

A high-throughput platform for the rapid screening of vitamin D status by direct infusion-MS/MS

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Abstract Vitamin D is an important fat-soluble pro-hormone with pleiotropic effects on human health, such as immunomodulation of the innate and adaptive immune system. There is an unmet clinical need for a rapid screening platform for 25-hydroxyvitamin D (25OH-D) determination without chromatographic separation that offers better precision and accuracy than immunoassays. Here, we introduce a high-throughput method for assessing vitamin D status from blood specimens based on direct infusion-MS/MS (DI-MS/MS) following click derivatization using 2-nitrosopyridine. We developed an optimized liquid-phase extraction protocol to minimize ion suppression when directly infusing serum or plasma extracts via a capillary electrophoresis system for quantitative determination of 25OH-D. Acceptable reproducibility (mean coefficient of variation = 10.9%, $n = 412$), recovery (mean = 102% at 15, 30, and 45 nmol/l), and linearity ($R^2 > 0.998$) were achieved for 25OH-D with lower detection limits (limit of detection ~1.2 nmol/l, $S/N \sim 3$), greater throughput (~3 min/sample), and less bias than a commercial chemiluminescence immunoassay prone to batch effects. There was mutual agreement in 25OH-D concentrations from reference blood samples measured by DI-MS/MS as compared with LC-MS/MS (mean bias = 7.8%, $n = 18$). We also demonstrate that this method could reduce immunoassay misclassification of vitamin D deficiency in a cohort of critically ill children ($n = 30$). **In conclusion**, DI-MS/MS offers a viable alternative to LC-MS/MS for assessment of vitamin D status in support of large-scale studies in nutritional epidemiology as well as clinical trials to rapidly screen individual patients who may benefit from vitamin D supplementation.

Supplementary key words critical care • direct infusion-MS/MS • infectious disease • interlaboratory performance • MS • nutrition • method validation • proficiency testing • vitamin D • vitamin D deficiency

Vitamin D, including cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2), represents a group of fat-soluble prohormones that are essential for human

health. Vitamin D is primarily derived by photo-conversion of 7-dehydrocholesterol in the skin from exposure to UV-B solar radiation as well as from diet and/or supplements (1). It is subsequently metabolized in the liver into 25-hydroxyvitamin D (25OH-D or calcidiol) that serves as the major and stable circulating reservoir of vitamin D prior to its activation into 1,25 dihydroxyvitamin D (calcitriol) and various other hydroxylated metabolites and their isomers (2). Other factors can also impact vitamin D nutritional status, including underlying medical conditions, abnormal fat absorption, aging, obesity, dark skin pigmentation, drug intake, and lifestyle, such as prolonged indoor activities (3).

Vitamin D deficiency is known to impair calcium homeostasis and bone mineralization in nutritional rickets and osteomalacia (4). In addition, vitamin D deficiency contributes to several extraskeletal pathologies relevant to cardiac, respiratory, neurological, and immune system dysfunction reflecting widespread expression of the vitamin D receptor in various tissues and cells (5, 6). In fact, the seasonal and latitude dependence on vitamin D deficiency has long been implicated in epidemic influenza with a periodic wintertime excess in infection and mortality (7). Similarly, low-circulating 25OH-D concentrations are associated with a greater risk of severe acute respiratory syndrome coronavirus 2 infection, severe illness, and mortality during the coronavirus disease 2019 (COVID-19) pandemic (8, 9) because of its function in regulating innate and adaptive immune responses. This is concerning given that a third and more than two-thirds of Canadians are estimated to be vitamin D deficient (25OH-D < 50 nmol/l) and insufficient (25OH-D < 75–80 nmol/l), respectively (10, 11), which is prevalent among older persons with dementia in long-term care facilities (12). Thus, rapid screening platforms that enable reliable assessment of vitamin D status in high-risk populations are needed to guide optimal prophylactic and early treatment strategies to prevent hospitalization from severe COVID-19 illness (<https://>

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vdmata.com/) since preinfection vitamin D deficiency increases disease severity and mortality (13).

The clinical significance of vitamin D deficiency has expanded demand for reliable tests for 25OH-D determination in blood specimens, regarded as the sum of 25OH-D₂ and 25OH-D₃ (14). Competitive protein-binding assays, radioimmunoassays, and chemiluminescence immunoassays have been established to analyze 25OH-D and other hydroxylated vitamin D metabolites in serum or plasma samples (15). However, the crossreactivity and reproducibility of commercial immunoassays used in clinical laboratories vary significantly between manufacturers, which has complicated the development of consensus 25OH-D cutoff concentrations to define vitamin D nutritional status (16). For these reasons, LC-MS/MS is recognized as the gold standard for quantitative 25OH-D determination because of its greater selectivity, lower detection limits, and improved precision as compared with immunoassays (17, 18). Yet, different column types, elution conditions, ion sources, and sample workup protocols with or without chemical derivatization have been developed that do not always resolve the 3-epimer of 25OH-D₃ (19–22). Longer run times (>10 min) in LC-MS/MS are often needed to separate 25-OH-D₃ from lower levels of its 3-*epi*-25OH-D₃ isomer using gradient elution with extra time for column re-equilibration. Nevertheless, the lack of isomer resolution does not significantly contribute to bias or clinical misdiagnosis in routine screening given its minor contribution to overall vitamin D status in most adults and children (23, 24).

