the desired concentration for the upper limit of the measuring ranges, the high concentrated native samples were spiked with 25OHD2 and 25OHD3. Final samples were prepared by dilution following specific ratios (10 + 0, 9 + 1, 8 + 2, 7 + 3, etc.). Recoveries were reported as the measured concentration recovered relative to the nominal concentration of the sample pools.

Sample stability

Stability of processed samples stored at 6 °C in the autosampler was investigated for a period of 17 days. Therefore, four different concentration levels from the precision experiment were re-measured (n=1 for each time point). Recoveries were calculated by comparing the measured values with the nominal concentration (t=0 d).

Additionally, the stability of spike solutions was assessed by utilizing spike solutions from three calibrator levels (low, mid, high) that were stored at −80 °C for a duration of 21 weeks. These spike solutions were then employed to prepare matrix-based levels, which were quantified using a calibrator set that was spiked with freshly prepared spike solutions.

Furthermore, matrix based calibrator and QC material stored at −80 °C was evaluated for a period of 33 weeks and recoveries were determined utilizing freshly prepared calibrator levels. All samples were prepared and measured once for each time point.

Stability can be guaranteed for a measurement interval of 2–28 days (x) for x–1 day, and for a measurement interval of >4 weeks (y) for y–1 week.

Uncertainty of measurements

Measurement uncertainty was determined according to the GUM [3] and was previously described in Taibon et al. [14]. The calculation considered the following steps: purity of the qNMR characterized reference material, weighing, preparation of stock, working, spike and calibrator solutions, preparation of ISTD solution, sample preparation of the calibrators, measurement of calibrators and generation of the calibration curve, preparation of unknown samples as well as the measurement and evaluation of sample results. The uncertainty estimation for the preparation of calibrators (unc_{cal}) was carried out using type B evaluation. For all other aspects, such as precision (unc_{prec}), type A evaluation was performed.

Reference or target values were determined by averaging multiple sample preparations conducted on different days. The measurement uncertainty was obtained by combining the uncertainty in calibrator preparation (unc_{cal}) with the uncertainty (standard deviation) of the mean of the measurement results (unc_{mean}). The resulting uncertainty was then multiplied by a coverage factor of k=2 for a confidence level of 95 %, assuming a normal distribution. For more detailed information, please refer to Supplemental Material 3.

Equivalence to JCTLM listed reference measurement procedures

Equivalence to JCTLM listed reference measurement procedures was demonstrated through the quarterly participation in the CDC Vitamin D Standardization-Certification Program (VDSCP). The reference measurements for the target value assignment of samples were performed in the CDC Vitamin D Reference Laboratory using the CDC ID-LC-MS/MS Reference Method for 25-hydroxyvitamin D2 and D3 [18] and/or by Ghent University using ID-LC-MS/MS Reference Method for 25-hydroxyvitamin D2 and D3 [19]. According to the CDC scheme, sample preparation must be conducted on two different days with duplicates on each day. Individual measurement results are then reported.

Furthermore, to show comparability to JCTLM listed reference laboratories, annual participation was carried out in the RELA (IFCC External Quality Assessment Scheme for Reference Laboratories in Laboratory Medicine) scheme. For this scheme, samples were prepared in triplicate on two separate days, and the mean of the measurement results (target value) along with the corresponding measurement uncertainty were reported.

Results

Traceability to SI units

The most important aspect of the reference measurement procedure is its traceability to the SI-unit kilogram, which has been established by utilizing Tecnazene as the qNMR ISTD which is directly traceable to the NIST PS1 (primary qNMR standard). Utilizing the above-mentioned parameters, n=6 measurements for 25-hydroxyvitamin D3 yielded an absolute content of 93.6 ± 0.8 % (k=2). For 25-hydroxyvitamin D2 an absolute content value of 83.7 ± 1.0 % (k=2) was determined with n=6 measurements.

Selectivity/specificity

Chromatographic separation of 25OHD2, 25OHD3 and their interferences 3-Epi-25OHD2 and 3-Epi-25OHD3 was achieved using a two-dimensional heart cut LC approach. The combination of Thermo Fisher Hypersil Gold C4 column and Phenomenex Kinetex F5 column achieved a satisfactory chromatographic resolution (R) of ≥1.7 for 25OHD2 and 3-Epi-25OHD2, and ≥2.0 for 25OHD3 and 3-Epi-25OHD3 in the second dimension.

