Analytical Methods

Quantifying vitamin D and its metabolites by LC/Orbitrap MS

Journal:	*Analytical Methods*
Manuscript ID:	AY-TEC-06-2015-001583.R1
Article Type:	Technical Note
Date Submitted by the Author:	06-Aug-2015

Complete List of Authors:	Raml, Reingard; Joanneum Research, HEALTH-Institute for Biomedicine and Health Sciences Ratzer, Maria; Joanneum Research, HEALTH-Institute for Biomedicine and Health Sciences Obermayer-Pietsch, Barbara; Medical University of Graz, Department of Internal Medicine, Division of Endocrinology and Metabolism Mautner, Anton; Joanneum Research, HEALTH-Institute for Biomedicine and Health Sciences Pieber, Thomas; Medical University of Graz, Department of Internal Medicine, Division of Endocrinology and Metabolism Sinner, Frank; Joanneum Research, HEALTH-Institute for Biomedicine and Health Sciences Magnes, Christoph; Joanneum Research, HEALTH-Institute for Biomedicine and Health Sciences

Analytical Methods

RSCPublishing

TECHNICAL NOTE

Quantifying vitamin D and its metabolites by LC/Orbitrap MS

Cite this: DOI: 10.1039/x0xx00000x

Reingard Raml[a], Maria Ratzer[a], Barbara Obermayer-Pietsch[b], Anton Mautner[a], Thomas R. Pieber[a,b], Frank M. Sinner[a,b] , Christoph Magnes[a*]

Received 00th June 2015,
Accepted 00th June 2015

DOI: 10.1039/x0xx00000x

www.rsc.org/

Establishing accurate und precise analytical methods for vitamin D species is of rising importance as awareness of the vitamin D deficiency problem grows. Here, we report an analytical method for quantifying vitamin D and relevant vitamin D metabolites by high-resolution mass spectrometry (HRMS). We combine a straightforward time saving sample preparation and chromatographic separation, employing gradients and column-switching technique, with high-resolution MS detection, extending its applicability to a broader range of relevant vitamin D metabolites. We calibrated the method to NIST standards and validated it for 25(OH)D_2 and 25(OH)D_3. We also present short- and long-term sample-stability data and proof-of-concept by applying the method to serum samples from a healthy patient library that were previously measured by immunoassay. Compared to the immunoassay data, our method showed good correlation of 25(OH)D_3 concentrations. Thus, we show that quantitative HRMS is now within reach of clinical diagnostics for vitamin D and its metabolites.

1. Introduction

It is currently estimated that almost 50% of the global population is affected either by vitamin D deficiency or even insufficiency, conditions that are linked to skeletal and organ problems, as well as cardiovascular diseases, diabetes, autoimmunity, cancer and increased mortality.[1–6]

Vitamin D supplements contain mostly vitamin D_3 and vitamin D_2, whereas the clinical indicator of vitamin D status is the total 25(OH)D concentration, [7] which is represented by the sum of the metabolites 25-hydroxyvitamin D2 [25(OH)D_2] and 25-hydroxyvitamin D3 [25(OH)D_3]. There are many analytical methods for the determination of 25(OH)D; nonetheless, the reliability and selectivity of the various methods is still a topic of lively discussions. [8–11]

Current methods for 25(OH)D determination are largely based on immunoassays or liquid chromatography in combination with mass spectrometry (LCMS). Immunoassays are favoured in clinical settings because they are inexpensive, technically simple, and allow high throughput, but the method can suffer from reduced accuracy and reliability. On the other hand, LCMS methods are increasingly being used (see review[12] and references therein) because they allow direct, specific measurement of vitamin D as well as the D_2 and D_3 metabolites. While LCMS methods do have some disadvantages, including the need for expensive equipment and specialised staff, lower sample throughput and possible interferences from 3-epimers of 25(OH)D, the methods are increasingly required for cross-validation of immunoassay results.

In addition to 25(OH)D, a number of further vitamin D metabolites are of potential importance for disease-specific diagnosis; including 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], which assists healing in tissues and bones,[13] while epimers of the different vitamin D metabolites may be involved in the selective hormonal regulation of various biological functions.[14–16] Thus, a method capable of quantifying more relevant vitamin D metabolites would be of great clinical benefit. Numerous methods were reported that used tandem MS/MS for the determination of vitamin D species.[12] Only three methods used high resolution mass spectrometry.[17–19] Bruce et al[17] compared MS/MS with HRMS for the quantification of 25(OH)D_2, 25(OH)D_3 and its epi-25(OH)D_3 and found slightly lower LLOQ when HRMS was applied. In a very recent publication[19] epi-25(OH)D_2 was also included. In a third report[18] an enzymatic derivatization was used in addition to high resolution mass spectrometry to increase the sensitivity of 25(OH)D_2 and 25-(OH)D_3 in serum.

