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Simultaneous determination of 24,25- and 25,26-dihydroxyvitamin D3 in serum samples with liquid-chromatography mass spectrometry – A useful tool for the assessment of vitamin D metabolism

Sieglinde Zelzer[a], Andreas Meinitzer[a], Dietmar Enko[a,b], Sebastian Simstich[a], Caroline Le Goff[c], Etienne Cavalier[c], Markus Herrmann[a,*], Walter Goessler[d]

[a] Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria
[b] Institute of Clinical Chemistry and Laboratory Medicine, General Hospital Hochsteiermark, Leoben, Austria
[c] Department of Clinical Chemistry, University of Liege, Liege, Belgium
[d] Institute of Chemistry, University of Graz, Graz, Austria

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ABSTRACT

Vitamin D status is typically assessed by the measurement of 25-hydroxyvitamin D (25(OH)D). However, in selected patient groups the sole determination of 25(OH)D has been proven insufficient for this purpose. The simultaneous measurement of additional vitamin D metabolites may provide useful information for a better evaluation of the vitamin D status. Therefore, we developed and validated a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of $25(OH)D_3$, $25(OH)D_2$, $24,25(OH)_2D_3$ and additionally $25,26(OH)_2D_3$, which was identified with a synthesized pure substance.

Pure and deuterated substances were used to prepare calibrators and internal standards for all target metabolites. Pre-analytical sample preparation comprised protein precipitation followed by liquid-liquid-extraction and derivatization with 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD) using 50 µL sample volume. Samples were analyzed on an Agilent HPLC 1260 system equipped with a silica-based Kinetex® 5 µm F5 100 Å core-shell column (150 × 4.6 mm) coupled to a Sciex 4500 mass spectrometer.

For all four metabolites, limit of detection (LoD) and limit of quantification (LoQ) ranged from 0.3 to 1.5 nmol/L and 1.0 to 3.1 nmol/L, respectively. Recovery varied between 76.1 % and 84.3 %. Intra- and inter-assay imprecision were < 8.6 % and < 11.5 %, respectively. The analysis of external and internal quality control samples showed good accuracy for $25(OH)D_3$, $25(OH)D_2$, $24(R),25(OH)_2D_3$ and $25,26(OH)_2D_3$. Method comparison studies with human samples that were also analyzed with two other LC-MS/MS methods showed close agreement. Finally, the present method has been shown capable of identifying patients with 24-hydroxylase deficiency, which proves its clinical utility.

1. Introduction

The growing awareness that vitamin D deficiency is highly prevalent and involved in many pathophysiological processes has triggered an enormous surge in vitamin D testing [1]. Scientific societies unanimously recommend to assess vitamin D status in patients at risk for vitamin D deficiency by measuring 25-hydroxyvitamin D (25(OH)D). However, in selected patient groups the analysis of additional vitamin D metabolites may provide helpful information beyond the sole measurement of 25(OH)D. Black Africans, for example, have approximately 40 % lower serum 25(OH)D concentrations than Caucasians [2–4], whereas parathyroid hormone concentrations, bone mineral density

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; $25(OH)D_3$, 25-hydroxyvitamin D_3; $25(OH)D_2$, 25-hydroxyvitamin D_2; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; C3-epi-$25(OH)D_3$, C3 epimer of 25-hydroxyvitamin D3; LC-MS/MS, liquid chromatography tandem mass spectrometry; HPLC, high performance liquid chromatography; VDBP, vitamin D binding protein; VMR, vitamin metabolite ratio; Ca, Calcium; NaCl, physiological saline solution; MeCN, acetonitrile; KOH, potassium hydroxide; tMBE, tert-methyl-butyl-ether; FA, formic acid; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; ESI, electro spray ionization; MRM, multiple reaction monitoring; ISTD, internal standards; LOD, limit of detection; LOQ, limit of quantification; RT, room temperature; DEQAS, Vitamin D External Quality Assessment Scheme; EQA, external quality assurance; SD, standard deviation; CV, coefficient of variation

* Corresponding author at: Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria.
E-mail address: markus.herrmann@medunigraz.at (M. Herrmann).

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and fracture risk are comparable. Also, pregnant women and patients in intensive care have lower 25(OH)D concentrations compared to the general population [5]. In addition, measuring serum 25(OH)D is not very helpful in patients with a disturbed vitamin D metabolism, such as in 1α-hydroxylase deficiency, 24-hydroxylase deficiency and sarcoidosis. Another limiting factor is the variable performance of most 25(OH)D immunoassays used by clinical laboratories [6]. These assays show variable cross-reactivity with vitamin D metabolites, such as 25-hydroxyvitamin D3 (25(OH)D_3), 25-hydroxyvitamin D_2 (25(OH)D_2) and 24,25-dihydroxyvitamin D_3 (24,25(OH)$_2D_3$) [6–8]. Differences in the efficacy to release vitamin D metabolites from vitamin D binding protein (VDBP), albumin and lipoproteins is another factor that contributes to the variable performance of 25(OH)D assays [9]. Finally yet importantly, approximately 0.5–3 % of all specimens contain heterophilic antibodies that cause significant interferences in immunoassays [10,11].