The evaluation of quantifier and qualifier transition for both analytes showed no interfering signals at the respective retention time in all tested matrices. The peak area of the residual unlabeled analyte from the injection of the isotopically labeled internal standard was found to be 0.3 % for 25OHD2 and 3.6 % for 25OHD3, which are well below the ≤20 % criteria.

Qualitative and quantitative matrix effects

The qualitative post-column infusion experiment showed no significant ion enhancement or suppression at the respective retention time for 25OHD2 or 25OHD3 and their ISTDs in all tested matrices.

In the quantitative experiment that involved comparing the slopes of standard lines, slopes for 25OHD2 were found to be 0.090 (95 % confidence interval [CI] 0.088 to 0.092) for the neat solution, 0.085 (95 % CI 0.083–0.086) for NIST SRM 2972a calibration solution, 0.089 (95 % CI 0.086–0.091) for the vitamin D depleted serum, 0.094 (95 % CI 0.089–0.098) for native human serum pool and 0.087 (95 % CI 0.086–0.088) for Li-heparin plasma. The coefficients of determination were 1.00 independent of the calibration matrix. For 25OHD3 slopes were found to be 0.070 (95 % CI 0.068–0.072) for neat solution, 0.067 (95 % CI 0.66–0.068) for NIST SRM 2972a calibration solution, 0.070 (95 % CI 0.067–0.072) for vitamin D depleted serum, 0.072 (95 % CI 0.069–0.076) for native serum pool and 0.069 (95 % CI 0.067–0.071) for Li-heparin plasma. Coefficients of determination were ≥0.999 independent of the calibration matrix. The overlapping confidence intervals of the slopes indicate no significant difference between them, suggesting the absence of matrix effects.

Recoveries for the vitamin D depleted serum, the native serum matrix and the Li-heparin plasma matrix were found to range between 101 and 105 % (25OHD2), and 97 and 104 % (25OHD3), respectively.

Precision, trueness and accuracy

Accuracy and precision of the method were determined by a 5-day validation experiment. Intermediate precision was found to be less than 4.0 % for 25OHD2 and less than 3.6 % for 25OHD3. Repeatability CV ranged from 1.6 to 3.3 % (25OHD2), and 1.4–2.9 % (25OHD3), respectively, over all concentration levels (Table 1).

Accuracy and trueness were demonstrated using certified secondary reference material from NIST (SRM 972a Level 3 and 4 for 25OHD3, SRM 972a Level 3 for 25OHD2) (Figure 1). In addition, trueness was evaluated in spiked vitamin D depleted serum and Li-heparin plasma. NIST SRM 972a Level 3 was found with a mean bias (n=6 preparations) of –1.0 % for 25OHD3. NIST SRM 972a Level 4 was found with a mean bias of 0.6 % for 25OHD2 and – 1.1 % for 25OHD3. In vitamin D depleted serum the mean bias was found to range between –2.6 % and 1.0 % for 25OHD2 and between –2.5 % and 0.9 % for 25OHD3, whereas for the plasma levels the mean bias was between –4.2 % and –4.1 % for 25OHD2 and –3.9 to 0.1 % for 25OHD3. High concentration levels were diluted with vitamin D depleted serum before sample preparation. The bias ranged from –0.4 to –1.0 % (25OHD2) and –1.8 to 0.9 % (25OHD3), respectively (Table 2).

Lower limit of the measuring interval (LLMI)

The LLMI was determined using spiked vitamin D depleted serum and corresponded to the lowest calibrator level (1.50 ng/mL for both analytes, 3.64 nmol/L for 25OHD2 and 3.74 nmol/L for 25OHD3). The mean bias (n=5 preparations) was 0.4 % and the CV was 3.5 % for 25OHD2. The evaluation for 25OHD3 showed a mean bias of 4.9 % and a CV of 1.7 %.

Linearity

Linearity was evaluated for an extended range of 20 % at the lower and upper end of the range. A linear regression model was chosen for 25OHD2 and 25OHD3 due to the random and equal distribution of residuals. The coefficients of determination were equal or better than 0.999 and 0.998, for 25OHD2 and 25OHD3, respectively. The measurement results of serially diluted samples showed a linear dependence with a correlation coefficient of 0.999 for 25OHD2, and 0.998 for 25OHD3. The recovery of the measured concentration relative to the nominal concentration ranged from 92 to 100 % for 25OHD2, and 98–106 % for 25OHD3.