Herein, we introduce for the first time a direct infusion (DI)-MS/MS protocol when using 2-nitrosopyridine (2-NO-Pyr) as a dienophile or click reagent (25) to enhance the ionization efficiency of labeled 25OH-D cycloadducts in serum or plasma extracts. This strategy enables high-throughput screening of vitamin D status that is not practical when relying on LC-MS/MS while offering better analytical and clinical performance than a commercial immunoassay system.

MATERIALS AND METHODS

Reference serum samples and plasma collection from critically ill children

Reference material National Institute of Standards and Technology (NIST) 972a consisting of human serum samples (L1-L4) with certified concentrations for vitamin D metabolites were purchased from the NIST (Gaithersburg, MD). Human serum samples with reference values reported for vitamin D metabolites (samples 566 to 580) as measured by a validated LC-MS/MS protocol were purchased from the Vitamin D External Quality Assessment Scheme (DEQAS, London, UK). Also, plasma samples from critically ill pediatric patients at McMaster Children's Hospital (from February 2021 until December 2021) were screened for vitamin D deficiency,

which was approved by Clinical Trials Ontario (#1761) and preregistered on ClinicalTrials.gov (NCT03742505). Informed consent was obtained from all participants in accordance with the Declaration of Helsinki principles. Blood samples were collected in EDTA-coated tubes and centrifuged at 2,000 *g* for 20 min at 4°C. The supernatant was transferred to a new uncoated tube and stored frozen at –80°C prior to analysis.

Liquid-phase extraction and chemical derivatization for 25OH-D analysis by DI-MS/MS

A separation-free protocol was developed for the rapid screening of 25OH-D from blood specimens as an indicator of vitamin D status using DI-MS/MS. Briefly, 32 µl of 1,000 nmol/l d₆-25OH-D₃ in ethanol was added to a glass amber vial followed by a 50 µl aliquot of thawed serum or plasma. Protein precipitation was initiated by adding 200 µl of methanol and 50 µl of water, followed by shaking for 5 min. The solution was then centrifuged for 5 min at 10,000 *g* under 4°C, and the supernatant was transferred to a new glass amber vial. To this solution, 200 µl of water, 25 µl of 1.0 M HCl, and 500 µl of hexane were added. Shaking was then performed for 5 min followed by centrifugation for 5 min at 10,000 *g* under 4°C. The upper hexane layer was transferred to a new amber glass vial and dried with a steady stream of nitrogen at room temperature. Then, 50 µl of a 10 mmol/l 2-NO-Pyr solution in acetonitrile was added, and the vials were vortexed. A hot block was used to heat the reaction at 60°C for 30 min. Derivatized plasma or serum extracts were then placed in the fridge to cool after which 400 µl of water, 25 µl of ammonium hydroxide, and 500 µl of hexane were added. Shaking was performed for 5 min followed by centrifugation for 5 min at 10,000 *g* under 4°C. The upper hexane layer was transferred to a new amber glass vial and dried under a flow of nitrogen at room temperature. Finally, dried serum or plasma extracts were then reconstituted to 25 µl by dissolution in 80% volume of methanol and were stored at 4°C prior to analysis by DI-MS/MS. Samples were measured in duplicate (i.e., two independent serum or plasma aliquots were processed and then analyzed once for each sample) for vitamin D screening purposes unless otherwise stated.

Instrumental DI-MS/MS operating conditions

DI-MS/MS was performed using an Agilent 7100 capillary electrophoresis (CE) system (Agilent Technologies, Inc, Mississauga, ON, Canada), which was used to inject and flush sample solutions into an Agilent 6550 quadrupole-TOF (Q-TOF) mass analyzer. A 75 cm (total length) uncoated fused-silica capillary (75 µm inner diameter and 360 µm outer diameter) from Polymicro Technologies, Inc (Phoenix, AZ) was used for DI after 1 cm of the polyimide outer coating was burnt off both ends of a capillary using a capillary window maker (MicroSolv Technologies, Inc, Leland, NC). The CE-MS system used an Agilent ESI coaxial sheath liquid interface with an Agilent 1260 Infinity isocratic pump and a 1260 Infinity degasser (Agilent Technologies, Inc) to deliver a sheath liquid consisting of 60:40 methanol-water with 0.1% volume of formic acid at a rate of 1 ml/min. A 1:100 splitter was used to reduce the sheath liquid volume to the sprayer to 10 µl/min. For real-time mass correction, reference ions for purine, hexamethoxyphosphazine (HP-0321), and hexakis(2,2,3,3-tetrafluoropropoxy)phosphazine (HP-0921) were spiked into the sheath liquid at 0.02% volume to provide constant mass signals at *m/z* 121.0509, 322.0481, and 922.0098, respectively. Optimized parallel reaction monitoring (PRM) transitions for