Here, we report the application of HRMS for the determination of 25(OH)D_2, 25(OH)D_3, epi-25(OH)D_3, their precursors vitamin D_2 and D_3 as well as another relevant metabolite 24,25 $(OH)_2D_3$. We have validated the method with emphasis on 25(OH)D_2 and 25(OH)D_3 and also provide short-term and long-term stability data.

To test our method on human serum samples, we carried out a proof-of-concept study: serum samples from a clinical trial were analysed by our method and immunoassay in parallel to compare the performance of the two methods.

2. Experimental

2.1 Chemicals and reagents

Reference standards for 25(OH)D_2 (25-hydroxyergocalciferol; 97.2% purity) and 25(OH)D_3 (25-hydroxycholecalciferol; 98.5% purity) were obtained from Cerilliant Corporation and were used for evaluation and recalculation of Sigma Aldrich standards via flow injection analysis (FIA)-HRMS measurements. Stock solutions for calibration and validation standards for 25(OH)D_2 (≥98% purity), 25(OH)D_3 (≥98% purity), 24,25$(OH)_2D_3$ (24,25-dihydroxycholecalciferol; ≥99% purity), vitamin D_2 (ergocalciferol, 99,9% purity) and vitamin D_3 (cholecalciferol, 99,9% purity) were purchased from Sigma Aldrich (St Louis, USA) and were prepared in ethanol (p.a.). [$^2H_6$]25(OH)D_2 (26,26,26,27,27,27-hexadeutero-25-hydroxyergocalciferol) and [$^2H_6$]25(OH)D_3 (26,26,26,27,27,27-hexadeutero-25-hydroxycholecalciferol) were purchased from Medical Isotopes (Pelham, USA) and used as internal standards (ISTDs). Serum-based 25(OH)D reference standards were purchased from Chromsystems (Munich, Germany; MassCheck® Serum Controls level I and II of the MassChrom® reagent kit for LC-MS/MS), from RECIPE (Munich, Germany; ClinCal® Serum Calibrators level 0 – 3) and from NIST (Gaithersburg, USA; Standard Reference Material 972 level 1 – 4). SRM 972 was used as an additional quality assurance tool for separation of 3-epi-25(OH)D_3. A serum matrix was obtained by collecting blood from 5 subjects in Vacuette® serum tubes (Greiner Bio-One, Kremsmünster, Austria). The blood was allowed to stand for 30 min, before being centrifuged at 2000 g for 15 min. The supernatant was pooled and stored at -75°C. Methanol and water used for LC-HRMS measurements were chromasolv grade, while sodium carbonate and *tert*-butyl methyl ether (TBME) were reagent grade and purchased from Sigma Aldrich.

2.2 Preparation of standard solutions

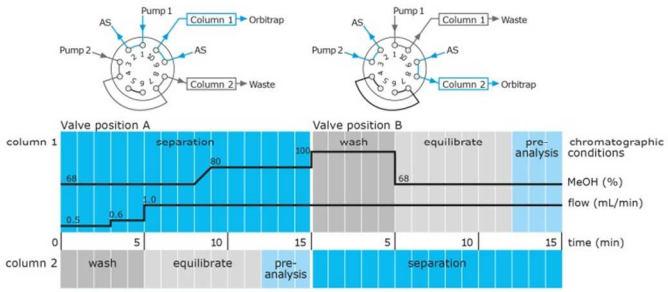
All stock solutions were prepared in ethanol and stored in amber glass vials at -75°C. Concentrations of Sigma stock solutions were verified against Cerilliant reference standard, which is calibrated to NIST standard reference material Lot 2972. Thus, 25(OH)D_2 and 25(OH)D_3 solutions (both 200 ng/mL) were prepared three times with Sigma stock solutions and three times with Cerilliant stock solution each. ISTD (final conc. 200 ng/mL) was added to all solutions. Each solution was analyzed six times via FIA-HRMS using a 50 µL injection loop. The area ratio of the samples prepared with Sigma stock was compared to the Cerilliant reference standard samples, and the concentration of the Sigma stock solutions were then corrected accordingly.
Final concentrations of the calibration standards ranging from 2.41 to 195.15 ng/mL for 25(OH)D_2 and 2.35 to 191.49 ng/mL for 25(OH)D_3 were prepared by serial 1:3 dilution steps in ethanol prior to use. A zero sample (ethanol with internal standard) and a blank sample (ethanol without internal standard) were also prepared to monitor the blank signal in the LC-HRMS system. Quality control standards (two levels) were prepared by spiking the pooled serum matrix with specific concentrations of vitamin D metabolites using Protein LoBind tubes (Eppendorf, Austria). These quality controls were included in each batch to test the robustness and accuracy of the method.