Sample stability

The stability of processed samples (autosampler stability) was determined at 6 °C by re-analyzing four different levels (three spiked stripped serum levels and NIST SRM 972a Level 4) with

Table 1: Detailed precision performance obtained by VCA analysis (n=60 measurements).

25OHD2						
Variance source	**CV, %**					
	4.50 ng/mL (10.9 nmol/L)	**80.0 ng/mL (194 nmol/L)**	**150 ng/mL (364 nmol/L)**	**NIST SRM 972a Level 3**	**NIST SRM 972a Level 4[a]**	**Patient sample**
Intermediate precision	**4.0**	**3.6**	**2.3**	**2.7**	–	**3.4**
Between-day	1.3	2.2	0.9	1.9	–	2.2
Between-calibration	1.6	0.0	1.2	1.1	–	1.9
Repeatability	**3.3**	**2.8**	**1.8**	**1.6**	–	**1.9**
Between-preparation	2.8	2.7	1.5	1.0	–	1.6
Between-injection	1.9	0.9	0.9	1.3	–	1.1
25OHD3						
Variance source	**CV, %**					
	4.50 ng/mL (11.2 nmol/L)	**80.0 ng/mL (200 nmol/L)**	**150 ng/mL (374 nmol/L)**	**NIST SRM 972a Level 3**	**NIST SRM 972a Level 4[a]**	**Patient sample**
Intermediate precision	**2.7**	**3.6**	**2.5**	**2.4**	**2.8**	**2.5**
Between-day	0.0	0.0	0.0	1.4	1.3	1.2
Between-calibration	0.7	2.1	1.8	1.4	1.7	1.5
Repeatability	**2.6**	**2.9**	**1.7**	**1.4**	**1.8**	**1.7**
Between-preparation	2.4	2.6	1.4	0.9	1.7	1.4
Between-injection	0.9	1.4	0.8	1.0	0.6	0.9

[a]Target value is provided only for 25OHD3. CV, coefficient of variation; RMP, reference measurement procedure. Conversion factor ng/mL to nmol/L: 2.42 for 25OHD2 and 2.50 for 25OHD3. The coefficients of variation for repeatability and intermediate precision, which were determined from the individual variances, are printed in bold.