Substantial improvements in the area of mass spectrometry have enabled researches to overcome most of the issues related to 25(OH)D immunoassays and accurately measure a range of vitamin D metabolites including 25(OH)D_3, C3 epimer of 25-hydroxyvitamin D3 (C3-epi-25(OH)D_3), 25(OH)D_2 or 24,25(OH)$_2D_3$. Quantifying these metabolites has provided new insights into vitamin D metabolism that may help to interpret patient results better. For example, Cavalier *et al.* have shown that interpreting 25(OH)D results in conjunction with 24,25(OH)$_2$D may improve the identification of vitamin D deficient subjects [12,13]. 24,25(OH)$_2$D is the principle catabolite of 25(OH)D that is only formed when sufficient amounts of 25(OH)D are available. 24-hydroxylase deficiency is another condition that influences the conversion of 25(OH)D into 24,25(OH)$_2$D and thus impacts the serum concentrations of both metabolites. The ratio between 25(OH)D and 24,25(OH)$_2$D (vitamin metabolite ratio; VMR) is also altered in these patients [14,15]. Identifying 24-hydroxylase deficient individuals is clinically relevant as they may develop symptomatic hypercalcemia and nephrocalcinosis, when 25(OH)D concentrations are normal or within the desirable range [16]. Therefore, vitamin D supplementation can be harmful in these patients. Until today, data about the prevalence of the 24-hydroxylase in the general population is lacking. The possibility to measure 24,25(OH)$_2$D by LC-MS/MS is a useful tool to identify individuals with genetic 24-hydroxylase defects, especially when 25(OH)D is detected in parallel [12,17]. 25,26-dihydroxyvitamin D3 (25,26(OH)$_2D_3$), another dihydroxylated vitamin D metabolite with the same mass as 24,25(OH)$_2D_3$, was first isolated in 1970 by DeLuca et al. [18]. So far, little is known about this compound and most available studies date back to the 1980s when HPLC with UV detection lacking sensitivity and specificity were used for its analysis. Existing results suggest that 25,26-dihydroxycholecalciferol is only active in the intestine [19–22]. However, these findings require confirmation with state-of-the-art methods.

Modern LC-MS/MS systems offer high sensitivity and specificity allowing the parallel measurement of multiple vitamin D metabolites. Therefore, this technology is considered the gold standard for vitamin D metabolite determination [17,23]. Previous studies have shown that LC-MS/MS can measure 25(OH)D_3, 25(OH)D_2 and C3-epi 25(OH)D simultaneously in a single analysis [12,17]. Adding 24,25(OH)2D to such a method would allow to calculate the VMR as a surrogate marker of 24-hydroxylase activity and to consider additional aspects of vitamin D metabolism [4,24].

Kaufmann et al measured the vitamin D metabolites 25(OH)D_3, 25(OH)D_2, 24,25(OH)$_2D_3$ and with an extended chromatography also 25,26(OH)$_2D_3$ in individuals with idiopathic infantile hypercalcemia. Their method enables to differentiate pathological states with CYP24A1 activity alteration [25].

The present study aimed to develop and validate a LC-MS/MS method for the simultaneous determination of 25(OH)D_3, 25(OH)D_2, 24,25(OH)$_2D_3$ and additionally 25,26(OH)$_2D_3$, which was identified with a synthesized pure substance.

2. Material and methods

2.1. Chemicals and reagents

LC-MS grade organic solvents (methanol, acetonitrile (MeCN)), and analytical grade chemicals (potassium hydroxide (KOH), n-heptane, tert-methyl-butyl-ether (tMBE) and formic acid (FA, (> 99 %)), were purchased from Merck (Darmstadt, Germany). Human albumin (20 %) was used from CSL Behring (Marburg, Germany); physiological saline solution (NaCl) from Fresenius Kabi (Graz, Austria); 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (> 97 %) was ordered from Sigma Aldrich (St. Louis, USA) and LC-MS grade water was purchased from Honeywell Riedel-de-Haën™ (Fisher Scientific GmbH, Schwerte, Germany).

Standards for 25(OH)D_3 (purity 97 %), 25(OH)D_2 (96.6 %) and 24(R),25(OH)$_2D_3$ (98 %) and internal standards for d6-25-hydroxyvitamin-D3 (d6-25OH)D_3), d3-25-hydroxyvitamin-D2 (d3-25(OH)D_2), d6-24(R),25-dihydroxyvitaminD3 (d6-24,25(OH)$_2D_3$), with 99 % purity (each with 1 mg) were ordered from Toronto Research Chemicals Inc. (Toronto, Canada). The 25(S),26(OH)$_2D_3$ (10 mg, 98 % purity) was synthesized by ENDOTHERM GmbH (Saarbrücken, Germany).

2.2. Preparation of calibrators and internal standards

2.2.1. Calibrators

For all target analytes, calibrator stock solutions with a concentration of 1 mg/mL were prepared by dissolving pure standards in the appropriate volume of methanol. These stock solutions and pure methanol were used to prepare a master mix solution with analyte concentrations of: 250 nmol/L (100 µg/L) of 25(OH)D_3, 50 nmol/L (20 µg/L) of 25(OH)D_2, 50 nmol/L (20 µg/L) of 24,25(OH)$_2D_3$ and 50 nmol/L (20 µg/L) of 25,26(OH)$_2D_3$. From this master mix 6 calibrators with the following concentrations were prepared by adding the appropriate amount of methanol: 7.80, 15.60, 31.25, 62.50, 125 and 250 nmol/L of 25(OH)D_3 and 1.50, 3.00, 6.00, 12.0, 24.0 and 48.0 nmol/L of 25(OH)D_2, 24,25(OH)$_2D_3$ and 25,26(OH)$_2D_3$.

2.2.2. Internal standards

Pure labeled internal standards (ISTD) were dissolved in methanol and ISTD stock solutions with the following concentrations were prepared and stored at –80 °C: 25 mmol/L d6-25OH)D_3, 0.25 mmol/L d3-25(OH)D_2 and 2.5 mmol/L d6-24,25(OH)$_2D_3$ and stored at –80 °C. These stock solutions were used to prepare the ISTD working solution of 150 nmol/L by adding methanol. All ISTD working solutions stored at 4 °C until sample preparation.

2.3. Quality controls (QCs) and samples for method validation

High and low serum controls from Recipe (RECIPE Chemicals + Instruments GmbH, Munich, Germany) were used for accuracy studies. An additional control sample in the mid-range was produced by mixing the two control samples 1 + 1. Aliquots (350 µL) were prepared in Eppendorf tubes (1.5 mL) and stored at −20 °C until analysis of the intra- and inter-assay imprecision.