2.3 Sample preparation

Serum sample or standard (200 µL) was spiked with ISTD (20 µL) in Protein LoBind tubes (final internal standard concentration 100 ng/mL). After equilibration at 2 - 8°C for 25 min, sodium carbonate buffer (40 µL, 0.25 M, aqueous, pH 9.0) was added as described by Tai et al.[20] Proteins were precipitated and vitamin D metabolites were extracted by addition of TBME (1mL) and by spinning the sample on a vortex mixer for 10 min at maximum speed followed by centrifugation at 10,000 g. The supernatant was transferred into a 1.5 mL Protein LoBind tube and evaporated to dryness by using a mini-speed vac (VWR, Vienna, Austria). The residue was reconstituted with ethanol (75 µL) and transferred into a 0.3 mL autosampling vial containing water (75 µL).

2.4 LC-HRMS conditions

All experiments were carried out on an Ultimate 3000 System (Dionex) comprising an autosampler with cooled tray and a column oven with switching unit coupled to a linear trap quadrupole or Exactive Orbitrap system (Thermo Fisher Scientific). The system was controlled by Xcalibur Software 2.1.0. The separation of the vitamin D metabolites was based on the method of Tai et al.[20] with some adaptations: Briefly, the chromatography was carried out on a Zorbax SB-CN column (4.6 mm x 250 mm, 5 µm particle diameter; Agilent Technologies) using a multi-step flow and solvent gradient. The oven temperature was set to 60°C. The injection volume was 50 µL. The start conditions were 68% MeOH at 1 mL/min. The flow was reduced to 0.5 mL/min from 6 to 9 min and set to 0.6 mL/min from 9 to 15 min. The flow and MeOH content were then increased to 1 mL/min and 80%, respectively, from 15 to 20 min, followed by a wash (100% MeOH from 20 to 25 min) and equilibration step (68% MeOH from 25 to 29 min) at 1 mL/min. The final chromatographic setup with the two-column switching approach is illustrated in Scheme 1.



Scheme 1. HPLC setup of the two-column-switching technique.

Positive atmospheric pressure chemical ionization (APCI)-HRMS was performed with the following parameters: APCI vaporizer temperature 350°C, capillary temperature 200°C, sheath gas pressure 30 arbitrary units (AU), auxiliary gas pressure 15 AU. Resolution was set to 60,000. The mass range for acquisition was limited to 300 – 500 m/z. Detection was performed by selecting accurate monoisotopic masses and the corresponding ion generated by the loss of water for 25(OH)D_2 (m/z 413.3414 and 395.3308), [$^2H_6$]25(OH)D_2 (m/z 419.3791 and 401.3685), 25(OH)D_3 (m/z 401.3414 and 383.3308), [$^2H_6$]25(OH)D_3 (m/z 407.3791 and 389.3685), 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ (m/z 417.3363 and 399.3257), vitamin D_2 (m/z 397.3465 and 379.3359) and vitamin D_3 (m/z 385.3465 and 367.3359) within a mass range of 6 ppm.

2.5 Validation of the method

Validation of the method was performed focusing on accuracy, precision, and stability. Four different validation standard levels, ranging from serum blank concentration to 100 ng/mL, were prepared by spiking the pooled serum matrix with specific concentrations of vitamin D metabolites using Protein LoBind tubes (Table 1). The spiked concentrations of the validation standards were

corrected for the endogenous concentration of the pooled serum matrix. For calculation of accuracy and intra-batch precision, the validation standards were extracted and analysed five times by using double injections. For calculation of inter-batch precision, the standards were extracted and analysed in three different batches by double injection. To evaluate the short-term stability of the vitamin D metabolites, two standard levels (QCa and QCc, 3 replicates, see Table 2) were prepared in serum. Each level was stored once in serum and once after extraction overnight at 4°C and at -75°C (incl. three freeze-thaw cycles). For long-term stability evaluation, these QCa and QCc samples (3 replicates) were stored once in serum and once after extraction over six months at -75°C. Stability samples were analysed using double injection. The area ratio of each level was compared to freshly prepared samples.

