NEW METHODS FOR IMPROVED DETERMINATION OF VITAMIN D METABOLIC MARKERS USING ADVANCED ANALYTICAL MASS SPECTROMETRY TECHNIQUES

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„Inmitten der Schwierigkeiten liegt die Möglichkeit“

Albert Einstein

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I. ZUSAMMENFASSUNG

Das Ziel der vorliegenden Arbeit war die Entwicklung neuer Methoden zur besseren Bestimmung von Vitamin D unter der Verwendung der analytischen Massenspektrometrie. Als Schwerpunkt wurden neue Derivatisierungs/und Trenntechniken entwickelt und deren Nutzen im Vergleich zu etablierten Vitamin D Messmethoden gezeigt. Vitamin D spielt eine Schlüsselrolle in verschiedenen Krankheitsbildern.

Die erste Methodenentwicklung basiert auf der quantitativen Bestimmung von sechs Vitamin D Metaboliten in einer Kohorte von 30 Patienten mit chronischer Lebererkrankung. Die Methodik beinhaltet einen neuartigen Derivatisierungsansatz, gefolgt von einer komplexen HPLC-MS/MS-Trennung und anschließender Quantifizierung der Vitamin D Metaboliten.

Zur gleichzeitigen Quantifizierung von 3α- und 3β-25(OH) -Vitamin D_3 aus getrockneten Bluttropfen wurde eine neue LC-MS/MS Methode entwickelt. Die Herstellung einer neuen künstlichen Vitamin D-freien Vollblut-Matrix aus gepoolten Spenderproben wurde erfolgreich realisiert, welche zur Imitierung der reellen Blutmatrix-Bedingungen für die Kalibrierung genutzt wurde.

Im nächsten Schritt wurde ein spezialisiertes Derivatisierungsreagenz selbst entwickelt, das der Bestimmung von Vitamin D in wässriger Reaktionsumgebung dient. Die Anwendung erfolgte auf verschiedenen wässrigen Zellsystemen unter physiologischen Bedingungen. Dieses neu entwickelte Derivatisierungsreagenz ermöglicht eine spezialisierte Nachweismethode von Vitamin D in wässriger Probenumgebung, ohne eine Veränderung der Probenumgebung selbst und deren Zusammensetzung.

II. ABSTRACT

The aim of the present work was to develop new methods for improved determination of vitamin D metabolic markers using advanced analytical mass spectrometry techniques. New derivatization and separation techniques were used and their benefit on the performance of the new methodology was shown in comparison to established vitamin D determination methods. Vitamin D plays a key role in different kind of diseases.

The first approach focused on simultaneously and quantitatively profiling multiple vitamin D metabolites in a cohort of 30 patients with chronic liver diseases (CLD). The development of this new methodology includes a novel derivatization approach for enhanced analyte response followed by complex HPLC-MS/MS separation and subsequently quantification of target compounds.

The first LC-MS/MS method for simultaneous quantification of 3α- and 3β-25(OH)-vitamin D_3 from dried blood spots (DBS) was established. The preparation of new artificial vitamin D-free whole blood from pooled donor samples was successfully achieved, which was used to mimic blood matrix conditions for calibration.

In the next step, a new specific derivatization reagent was developed, which was used for determination of vitamin D by derivatization in an aqueous reaction environment. The application of this new derivatization reaction was performed with different aqueous cell systems under their physiological conditions. This specialized technique is for investigating vitamin D metabolites in aqueous environments, in particular where the sample integrity cannot be disturbed.

III. ABBREVIATIONS

25(OH)D	25-hydroxyvitamin D_3
AMPs	antimicrobial peptides
ANOVA	analysis of variance
APCI	atmospheric-pressure chemical ionization
CID	collision-induced dissociation
CKD	chronic kidney disease
CLD	chronic liver disease
CN	cyano
CYPs	cytochromes P450
CV	coefficients of variation
CVD	cardiovascular disease
DBS	dried blood spots
DBP	vitamin D binding protein
Diasorin	chemiluminescence assay
ER	endoplasmic reticulum
ESI	electrospray ionization
ELISA	enzyme-linked immunosorbent assay
FDA	U.S. food and drug administration
FGF-23	fibroblast growth factor 23
HSA	human serum albumin
LC-MS/MS	liquid chromatography tandem mass spectrometry
HOMO	highest occupied molecular orbital
HPLC	high-performance liquid chromatography
LDL	low-density lipoprotein

III. ABBREVIATIONS

LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LUMO	lowest occupied molecular orbital
MDBP	12-(maleimidyl)-dodecyl-tri-*n*-butylphosphonium
MBOTAD	4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione
MRM	multiple reaction monitoring
ng/mL	nanograms per milliliter
nmol/L	nano mol per liter
1-OHase	1α-hydroxylase
PBMC	peripheral blood mononuclear cells
PFP	pentafluorphenyl
pg/mL	picograms per milliliter
PP	protein precipitation
PTAD	4phenyl-1,2,4-triazoline-3,5-dione
PTH	parathyroid hormone
QqLit	quadrupole-linear ion trap spectrometer
RIA	radioimmunoassay
SLE	supported liquid extraction
SPE	solid phase extraction
µg/mL	micrograms per milliliter
UHPLC	ultra-high-performance liquid chromatography
UVB	ultraviolet B electromagnetic radiation (wavelength from 10 – 400 nm)
VDR	vitamin D receptor
VDREs	vitamin D receptor response elements

IV. INTRODUCTION

VITAMIN D METABOLISM

Vitamin D as a general term, is a description for a large class of secosteroids, of which the two most important are vitamin D_2 and D_3. Both are chemically very similar, while the major difference comes from their different biological origins; vitamin D_3 is photosynthesized in mammals in their skin [1] and vitamin D_2 mainly occurs in mushrooms [1]. In the present work, the generic term vitamin D describes the vitamin D_3 form. The impact of vitamin D in health is undisputed. Vitamin D deficiency has been widely associated with many problematic health conditions such as diabetes, depression, chronic liver disease, Alzheimer's disease, cancer and multiple sclerosis [2–12]. Vitamin D and their metabolites have effects on numerous physiological functions including the inhibition of growth of cancer cells and protection against certain immune mediated disorders [13,14]. The strongest evidence for a negative effect caused by vitamin D deficiency is on skeletal and bone health described [1,15,16]. Through the complex vitamin D metabolism a number of different metabolites over a wide dynamic concentration range are generated and observed. Vitamin D synthesis starts in the skin by sunlight photosynthesis, that means in detail that the UVB radiation catalyzes the conversion of 7-dehydrocholesterol to previtamin D, which then thermally converts to vitamin D (Figure 1). This causes problems because of the limitation of self-production of vitamin D in the human body, the melanin in human skin blocks UVB radiation, comparable to clothing and sunscreen. Seasonal effects and latitude are responsible for variations in the intensity of UVB radiation from sunlight linked to lower vitamin D production in skin [17]. The first step in the metabolism pathway of vitamin D is the conversion to 25(OH)D by CYP with 25-hydroxylase activity (Figure 1). 25-hydroxyvitamin D is the major circulating metabolite and it is biologically inactive although it is commonly used for assessing vitamin D status. The metabolite 25(OH)D undergoes further oxidation in the kidney to the biological active metabolites $1,25(OH)_2D$ and $24,25(OH)_2D$ by CYP24A1 [1,18] (Figure 1).

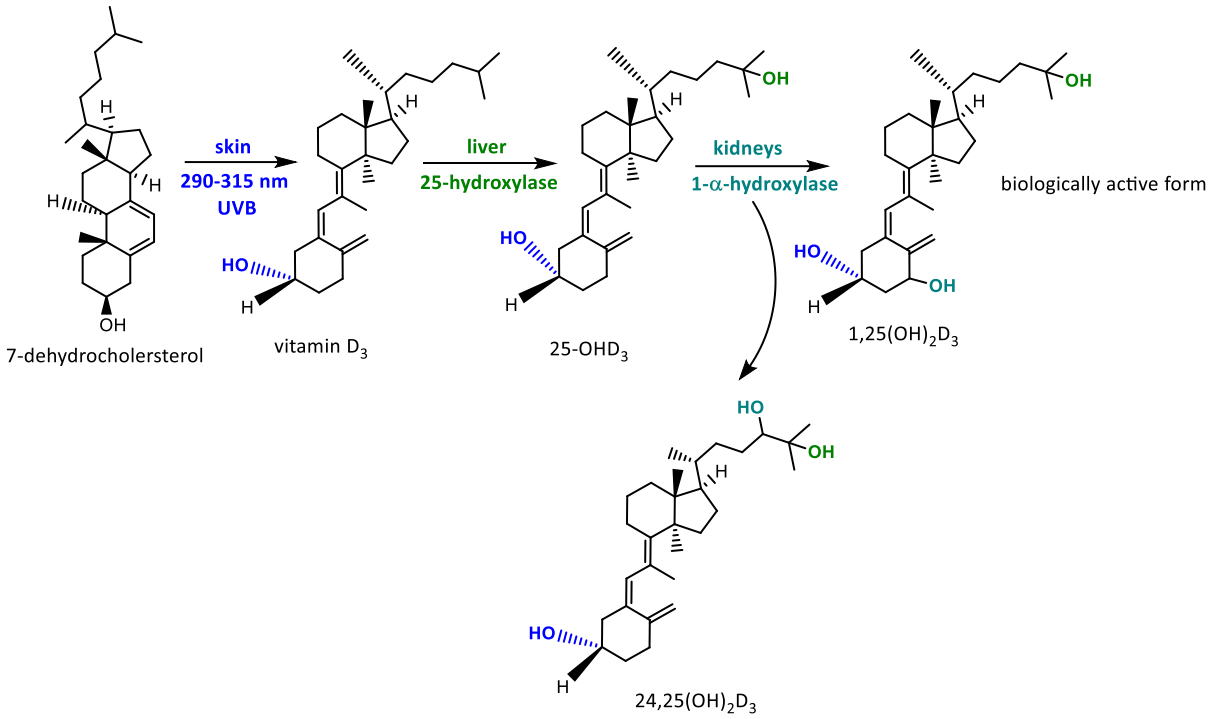

Figure 1: Vitamin D biosynthesis and metabolism.

Megalin is located in the kidney and a member of LDL (low-density lipoprotein) receptor gene family. As a multiligand binding receptor, megalin plays an essential role in endocytic internalization of 25(OH)D [14,19]. The responsible enzyme for all processes is cytochrome P450 mixed-function oxidases (CYPs) [20] (Figure 2). CYPs are the major enzymes which usually consists of about 500 amino acids. Their key role in drug metabolism has already been described in detail, accounting for about 75% of the total metabolism [21]. These CYPs are normally located in the endoplasmic reticulum (ER) or in mitochondria [20]. All mitochondrial P450 enzymes transfer electrons from NADPH to NADPH-ferrodoxin reductase through ferrodoxin, during the 1α–hydroxylase reaction [14,20]. A mitochondrial P450 enzyme can hydroxylate mono and dihydroxylated vitamin D species (25(OH)D and 1,25$(OH)_2$D) [22]. Established 24-hydroxylase in vitamin D metabolism is only CYP24A1, special feature of this enzyme is 24-hydroxylase and 23-hydroxylase activity, in different ratios depending on enzyme task [20,23]. The principal role of CYP24A1 in vitamin D metabolism seems to be the prevention of accumulation of toxic levels of the vitamin D vitamers [20,24]. The number of malignancies has increased in last decade and great efforts have been perused to develop inhibitors of CYP24A1 because of described and established increased CYP24A1 expression in malignant cells [25]. The aim is to increase endogenous 1,25$(OH)_2$D levels in tumors in the hope of increasing the anti-proliferative effect of 1,25$(OH)_2$D in treated cells [25].

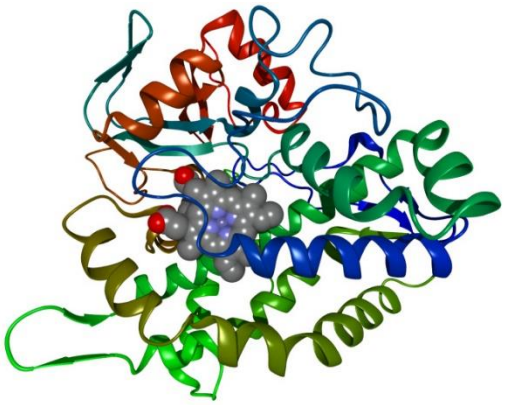

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Figure 2: Molecule structure of Cytochrom P450.

The stimulation of 1,25(OH)D production by estrogens alone or combined with androgens or progesterone was observed [26–28]. Surprisingly estrogens suppress 24,25(OH)2D synthesis [28]. An additional hormone, namely prolactin seems to stimulate 1,25(OH)D production [14]. For regulation of vitamin D metabolism, increased fibroblast growth factor 23 (FGF-23) concentration reduce levels of $1,25(OH)_2D$ by inhibiting synthesis and promoting catabolism of $1,25(OH)_2D$ [29]. A vitamin D analog, namely CTA018 is now in phase II of a clinical trial for secondary hyperparathyroidism (Cytochroma) because of shown selective antagonist activity for CYP24A1 expression and VDR agonist activity [20,30]. The dihydroxylated species $1,25(OH)_2D$ is a ligand for the vitamin D receptor (VDR). The vitamin D receptor described as a transcription factor and member of the steroid hormone nuclear receptor family is found in nearly every tissue and binds to sites in the DNA, which are called vitamin D response elements (VDREs). In the DNA there are thousands of these binding sites, which regulate hundreds of genes in a cell-specific fashion [20]. In addition, nongenomic actions were observed, $1,25(OH)_2D$ exerts effects that are too rapid to involve genomic actions, for example the rapid stimulation of intestinal transport in a vitamin D replete chick [31] or effect on chondrocytes in the growth plate [31] and in keratinocytes in the skin [32]. Over the years the human metabolism of vitamin D has been extensively studied and more than 50 circulating vitamin D metabolites have been described [33–36]. All vitamin D compounds are predominantly bonded to vitamin D binding protein (DBP) for circulation [9,20,21]. An alternative pathway for vitamin D activation is recently described by Slominski et al. [27]. In keratinocytes the 20-hydroxylation of vitamin D by CYP11A1 was identified, the product 20(OH)D and their metabolite $20,23(OH)_2D$ appear to demonstrate similar activity to 1,25(OH)D [37].

A different metabolomic pathway is observed by 3-epimerase, which isomerizes the C-3 hydroxyl group of the A ring of all natural vitamin D metabolites from the α to β orientation [20] (Figure 3). The first 3-epimerase activity was identified in keratinocytes by production of metabolite 3-epi-1,25$(OH)_2$D [38]. The effect of 3-epimerase was extended and observed in different cells as colon cancer cells, parathyroid cells, osteo-blasts, and hepatocyte-derived cells [20,39]. First differences in the activity and biochemical behavior between α and β epimers were observed for 3-epi-25(OH)D, the metabolite 3-epi-25(OH)D has reduced binding to DBP relative to 25(OH)D and the dihydroxylated form epi-1,25$(OH)_2$D has reduced affinity for VDR relative to 1,25(OH)D, additional differences in transcriptional activity and biological activity is currently a research focus [40].

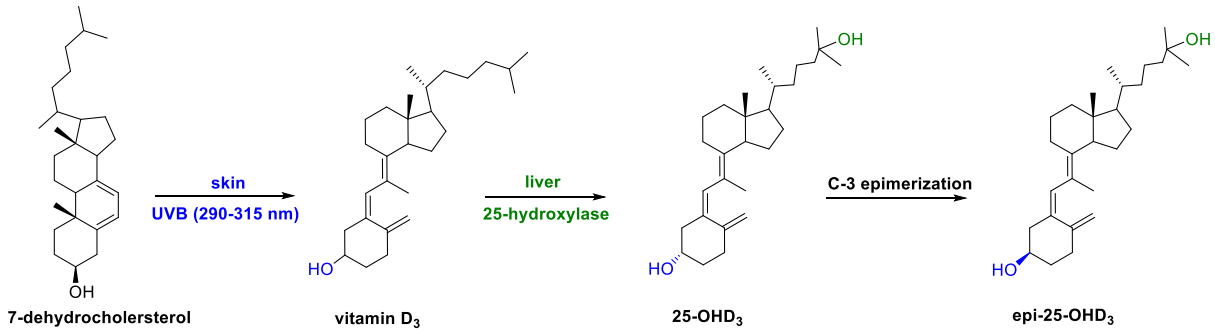

Figure 3: Epimerisation process in metabolomic pathway.

Furthermore different vitamin D species are generated by 24-hydroxylase during deactivation of 1,25$(OH)_2$D to trihydroxylated metabolites such as 1,24,25$(OH)_3$D, which then undergo further metabolism including oxidation and conjugation [13]. For example, 1,25$(OH)_2$D can be oxidized to 1,25(OH)D- 26,23-lactone and calcitroic acid [41].

As mentioned above, the 25(OH)D metabolite is used, instead of biologically active metabolites 1,25$(OH)_2$D and 24,25$(OH)_2$D, to determine the vitamin D status of an individual. The reason is the high concentration in human blood and sufficiently long half-life time of approximately 19 days in circulation to reflect a realistic vitamin D sufficiency or deficiency in individuals [1,18]. Other downstream metabolites especially 1,25$(OH)_2$D and 24,25$(OH)_2$D are often transient species, which are usually present at much lower physiological concentrations.

For a metabolic profiling, defined here as the quantification of metabolites involved in the same metabolic pathway, it is necessary to catch all of these relevant metabolites.

VITAMIN D ANALOGS

Over the years many clinically used analogs have been synthesized [42]. Development of different kinds of analogs starts with prodrugs requiring further metabolism to be in an active state. Alphacalcidol for example is approved in Europe and Japan for treatment of osteoporosis and Doxercalciferol is used in USA for treatment of secondary hyperparathyroidism. The early analog of $1,25(OH)_2D$ is 26,27 F_6-$1,25(OH)_2D$, namely falecalcitriol, which is approved for three different treatments: osteoporosis, secondary hyperparathyroidism and hypoparathyroidism. The metabolism is reduced for these analogs because of the structural features of the fluoride component in the side chain, it resists further metabolism and retains their biological activity [43]. Many other analogs were designed for reducing DBP affinity, increasing clearance in hope to reduce the impact on intestinal calcium transport and bone resorption [20].

VITAMIN D AND CLINICAL APPLICATIONS

The literature assessing the relationship between vitamin D and different human disease is vast, and in the following paragraphs only several points are summarized. The optimal 25(OH)D threshold for supplementation therapy is frequently debated [44,45]. PTH has been used to define optimal vitamin D intake, in correlation to decreases of the PTH level with vitamin D intake [44]. According to established treatment data, a 25(OH)D value of 50 nmol/L represents one threshold value for optimal vitamin D nourishment [45].

Vitamin D adequacy and the skeleton; the key role of vitamin D in prevention of rickets, osteomalacia, osteoporosis and fractures is undisputed [46,47]. The task of vitamin D is to provide adequate levels of calcium and phosphate in bone and skeletal development, the effects on bone development and remodeling have direct and indirect in nature. The greater question is, which vitamin D level is sufficient to take optimized precaution and protection against bone diseases and the aging process [20].

Patients with obesity, diabetes mellitus and metabolic syndrome suffer from low 25(OH)D blood concentration levels in comparison to healthy individuals. The adipocytes express the VDR, in addition the $1,25(OH)_2D$ metabolite promotes increased lipogenesis and decreased lipolysis [20,48].

Recent research demonstrated insulin resistance is associated with vitamin D deficiency [49]. The first clinical trials with diabetes mellitus patients showed a benefit from the vitamin D administration, but longer and larger randomized clinical trials are still needed [20,50,51].

Cancer development and progress and metastasis can be prevented through $1,25(OH)_2D$, the results were obtained from animal and cell culture studies [20]. Cell specific mechanism induced by $1,25(OH)_2D$ are responsible for suppressing tumor development and slowing the progress of growth. The inhibition of proliferation by blocking targets in the cell cycle or interference with signaling by growth factors, which will induce apoptosis, stimulation of DNA damage repair, prevention of tumor angiogenesis and inhibition of metastasis can be applied for suppression [20].

Vitamin D deficiency is associated with cardiomyopathy [52]. Different epidemiologic studies were performed to establish the association of increased cardiovascular disease (CVK) risk with reduced 25(OH)D levels in the blood [53]. A large randomized clinical trial is required to clarify the role of vitamin D and their metabolites in the prevention or treatment of cardiovascular disease.

The immunological phenotype and the activation state of immune cells determine their vitamin D metabolism. Vitamin D is an important determinant of cellular differentiation [15,54–58]. The immune system is comprised of two types of immunity, innate and adaptive. Adequate vitamin D blood levels promote the innate immune response. Innate immune response involves the activation of Toll-like receptors and leads to induction of antimicrobial peptides (AMPs), such as cathelicidin, which kill the organism. The expression of cathelicidin is induced by $1,25(OH)_2D$ [59]. In addition, vitamin D exerts an inhibitory action on the adaptive immune system and response. The adaptive immune response initiated by macrophages and vitamin D influence the differentiation of T and B lymphocytes [20]. Correlation between vitamin D deficiency and tuberculosis is well known [60], however there is no clinically approved vitamin D analog or drug for immune modulation.

VITAMIN D ANALYSIS

The vitamin D metabolite analysis has been historically dominated by immunoassays and receptor binding assays [18,61]. The general procedure of using antibodies as selection agents for isolation and measuring specific analytes has been reported since the 1960's [62]. Classical immunoassays used one antibody as a capture agent and a second antibody combined with a detectable label was used as the detection or reporter reagent [62]. Development of automated 25(OH)D immunoassays from various manufacturers are available, but recent experiments showed unsatisfied accuracy and precision [63,64]. In addition two main difficulties in immunoassay measurement of vitamin D are challenging, the strong bond of vitamin D to their bonding protein and the generation of antibodies against small antigenic molecules in the assay [65]. The limitation of the common immunoassays are the cross-activity of the antibodies and non-equimolar recognition of the different vitamin D species such as $25(OH)D_3$ and $25(OH)D_2$ [66].

Additional improved assays with nonradioactive detection, such enzyme-linked immunosorbent assay (ELISA) and chemiluminescence on the Liaison have been introduced. However, all of these assays are not able to isolate a specific analyte for further characterization or investigation, which led to increasing interest in applying the specificity of antibody-antigen reactions to analytical and separation sciences [62]. The common interest increased on developing immunoaffinity isolation procedures as an analytical technique with the aim to isolate a specific analyte through the antibody selectivity. The major advantage of antibody-based separation/extraction is the specificity of the antibodies which enabled an isolation of target analyte from complex biological matrices and may be followed analyses by a second technique, such as high-performance liquid chromatography (HPLC) [62]. Current clinically applied 25(OH)D immunoassays employ polyclonal or monoclonal antibodies directed against the target metabolite 25(OH)D. But the existing competition between the 25(OH)D capture antibody and the vitamin D bonding protein in a patient sample makes these kind of assays difficult to control. An acetonitrile extraction step is necessary to release all vitamin D metabolites from vitamin D bonding protein and possibly sources of interferences, including heterophile antibodies, before incubation with the capture antibody [65]. The available automated immunoassays are not justified for such an aggressive extraction step, which may explain the variable performance between different assays [65].

Previous experiments with homogenous 1-step assays, that lead to incomplete separation of 25(OH)D from the vitamin D bonding protein, demonstrated poor agreement with LC-MS/MS measurements [65,67].

Unsurprisingly in recent years, LC-MS/MS has been established as the gold standard technique for vitamin D metabolic profiling because of the technique's inherent analytical specificity and sensitivity [18,68,69]. In addition, the increasing flow of routine samples prompted further developments of robust high-throughput methodologies. LC-MS/MS is often chosen for separation of vitamin D metabolites via chromatographic isolation, which allows direct quantification of 25(OH)D without the need for antibody capture or protein interaction with vitamin D binding proteins [64]. Over the time many different approaches for vitamin D determination were published under various measurement requirements, although a generally accepted reference method for 25(OH)D determination was still lacking. The resulting substantial disagreement was illustrated by the results from the DEQAS (Vitamin D Quality Assessment Scheme) external quality assurance scheme (October 2008 distribution). Different candidate reference methods were developed [66,70], but only the release of an NIST standard reference material (SRM 972) gained acceptance. This new reference material is used in clinical approaches to facilitate a harmonization across all different assay's for 25(OH)D determination [65]. In comparison tests for performance and agreement in vitamin D determination, different LC-MS/MS methods showed excellent agreement between measured vitamin D values (25(OH)D)[65,71]. In the case of immunoassays, variable performance was obtained and not all assays demonstrated the ability to meet the needs of the clinical laboratories. The published relatively poor performance in measuring 25(OH)D at lower concentrations (8 µg/L= 20 nmol/L) leads to inaccuracies [65]. In addition, it must be realized that the immunoassays do not recognize the C-3 epimers of vitamin D [20] that leads to positive bias and overestimation of vitamin D level. This effect may have a limited impact on treatment decisions in vitamin D deficiency and disease, but can influence the view of the whole (metabolic) picture in research.

The vitamin D LC-MS/MS methods are comprised of a choice of sample preparation steps to purify the vitamin D metabolites followed by a separation step via liquid chromatography to separate target metabolites from residual matrix components, isomers and epimers before mass spectrometric determination. The first step, the sample preparation, especially sample cleaning showed improved signal to noise ratio values in vitamin D measurements [69,72–74].

In recent years, many sample preparation techniques for sample clean-up in complex matrices such as food, serum, plasma and urine have been established [18,72,73,75,76]. Because of the nature and binding behavior of vitamin D metabolites, different methods such as protein precipitation, solid phase extraction (SPE) [76–82], liquid-liquid extraction (LLE) [34,83–86] and supported liquid extraction (SLE) are established to enable a specific extraction of target metabolites from their interfering matrix components, such as high-abundant proteins and phospholipids. The LLE method separates compounds, such as vitamin D from proteins and phospholipids in their natural sample environment, based on their relative solubility in two different immiscible liquids, usually water or sample liquid such as serum or plasma (water based) and an organic solvent. It is a specialized extraction of vitamin D metabolites from one liquid into another liquid phase and retaining interfering compounds in the previous starting liquid (Figure 4). In contrast, the SPE extraction method is a sample preparation process where vitamin D gets separated from other compounds in the biological sample according to their physical and chemical properties. Separation principal is the affinity of vitamin D dissolved in a liquid (biological sample) for a solid through which the sample is passed to separate desired vitamin D from non-desired components. Depending on type of SPE (normal or reversed phase) desired vitamin D metabolites are retained on the stationary phase and must be eluted with organic solvent or go through solid phase without interactions with the phase (Figure 4). The recovery rates of these different extraction methods were determined and showed acceptable values [79,87–90]. The LLE and SPE extraction methodologies have a big disadvantage in sample preparation time, which leads to limited sample throughput, and generally sample preparation is time consuming and costly. Fully automated methodologies uses several disjointed steps, but still high investments and maintenance are necessary for the devices [87]. Recently customer adaptions were performed with SLE or SPE 96 well-plates, which also simplifies the extraction protocol and can be automated with pipetting robots to save time and increase the sample extraction throughput [88–90]. Developments to simplify sample preparation/extraction for vitamin D analysis in human bio-fluids or tissues are still a major research focus because of the need to solely isolate target vitamin D compounds from lipoproteins and protein and in addition to release vitamin D from VDP. In addition, ion suppression can be a major problem under these circumstances, but it can be minimized through the use of stable isotope standards of vitamin D compounds, which are essential to correct analytical issues caused by ion suppression effects [61,91].

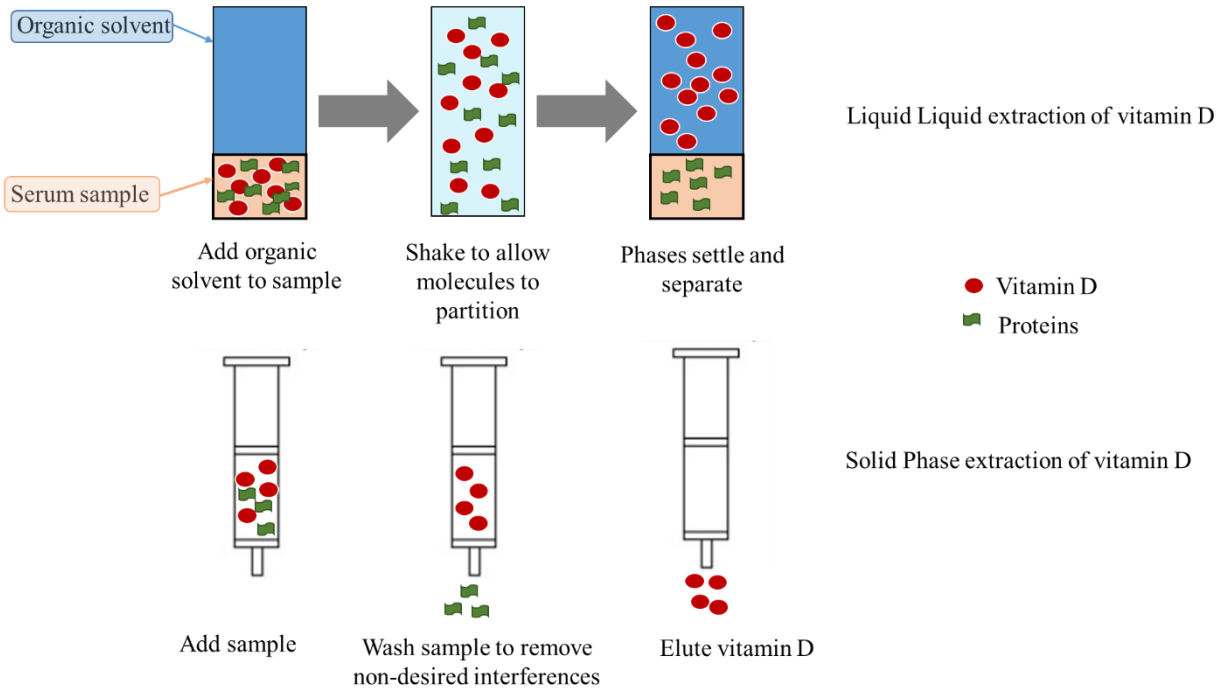

Figure 4: Extraction scheme of LLE (liquid liquid extraction) and SPE (solid phase extraction).