External quality control samples for the assessment of analytical accuracy were obtained from the Vitamin D External Quality Assessment Scheme (DEQAS) (UK). For these samples target values for 25(OH)D_3, 25(OH)D_2, and 24,25(OH)$_2D_3$ are available. The DEQAS external quality assurance (EQA) program includes 5 samples 4 times per year. In addition, we obtained 40 well characterized patient samples from the Department of Clinical Pathology at Bolzano Hospital (Italy), where they were previously analyzed on a Shimadzu 8040 LC-MS/MS system using the 25-OH Vitamin D2/D3 LC-MS/MS ClinMass® kit from Recipe. The superior analytical performance of this method has been demonstrated in multiple cycles of the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPQAP). From these samples, a subset of 20 samples 24,25(OH)$_2D_3$ was also

measured with a validated in-house LC-MS/MS method at the Department of Clinical Chemistry, University of Liege (Belgium) [4]. Finally, a pooled serum, obtained from healthy individuals, was prepared for daily internal laboratory control and for stability studies. Daily QC was judged on the basis of lab specific performance data. Long term QC results were used to calculate lab specific mean, SD and CV for each QC material. Samples were rejected if they violated one of the Westgard rules that the lab regularly uses for this purpose.

2.4. Sample preparation

In order to ensure a similar matrix composition, different procedures were adopted for calibrators and samples/controls. An aliquot of 50 µL of calibrator were mixed with 25 µL ISTD working solutions in glass tubes and completely dried down under a gentle stream of nitrogen. The dried calibrators were re-suspended with 50 µL of a 7 % albumin-NaCl solution. Instead, 50 µL of samples and controls were used to re-suspend 25 µL of dried ISTD. By this procedure all components were introduced in a similar matrix with the same concentration of ISTD, thus reducing potential matrix effects.

After adding 10 µL KOH (2 M), samples were extracted with 2 mL of 1 + 1 (v + v) n-heptane + tMBE under constant agitation for 10 min on a programmable rotator (BioSan Multi RS-60, ProfiLab24 GmbH, Berlin, Germany). Subsequently, this mixture was centrifuged at 4000*g* for 5 min at room temperature (RT) and frozen for 30 min at −80 °C for an easier decanting. The supernatant was evaporated with nitrogen and the residue was reconstituted in 50 µL acetonitrile containing 0.5 mg/mL PTAD for derivatization. After a 10-min incubation, the excess PTAD was quenched with 50 µL formic acid (0.1 %). At the end, samples were placed in the autosampler for analysis. In a working shift of eight hours 100 samples can be prepared.

2.5. LC-MS/MS analysis

Analyses were performed on a LC-MS/MS system composed of an Agilent 1260 infinity high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, USA) and an AB SCIEX QTRAP 4500 mass spectrometer equipped with an AcQuRate Pulse Counting CEM and Analyst 1.6.2 software (SCIEX, Framingham, Massachusetts, USA). The HPLC system included a binary loading pump, a quaternary eluting pump, a high performance autosampler, a degasser, a temperature controlled column compartment, a diode array detector, and six switching valves.

After appropriate preparation, 10 µL of calibrators, controls and samples were introduced into the HPLC system. First, analytes were pre-concentrated by online SPE (solid phase extraction) using a POROS™ R1 20 µm stainless steel column, 2.1 × 30 mm, (Thermo Scientific™), and subsequently transferred to a Kinetex® 5 µm F5 100 Å analytical LC column 150 × 4.6 mm (Phenomenex, Torrance, CA, USA). An acetonitrile/water gradient was used for elution. The complete HPLC protocol is listed in Table 1.

The eluent from the HPLC system was introduced into the ion source of the mass spectrometer operated in positive ion mode. After electro spray ionization (ESI), analytes were measured in multiple reaction monitoring (MRM) mode with a scan time of 0.9351 s. The instrument settings were as follows: electro spray needle voltage 5500 V, ion source temperature 650 °C, ion source gas 1 50 psi, and ion source gas 2 70 psi, curtain gas 40 psi; and collision gas 9 psi; respectively. For all analytes the mass to charge ratios (*m/z*) are listed in Table 2. Analyte concentrations were calculated using Analyst® 1.6.2 Software (SCIEX).

Linear 6-point calibration curves for all target analytes were constructed. For each calibrator the analyte/ISTD peak area ratio was plotted against the nominal concentration of each compound, whereby d6-24(R),25-dihydroxyvitamin D3 was used as internal standard for $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$. Linear regression analysis was used to obtain slope, intercept and correlation coefficient (r^2). The acceptance criteria of the recalculated measured values of each calibration point were ≤15 %.

Table 1
HPLC protocol of the two column method. Samples were loaded on column 1 with mobile phase C, methanol/water (30:70) with the quaternary pump. The binary solvent system consisted of 0.1% formic acid in acetonitrile (95%) (mobile phase A) and 0.1% formic acid in water (mobile phase B) for chromatographic separation on column 2.

Time	Binary pump		Flow	Time	Quaternary pump	Flow
	Mobile phase A	Mobile phase B			Mobile phase C	
(min)	(%)	(%)	(mL/min)	(min)	(%)	(mL/min)
0.00	52.6	47.4	0.7	0.00	100	1.0
6.50	79.6	20.4	0.7	0.10	100	2.0
9.50	100	0.0	0.7	1.60	100	0.1
11.60	100	0.0	1.4	10.10	100	2.0
15.10	52.6	47.4	0.7	12.20	100	0.1
17.00	Controller Stop			16.90	100	0.1
				17.00	Controller Stop	

Table 2
Analytical performance of the vitamin D metabolite LC-MS/MS method.

	$25(OH)D_3$	$25(OH)D_2$	$24,25(OH)_2D_3$	$25,26(OH)_2D_3$
m/z ratio	558.4/298	570.2/298	574.2/298	574.1/298
Linear range (nmol/L)	7.8–250	1.5–48	1.5–48	1.5–48
Correlation coefficient (r^2)	0.999	0.997	0.998	0.997
Intra-day precision (CV %)	1.8–7.0	1.3–4.0	2.8–8.6	2.2–8.1
Inter-day precision (CV %)	2.9–7.9	3.5–7.2	1.2–11.5	7.6–9.6
LoD (nmol/L)	1.5	0.3	0.3	0.3
LoQ (nmol/L)	3.1	1.0	1.0	1.0
Recovery (%)	76.1	78.9	79.5	84.3

m/z = mass to charge ratio; LoD = limit of detection; LoQ = limit of quantification; CV = coefficient of variation.