Table 1. LC-HRMS method validation results for accuracy and precision of $25(OH)D_2$ and $25(OH)D_3$.

Validation Standard	Expected Conc. [ng/mL]	Detected mean [ng/mL]	Accuracy[a] [%]	Intra-batch precision[a] [%]	Inter-batch precision[b] [%]
$25(OH)D_2$					
unspiked		0.7[c]	-	-	-
1	5.58	5.84[a]	105	3.6	3.5
2	49.49	50.94[a]	103	1.0	1.7
3	98.27	97.60[a]	99	2.0	2.7
$25(OH)D_3$					
unspiked		15.59[c]	-	-	-
1	20.34	21.02[a]	103	2.1	2.1
2	63.09	63.51[a]	101	1.4	3.0
3	110.58	107.48[a]	97	2.1	2.9

[a] mean of n=5, double injections
[b] mean of n=3, double injection
[c] mean of n=6, double injection

Table 2. Accuracy of $25(OH)D_2$ and $25(OH)D_3$ analysis by LC-HRMS of different serum control standards.

	$25(OH)D_2$			$25(OH)D_3$		
Serum control standard	Certified conc. [ng/mL]	Detected mean[a] [ng/mL]	Accuracy[a] [%]	Certified conc. [ng/mL]	Detected mean[a] [ng/mL]	Accuracy[a] [%]
NIST SRM 972						
Level 1	0.6±0.2[b]	-	-	23.9±0.8	24.31	102
Level 2	1.71±0.08[b]	0.90	53	12.3±0.6	11.52	94
Level 3	26.4 ±2.0	26.67	101	18.5±1.1	19.40	105
Level 4	2.4±0.21	2.10	87	33.0±0.8	33.96	103
ChromSystem MassCheck® Serum Control						
Level I	17.4±3.5	18.78	108	16.7±3.5	16.80	101
Level II	38.7±7.7	41.98	108	38.4±7.7	39.76	104
Recipe ClinCal®Serum Calibrators						
Level 0	4.19	3.50	83	0.316[b]	0.01	4
Level 1	7.61	6.81	89	9.64	9.02	94
Level 2	24.00	24.27	101	26.00	26.06	100
Level 3	62.9	70.08	111	71.40	71.44	100

[A] mean of n=3, double injections
[B] conc. below LLOQ

2.6 Human serum samples

A patient library of 30 representative human serum samples from apparently healthy persons was supplied by IDS. The 30 samples, previously analysed by using the IDS-iSYS 25-hydroxy vitamin D immunoassay (IDS), were analysed using our LC/HRMS method in order to compare our method with the immunoassay method.

3. Results and discussion

3.1 Method development

The method has a simplified sample preparation and chromatographic separation compared to the method of Tai et al[20] and we have extended it to other substances relevant to vitamin D deficiency.

3.1.1 Sample preparation

Our samples were prepared with a single liquid-liquid extraction by using tert-butyl methyl ether, which shows excellent extraction efficiency [21] and high volatility for fast evaporation. The extraction volume was thereby reduced from 16 to only 1 mL, which allowed us to use lower-cost consumables such as 1.5 mL plastic tubes, and also dramatically shortened the evaporation time. The use of Protein LoBind tubes for the whole sample preparation process was crucial for preparing extracts with no significant interferences at the lower limit of quantification (LLOQ) because it avoids adsorption of analytes onto the tube surface.

3.1.2 Liquid chromatography

The goals of optimizing the LC conditions were to (1) keep the high resolving power of the method of Tai et al. [20] and (2) to extend the method to the separation of other relevant substances such as di-hydroxyvitamin D and vitamin D, and (3) simultaneously achieve acceptable run times. Our method, combining flow and MeOH gradient, allowed us to separate vitamin D_3-epimers with the same monoisotopic mass (m/z 401.3414: $25(OH)D_3$ and 3-epi-$25(OH)D_3$) in half the time of the initial method (see Figure 1). Briefly, a flow rate of 1 mL/min and 68% MeOH were applied as starting conditions at sample injection. The flow rate was then reduced to 0.5 mL/min for the separation of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ and set to 0.6 mL/min for the separation of $25(OH)D_2$ and $25(OH)D_3$. For the elution of vitamins D_2 and D_3 the flow rate and the MeOH content were increased to 1 mL/min and 80%, respectively, followed by a wash and equilibration step.