2-NO-Pyr cycloadducts of 25OH-D3, 25OH-D2, and d6-25OH-D3 were implemented to lower detection limits as summarized in [supplemental Table S1](#). The Q-TOF system was operated in positive ion mode, and ion source conditions were as follows: gas temperature at 225°C, drying gas flow rate at 1 l/min, nebulizer pressure at 60 psi, sheath gas temperature at 125°C, sheath gas flow rate at 2 l/min, Vcap at 3,000 V, nozzle voltage at 2,000 V, and fragmentor at 150 V. During sample introduction, the nebulizer was set to 10 psi, and a sample vial was flushed at 950 mbar for 10 s. The CE instrument automated injections of plasma/serum extracts, calibrant solutions, and/or blanks corresponding to about 1.1 µl of total sample volume loaded onto a bare fused-silica capillary. After sample injection, a vial containing 80% volume of methanol was placed at the capillary inlet, the nebulizer pressure was set back to 60 psi, and a pressure of 100 mbar was applied for 2.3 min. This procedure was repeated by programming a high-pressure sample introduction followed by a low-pressure sample infusion using Agilent Mass Hunter software. The quadrupole was set to a narrow mass window (m/z 1.3), and the acquisition rate for both MS and MS/MS was set to 3 spectra/s. Also, the mass range for MS/MS was set from m/z 50 to 600, whereas the range for full-scan MS spectra was set from m/z 50 to 1,700.

Calibrants, method validation, and interlaboratory comparison study

Calibrants containing 25OH-D3 and 25OH-D2 were prepared at 10 different concentration levels (5, 10, 15, 20, 25, 50, 75, 100, 200, and 400 nmol/l) in 60% volume methanol with d6-25OH-D3 as a stable-isotope internal standard. All calibrant solutions were measured in triplicate by DI-MS/MS. Briefly, 32 µl of a 1,000 nmol/l stock of d6-25OH-D3 in 60% volume methanol was added to 1.5 ml amber vials. Aliquots (5, 10, 15, 20, 25, 50, 75, 100, 200, and 400 µl, respectively) of stock solutions containing 100 nmol/l of 25OH-D3 and 25OH-D2 in 80% volume methanol were transferred to the vials. The solutions were then dried under a gentle stream of nitrogen before being reconstituted with 200 µl of methanol, 300 µl of water, 25 µl of 1.0 M HCl, and 500 µl of hexane. Extraction and click derivatization steps with 2-NO-Pyr were performed identically to those aforementioned for reconstituted serum or plasma extracts having a final volume of 25 µl. Intraday precision samples were prepared from normal human serum purchased from Sigma-Aldrich, Inc. Samples were analyzed sequentially in batches of 20, which were separated by the analysis of a single blank solution to check for sample carryover. A fresh solution vial was used for infusion after every 10 sample injections to minimize sample carryover. Interday precision samples were analyzed over 3 days under conditions to simulate real-world laboratory operating conditions. On each day, a new fused-silica capillary was prepared, the ion source was cleaned, and the instrument was mass tuned. Each day, analysis began by running a blank, a 6-point calibration curve, and 10 serum extract samples. This procedure was repeated five times resulting in the analysis of 5 blanks, 30 calibrants, and 50 serum samples. Spike and recovery experiments were performed by spiking 25OH-D metabolites at 0, 15, 30, and 45 nmol/l in Sigma serum samples in triplicate. The percent recovery was calculated by subtracting the concentration of the nonspiked samples from that of the spiked samples and then dividing by the known spiked concentration. Stability of 25OH-D cycloadducts was investigated by processing DEQAS serum samples (576–580) and analyzing them after 0, 1, and 2 repeat freeze-thaw cycles when stored

at –80°C. External validation was performed to evaluate the accuracy of DI-MS/MS as compared with a commercial chemiluminescence immunoassay system (Qualigen Therapeutics, Inc, Carlsbad, CA) by analyzing reference serum samples from NIST and DEQAS. This Qualigen immunoassay system is currently approved by the Food and Drug Administration and Health Canada, which has been the assay of choice for critical care in hospital sites that do not offer rapid 25OH-D screening by their local clinical laboratories (26). An intermethod comparison of DI-MS/MS with the Qualigen immunoassay was also performed on plasma samples analyzed from a cohort of critically ill pediatric patients ($n = 30$). Quality control samples used in the immunoassay were prepared by pooling 10 µl of plasma from each of the pediatric patient samples.

Data processing and statistical analysis

All data acquired by DI-MS/MS were analyzed with the Agilent Mass Hunter Workstation Software (Qualitative Analysis, version B.06.00; Agilent Technologies, Inc). Ion chromatograms were extracted in profile mode with a 50 ppm mass window and integrated after smoothing using a Gaussian function (function width: 15; gaussian width: 10). Peak areas were transferred to Excel (Microsoft Office, Edmond, WA) for the calculation of relative peak areas using d6-25OH-D3 to correct for differences in injection volume, ion suppression, and overall sample workup. All chromatograms were depicted using the Igor Pro 5.0 software (WaveMetric, Inc, Lake Oswego, OR). Analysis of external calibration data, calculation of figures of merit, and generation of control charts were performed using Microsoft Excel. Box plots, Passing-Bablok regressions, Bland-Altman percent difference plots, and empirical cumulative distribution function plots were generated in R, version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Mountain percent difference plots were generated using MedCalc (MedCalc Software, Ostend, Belgium). Further details on chemicals and reagents, Qualigen immunoassay protocol for vitamin D screening, as well as the synthesis and characterization of 2-NO-Pyr are outlined in the [Supplemental Experimental](#) section.