The mentioned effect of isomeric and isobaric noise present in all serum or plasma samples can lead to an overestimation of vitamin D values if the compounds are not properly separated by liquid chromatography [91]. Furthermore matrix effects from the epimeric species, which is metabolically generated lead to similarly described issues, plus the chromatographic and mass spectrometric challenge by having the same charge/mass ratios and same polarity of epimers.

INTERFERENCES, ISOBARS AND EPIMERS IN VITAMIN D MEASUREMENTS

Screening of vitamin D metabolites from human bio-fluids such as plasma or serum using mass spectrometry are not without challenges. First of all the lipophilic nature of the analytes and their tight binding to their carrier protein [71,72] inducing isobaric and isomeric interferences in serum/plasma [73], lipoproteins and phospholipids are non-desired compounds, which are not always completely extracted through previously performed extractions. Furthermore, low ionization efficiencies and nonspecific water losses for MRM scan type (multiple reaction monitoring) inhibit mass spectrometric analysis [66].

The previously mentioned metabolic production of 25(OH)D epimers, namely 3-α–epi-25(OH)D_3 and 3-β–epi-25(OH)D_3 at position C3 in structure leads to challenge. The 3-α-epi-25(OH)D_3 has the potential to influence measured levels of 25(OH)D_3 to a positive bias [39,40,92–96], resulting in overestimation of vitamin D levels (Figure 5). While the biological function of the 3α epimer remains unknown, its presence needs to be accurately captured and separated from the main 3β epimer [39,92,94,96,97].Previous studies have shown values of interfering epimer in infant blood and also in adults blood samples [34,92,98]. This kind of epimers are positional epimers, the hydroxyl group at C3 is in the front or back position, and therefore they have the same molecular weight, which is explained by same elemental structure. The difference of these epimers at all are so small, but have a big effect on their measurement and detection. The mass spectrometric problems originate by the above mentioned same mass to charge ratio of these epimers, which leads to the same MRM transition. It should be added that the liquid chromatography separation is also challenging because of similar polarity and similar chromatographic interactions. Optimized chromatographic techniques and mass spectrometric settings are necessary to enable quantification of the real 25(OH)D_3 level. In addition, it can be important in the future to know the concentration of interfering 3-α-epi-25(OH)D_3. Research focuses on elucidation of biological activity of 3-α-epi-25(OH)D_3 and changes of concentration distribution with age [94,95,98].

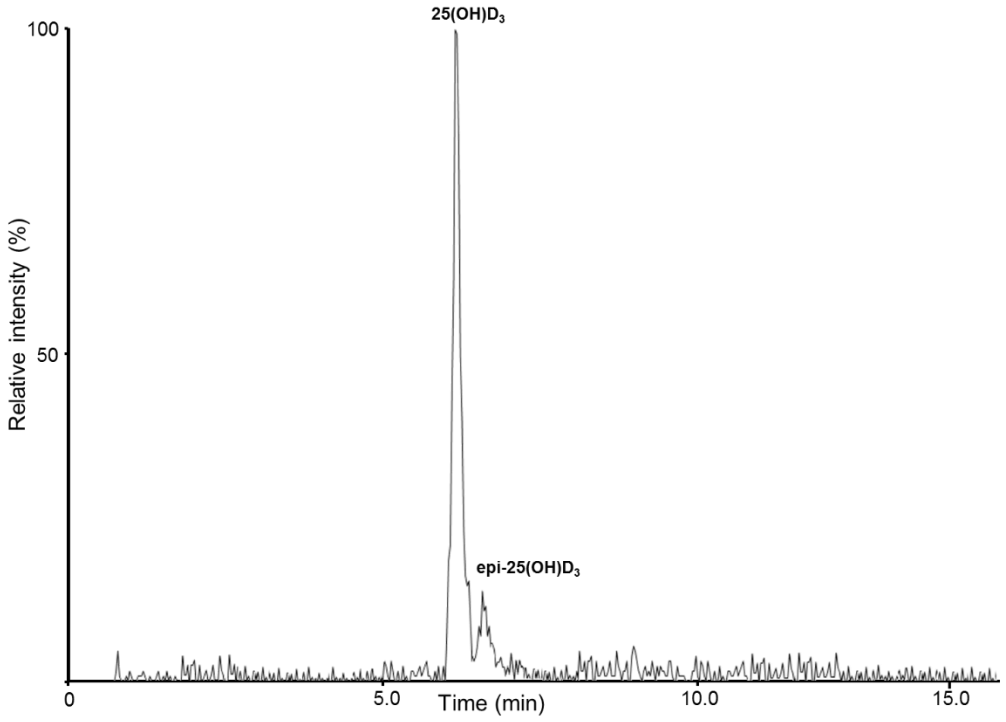

Figure 5: Typical MRM chromatogram of 25(OH)D_3.

A different problem increased through the obtained detection limits for vitamin D metabolites. Especially low-abundant vitamin D compounds, such as dihydroxylated species, are often not sufficiently low to access physiological levels by LC-MS/MS measurements. The reason is their low circulating concentration in blood and the short half-life time in blood of 19 hours in contrast to 19 days for 25(OH)D. This often limits the determination of vitamin D to the main metabolite 25(OH)D to define vitamin D status and assess vitamin D deficiency. More generally all vitamin D metabolites show low ionization efficiency under electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) conditions, which are the two most often applied ionization techniques for vitamin D analysis by LC-MS/MS [79,81,84,86,97,99–102]. To improve the ionization properties of vitamin D metabolites, derivatization was applied, with the goal to introduce chemical functionalities that promote the ionization process [99,102,103]. An additional advantage through the introduction of new functionalities into the molecule is the shift to a higher mass range. The detected mass to charge values of vitamin D metabolites were shifted to mass ranges, where chemical noise in ESI and APCI-MS is significantly lower and isobaric interferences are less present [91,99]. Diels Alder reactions in vitamin D analysis using Cookson-type reagents (e.g. 4-phenyl-1,2,4-triazoline-3,5-dione, PTAD) are implemented [76,77,81,104].

From chemical point of view Diels–Alder reaction describes an organic chemical reaction between a conjugated diene and a substituted dienophile to build a substituted cyclohexene system by a [4+2] cycloaddition [105,106]. Diels–Alder reactions are governed by orbital symmetry considerations, which indicate a suprafacial/suprafacial interaction of the 4π electron system of the diene and the 2π electron system of the dienophile [106]. The stereochemical information is retained in product of Diels–Alder reactions, the reactions proceed stereospecific and selective. The introduced functionality forms a new target product, which allows simplified ionization of vitamin D compounds. Recently developed new derivatization reagent produce preformed ions by derivatization reaction [99,107]. The new commercial derivatization reagent is also based on the Diels-Alder reaction and called Amplifex (Figure 6). The advantage in comparison to common derivatization reagents is posing a permanently-charged quaternary ammonium group, which significantly improves ionization efficiency in comparison to PTAD [99,107]. The reason for that big improvement in ionization efficiency is the production of preformed ions. This positive charge enabled much easier detection of vitamin D metabolites because the new preformed vitamin D ions are transferred much more efficiently into the gas phase by electrospray. But in all cases of vitamin D analysis, difficulty arose during the derivatization reaction from the two possible sides of attack of the cis-diene moiety of vitamin D from derivatization reagent [77,79] (Figure 6). This problem is published for different derivatization reagents [79,99,101]. The reagent can link to the molecule from α and β sides of vitamin D metabolites, all vitamin D metabolites react on this cis-diene moiety, therefore for all metabolites two epimeric products for each compound are possible. As a result, two peaks may be expected for each compound in the MRM ion chromatograms. Nevertheless comparisons report an increase of the response factor by 100-1000-fold over the non-derivatized compounds [103]. PTAD (4-phenyl-1,2,4-triazoline-3,5-dione), which is commonly used for vitamin D derivatization, has been shown to exhibit limited stability [77] therefore, improved versions were recently developed [76,108,109].

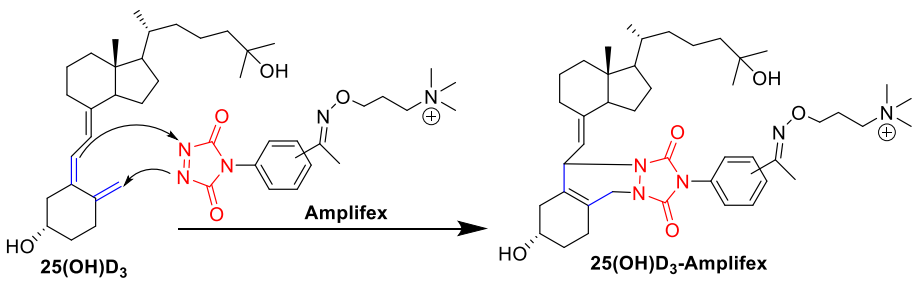

Figure 6: Epimeric products occur in the Diels–Alder derivatization reaction with vitamin D.

Although all derivatization reagents have a limitation in their application; they all react in non-aqueous environment. Any traces of water leads to non-reaction or interruption of the reaction [58,77,99,110]. The sample preparation must be strictly performed to obtain a dried sample extract followed by derivatization, sample integrity and sample environment undergoes changes through the whole sample preparations steps like extraction and drying. Furthermore these steps are costly and time consuming, but in ratio to obtained improved ionization efficiency valuable. Further developments in derivatization reagents and their reactions are needed to allow aqueous vitamin D analysis in their physiological nature and environment to support the implementation of specialized techniques for investigating vitamin D metabolites in the future, in particular where the sample integrity cannot be disturbed.

OBJECTIVES

The objective of this study was to demonstrate the possibility of advanced analytical mass spectrometry techniques and develope new solutions for existing challenges. The experimental strategy was first to establish a conventional quantification method for six different vitamin D metabolites, including low abundant species, by using common Diels–Alder derivatization process. The new method was planned with a SLE extraction step in 96-well plates to clean samples from interfering compounds such as described above, followed by a drying step and the optimized derivatization protocol with Amplifex, the new derivatization reagent with a permanently charged functional group for improved ionization efficiency of vitamin D metabolites. A complex chromatographic separation method for these vitamin D metabolites was developed. The assay validation was designed following FDA (U.S. Food and Drug Administration) guidlines. Afterwards the new assay was successfully tested on a chronic liver disease (CLD) study with 112 patients. Furthermore, the assay was extended to Colecalciferol, the seventh vitamin D metabolite.

The first investigation of the new purification and separation technologies for quantification of 25(OH)D epimers (3-α–epi-25(OH)D_3 and 3-β–epi-25(OH)D_3) from DBS (dried blood spots) was planned in terms of proper quantification of 25(OH)D_3 levels and quantification of interfering 3-α–epi-25(OH)D_3. Challenge in this approach was the low planned sample (whole blood) volume for determination of vitamin D levels and the extraction step from filter paper. Vitamin D free serum is commercially available, but not for free whole blood, which made dried blood spot calibration much more complicated in comparison to serum calibration. It should be mentioned that dried blood spot calibration is commonly performed as a standard addition method, because of the lack of vitamin D free whole blood for calibration. The dried blood spot calibration strategy was newly designed, based on artificial whole blood matrix, which was in-house prepared and developed. Challenging chromatographic separation and mass spectrometric settings were performed and optimized. Assay performance was planned to compare with previously developed serum assay and Diasorin a chemiluminescent immunoassay (CLIA). The application of this assay was successfully performed on 10 donor samples.

Finally, a new derivatization label MDBP (12-(maleimidyl)dodecyl-tri-n-butylphosphonium) was synthesized, within in four-step reaction, for the 25(OH)D3 derivatization in aqueous environments, which was unfortunately not possible with the available derivatization reagents.

The unique feature of this derivatization reagent is the solvent-(including water!) and matrix-independent derivatization of vitamin D metabolites. The new reagent is based on an ionic liquid and was planned to be used for derivatization of vitamin D in cellular fractions, in natural aqueous environment without sample extraction. Collision-induced dissociation spectra was planned to identify more specific MRM transition in comparison to commonly used unspecific water loss transition for vitamin D compounds. The chosen cells for the application were T cells, B cells, helper T cells and macrophages. An additional successful study was the monitoring of uptake of 25(OH)D_3 in extra- and intracellular regions of PBMC (peripheral blood mononuclear cells) cellular systems.

V. SUMMARY AND CONCLUSIONS

The number and frequency of measurement of vitamin D levels in humans has significantly increased over the last decade, with the aim to describe public health in populations and to support clinical diagnosis and monitoring of various diseases in individuals. Most current assays are limited to the main metabolite 25-hydroxyvitamin D, because of the previously mentioned low ionization efficiency of the vitamin D compounds and the low physiological concentration levels of other metabolites in blood.

The simultaneous quantification of multiple vitamin D metabolites in human serum was measured with the aim to determine diverse high and low abundant vitamin D metabolites with the same performance as that obtained for $25(OH)D_3$. In the literature, described assays for determination of vitamin D which used a derivatization protocol are limited to Cookson type reagents as the derivatization compound [77,81,81,101,111,112]. Cookson type reagents contain a molecular structure, which allows generation of more specific ions in the ion source of a mass spectrometer, compared to normal non-derivatized vitamin D metabolites. This leads to an improved ion efficiency of 100-1000-fold compared to native vitamin D [113]. Vitamin D molecules in the non derivatized form exhibit unspecific fragmentations from water loss or less specific “picket fence” signals in the lower *m/z* range from backbone dissociations [58,61]. The use of a derivatization reagent such as Amplifex, which already contains a permanently charged functionality, is a more general improvement for mass spectrometric analysis. Through the reaction of Amplifex with vitamin D metabolites, ions were obtained which are much more efficiently transferred into the gas phase during electrospray ionization. This effect leads to an improvement of 1000-10000-fold of ionization efficiency [99,107,110]. We applied this new idea of the derivatization reagent on multiple vitamin D metabolites to simultaneous quantify them in human serum at low physiological levels. The derivatization protocol was optimized for considerably improving the derivatization reagent’s performance by eliminating the formation of multiple diastereomers through chemical derivatization process [79,99]. The chemotyping assay validation was performed under FDA guidlines and transferred to a study with 112 chronic liver disease patients, provided by the Department of Medicine II, Saarland University Medical Center (Homburg).

Good performance in terms of accuracy, precision and linearity was demonstrated and additional experiments to evaluate matrix effects, stability and ruggedness were performed. Method comparison was carried out by means of data from a routine laboratory using Diasorin chemiluminescence assay and showed good correlation. In addition it should be mentioned that the new chemotyping assay has the potential to become an automated process. All steps in the new methodology are easy to adapt as an automated assay, which has a lot of benefits for routine measurement of vitamin D metabolites such as less time consuming in sample preparation. For interpretation the metabolomic picture of health status of individuals seems to be necessary to catch and quantify all possible relevant metabolites, which our results in this study confirmed, for example by detection of the interfering 3-α-epi-25(OH)D species in all analyzed samples.

In next step of the project, an alternative sample preparation method based on extraction of vitamin D epimers from DBS (dried blood spots) was developed, with the aim to quantify the individual epimers of $25(OH)D_3$. This assay is the first LC-MS/MS method for simultaneous quantification of 3α- and 3β-$25(OH)D_3$ from dried blood spots. Described literature understand the relevance of interfering epimers such as 3α-25(OH)D in analytical measurements, but the biological relevance is still under discussion, whereas existing methods are limited by avoiding interference of these compounds, but less quantification of such compounds [34,95,96,98]. The aim should be to know concentrations of all possibly relevant metabolites, also if the data are not sufficient to understand the whole biological process at that time, in the future it may have a bigger impact on understanding the whole metabolic process and development of disease, based on metabolite target distribution. Based on the results of the chemotyping study with chronic liver disease patients, we established that both epimers, namely 3α- and 3β-$25(OH)D_3$ were present in all analyzed samples, leading to development of a new method enabling simultaneous quantification of both metabolites from dried blood spot (DBS). DBS are commonly used for newborn screening especially for the analysis of vitamin D levels in infant blood and these samples are particularly likely to contain a significant level of interfering 3α-epimer [94,114,115]. DBS approaches showed in general different advantages in comparison to vessel or vein puncture blood because of their small sample volume and non-challenging storage conditions.

From a mass spectrometric point of view, the two epimers $25α(OH)D_3$ and $25β(OH)D_3$ are virtually impossible to differentiate, as they exhibit nearly identical dissociation behavior upon collision induced dissociation (CID) [39,92,96,114].

Without a proper separation of 25α(OH)D_3 from 25β(OH)D_3 by liquid- chromatography, co-elution is unavoidable and leads to described overestimation of vitamin D levels [92,96,97]. The challenge in this methodology is the new sample preparation by new matrix, means new possibly interfering compounds, of dried blood spot filter cards. A specialized extraction method was necessary, also by less sample volume of 50 µL blood spot. After successful extraction the established chemical derivatization was applied. Furthermore, proper calibration of DBS samples is much more difficult to achieve than for liquid serum or plasma samples [116,117]. Because of the lack of vitamin D free whole blood, standard addition calibration strategy is commonly used for DBS vitamin D analysis. The idea was to develop a new artificial whole blood matrix for calibration which contains no vitamin D species, with knowledge that virtually all 25(OH)D in whole blood is distributed in the serum compartment (>98%) and completely excluded from intra-cellular and membrane components of red blood cells [116], we isolated red blood cells from human donor samples and reconstituted them in vitamin D antibody-purified serum (in natural ratio of 45:55 (v/v) to give the artificial vitamin D-free whole blood. This should mimic the endogenous matrix more so than previous approaches and also provide a whole blood behavior on the collection papers that closely resembles that of patient samples, it also avoided matrix effects and averaged out inter-individual variations of endogenous components [92]. This extended the application range of the DBS assay to lower concentrations, in comparison to the commonly used standard addition method [117–120,112]. Comparison between serum LC-MS/MS and DBS-LC-MS/MS measurements and serum Diasorin chemiluminescence assay demonstrated good agreement in all experiments between compared assays. Limitation of the method is the composition of artificial whole blood matrix. The real human serum ingredients are electrolytes and nutrients, hormones or waste products besides water (91%) and proteins (7%) [118]. However, with respect to the exact composition of the artificial vitamin D-free blood, as only human serum albumin solution (HAS, 7% w/v saline) was added to mimic vitamin D-free whole blood. In summary this DBS method provides a robust alternative to conventional serum or plasma analyses, in particular it can be readily expanded to the other vitamin D metabolites as needed, as the analyte derivatization step after DBS extraction provides virtually equal analytical figures of merit for all mono- and dihydroxylated metabolites of vitamin D [92,99].

Finally, a new derivatization reagent MDBP (12-(maleimidyl)dodecyl-tri-n-butylphosphonium bromide) was developed, which shows a great advantage in comparison to all available derivatization reagents for vitamin D determination.

V. SUMMARY AND CONCLUSIONS

The limitations of available derivatization reagents lie in their required non-aqueous reaction environment and in their stability [18,58,81,92,101,110]. Improvements on published derivatization reagents are on their molecular stability and long term stability, neither on water-stability and aqueous reaction [103,113]. Specialized vitamin D analysis, such as in cellular fractions under aqueous natural conditions were limited to native, non- derivatized vitamin D metabolites, which leads to non-sufficient LOD (limit of detection) limits to assess low metabolite concentrations. An additional problem occurs in specialized approaches and applications, which are focused on the determination of spatial distributions of vitamin D metabolites, such as in MALDI-Imaging technologies and samples which need to preserve their integrity [58,110]. The new derivatization label is based on an ionic liquid, which enables a high stability environment in both organic and non-organic solvents, in contrast with all other derivatization reagents. The derivatization protocol using a reagent that is permanently positively charged pioneered the development of a new derivatization label. The positive effect on mass spectrometric performance was also obtained by binding a permanently charged quaternary phosphonium ion functional group to the vitamin D target compound, in contrast to the previously used reagent with a permanently charged quaternary ammonium ion functional group. An exchange of nitrogen to carbon in the hetero atom ring at the attack position for the Diels−Alder reaction of commonly used derivatization reagents, which we developed, have two main results: desired stability against water leads to an autonomous derivatization reaction environment and necessity of higher reaction time because of less reactivity of derivatization reagent. The performance of our new derivatization label MDBP is comparable to the other available derivatization reagents, however the stability of MDBP is much higher in comparison with the other derivatization reagents, although the lower reactivity of MDBP as demonstrated be the longer reaction time of 24 hours is still acceptable. A new electrospray liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS) method for determining 25(OH)D_3 by MDBP derivatization was developed and applied directly in aqueous cellular systems, which were analyzed in their natural environment without any sample preparation.

The aim of this developed method is measuring vitamin D in physiological conditions without sample extraction and preparation. The proof-of-concept for the MDBP assay was demonstrated by measuring the time-dependent uptake of 25(OH)D_3 into the investigated cells (T cells, helper T cells, B cells and marcophages).

All cells were analyzed in different activation forms, which means un-activated and activated forms to demonstrate applicability of the new label and method. This specialized technique is beneficial for investigating vitamin D metabolites in aqueous environments, in particular where the sample integrity cannot be disturbed.

OUTLOOK

This thesis describes a significant step towards establishing different approaches for determining vitamin D metabolites in body fluids using LC-MS/MS. The developed techniques, especially the derivatization approaches, have proven to be a very useful tool to solve the studied analytical challenges of vitamin D, which are mainly due to the lipophilic nature of the compound, low ionization efficiency and versatility. Nevertheless, vitamin D research encompasses a wide range of experiments, new outcomes and development. Our experiments showed promising results and proved to be extendable to other vitamin D metabolites, which are overlooked or unknown to date. The obtained concentration ranges of the measured metabolites enabled the search for new low abundant vitamin D metabolic products and epimers. The workflow for the large number of samples, combining sample extraction and the derivatization reaction, followed by mass spectrometry detection has to be fully automated to significantly reduce analysis time and considerably decrease the cost per measurement and enable clinical routine measurements.

Dried blood spot filter cards seem to be the future means of sample collection because of the low cost and high sample stability. Furthermore easy application for the analyst and for the patient permits determination of vitamin D levels for metabolic profiling studies. The use of DBS enables an easy way to determine a large number of different target metabolites, which have the potential to aid the understanding of the pathobiological function of vitamin D in healthly and disease states, therefore the assay needs to be extended and validated by different interested target compounds.

The new MDBP derivatization technique may be equally useful in imaging mass spectrometry, where it could be used for enhancing signal responses of spatially localized vitamin D metabolites on wet tissue surfaces, without destroying the integrity of the tissue surface.

Our results undoubtedly support the implementation of new derivatization reagents for varied uses as matrix or derivatization reagents in specialized approaches performed in aqueous reaction environments.

In future research with respect to vitamin D, extension to wider aspects and chemotyping assays, which includes different specific target compounds such as other vitamins, hormones and neurotransmitter, is expected. The correlations between these metabolite distributions and diseases is of great interest to prevent disease and retain a healthy state, but there is a need for more specific and sensitive methods.

It is conceivable that high resolution mass spectrometry conquers the field of further vitamin D analysis, in particular if other metabolites of interest are included in that assay. The early progress is already noted by recently published articles on vitamin D determination by high resolution mass spectrometry [120–124]. In general, quantification of small molecules, commonly involves triple-quadrupole instruments (QQQs) operated at unit mass resolution. The described methods showed the feasibility of LC-MS/HR-MS for small-molecule quantification in routine laboratory testing for vitamin D metabolites with the major benefit of less sample preparation.

A different approach for easy and fast vitamin D measurement can be applied through ion mobility spectrometry to remove isobars and interferences [119], but current commercial IMS instrumentation often lacks the performance necessary to routinely resolve small molecule isomers with minor structural differences, including stereoisomers. The ion mobility spectrometry (IMS) technique is able to separate gas-phase ions based on differences in their size, shape, and charge. A distinct advantage to IMS is its short analysis time. But no method is described that allows the separation of 3α- and 3β-25(OH)D_3. The use of a chiral modifier may support the separation of these epimers in ion mobility spectrometry.

The development of new methods to measure free non-bonded vitamin D are limited [58,120–124]. The MDBP derivatization approach also allows determination of solely free vitamin D since no extraction with organic solvents, such as acetonitrile which is commonly used to release vitamin D from the vitamin D binding protein, is used.

Determination of free non bonded vitamin D in saliva could be a new tool for assessing vitamin D status [58], because the vitamin D values in saliva may better reflect the true vitamin D status in humans. The correlation between circulating vitamin D binding protein, total, free and bioavailable 25-hydroxyvitamin D and disease and healthy states must be clarified in the future. Recent research confirmed that measured levels of free and total 25-hydroxyvitamin D were strongly correlated [122].

Hypothetically it would be possible to develop a new method for saliva to assess vitamin D deficiency in a few seconds with an easy device, which enables individual self-monitoring, such as is routinely performed with insulin measuring instruments.

This idea of self-monitoring vitamin D levels can also transferred to blood analysis, but the potential method to measure a sample without any puncture or injury will be more readily accepted by patients. In addition risk of infection or injury is higher compared to a saliva-based method.

In summary, I am convinced that the field of metabolomic chemotyping, in particular vitamin D chemotyping will greatly expand in the near future and will help us to explain phenomena, which we did not understand for decades, or heal diseases, which are currently untreatable. In the future, the aim should be focused on the assessment of the early health status of the individual patient with all relevant metabolites, and surveillance of these values of whole metabolomic fingerprint through the individual's life to monitor the changes to maintain a healthy state and prevent or minimize disease states. This new approach will help to easily pinpoint metabolomic errors and understand the formation of phenotypes.

Recently published articles about vitamin D and cancer captured my attention. Ultraviolet radiation (UVB) acts as a double edged sword inducing skin cancer and producing vitamin D. The authors described in detail the inverse correlation of development of skin cancer and the production of vitamin D, for both processes UVB is the responsible effector[125]. The need of UVB radiation for production of vitamin D and the fear and danger of developing skin cancer leads to a big challenge for humans. Surprisingly the bioactive calcitriol (1,25(OH)2D) and other bioactive interesting CYP11A1-derived hydroxyderivatives of vitamin D showed anti-melanoma activities and protective properties such as photo protective and anti-carcinogenic effects such as inhibitory effects on proliferation, plating efficiency and anchorage-independent growth of cultured human melanomas *in vitro*, as well as *in vivo* inhibition of tumor growth by vitamin D (20(OH)D) [125]. In summary, biologically active vitamin D can effect melanoma genesis and disease progression. This idea expands to include vitamin D in melanoma management because of the stated beneficial effects for patients. Using vitamin D for melanoma therapy is a future consideration, but the question is still which form of active vitamin D is suitable would be most effective. A new creative approach is necessary to avoid or reduce toxic side effects and very expensive checkpoint immunotherapy in the fight against cancer.

V. SUMMARY AND CONCLUSIONS

The vision is to control tumor growth and to obtain a positive disease developement by using biologically active vitamin D as therapeutic approach. Vitamin D seems to be an important effector for cancer development and cancer growth. The complicated situation of danger and need of UVB radiation can solve by vitamin D supplementation to avoid the risk of non-protective sun exposure. The protection of sun exposure is obtained by using sun creams, but this leads to non-production of vitamin D in the skin. It would be perfect to develop a sun cream, which already contains preformed vitamin D and can diffuse through the skin and regulate the healthy vitamin D metabolism while maintaining the same protection as normal sun cream to avoid or minimize the risk of skin cancer. Such a sun cream shows an important benefit of vitamin D production without supplementation. The early prevention can start with such a sun creme containing vitamin D to prevent skin cancer and to regulate vitamin D metabolism. Importantly, if cancer breaks out independent of location in human body, vitamin D has in general anti-melanoma effects to the cancer growth and disease, therefore a high vitamin D level can reduce or mitigate the progression of disease and should be considered as a therapeutic approach in the future. Different ways in maintaining a healthy state and treatment of cancer should be established with the aim to obtain and retain the individual or personalized vitamin D level, to reduce the use of toxic therapeutics with many side effects for patients. Sources of vitamin D could be supplements, nourishments or maybe in the future topical creams.

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VII. LIST OF PUBLICATIONS

The results of the dissertation have been published in the following peer-reviewed journals:

1. M.J. Müller, C.S. Stokes, F. Lammert, D.A. Volmer, Chemotyping the distribution of vitamin D metabolites in human serum, *Sci Rep.*, **2016**, 6, 21080.

2. M.J. Müller, C.S. Stokes, D. A. Volmer, Quantification of the 3α and 3ß epimers of 25-hydroxyvitamin D3 in dried blood spots by LC-MS/MS using artificial whole blood calibration and chemical derivatization, *Talanta*, **2017**, 167, 398-404.

3. M.J. Müller, H. Bruns, D.A. Volmer, Direct aqueous measurement of 25-hydroxyvitamin D levels in a cellular environment by LC-MS/MS using the novel chemical derivatization reagent MDBP, *Analytical and Bioanalytical Chemistry*, **2017**,10, 2705-2714.

Additionally, the following review article was included in the dissertation as an introduction to the research field but does not contain any relevant research results:

4. M.J. Müller, D.A. Volmer, Mass Spectrometric Profiling of Vitamin D Metabolites beyond 25-Hydroxyvitamin D, *Clin Chem*., **2015**, 61 (8),1033-48.

PUBLICATION 1

Mass Spectrometric Profiling of Vitamin D Metabolites beyond 25-Hydroxyvitamin D

M.J. Müller, D.A. Volmer.