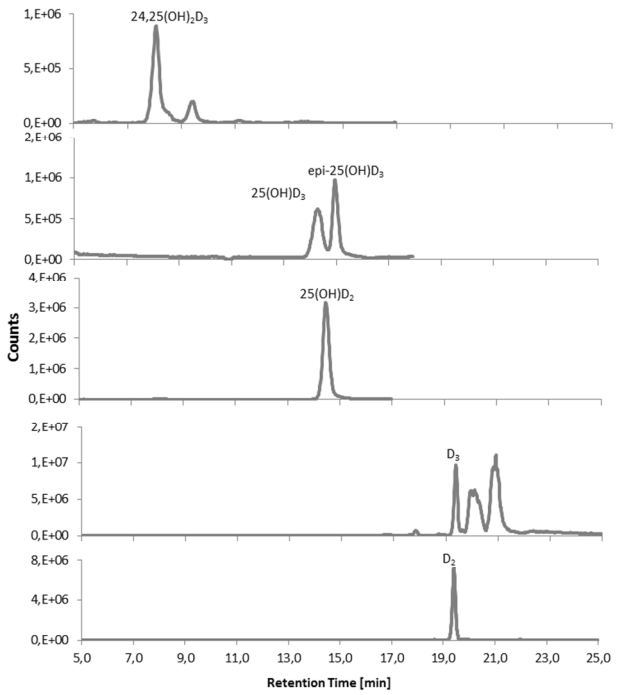


Figure 1. Chromatogram of a serum sample spiked with 24,25 $(OH)_2D_3$, $25(OH)D_3$, epi-$25(OH)D_3$, $25(OH)D_2$, D_3 and D_2.

In order to efficiently utilize the time taken by column cleaning and equilibration, we adopted a two-column-switching approach (Scheme 1). Briefly, during analysis of one sample on column 1 the second column is cleaned and equilibrated. The chromatographic process can be therefore divided into two equal parts – the analytical separation part on column 1 and the cleaning, equilibration and pre-separation part on column 2. By using this approach the run time was reduced to only 15 min while maintaining excellent resolution of all interfering metabolites of vitamin D.

3.1.3 High resolution mass spectrometry

The final part of method development comprised the mass spectrometric detection, where HRMS was applied to vitamin D analysis. The high resolution (up to 1:100,000) enabled selective detection of compounds with the same nominal molecular mass but different elemental composition without the need for fragmentation. 25(OH)D, in particular, is known to undergo multiple fragmentation in the collision cell, with only a few percent of the 25(OH)D ion current contributing to the MS/MS transition, leading to drastic sensitivity loss. [21]

The ion-trap design of the Orbitrap system allows for detection in full-scan mode at the same sensitivity as in single-ion monitoring mode. Acquisition of full scan spectra enables, therefore, the simultaneous detection of all ionized adducts of 25(OH)D, such as $[M+H]^+$, $[M+Na]^+$, $[M-H_2O+H]^+$, etc. As shown in Figure 2, dominant $[M-H_2O+H]^+$ in addition to the $[M+H]^+$ of $25(OH)D_3$ and the corresponding isotopic labelled ISTD were present in the acquired spectra. Both adducts were selected and combined for quantification purposes to maximize sensitivity.

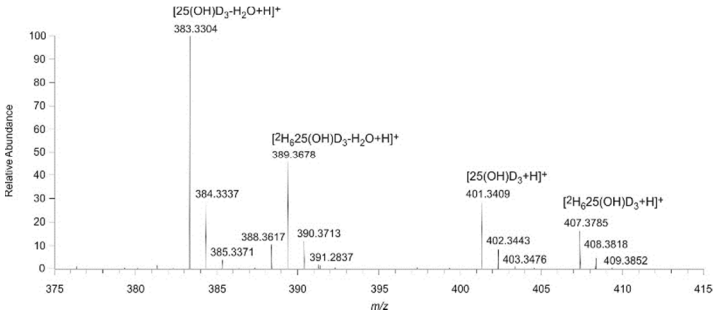


Figure 2. HRMS spectra of $25(OH)D_3$ and the internal standard $[^2H_6]25(OH)D_3$.