RESULTS

A DI-MS/MS protocol for rapid screening of vitamin D status

2-NO-Pyr was first synthesized via a two-step procedure that was characterized by ¹H-NMR and MS/MS ([supplemental Fig. S1](#)). Sample matrix effects in ESI can be minimized in DI-MS/MS when using stable-isotope internal standard(s) together with optimal sample workup procedures. In our case, a modified liquid-phase extraction protocol (25) using a 50 µl aliquot of serum or plasma was developed to significantly reduce ion suppression by 7-fold to 8-fold because of excess reagent and reaction byproducts ([supplemental Fig. S2](#)) following click derivatization when incorporating an alkaline aqueous back extraction step in hexane. Chemical labeling introduces a cationic pyridinium moiety, which enhances solute ionization efficiency by over 10-fold under positive ion mode detection relative to unlabeled 25OH-D ([supplemental Fig. S3](#)) with

quantitative 25OH-D cycloadduct formation (> 95%) via a Diels-Alder reaction (10 mmol/l of 2-NO-Pyr, 60°C at 30 min). **Figure 1A** highlights that a CE instrument was configured to perform DI by programming a repeated sequence comprising a long hydrodynamic sample volume injection (~1.1 µl on-capillary) followed by a low-pressure (100 mbar or 10 kPa) solvent flush with 80% volume of methanol when coupled to a coaxial sheath liquid interface. The effective duty cycle was 3.3 min per sample including delay times for automatic changing of vials on the sample carousel, where labeled 25OH-D3, 25OH-D2, and d6-25OH-D3 cycloadducts are monitored via PRM (**supplemental Table S1**) as shown in a representative MS/MS spectrum (**Fig. 1B**). Moreover, data processing after Gaussian smoothing was found to generate normal peaks (13 data points collected over a base peak width of ~1 min; **supplemental Fig. S4**) with data normalized to d6-25OH-D3 (1,280 nmol/l) to correct for variations in injection volume, ion suppression, and sample handling. Overall, we found that a scan frequency of 3 spectra/s with data acquisition over ~1 min was an optimal balance for ensuring adequate sensitivity, reproducibility, and sample throughput, whereas data transformation

via Gaussian smoothing allowed for more consistent peak integration.

Method validation of DI-MS/MS for reliable 25OH-D determination

Next, method validation of the DI-MS/MS assay was conducted, including a repeat analysis of Sigma serum extracts continuously over a 24 h period that generated good reproducibility for 25OH-D3 quantification (mean coefficient of variation [CV] = 10.9%, $n = 412$) without evidence of sample carryover for blank samples ($n = 20$) analyzed intermittently as shown in the control chart together with selected extracted ion electropherograms (**Fig. 1C, D**). Overall, excellent linearity ($R^2 > 0.998$) was achieved for 10-point calibration curves measured in triplicate over an 80-fold linear dynamic range (5–400 nmol/l) for both 25OH-D3 and 25OH-D2 (**Fig. 1E, F**) with quantification limits ($S/N \sim 10$) and detection limits ($S/N \sim 3$) of about 4 and 1 nmol/l, respectively (**Table 1**). Moreover, spike-recovery studies (at 15, 30, and 45 nmol/l in triplicate) confirmed acceptable accuracy for serum 25OH-D3 and 25OH-D2 determination by DI-MS/MS with a mean recovery of (102 ± 12)% ranging from 92% to 113% (**Fig. 1G; Table 1**).