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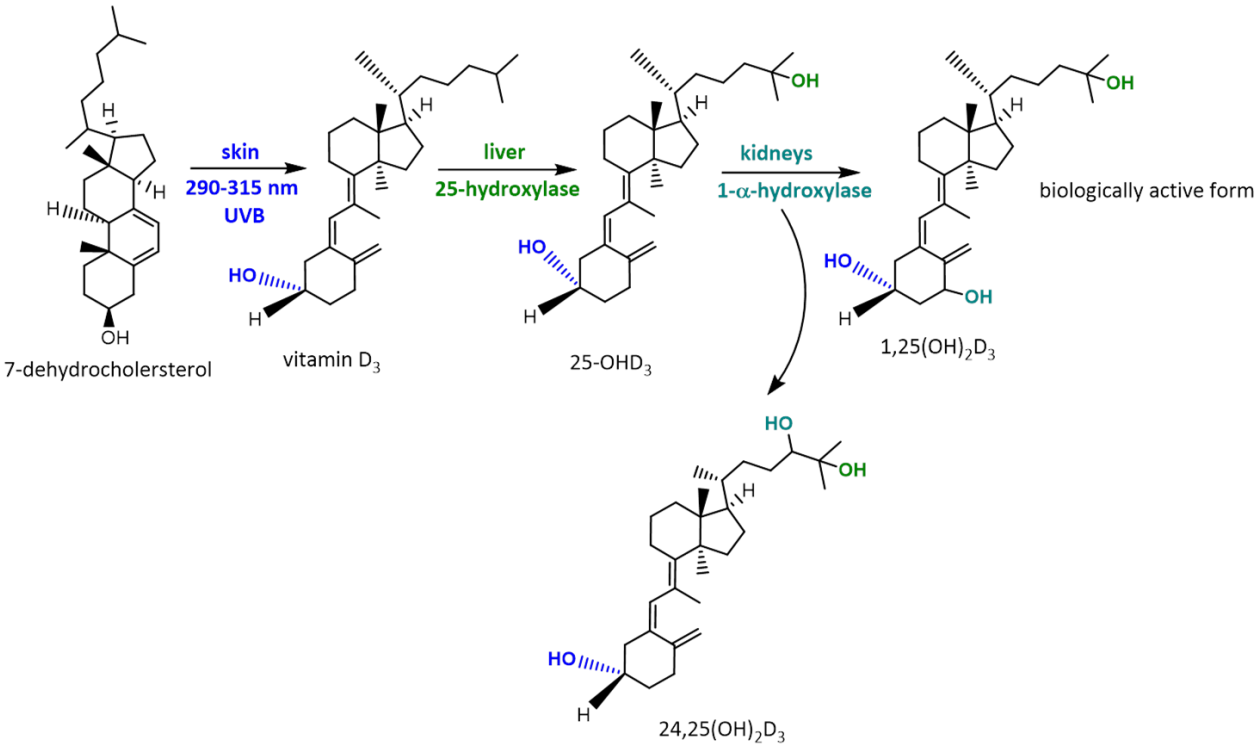

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Review

Mass Spectrometric Profiling of Vitamin D Metabolites beyond 25-Hydroxyvitamin D

Miriam J. Müller[1] and Dietrich A. Volmer[1*]

BACKGROUND: The frequency of measurements of vitamin D in the human population has significantly increased over the last decade because vitamin D has now been linked to many diseases, in addition to its established role in bone health. Usually, serum 25-hydroxyvitamin D concentrations are measured to assess the vitamin D status of individuals. Unfortunately, many studies investigating links between vitamin D and disease also use only this single metabolite. Intricate correlations with other vitamin D metabolites or dynamic effects of downstream metabolites may therefore be overlooked. Fortunately, powerful LC-MS/MS approaches have recently become available that can simultaneously quantify the concentrations of multiple vitamin D metabolites. These approaches are challenging, however, because of inherent instrumental problems with detection of vitamin D compounds and the low concentrations of the metabolites in biological fluids.

CONTENT: This review summarizes recent mass spectrometry assays for the quantitative measurement of multiple vitamin D metabolites and their application in clinical research, with a particular focus on the low-abundance downstream metabolic species generated after the initial hydroxylation to 25-hydroxyvitamin D.

SUMMARY: To study the pathobiological effects and function of vitamin D metabolites in disease, in particular in low-abundance species beyond 25-hydroxyvitamin D, we need to know their concentrations. Although detection of these vitamin D species is challenging, a number of recent mass spectrometry assays have successfully demonstrated that LC-MS/MS methods can quantify multiple vitamin D compounds over a wide dynamic range individually or as part of multimetabolite assays.

The term vitamin D describes a large class of secosteroids, of which the 2 most important representatives are vitamin D_2 and D_3. While chemically very similar, both vitamers have entirely different biological origins; whereas D_3 is photosynthesized in mammals in their skin *(1)*, D_2 mainly occurs in mushrooms *(1)*. Here we use the generic term vitamin D to describe the "human species" D_3. D_2 is, of course, also active in humans and is sometimes used as food supplement and, therefore, D_2 is always specifically labeled in this review. Vitamin D is best known for its vital role in bone health *(1)*, but it has been linked to a much wider range of diseases, including diabetes, cancer, multiple sclerosis, depression, and cardiovascular diseases *(2)*.

Traditionally, the measurement of vitamin D compounds has been performed using methods such as immunoassays *(3)* or liquid chromatography *(4)*; in fact, 90% of routine analyses today are performed by immunoassay techniques. In recent years, LC-MS/MS has been established as the gold standard technique for vitamin D analysis because of the technique's inherent analytical specificity and sensitivity *(5)*. Measurements of vitamin D species from biological fluids such as plasma or serum using mass spectrometry are not without challenges, however, for several reasons, including the lipophilic nature of the analytes, their tight binding to the carrier protein (e.g., vitamin D binding protein [DBP][2]), abundant isobaric and isomeric interferences in serum/plasma *(6)*, and the low ionization efficiencies for mass spectrometric analyses *(7)*. As a result, LC-MS/MS assays described in the literature for vitamin D compounds often permit only quantitative analysis of the main metabolite, 25-hydroxyvitamin D [25(OH)D], whereas detection capability is too low to access the required physiological concentrations of low-abundance metabolites such as

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[2] Nonstandard abbreviations: DBP, vitamin D binding protein; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, active 1,25-dihydroxyvitamin D; 1-OHase, 25-hydroxylase D-1α-hydroxylase; FGF-23; fibroblast growth factor 23; 24-OHase, 25-hydroxyvitamin D-24 hydroxylase; PP, protein precipitation; LLE, liquid–liquid extraction; SPE, solid phase extraction; MRM, multiple reaction monitoring; ESI, electrospray ionization; APCI, atmospheric-pressure chemical ionization; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; 3-epi-$1,25(OH)_2D$, C-3 epimer of $1,25(OH)_2D$; LLOQ, lower limit of quantification; UHPLC, ultra-high-performance liquid chromatography; ρ_c, Lin concordance correlation coefficient; LOD, limit of detection; C-18, cctadecyl; CN, cyano; IDS, Immunodiagnostic Systems; PFP, pentafluorophenyl; HL-60, human promyelocytic leukemia cells; 25(OH)DS, vitamin D-3β-sulfate; CKD, chronic kidney disease; FGF23, fibroblast growth factor 23; QqQ, triple quadrupole; DAPTAD, 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione; *d*-DAPTAD, d_6- 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione; PBMC, peripheral blood mononuclear cell; APPI, atmospheric pressure photoionization.

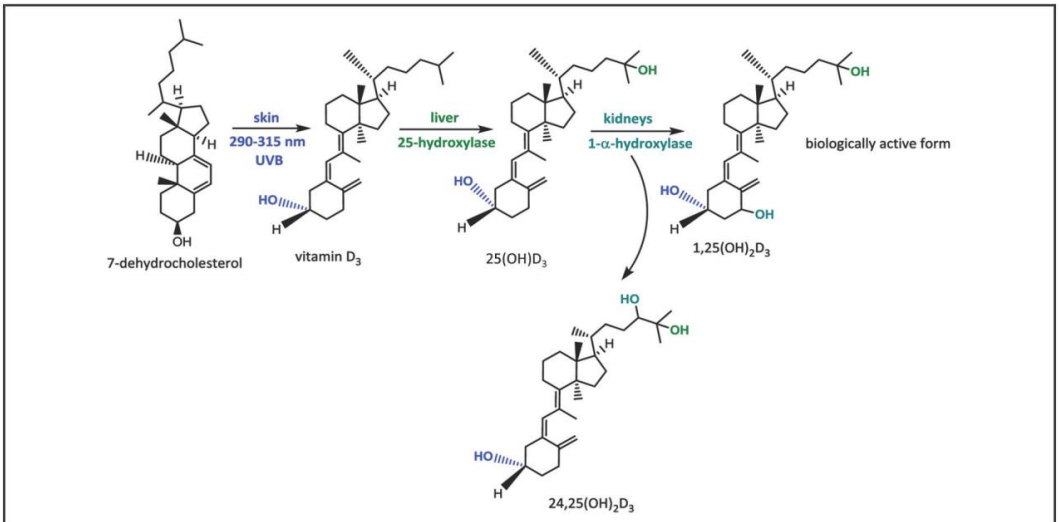

Fig. 1. Abbreviated metabolic pathway for vitamin D.

1,25-dihydroxyvitamin D [1,25(OH)$_2$D] in human serum or plasma.

Ideally, a true gold standard method for all vitamin D metabolites would cover all relevant high- and low-abundance species, using a universal and standardized analytical method. Although no simple assay solution for this purpose presently exists, several promising techniques have been reported over the last few years that demonstrate the future potential of mass spectrometry for comprehensive profiling of vitamin D metabolites *(8, 9)*. The role of LC-MS/MS in the determination of 25(OH)D has recently been reviewed by us *(7)*. The present review focuses on the mass spectrometric analysis of low-abundance vitamin D species beyond 25(OH)D and its future role in profiling and applications as well as clinical metabolomics.

Vitamin D Metabolism

The generation of vitamin D starts in the skin by sunlight photosynthesis. UVB radiation catalyzes conversion of 7-dehydrocholesterol to previtamin D, which then thermally converts to vitamin D. Excessive sun exposure degrades previtamin D and vitamin D into inactive photoproducts *(1)*. Vitamin D compounds predominantly bind to DBP, and to a lesser extent to albumin and lipoproteins, for circulation *(10)*. Vitamin D metabolism generates a number of different metabolites over a wide dynamic concentration range *(2, 11)*.

The first step in the metabolic pathway of vitamin D is conversion to 25(OH)D (Fig. 1) by vitamin D-25-hydroxylase. Although 25(OH)D is the major circulating form of vitamin D, it is biologically inactive. It then undergoes further oxidation in the kidney to the active 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] via 25-hydroxylase D-1α-hydroxylase (1-OHase). Renal production is influenced by multiple factors, including concentrations of serum phosphorus, calcium, and fibroblast growth factor 23 (FGF-23). 1,25(OH)$_2$D also regulates its own synthesis through negative feedback; it also influences secretion of parathyroid hormone and expression of 25-hydroxyvitamin D-24 hydroxylase (24-OHase) *(1)*.

The human metabolism of vitamin D has been extensively studied, and >50 of circulating vitamin D metabolites have been described *(12)*. The actual number is even higher because of additional biochemical processes such as epimerization and lactonization *(11)*. As mentioned above, 25(OH)D is used to determine the vitamin D status of an individual, because only 25(OH)D has sufficiently high concentrations to be measured by routine assays and a sufficiently long half-life (approximately 19 days) in circulation to reflect sufficiency or deficiency in individuals *(1)*. Other downstream metabolites are often transient species that are usually present at much lower concentrations.

Importantly, to study the pathobiological role of vitamin compounds—including important low-abundant species—we need to know their concentrations. Therefore, we require very sensitive analytical methods to quantify the different vitamin D metabolites in relevant human tissues, which at the same time are selective enough to differentiate the various metabolic species.

Limitations of LC-MS/MS Assays

Current LC-MS/MS assays for vitamin D and its metabolites exhibit several areas of concern with the potential to negatively affect detection sensitivity, accuracy, and imprecision. These primarily relate to protein binding, ionization properties, and stabilities and interferences from isomers and isobars, as recently reviewed *(7)*. Briefly, the endogenous properties of vitamin D, such as its lipophilic nature and tight binding to DBP and albumin, have the potential to cause substantial matrix effects during sample extraction, which is usually performed using protein precipitation (PP), liquid–liquid extraction (LLE), solid-phase extraction (SPE), or combination of these techniques *(13, 14)*. Stable isotope standards of vitamin D compounds are essential to correct for analytical issues caused by ion suppression effects *(7)*. The isomeric and isobaric noise present in samples such as serum or plasma can lead to overestimation of 25(OH)D concentrations if compounds are not properly separated by liquid chromatography or if unspecific transitions are used for multiple reaction monitoring (MRM) during LC-MS/MS. Qi et al. recently provided a comprehensive characterization of isobaric and isomeric interferences in human serum, which have the potential to influence measured levels of 25(OH)D *(6)*.

Similarly, exogenous compounds such 1α-hydroxyvitamin D_3 can inflate measured 25(OH)D concentrations when given to patients during management of secondary hyperparathyroidism in chronic kidney disease *(13)*. Finally, the C-3 epimer, 3-epi-25(OH)D, will also give overestimated 25(OH)D concentrations if not properly resolved by chromatography (vide infra) *(7, 15, 16)*.

Furthermore, because these molecules exhibit low ionization efficiency under electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) conditions, which are the 2 most often applied ionization techniques for vitamin D analysis by LC-MS/MS, detection limits obtained for vitamin D metabolites by LC-MS/MS are often not sufficiently low to access physiological concentrations of low-abundant vitamin D compounds. This often limits LC-MS/MS analysis to measurement of 25(OH)D in clinical assays. To improve ionization properties, derivatization can be applied to introduce chemical functionalities that promote ionization *(17)*, as well as shifting detected *m/z* values to higher ranges, where chemical noise in ESI and APCI-MS is significantly lower. Usually, Diels Alder reactions using Cookson-type reagents [e.g., 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD)] are implemented for this purpose, giving reported increases of response of factor 100–1000 over the nonderivatized compounds *(17)*. PTAD has been shown to exhibit limited stability *(18)*; improved versions were reported by Higashi et al. *(17)*.

The same group also described an isotope-coded differential analog of PTAD for relative quantification *(19)*. Furthermore, a commercial derivatization reagent (Amplifex) is now available, which possesses a permanently charged quaternary ammonium group that substantially improves ionization efficiency *(20)*.

Analysis of Individual Vitamin D Species

In this section, we briefly review individual LC-MS/MS methodologies for quantitative analysis of important low-abundant vitamin D species, including $1,25(OH)_2D$, 3-epi-25(OH)D, $24,25(OH)_2D$, and other dihydroxylated species as well as transformation and conjugation products such as lactones and sulfates. The actual vitamin D species, i.e., the precursor compound before hydroxylation in the liver, has rarely been measured, except for a few studies concerned with breastfeeding mothers *(21)*.

25(OH)D

The quantitative analysis of the main metabolite 25(OH)D has recently been thoroughly reviewed *(7)* and assays limited to 25(OH)D are therefore not included here.

$1,25(OH)_2D$

$1,25(OH)_2D$ is the biologically active form of vitamin D (Fig. 1). As seen with 25(OH)D, epimerization can lead to high concentrations of the C-3 epimer, 3-epi-$1,25(OH)_2D$, in samples *(22)*. Also, 3-epi-$1,25(OH)_2D_2$ must be considered if vitamin D_2 was supplemented. The main role of $1,25(OH)_2D$ is the maintenance of calcium and phosphorus concentrations in blood *(1)*. For clearance, $1,25(OH)_2D$ degrades into several water soluble products with calcitriol acid as the final product excreted into bile. This catabolism leads to intermediates of vitamin D with progressively higher water solubility and lower or negligible biological activity *(23)*.

Concentrations of $1,25(OH)_2D$ are often measured for diagnosis of renal diseases and hypercalcemic syndromes associated with chronic granulomatous disorders as well as inborn and acquired disorders of 25(OH)D metabolism *(24)*. The mass spectrometry measurement problems discussed in the previous section equally apply to this vitamin D species, in particular the low ionization efficiency, which was improved by derivatization in one study *(19)*. The problem is further amplified by the short half-life of circulating $1,25(OH)_2D$ of only 4–6 h and the low physiological concentrations in the picomolar range *(2)*. In the case of $1,25(OH)_2D$, particular care must be taken to chromatographically separate it from other dihydroxylated vitamin D species [e.g., $23,25(OH)_2D$, $25,26(OH)_2D$, $24,25(OH)_2D$, and 4β,$25(OH)_2D$], since these isomers

exhibit the same molecular weight and mass-to-charge ratios of the $[M+H]^+$ precursor compounds, and will give very similar product ion spectra upon collision-induced dissociation.

Kissmeyer et al. reported an LC-MS/MS assay for $1,25(OH)_2D$ in rat and pig serum. The authors used adduct formation with ammonium to achieve a lower limit of quantification (LLOQ) of 48 pmol/L from 1 mL of serum *(25)*. More recently, quantification of $1,25(OH)_2D$ in human serum was reported by Casetta et al., who used stable Li^+ adducts to improve LLOQ values to 36 pmol/L with acceptable CVs of 5%–15% *(26)*.

Because of the poor ionization efficiencies seen in ESI and APCI analysis from the lack of readily ionizable polar groups, Aronov et al. implemented a Cookson-type reagent, to obtain an LLOQ of 25 pmol/L for $1,25(OH)_2D$ using ultra–high-performance liquid chromatography (UHPLC)–ESI-MS/MS *(18)*. Duan et al. demonstrated that ionization efficiency and stability of the PTAD–vitamin D metabolite improved appreciably over the unmodified compounds *(27)*. The method exhibited accurate (error <13.8%) and precise (CV <14.1%) concentration data, with an LLOQ of 5 pg/mL using an ESI triple-quadrupole mass spectrometer. Hedman et al. implemented the commercial derivatization reagent Amplifex *(20)* for 20 patient samples and compared Amplifex to PTAD and a standard RIA. Using only 200 μL of serum, the limit of detection (LOD) was reported at 2 pg/mL. For comparison with RIA, they combined the derivatization results of vitamin D_2 and D_3 because the RIA does not distinguish the 2 forms. A Lin concordance correlation coefficient (ρ_c) was determined for Amplifex/RIA comparison (ρ_c= 0,92; n = 20) and Amplifex/PTAD comparison (ρ_c= 0,88 n = 12). The Amplifex method generally correlated well with RIA and PTAD methods, with limitations at the low concentration standards, where the PTAD method was not as sensitive as Amplifex. In contrast, LODs for PTAD were 15 pg/mL for $1,25(OH)_2D_3$ and 30 pg/mL for $1,25(OH)_2D_2$.

The general assay performance can also be increased by improving sample preparation via analyte enrichment by immunoaffinity extraction *(28)*. Yuan et al. used an ImmunoTube LC-MS/MS kit from Immundiagnostik for affinity purification to remove isobaric and other matrix interferences present in human serum *(28)* and obtained LLOQ of 8.2 pmol/L for $1,25(OH)_2D_3$ and 9.1 pmol/L for $1,25(OH)_2D_2$. An even lower LLOQ of 3 pmol/L was achieved by Strathmann et al. *(29)* after protein precipitation and affinity extraction using solid-phase-bound anti-$1,25(OH)_2D$ monoclonal antibody from Immunodiagnostic Systems (IDS) and derivatization with PTAD. Chan and Kaleta recently developed a quantitative LC-MS/MS method for $1,25(OH)_2D_{2/3}$ using solid-phase extraction and Amplifex derivatization. These authors compared the performance to an immunoextraction assay (ALPCO ImmunoTube $1,25(OH)_2$ vitamin D extraction kit) *(30)*. The LOD using SPE-Amplifex was 1.9 pg/mL for $1,25(OH)_2D_2$ and 2.7 pg/mL for $1,25(OH)_2D_3$, respectively; the LOQ was 4 pg/mL for both metabolites. The affinity extraction method gave an LOD of 2.7 pg/mL for $1,25(OH)_2D_2$ and 1.7 pg/mL for $1,25(OH)_2D$; the LOQ was 4 pg/mL for both metabolites.

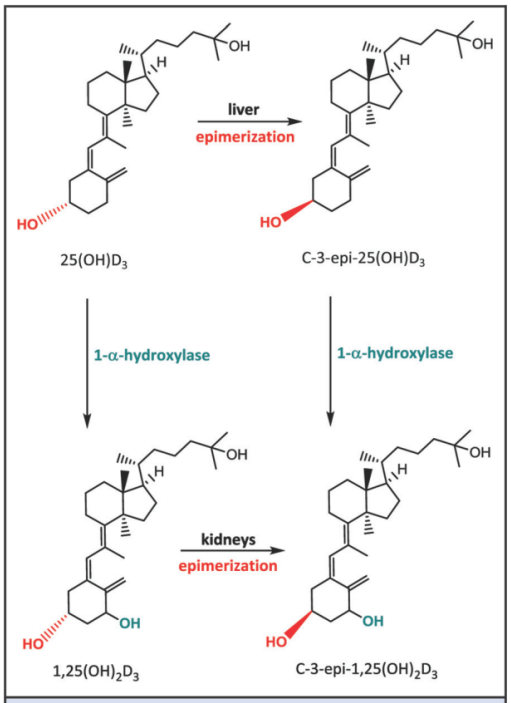

Fig. 2. Epimerization reactions of vitamin D metabolites.

3-EPI-25(OH)D

Additional vitamin D compounds are introduced by epimeric effects (Fig. 2). Enzyme activity appears to be unidirectional and dependent on the presence of hydroxyl groups at C-1 or C-25, independent of cytochrome P450 enzymes (CYP 24, 27A1, and 27B1) and 3(α,β)-hydroxysteroid epimerase *(31)*.

Generally, 3-epi-25(OH)D concentrations decrease with age; concentrations are often high in infants *(32)*. It was hypothesized that hepatic immaturity may play a role in epimer production *(22)*, but substantial concentrations have also been found in adults *(33)*. Lensmeyer et al. analyzed serum from 214 patients (age >20 years) and published concentrations of 3-epi-25(OH)D ranging from 0.25–59.3 nmol/L *(15)*.

As mentioned above, 3-epi-25(OH)D is a stereoisomer of 25(OH)D, and their mass spectrometric behaviors are very similar. The epimer will therefore lead to an overestimation of vitamin D during measurement if not properly separated before mass spectrometric analysis. Interestingly, it was recently shown that the molar ESI response for 3-epi-25(OH)D was higher than that for regular 25(OH)D *(34, 35)*. Importantly, even if the biochemical role of 3-epi-25(OH)D has not yet been clarified, it should be properly differentiated from 25(OH)D and individually measured because its concentrations may be important in the future for diagnostic purposes. Therefore, it is important that 3-epi-25(OH)D quantification not be performed on the basis of the stable isotope standard of 25(OH)D, because this will lead to inaccurate results. Rather, a stable isotope internal standard of 3-epi-25(OH)D, which has recently become commercially available, should be used for this purpose *(35)*.

For liquid chromatographic separation of the epimers, different stationary-phase chemistries have been investigated. Bedner and Phinney developed an LC-APCI-MS/MS method for quantification of 25(OH)D_3, 25(OH)D_2, and 3-epi25(OH)D_3 for SRM 972 and 909c reference materials and compared 3 different HPLC columns, namely cctadecyl (C-18), cyano (CN), and pentafluorophenyl (PFP)*(36)*. The latter column was able to provide better resolution for the pair 25(OH)D_3/3-epi25(OH)D for SRM 909c, which contained matrix components interfering with the deuterated internal standard of 25(OH)D. Singh et al. successfully used a 5-dinitrobenzoyl-(R)-phenylglycine column *(22)*; Lensmeyer et al. implemented a cyanopropyl stationary phase *(15)*, while Shah et al. improved the separation using a chiral column *(13)*. Furthermore, Higashi et al. demonstrated that, after acetylation of the 3-OH group, a conventional C-18 column could be used for separation of the C-3 epimer *(37)*; these authors determined 3-epi-25(OH)D from neonatal dried blood spots after PTAD derivatization. In recent years, PFP columns appear to be the favored choice for separation of the 2 epimers *(29, 38–40)*.

24,25(OH)$_2$D

The dihydroxylated 24,25(OH)$_2$D metabolite is usually considered a catabolic species, the first step in the clearance pathway of 25(OH)D *(41)*. There have been proposals, however, that this metabolite has effects of its own. For example, 24,25(OH)$_2$D and 1,25(OH)$_2$D have been reported to directly stimulate calcification of bone synergistically with PTH *(42)* and that 24,25(OH)$_2$D decreased the number and perimeter of resorption sites in bone *(43)*. Furthermore, a link between increased bone volume and 24,25(OH)$_2$D was reported *(44)* as well as the critical role of 24,25(OH)$_2$D for healing processes in bones and tissues *(45)*. Contributory roles in other pathobiological processes have also been described for 24,25(OH)$_2$D *(46)*.

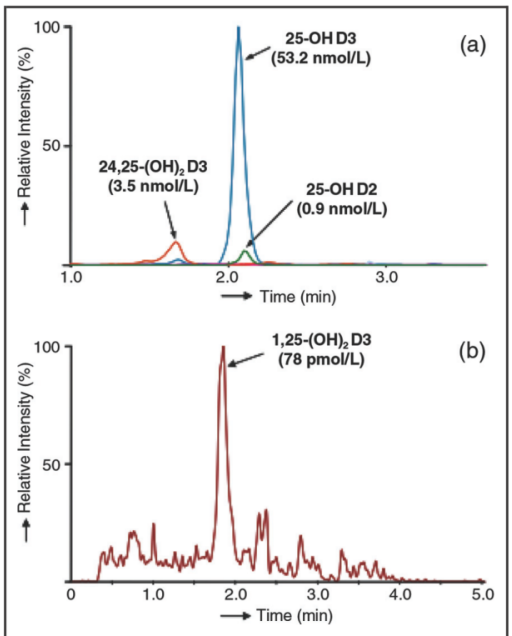

Fig. 3. Typical MRM chromatogram for several vitamin D metabolites from plasma after SPE and PTAD derivatization; (a), vitamin D metabolites 24,25(OH)$_2$D$_3$, 25(OH)D$_3$, and 25(OH)D$_2$; (b), low-abundance vitamin D metabolite 1,25(OH)$_2$ D$_3$ [reprinted with permission from Ding et al. *(14)*].

Analytically, 24,25(OH)$_2$D is much easier to access than 1,25(OH)$_2$D because the circulating concentrations in blood are approximately 1000× higher than those of 1,25(OH)$_2$D *(47)*. The ionization efficiency is, again, low and can be improved by derivatization *(14, 17)*. LC-MS/MS assays for 24,25(OH)$_2$D have been described, usually as part of methods for multiple vitamin D species (Fig. 3). Several individual assays have been reported, however, including studies by Higashi et al., who identified 3-epi-24,25(OH)$_2$-D-24-glucuronide in bile of rats, which were administered pharmacological doses of 24,25(OH)$_2$D *(48)*. The authors separated 3-epi-24(*R*),25(OH)$_2$D and 24(*R*),25-(OH)$_2$D (the latter is the biologically active form) using a cyclodextrin-bonded chiral column *(48)*. Furthermore, 281 multiple sclerosis patients and 22 healthy individuals were analyzed for 1,25(OH)$_2$D and 24,25(OH)$_2$D *(27)*; it was discovered that the concentrations of both compounds were significantly lower in the patients compared to healthy individuals.

Wagner et al. suggested the use of serum 24,25$(OH)_2$D in conjunction with 25(OH)D as a novel marker for 25(OH)D catabolism and predictor of serum 25(OH)D response to vitamin D supplementation *(49)*. Different studies measured 24,25$(OH)_2$D quantitatively in addition to 25(OH)D, with serum concentrations ranging from 0.7 to 24 nmol/L; these concentrations positively correlated with serum 25(OH)D concentrations *(18, 27, 40, 49)*. For all methods, it is important to consider that the natural (*R*)-form and not the (*S*)-epimer is metabolically active *(50)*. Wagner and Tsugawa have reported an LC-MS/MS method for 25(OH)D, with which 24,25$(OH)_2$D was also captured, albeit with suboptimal analytical sensitivity *(49)*. To enhance ionization efficiency, Cookson reagents were implemented; for example, Higashi et al. described a dedicated LC-MS method for 24,25$(OH)_2$D from human plasma using MBOTAD (4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione) and deuterated 24,25$(OH)_2$D as internal standard *(48)*.

OTHER DIHYDROXYLATED SPECIES

In addition to 1,25$(OH)_2$D and 24,25$(OH)_2$D, a few other dihydroxylated species have been measured by mass spectrometry. Wang et al. identified 4β,25$(OH)_2$D at similar concentration levels as compared to 1,25$(OH)_2$D *(51)*. The authors suggested that some literature methods might overestimate 1,25$(OH)_2$D concentrations due to coeluting 4β,25$(OH)_2$D. Yuan et al. demonstrated 23,25$(OH)_2$D as potentially interfering compound, but no clinically relevant concentrations were found in patient samples *(28)*.

25(OH)D-26,23-LACTONE AND 1,25$(OH)_2$D-23,26-LACTONE

Further vitamin D species are generated by 24-hydroxylase during deactivation of 1,25$(OH)_2$D to 1,24,25$(OH)_3$D, which then undergo further metabolism including oxidation and conjugation *(12)*. For example, 1,25$(OH)_2$D can be oxidized to 1,25(OH)D-26,23-lactone and calcitroic acid *(52)*. 25(OH)D-26,23-lactone has received some attention because of high concentrations in plasma of animals that were supplemented with large amounts of vitamin D measured by HPLC-UV (254 nm) using Zorbax Sil silicic acid column *(53)*. 25(OH)D-26,23-lactone suppresses serum calcium concentrations in competition with 1,25$(OH)_2$D *(54)*. Careful experiments by Napoli and Horst have clearly ruled out the possibility that 25(*S*),26$(OH)_2$D was the biosynthetic precursor of the lactone *(53, 55)*. The lactone structure was confirmed by chemical synthesis *(56)*. To our knowledge, no analytical assay exists for determination of 1,25$(OH)_2$D-23,26-lactone and 25(OH)D-26,23-lactone. It is potentially of interest to measure these lactones, however, because 1,25$(OH)_2$D-23,26-lactone was shown to exhibit biological function, including inhibition of human promyelocytic leukemia cells (HL-60) differentiation induced by 1,25$(OH)_2$D *(54)* 1,25$(OH)_2$D-26,23-lactone also stimulated intestinal calcium absorption and significantly decreased serum calcium concentration in rats; as well, 1,25$(OH)_2$D-26,23-lactone increased alkaline phosphatase activity and stimulated collagen synthesis and mineralization in vivo *(54)*.