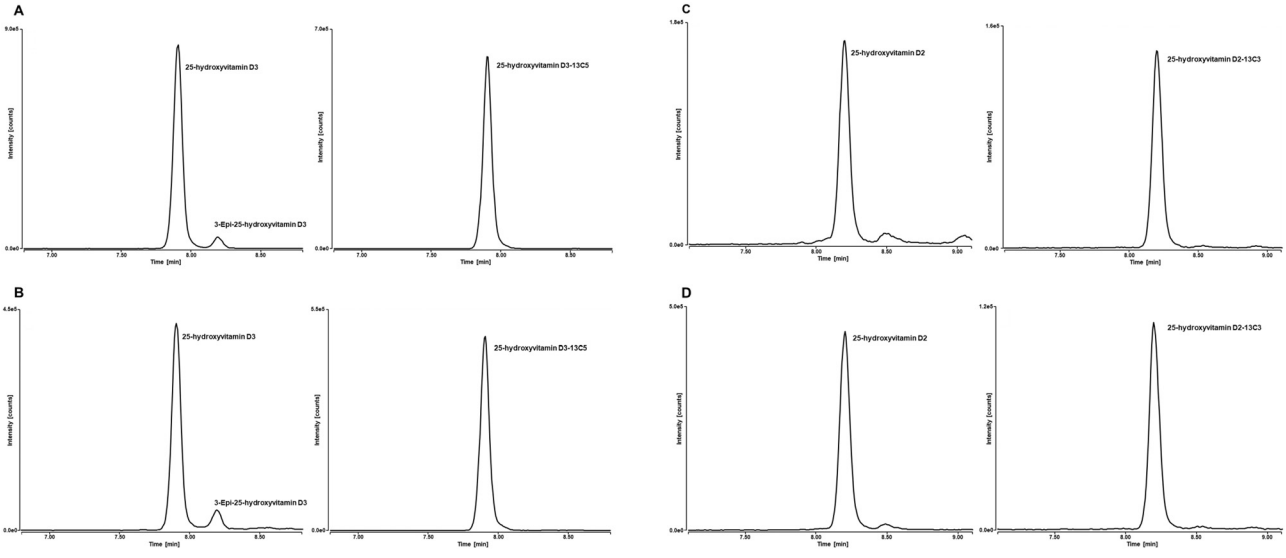

Figure 1: LC-MS/MS analytical readouts. (A) Chromatogram of the NIST SRM 972a with a 25OHD3 concentration of 19.8 ng/mL (49.4 nmol/L). Analyte (25OHD3) left and internal standard (25OHD3-13C5) right. (B) Chromatogram of a native patient sample with a 25OHD3 concentration of 12.5 ng/mL (31.2 nmol/L). Analyte (25OHD3) left and internal standard (25OHD3-13C5) right. (C) Chromatogram of the NIST SRM 972a with a 25OHD2 concentration of 13.2 ng/mL (32.0 nmol/L). Analyte (25OHD2) left and internal standard (25OHD2-13C3) right. (B) Chromatogram of a native patient sample with a 25OHD2 concentration of 44.5 ng/mL (108 nmol/L). Analyte (25OHD2) left and internal standard (25OHD2-13C3) right.

Table 2: Bias evaluation results (n=6 measurements).

	25OHD2			
	Nominal concentration, ng/mL (nmol/L)	Bias evaluation results		
		Bias, %	SD, %	95 % CI
Surrogate matrix				
Level 1	4.57 (11.1)	−2.6	3.2	−5.1 to −0.1
Level 2	81.2 (197)	1.0	2.6	−1.1 to 3.1
Level 3	152 (368)	−2.1	1.4	−3.3 to −1.0
Dilution 1	100 (242)	−1.0	1.4	−2.2 to 0.1
Dilution 2	151 (366)	−0.4	1.4	−1.3 to 0.5
Secondary reference material				
NIST SRM 972a Level 3	13.2 (32)	0.6	1.8	−0.9 to 2.0
NIST SRM 972a Level 4[a]	–	–	–	–
Li-heparin plasma				
Level 1	4.57 (11.1)	−4.2	3.0	−6.6 to −1.9
Level 2	81.2 (197)	−4.1	1.7	−5.4 to −2.8
Level 3	152 (368)	−4.2	1.3	−5.2 to −3.2
	25OHD3			
Surrogate matrix				
Level 1	4.48 (11.2)	−0.4	2.9	−2.7 to 1.9
Level 2	79.7 (199)	−0.1	2.4	−2.0 to 1.8
Level 3	149 (372)	−2.5	1.4	−3.6 to −1.4
Dilution 1	99.9 (249)	−1.8	1.7	−3.2 to −0.4
Dilution 2	150 (374)	0.9	2.2	−0.9 to 2.6
Secondary reference material				
NIST SRM 972a Level 3	19.8 (49.4)	−1.0	2.6	−3.1 to 1.1
NIST SRM 972a Level 4[a]	29.4 (73.4)	−1.1	2.6	−3.1 to 0.9
Li-heparin plasma				
Level 1	30.0 (74.9)	0.1	0.8	−0.5 to 0.7
Level 2	105 (262)	−3.9	1.8	−5.4 to −2.5
Level 3	175 (437)	−2.5	2.9	−4.8 to −0.2

[a]Target value is provided only for 25OHD3. Measurements were performed in triplicate within part A and part B (n=6 measurements). The bias and corresponding confidence intervals were calculated using the individual sample biases of n=6 preparations. Conversion factor ng/mL to nmol/L: 2.42 for 25OHD2 and 2.50 for 25OHD3.

freshly prepared calibrators. Processed samples were found to be stable for 16 days with recoveries ranging from 93 to 105 % for 25OHD2 and 95–103 % for 25OHD3.

The stability of neat spike solutions was evaluated based on three calibrator levels, which were stored at −80 °C. Vitamin D depleted serum was spiked with these solutions and the recovery was determined using freshly prepared calibrators. It was found that the neat spike solutions remained stable when stored at −80 °C for 21 weeks. Recoveries ranged from 98 to 107 % for 25OHD2 and 94–106 % for 25OHD3.

Stability of matrix-spiked calibrator and QC material were evaluated using four different levels (three spiked stripped serum levels and NIST SRM 972a Level 4). Samples were found to be stable for 33 weeks, when stored at −80 °C. Recoveries ranged from 97 to 104 % for both analytes.