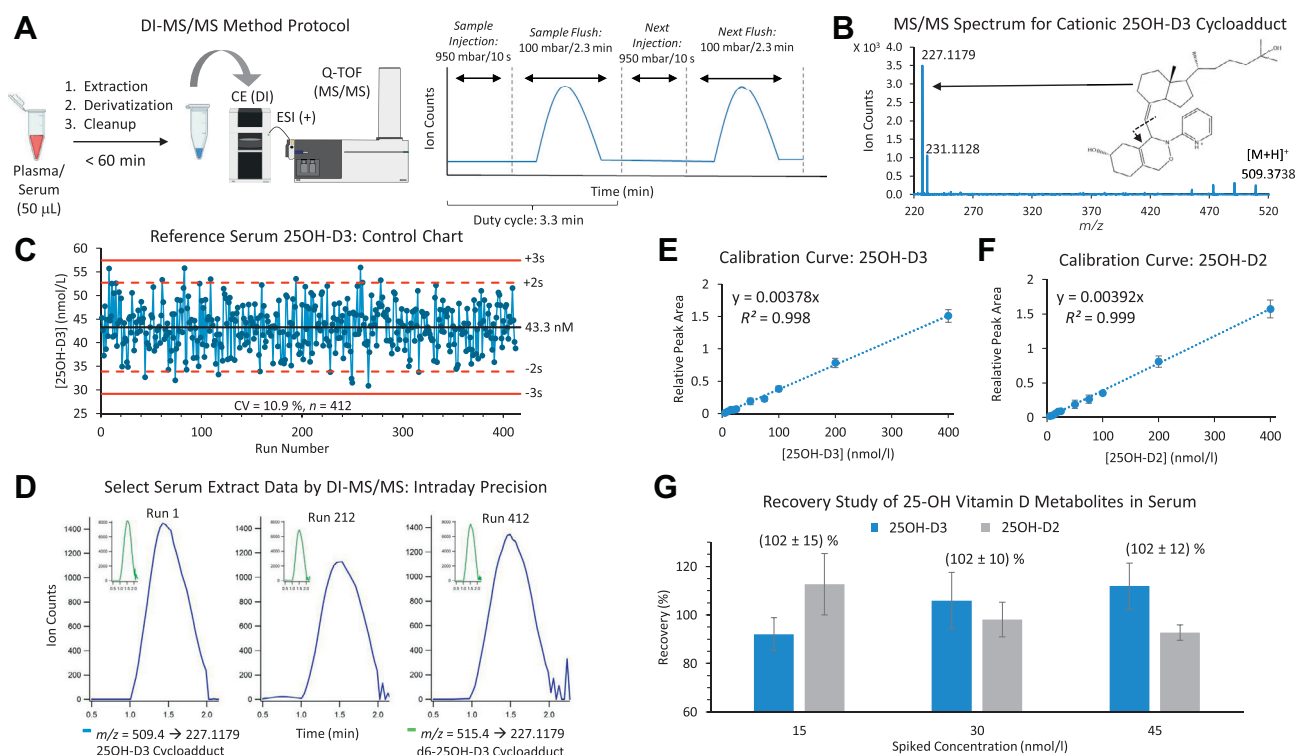


Fig. 1. Schematic depicting the rapid screening of vitamin D status by DI-MS/MS following 2-NO-Pyr click derivatization and liquid-phase extraction of blood specimens. **A:** A CE system was used to automate sample injection and infusion into coaxial sheath liquid interface with an effective duty cycle of 3.3 min/sample. **B:** PRM was used to measure product (i.e., quantifier) ions following collision-induced dissociation of labeled 25OH-D cycloadducts under positive ion mode detection. **C:** Acceptable intraday precision (mean CV = 10.8%) was achieved with continuous analysis of standard serum extracts ($n = 412$) and blank ($n = 20$) samples over a period of 24 h after data smoothing and normalization of ion responses to a stable isotope internal standard. **D:** Representative traces of the first, middle, and final runs of the intraday precision study for the product ion of 25OH-D3 and its matching deuterated IS after data smoothing. Good linearity ($R^2 > 0.998$) was achieved for calibrants over an 80-fold linear dynamic range for **(E)** 25OH-D3 and **(F)** 25OH-D2. **G:** Spike and recovery studies in standard serum samples confirmed acceptable method accuracy for 25OH-D3 (103 ± 12)% and 25OH-D2 (101 ± 12)% quantification at different concentration levels (15, 30, and 45 nmol/l). DI-MS/MS, direct infusion-MS/MS; 2-NO-Pyr, 2-nitrosopyridine; CE, capillary electrophoresis; PRM, parallel reaction monitoring; CV, coefficient of variation.

TABLE 1. Summary of key figures of merit for the determination of 25OH-D in plasma and serum extracts by DI-MS/MS with 2-NO-Pyr click derivatization

Figures of merit	25OH-D3	25OH-D2
Mean recovery (range) ^a	103% (92–112%)	101% (93–113%)
Precision (intraday; interday) ^b	10.9%; 10.5%	N/A; N/A
Linearity (slope; r^2) ^c	0.0037; 0.992	0.0037; 0.996
LOQ ($S/N = 10$)	3.9 nmol/l (1.7 pg)	3.6 nmol/l (1.6 pg)
LOD ($S/N = 3$)	1.2 nmol/l (0.52 pg)	1.1 nmol/l (0.50 pg)
% Detects in reference blood samples ($n = 18$) ^d	100%	21%
% Detects in pediatric plasma samples ($n = 30$) ^e	100%	43%

LOD, limit of detection; LOQ, limit of quantification; N/A, not available.

^aSpike-and-recovery experiments performed at three concentration levels (15, 30, and 45 nmol/l) in triplicate, showing the average and range of 25OH-D recoveries.

^bIntraday precision, summarized as coefficient of variation, was determined by repeatedly analyzing a processed plasma sample ($n = 412$) over 24 h. Interday precision was determined by sequentially analyzing a processed plasma sample 50 times a day for 3 days. The blood samples used for these experiments had 25OH-D2 below the LOQ of the DI-MS/MS method.

^cExternal calibration curves were normalized to d6-25OH-D3 over a 12.5-fold calibration range using six calibrants where slope units are in $(\text{nmol/l})^{-1}$.

^dDetection frequency >LOD in reference materials from NIST and DEQAS ($n = 18$).

^eDetection frequency >LOD in VITdALIZE-KIDS plasma samples ($n = 30$).