2.6. Method validation

Method validation was based on the recommendations published by the Food and Drug Administration (FDA) [26].

The limit of detection (LOD) was defined as the lowest concentration, which produced a signal at least three times higher than the average background noise. The limit of quantification (LOQ) was defined as the lowest concentration that allowed quantification with an imprecision of < 10 % [27]. Both indices were determined by analyzing serial dilutions of the lowest calibrator. Each dilution was measured 5 times.

Intra- and inter-assay imprecision were assessed for all target analytes by measuring three different Recipe controls materials with low, middle and high concentrations on 5 consecutive days. In these samples the concentrations of all four target metabolites were as follows: $25(OH)D_3$: 40.5, 81.2 and 120.5 nmol/L; $25(OH)D_2$: 37.1, 71.1 and 105 nmol/L; $24,25(OH)_2D_3$; 1.5, 3.2 and 4.8 nmol/L; $25,26(OH)_2D_3$: 1.2, 1.9 and 2.5 nmol/L.

Recovery was determined for all target analytes by adding either 50 µL of calibrator 2 (15.6 nmol/L for $25(OH)D_3$, and 3.0 nmol/L for $25(OH)D_2$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$) or calibrator 4 (62.5 nmol/L for $25(OH)D_3$, and 3.0 nmol/L for $25(OH)D_2$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$) to a pooled serum with known concentrations for each vitamin D metabolite. Each spiking experiment was performed in quintuplicate. The recovery was calculated as percentage of the measured concentration in relation to the expected concentration.

Accuracy was assessed by measuring DEQAS samples with known target concentrations for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ [28].

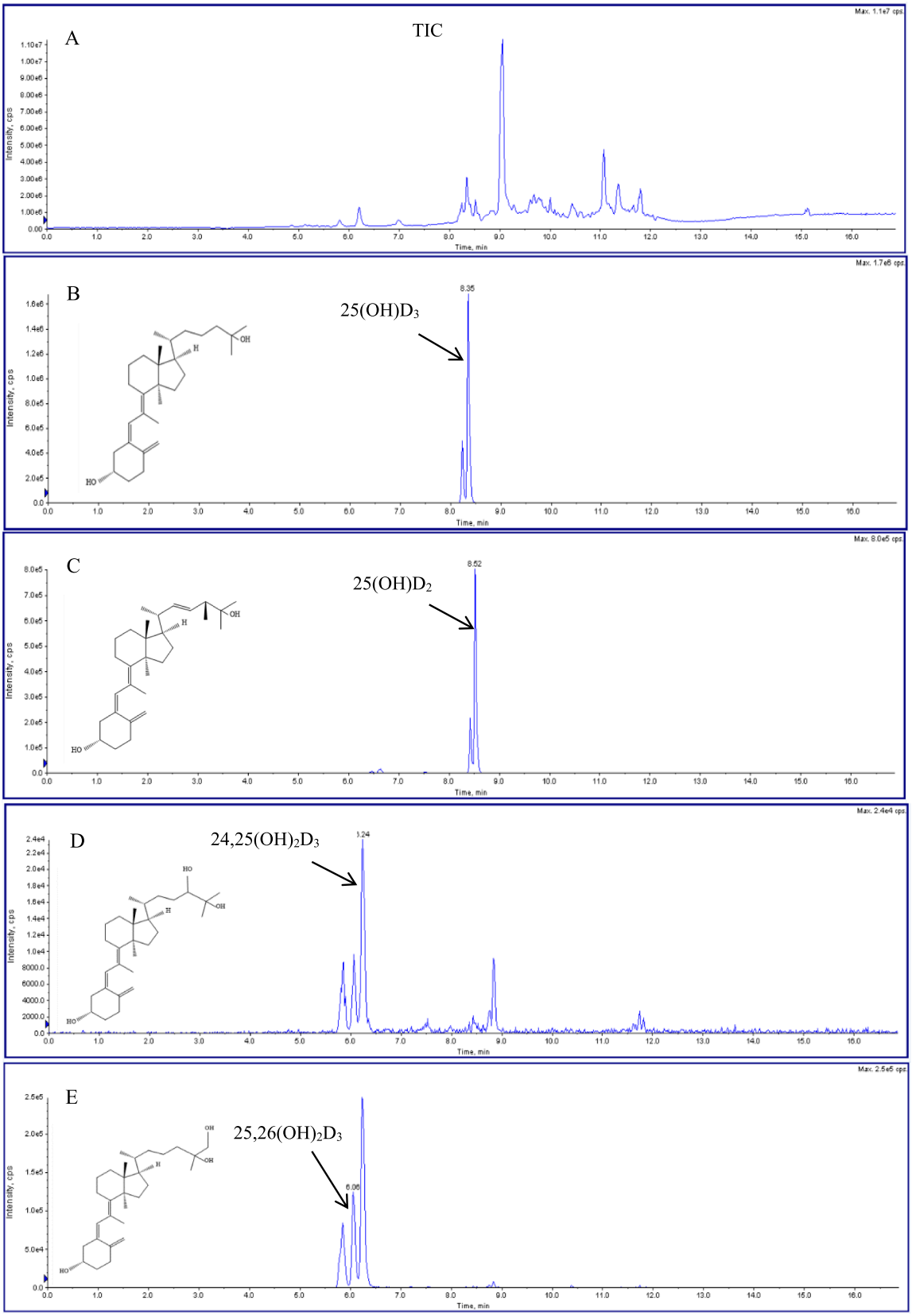

Fig. 1. LC-MS/MS chromatograms from the Recipe control level II using the vitamin D metabolite method. (A) total ion chromatogram (TIC); (B) 25(OH)D_3 concentration:105 nmol/L; (C) 25(OH)D_2 concentration: 103 nmol/L; (D) 24,25(OH)$_2D_3$ concentration: 2.2 nmol/L; (E) 25,26(OH)$_2D_3$ concentration: 1.2 nmol/L.

In addition, method comparisons were performed by measuring 40 randomly selected patient samples that were previously analyzed with an alternative LC-MS/MS method as described above. A subset of 20 samples was additionally analysed with a third LC-MS/MS method capable of detecting 24,25(OH)$_2D_3$ [4].