Uncertainty of results

The overall uncertainties for a single measurement for 25OHD2 ranged from 2.3 to 3.9 % and from 2.4 to 3.6 % for 25OHD3 (Table 3). By multiplying the overall uncertainty by a coverage factor of k=2 (corresponding to a confidence level of 95 % assuming a normal distribution), the corresponding expanded uncertainty varied between 5.0 % and 8.1 % (25OHD2) and 5.0–7.4 % (25OHD3), respectively. These uncertainties can be reduced by performing multiple measurements.

For the reference or target value assignment, multiple sample preparations were carried out for each sample on at least 2 days. The average of these results, calculated using n=6, was used. The overall uncertainties were found to range from 0.5 to 1.7 %, with expanded uncertainties ranging from 2.1 to 3.9 % for 25OHD2. For 25OHD3, the overall uncertainties ranged from 0.7 to 1.4 %, with expanded uncertainties ranging from 2.1 to 3.2 %, as shown in Table 4.

Equivalence to JCTLM listed reference measurement procedures

Equivalence to JCTLM listed RMPs was demonstrated through the participation in the CDC Vitamin D Standardization-Certification Program as well as the RELA scheme, as illustrated in Table 5.

Over a period of a year (Q2 2023 to Q1 2024) 40 samples were measured with n=2 preparations over 2 days, resulting in n=4 preparations per sample. Sample means for total 25OHD (sum of 25OHD2 and 25OHD3) are then compared to target values (nmol/L) provided by the CDC. The Passing-Bablok regression showed a very good agreement and yielded a regression equation with a slope of 1.02 (95 % CI 1.00 to 1.05), an intercept of −0.9 (95 % CI −2.5 to −0.7) and the Bland-Altman plot with a bias of 0.6 % (95% CI -0.44 to 1.7) (Figure 2).

Table 3: Combined measurement uncertainty estimation for a single measurement.

	25OHD2					
	Level					
	4.50 ng/mL (10.9 nmol/L)	80.0 ng/mL (194 nmol/L)	150 ng/mL (364 nmol/L)	NIST SRM 972a Level 3	NIST SRM 972a Level 4[a]	Patient sample
Type B uncertainty calibrator preparation, CV (%)	**1.0**	**0.9**	**0.9**	**0.9**	–	**0.9**
Characterization of reference material	0.5	0.5	0.5	0.5	–	0.5
Preparation of						
Stock solution	0.61	0.61	0.61	0.61	–	0.61
Working solution	0.64	0.64	0.64	0.64	–	0.64
Spike solution	0.82	0.73	0.70	0.71	–	0.73
Matrix-based calibrator	0.97	0.89	0.87	0.87	–	0.89
Type A uncertainty intermediate precision, CV (%)	**3.9**	**3.6**	**2.3**	**2.7**	–	**3.4**
Total measurement uncertainty (k=1), CV (%)	**4.1**	**3.7**	**2.5**	**2.9**	–	**3.5**
Expanded measurement uncertainty (k=2), CV (%)	**8.1**	**7.4**	**5.0**	**5.7**	–	**7.1**
	25OHD3					
	Level					
	4.50 ng/mL (11.2 nmol/L)	80.0 ng/mL (200 nmol/L)	150 ng/mL (374 nmol/L)	NIST SRM 972a Level 3	NIST SRM 972a Level 4[a]	Patient sample
Type B uncertainty calibrator preparation, CV (%)	**0.9**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**
Characterization of reference material	0.4	0.4	0.4	0.4	0.4	0.4
Preparation of						
Stock solution	0.44	0.44	0.44	0.44	0.44	0.44
Working solution	0.49	0.49	0.49	0.49	0.49	0.49
Spike solution	0.71	0.60	0.57	0.60	0.60	0.57
Matrix-based calibrator	0.87	0.79	0.76	0.79	0.79	0.77
Type A uncertainty intermediate precision, CV (%)	**2.7**	**3.6**	**2.5**	**2.4**	**2.8**	**2.5**
Total measurement uncertainty (k=1), CV (%)	**2.8**	**3.7**	**2.6**	**2.6**	**2.9**	**2.7**
Expanded measurement uncertainty (k=2), CV (%)	**5.6**	**7.4**	**5.2**	**5.0**	**5.8**	**5.3**

[a]Target value is provided only for 25OHD3. Uncertainties are expressed as CVs, for better interpretability. In all formulas, the terms "unc" corresponds to standard deviations. CV, coefficient of variation. Conversion factor ng/mL to nmol/L: 2.42 for 25OHD2 and 2.50 for 25OHD3. The measurement uncertainty of the whole approach for a single measurement, estimated as a combination of the uncertainty of calibrator preparation (type B uncertainty) and the uncertainty of the precision experiment (type A uncertainty), is given in bold.