VITAMIN D SULFATES

Vitamin D-3β-sulfate [25(OH)DS] has been synthesized by Reeve et al. using pyridine sulfur trioxide as the sulfate donor *(57)*. The biological activity of vitamin D sulfate was determined in vitamin D–deficient rats *(58)* and shown to be very low or absent at physiological concentrations; the sulfate exhibited <5% of the activity of vitamin D to mobilize calcium from bone and only approximately 1% to stimulate calcium transport or support bone calcification. Nagubandi et al. administered larger doses of vitamin D sulfate and demonstrated that biological activity increased at higher concentrations *(58)*. Higashi et al. suggested that quantification of 25(OH)DS was potentially helpful in assessing vitamin D status, especially in infants *(19)*. The authors developed a simple and analytically sensitive method for 25(OH)DS in plasma using mixed-mode SPE and LC-MS/MS of the $[M-H]^-$ ions in negative ionization mode. The LLOQ was reported as 2.5 ng/mL *(19)*.

NON-PROTEIN-BOUND VITAMIN D SPECIES

Almost all circulating vitamin D metabolites are bound to plasma proteins, mainly to DBP (approximately 85%) and albumin (approximately 14.6%). Only a small amount is freely available (0.4%) *(59)*. There is increasing interest in the measurement of free, non–protein-bound vitamin D metabolites, because they may better reflect the true vitamin D status in humans. Van Hoof et al. measured non–protein-bound plasma 1,25$(OH)_2$D concentrations *(60)*. These authors developed a symmetric dialysis method by placing identical samples on both sides of a membrane and adding tritium-labeled 1,25$(OH)_2$D to one side. The rate of transfer of the radiolabeled compound through the membrane was then related to the free amount of plasma 1,25$(OH)_2$D *(60)*.

Multimetabolite LC-MS/MS Assays

Currently, most research examining the link between disease and vitamin D focuses on the main vitamin D compound 25(OH)D, and intricate correlations with other metabolites or dynamic effects may therefore be overlooked. Multimetabolite LC-MS/MS methods have the ability to uncover such correlations, because all biologically relevant species are determined simultaneously within a single analysis, thus readily allowing interpretation of the vitamin D metabolite distributions as a func-

tion of factors such as disease phenotype, progression, and treatment procedure. A single multimetabolite method offers the additional advantage of avoiding interassay analytical accuracy and imprecision issues when data from different assays are used for comparisons.

From such a multimetabolite assay, an inverse correlation between 1,25(OH)$_2$D rather than 25(OH)D and pneumonia severity was observed, suggesting greater importance be placed on the biologically active form of vitamin D in these patients *(61)*. A number of studies have shown that serum 3-epi-25(OH)D *(15, 22, 33)* and 24,25(OH)$_2$D *(18, 27, 40, 49)* are highly correlated with 25(OH)D concentrations. Wagner et al. have further demonstrated that the ratio of serum 24,25(OH)$_2$D to 25(OH)D is predictive of 25(OH)D response to supplementation *(49)*. Also, the serum concentration of 24,25(OH)$_2$D, its ratio to other metabolites, and the rate of turnover of 25(OH)D have been proposed as alternative markers of vitamin D status *(62)*. Bosworth et al. have characterized chronic kidney disease (CKD) using metabolic and catabolic vitamin D species. The authors illustrated that chronic CKD is a state of stagnant vitamin D metabolism characterized by decreases in both 1,25(OH)$_2$D production and vitamin D catabolism *(63)*. More recently, a vitamin D supplementation study in patients with CKD reported an inverse correlation between lower 24,25(OH)$_2$D and increased FGF23 concentrations, consistent with declining 24-hydroxylase activity and resistance to induction by FGF23, a CKD risk marker *(64)*. For measuring these metabolic vitamin D fingerprints (vitamin D "chemotypes"), a number of analytical mass spectrometry assays have been described that determine multiple vitamin metabolites simultaneously. A relatively simple task is the differentiation of the various vitamer species of vitamin D. For example, LC-MS/MS assays routinely separate 25(OH)D$_2$ and 25(OH)D$_3$ *(65–67)*. In general, the individual vitamers of all relevant species, such as D$_2$/D$_3$, 25(OH)D$_2$/25(OH)D$_3$ and 1,25(OH)$_2$D$_2$/1,25(OH)$_2$D$_3$, are easily separated on reversed-phase HPLC columns on the basis of differences of hydrophobicity.

More interesting, of course, are profiling approaches that measure as many of the individual metabolites simultaneously as possible in human samples. We have summarized the most important recent studies for quantifying multiple vitamin D species in Table 1. To aid the reader and contrast the different techniques, we have highlighted, for each assay, the vitamin D species monitored, the internal standard that was used, whether or not derivatization was used, the sample type and volume, sample preparation method, HPLC stationary and mobile phases, type of mass spectrometer and ionization technique, *m/z* transitions if multiple reaction monitoring (MRM) implemented, linear dynamic range, LLOQ, and precision of analysis.

In general, almost all of the assays in Table 1 use ESI for ionization and triple-quadrupole (QqQ) instruments as the mass analyzer. Most authors have used relatively unspecific H$_2$O loss transitions in MRM for reasons of signal intensity *(7)*. Improved HPLC separation was often achieved using PFP columns as compared to the commonly used C-18 columns in other areas such as drug metabolism. Protein precipitation was initially carried out in all assays, followed mostly by SPE, with a few authors preferring LLE. The reported LLOQ values ranged from 62 nmol/L to 3.671 pmol/L. Challenges during method development arose for separation of the vitamin D metabolites if derivatization chemistry was employed, as recently shown by Burild et al. *(68)*. These authors were not able to separate PTAD-derivatized 3-epi-25(OH)D from 25(OH)D using different stationary phases (C-18, PFP). PFP phases have been reported to enhance selectivity for 3-epi-25(OH)D, but this has been demonstrated only for the native, underivatized vitamin D metabolites *(39)*.

An entirely new approach was the use of isotope-coded derivatization reagents: 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) and its isotope-coded analog: $^2H_4$-DAPTAD [*d*-DAPTAD]), which was developed by Ogawa et al. for differential quantification of urinary vitamin D metabolites using LC-ESI-MS/MS. The method was successfully applied to urine samples and detected increases of urinary 25(OH)D and 24,25(OH)$_2$D concentrations after vitamin D supplementation (Fig. 4). The study was performed with 8 healthy male study participants (age 22–35 years) after daily oral administration of 1000 IU vitamin D for 7 days *(69)*.

Almost all assays in Table 1 used human biofluids, except the method by Lipkie et al. for vitamin D$_2$, vitamin D$_3$, 25(OH)D$_2$, and 25(OH)D$_3$ in soft tissues, which addressed distribution within rat tissues. These authors analyzed liver, gastrocnemius muscle, and epididymis fat. The 25(OH)D concentrations did not differ significantly between tissues, but vitamin D concentrations were higher in epididymis fat than in gastrocnemius muscle and liver *(70)*.

Finally, 2 studies directly measured multiple low-abundance vitamin D species from serum without using signal-enhancing immunoaffinity extraction or derivatization. A fully automated measurement assay for analysis of D$_2$/D$_3$, 25(OH)D$_2$/D$_3$, 1,25(OH)$_2$D$_2$/D$_3$, and 24,25(OH)$_2$D$_3$ from human serum (spiked pooled human serum) was shown by Mena-Bravo et al., who performed hyphenated on-line SPE of serum and LC-MS/MS *(71)*. Although the excellent quantitative performance, particularly for the dihydroxylated vitamin D species, was not specifically mentioned by the authors, we suggest that this was likely due to the focused elution bands of chromatographic peaks achieved by the online

Table 1. Summary of the most important recent studies for quantifying multiple vitamin D species.

	Vitamin D species	Internal standard	Derivatization	Sample type amount	Sample treatment	HPLC column	Instrument	Mobile phase	Ionization	*m/z* Transition	Linear range, LLOQ	Precision % RSD	Reference
1 2 3 4 5	25(OH)D_3 25(OH)D_2 24,25$(OH)_2D$ 1,25$(OH)_2D_3$ 1,25$(OH)_2D_2$	$^2H_6$-25(OH)D_3 $^2H_6$-1,25$(OH)_2D_3$	PTAD	Serum 500 μL	PP, SPE, LLE	C-18	QqQ	(A) MeCN:H_2O (10:90 v/v) + 0.1% FA[a]	ESI	1. 558 → 298 2. 570 → 298 3. 574 → 314 4. 586 → 314 5. 574 → 298	Linear range: undisclosed LLOQ: 1. 62 pmol/L 2. 61 pmol/L 3. 60 pmol/L 4. 58 pmol/L 5. 60 pmol/L	Intraday: 1.6%-4.8% Interday: 5%-16%	Aronov et al. 2008 *(18)*
1 2 3	25(OH)D_3 25(OH)D_2 24,25$(OH)_2D$	$^2H_6$-25(OH)D_3	None	Plasma 100 μL	PP, SPE	C-18	QqQ	MeOH:H_2O (95:5 v/v)	APCI	1. 401 → 257 2. 413 → 355 3. 417 → 363	Linear range: 1. 62-250 nmol/L 2. 61-242 nmol/L 3. 60-240 nmol/L LLOQ: 1. 62 nmol/L 2. 61 nmol/L 3. 60 nmol/L	Intraday: 2.5%-5.7% Interday: 4.5%-5.1%	Tsugawa et al. 2005 *(67)*
1 2 3 4 5	Vitamin D_3 Vitamin D_2 25(OH)D_3 25(OH)D_2 1,25$(OH)_2D_3$	$^2H_6$-25(OH)D_3	None	Serum 1 mL	PP, LLE	C-18	QqQ	(A) MeCN:H_2O (90:10 v/v) 5 mmol/L ammonium formate (B) MeOH 5 mm ammonium formate	ESI	1. 385 → 159 2. 397 → 159 3. 383 → 159 4. 413 → 395 5. 399 → 226	Linear range: 1. 2.6-1302 nmol/L 2. 2.5-1260 nmol/L 3. 2.5-1250 nmol/L 4. 2.4-1213 nmol/L 5. 1.2-60.1 nmol/L LLOQ: 1. 0.8 nmol/L 2. 1.5 nmol/L 3. 1.5 nmol/L 4. 1.0 nmol/L 5. 1.2 nmol/L	Intraday: 3.17%-6.76% Interday: 5.07%-11.53%	Priego Capote et al. 2007 *(73)*
1 2 3 4 5	25(OH)D_3 25(OH)D_2 1,25$(OH)_2D_3$ 1,25$(OH)_2D_2$ 24,25$(OH)_2D$	$^2H_6$-25(OH)D_3 $^2H_6$-25(OH)D_2 $^2H_6$-1,25$(OH)_2D_3$ $^2H_6$-1,25$(OH)_2D_2$	PTAD	Plasma 200 μL, 50 μL	PP, SPE	C-18	QqLIT	(A) 0.1% FA in H_2O 5 mmol/L ammonium formate (B) 0.1% FA in MeOH	ESI	1. 607 → 289 2. 619 → 289 3. 623 → 289 4. 623 → 314 5. 635 → 314	1. 6.2-750 pmol/L 2. 6.1-727 pmol/L 3. 0.2-720 pmol/L 4. 0.5-120 pmol/L 5. 0.2-117 pmol/L LLOQ: 1. 25 pmol/L 2. 24 pmol/L 3. Undisclosed 4. 48 pmol/L 5. 23 pmol/L	Intraday: 1.6%-4.1% Interday: 3.7%-6.8%	Ding et al. 2010 *(14)*

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Table 1. Summary of the most important recent studies for quantifying multiple vitamin D species. (*Continued from page 1040*)

	Vitamin D species	Internal standard	Derivatization	Sample type amount	Sample treatment	HPLC column	Instrument	Mobile phase	Ionization	*m/z Transition*	Linear range, LLOQ	Precision % RSD	Reference
1	25(OH)D_3	$^2H_6$-25(OH)D_3	PTAD	Serum 200 μL	PP, SPE	C-18	QqQ	(A) 2 mmol/L ammonium formate in 60% MeOH (B) 2 mmol/L ammonium formate in 97% MeOH	ESI	1. 558 → 289 2. 570 → 289 3. 574 → 289 4. 574 → 314	Linear range: 1. 0.25–125 nmol/L 2. 0.24–121 nmol/L 3. 0.12–60 nmol/L 4. 0.012–6 nmol/L LLOQ: 1. 0.25 nmol/L 2. 0.24 nmol/L 3. 0.12 nmol/L 4. 0.012 nmol/L	Intraday: 4.3%–12.2% Interday: 3.5%–14.1%	Duan et al. 2010 (27)
2	25(OH)D_2	$^2H_6$-1,25(OH)$_2D_3$											
3	1,25(OH)$_2D_3$												
4	24,25(OH)$_2$D												
1	25(OH)D_3	d$_3$-Stanozolol[c]	None	Serum 1 mL	PP, LLE	C-18 x chiral[b]	QqQ	(A) 0.1% FA in MeCN (B) 0.1% FA in H_2O	ESI	(1 + 2 + 4 + 5) 401 → 383 365 159 3. 413 → 395 377 355	Linear range: 1. 1.3–210.6 nmol/L 2. Undisclosed 3. 1.2–204.5 nmol/L LLOQ: Undisclosed	Intraday: 2.1%–9.1% Interday: 4.1%–12.1%	Shah et al. 2011 (13)
2	3-epi-25(OH)D_3												
3	25(OH)D_2												
4	1-α(OH)D_3												
5	7-α(OH)4-cholesten-3-one												
1	25(OH)D_3	d$_3$-Stanozolol[c]	None	Serum 1 mL	PP, LLE	C-18 x chiral[b]	QqQ	(A) ACN + 0.3% FA (B) ACN + 0.1% FA 2 mmol/L ammonium acetate	ESI	1. 401 → 383 365 159 2. 403 → 385 367 159 3. 401 → 377 355 383 4. 413 → 395 377 355 5. 399 → 135 381 6. 411 → 151 7. 385 → 159 8. 397 → 159	Linear range: 1. 0.5–100 nmol/L 2. 0.1–100 nmol/L 3. 0.1–100 nmol/L 4. 0.1–100 nmol/L 5. 0.015–1 nmol/L 6. 0.015–1 nmol/L 7. 0.5–100 nmol/L 8. 0.5–100 nmol/L LOD: 1. 0.025 nmol/L 2. 0.025 nmol/L 3. 0.05 nmol/L 4. 0.05 nmol/L 5. 0.010 nmol/L 6. 0.010 nmol/L 7. 0.05 nmol/L 8. 0.05 nmol/L	Intraday: 1.3%–11.3% Interday: 2.2%–13.3%	Shah et al. 2012 (72)
2	25(OH)D_2												
3	3-epi-25(OH)D_3												
4	3-epi-25(OH)D_2												
5	1,25(OH)$_2D_3$												
6	1,25(OH)$_2D_2$												
7	Vitamin D_3												
8	Vitamin D_2												

Continued on page 1042

Table 1. Summary of the most important recent studies for quantifying multiple vitamin D species. (*Continued from page 1041*)

	Vitamin D species	Internal standard	Derivatization	Sample type amount	Sample treatment	HPLC column	Instrument	Mobile phase	Ionization	*m/z Transition*	Linear range, LLOQ	Precision % RSD	Reference
1	1,25(OH)$_2$D$_3$	^{2}H$_6$-1,25(OH)$_2$D$_3$	None	Serum 500 μL	Immunoaffinity extraction 1. Immuno Tubes 2. IDS antibody and SPE	C-18	QqQ	(A) MeOH:H$_2$O 50 mmol/L lithium acetate (70:29:1 v/v/v) (B) MeOH 50 mmol/L lithium acetate	ESI	1. 423 → 369 2. 435 → 381 3. 423 → 367 4. 423 → 367 5. 423 → 369 349	Linear range: 1. 8-495 pmol/L 2. 9-496 pmol/L LLOQ: 1. 8 pmol/L	Intraday: 2.5%-5% Interday: 3%-6.4%	Yuan et al. 2011 (*28*)
2	1,25(OH)$_2$D$_2$	^{2}H$_6$-1,25(OH)$_2$D$_2$											
3	24S,25(OH)$_2$D												
4	24R,25(OH)$_2$D												
5	23R,25(OH)$_2$D												
1	25(OH)D$_3$	^{2}H$_6$-25(OH)D$_3$	PTAD	Plasma 1 mL	PP, LLE	C-18	QqQ	(A) 0.1% FA in H$_2$O (B) MeCN	ESI	1. 558 → 298 2. 574 → 298 3. 574 → 314 4. 574 → 314	Linear range: 1. 6.2-200 nmol/L 2. 1.2-38 nmol/L 3. 0.06-1.9 nmol/L LLOQ: 1. 0.13 nmol/L 2. 0.12 nmol/L 3. 0.06 nmol/L	Intraday: 1.1%-1.2.4% Interday: 1%-13.8%	Wang et al. 2011 (*51*)
2	24,25(OH)$_2$D												
3	1,25(OH)$_2$D$_3$	^{2}H$_6$-1,25(OH)$_2$D$_3$											
4	β,25(OH)$_2$D												
1	25(OH)D$_3$	^{2}H$_6$-25(OH)D$_3$	None	Serum 100 μL	PP, LLE	PFP	QqQ	MeOH:H$_2$O (72:28 v/v)	APCI	1. 383 → 365 2. 383 → 365 3. 395 → 377	Linear range: 1. 12-150 nmol/L 2. 4-50 nmol/L 3. 8-100 nmol/L LLOQ: Undisclosed	Total imprecision: ≤10% when concentrations ≥20 nmol/L	Schleicher et al. 2012 (*39*)
2	3-epi-25(OH)D$_3$	2H$_3$-25(OH)D$_2$											
3	25(OH)D$_2$												
1	25(OH)D$_3$	^{2}H$_6$-25(OH)D$_3$	None	Serum 200 μL	PP, SPE	PFP	QqQ	MEOH + 0.5 mmol/L ammonium acetate	APCI	1. 401 → 159 2. 413 → 159 3. 417 → 381 4. 401 → 159	Linear range: 1. 4-265.3 nmol/L 2. 3.9-183.6 nmol/L 3. 2.8-129.9 nmol/L 4. 2.0-133.8 nmol/L LLOQ: 1. 4 nmol/L 2. 3.9 nmol/L 3. 2.8 nmol/L 4. 2.0 nmol/L	Intraday: <5% Interday: <4%	Baecher et al. 2012 (*40*)
2	25(OH)D$_2$												
3	24,25(OH)$_2$D												
4	3-epi-25(OH)D$_3$												
1	25(OH)D$_3$	^{2}H$_6$-25(OH)D$_3$	PTAD	Serum 100 μL	PP, SPE	C-18	QqQ	(A) 0.1% FA in H$_2$O 5 mmol/L methylamine (B) 0.1% FA in MeOH 5 mmol/L methylamine	Jetstream ESI	1. 607 → 298 2. 591 → 298 3. 623 → 298	LOD: 1. 0.02 nmol/L 2. 0.2 nmol/L 3. 0.2 nmol/L	Intraday: 2.3%-7.4% Interday: 2.6%-10.2%	Burild et al. 2014 (*68*)
2	Vitamin D$_3$												
3	24,25(OH)$_2$D												

Continued on page 1043

Table 1. Summary of the most important recent studies for quantifying multiple vitamin D species. (*Continued from page 1042*)

	Vitamin D species	Internal standard	Derivatization	Sample type amount	Sample treatment	HPLC column	Instrument	Mobile phase	Ionization	*m/z Transition*	Linear range, LLOQ	Precision % RSD	Reference
1	25(OH)D_3	$^2H_3$-25(OH)D_3	None	Serum 100 μL	PP, 96-well-plate SPE	CN	QqQ, HRMS	(A) 0.1% FA in H_2O (B) 0.1% FA in MeOH	ESI	1. 401 → 383 2. 413 → 395 3. 401 → 383	LLOQ QpQ: 1. 3.4 nmol/L 2. 6.2 nmol/L LLOQ HRMS: 1. 2.1 nmol/L 2. 1.7 nmol/L	Intraday: 2% Interday: 1%–4.9%	Bruce et al. 2013 (*74*)
2	25(OH)D_2												
3	3-epi-25(OH)D_3	$^2H_3$-3-epi-25(OH)D_3											
1	25(OH)D_3		DAPTAD, d-DAPTAD	Urine 1 mL	PP, SPE	C-18	QqQ	(A) 10 mmol/L ammonium formate in MeOH (8:3 v/v) (B) 10 mmol/L ammonium formate in MeOH	ESI	1. 619 → 341 2. 635 → 341 Undisclosed	Linear range: 1. 4–100 nmol/L 2. 4–200 nmol/L	Intraday: 2.9%–9.5% Interday: 2.6%–7.4%	Ogawa et al. 2014 (*69*)
2	24,25(OH)$_2$D												
3	1,25(OH)$_2$$D_3$												
4	3-epi-25(OH)D_3												
5	3-epi-24,25(OH)$_2$$D_3$												
1	Vitamin D_3	$^2H_6$-Vitamin D_3	PTAD	Soft tissues	PP, SPE	C-18	QqQ	(A) 0.1% FA in H_2O 5 mmol/L methylamine (B) 0.1% FA in ACN 5 mmol/L methylamine (C) EtOAC	ESI	1. 591 → 298 2. 603 → 298 3. 607 → 298 4. 619 → 298	Linear range: 0.1–1000 ng/mL LOD: 0.1 ng/g tissue	Intraday: 9.6%–10%	Lipkie et al. 2013 (*70*)
2	Vitamin D_2	$^2H_3$-Vitamin D_2											
3	25(OH)D_3	$^2H_6$-25(OH)D_3											
4	25(OH)D_2	$^2H_3$-25(OH)D_2											
1	Vitamin D_3	$^2H_6$-Vitamin D_3	None	Serum 1 mL	PP, SPE	C-18	QqQ	5 mmol/L ammonium formate 85:15 MeOH: H_2O (v/v)	ESI	1. 385 → 107 2. 397 → 107 3. 383 → 159 4. 395 → 133 5. 399 → 147 6. 411 → 133 7. 399 → 121	Calibration range: 1. 1 pg/mL–500 ng/mL 2. 50 pg/mL–500 ng/mL 3. 1.2 pg/mL–250 ng/mL 4. 0.25 ng/mL–250 ng/mL 5. 4 pg/mL–5 ng/mL 6. 50 pg/mL–50 ng/mL 7. 5 pg/mL–50 ng/mL LOQ: 1. 1 pg/mL 2. 50 pg/mL 3. 1.2 pg/mL 4. 250 pg/mL 5. 4 pg/mL 6. 50 pg/mL 7. 5 pg/mL	Intraday: 2.9%–9.5% Interday: 2.6%–7.4%	Mena-Bravo et al. 2015 (*71*)
2	Vitamin D_2	$^2H_3$-Vitamin D_2											
3	25(OH)D_3	$^2H_6$-25(OH)D_3											
4	25(OH)D_2	$^2H_3$-25(OH)D_2											
5	1,25(OH)$_2$$D_3$	$^2H_6$-1,25(OH)$_2$$D_3$											
6	1,25(OH)$_2$$D_2$	$^2H_6$-1,25(OH)$_2$$D_3$											
7	24,25(OH)$_2$D	$^2H_6$-24,25(OH)D_3											

[a] FA, formic acid.
[b] Combination of C-18 and chiral column.
[c] No corresponding vitamin D species in the first column.

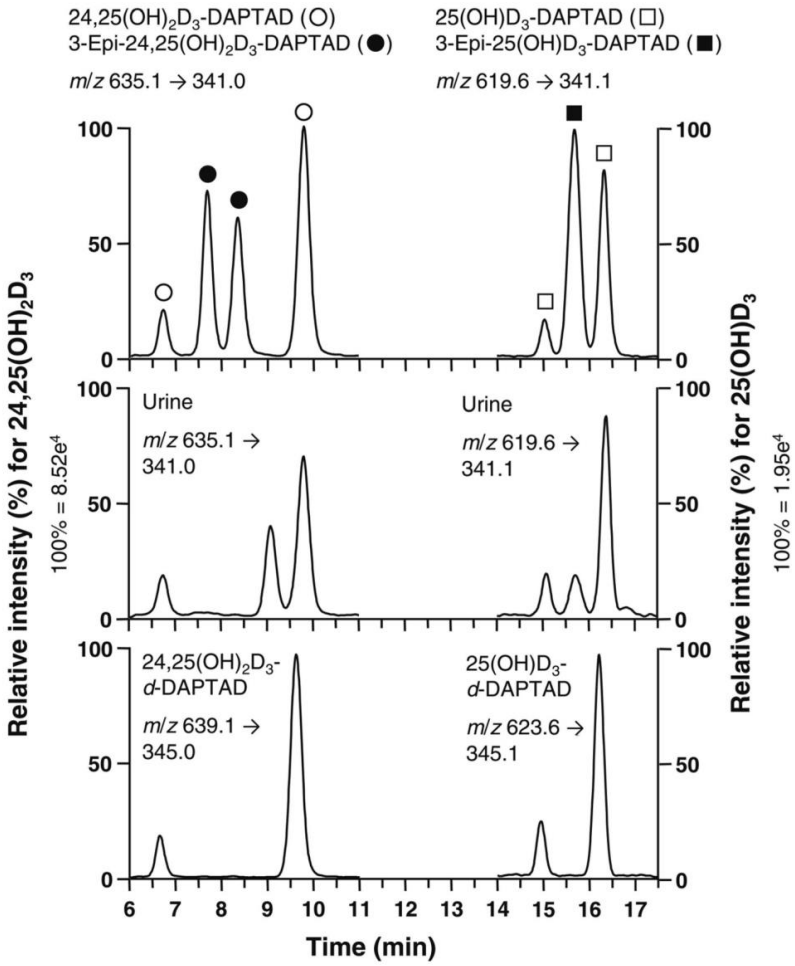

Fig. 4. Chromatograms of vitamin D_3 metabolites derivatized with DAPTAD and d_4-DAPTAD.

(Top) authentic standards of 25(OH)D_3 and 3-epi-25(OH)D_3, as well as 24,25(OH)$_2D_3$ and 3-epi-24,25(OH)$_2D_3$; (middle) DAPTAD-derivatized urine sample; (bottom) *d*-DAPTAD-derivatized standards 25(OH)D_3 and 24,25(OH)$_2D_3$; measured by LC-MS/MS [reprinted with permission from Ogawa *et al. (69)*].

SPE setup. Shah et al. directly determined 8 vitamin D species *(72)* from larger volumes (1 mL) of human serum than those used in other studies for LLE, which was then dried and reconstituted in 200 μL of solvent, effectively giving a 5-fold enrichment factor.

Outlook: Vitamin D as Part of Larger Metabolomics Studies

In clinical research, metabolic phenotyping studies are increasingly being performed. These techniques have the potential to be of great value for clinical metabolomics. Some studies have already demonstrated the contributing role of vitamin D metabolites as part of larger metabolic networks. For example, metabolic phenotyping by O'Sullivan et al. revealed a phenotype that was responsive to vitamin D supplementation, as shown in a double-blind, randomized placebo-controlled dietary intervention of 160 participants who received 15 μg vitamin D_3 or placebo daily for 4 weeks. The vitamin D supplementation significantly increased the serum 25(OH)D concentrations to an endpoint concentration of 78.1 (20.0)

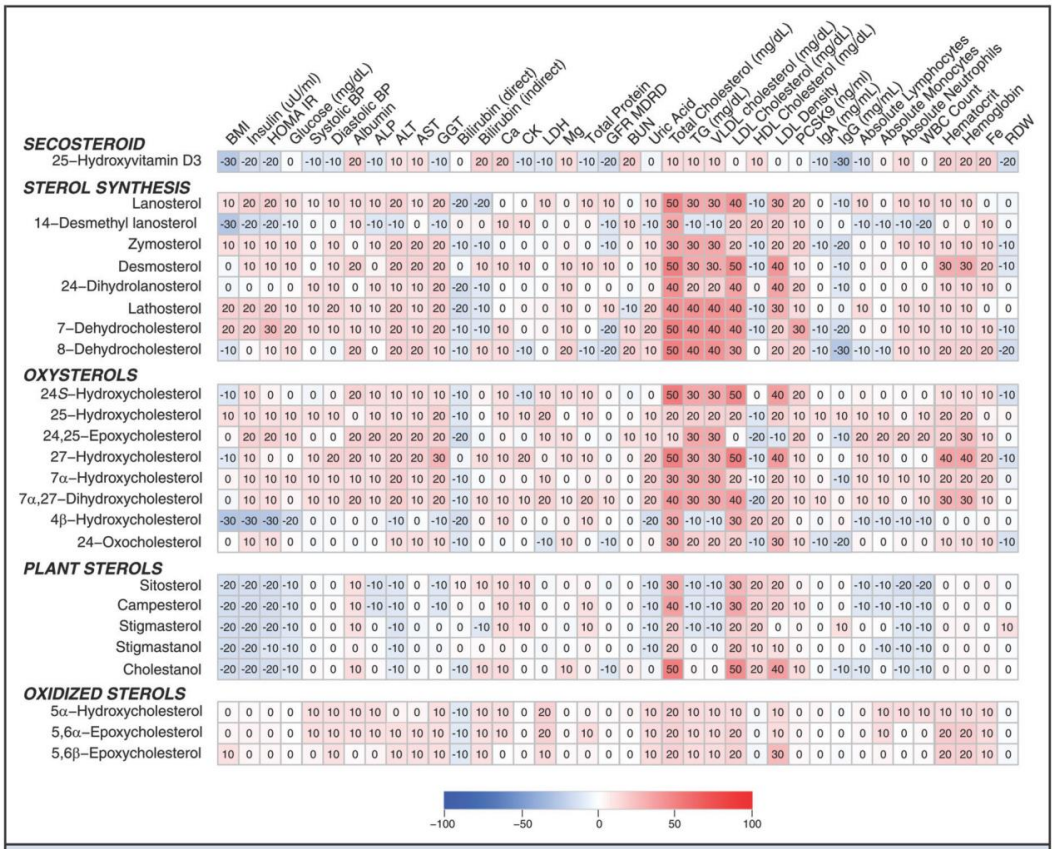

Fig. 5. Pairwise correlations between serum levels of analytes and clinical phenotypes.