Also, intermediate precision when measuring serum extracts over 3 days by DI-MS/MS after daily preventative maintenance and mass tuning demonstrated adequate reproducibility (mean CV = 10.5%, $n = 150$; supplemental Fig. S5). Also, analysis of 25OH-D from plasma extracts after two repeat freeze-thaw cycles confirmed good chemical stability without degradation of labeled vitamin D cycloadducts, thus allowing for repeat analyses of processed samples after storage (supplemental Fig. S6).

Interlaboratory comparison for serum 25OH-D determination in reference samples

We next performed an external validation of DI-MS/MS for 25OH-D determination by analyzing serum specimens from two quality assessment providers (DEQAS, $n = 15$; NIST, $n = 3$) in duplicate, which were measured independently by LC-MS/MS as a reference method. In addition, these samples were also analyzed in duplicate using a commercial Qualigen immunoassay system. Figure 2A, B highlights that there was good mutual agreement in 25OH-D quantification by DI-MS/MS as compared with LC-MS/MS with acceptable linearity (slope = 1.17) and a mean bias of 7.8% ($n = 18$). As expected, this positive bias was mainly attributed to coelution of 3-*epi*-25OH-D3 to total 25OH-D as measured by DI-MS/MS, which was confirmed after adjustment for the 3-*epi*-mer that reduced the overall bias relative to LC-MS/MS to only 1.5% (supplemental Fig. S7). To date, automated immunoassays with bias <10% have been reported to be applied safely in clinical practice (13). However, an intermethod comparison of the Qualigen immunoassay and LC-MS/MS for the same reference blood samples confirmed a larger mean bias of 12.9% (supplemental Fig. S8) reflecting lower method selectivity. In fact, there was also appreciable positive bias (42%) for the Qualigen immunoassay from 3-*epi*-25OH-D3 that was highly enriched in the NIST L4 sample with nearly equimolar

concentrations to 25OH-D3 (supplemental Fig. S9). This atypical reference sample was excluded from the interlaboratory comparison as both the immunoassay and DI-MS/MS were prone to major bias relative to LC-MS/MS that offers isomer resolution. In addition, the Qualigen immunoassay was prone to batch effects when using five different reagent lots (supplemental Fig. S10) with larger variability for the repeated analysis of pooled plasma samples as quality control (mean CV = 18%, $n = 52$) over a period of 10 months.

Impact of method bias when screening for vitamin D-deficient critically ill children

Figure 2C depicts a Mountain plot overlay that illustrates that DI-MS/MS (−4.1%) generates a lower median bias than the Qualigen immunoassay (−9.6%) when both were compared to LC-MS/MS for the reference serum samples from NIST and DEQAS analyzed ($n = 18$). The greater extent of bias for the Qualigen immunoassay has important consequences when classifying vitamin D-deficient (<50 nmol/l) children who may clinically benefit from vitamin D supplementation, yet may not be recruited if relying on immunoassay results alone. For example, Figure 2D depicts empirical cumulative frequency distribution plots for plasma 25OH-D concentrations from pediatric patients ($n = 30$) for their potential recruitment in a randomized controlled trial when using the Qualigen immunoassay or DI-MS/MS as the primary screening method. As expected, there was a lower mean 25OH-D concentration reported for all potential participants (−5.0 nM) for DI-MS/MS relative to the Qualigen immunoassay (59.7 vs. 64.7 nmol/l), which corresponded to a larger fraction of participants being classified as vitamin D deficient (13 of 30 or 43% vs. 5 of 30 or 16.7%). Plasma 25OH-D concentration was also over a wider range in this cohort when measured by DI-MS/MS as compared with the Qualigen immunoassay because of its higher limit of quantification