Additionally, we investigated diagnostic utility by analyzing four

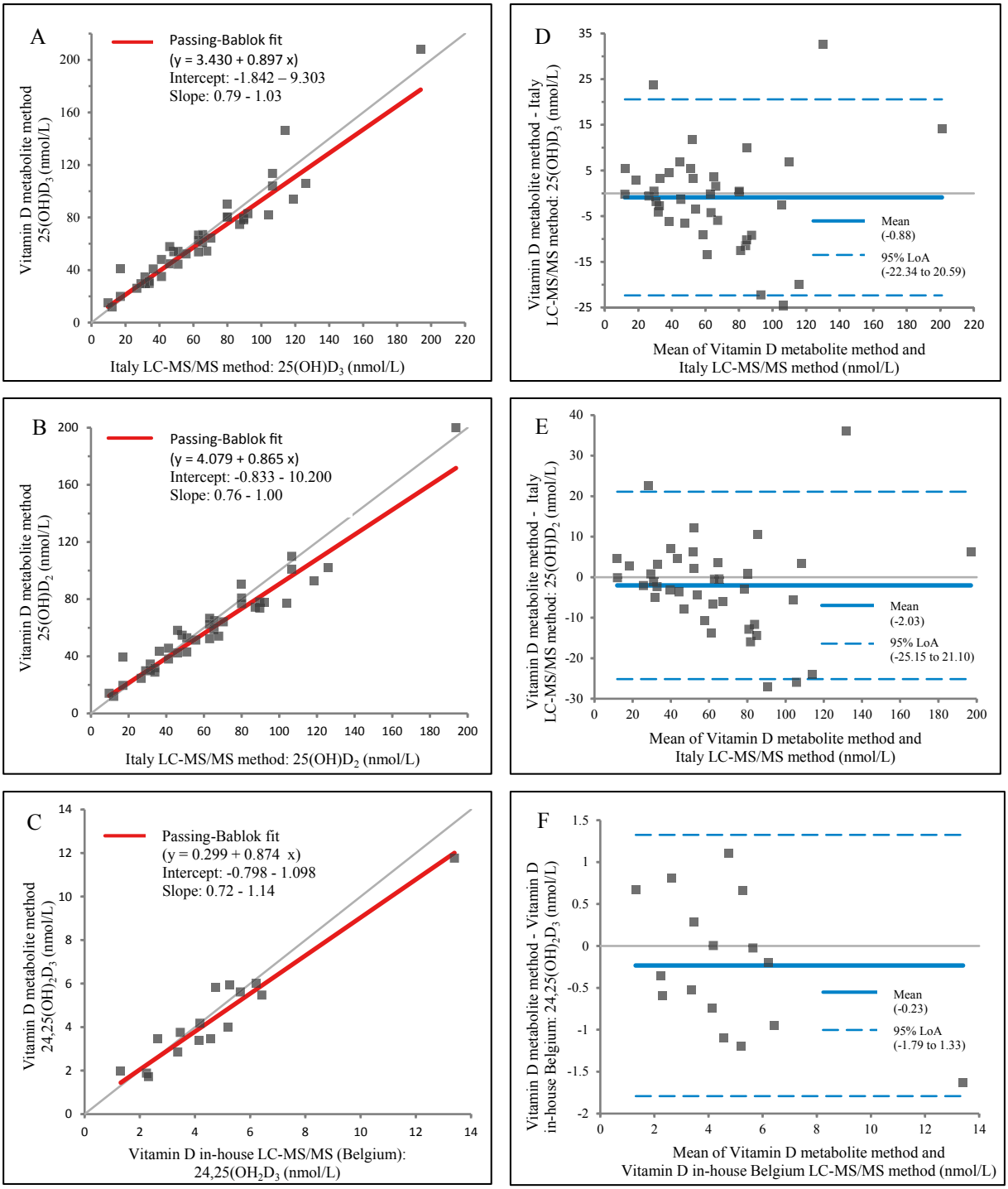

Fig. 2. Method comparison results of the vitamin D metabolites. Passing Bablok Regression analyses are shown in Figure A–C. 25(OH)D_3 and 25(OH)D_2 compared with our vitamin D metabolite method and the 25-OH-Vitamin D_2/D_3 LC-MS/MS assay (Recipe) measured in Bolzano, Italy (A-B); Regression analyses from 24,25$(OH)_2D_3$ compared with our vitamin D metabolite method and the LC-MS/MS method (Liege, Belgium (C). Bland-Altman analysis of our LC-MS/MS serum assay versus the Italy LC-MS/MS method (D-E) and versus the vitamin D in-house LC-MS/MS method Belgium) (F). LoA = Limit of Agreement.

serum samples from another study (approved by the Ethical Committee of the Medical University of Graz; EK 31-344 ex 18/19) with 25(OH)D serum concentrations > 100 nmol/L. One of these patients suffered from 24-hydroxylase deficiency which was confirmed by genetic testing. Written informed consent was provided by all subjects.

Sample stability was assessed using a drug-free serum pool, which was analyzed immediately after preparation. Short term stability was determined by measuring 12 aliquots from native serum that were kept for one day at room temperature. For the analysis of long-term stability the remaining native pool serum was split in aliquots of 250 µL. One half of these aliquots were stored at −20 °C whereas the other half was kept at −80 °C until analysis after 14, 28, 56 and 168 days. At each day, 12 aliquots per storage condition were processed and analyzed in parallel.

2.7. Statistical analysis

All statistical analyses were performed with Analyse-IT software

Table 3
Performance comparison of the vitamin D metabolite method using twenty subsequent DEQAS samples of the 2019–2020 cycle.