Values are partial correlation coefficients × 100 after adjustment for age, sex, and ethnicity between the indicated pairs of analytes and clinical parameters; *r* values for individual comparisons are depicted using a bipolar color progression as indicated by the scale below the figure. Only traits for which at least one *r* value was greater than or equal to ±10 are shown [reproduced with permission from Stiles *et al. (79)*]. BMI, body mass index; HOMA IR, homeostatic model assessment of insulin resistance; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; CK, creatine kinase; LDH, lactate dehydrogenase; GFR MDRD, glomerular filtration rate according to the Modification of Diet in Renal Disease formula; BUN, blood urea nitrogen; TG, triglycerides; WBC, white blood cell.

nmol/L, but there was no detectable effect on the markers of metabolic syndrome *(75)*. Similarly, Stepien et al. studied vitamin D supplementation *(76)* to characterize the peripheral blood mononuclear cell (PBMC) metabolomics profile following short-term vitamin D supplementation, and to investigate effects attributable to sex. Higher vitamin D status showed no effect on the fatty acid profile of PBMCs, but inflammatory biomarkers and adipokines correlated positively with stearic acid but not with palmitic acid concentrations. No sex-specific metabolites were observed. Ahonen et al. identified 25(OH)D in mouse brain. These authors developed an UHPLC-atmospheric pressure photoionization (APPI)-MS/MS method for simultaneous quantitative analysis of 7 oxysterols and 25(OH)D (LOQ 1.0–5.0 ng/mL) in mouse brain and a cell line (wild-type SH-SY5Y human neuroblastoma) *(77)*.

Finkelstein et al. demonstrated how vitamin D status affected serum metabolomics profiles. The aim of the study was the evaluation of differences of metabolite profiles between low and high 25(OH)D groups. The authors concluded that lower maternal vitamin D status was associated with alterations of biochemical parameters, which may be associated with inflammation, oxidative stress, fatty acid β-oxidation, and the gut microbiome *(78)*. Lucock et al. demonstrated that vitamin D

receptor polymorphisms, which are affected by thyroid-stimulating immunoglobulin early in life, also determine the relevance of dietary sources of vitamin D in defining insulin concentrations at later stages in life *(79)*.

Finally, Stiles et al. described population-based analytical measurements of >60 sterol and vitamin D derivatives and the correlation with genotype/phenotype information for a clinical cohort of 3230 individuals. The authors used LC-APCI-MS and found 27 sterols and vitamin D derivatives that were consistently detected with marked interindividual variation in their serum levels; the study also revealed genetic, anatomic, and clinical phenotypes that were associated with several of these lipids (Fig. 5) *(80)*.

Conclusions

Substantial advancements have been made in recent years in the measurement technology for vitamin D and its metabolites from biological fluids, with LC-MS/MS methods undoubtedly being the current gold standard technique for quantitative analysis. Importantly, to understand the pathobiological role and function of vitamin D, we need to know the concentrations of all relevant vitamin D metabolic species. Measuring 25(OH)D will always give only an incomplete picture that may or may not correlate with function in disease; rather, in our opinion, comprehensive chemical fingerprint (chemotype)/disease phenotype correlations are required in the future for vitamin D research. Unfortunately, the current state-of-the-art of mass spectrometric measurement assays do not allow quantitative measurement of all relevant species at the required LLOQ limits without using additional derivatization reagents to enhance response for the low-abundance metabolites. This derivatization step may become obsolete in the future, however, if more sensitive mass spectrometers become available or if more selective sample preparation procedures are used.

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PUBLICATION 2

Chemotyping the distribution of vitamin D metabolites in human serum.

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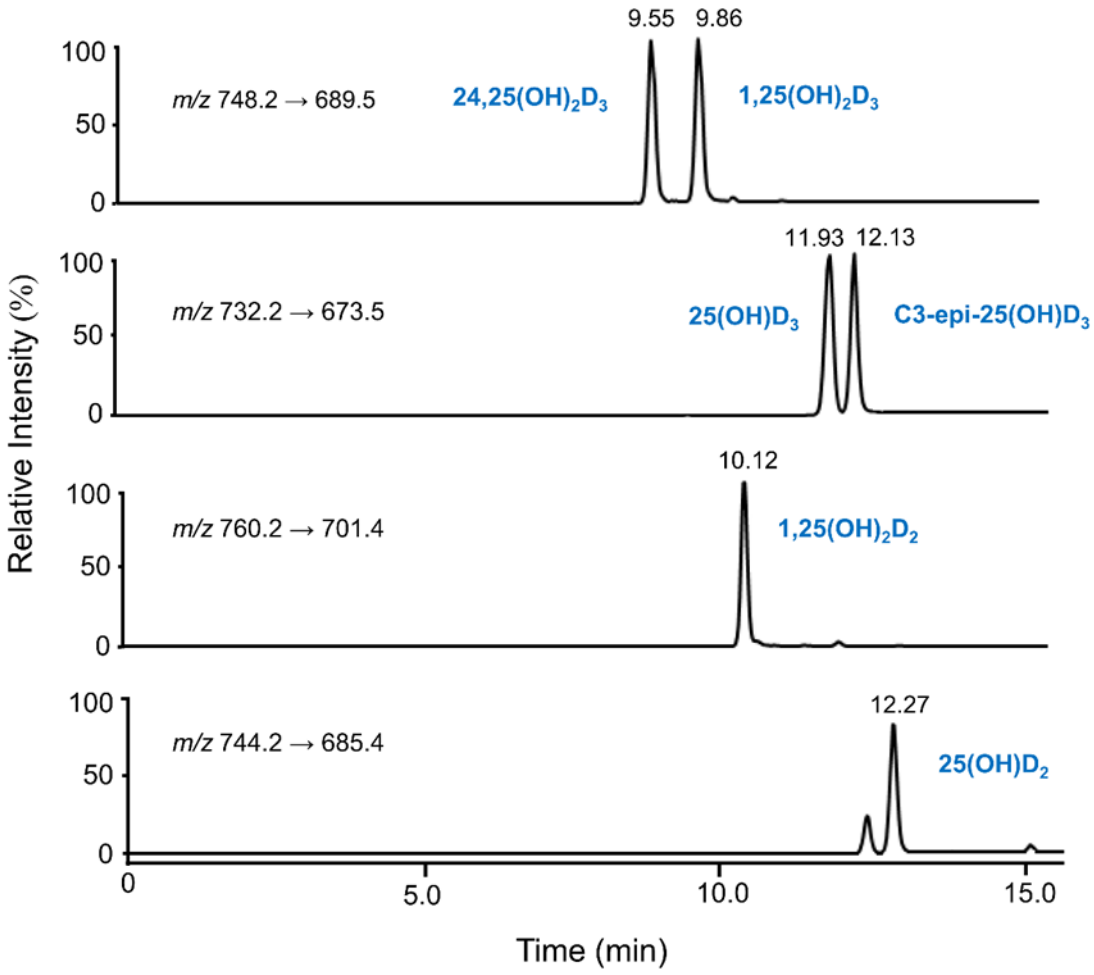

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Chemotyping the distribution of vitamin D metabolites in human serum

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Most studies examining the relationships between vitamin D and disease or health focus on the main 25-hydroxyvitamin D_3 (25(OH)D_3) metabolite, thus potentially overlooking contributions and dynamic effects of other vitamin D metabolites, the crucial roles of several of which have been previously demonstrated. The ideal assay would determine all relevant high and low-abundant vitamin D species simultaneously. We describe a sensitive quantitative assay for determining the chemotypes of vitamin D metabolites from serum after derivatisation and ultra-high performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (UHPLC-ESI-MS/MS). We performed a validation according to the 'FDA Guidance for Industry Bioanalytical Method Validation'. The proof-of-concept of the method was then demonstrated by following the metabolite concentrations in patients with chronic liver diseases (CLD) during the course of a vitamin D supplementation study. The new quantitative profiling assay provided highly sensitive, precise and accurate chemotypes of the vitamin D metabolic process rather than the usually determined 25(OH)D_3 concentrations.

A debate over healthy levels of vitamin D has been waging in the scientific and popular press in recent years. While the vital role of vitamin D in bone health is clearly established[1], its functional involvement in other diseases such diabetes, cancer, multiple sclerosis, depression, hepatic, renal and cardiovascular diseases is currently the subject of intense research[2,3], particularly because vitamin D receptor is present in virtually all organs[4]. Typically, the 25-hydroxyvitamin D (25(OH)D) metabolite is used for assessing vitamin D status, because it can be readily measured due to its long half-life and high concentration in blood, as well as the fact that the 25(OH)D level represents the optimum marker for substrate availability rather than the tightly regulated levels of other downstream metabolites[5]. In the photosynthesis and metabolism of vitamin D, the substrate vitamin D is transported to the liver, where it undergoes the first hydroxylation step at C-25 (catalysed by CYP2R1 and CYP27A1) to give 25(OH)D. This is followed by CYP27B1 conversion to the active form 1,25$(OH)_2$D in the kidneys[1]. 1,25$(OH)_2$D suppresses gene transcription by binding to vitamin D receptor (VDR), with CYP3A4 being the most important cytochrome P450 species supressed by 1,25$(OH)_2$D[6]. It has been shown that patients, who were supplemented with vitamin D, exhibited increased clearance of atorvastatin (substrate of CYP3A4)[7]. While CYP24A1 activity dominates the 1,25$(OH)_2$D/25(OH)D catabolism in the healthy kidney, CYP3A4 dominates in the liver and small intestine, because of the higher level of basal and induced enzyme expression[8]. A correlation of plasma levels of vitamin D metabolites and intestinal CYP3A4 activity showed only low levels of vitamin D metabolites in patients with cirrhosis as a result of the reduction of CYP3A protein expression[9]. Intra and inter-individual differences of circulating vitamin D levels and associated intestinal CYP3A4 activities may also contribute to variability of oral bioavailability of vitamin D; utilizing these effects may allow customised supplementation of vitamin D in the future, if the CYP genotype status of an individual is known. Recently, Binkley *et al.* nicely demonstrated a mathematical model using baseline and 6 months metabolite levels of vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$ to allow efficient "treat-to-target" prediction of 25(OH)D_3 levels[10].

In this simplified assessment, however, dynamic effects of downstream metabolites are overlooked. For example, after Holick *et al.*[11] discovered 24,25$(OH)_2D_3$ as clearance product of vitamin D, it was initially assumed that 24,25$(OH)_2D_3$ is merely a catabolite without biological activity. Today, we know that 24,25$(OH)_2D_3$ plays crucial roles in intra-membrane/endochondral bone formation and bone fracture repair[12]. Decreased activity of 24,25$(OH)_2D_3$ has also been associated with increased risk of mortality in renal patients[13]; it has also been

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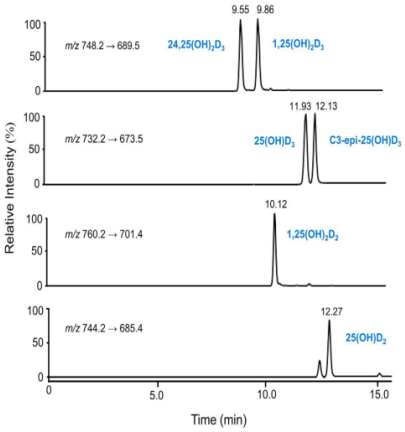

Figure 1. LC-MS/MS chromatograms for the investigated vitamin D metabolites acquired in MRM mode (see Supplementary Table S1 for details on the MS/MS data acquisition routine): MRM traces for the six investigated metabolites (standard solution at 0.2 µg/mL each; the calculated chromatographic resolution (R_s) for the peak pair 3-epi-25(OH)D/25(OH)D was $R_s = 1.51$, demonstrating full baseline resolution for the two epimers under the applied chromatographic conditions; $R_s = (t_{R1} - t_{R2})/(w_{b2} + w_{b1})/2$ with t_{r1} and t_{r2} the retention times of the two epimers and w_{b1} and w_{b2} the peak widths at baseline).

suggested that 24,25(OH)D_3 is biologically active because it is recognised by the kidneys and converted to 1,24,25(OH)$_3$$D_3$ and 24-oxo-25(OH)D_3 in rats[14].

The importance of dynamic changes of vitamin D concentrations and intra/inter-individual variability after administration of fixed supplementation doses has also been demonstrated in several recent studies. For example, Bosworth *et al.* observed decreased vitamin D catabolism and 1,25(OH)$_2$$D_3$ production in chronic kidney disease (CKD)[13]. Stubbs *et al.* showed CKD patients' altered ability to increase serum 24,25(OH)$_2$$D_3$ after vitamin D_3 therapy, suggesting decreased 24-hydroxylase activity[15]. de Boer *et al.* investigated associations of estimated glomerular filtration rate (GFR) with circulating 24,25(OH)$_2$$D_3$ levels across populations with wide range of GFRs and found that lower estimated GFR was associated strongly with reduced vitamin D catabolism, as measured by 24,25(OH)$_2$$D_3$[16]. Berg *et al.*[17] suggested the ratio of serum 24,25(OH)$_2$$D_3$ to 25(OH)D_3 (=vitamin D metabolite ratio, VMR) as a new candidate biomarker for vitamin D status, while Wagner *et al.* demonstrated that serum 24,25(OH)$_2$$D_3$/25(OH)$D_3$ ratios were predictive of 25(OH)D_3 response to supplementation[18]. Finally, no significant differences for serums concentrations of 25(OH)D_3 was observed by Duan *et al.*[19] between a cohort of healthy subjects and multiple sclerosis patients, but levels of 1,25(OH)$_2$$D_3$ and 24,25(OH)$_2$$D_3$ were significantly lower in the patient group as compared to the healthy group.

Correlations of vitamin D metabolite distributions ('chemotypes') and disease/health phenotypes therefore are an interesting topic to study, requiring metabolomics techniques for vitamin D metabolites for measurement. Of course, these assays would need to determine all relevant high and low-abundant vitamin D species simultaneously with equal analytical figures of merit. Commonly applied techniques for vitamin D are immunoassays and liquid chromatography-tandem mass spectrometry (LC-MS/MS)[20]. These methods are often restricted to 25(OH)D_3 because of limited sensitivity and specificity[20,21]. Fortunately, recent technical advances have permitted analysis of metabolites in the metabolic cascade beyond 25(OH)D_3, including lower abundant species[21,22].

In the present study, we developed a new assay for quantitative measurement of vitamin D chemotypes based on ultra-high performance liquid chromatography (UHPLC)-MS/MS. We performed a validation according to the U.S. Food and Drug Administration (FDA)'s 'Guidance for Industry Bioanalytical Method Validation' and demonstrated the assay's performance by following patients with chronic liver diseases (CLD) during a vitamin D supplementation study.

Results

Important features of the new vitamin D chemotyping assay were: (*1*) it required only 50 µL of human serum and was conducted in parallel 96-well plate format; (*2*) metabolites were derivatised for enhanced response. The derivatisation protocol was developed for considerably improving the reagent's performance by eliminating the formation of multiple diastereomers; (*3*) stable isotope standards were used for all investigated metabolites, to correct for systematic errors from matrix interferences and differential ionisation properties of metabolites.

The chemotyping method for each serum sample comprised extraction of 50 µL of serum by supported liquid extraction (SLE), ultra-high-performance liquid chromatography separation using a fluorinated HPLC column and detection and quantification by tandem mass spectrometry using specific multiple reaction monitoring (MRM) transitions for each investigated metabolite (see Table S1, Supplementary Information, for optimised conditions). A representative LC-MS/MS chromatogram is shown in Fig. 1. Importantly, baseline chromatographic resolution was achieved for all species, including the 25(OH)D_3 epimers; previously, baseline resolution was not achieved for the PTAD-derivatised 25(OH)D_3 epimers[23]. The assay was then applied to a cohort of CLD patients. The measured chemotypes for all investigated patients are summarised in Figure S1 (Supplementary

Metabolite	LOQ (serum) (ng/mL)	LOD[1] (serum) (pg/mL)	Linearity (ng/mL)	Precision intra-day (CV, %)	Precision inter-day (CV, %)
$25(OH)D_3$	1	0.05	1–100	2.1	5.3
$25(OH)D_2$	1	0.05	1–100	2.4	5.1
3-epi-$25(OH)D_3$	0.25	0.04	0.25–65	1.9	4.1
$1,25(OH)_2D_3$	0.01	0.04	0.01–0.5	3.9	5.4
$1,25(OH)_2D_2$	0.01	0.04	0.01–0.5	3.6	6.0
$24,25(OH)_2D_3$	0.1	0.05	0.1–25	3.2	4.3

Table 1. Limits of quantification (LOQ), limits of detection (LOD), linear dynamic range as well as intra- and inter-day precision. [1]$S/N = 3$.

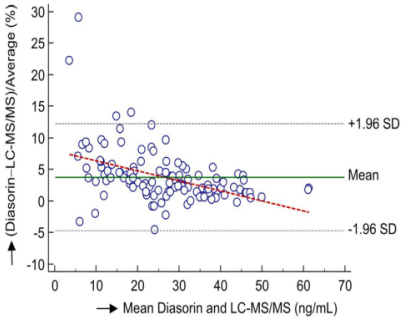

Figure 2. Bland-Altman analysis for the newly developed UHPLC-MS/MS method *versus* the Diasorin chemiluminescence assay for the $25(OH)D_3$ levels using all investigated patient samples. The analysis exhibited minor concentration-dependent bias at low concentrations (<10 ng/mL) in comparison to the Diasorin chemiluminescence assay, showing that the difference between assays was higher at very low concentrations of $25(OH)D_3$.

Information). For the FDA-compliant validation of the chemotyping assay[24], vitamin D-free blank serum samples were spiked with varying amounts of the investigated vitamin D compounds. Tables S1-S4 (Supplementary Information) summarise the full validation results.

Analyte recovery from the SLE material was determined by spiking vitamin D-free human serum with $25(OH)D_3$, $25(OH)D_2$ and 3-epi-$25(OH)D_3$ at 50 ng/mL; and $1,25(OH)_2D_3$, $1,25(OH)_2D_2$ and $24,25(OH)_2D_3$ at 50 pg/mL. After performing the extraction, the corresponding isotope standards were added to the extracts ($n = 3$). The individual recoveries were: $25(OH)D_3$, 74%; $25(OH)D_2$, 70%; 3-epi-$25(OH)D_3$, 72%; $1,25(OH)_2D_3$, 64%; $1,25(OH)_2D_2$, 62%; $24,25(OH)_2D_3$, 68%.

Precision of the assay was excellent, between 1.9–3.9% RSD (intra-day) and 4.1–6.0% (inter-day). The upper and lower limits of the calibration range for all metabolites were chosen, so that all patient samples fell into the target range. Linear regression analysis demonstrated excellent linearity over the calibration range (10 pg/mL–100 ng/mL, $R^2 > 0.998$). Limits of quantification (LOQ) for all compounds were between 10 pg/mL and 1 ng/mL, as determined by the implemented lowest calibrators. Limits of detection (LOD) were several orders of magnitude lower (Table 1) and demonstrated that quantitative measurements are readily possible at much lower levels, after extending the calibration range to lower concentrations. Six in-house calibration standards in vitamin D-free serum were measured using five determinations per concentration; none of these measurements deviated by more than 11% (20% is acceptable[24]). Quality control samples at five concentrations were measured in duplicate and showed only small deviations between 5–8% (15% is acceptable[24]).

Ion suppression effects were negligible (between 0.01–1.7% at 10 ng/mL, depending on the individual metabolites).

Stability experiments under different storage conditions (room temperature, 4 °C, −80 °C) demonstrated only limited sample degradation (<6.8%) under all studied conditions (Table S4, Supplementary Information; for more information).

Unequivocal assignment of analyte peaks in the MRM chromatograms of samples to the investigated metabolites, in particular for the closely-eluting dihydroxylated isomers $1,25(OH)D_3$ and $24,25(OH)_2D_3$, was achieved by matching the retention times of the extracted ion chromatograms (using the quantifier ions) with those of the isotope standards from a separate calibrator sample. These numbers were always within ± 0.15 min or smaller.

In addition, the quantification results for $25(OH)D_3$ were compared to results from a routine clinical vitamin D assay for accuracy assessment. Bland-Altman analysis for $25(OH)D_3$ exhibited minor concentration-dependent bias at low concentrations (<10 ng/mL) in comparison to the chemiluminescence assay (Fig. 2), showing that the difference between assays was higher at very low concentration levels for $25(OH)D_3$, similar to previous results[25,26].

For the CLD patient samples, circulating concentrations of $25(OH)D_3$ were between 7–60 ng/mL and values for $1,25(OH)_2D_3$ were in the range of 10–100 pg/mL; levels of $24,25(OH)_2D_3$ were approximately an order of magnitude lower than $25(OH)D_3$ for most patients. The vitamin D_2 metabolites $25(OH)D_2$ and $1,25(OH)_2D_2$ were not found in the investigated CLD patient samples. The 3-epi-$25(OH)D_3$ species was present in all samples between 0.1–4.5 ng/mL, demonstrating the importance of separating 3-epi-$25(OH)D_3$ from $25(OH)D_3$ to avoid overestimation of vitamin D status (Fig. 1).

Discussion

In the present study, we developed profiling techniques for capturing the dynamic chemotypes of vitamin D metabolites in human biofluids. Serum $25(OH)D_3$ levels are routinely used as a measure for vitamin D status[27], due to $25(OH)D_3$'s long half-life, relatively high concentration and clear relation to serum vitamin D_3[28]. Here, the goal was to measure multiple high and low abundant vitamin D metabolites with the same performance as that obtained for $25(OH)D_3$. The new developed method was specific, sensitive and precise for simultaneous quantification of six vitamin D metabolites using a serum volume of only 50 μL in parallel 96-well plate format. The newly developed cold-temperature derivatisation combined with high resolution chromatographic separation was very simple to perform and avoided previous problems with diastereomer formation and epimer resolution[25,29,30]. Stable isotope standards were used for all investigated metabolites, to correct for systematic errors from matrix interferences and differential ionisation properties of metabolites.

One important further aspect of metabolite derivatisation is that the converted metabolites showed similar electrospray ionisation (ESI) response factors (0.93–0.99). This was not surprising considering the permanently-charged quaternary ammonium function, which was expected to dominate the remainder of the hydrophobic structures and provide a response-levelling effect. Because of this response equalisation, other vitamin D metabolites, conjugates or yet undiscovered vitamin D compounds can be readily quantified with analytical figures of merit expected to be similar to those described above even without using dedicated stable isotope standards.

We applied the assay to a subset of patients for the validation of the assay and preliminary characterisation. Importantly, these patients served as their own controls as we compared all vitamin D values before, during and after a six-month supplementation. This procedure provided patient specific chemotypes of vitamin D metabolites at each of the investigated time points and allowed both intra-individual as well as inter-individual comparisons. For example, the separate determination of 3-epi-$25(OH)D_3$ from $25(OH)D_3$ provided accurate vitamin D status, but, more importantly, it reveals considerable differences in the 3-epi-$25(OH)D_3$ to $25(OH)D_3$ ratios. The absolute concentration levels for 3-epi-$25(OH)D_3$ mentioned in the previous section hide the fact that these levels corresponded to strongly varying amounts of the 3-epimer relative to $25(OH)D_3$. In the investigated patients, these values corresponded to between 1.6 and 12% of the individuals' $25(OH)D_3$. Similar subject-specific ratios have also been observed in previous studies[31].

The assay was able to capture serum concentrations of patients with severe vitamin D deficiency (*i.e.*, serum $25(OH)D_3 \leq 10$ ng/mL), which was present in nine (30%) of patients included in the analysis. A clear inverse linear correlation was observed between baseline $25(OH)D_3$ and response to supplementation: patients with the lowest $25(OH)D_3$ at baseline exhibited the largest increase in $25(OH)D_3$ ($P < 0.001$; Fig. 3a). Additionally, lower baseline $24,25(OH)_2D_3$ correlated with larger changes to $25(OH)D_3$ concentrations ($P = 0.005$; Fig. 3b). This finding however, cannot be generalised to the entire sample. Three women with severe vitamin D deficiency had much higher baseline $24,25(OH)_2D_3$, though these concentrations were not detected in men (Fig. 3c). Interestingly, these three supplemented women attained serum $25(OH)D_3$ levels within the normal range, which coincided with decreased $24,25(OH)_2D_3$ concentrations. In contrast, patients with lower baseline $24,25(OH)_2D_3$ demonstrated increases in response to increasing $25(OH)D_3$ levels. Additionally, patients with a greater response to vitamin D supplementation tended to have lower concentrations of 3-epi-$25(OH)D_3$, as shown in Fig. 3d ($P < 0.001$). Importantly, the measured chemotypes include accurate C-3 epimer contributions for 3-epi-$25(OH)D_3$ and are not skewed by inter-epimer response variations as reported before[22].

In conclusion, we developed a highly sensitive, precise and accurate assay for simultaneous determination of six relevant vitamin D metabolites in human serum over a wide dynamic range. The assay provided chemotypes of the vitamin D metabolic process rather than the usually determined $25(OH)D_3$ concentration; the latter gives an incomplete picture that may or may not correlate with function or disease. The chemotypes can be extended to include other vitamin D compounds without compromise as the implemented procedure provides similar performance for all metabolites. Furthermore, the assay can be readily extended to quantitative measurements at concentration levels several orders of magnitude lower than described here after appropriate re-validation; the limits of quantification reported here were determined by calibration solutions chosen for the specific application. In summary, the new metabolomics technique is believed to be a useful tool with the potential to aid the understanding of the pathobiological function of vitamin D in health and disease, and to predict individual response to vitamin D supplementation.

Methods

Materials. Standards of $25(OH)D_{2/3}$, 3-epi-$25(OH)D_3$, $1,25(OH)_2D_{3/2}$ and $24,25(OH)_2D_3$ and methanol (HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Stable isotope-labelled d_6-$25(OH)D_3$ and d_6-$1,25(OH)_2D_3$ were from Chemaphor (Ottawa, ON, Canada); d_6-3-epi-$25(OH)D_3$, d_6-$25(OH)D_2$, d_6-$1,25(OH)_2D_2$ and d_6-$24,25(OH)_2D_3$ from Endotherm (Saarbrücken, Germany). Stock solutions were prepared in methanol (0.1 mg/mL); working solutions were obtained prior to use by dilution. Organic-free water was generated by a Millipore (Bedford, MA, USA) Direct-Q8 purification system. Lyophilised ClinCal and ClinCheck 25-OH-Vitamin D_2/D_3 serum calibrators (level 0-III) and quality control sera (level I, II) were from Recipe (Munich, Germany) and reconstituted in water prior to use. Vitamin D-free human serum as calibration and

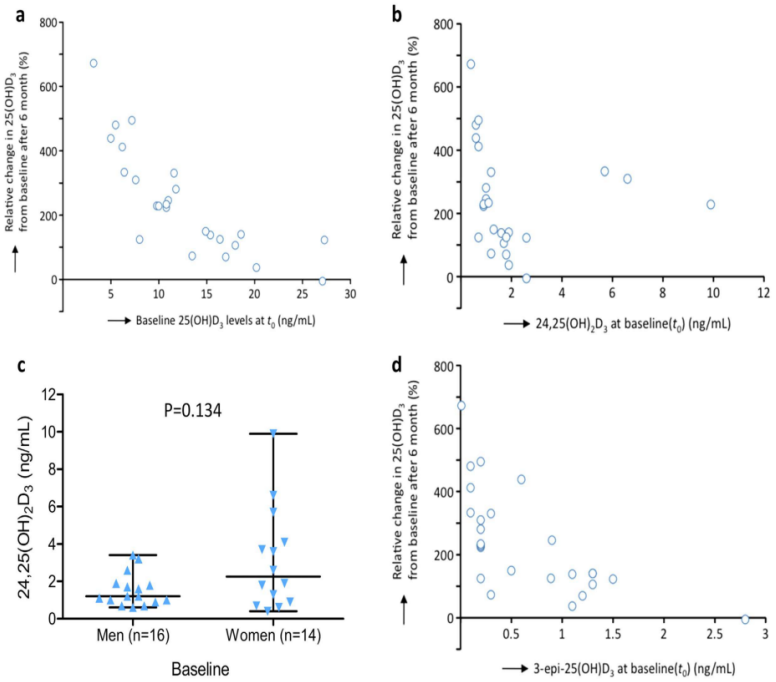

Figure 3. Non-parametric correlations between baseline vitamin D metabolites and response to vitamin D supplementation after six months: (**a**) the relative change in serum $25(OH)D_3$ (in response to vitamin D supplementation) correlated inversely with baseline $25(OH)D_3$ concentrations ($r = -0.86$, $P < 0.001$; $n = 25$); (**b**) similarly, an inverse correlation between relative change in serum $25(OH)D_3$ and baseline $24,25(OH)_2D_3$ was observed ($r = -0.54$, $P = 0.005$; $n = 25$); (**c**) baseline $24,25(OH)_2D_3$ concentrations were non-significantly higher in women with a median value of 2.3 (0.4–9.9 ng/mL) as compared to men, 1.2 (0.6–3.4 ng/mL); (**d**) patients with lower 3-epi-$25(OH)D_3$ concentrations at baseline tended to have a larger response to vitamin D supplementation ($r = -0.75$, $P < 0.001$; $n = 25$).

quality control matrix was purchased from Golden West Biologicals (Temecula, CA, USA). Supported liquid extraction 96-well AC micro-extraction plates were from Tecan (Männedorf, Switzerland). Amplifex reagent was obtained from Sciex (Concord, ON, Canada).

CLD patient samples. Serum samples of patients with chronic liver diseases (CLD) of various etiologies were used from a study at the Department of Medicine II, Saarland University Medical Center (Homburg, Germany)[32]. Blood samples were collected, the serum was extracted, aliquoted and frozen at −80 °C. Haemolysis of blood samples was minimised as outlined in the sample collection, handling and transport SOP of the department's laboratory. The samples were neither subjected to prolonged light exposure, nor to freeze/thaw cycles. One aliquot per patient was thawed out shortly before conducting the LC-MS/MS analyses. Furthermore, studies have shown that the vast majority of haemolysed specimens (~95%) only slightly haemolyse under typical collection, transport, handling and storage conditions[33]. All patients had their serum $25(OH)D_3$ levels assessed at baseline (t_0). Those with vitamin D deficiency ($25(OH)D_3 < 30$ ng/mL) were supplemented with vitamin D_3 at 20,000 IU/week (Dekristol, Jenapharm, Jena, Germany) for six months. All other patients were monitored as controls. Serum $25(OH)D_3$ and metabolite levels were measured at different time points during the study (t_1, t_2 and t_3, at 3, 6 and 12 months, respectively). All patients provided written informed consent and the study was approved by the local research ethics committee (Ärztekammer des Saarlandes, ref. 57/11). The study was conducted in accordance with the good clinical practice guidelines as defined in the Declaration of Helsinki. A subset of 30 patients (120 samples) was used for the validation of the assay and preliminary characterisation.