The spectra were monitored in full scan mode (m/z 300-500) at a resolution of 60,000, which resulted in 30-80 spectra per peak, which was clearly sufficient for 25(OH)D quantification. Another advantage of HRMS is that $[M-H2O+H]^+$ of the internal standard 25(OH)D2-d6 at m/z 401.3685 is completely separated from $[M+H]^+$ of 25(OH)D3 at m/z=401.3414 and no isobaric interference occurs. In addition, the full-scan mode in combination with accurate mass data with deviations below 2 ppm enabled the quantification of target substances together with post-acquisition data-mining. As all of the information is present in the acquired spectrum, posterior screening for other vitamin D metabolites of interest and semi-quantitative post-analysis of additional substances was possible without the need for further analyses.

3.2 Method validation

3.2.1 Accuracy and precision

The $25(OH)D_2$ and $25(OH)D_3$ analysis was validated with special emphasis on accuracy, precision, and sample stability. Isotope-labelled ISTDs were used to achieve best quantification performance. Calibration was accomplished by means of 5-point calibration curves in the same range as other common 25(OH)D assays of approx. 2.4 to 200 ng/mL for $25(OH)D_2$ and $25(OH)D_3$, whereas a linear response was achieved for both compounds with $r^2 \geq 0.999$. The LLOQ was set to the lowest calibration point and the limit of detection (LOD, signal to noise ratio of 3) was estimated for $25(OH)D_2$ at 0.1 ng/mL in serum due to a signal to noise ratio >150 at 5 ng/mL in spiked serum. The corresponding LOD value for $25(OH)D_3$ could not be determined as no $25(OH)D_3$ free serum sample was available. The S/N ratio of the unspiked 25(OH) D3 QC level (15.59 ng/mL, Table 1) was >50. Therefore, an LOD of 0.9 ng/mL can be estimated. The validation results for accuracy and precision for both analytes, as summarized in Table 1, show the potential of LC-HRMS using Orbitrap technology for quantification. The accuracy was between 97-105% and the precision between 1.0 and 3.6% for the two compounds. These results are comparable to published triple quadrupole LC-MS/MS methods.[21–23]

3.2.2 Sample stability

The stability of $25(OH)D_2$ and $25(OH)D_3$ was evaluated in serum samples as well as in the extracted form. Excellent short-term stability was observed for $25(OH)D_2$ and $25(OH)D_3$ in serum, with recoveries between 97-101% and 102-104% at 4°C, and 96-102% and 102-103% at -75°C, respectively, including multiple freeze-thaw cycles. This is in confirmation with earlier reported results[24]. Comparable results were

obtained in the extracted form for 25(OH)D_2 and 25(OH)D_3; 97-105% and 96-97% at 4°C, and 99-100% and 97-99% at -75°C, respectively. Furthermore, long-term stability was observed for 25(OH)D_2 and 25(OH)D_3 in serum, with recoveries between 93-100% at -75°C after 6 months. Excellent results were obtained in the extracted form for 25(OH)D_2 and 25(OH)D_3; 100-102% and 96-103% at -75°C, respectively, after 6 months.

3.2.3 Validation of quantification of other vitamin D metabolites

Since there are no corresponding isotopically labelled ISTDs in the sample, the quantification properties of the method for untargeted vitamin D compounds was cross checked by spiking. All 25(OH)D validation standards were additionally spiked with 24,25$(OH)_2D_3$, vitamin D_2 and vitamin D_3 in the same concentration range as described for 25(OH)D_2 and 25(OH)D_3. [$^2H_6$]25(OH)D_3 was used for their quantification. The results of this semi-quantitative investigation for the accuracy and precision are shown in Table S1 in the Supplementary Information. Here, accuracy and precision lay in a wider range of 83 to 115% and 3.4 to 22.1%, respectively for all compounds. The short term stability of 24,25$(OH)_2D_3$, vitamin D_2 and vitamin D_3 was found to be similar to that of 25(OH)D; i.e., are stable at 4°C overnight and for three freeze-thaw cycles at -75°C. In serum as well as in the extracts, all additional vitamin D compounds were stable at 4°C, with accuracies of 93 – 113% (serum) and 75-119% (extracts), and at 75°C 94-103% (serum) and 86-121% (extracts).