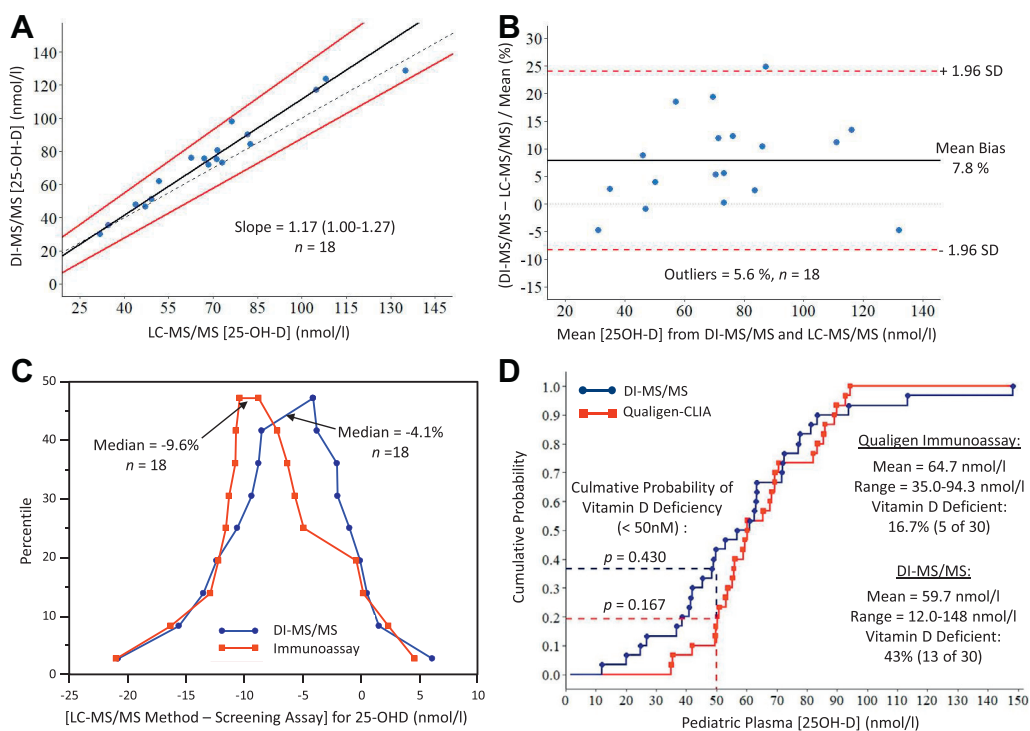


Fig. 2. An intermethod comparison between DI-MS/MS and validated LC-MS/MS methods for 25OH-D quantification using reference blood samples from DEQAS and NIST ($n = 18$) that were measured in duplicate. Overall, good mutual agreement was demonstrated as reflected by (A) a Passing-Bablok regression showing a slope of 1.17 within 95% confidence limits of the line of equality and a (B) a Bland-Altman percent difference plot with a mean bias of 7.8% with only one outlier exceeding agreement limits. C: A Mountain plot overlay highlights the greater median bias of a commercial Qualigen immunoassay system relative to DI-MS/MS when reporting circulating 25OH-D concentrations from reference blood samples ($n = 18$) independently measured by LC-MS/MS. D: An empirical cumulative distribution plot overlay of plasma 25OH-D concentrations measured in a cohort of hospitalized pediatric patients ($n = 30$) illustrating the impact of method bias when screening for vitamin D nutritional status. A greater fraction of patients was classified as vitamin D deficient (25OH-D <50 nmol/l) when screening is performed by DI-MS/MS (43%) as compared with the Qualigen immunoassay (16.7%), which impacts clinical treatment decisions because of misclassification.

(~32 nmol/l) that is also prone to matrix effects in blood samples. As a result, precise and accurate screening for 25OH-D determination is critical to authenticate vitamin D-deficient children likely more responsive to therapeutic vitamin D interventions.

DISCUSSION

To the best of our knowledge, our work represents the first validated DI-MS/MS protocol reported for the rapid screening of vitamin D deficiency. Most assays for assessment of vitamin D status from blood specimens have relied on immunoassays or more selective LC-MS/MS instrumental methods (14, 15). Alternatively, direct or flow infusion coupled to high-resolution MS offers a robust approach for metabolite profiling with higher throughput than chromatographic separations (27). Moreover, troubleshooting is minimized as issues related to column conditioning, changes in solute retention time, and sample carryover effects are largely avoided. As a versatile high-resolution mass analyzer, Q-TOF-MS is gaining interest in the clinical laboratory for comprehensive drug screening applications in toxicology (28), whereas PRM is optimal for targeted

analysis of compounds with greater confidence in their annotation than multiple reaction monitoring (29). In this work, a CE-MS system with software control was utilized to automate repeat sample injections and infusion processes without voltage application, rendering this separation-free method easily transferable to other laboratories without specialized flow injection or sequential injection equipment. Each blood or calibrant extract was infused until a steady-state signal was reached (~1 min) followed by Gaussian smoothing of raw data to improve the reproducibility of peak integration. Overall, click derivatization and liquid-phase extraction were critical to boost ionization efficiency and reduce matrix-induced ion suppression effects when analyzing low nanomolar levels of 25OH-D by DI-MS/MS. The inherent stability of labeled vitamin D cycloadducts in processed samples after repeat freeze-thaw cycles by DI-MS/MS is consistent with the long-term chemical stability of native 25OH-D reported in frozen serum from biorepositories (30). Also, reference serum samples can be shipped under ambient conditions without bias when analyzed by LC-MS/MS unlike immunoassays that are more susceptible to preanalytical variance (31).


In this work, method validation confirmed that DI-MS/MS can reliably quantify 25OH-D₂ and 25OH-D₃ with better selectivity and lower detection limits than the Qualigen immunoassay that measures total 25OH-D. The figures of merit (Table 1) in terms of precision and recovery are comparable with LC-MS/MS using chemical derivatization with 2-NO-Pyr, which required a 13 min gradient elution solvent program without 3-epimer resolution (25). Importantly, our method forgoes chromatographic separation of regioisomers since two diastereomeric cycloadducts (1:4 ratio) are generated after click derivatization that further complicates data processing (25). As expected, the major source of bias in our method was from coelution of 3-*epi*-25OH-D₃ as an isomeric interference, which also contributed to significant antibody crossreactivity in the Qualigen immunoassay. The bioactivity of 3-*epi*-25OH-D₃ is reported to be weaker than 25OH-D₃, and its concentration is proportionally lower on average than 25OH-D₃ in adults (5.9%) as compared with infants (<1 year, 21%) (32). Furthermore, oral vitamin D supplementation relative to UV irradiation increases the proportion of 3-*epi*-25OH-D₃ in mice but not humans (33). Thus, the absence of epimer resolution when using DI-MS/MS and some LC-MS/MS methods is not anticipated to impact the routine screening of vitamin D deficiency in most patients (22, 23).