Sample preparation. Serum calibrators, quality control and patient samples were extracted in parallel by means of supported liquid extraction (SLE) using 96-well micro-extraction plates (AC extraction plates). The extraction method for $25(OH)D_{3/2}$, 3-epi-$25(OH)D_3$, $1,25(OH)_2D_{3/2}$ and $24,25(OH)_2D_3$ was adapted from our previously reported technique for $25(OH)D_3$[26]. The following internal standards and buffer solutions were used: (A) internal standard: d_6-$25(OH)D_{3/2}$ (50 ng/mL), d_6-3-epi-$25(OH)D_3$ (25 ng/mL), d_6-$1,25(OH)_2D_{3/2}$ (0.20 ng/mL) and d_6-$24,25(OH)_2D_3$ (15 ng/mL) in acetonitrile; (B) extraction buffer (0.2 M sodium carbonate/sodium hydrogen carbonate 1:1 (v/v) in water/ acetonitrile 95:5 v/v); (C) washing buffer (water/methanol 90:10 v/v); and (D) elution buffer (water/ methanol 10:90 v/v). A mixture of internal standard and extraction buffer (A/B 1:2 v/v)

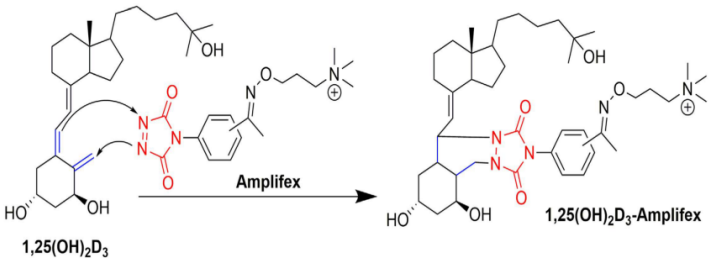

Figure 4. Chemical derivatisation reaction using the of Amplifex diene label. Example shown for $1,25(OH)_2D_3$.

(150 μL) was transferred to each extraction well and 50 μL of serum was added. To release vitamin D metabolites from the binding proteins, horizontal shaking (Eppendorf Thermomixer, Hamburg, Germany) was performed (10 min); the supernatant was discarded, and the extraction phase washed with the washing buffer (200 μL) by horizontal shaking (2 min). Finally, all vitamin D metabolites were eluted with elution buffer (200 μL) by horizontal shaking (5 min). Drying of serum sample extracts was performed using an Eppendorf concentrator (model 5301) under vacuum conditions at 30 °C; the drying time depended on the number of samples (5 min-2 h).

Derivatisation reaction. The reagent used for derivatisation of the vitamin D compounds was Amplifex Diene Reagent Kit, consisting of two components, Diene Reagent and Diene Dilution. For derivatisation, 900 μL of Diene Diluent solution was added to the Diene reagent vial and the mixture was vortexed. 50 μL of the derivatisation mixture was added to the dried serum sample extracts, followed by vortexing for 20 s and incubation for 30 min in a fridge at 4 °C. A difficulty arose during the derivatisation from the two possible sides of attack of the *cis*-diene moiety of vitamin D compounds (Fig. 4)[25,34]. The reagent can link to the molecule from both α and β sides of vitamin D compounds, giving two epimeric products for each compound. As a result, two peaks may be expected for each compound in the MRM ion chromatograms. We observed a kinetic effect and utilised it to improve the reaction: derivatisation under cold conditions at 4 °C directed the reaction favourably into a single epimeric species. To our knowledge, derivatisation at low temperatures and forcing the reaction into predominantly a single epimeric species has not yet been described. Because dedicated stable isotope standards were utilised for all metabolites, this differential formation had no impact on the accuracy of the determination. Hedman *et al.* developed a highly sensitive assay for $1,25(OH)_2D_{3/2}$ using Amplifex labeling without specifically mentioning the epimeric products[35]. Other derivatisation procedures for vitamin D also showed epimeric products, *e.g.* after PTAD derivatisation[25,34] and usually either the major product was used for quantification or the flow rate was reduced to try eluting both species in a single peak. Importantly, derivatisation shifted the molecular weights to higher *m/z* regions with less isomeric and isobaric interference as compared to the native molecules[36,37]. The final step comprised termination of the reaction by adding 50 μL of methanol/ deionised water (50:50 v/v) and vortexing for 20 s, followed by transfer to the HPLC autosampler vials.

Liquid chromatography-mass spectrometry. Five microliter of each sample was injected in triplicate into a Dionex Ultimate 3000 UHPLC system (Thermo-Fisher, Bremen, Germany). Separations of the six vitamin D metabolites were performed on a Phenomenex (Torrance, CA, USA) Kinetex PFP 100 A column (100×2.1 mm, $d_p = 2.6$ μm) at 25 °C and flow rate of 0.4 mL/min using gradient elution. The mobile phases were: (A) water + 0.1% formic acid and (B) methanol + 0.1% formic acid. The gradient started at 50% B and was held for 0.3 min, then increased to 70% and held there for 7 min, and further increased to 85% B and held for 1.2 min. Finally, B was increased to 90% and held for 4 min before returning to the initial conditions and re-equilibration for 5 min. The UHPLC system was coupled to a Sciex QTRAP 5500 quadrupole-linear ion trap (QqLIT) mass spectrometer via electrospray ionisation (ESI) operated in positive ion mode. Ion source and MS parameters were as follows: source temperature: 550 °C, curtain gas: 50 psi, gas 1: 45 psi, gas 2: 45 psi, collision exit potential: 20 V and collision energy: 40–43 V declustering potential: 0–130 V, optimised for each of the individual vitamin D metabolites (Table S1, Supplementary Information). Quantification of $25(OH)D_{3/2}$, 3-epi-25(OH) D_3, $1,25(OH)_2D_{3/2}$ and $24,25(OH)_2D_3$ was performed by multiple reaction monitoring (MRM) using the $[M + H]^+ \rightarrow [(M + H) - (N(CH_3)_3)]^+$ transitions for all measured vitamin D metabolites with 200 ms dwell time per transition (Table S1, Supplementary Information). The overall chromatographic run time including column wash/equilibration was 15 min per sample. Data acquisition was performed using Analyst software 1.6 (Sciex) and MultiQuant 2.1.1 (Sciex) for quantification of all vitamin D metabolites. Linear regression analysis was performed by the least-squares method to evaluate the calibration curve of each analyte as a function of its serum concentration.

Chemiluminescence immunoassay. Serum levels of $25(OH)D_3$ of the CLD patients samples were determined in Saarland University Medical Center´s central laboratory (Department of Clinical Chemistry and Laboratory Medicine) using the Liaison 25-OH Vitamin D chemiluminescence immunoassay (DiaSorin, Dietzenbach, Germany).

FDA Guidance for Industry-Bioanalytical Method Validation. We performed the method validation in accordance with guidelines of the United States Food and Drug Administration (FDA)[24];

Cross-validation. assay was compared to validated reference method (chemiluminescence assay) using incurred samples.

Selectivity. blank samples of appropriate biological matrix (serum) were obtained from different sources (human serum, vitamin D-free human serum, horse serum).

Reproducibility. reproducibility was assessed by replicate measurements using the new assay, including quality controls and incurred samples (Table S1, Supplementary Information).

Calibration curve. calibration curves were generated for each analyte in the sample in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. Six concentrations of standards were chosen on the basis of the concentration range expected in the conducted study; analysed in triplicate in each run. (Tables S2 and S3, Supplementary Information).

Accuracy, precision and recovery. a minimum of three concentrations in the range of the expected study sample concentrations was performed and measured using a minimum of five determinations per concentration (results should not deviate by more than 20%). Recovery experiments were performed by comparing analytical results for extracted samples at three concentrations (low, medium, high) with non-extracted standards that represent 100% recovery (Table S3, Supplementary Information).

Quality control samples. three concentrations (low level, medium level, high level) in triplicate were incorporated into each run. At least 67% of them (in total) should be within 15% of their respective theoretical values and 50% (at each level) should be within 15% of their theoretical concentrations (Table S3, Supplementary Information).

Matrix effects (ion suppression). Matrix effects were evaluated for five different serum samples at three different concentration levels: high (50 ng/mL), medium (30 ng/mL), low (10 ng/mL), as described by Matuszewski *et al.*[38] The experimental strategy comprised a first set of serum samples, which were fortified after SLE with analytes and internal standards at three different concentration levels. The second set of samples consisted of pure analytes and internal standards at three different levels in mobile phase without performing SLE. The absolute matrix effect was calculated by dividing the mean peak areas of each metabolite from the first set (with SLE) by the mean of each metabolite peak area of the second set (without SLE). The obtained values (expressed in percent) were subtracted from 100% to give the amount of ion suppression in the presence of matrix.

Stability study. Stability was tested using vitamin D-free blank serum ($n = 5$) spiked with the metabolites of interest at three concentration levels: high (50 ng/mL), medium (30 ng/mL), low (10 ng/mL). Short-term temperature stability was evaluated for 24 h at room temperature, for 24 h at 4 °C within the autosampler, and for 24 h at −80 °C in the freezer. Additionally, three freeze/thaw cycles at −20 °C and a long-term stability experiment (20 d) were performed (Table S4, Supplementary Information). On the day of the LC-MS/MS analysis, the internal standard was added and analysis performed in an identical manner to the patient samples as described above; LC-MS/MS experiments were performed in duplicate for each sample. The calculated concentrations for each stability experiment were compared to the measured sample concentration of the day of preparation.

Ruggedness. The robustness of the method was examined by systematically modifying several experimental parameters. The HPLC flow rate was varied between 0.3–0.5 mL/min (regular 0.4 mL/min) using two different mixtures of vitamin D metabolites at different levels: 10 and 40 ng/mL for $25(OH)D_{3/2}$ and 3-epi-$25(OH)D_3$, and 10 and 40 pg/mL for the dihydroxylated species $1,25(OH)_2D_{3/2}$ and $24,25(OH)_2D_3$. Additionally, the column temperature was investigated between 20–30 °C (regular 25 °C). In these experiments, one parameter was held constant, while the other was varied: first the HPLC flow rate was set to 0.3 mL, while the column temperature was set to 20, 25 and 30 °C. This procedure was repeated for the flow rates of 0.4 and 0.5 mL/min. In all ruggedness-testing experiments, the differences of peak areas in comparison to the standard settings were always less than 4%.

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Author Contributions

M.J.J. performed all analytical experiments, analysed and interpreted the data, and drafted the manuscript. C.S.S. designed the supplementation study, collected patient samples and contributed to interpretation of the data. F.L. contributed to the design of the supplementation study and interpretation of the data. D.A.V. conceived, designed and coordinated the study, analysed and interpreted the data, and drafted the manuscript. All authors revised the manuscript for important intellectual content and approved the final version for publication.

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Supplementary Information

Chemotyping the distribution of vitamin D metabolites in human serum

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This Supplementary Information contains Tables S1-S4 and Supplementary Figure S1.

Table S1. Optimised MS/MS (MRM) settings for the derivatised vitamin D metabolites.[1]

Metabolite	Collision energy (eV)	Declustering potential (volts)	Precursor ion (*m/z*)	Product ion (*m/z*)
25(OH)D_3	40	100	732.2	673.5
25(OH)D_2	43	55	744.2	685.4
3-epi-25(OH)D_3	41	10	732.2	673.5
1,25$(OH)_2D_3$	43	130	748.2	689.5
1,25$(OH)_2D_2$	42	0	760.2	701.4
24,25$(OH)_2D_3$	42	130	748.2	689.5

[1]Settings for Sciex 5500 QTRAP instrument.

Table S2. In-house calibration standards prepared in vitamin D-free serum.

Metabolite	Calibration level					
	0 (ng/mL)	I (ng/mL)	II (ng/mL)	III (ng/mL)	IV (ng/mL)	V (ng/mL)
25(OH)D_3	1	10	25	50	70	100
25(OH)D_2	1	10	25	50	70	100
3-epi-25(OH)D_3	0.25	2	10	25	45	65
1,25$(OH)_2D_3$	0.01	0.10	0.15	0.20	0.25	0.5
1,25$(OH)_2D_2$	0.01	0.10	0.15	0.20	0.25	0.5
24,25$(OH)_2D_3$	0.1	1.5	5	10	15	25
Deviation (%)	11	10	8	6	8	6

Table S3. Implemented quality control (QC) samples.

QC		Theoretical (ng/mL)	Measured deviation (%)
In-house (all six metabolites in each QC)			
Level I		10	7
Level II		30	8
Level III		70	5
Recipe[1]			
Level I	25(OH)D_3	20.5	8
	25(OH)D_2	16.3	
Level II	25(OH)D_3	44.3	6
	25(OH)D_2	36.6	

[1]Recipe QC samples were commercially available only for 25(OH)D_3 and 25(OH)D_2.

Table S4. Compound stabilities for the investigated vitamin D metabolites in serum under different sample storage conditions. Shown is the span of observed decrease values in % for the five measured samples at each investigated concentration in comparison to the concentration measured after initial preparation (each sample was measured in duplicate).

Storage condition (*n*=5 samples ea.)	**25(OH)D_3 decrease (%)**	**3-epi-25(OH)D_3 decrease (%)**	**1,25$(OH)_2D_3$ decrease (%)**	**24,25$(OH)_2D_3$ decrease (%)**
24 h RT				
High[1]	2.7 - 5.2	2.7 - 5.9	2.9 - 5.3	2.9 - 5.4
Medium	2.1 - 6.4	3.3 - 5.7	2.8 - 5.6	3.9 - 5.9
Low	2.5 - 5.1	4.0 - 4.7	3.2 - 6.4	2.9 - 5.8
24 h 4°C				
High	0.2 - 3.6	0.2 - 6.7	0.6 - 6.8	0.2 - 5.8
Medium	0.5 - 5.4	0.7 - 3.3	0.4 - 5.1	0.8 - 3.3
Low	0.3 - 2.0	0.3 - 1.2	0.3 - 2.3	0.5 - 2.8
24 h 80°C				
High	0.3 - 2.7	0.5 - 2.5	0.3 - 2.1	0.4 - 1.7
Medium	0.4 - 1.1	0.3 - 2.2	0.2 - 2.3	0.4 - 2.9
Low	0.5 - 2.3	0.5 - 3.2	0.8 - 2.0	0.7 - 2.1
3 Freeze/thaw cycles				
High	1.1 - 5.3	1.8 - 5.3	1.3 - 4.8	1.2 - 5.8
Medium	1.1 - 4.3	1.3 - 4.1	1.2 - 5.5	1.2 - 4.1
Low	1.3 - 5.8	1.3 - 3.2	1.0 - 5.3	1.5 - 5.7
Long-term (20 d)	0.1 - 5.5	0.2 - 5.0	0.2 - 5.5	0.1 - 5.5

[1]High (50 ng/mL), medium (30 ng/mL), low (10 ng/mL) of each metabolite.

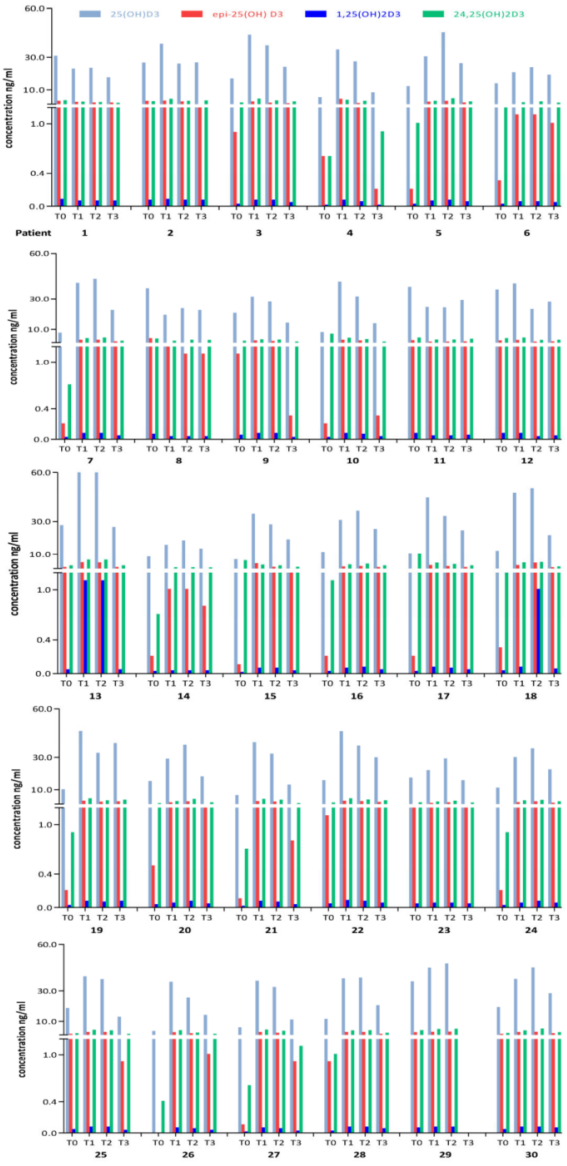
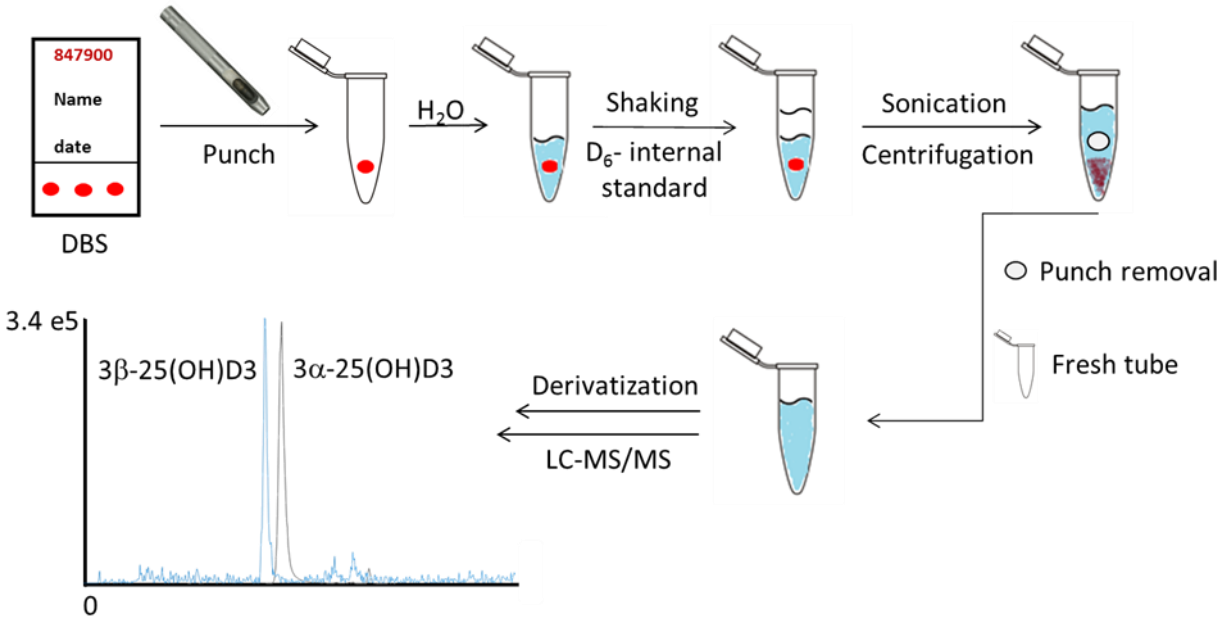


Figure S1: Serum vitamin D metabolite levels of 30 selected CLD patient samples at the four investigated time points (t_0-t_3) over the course of the vitamin D_3 supplementation study. Only patients with 25(OH)D_3 levels <30 ng/ml received supplementation. All other patients were monitored as controls.

PUBLICATION 3

Quantification of the 3α and 3β epimers of 25-hydroxyvitamin D 3 in dried blood spots by LC-MS/MS using artificial whole blood calibration and chemical derivatization.

M.J. Müller, C.S. Stokes, D.A. Volmer, *Talanta.*, **2017**, 165, 398-404.



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Quantification of the 3α and 3β epimers of 25-hydroxyvitamin D_3 in dried blood spots by LC-MS/MS using artificial whole blood calibration and chemical derivatization

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ABSTRACT

While the biological function of the 3α epimer of 25-hydroxyvitamin D_3 (25(OH)D_3) remains unknown, its presence needs to be accurately captured and separated from the main 3β epimer, to avoid positive bias in vitamin D status analyses. Several recent LC-MS/MS assays for 25(OH)D_3 successfully separate the 3α and 3β epimers by chromatography. Unfortunately, none of the existing LC-MS/MS assays, which utilize dried blood spots (DBS) as sampling/storage vessels, is able to quantify the individual epimers. DBS are often used for analysis of infant blood, however, and these samples are particularly likely to contain significant levels of interfering 3α epimer. Furthermore, proper calibration of DBS samples is much more difficult to achieve than for liquid serum or plasma samples. We addressed this important issue by creating an artificial vitamin D-free whole blood for calibration and then quantified 3α- and 3β-25(OH)D_3 levels from DBS. After chemical derivatization, the vitamin D epimers were separated on a PFP column and concentrations determined by electrospray ionization LC-MS/MS on a triple quadrupole mass spectrometer. Calibration with artificial whole blood showed improved precision over standard addition (7.6 *versus* 31.5% RSD for 3β-25(OH)D_3). The limits of quantification for 3β-25(OH)D_3 and for 3α-25(OH)D_3 were 1.0 and 0.1 ng/mL, respectively. Excellent intra/interday precisions between 2.1 and 2.2% CV (intra) and 4.4–5.3% CV (inter) were established for 3β-25(OH)D_3 and 3α-25(OH)D_3. For 3β-25(OH)D_3, only small concentration-independent bias and deviation of < 3.3 ng/mL were seen between serum LC-MS/MS and DBS-LC-MS/MS measurements; analyses of 3α-25(OH)D_3 showed deviations of < 0.8 ng/mL in all experiments.

1. Introduction

The concentration of the main hepatic vitamin D metabolite, 25-hydroxyvitamin D (25(OH)D) in human serum or plasma is commonly used to determine vitamin D status [1]. The term 'vitamin D status' refers to the combined concentrations of the 25-hydroxy metabolites of vitamin D_2 and D_3. In this study, we are concerned with vitamin D_3. Recent studies have demonstrated the capability of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to capture a wide range of other vitamin D metabolites beyond 3β-25(OH)D_3 [2], including a stereoisomeric variation resulting from configuration reversal (β→α) at C-3 (=3α-25(OH)D_3) (*NB*: 3α- and 3β-25(OH)D_3 are both chemical epimers; the vitamin D community, however, commonly refers only to the 3α species as the epimer, while the more common 3β species is often called the non-epimeric form). This 3α epimer has been shown to originate from an alternate metabolic pathway of vitamin D_3 [3], the biological relevance of which is not yet understood [4]. From a mass spectrometric point of view, the 2 epimers are virtually impossible to differentiate, as they exhibit nearly identical dissociation behavior upon collision induced dissociation (CID). Fortunately, various liquid chromatography procedures have shown sufficient selectivity to successfully separate the 2 species [5–8]. Because the metabolic products of 3α-25(OH)D_3 do not exhibit the calcemic effects of the regular vitamin D species, this separation of 3α- from 3β-25(OH)D_3 during analysis is mandatory, to avoid the risk of overestimating vitamin D status [8]. This is particularly important for infant samples, where concentrations of 3α-25(OH)D_3 can be very high. For example, Singh et al. showed 3α-25(OH)D_3 levels of up to

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; ANOVA, analysis of variance; CID, collision-induced dissociation; DBS, dried blood spots; ESI, electrospray ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOQ, limit of quantification; MRM, multiple reaction monitoring; PFP, pentafluorophenyl; UHPLC, ultra high performance liquid chromatography

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40% of total plasma 3β-25(OH)D in infants [9] and van den Ouweland et al. reported 15–55% contribution of 3α- relative to 3β-25(OH)D_3 serum concentration in newborns [8].

Infant blood is often collected on filter cards as dried blood spots and several assays have reported DBS for measurement of 3β-25(OH)D_3 in infant samples, in particular the seminal studies presented by Eyles and coworkers [10–13]. Kvaskoff et al. suggested a minimum volume of 50 µL of blood per blood spot, as smaller volumes lead to spurious results [13]. DBS are not limited to infant samples, however, as blood sampling *via* DBS as part of larger cohort studies in adults have readily permitted sampling at the participants' home without the need for healthcare professionals; samples can then be shipped *via* regular mail [14], as it has been shown that 3β-25(OH)D_3 in DBS remains stable for several years at room temperature [11]. DBS exhibit a particular advantage for the laboratory personnel involved in blood collection and analyses, as DBS present only a minimal biohazard, due to the inactivation of most infectious agents during the drying process [15].

To our knowledge, none of the existing DBS-LC-MS/MS methods for 25(OH)D_3 are able to quantitatively determine both 25(OH)D_3 epimers. Knowing the concentrations of both species is important, however, as not only could such a procedure eliminate the 3α epimer for accurate 3β-25(OH)D_3 quantification as described above, but this would also allow functional assessment of the individual C-3 epimer species and correlation of epimer levels with clinical phenotype. Higashi et al. eliminated the interfering 3α epimer during LC-MS/MS analysis from DBS by applying a 2-step derivatization procedure that also enhanced response for 25(OH)D_3 [16], which was recently further improved [17]. One of the major difficulties with this approach is that the previously collected whole blood sample is used as sample matrix in dried form. These dried blood spots contain endogenous levels of the target vitamin D compounds. Similarly, most DBS calibration routines for vitamin D use whole blood as matrix and calibrate *via* the standard addition method [11,13,14,18–20]. The baseline concentration of 25(OH)D_3 is measured in these samples and then used as zero point in the calibration curve; the calibrator samples contain specific amounts of added analyte above the baseline blood levels [21]. This approach was pioneered by Kvaskoff et al. for 25(OH)D_3 [11]. The linear dynamic range of this method, however, is limited at the lower end of the curve. Furthermore, person-specific blood interferences from effects of age, smoking or general health status are not properly captured using this approach. Higashi et al. developed an improved method for DBS calibration based on artificial vitamin D-free blood, by adding albumin/saline solution to the isolated red blood cell component of whole blood [16]. This was a very elegant solution, as Kvaskoff et al. had previously shown *via* radioactive ^{125}I-25(OH)D_3 labeling experiments, that virtually all 25(OH)D_3 in whole blood is distributed in the serum compartment (> 98%) and completely excluded from intra-cellular and membrane components of red blood cells [13]. The method exhibited a limitation, however, with respect to the exact composition of the artificial vitamin D-free blood, as only human serum albumin solution (HAS, 7% w/v saline) was added to mimic vitamin D-free whole blood. Human serum contains 91% water and up to 7% proteins; namely, 62% albumin and 38% different globulins such as α1-globulin, α2-globulin, β-globulin and γ-globulin; the residual 2% are electrolytes and nutrients, hormones or waste products [22]. Therefore, we developed a new approach for DBS calibration using an improved artificial vitamin D-free whole blood, which differed to Higashi et al.'s whole blood in that it used vitamin D-free serum instead of HAS/saline solution.

Herein we report an LC-MS/MS assay that specifically targets both 3α- and 3β-25(OH)D_3 metabolites from DBS, with a particular calibration routine based on artificial vitamin D-free blood. This artificial blood closely resembles natural whole blood with respect to the chemical composition. We demonstrate enhanced performance with this artificial blood DBS calibration routine in comparison to a procedure based on a calibration *via* standard addition. To evaluate the new assay, we compared 3α- and 3β-25(OH)D_3 levels in DBS of human blood donors to those measured in serum. In addition, we compared the measured serum vitamin D levels to results obtained from a routine clinical vitamin D assay for accuracy assessment.

2. Experimental

2.1. Chemicals and materials

Standards of 3α-25(OH)D_3, 3β-25(OH)D_3 and human serum albumin (HSA) were purchased from Sigma-Aldrich (Steinheim, Germany), stable isotope-labelled d_6-3β-25(OH)D_3 from Chemaphor (Ottawa, ON, Canada), d_6-3α-25(OH)D_3 from Endotherm (Saarbrücken, Germany), and Amplifex Diene Reagent Kit from Sciex (Darmstadt, Germany). Stock solutions were prepared in methanol (0.1 mg/mL); working solutions were prepared prior to use by dilution. Organic-free water was generated by a Millipore (Bedford, MA, USA) Direct-Q8 purification system. HPLC-MS grade methanol, formic acid and acetonitrile were obtained from Sigma-Aldrich. Antibody-purified vitamin D-free human serum was acquired from Golden West Biologicals (Temecula, CA, USA). Supported liquid extraction 96-well AC plates were purchased from Tecan (Männedorf, Switzerland).

2.2. Blood samples

Whole blood and serum samples were obtained from 10 donors from the Blood Donation Center Saar-Pfalz (Saarbrücken, Germany). Samples were neither subjected to prolonged light exposure nor multiple freeze/thaw cycles. The donors provided written informed consent. The local research ethics committee (Ärztekammer des Saarlandes, Saarbrücken) approved the use of the anonymous samples for this study without requiring dedicated ethical permission.

2.3. Dried blood spot preparation

After spotting 50 µL of whole blood on Whatman (Cardiff, UK) 903 specimen collection papers, samples were air dried and stored at room temperature for 48 h before extraction. The assay calibrants (standard addition and artificial vitamin D-free blood) were deposited, dried, extracted and derivatized in the same fashion as the donor samples.