3.2.4 Performance of the 25(OH)D analysis

The performance of our LC-HRMS method was tested by analysing various serum-based control standards, including SRM 972 (NIST), MassCheck® Serum Control (ChromSystems) and ClinCal® Serum Calibrator (RECIPE). The results as shown in Table 3 correspond well with the certified concentrations. Accuracies for NIST Standards ranging from 94 - 105% for 25(OH)D_3 and 101% for 25(OH)D_2 for the level above LLOQ (level 3). For the commercially available serum control standards (ChromSystems, RECIPE) the detected levels fit to the expected nominal concentrations.

3.2.5 Vitamin D in human serum samples

We applied our method to the serum samples of 30 healthy subjects from a patient library supplied by IDS. Vitamin 25(OH)D levels of the samples were previously measured by using the iSYS immunoassay (IA). Data from the two methods are plotted in Figure 3, showing good correlation.

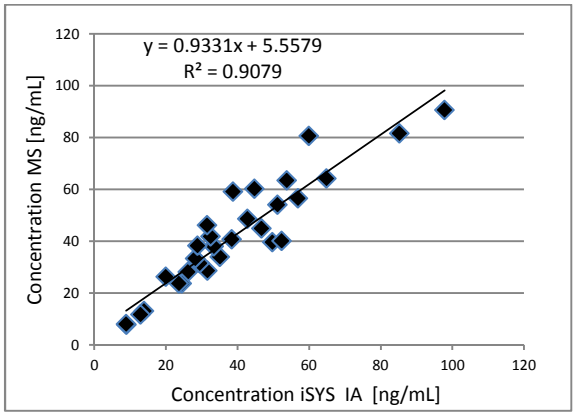


Figure 3. Comparison of iSYS (IDS) and LC-MS data for 30 individual measurements of 25(OH)D in serum samples. LC-MS data are plotted as the sum of the 25(OH)D_2 and 25(OH)D_3 concentrations.

4. Conclusions

We have presented a new method to quantify vitamin D and relevant vitamin D metabolites using LC/HRMS. The method shows excellent precision and accuracy and it clearly shows the potential of HRMS for accurate and precise quantification of vitamin D and its metabolites. Moreover, the simplicity and accessibility of the method render it potentially applicable for use in routine clinical analysis.

Acknowledgements

This work was supported by BioPersMed (COMET K-project 825329), which is funded by the Federal Ministry of Transport, Innovation and Technology (BMVIT) and the Federal Ministry of Economics and Labour/the Federal Ministry of Economy, Family and Youth (BMWA/BMWFJ) and the Styrian Business Promotion Agency (SFG).
We thank IDS for providing the patient library and Alison Green for help with writing the manuscript.

Notes and references

[a] Joanneum Research, HEALTH – Institute for Biomedicine and Health Sciences, Neue Stiftingtalstrasse 2, 8010 Graz, Austria.
[b] Medical University of Graz, Department of Internal Medicine, Division of Endocrinology and Metabolism, Auenbruggerplatz 15, 8036 Graz, Austria

1 M. F. Holick, *Mayo Clin. Proc.*, 2006, **81**, 353–73.

2 E. Hyppönen, E. Läärä, A. Reunanen, M. R. Järvelin and S. M. Virtanen, *Lancet*, 2001, **358**, 1500–3.

3 E. Van Etten, B. Decallonne, L. Verlinden, A. Verstuyf, R. Bouillon and C. Mathieu, *J. Cell. Biochem.*, 2003, **88**, 223–6.

4 E. Giovannucci, Y. Liu, E. B. Rimm, B. W. Hollis, C. S. Fuchs, M. J. Stampfer and W. C. Willett, *J. Natl. Cancer Inst.*, 2006, **98**, 451–9.

5 P. Tuohimaa, *Nutr. Rev.*, 2008, **66**, S147–52.

6 M. Pfeifer, B. Begerow, H. W. Minne, D. Nachtigall and C. Hansen, *J. Clin. Endocrinol. Metab.*, 2001, **86**, 1633–7.

7 K. M. Seamans and K. D. Cashman, *Am. J. Clin. Nutr.*, 2009, **89**, 1997S–2008S.

8 A. Prentice, *Nutr. Rev.*, 2008, **66**, S153–64.

9 N. Binkley, D. Krueger, C. S. Cowgill, L. Plum, E. Lake, K. E. Hansen, H. F. DeLuca and M. K. Drezner, *J. Clin. Endocrinol. Metab.*, 2004, **89**, 3152–3157.