In addition, the secondary reference material SRM 2970 from NIST was measured in each sequence (n=8 results). We achieved a mean 25OHD2 concentration of 23.9 ng/mL (57.9 nmol/L) with an average bias of 1.6 % and a mean 25OHD3 concentration of 9.77 ng/mL (24.4 nmol/L) with an average bias of 1.4 % (Table 6).

Furthermore, participation in the RELA scheme showed a good agreement with results from JCTLM listed laboratories. Results are shown only for 25OHD3 in ng/mL (Table 7).

Discussion

Our candidate RMP was developed for the simultaneous quantification of 25OHD2 and 25OHD3 in human serum and

Table 4: Combined measurement uncertainty estimation for target value assignment (n=6).

	25OHD2					
	Level					
	4.50 ng/mL (10.9 nmol/L)	80.0 ng/mL (194 nmol/L)	150 ng/mL (364 nmol/L)	NIST SRM 972a Level 3	NIST SRM 972a Level 4[a]	Patient sample
Type B uncertainty calibrator preparation, CV (%)	**1.0**	**0.9**	**0.9**	**0.9**	–	**0.9**
Characterization of reference material	0.5	0.5	0.5	0.5	–	0.5
Preparation of						
Stock solution	0.61	0.61	0.61	0.61	–	0.61
Working solution	0.64	0.64	0.64	0.64	–	0.64
Spike solution	0.82	0.73	0.70	0.71	–	0.73
Matrix-based calibrator	0.97	0.89	0.87	0.87	–	0.89
Type A uncertainty intermediate precision, CV (%)	**1.4**	**0.9**	**0.5**	**1.1**	–	**1.7**
Total measurement uncertainty (k=1), CV (%)	**1.7**	**1.3**	**1.0**	**1.4**	–	**2.0**
Expanded measurement uncertainty (k=2), CV (%)	**3.4**	**2.6**	**2.1**	**2.8**	–	**3.9**
	25OHD3					
	Level					
	4.50 ng/mL (11.2 nmol/L)	80.0 ng/mL (200 nmol/L)	150 ng/mL (374 nmol/L)	NIST SRM 972a Level 3	NIST SRM 972a Level 4[a]	Patient sample
Type B uncertainty calibrator preparation, CV (%)	**0.9**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**
Characterization of reference material	0.4	0.4	0.4	0.4	0.4	0.4
Preparation of						
Stock solution	0.44	0.44	0.44	0.44	0.44	0.44
Working solution	0.49	0.49	0.49	0.49	0.49	0.49
Spike solution	0.71	0.60	0.57	0.60	0.60	0.57
Matrix-based calibrator	0.87	0.79	0.76	0.79	0.79	0.77
Type A uncertainty intermediate precision, CV (%)	**1.4**	**1.0**	**1.3**	**0.7**	**1.0**	**1.2**
Total measurement uncertainty (k=1), CV (%)	**1.6**	**1.3**	**1.5**	**1.1**	**1.3**	**1.5**
Expanded measurement uncertainty (k=2), CV (%)	**3.2**	**2.6**	**3.0**	**2.1**	**2.6**	**2.9**

[a]Target value is provided only for 25OHD3. Conversion factor ng/mL to nmol/L: 2.42 for 25OHD2 and 2.50 for 25OHD3. The measurement uncertainty of the whole approach for target value assignment, estimated as a combination of the uncertainty of calibrator preparation (type B uncertainty) and uncertainty of replicate measurements (type A uncertainty), is given in bold.

plasma through SLE clean-up and a 2D-heart cut ID-LC-MS/MS method.

Although certified standard reference materials for 25OHD3 and 25OHD2 are available (SRM 2972a calibration solutions [21]), our new candidate reference measurement procedure employed SI traceable qNMR-characterized reference materials to prepare matrix-based calibrators. This approach was necessary because the concentration of NIST SRM 2972a was not suitable for spiking at the concentration ranges required by our method. Furthermore, we observed no significant difference between the NIST SRM 2972a and our qNMR-characterized reference material, as the slopes of calibrator samples were comparable.