2.4. Calibration curves based on artificial vitamin D-free whole blood

In this study, we utilized new vitamin D-free whole blood for DBS analysis. Briefly, we isolated red blood cells from human donor samples and reconstituted them in vitamin D antibody-purified serum to give the artificial vitamin D-free whole blood. Experimentally, we extracted and washed the cell components (*e.g.*, red blood cells, leukocytes and thrombocytes) and combined them with a commercial vitamin D-free serum. This should approximate the endogenous matrix more so than previous approaches and also provide a whole blood behavior on the collection papers that closely resembles that of patient samples. A literature method for artificial vitamin D-free blood preparation was used [16], however, after major modification with respect to the plasma/serum compartments. Whole blood samples of 10 donors were pooled (10 mL total), centrifuged (11,200*g*, 10 min) at room temperature, and the supernatant discarded. The separated cellular components (red blood cells, leukocytes and thrombocytes) were washed with saline solution (25 mL), followed by centrifugation (11,200*g*, 10 min). The supernatant was discarded and the procedure repeated 5 times. The generated red blood cells were diluted in 5% (w/v) human serum albumin (HSA)/saline solution (0.9% NaCl in water to approximate the osmolarity of NaCl in blood). Subsequently, these cellular components were added to commercial vitamin D-free human serum at a ratio of 45:55 (v/v) (based on the natural composition of whole blood [23]),

Table 1
Limit of quantification (LOQ), linear dynamic range and intra/interday precision.

Epimer	LOQ (ng/mL)	Linearity (ng/mL)	Intraday (CV, %)		Interday (CV, %)		RSD (%)	
			Serum	DBS	Serum	DBS	Artific. blood	Standard addition
3β	1.0	1.0–100.0	2.1	2.4	5.3	7.4	7.6	31.5
3α	0.1	0.1–65.0	2.2	2.5	4.4	6.3	6.0	27.3

resulting in 10 mL of artificial vitamin D-free whole blood as a homogenous mixture of serum and blood cells (Fig. S1, Supporting Information). This new vitamin D-free whole blood matrix was fortified with the investigated epimers (see below) and samples then spotted onto the specimen collection paper (50 µL).

2.5. Calibration curves based on standard addition

Standard addition was also performed here for comparison as it is the commonly used methodology for DBS calibration using LC-MS/MS [11]. Human blood was collected and mixed for 5 min using an IKA (Staufen, Germany) KS 130 Basic orbital shaker. Subsequently, 7 1-mL aliquots were pipetted into individual Eppendorf tubes. Each aliquot received 5 µL of methanol solution containing 3β- and 3α-25(OH)D_3, to obtain the final blood concentrations (Table 1); furthermore, 1 aliquot received 5 µL of methanol without any vitamin D metabolites, to provide the baseline sample. All aliquots were mixed and deposited (50 µL) on fresh filter paper.

2.6. Calibration curves based on vitamin D-free serum

Calibration curves were obtained for 3α- and 3β-25(OH)D_3 by spiking vitamin D-free serum matrix with known concentrations of both analytes. Six concentrations were chosen spanning a range based on levels seen in a previous study [24]: 1, 10, 25, 50, 70 and 100 ng/mL for 3β-25(OH)D_3, and 0.1, 2, 10, 25, 45 and 65 ng/mL for 3α-25(OH)D_3.

2.7. Reproducibly

For all investigated calibration routines (artificial blood, standard addition, serum calibration), 3 quality controls (low: 10 ng/mL, medium: 30 ng/mL, high: 70 ng/mL) were incorporated into each calibration curve and measured in triplicate.

2.8. Precision

Precision was assessed by repeatability (intraday precision) and intermediate precision (interday precision). Precision was determined from 3 same-day replicates (intraday), and daily measurements over a period of 1 wk (interday). The results were expressed as %RSD.

2.9. Cross-validation

The assay was compared to a reference method (immunoassay) for 25(OH)D_3 and to an established serum LC-MS/MS method for 3α- and 3β-25(OH)D_3. For the immunoassay, donor serum levels of 3β-25(OH)D_3 were determined at Saarland University Medical Center´s central laboratory (Department of Clinical Chemistry and Laboratory Medicine) using the Liaison 25-OH Vitamin D chemiluminescence immunoassay (goat polyclonal antibody; DiaSorin, Dietzenbach, Germany). The lower limit of quantification of this assay was 4 ng/mL; intra- and interday pecisions were 4.9% and 7.5% RSD, respectively [25].

2.10. Selectivity

Blank samples of different biological matrices (serum, pooled whole blood, artificial whole blood) were investigated.

2.11. Matrix effects (ion suppression)

Matrix effects were evaluated from pooled whole blood (from 10 donor samples) and artificial vitamin D-free whole blood at 2 different concentrations: 10 and 30 ng/mL. The described strategy comprised a first set of samples, which were fortified after extraction with analytes and internal standards at the specific concentrations as described by Matuszewski et al. [26]. The second set of samples consisted of analyte and internal standards in mobile phase without extraction. The third set of samples consisted of the same pooled whole blood as per the first sample set, but with no additional added analytes except for internal standards, to evaluate existing 25(OH)D_3 levels. Correction for endogenous concentrations of whole blood was performed by subtracting the third set levels (unfortified whole blood, extracted) from the first set levels of the whole blood series. The absolute matrix effect was calculated by dividing the mean peak area of each metabolite from the first set by the mean of each metabolite peak area of the second set. These values were subtracted from 100% to give the amount of ion suppression in the presence of matrix (in %) for both matrices (donor whole blood, artificial vitamin D-free whole blood).

2.12. Extraction method for dried blood spots

A previously described method by Eyles et al. was modified for DBS extraction [11]: a circular area of 5 mm diameter (corresponding to 4.8 µL of whole blood) was punched from the collection paper using a BGS (Wermelskirchen, Germany) hole punch; 3 DBS samples were used for each donor and calibration. The punch was cleaned with methanol between each DBS. The 5 mm discs were placed into Eppendorf tubes, 50 µL of water were added and the tubes were shaken for 30 min at 60° using an Eppendorf (Hamburg, Germany) Thermomixer. Subsequently, 500 µL of an acetonitrile solution of the deuterated analogs (50 ng/mL, 125 nmol/L) of the C-3 epimers were added as internal standard to each tube, followed by vortexing, sonication in a warm water bath for 2 min, vortexing and centrifugation (11.200*g*, 5 min). The supernatant was decanted into Eppendorf tubes and evaporated to dryness under vacuum conditions.

2.13. Extraction method for serum samples

The extraction method for 3α- and 3β-25(OH)D_3 was adapted from our previous method [27]. All samples, specifically serum calibrators, quality control and donor serum samples, were extracted in parallel by means of supported liquid extraction (SLE) using Tecan 96-well microextraction plates (AC extraction plates). For extraction, the following internal standards and buffer solutions were used: (A) internal standard: d_6-3α-25(OH)D_3 and d_6-3β-25(OH)D_3 (50 ng/mL, 125 nmol/L) in acetonitrile, (B) extraction buffer (0.2 M sodium carbonate/sodium hydrogen carbonate 1:1 (v/v) in water/acetonitrile 95:5 v/v), (C) washing buffer (water/methanol 90:10 v/v), and (D)

elution buffer (water/methanol 10:90 v/v). The mixture of internal standard and extraction buffer (A/B 1:2 v/v) (150 µL) was transferred to each extraction well and 50 µL of sample were added. To release the 3α- and 3β-25(OH)D_3 metabolites from the binding proteins, horizontal shaking (Eppendorf Thermomixer) was performed (10 min); the supernatant was discarded, and the extraction phase washed with the washing buffer (200 µL) by horizontal shaking (2 min). Finally, the vitamin D metabolites were eluted with elution buffer (200 µL) by horizontal shaking (5 min). Drying of serum sample extracts was performed using an Eppendorf 5301 concentrator under vacuum conditions at 30 °C.

2.14. Derivatization

The dried samples resulting from all described experiments (quality controls, calibration and donor samples [DBS+serum]) were derivatized with Amplifex Diene Reagent, as described previously [24]. In the present study, the previous method was re-validated, to extend LOQ for 3α-25(OH)D_3 to 0.1 ng/mL (Table 1). For derivatization, Diene Diluent solution was added to the Diene reagent vial and the mixture was vortexed. Fifty µL of the derivatization mixture were added to the dried blood spot extracts, followed by vortexing for 20 s, and incubation for 30 min.

2.15. LC-MS/MS

Each dried blood spot and serum sample was injected (5 µL) in triplicate into a Dionex Ultimate 3000 UHPLC system (Thermo-Fisher, Bremen, Germany). The separation of 25(OH)D_3 epimers were performed on a Phenomenex (Torrance, CA, USA) Kinetex PFP F5 100A column (100×2.1 mm, d_p=2.6 µm) at 25 °C. The flow rate was 0.4 mL/min using gradient elution. The mobile phases were: (A) water+0.1% formic acid and (B) methanol+0.1% formic acid. The gradient started at 50% B and was held for 0.3 min, then increased to 70% and held there for 7 min, and subsequently further increased to 85% B, and held for 1.2 min. Finally, B was increased to 90%, held for 4 min before returning to the initial conditions for re-equilibration for 5 min. These chromatographic conditions were chosen to achieve baseline resolution for the epimer pair (Fig. 1). This was important as the mass spectral dissociation patterns of both epimers were virtually identical. The UHPLC system was coupled to a Sciex QTRAP 5500 quadrupole-linear ion trap mass spectrometer *via* electrospray ionization (ESI). Quantification of derivatized 25(OH)D_3 epimers was performed by multiple reaction monitoring (MRM) using the $[M]^+ \rightarrow [M\text{-}N(CH_3)_3]^+$ transitions with 200 ms dwell time per transition. The instrumental parameters are summarized in Table S1 (Supporting information). The accuracy of the LC-MS/MS method was investigated by comparing the serum results with those of the Diasorin chemiluminescence assay. The correlation indicated only minor, concentration-independent deviations of < 2.4 ng/mL (Fig. S2, Supporting information).

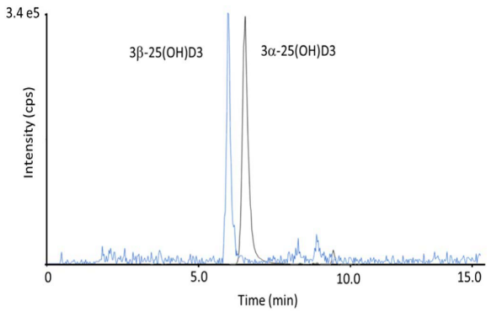

Fig. 1. LC-MS/MS chromatograms of equimolar amounts of the 3α- and 3β-25(OH)D_3 epimers in MRM mode of a fortified DBS artificial whole blood sample. The calculated chromatographic resolution was R_s=1.49, demonstrating baseline resolution for 3α and 3β species ($R_s=2(t_{r2}-t_{r1})/(w_{b2}+w_{b1})$, with t_{r1} and t_{r2} the retention times of the 2 epimers, and w_{b1} and w_{b2} the peak widths at baseline).

2.16. Statistical analyses

A one-way repeated measures ANOVA was conducted using the SPSS version 20.0 software (IBM, Munich, Germany) to assess whether there were significant differences in quantified 25(OH)D_3 levels when using the following three methods: DBS-LC-MS/MS, serum LC-MS/MS and serum DiaSorin. Three paired sample *t*-tests were then used to conduct post-hoc comparisons between each of the methods. The above tests were adjusted for multiple testing using the Bonferroni correction. A paired *t*-test was used to compare the 3α-25(OH)D_3 concentrations available for the following 2 methods: serum LC-MS/MS and DBS-LC-MS/MS. The normal distribution of all data and differences in the Bland-Altman analyses was confirmed by applying the Shapiro-Wilk test using the GraphPad Prism version 5.0 software (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

It was recently shown that the composition of blood and blood volume strongly affected collection on DBS specimen papers [13]. This topic was addressed here by developing a new calibration method based on vitamin D-free artificial whole blood, which utilized pooled donor blood samples (see Experimental Section) to average out inter-individual variations of the blood components. Before implementation, we verified that the new artificial whole blood was free of any residual traces from the 25-hydroxylated vitamin D species (the MRM traces for these analyses are shown in Fig. S3, Supporting information).

3.1. Comparison of standard addition calibration versus artificial vitamin D-free whole blood as matrix for DBS calibration

In the first set of experiments, we performed a systematic comparison of the commonly used mass spectral calibration strategy for DBS (=standard addition) to our newly developed method based on artificial vitamin D-free whole blood as matrix for DBS calibration. Using standard addition calibration, 3β-25(OH)D_3 levels could be readily quantified, but we were unable to capture the 3α-25(OH)D_3 levels in 4 of the 10 donor samples in dried blood spots, because these concentrations were outside the linear range. Very low concentrations of 3α-25(OH)D_3 were invisible in our experiments, because the baseline signal was higher than that of 3α-25(OH)D_3 in those samples, making this calibration method unfit-for-purpose for the intended application. This is a general limitation of calibration by standard addition, even though it is much easier and faster to perform as compared to the artificial vitamin-free whole blood-based approach. For the artificial vitamin D-free whole blood calibration, quantification of 3α- and 3β-25(OH)D_3 levels from all 10 donor samples was readily achieved, making the method fit-for-purpose for 3α-25(OH)D_3 quantification. Bland-Altman analysis of artificial vitamin D-free blood calibration *versus* standard addition for 3α-25(OH)D_3 and 3β-25(OH)D_3 levels exhibited minor negative concentration-dependent bias with maximum deviation of 4.1 ng/mL (10.25 nmol/L) for 3β-25(OH)D_3 and 1.6 ng/mL (4 nmol/L) for 3α-25(OH)D_3 levels (Fig. 2a). In terms of precision, lower relative standard deviations were seen with artificial blood (7.6% and 6.0% RSD for 3β-25(OH)D_3 and 3α-25(OH)D_3, respectively) in comparison to standard addition (31.5% RSD for 3β-25(OH)D_3 and 27.3% RSD for 3α-25(OH)D_3). We believe that the extra effort and cost needed to prepare vitamin D-free artificial whole blood as calibration matrix for dried blood spot analyses is worthwhile, because the procedure closely approximates the composition of whole blood. Furthermore, it provides improved analytical precision and lower limits of quantification in comparison to standard addition, as needed

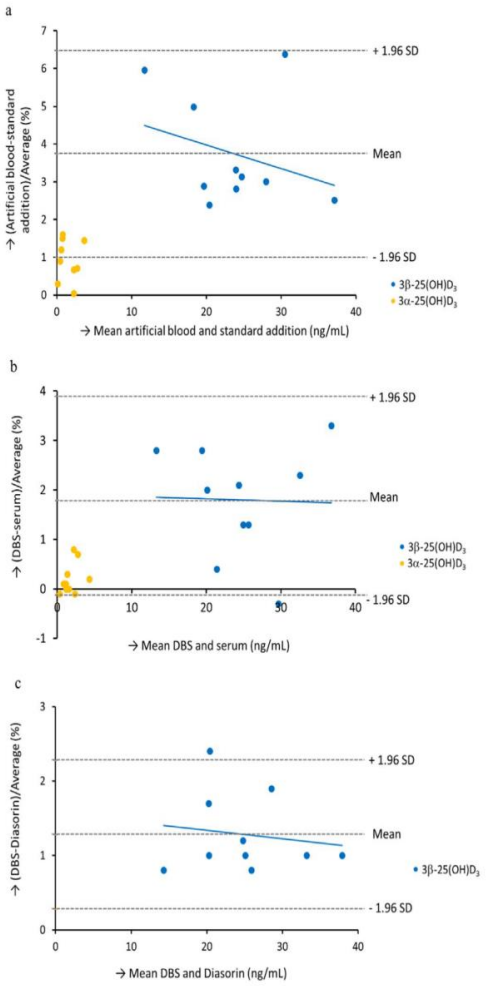

Fig. 2. Comparison of dried blood spot calibration strategies: **a** new artificial whole blood matrix *versus* standard addition. **b** Bland-Altman analysis of the LC-MS/MS serum assay data *versus* the newly developed DBS method for 3α- and 3β-25(OH)D_3 quantification. **c** Comparison of Diasorin serum data for 3β-25(OH)D_3 *versus* DBS levels. (Note: means and limits of agreement for −1.96 SD to +1.96 SD are only shown for the 3β-25(OH)D_3 species (dotted lines); the regression line for 3α-25(OH)D_3 is not shown in the diagrams.)

to capture the low levels of 3β-25(OH)D_3 as well as the generally low concentrations of 3α-25(OH)D_3.

3.2. Comparison of whole blood 3α- and 3β-25(OH)D_3 versus serum levels measured with Diasorin immunoassay and LC-MS/MS

The results of the quantification for 25(OH)D_3 in whole blood were compared to measured concentrations in serum of the same samples from a routine clinical assay for 3β-25(OH)D_3 (Diasorin immunoassay) and to our previously reported LC-MS/MS reference method [24], to provide a means of assessing the general accuracy of the DBS-LC-MS/MS approach. The immunoassay did not capture the levels of 3α-25(OH)D_3, thus permitting only the investigation of 3β-25(OH)D_3 for the comparison.

Bland-Altman analysis demonstrated agreement between serum LC-MS/MS and DBS-LC-MS/MS for all investigated concentrations (Fig. 2b; all variables included in the Bland-Altman plots in Fig. 3a-c

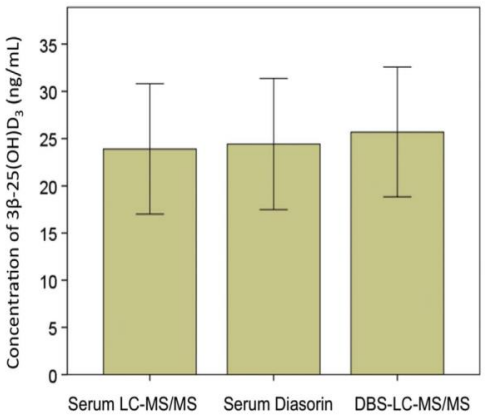

Fig. 3. Bar chart displaying mean ± SD for 3β-25(OH)D_3 concentrations based on 10 donor samples quantified using serum LC-MS/MS, serum DiaSorin and DBS-LC-MS/MS.

were normally distributed). For all 3β-25(OH)D_3 levels, only small concentration-independent bias and deviation of < 3.3 ng/mL (equal to 8.2 nmol/L) were seen between serum LC-MS/MS and DBS-LC-MS/MS (9.4% relative difference); the 3α-25(OH)D_3 levels showed deviation of < 0.8 ng/mL (2 nmol/L, 7.9%). To further assess the accuracy of the new DBS approach, a comparison of 3β-25(OH)D_3 levels in serum (*via* the Diasorin chemiluminescence assay) to the DBS whole blood data was also performed, exhibiting small deviations (< 1.8 ng/mL; 4.8 nmol/L) and no concentration-dependent bias (Fig. 2c). The 3β-25(OH)D_3 concentrations measured from serum and DBS samples corresponded to an overall bias of 0.5 ng/mL (2.1%) between the assays. In comparison, the 3α-25(OH)D_3 values for serum and DBS corresponded to 0.1 ng/mL (5.2%) bias.

One-way repeated measures ANOVA showed a significant difference, however, between the 3 methods, with regards quantification of 3β-25(OH)D_3 concentrations, F(1.08–9.72)=12.98, p=0.005. Paired *t*-tests indicated significant differences between serum LC-MS/MS (23.9 ± 6.9 ng/mL) and DBS-LC-MS/MS (25.7 ± 6.9 ng/mL), p=0.002, and between serum DiaSorin (24.4 ± 6.9 ng/mL) and DBS-LC-MS/MS (25.7 ± 6.9 ng/mL), p < 0.0001. The 2 serum methods (LC-MS/MS and DiaSorin), however, did not significantly differ from each other (p > 0.05).

The 3α-25(OH)D_3 concentrations did not differ significantly (p > 0.05), when comparing serum LC-MS/MS (1.8 ± 1.1 ng/mL) with DBS-LC-MS/MS (1.9 ± 1.2 ng/mL).

The mean ± SD for the 2 vitamin D metabolites using the aforementioned methods are summarized graphically in Fig. 3.

Some of the observed differences between serum and DBS assays, in particular for 3β-25(OH)D_3, could, in theory, be attributed to the small concentration differences of 25(OH)D_3 in serum *versus* whole blood, assuming complete recoveries from the DBS and serum matrices in our assays. LC-MS/MS studies by Newman et al. [15] and Larkin et al. [19], however, found slightly smaller values for 3β-25(OH)D_3 in DBS as compared to serum. Larkin et al. showed DBS measurements to be on average 1.1 ng/mL lower than serum analyses, with the observed differences statistically not being significant. Interestingly, the authors also performed whole blood analyses by LC-MS/MS and found that DBS concentrations of 3β-25(OH)D_3 were on average 3.2 ng/mL lower than in whole blood, which corresponded to a statistically significant 11% difference [19]. The authors point out that the whole blood levels may not have been adequately corrected for the whole blood hematocrit value, but also mention the possibility of some degradation during drying of the blood spots, as originally suggested by Eyles and coworkers [10]. The latter study found excellent correlation between

measured concentrations in DBS and cord blood. Subsequently, Kvaskoff *et al.* attributed lower recoveries from DBS predominantly to diminished blood absorption in spots of less than 50 µL in size [13].

Overall, we agree with Larkin et al. that in the absence of proper reference materials for the investigated sample matrices, we can only measure differences between the methods, but are unable to obtain a true measure that allows us to assign the bias to one of the methods, *e.g.* the dried blood spots. Importantly, the variability between our 3 implemented methods was well below the reported 15–20% variability, which has been shown to occur between some methods for 3β-25(OH)D_3 [28].

Reproducibly was demonstrated with quality control samples, which were analyzed using all implemented analytical approaches (artificial blood matrix for DBS, standard addition for DBS, serum LC-MS/MS) at 3 levels (low, medium, high; see Section 2), showing negligible deviations (< 5%) from the specified values.

Precision of the assay was excellent, ranging from 2.1% to 7.4% RSD (Table 1). Six in-house calibration standards prepared for the matrices (dried blood spots, serum) were measured using 3 measurements per concentration. The upper and lower limits of the calibration range were chosen so that all donor samples fell into the target range. Linear regression analysis demonstrated excellent linearity over the calibration range (0.1–100.0 ng/mL, R^2 > 0.997). Limit of quantification (LOQ) for both compounds were between 0.1 and 1 ng/mL, as determined by the implemented lowest calibrators.

Ion suppression effects by the matrices were very small, between 2.3 and 3.9% at 10 ng/mL for pooled whole blood as compared to the artificial vitamin D-free blood, which exhibited similarly minor signal suppression between 2.0 and 3.1% (Table S2, Supporting information). These results demonstrate close similarity between our new artificial vitamin D-free blood as calibration matrix and real whole blood from humans, further substantiating the observations described above, showing how closely the new matrix resembles human whole blood.

The chromatograms provided baseline chromatographic resolution of the 2 epimers (Fig. 1). This was important, as 3α- and 3β-25(OH)D_3 exhibited virtually identical MS behavior, which would lead to overestimation of vitamin D status if the species were not adequately separated. Also, for those assays that do not derivatize the analytes, it is important to remember that the molar ESI responses of 3α- and 3β-25(OH)D_3 differ [29,30], which necessitates the use of isotope standards for both epimer species. In our assay, response factors of metabolites were equalized by means of the permanently-charged quaternary ammonium group of the reagent label [22]; in addition, dedicated isotope standards for both epimers were implemented.

All measured dried blood spot values were corrected for mean hematocrit value of 0.43 (independent of sex), because 25(OH)D_3 is almost completely excluded from the erythrocytes [13]. In the blood samples investigated here, 3α epimer concentrations were between 2.0% and 13.1% of 25(OH)D_3 levels. Similar subject-specific ratios have previously been observed in adults [31,32]. The assay was able to capture 3α- and 3β-25(OH)D_3 from DBS at lower LOQ of 1.0 and 0.1 ng/mL, respectively. When referring to accepted cut-off values for vitamin D deficiency (*e.g.* 3β-25(OH)D < 20 ng/mL [33]), which in many cases is used to guide clinical decision making, a discrepancy for 3 of the 10 donor samples occurred between the 3 methods. The discrepancy however, resulted in only minor differences above and below this threshold. Of note, samples were collected in the summer season and the seasonal dependence of vitamin D deficiency has been described previously [34–36].

4. Conclusions

To our knowledge, 3α-25(OH)D_3 levels have not been previously quantified from dried blood spots, making this the first LC-MS/MS method for simultaneous quantification of 3α- and 3β-25(OH)D_3 from DBS. The new method provides a robust alternative to conventional serum or plasma analyses. In-house prepared artificial vitamin D-free whole blood from pooled donor samples was used to mimic blood matrix conditions for calibration. This extended the application range of the DBS assay to lower concentrations, in comparison to the commonly used standard addition method. It also avoided matrix effects and averaged out inter-individual variations of endogenous components. Good agreement of 3α- and 3β-25(OH)D_3 concentrations from dried blood spots was achieved in comparison to a serum LC-MS/MS method, demonstrating the suitability of the artificial whole blood approach, in particular for very low levels of the metabolites. The DBS assay is particularly useful for long-term cohort studies in pediatric populations or adults, where multiple time points are required, or for application in remote or rural clinics. The assay can be readily expanded to other vitamin D metabolites as needed, as the analyte derivatization step after DBS extraction provides virtually equal analytical figures of merit for all mono- and dihydroxylated metabolites of vitamin D [19].

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2016.12.081.

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Supporting Information

Quantification of the 3α and 3β epimers of 25-hydroxyvitamin D_3 in dried blood spots by LC-MS/MS using artificial whole blood calibration and chemical derivatization

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This Supporting Information contains Figures S1-S3, and Tables S1 and S2.

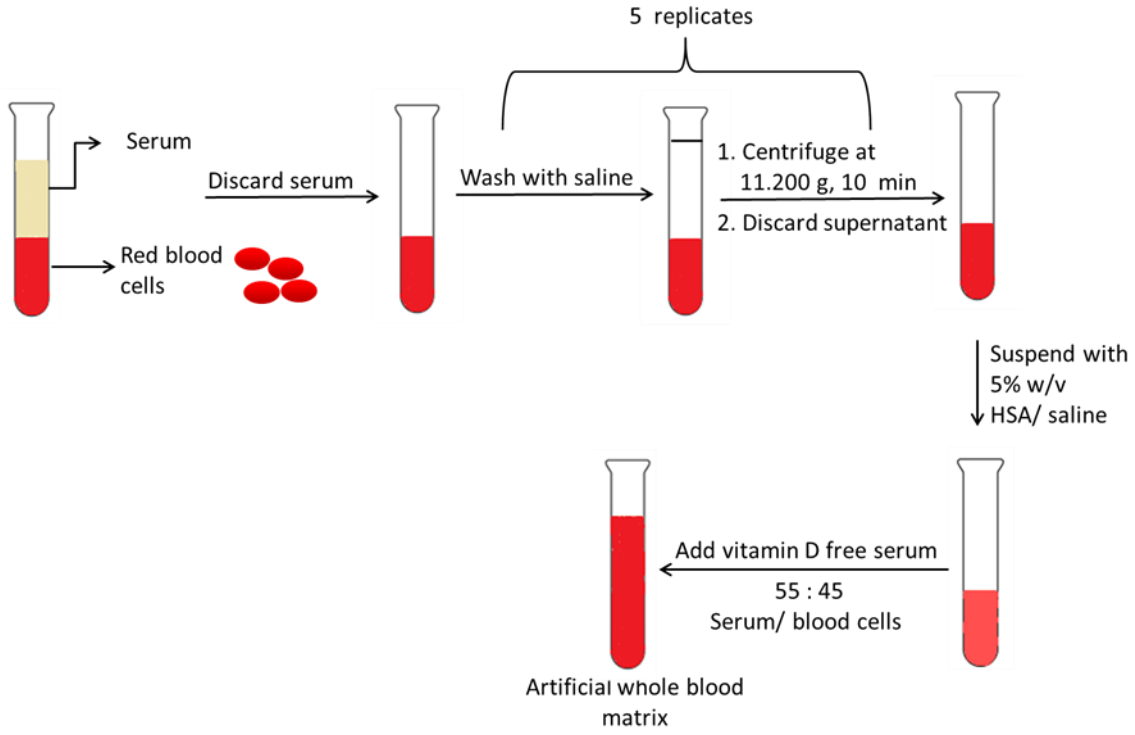

Fig. S1. In-house preparation of vitamin D-free artificial whole blood matrix for calibration.

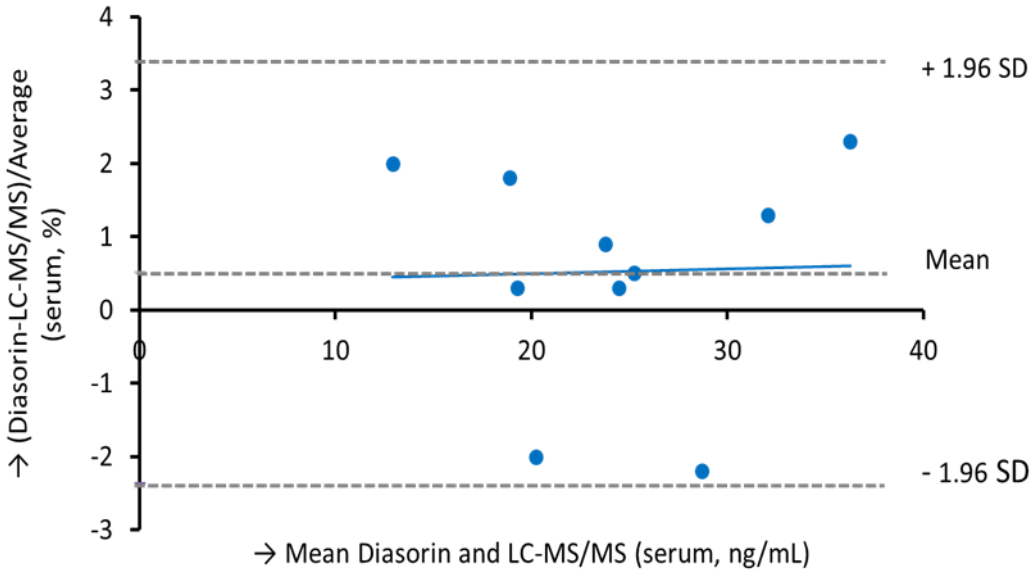

Fig. S2. Bland-Altman analysis for serum LC-MS/MS *versus* serum Diasorin chemiluminescence assay for 3β-25(OH)D_3.