Sample	total 25(OH)D*			$24,25(OH)_2D_3$			$25,26(OH)_2D_3$
	Target values	Results	Bias	Target values	Results	Bias	Our method
	Mean ± SD (nmol/L)		(%)	Mean ± SD (nmol/L)		(%)	(nmol/L)
551	64.5 ± 1.0	65.7	+ 1.9	5.2 ± 0.7	5.1	−1.3	1.4
552	63.6 ± 0.9	65.4	+ 2.8	4.5 ± 0.6	4.2	−6.7	1.6
553	50.2 ± 0.6	54.0	+ 7.6	3.2 ± 0.5	3.0	−6.3	1.3
554	39.0 ± 0.5	41.5	+ 6.4	2.4 ± 0.4	2.4	0.0	1.1
555	167 ± 2.2	170	+ 1.7	18.0 ± 2.3	17.3	−3.9	4.5
556	66.2 ± 0.9	73.2	+ 10.6	5.0 ± 0.7	5.7	+ 14.0	1.8
557	100 ± 1.4	109	+ 9.0	8.2 ± 0.9	9.1	+ 10.9	2.7
558	37.5 ± 0.5	41.3	+ 10.1	1.9 ± 0.3	1.8	−5.3	1.0
559	79.4 ± 1.0	81.2	+ 2.3	5.9 ± 0.8	6.3	+ 6.8	2.4
560	58.2 ± 2.7	63.1	+ 8.4	3.4 ± 0.5	3.3	−2.9	1.7
561	66.5 ± 0.9	63.3	+ 8.0	5.1 ± 0.7	4.7	−7.9	1.8
562	47.9 ± 0.6	45.1	−5.8	2.9 ± 0.4	3.1	+ 6.9	1.7
563	88.8 ± 1.2	82.5	−7.1	6.3 ± 0.8	7.1	+ 12.6	1.8
564	43.4 ± 0.7	39.6	−8.8	4.6 ± 0.5	4.9	+ 6.5	1.7
565	76.3 ± 1.0	71.2	−6.7	4.3 ± 0.4	3.9	−9.5	1.7
566	68.4 ± 1.0	68.8	+ 0.6	4.7 ± 0.6	4.8	+ 2.1	1.2
567	63.1 ± 0.7	58.2	−8.4	3.4 ± 0.5	3.3	−2.4	1.2
568	31.7 ± 0.5	34.7	+ 9.5	1.9 ± 0.4	1.8	+ 5.9	0.6
569	82.6 ± 1.1	89.7	+ 8.6	7.1 ± 1.2	7.3	+ 2.8	1.6
570	135 ± 2.1	147	+ 9.2	12.3 ± 1.8	12.7	+ 3.3	2.8

* total 25(OH)D is the sum of $25(OH)D_3$ and $25(OH)D_2$ concentrations including the C3-epimer.

Ltd. (Leeds, UK) for Microsoft Excel®. Intra- and inter-day precision for each concentration tested were assessed by calculating mean, standard deviation (SD) and coefficient of variation (CV) of the replicate analyses. Bland-Altman and Passing-Bablok regression analyses were performed for method comparison. VMRs were calculated as the ratio between $24,25(OH)_2D_3/25(OH)D_3$/ x 100 (VMR 1, %); the ratio between $25,26(OH)_2D_3/25(OH)D_3$/ x 100 (VMR 2, %) and the ratio between $25,26(OH)_2D_3/24,25(OH)_2D_3$ (VMR 3). The following formula was used for bias calculations: mean bias (%) = measured value – expected value/expected value × 100.

3. Results and discussion

With our newly developed method $25(OH)D_3$, $25(OH)D_2$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ are adequately separated with retention times of 6.06, 6.24, 8.35, and 8.52 min, respectively (Fig. 1). The general performance characteristics are shown in Table 2. For all target analytes the method is linear over the investigated concentration ranges with coefficients of determination (r^2) ≥ 0.997. LOD and LOQ ranged between 0.3 and 1.5 nmol/L and 1.0–3.1 nmol/L, respectively. Recovery for all four metabolites, ranged between 76.1 % and 84.3 %. Intra- and interday imprecision for all vitamin D metabolites tested were < 9 % and < 12 %, respectively. The linear range covers well clinical needs and allows the quantification of very small analyte concentrations. Intra- and interday imprecision for all four metabolites ranged between 1. 8–8.6 % and 1.2–11.5 %, respectively, which is within the expectations for a method used in clinical laboratories.

Accuracy was assessed by Passing Bablok Regression analyses, which showed good agreement with the other two LC-MS/MS methods (Fig. 2). None of the target analytes showed significant proportional bias. The slope of regression lines ranged between 0.79 and 1.03 for 25-hydroxyvitamin D_3, between 0.76 and 1.00 for 25-hydroxyvitamin D_2, and 0.72 and 1.14 for $24,25(OH)_2D_3$. Furthermore, no constant bias was detected for any of the measured metabolites, as the 95 confidence intervals of the intercept included zero. Bland-Altman method comparisons of our method with the methods from RECIPE (Italy) and LIEGE (Belgium) revealed very small mean biases for $25(OH)D_3$ of −0.88, for $25(OH)D_2$ of −2.03, and for $24,25(OH)_2D_3$ of −0.23 nmol/L (P < 0.001), respectively (Fig. 2, D–F). Accuracy was further assessed by analysing 20 samples from the DEQAS program with target values for $25(OH)D_3$, $25(OH)D_2$, $24,25(OH)_2D_3$. The results obtained with our method are consistently within the allowable limits of performance established by DEQAS (Table 3). DEQAS program also provides target values for C3-epi-$25(OH)D_3$ but our method does not separate this epimer.

The recovery of our method ranges between 76 and 84 %, which appears slightly lower than expected. However, the approach that we used for the assessment of recovery differed from previously published reports [4,26]. Recovery was determined by adding internal standards directly to the samples without prior solution in an organic solvent, and before sample extraction and derivatization, ensuring that the ISTD runs through the entire analytical processes. Other groups determine recovery by adding a known amount of analyte just at the derivatization step, which excludes an important part of the procedure [4,29].

In line with a previous study [30], all analytes tested showed good stability for short term sample storage. Additionally, after 56 days, only minor changes occurred which not exceed 15 % (Fig. 3). Longer storage instead resulted in a substantial increase in $24,25(OH)_2D_3$ between 20 and 30 %, while there is only a slightly increase in $25,26(OH)_2D_3$ between 10 and 12 %.