Overall, a mean bias of 7.8% was determined when analyzing reference serum samples by DI-MS/MS as compared with LC-MS/MS, which was in turn lower than the Qualigen immunoassay (12.9%). Sources of bias in the Qualigen immunoassay can also arise from spectral interferences because of turbidity from lipidemia (34) or elevated total protein levels as blood samples were only diluted 3-fold in buffer prior to analysis unlike the sample workup protocol developed for DI-MS/MS. Also, the lot-to-lot variability contributed to greater variability when using the Qualigen immunoassay, which may be attributed to manufacturing inconsistencies (e.g., antibody specificity) and reagent kit stability during storage (35). As a result, immunoassays are less favorable for large-scale epidemiological studies or longitudinal clinical trials as compared with more robust instrumental methods, such as DI-MS/MS. A major consequence of the larger bias and higher limit of quantification of the Qualigen immunoassay as a primary screening method for 25OH-D was the much lower fraction of children who were classified as vitamin D deficient when compared with DI-MS/MS. A growing number of randomized clinical trials indicate the potential value of rapid screening of vitamin D deficiency with prompt administration of loading doses to improve clinical outcomes during hospitalization during the COVID-19 pandemic (<https://vdm-meta.com/>). However, the lack of fast and accurate assays for 25OH-D impacts the scientific validity of clinical trials by recruiting ineligible patients who are vitamin D sufficient while excluding

vitamin D-deficient patients from benefiting because of their misclassification. Also, therapeutic monitoring for potential vitamin D toxicosis (25OH-D >375 nmol/l) is also warranted to prevent rare complications (e.g., hypercalcemia) from excessive vitamin D intake (36).

In summary, 25OH-D remains the most widely measured biomarker of vitamin D nutrition implicated in numerous chronic human disorders (e.g., cancer, cardiovascular disease, autoimmune diseases), post-operative recovery from surgery, as well as susceptibility to microbial infections. We have introduced a DI-MS/MS protocol for the rapid screening of 25OH-D with greater sample throughput and practicality than LC-MS/MS, which also offers less bias and lower detection limits than the Qualigen immunoassay. Automated programming of infusion and flushing steps using a CE instrument for DI-MS/MS provided an effective duty cycle of 3.3 min/sample allowing for continuous operation with minimal troubleshooting. A major limitation was interference from 3-*epi*-25OH-D when analyzing plasma or serum extracts; however, this lower abundance isomer contributes only a modest extent of positive bias that is acceptable for routine screening without misclassifying vitamin D status. Also, a multistep sample workup procedure greatly reduced matrix-induced ion suppression following click derivatization, which can benefit from faster processing with automation. Other mass analyzers, scanning modes, and ion sources may also be used for high-throughput screening of vitamin D status using DI-MS/MS based on this protocol. For instance, greater sensitivity and lower detection limits for 25OH-D and other lower abundance vitamin D metabolites are anticipated when coupling CE to a triple quadrupole mass analyzer with multiple reaction monitoring in conjunction with a very low sheath flow/nanospray CE-MS interface (37). Importantly, rapid 25OH-D screening by DI-MS/MS can assist in developing personalized vitamin D therapeutic interventions to reduce the burden of COVID-19 on hospitalization and mortality for emerging severe acute respiratory syndrome coronavirus 2 variants, including other respiratory infections. This platform is attractive in support of clinical trials for reliable classification of high-risk vitamin D-deficient patients in a critical care setting as well as large-scale epidemiological studies for biomonitoring vitamin D status on a population level. Future work will be engaged to adapt this DI-MS/MS method to Good Clinical Laboratory Practice and Clinical Laboratory Improvement Amendments compliance for the purpose of reporting clinical values.

Data availability

The data supporting this study are available in the article, the supplemental data, or available from the corresponding author upon reasonable request. 

Supplemental data

This article contains [supplemental data](#) (25, 38, 39).

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Author contributions

P. B. M. and D. M. conceptualization; P. B. M. and D. M. methodology; E. H. and P. B. M. formal analysis; E. H. and P. B. M. data curation; E. H. and P. B. M. writing—original draft; E. H., E. F., L. P., K. C., K. H., D. M., and P. B. M. writing—review & editing; L. P. and K. H. project administration; P. B. M. and D. M. funding acquisition.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

CE, capillary electrophoresis; COVID-19, coronavirus disease 2019; CV, coefficient of variation; DEQAS, Vitamin D External Quality Assessment Scheme; DI-MS/MS, direct infusion-MS/MS; NIST, National Institute of Standards and Technology; 2-NO-Pyr, 2-nitrosopyridine; 25OH-D, 25-hydroxyvitamin D; PRM, parallel reaction monitoring; Q-TOF, quadrupole-TOF.

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