For the measurement of 25OHD2 and 25OHD3 in human serum, liquid chromatography-isotope dilution tandem mass spectrometry methods [18–20] have been reported as reference measurement procedures and listed by the JCTLM. Table 5 provides an overview of these methods, including details

Table 5: Method performance of JCTLM listed ID-MS based RMPs.

Reference	Measurement technique	Sample preparation	Sample volume	Applicable range	Expected uncertainty (k=2)
			25OHD2		
[20]	ID-LC-MS/MS	LLE	Approx. 2 g	0.857 ng/g	2.4 %
[19]	ID-LC-MS/MS	LLE	250–500 µL	1.2–280 nM	3.9 %
[18]	ID-LC-MS/MS	LLE	0.25–1 g	1–10 nM	3.1–3.8 %
This method	ID-LC-MS/MS	SLE	200 µL	3.64–436 nM	2.1–3.9 %
			25OHD3		
[20]	ID-LC-MS/MS	LLE	Approx. 2 g	6.311–26.972 ng/g	2–2.1 %
[19]	ID-LC-MS/MS	LLE	250–500 µL	1.1–600 nM	3.4 %
[18]	ID-LC-MS/MS	LLE	0.25–1 g	10–100 nM	2.7–3.9 %
This method	ID-LC-MS/MS	SLE	200 µL	3.74–449 nM	2.1–3.0 %

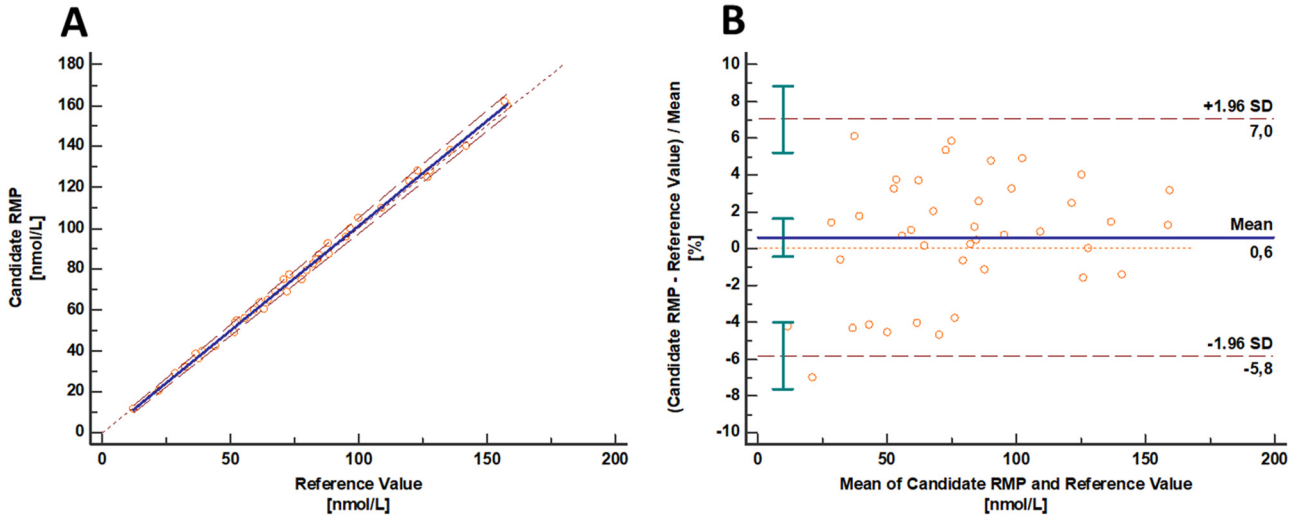

Figure 2: 25OHD total results (nmol/L) for the quarterly participation in the Vitamin D Standardization-Certification Program (VDSCP) provided by CDC. (A) Passing-Bablok regression analysis: Slope 1.02 (95 % CI 1.0–1.05), intercept −0.9 (95 % CI −2.5 to −0.7). (B) Bland-Altman plot mean bias 0.6 %.

Table 6: Trueness of the candidate reference measurement procedure for quantitation of 25OHD2 and 25OHD3 using NIST SRM 2970.