10 G. D. Carter, *Clin. Chem.*, 2009, **55**, 1300–1302.

11 G. D. Carter, *Curr. Drug Targets*, 2011, **12**, 19–28.

12 J. M. W. van den Ouweland, M. Vogeser and S. Bächer, *Rev. Endocr. Metab. Disord.*, 2013, **14**, 159–84.

13 E.-G. Seo, *Endocrinology*, 1997, **138**, 3864–3872.

14 M. Kamao, S. Tatematsu, S. Hatakeyama, T. Sakaki, N. Sawada, K. Inouye, K. Ozono, N. Kubodera, G. S. Reddy and T. Okano, *J. Biol. Chem.*, 2004, **279**, 15897–907.

15 R. J. Singh, R. L. Taylor, G. S. Reddy and S. K. G. Grebe, *J. Clin. Endocrinol. Metab.*, 2006, **91**, 3055–61.

16 H. C. M. Stepman, A. Vanderroost, D. Stöckl and L. M. Thienpont, *Clin. Chem. Lab. Med.*, 2011, **49**, 253–6.

17 S. J. Bruce, B. Rochat, A. Béguin, B. Pesse, I. Guessous, O. Boulat and H. Henry, *Rapid Commun. Mass Spectrom.*, 2013, **27**, 200–6.

18 J. Abdel-Khalik, P. J. Crick, G. D. Carter, H. L. Makin, Y. Wang and W. J. Griffiths, *Biochem. Biophys. Res. Commun.*, 2014, **446**, 745–750.

19 G. Liebisch and S. Matysik, *J. Lipid Res.* , 2015, **56** , 1234–1239.

20 S. S.-C. Tai, M. Bedner and K. W. Phinney, *Anal. Chem.*, 2010, **82**, 1942–8.

21 P. A. Aronov, L. M. Hall, K. Dettmer, C. B. Stephensen and B. D. Hammock, *Anal. Bioanal. Chem.*, 2008, **391**, 1917–1930.

22 X. Duan, B. Weinstock-Guttman, H. Wang, E. Bang, J. Li, M. Ramanathan and J. Qu, *Anal. Chem.*, 2010, **82**, 2488–97.

23 Z. Maunsell, D. J. Wright and S. J. Rainbow, *Clin. Chem.*, 2005, **51**, 1683–90.

24 B. W. Hollis, *Am. J. Clin. Nutr.*, 2008, **88**, 507S.

25 I. H. de Boer, M. C. Sachs, P. a Cleary, A. N. Hoofnagle, J. M. Lachin, M. E. Molitch, M. W. Steffes, W. Sun, B. Zinman and J. D. Brunzell, *J. Clin. Endocrinol. Metab.*, 2012, **97**, 4780–8.

26 I. H. de Boer, M. C. Sachs, M. Chonchol, J. Himmelfarb, A. N. Hoofnagle, J. H. Ix, R. a. Kremsdorf, Y. S. Lin, R. Mehrotra, C. Robinson-Cohen, D. S. Siscovick, M. W. Steffes, K. E. Thummel, R. P. Tracy, Z. Wang and B. Kestenbaum, *Am. J. Kidney Dis.*, 2014, **64**, 187–197.

Table S1. LC-HRMS method validation results for accuracy and precision of $24,25(OH)_2D_3$, vitamin D_2 and vitamin D_3 (N.D. = not detected)

Validation standard	Expected Conc. [ng/mL]	Detected mean [ng/mL]	Accuracy [%]	Intra-batch precision [%]	Inter-batch precision [%]
$24,25(OH)_2D_3$					
0	0.0	n.d.			
1	5.0	3.63[a] 4.71[b]	73	15.4	22.1
2	50.0	47.15[a] 52.47[b]	94	3.9	9.9
3	100.0	92.20[a] 103.13[b]	92	3.3	9.5
Vitamin D_2					
0	0.0	n.d			
1	7.69	8.62[a] 8.41[b]	112	4.8	7.1
2	52.69	54.14[a] 56.18[b]	103	4.1	5.8
3	102.69	101.49[a] 111.41[b]	99	3.6	6.0
Vitamin D_3					
0	0.0	n.d			
1	23.01	25.91[a] 24.34[b]	113	4.9	10.2
2	68.01	78.44[a] 80.97[b]	115	3.8	3.4
3	118.01	126.40[a] 138.51[b]	107	3.9	5.9

[a] mean of n=5, double injection
[b] mean of n=3, double injection

We developed a HPLC-HRMS method for the determination of 25(OH)D_2, 25(OH)D_3, epi-25(OH)D_3, vitamin D_2 and D_3 as well as 24,25 $(OH)_2D_3$.