In order to prove the clinical usability of our method, serum concentrations of all vitamin D metabolites were measured in four different serum samples. One of these samples was from a patient with a homozygous R396W mutation in the CYP24A1 gene, which causes a loss of function. This patient showed a markedly low $24,25(OH)_2D_3$ concentration, an abnormal VMR 1 and chronic hypercalcemia of 3.7 mmol/L (Table 4). This pattern is typical for the 24-hydroxylase deficiency [25,31].

The results from the validation protocol reported above demonstrate that the present LC-MS/MS method is capable of measuring four vitamin D metabolites with good sensitivity, selectivity and precision. This conclusion is supported by method comparisons with the 25-OH Vitamin D2/D3 LC-MS/MS ClinMass® Komplettkit from RECIPE and the validated in-house LC-MS/MS method at the University of Liege [4]. Despite a lack of commercial serum controls for $24,25(OH)_2D_3$, we could demonstrate that our method accurately quantifies this metabolite in EQA samples from the DEQAS program. In a recent study from Carter et al. [32], a plot of 55 DEQAS samples based on participants'

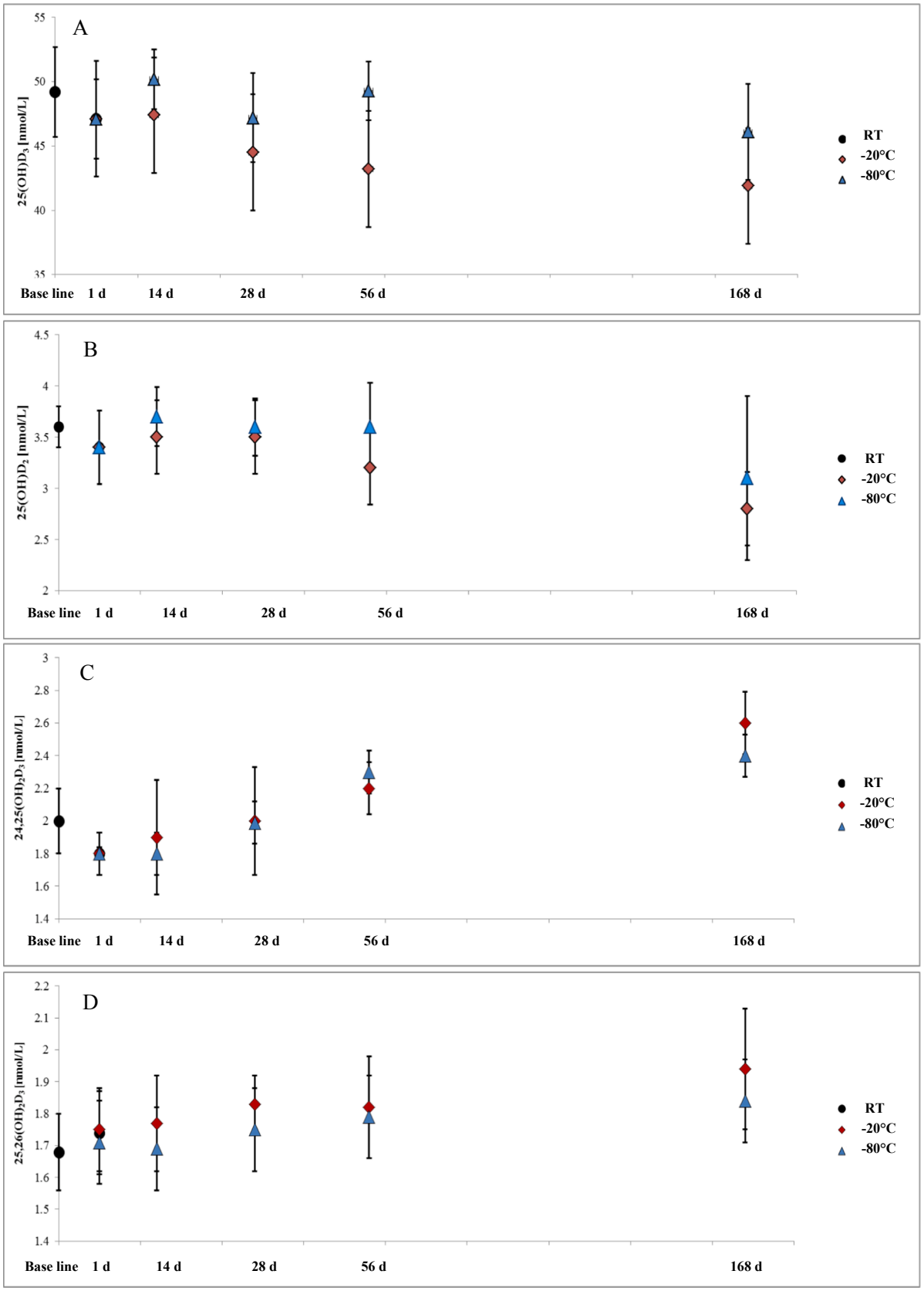

Fig. 3. Storage stability of vitamin D metabolites over 168 days at −20 °C and −80 °C. Stability at room temperature (RT) was assessed for one day only because samples are typically frozen when kept for longer periods. For each data point, 12 aliquots were processed and analyzed in parallel and the mean ± standard deviation (SD) was calculated. (A) $25(OH)D_3$, (B) $25(OH)D_2$, (C) $24,25(OH)_2D_3$, (D) $25,26(OH)_2D_3$.

Table 4
Serum concentrations of 4 vitamin D metabolites, calcium, and the calculated VMRs 1–3* in 4 patients.