	Number of measurements	Target ± U, ng/mL, nmol/L	Bias (95 % CI), %
25OHD2	8	23.5 ± 0.3 (56.9 ± 0.8)	1.6 (0.5–2.6)
25OHD3	8	9.63 ± 0.31 (24.0 ± 0.8)	1.4 (−0.3 to 3.2)

on measurement techniques, sample preparation, sample size, applicable range and expected uncertainty. Most methods listed by the JCTLM employ concentration-matched ISTD. Samples are initially measured using a routine method to determine the appropriate ISTD concentration, with the goal of achieving an analyte to ISTD ratio close to one.

Our RMP demonstrates comparable performance and can be implemented without the need for preliminary measurements. We utilized an 8-point calibration curve without requiring individual ISTD concentration or sample volume adjustments. All samples, including matrix-based calibrators, quality controls, and unknowns, received the same ISTD amount and underwent identical preparation. This approach resulted in excellent precision and minimal bias. Moreover, our method requires a reduced sample volume and employs SLE as a relatively simple and automatable sample preparation technique.

A key advantage of our RMP is its ability to simultaneously quantify 25OHD2 and 25OHD3 within 11 min per sample, eliminating the need for duplicate injections. To resolve the isobaric and diastereomeric interferences (3-Epi-25OHD2 and 3-Epi-25OHD3) from 25OHD2 and 25OHD3, we

Table 7: External quality control for reference laboratories – RELA scheme. Only provided for 25OHD3.

	Laboratory	Sample A, ng/mL	Expanded uncertainty, ng/mL	Sample B, ng/mL	Expanded uncertainty, ng/mL	Method
Year 2022	A[a]	21.9	0.9	39.6	1.7	ID/LC/MS/MS
	B[a]	22.13	1.56	41.33	2.5	ID/LC/MS/MS
	Roche	21.7	0.436	40.1	0.850	ID/LC/MS/MS
Year 2023	A[a]	62.5	2.62	19.3	0.812	ID/LC/MS/MS
	C[a]	61.56	1.48	20.24	0.63	ID/LC/MS/MS
	Roche	62.6	1.22	18.9	0.324	ID/LC/MS/MS

[a]JCTLM, listed services. Values are reported in ng/mL in accordance with the data provided at the RELA, homepage. Conversion factor ng/mL to nmol/L: 2.42 for 25OHD2 and 2.50 for 25OHD3.

used a two-dimensional heart-cut LC-MS/MS method. This approach employs two orthogonal stationary phases, achieving satisfactory chromatographic resolution for 25OHD2 from 3-Epi-25OHD2, and for 25OHD3 from 3-Epi-25OHD3 in the second dimension, thus preventing overestimation.

During our validation process, we have successfully demonstrated the accurate measurement of NIST SRM 972a. Additionally, we have implemented NIST SRM 2970 and spiked samples as quality controls within each of our measurement sequences, ensuring that the method delivers reliable and accurate measurements. The equivalence of the candidate RMP to JCTLM-listed RMPs was further demonstrated through participation within the CDC VDSCP and the RELA scheme, proving the RMP to be equivalent to JCTLM listed RMPs.

Conclusions

We developed a candidate RMP for the simultaneous quantification of 25OHD2 and 25OHD3 in human serum and plasma by utilizing qNMR characterized SI-traceable primary calibrators. This was achieved using SLE clean-up and a 2D-heart cut ID-LC-MS/MS method. It has been verified that the candidate RMP meets the criteria for accuracy, precision, selectivity, and specificity required for a higher-order RMP. The performance of this method enables the standardization of routine assays, ensuring traceability and accurate measurement of these analytes in human samples.

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Research ethics: All procedures were in accordance with the Helsinki Declaration. All samples used were exclusively anonymized samples.

Informed consent: Not applicable.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Use of Large Language Models, AI and Machine Learning Tools: Roche Chat, Roche's artificial intelligence (AI) Technology, was used to improve the language of the manuscript.

Conflict of interest: Kerstin Kandler, Neeraj Singh, Judith Taibon, Christian Geletneky, Elie Fux and Andrea Geistanger are all employees of Roche Diagnostics GmbH. Friederike Bauland is an employee of Chrestos Concept GmbH & Co. KG, Essen, Germany. Roche employees holding Roche non-voting equity securities (Genussscheine): Kerstin Kandler, Judith Taibon, Christian Geletneky, Andrea Geistanger, Elie Fux.

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Data availability: The raw data can be obtained on request from the corresponding author.

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