Patient N	25(OH)D_3 (nmol/L)	25(OH)D_2 (nmol/L)	24,25(OH)$_2D_3$ (nmol/L)	25,26(OH)$_2$ D_3 (nmol/L)	VMR1 (%)	VMR2 (%)	VMR3	Ca (nmol/L)
1	166	3.3	0.2	4.0	0.1	2.4	20.0	3.7
2	445	0.3	6.5	11.8	1.5	2.7	1.8	2.5
3	182	1.0	11.1	7.5	6.1	4.1	0.7	2.4
4	134	1.8	10.9	8.7	8.1	6.5	0.8	2.4

* VMR1 = 24,25(OH)$_2D_3$/25(OH)D_3/ x 100; VMR2 = 25,26(OH)$_2D_3$/25(OH)D_3/ x 100; VMR3 = 25,26(OH)$_2D_3$/24,25(OH)$_2D_3$.

results for 24,25(OH)$_2D_3$ and NIST assigned values for 25(OH)D_3 demonstrated a correlation of R^2 = 0.896 and an x-intercept of 8 nmol/L. In this study, we have analyzed the results from the 20 DEQAS samples together with the patients from Table 4. 24,25(OH)$_2D_3$ concentrations correlate significantly with 25(OH)D_3 (24,25(OH)$_2D_3$ = −0.789 × 25(OH)D_3 + 0.086; R^2 = 0.851; P < 0.0001). A similar linear relationship is observed for 25,26(OH)$_2D_3$ and 25(OH)D_3 (25,26(OH)$_2D_3$ = −0.758 × 25(OH)D_3 + 0.039; R^2 = 0.661; P < 0.0001). Both correlations are in line with previous studies [21,25].

Our method does not separate C3-epimers. Based on a previous publication from our group C3-epimers contribute significantly to total 25(OH)D concentrations [5] and their clinical relevance is still a matter of debate. In addition, C3-epimers co-elute with the 25(OH)D. If C3-epi-25(OH)D is added to the target value, the mean bias is significantly higher (3.9 %) compared to our 25(OH)D values (2.2 %).

In recent years, the parallel measurement of 25(OH)D and additional vitamin D metabolites has gained growing interest [33]. 24,25(OH)$_2$D, for example, is the primary metabolite of 25(OH)D and can be considered a functional marker of vitamin D metabolism that is only produced when cells are supplied with sufficient amounts of the precursor 25(OH)D [11,24]. Under physiological conditions the 24,25(OH)$_2$D concentration ranges at approximately 10 % of the 25(OH)D. When considered in conjunction with 25(OH)D and other vitamin D metabolites, 24,25(OH)$_2$D adds valuable information beyond analysis 25(OH)D only. It is a helpful marker for the identification of patients with functional vitamin D deficiency [13,24]. However, its utility for guiding vitamin D supplementation therapy is a matter of ongoing debate [34,35]. Furthermore, individuals with *CYP24A1* deficiency typically have a very low VMR, which indicates their inability to catabolize 25(OH)D and 1,25(OH)$_2$D [36]; Amongst the patient samples tested in the present study, the one with 24-hydroxylase deficiency exhibited an very low 24,25(OH)$_2D_3$ concentration (0.2 nmol/L) and a VMR 1 of 0.1 %. In comparison, Fabregat-Cabello et al reported VMR 1 ranges in healthy individuals between 6.8 and 8.2 %. In contrast, individuals with chronic kidney disease and hemodialyzed patients have markedly lower VMR 1 ratios ranging between 1.44 and 2.34 and 0.52–0.74 %, respectively [4]. Typically, patients with *CYP24A1* deficiency exhibit calcium levels in the upper normal range or higher combined with inappropriately high levels of 25(OH)D and 1,25(OH)$_2$D, whereas parathyroid hormone is low or even suppressed [16,31]. Our patient showed a similar pattern. In addition, the VMR can help to distinguish *CYP24A1* deficiencies from idiopathic infantile hypercalcemia type II, which is caused by a mutation of the SLC34A1 gene and a defective sodium-dependent phosphate transporter 2A [16,25,37].

In the chromatogram shown in Fig. 1, we observed an additional peak next to that of 24,25(OH)$_2D_3$. Speculations that this peak might represent 25,26(OH)$_2D_3$ were confirmed by synthesizing this dihydroxylated metabolite and analyzing it with our method. When compared to non-mutated subjects, our 24-hydroxylase deficient patient exhibited a markedly lower 24,25(OH)$_2D_3$ serum concentration. However, quantitating the exact 24,25(OH)$_2D_3$ concentration, free from contamination of 25,26(OH)$_2D_3$, changed VMR 1 from 0.025 to 0.001. This observation confirms results by Kaufmann et al. in a series of patients with different types of 24-hydroxylase deficiency [25]. Based on these results it appears that the clinical utility of VMR 1 improves when 24,25(OH)$_2D_3$ is measured without contamination from 25,26(OH)$_2D_3$. Furthermore, the separation of 24,25(OH)$_2D_3$ and 25,26(OH)$_2D_3$ allows the calculation of VMR 3, which also seems to be highly discriminative between patients and healthy controls.

Until today, several multiplex methods for the determination of vitamin D metabolites have been published, e. g. Kassim et al; Satoh et al or Oberson et al [38–40], and the already mentioned method from Kaufmann et al [25]. Through the incorporation of a derivatization step we could increase sensitivity and thus reduce sample volume to 50 µL. Previously published methods without derivatization require substantially larger sample volumes between 200 and 500 µL, which limits a wider use in clinical practice [38,41]. In addition, the derivatization step also improves chromatographic separation of the two dihydroxylated metabolite 24,25(OH)$_2D_3$ and 25,26(OH)$_2D_3$, which is required for a reliable detection of 24-hydroxylase deficiency.

4. Conclusion

Our newly developed LC-MS/MS method is capable of accurately measuring four different vitamin D metabolites, which are helpful in identifying innate and acquired dysfunctions of vitamin D metabolism. In particular, it allows calculation of the VMR by a single analysis. The analytical and diagnostic performance has been shown to be satisfactory for clinical purposes. Future studies should evaluate if the simultaneous measurement of multiple vitamin D metabolites improves patient management and outcome.

CRediT authorship contribution statement

Sieglinde Zelzer: Methodology, Validation, Investigation, Formal analysis, Writing - original draft. **Andreas Meinitzer:** Methodology, Writing - review & editing, Project administration, Supervision. **Dietmar Enko:** Formal analysis, Writing - review & editing. **Sebastian Simstich:** Data curation, Writing - review & editing. **Caroline Le Goff:** Formal analysis, Data curation. **Etienne Cavalier:** Writing - review & editing. **Markus Herrmann:** Conceptualization, Writing - review & editing, Visualization, Resources. **Walter Goessler:** Writing - review & editing, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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