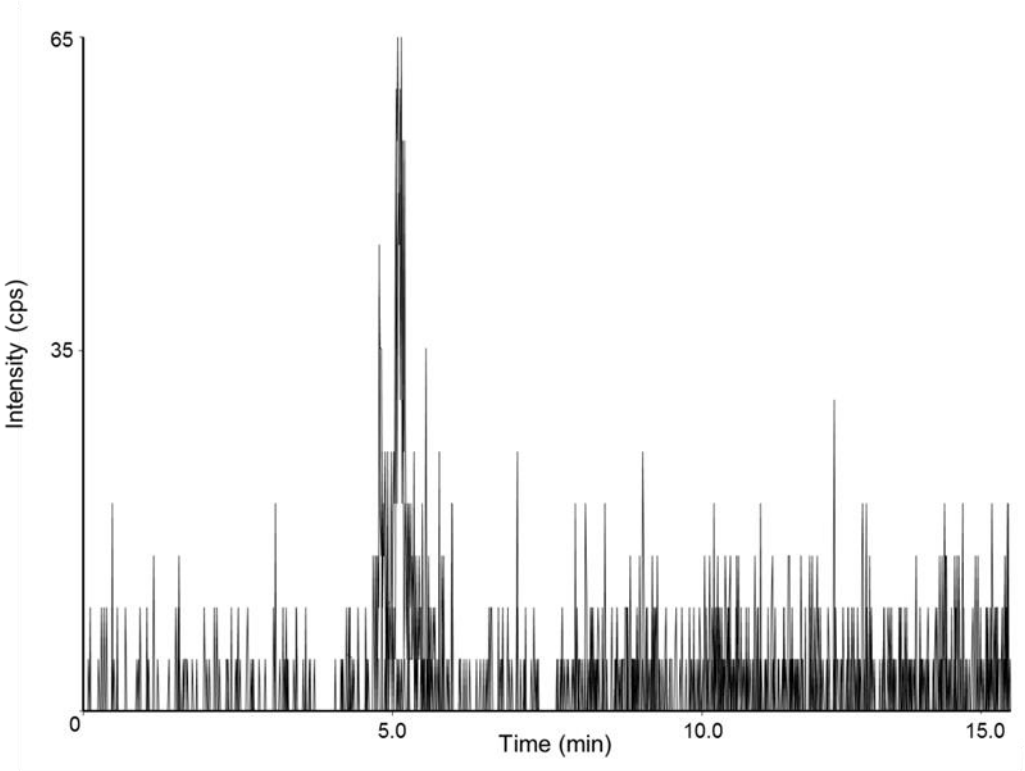

Fig. S3. Combined LC-MS/MS (MRM) traces for the 3α and 3β epimers of 25(OH)D_3 from an extracted blank sample of artificial whole blood (the retention times of 3β- and 3α-25(OH)D_3 are t_r= 6.05 and 6.53 min, respectively).

Table S1. Optimized MS/MS (MRM) settings for the derivatized vitamin D epimers.

25(OH)D_3 epimer	**Collision energy (eV)**	**Declustering potential (V)[1]**	**Precursor ion (*m/z*)**	**Product ion (*m/z*)**
3β	40	100	732.2	673.5
3α	41	10	732.2	673.5

[1]the observed differences for the optimized declustering potentials can be explained by different physicochemical properties of the epimers, as recently described by van den Ouweland *et al.* (J. Chromatogr. B 967 (2014) 195-202).

Table S2. Matrix effects (ion suppression in %) during the LC-ESI-MS/MS (MRM) analysis of pooled whole blood and artificial vitamin D-free whole blood.

Ion suppression (%)				
25(OH)D_3 epimer	**Pooled whole blood**		**Artificial whole blood**	
	10 ng/mL	**30 ng/mL**	**10 ng/mL**	**30 ng/mL**
3β	3.8	2.3	2.3	2.0
3α	2.6	3.9	3.1	2.6

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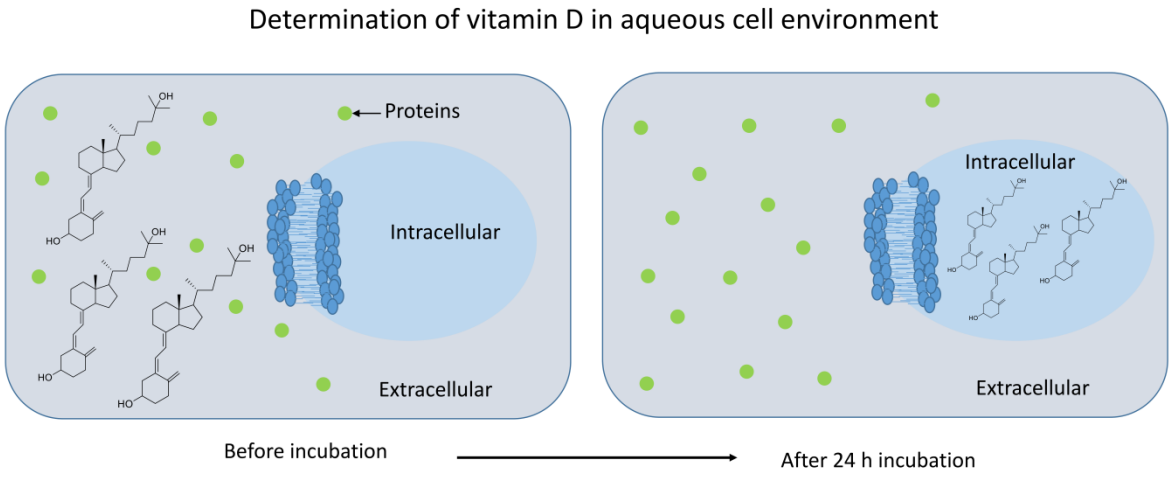

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RESEARCH PAPER

Direct aqueous measurement of 25-hydroxyvitamin D levels in a cellular environment by LC-MS/MS using the novel chemical derivatization reagent MDBP

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Abstract Vitamin D measurements in biological fluids by mass spectrometry are challenging at very low concentration levels. As a result, chemical derivatization is often employed to enhance the ionization properties of low abundant vitamin D compounds. Cookson-type reagents such as 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) or similar derivatives work well but require careful, water-free experimental conditions, as traces of water inactivate the reagent and inhibit or stop the derivatization reactions, thus making quantitative measurements in aqueous samples impossible. We describe a novel electrospray liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for determining 25-hydroxyvitamin D_3 (25(OH)D_3) directly in aqueous cellular systems using a new derivatization reagent, the ionic liquid 12-(maleimidyl)dodecyl-tri-*n*-butylphosphonium bromide (MDBP). The proof-of-concept for the MDBP assay was demonstrated by measuring the levels of 25(OH)D_3 in four different human cell types, namely T cells, helper T cells, B cells, and macrophages. In addition to the ability to determine the levels of 25(OH)D_3 directly in aqueous samples, the cellular integrity was maintained in our application. We show the time-dependent uptake of 25(OH)D_3 into the investigated cells to demonstrate the applicability of the new label. Furthermore, the MDBP derivatization technique may be equally useful in imaging mass spectrometry, where it could be used for response enhancements of spatially localized vitamin D metabolites on wet tissue surfaces, without destroying the integrity of the tissue surface.

Keywords Vitamin D · LC-MS/MS · Chemical derivatization · Cellular uptake

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Introduction

The levels of vitamin D are routinely monitored in human blood because of vitamin D's vital function in bone metabolism [1], as well as its potential role in diseases such as diabetes [2], multiple sclerosis [3], depression [4], and chronic liver diseases [5]. There are many assays available for determination of the main vitamin D metabolite, 25-hydroxyvitamin D_3 (25(OH)D_3), as well as other metabolites [6]. Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) is currently considered the gold standard technique for vitamin D measurements [7]. The technique still has several limitations, however, primarily linked to the low circulating levels of some vitamin D metabolites and the large dynamic ranges, spanning over several orders of magnitude from picomolar to nanomolar levels. Chemically, the molecule's limitation for sensitive mass spectrometric detection is due to the lack of suitable functionalities of sufficient basicity for efficient ionization via electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). As a result, very sensitive mass spectrometry instruments are required to overcome this inherent imperfection, if levels have to be determined at trace levels in biofluids. Alternatively, the vitamin D molecules can be chemically derivatized into better responding analogs by introducing readily ionizable groups or permanently charged moieties into the molecule. Chemical derivatization is common in GC-MS [8] and has

also been frequently implemented for vitamin D analyses by using Cookson-type triazoline-dione and related reagents. For example, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) leads to 100–1000-fold increased ionization efficiency over the non-derivatized vitamin D compounds [9]. Derivatization occurs via [4 + 2] Diels–Alder reaction; the dienophile moiety of the reagent reacts with the s-*cis*-diene group at C-5/C-6 and C-10/C-19 of vitamin D [10–13]. This diene location is well placed within the core structure of vitamin D compounds, as all metabolites are equally transformed. The transformed compounds also give rise to characteristic product ions during MS/MS [14, 15], whereas native vitamin D molecules exhibit unspecific fragmentations from water loss or less specific "picket fence" signals in the lower *m/z* range from backbone dissociations [7]. In addition, significant levels of isobaric interference have been described for the underivatized vitamin D compounds [15]. The derivatization step shifts the *m/z* values for the derivatized products to regions of higher *m/z* and fewer interferences [16]. A fundamental limitation of all existing commercial and non-commercial derivatization reagents for vitamin D is the instability of the reagent in the presence of water. As the working solutions for the reagent are only active for a short period of time (hours), they must be used up rather quickly, which is an unsatisfactory drawback, as some commercial derivatization reagents only come in large package sizes. That is, once prepared, the entire batch (e.g., 50 samples) must be fully expended. Moreover, traces of water in the sample extracts inactivate the reagent completely and inhibit the derivatization reaction. Sample extraction must therefore be followed by a careful and complete drying step [17]. *Ipso facto*, the integrity of the sample matrix cannot be preserved in vitamin D analysis, which is a limitation if vitamin D levels are to be determined in aqueous environments. One example for such an application is the analysis of extra- and intracellular concentrations of vitamin D. Another potential application is the in situ mass spectrometric derivatization of vitamin D on tissue surfaces, where the spatial location of vitamin D is to be measured and the sample therefore cannot be dissolved. We described a new derivatization label (12-(maleimidyl)dodecyl-tri-*n*-butylphosphonium, MDBP) for 25(OH)D_3 with the unique feature of solvent- (including water!) and matrix-independent derivatization. The proof-of-concept for the technique is demonstrated in this study by showing relative quantification of 25(OH)D_3 levels inside and outside of different human cells.

Experimental

Chemicals

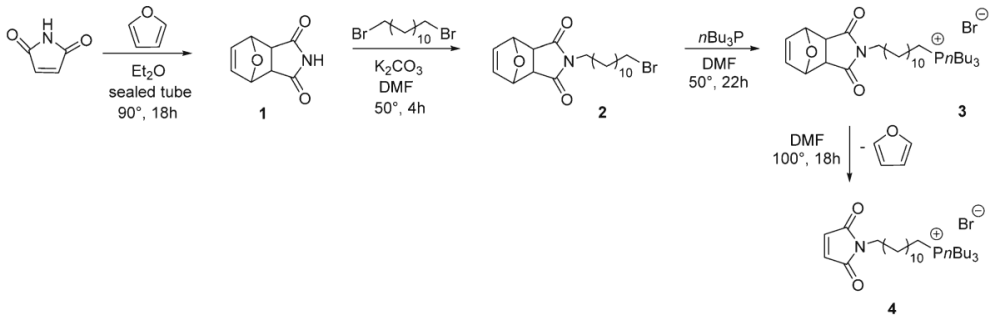
Tri-*n*-butylphosphine, 1,12-dibromododecane, furan, maleimide, *N,N*-dimethylformamide (DMF), potassium carbonate (K_2CO_3), magnesium sulfate, diethyl ether (Et_2O), dichloromethane (DCM), 25-hydroxyvitamin D_3 and methanol (HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Isotope-labelled d_6-25-hydroxyvitamin D_3 was from Chemaphor (Ottawa, ON, Canada), and Amplifex diene reagent was from Sciex (Darmstadt, Germany). Stock solutions of the analyte were prepared in methanol (0.1 mg/mL); working solutions were prepared prior to use by dilution. Organic-free water was generated by a Millipore (Bedford, MA, USA) Direct-Q8 purification system.

Cell types

Different human cell types were obtained from Medical University Erlangen from two human donors. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of buffy coat preparations from the peripheral blood of healthy donors. T cells (CD3+), helper T cells (CD4+, a subpopulation of T cells), and B cells (CD19+) were purified by positive or negative selection using microbeads according to the manufacturer's instructions (Miltenyi Biotech, Bergisch-Gladbach, Germany). The purity was >95% as determined by flow cytometry. To obtain macrophages, monocytes were isolated by adherence on plastic and cultured in the presence of M-CSF (50 ng/ml). Macrophages were detached with EDTA (1 mM, Sigma-Aldrich) after 6 days of culturing. Phenotype was evaluated by expression of surface markers CD68, CD163, HLA-DR, and CD11b. T cells were stimulated with human T-activator CD3/CD28 beads at a bead-to-cell ratio of 1:1 (Invitrogen, Carlsbad, CA, USA) for 2 days. For B activation, CD19+ cells were incubated in round-bottom microplates (96-well) with Pokeweed mitogen (10 μg/ml, Sigma-Aldrich) for 72 h. Macrophages were activated with lipopolysaccharide (10 ng/ml, LPS, Sigma-Aldrich) overnight. Samples were collected upon approval by the local research ethics committee (ethics number: 36_12 B) and patients' written informed consent.

Synthesis of 12-(maleimidyl) dodecyl-tri-*n*-butylphosphonium bromide

Synthesis of the ionic liquid was performed in four steps (Scheme 1) using a modified procedure previously reported in the literature [18]. The precursor 3,6-exo-tetrahydrophthalimide **1** was obtained through reaction of maleimide (2.0 g, 20.6 mmol) and furan (2.10 g, 31 mmol) in 18 h. The reaction was performed in 30 mL diethylether in a pressure stable tube at a temperature of 90 °C, which was held constant in a compartment dryer. After cooling the mixture to room temperature, one precipitated as a white crystalized product. The crystals were filtered and washed five times with 10 mL of cold diethylether to remove unreacted maleimide. A



Scheme 1 Synthetic route for preparation of maleimide-modified phosphonium (MDBP)

yield of 77% was achieved (^{1}H NMR properties: $CDCl_3$, 400 MHz, δ(ppm) = 7.19 (broad singlet, 1H), 6.63 (doublet, 2H), 5.25 (doublet, 2H), 2.83 (singlet, 2H)). High-resolution mass spectrometry (ESI-FTICR) showed the permanently charged molecular ion M^+ at *m/z* 165.079). In the next step, compound **1** (1.056 g, 6.41 mmol) and 1,12-dibromododecane (8.2 g, 25.01 mmol) were dissolved in 70 mL of dry DMF, 0.58 g of K_2CO_3 (4.094 mmol) were added, and the mixture was heated to 50 °C in a compartment dryer for 4 h. Subsequently, the mixture was washed with water to remove DMF and the intermediate product extracted with dieethylether (4 × 20 mL). The organic layer was dried with magnesium sulfate, solvent removed using a rotary evaporator, and purified by flash chromatography using a 2-step eluent (DCM/hexane 50:50 *v/v*, then DCM/methanol 95:5) to isolate white crystals of **2** (12-(3,6-endoxo-Δ4-tetrahydrophtalimide) bromododecane) with a yield of 65% (^{1}H NMR: $CDCl_3$, 400 MHz; δ(ppm) = 5.58 (doublet, 2H), 4.90 (doublet, 2H), 3.33 (triplet, 2H), 2.78 (triplet, 2H), 1.43-1.30 (multiplet, 4H), 1.20 (broad, 16H); MS (ESI), M^+ at *m/z* 411.135). Subsequently, in step 3, tri-*n*-butylphosphine (1.801 g, 8.90 mmol) was dropwise added to dissolve **2** (3.337 g, 8.12 mmol) in 65 mL of dry DMF. The mixture was heated to 50 °C under argon in a compartment dryer for 22 h. DMF was removed in a Schlenk apparatus. The resulting viscous oil was dissolved in DCM (3 mL) and titrated with Et_2O (20 mL). The resulting emulsion was cooled to −24 °C, and a two-phase separation of the product was performed by decanting the solvent. The extraction procedure was repeated five times. To obtain **3** (12-(3,6-endoxo-Δ4-tetrahydrophtalimide)dodecyl-tri-*n*-butylphosphonium bromide), which is a viscous lightly yellow oil, residual solvent was removed using the Schlenk apparatus with a yield of 86% (^{1}H NMR: $CDCl_3$, 400 MHz δ (ppm) = 5.24 (singulet, 2H), 3.42 (triplet, 2H), 2.82 (singulet, 2H), 2.40 (multiplet, 8H), 1.48 (multiplet, 20H), 1.21 (multiplet, 12H); MS (ESI); *m/z*: 534.460). Next, **3** (1.44 g, 2.35 mmol) was dissolved in DMF (14 mL) and heated in an open flask to 100 °C for 18 h to remove furan. DMF was removed in the Schlenk apparatus. The mixture was dissolved in DCM (3 mL) and titrated with Et_2O (20 mL). The resulting emulsion was cooled to −24 °C, and two-phase separation was performed by decanting the solvent; extraction was performed five times. The final product **4** (12-(maleimidyl)dodecyl-tri-*n*-butylphosphonium bromide) is a clear brown viscous liquid, which was obtained by removal of residual solvent using the Schlenk apparatus with yield of 81%. ^{1}H NMR analysis (see Electronic Supplementary Material (ESM) Fig. S1) confirmed that the reaction was quantitative ($CDCl_3$, 400 MHz δ (ppm) = 5.28 (singulet, 2H), 2.43 (multiplet, 12H), 1.52 (multiplet, 20H), 1.29 (multiplet, 10H), 0.96 (triplet, 9H); MS (ESI), M^+ at *m/z* 463.255).

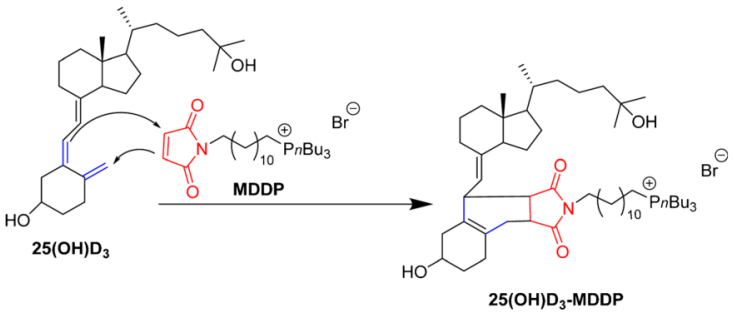
Derivatization with 12-(maleimidyl) dodecyl-tri-*n*-butylphosphonium bromide

The derivatization strategy comprised a Diels–Alder reaction under physiological conditions in an aqueous environment, converting 25(OH)D_3 into a permanently charged quaternary phosphonium ion (Scheme 2a). For derivatization, 50 μL of the 25(OH)D_3 solutions (50 ng/mL in methanol) was added to 50 μL of MDBP solution (0.1 g/mL in methanol). The reaction time was varied between 1, 2, 5, 10, 24, 48, and 72 h to establish the point of quantitative turnover. Additionally, these timed experiments were performed at room temperature and at 40° to evaluate possible thermal effects. Stable isotope standards were added prior to derivatization.

Assay performance for cellular samples

The different cell samples were aliquoted, but no further sample preparation such as extraction or drying was necessary for derivatization with the MDBP label. Each cellular system, that is, the four different cell types in activated and non-activated forms, was measured first as a control to determine blank 25(OH)D_3 values, which may have originated from natural sources. A second set of all samples was generated by spiking the cellular systems with 50 ng/mL of 25(OH)D_3 and internal standard d_6-25(OH)D_3 (10 ng/mL). Finally, a third cellular set was spiked with 50 ng/mL of 25(OH)D_3 and internal standard d_6-25(OH)D_3 (10 ng/mL) for comparative derivatization with

Scheme 2 Derivatization reaction of 25(OH)D_3 using the MDBP label



Amplifex, without the usually applied extraction and drying steps for the reagent [17], to provide a comparison of the performance of the labels in aqueous solution. In general, we observed similar analytical figures of merit for MDBP as for Amplifex. The limit of detection (LOD) for 25(OH)D_3 after derivatization with Amplifex was 0.05 ng/mL [14], in comparison to 0.10 ng/mL determined here using MDBP. The intra-day precision (CV) for 25(OH)D_3-MDBP was <7% RSD at concentrations of 30 and 50 ng/mL 25(OH)D_3, which were spiked into the cellular samples ($n = 3$ each), similar to values observed for 25(OH)D_3-Amplifex, i.e., 2.1% (intra-day) to 5.3% (inter-day) [14].

Monitoring 25(OH)D_3 in human cell systems

Time-dependent experiments were performed to monitor the transfer of 25(OH)D_3 from extracellular regions into the cells. Cell samples from two donors were obtained and aliquoted. PBMC were isolated by density gradient centrifugation of buffy coat preparations from the peripheral blood of healthy donors. Cells were cultured in RPMI 1640 media (Biochrom, Berlin, Germany) supplemented with glutamine (2 mM; Sigma-Aldrich), 10 mM HEPES, 13 mM $NaHCO_3$, 100 μg/mL streptomycin, 60 μg/mL penicillin (all Biochrom), and 10% FCS (Sigma-Aldrich). PBMC (5×10^6 cells/mL) were spiked with 50 ng/mL of 25(OH)D_3 solution and incubated for 24 h. In the first set, the supernatant was separated directly after spiking with 25(OH)D_3, and the cells and the supernatant were separately derivatized with MDBP label and measured. The second set was incubated for 24 h after spiking with 25(OH)D_3, followed by separation of supernatant from cells and derivatization and measurement of supernatant and cells separately. A third set of the same cells was spiked and incubated for 24 h, followed by derivatization and measurement of the sum of cells and supernatant.

Liquid chromatography-electrospray ionization-tandem mass spectrometry

Each cellular sample (5 μL) was injected in duplicate into a Dionex Ultimate 3000 UHPLC system (Thermo, Bremen, Germany). Separation of 25(OH)D_3-MDBP from interfering compounds was performed on a Phenomenex (Torrance, CA, USA) Kinetex PFP 100A column (100 × 2.1 mm, $dp = 2.6$ μm) at 25 °C. The flow rate was 0.4 mL/min using gradient elution. The mobile phases were: (A) water + 0.1% formic acid and (B) methanol + 0.1% formic acid. The gradient started at 50% B and was held for 0.3 min, then increased to 70% and held there for 7 min, further increased to 85% B, and held for 1.2 min. Finally, B was increased to 90% and held for 4 min before returning to the initial conditions for re-equilibration for 5 min. The LC system was coupled to a Sciex QTRAP 5500 quadrupole-linear ion trap mass spectrometer via electrospray ionization (ESI). Ion source and MS parameters optimized for 25(OH)D_3-MDBP were as follows: source temperature, 50 °C; curtain gas, 55 psi; gas 1, 16 psi; gas 2, 0 psi; collision exit potential, 22 V; collision energy, 59 V; declustering potential, 120 V; collision gas pressure, medium; ion spray voltage, 5500 V. Analyses were performed by multiple reaction monitoring (MRM) using the m/z 866.7 → 369.3 transition for 25(OH)D_3, with 200 ms dwell time. Data acquisition was performed using Analyst software 1.6 (Sciex).

Results and discussion

In this study, we performed derivatization of 25(OH)D_3 in different types of cells (T cells, helper T cells, B cells, and macrophages) using a novel derivatization label (MDBP), which was chosen for the particular purpose of enabling derivatization in aqueous environments. The new label MDBP was compared to a well-established derivatization technique for vitamin D metabolites based on the commercial Amplifex reactions [14]. In the comparison, identical aqueous samples and sample preparation conditions were chosen. As proof-of-concept application for the new label, the time-dependent uptake of 25(OH)D_3 into cells was investigated by means of relative quantification.

Important features of the new label were: (*1*) It allows derivatization independent from solvent and sample matrix, including aqueous environments; (*2*) it provides significantly

improved long-term stability of reagent solutions as compared to PTAD or Amplifex; (*3*) it introduces a permanently charged moiety into the derivatized molecules, thus significantly increasing the ionization efficiency in ESI; (*4*) it is significantly cheaper than commercial diene labels; and (*5*) it preserves the integrity of the biological sample, including spatial differences of metabolite locations, if required.

Derivatization via Diels–Alder reaction using ionic liquids → MDBP

Diels–Alder reactions are routinely performed using ionic liquids as solvents, reaction media, or organo-catalysts [19–21]. In the present study, the ionic liquid MDBP was synthesized and used for the first time as a derivatization reagent in analytical chemistry applications, specifically for Diels–Alder conversion of 25(OH)D_3 in aqueous environments. MDBP works as a dienophile, similar to Cookson-type reagents, but exhibits one important structural difference, viz., it does not utilize the C═O activated N═N bonding element, but rather a less reactive C═C moiety (Scheme 2). While this offers significant advantages with respect to stability in aqueous environments, it significantly slows down the reaction with 25(OH)D_3 and substantially lengthens the reactions times (vide infra).

The reason for the reduced reactivity can be readily explained by the mismatched orbital energy gaps during the Diels–Alder reaction using the activated C═C bond. Diels–Alder reactions are governed by orbital symmetry considerations ([4π + 2π] cycloaddition) generated through the suprafacial/suprafacial interaction of the 4π electron system (diene) with the 2π electron system (dienophile) [22]. For reactions to occur at reasonable speeds, the energy levels of the highest occupied molecular orbital (HOMO) of the diene and the lowest unoccupied molecular orbital (LUMO) of the dienophile have to be close to each other to guarantee overlap of π-orbitals. For regular Diels–Alder reactions, electron-withdrawing groups at the dienophile lower the LUMO, which is achieved by the conjugated keto groups. For C═C, the energy gap of HOMO and LUMO is larger than for N═N, which will make overlap more difficult and increase the activation energy of the reaction [23, 24]. This leads to increases of temperature or time to achieve similar yields as compared to N═N.

Time and temperature dependence of derivatization

The outcome of the MDBP derivatization reaction was initially investigated to establish whether quantitative turnover of the process can be reached. The reaction time was varied between 1 and 72 h (see Experimental and Fig. S3, ESM). The initial signal of *m/z* 401 for the $[M + H]^+$ ions of underivatized 25(OH)D_3 was used as marker for the reaction progression (set to 100%, i.e. 0% of product at $t = 0$). After 5 h, 32% of 25(OH)D_3-MDBP was formed and 73% at 10 h. After 24 h, >98% of 25(OH)D_3-MDBP was seen; longer reaction times only gave negligible improvements at times up to 72 h. The higher temperature of 40 °C showed only minor improvements in comparison to room temperature. Reaction conditions were therefore chosen as follows: derivatization time 24 h at room temperature. The 24-h reaction time is an important drawback of MDBP, as reaction times for quantitative derivatization of 25(OH)D_3 with Amplifex were only 30 min for comparable turnover.

The major advantage of using the MDBP reagent is the stability of its solutions over extended periods of time (several weeks), allowing storage and reuse of prepared mixtures. Reagents such as PTAD usually have to be used up within 2 h. As mentioned before, PTAD and Amplifex require fully water-free organic extracts for derivatization and can therefore not be used without destroying the sample matrix, which is disadvantageous in certain applications, where the biological environments are directly probed (see below).

Several peculiarities encountered with chemical derivatization of 25(OH)D_3 have been previously reported for vitamin D metabolites [14, 25, 26]. In particular, the formation of two epimeric products for 25(OH)D_3 from the two possible sides of attack of the *cis*-diene moiety of vitamin D (Scheme 2) results in two products and thus two peaks in the MRM chromatograms. The same was observed with MDBP (Fig. 2). The major epimeric product was chosen for quantification of 25(OH)D_3, as performed with PTAD or other reagents [6, 25]. The direct comparison with Amplifex reagent demonstrated the unique advantage of MDBP, that is, its stability in water. Amplifex did not give any signals for derivatized 25(OH)D_3 (Fig. S3, ESM). Obviously, this was expected, as reagents such as PTAD or Amplifex do not to react in aqueous samples because of the high reactivity of the activated N═N dienophile moiety (vide supra).

Importantly, the MDBP approach for 25(OH)D_3 was as sensitive as the labelling technique with Amplifex. We observed an approx. 1000-fold increase of ionization efficiency of 25(OH)D_3-MDBP in comparison to underivatized 25(OH)D_3 (Fig. S2, ESM), which corresponded well to the enhancements previously seen with Amplifex-labelled 25(OH)D_3 [14, 27]. Equally, ESI response factors were very similar for both reagents, 0.98 for MDBP and 0.95 for Amplifex. Obviously, these effects are due to the permanent charges of the derivatized analytes, i.e., quaternary ammonium ions for Amplifex-transformed species and phosphonium ions for MDBP. These preformed ions are much more efficiently transferred into the gas phase during ESI and also equalize the response factors between derivatized molecules. The more commonly used PTAD does not give preformed ions after derivatization, and detection sensitivity therefore is lower, with enhancement factors on the order of 10–100 over the underivatized species [25, 26, 28]. The enhancement and response-levelling properties of MDBP and Amplifex have the beneficial side effect of applying equally to all major vitamin D metabolites [14], which will readily allow transferring

the described MDBP assay to low-abundant species such as 1,25(OH)2D_3.

Collision-induced dissociation spectra of 25(OH)D_3-MDBP

Fragmentation of the derivatized 25(OH)D_3 metabolite exhibited a distinct dissociation pattern (Fig. 1). In comparison, for the native $[M+H]^+$ ion of 25(OH)D_3 molecule, unspecific water losses dominate the collision-induced dissociation (CID) spectra at lower collision energies. Activation at higher energies provides a wide spectrum of "picket fence" ions, which, unfortunately, also exhibit only limited specificity, as all vitamin D compounds show very similar product ions [7]. CID of the $[M]^+$ ion of 25(OH)D_3-MDBP (*m/z* 866, $C_{55}H_{97}NO_4P^+$; Fig. 1) generated three main product ions at *m/z* 387, 369, and 286. The calculated elemental formula for *m/z* 387 was $C_{27}H_{45}$, based on mass measurement at high mass accuracy; equally, *m/z* 369 corresponded to $C_{27}H_{47}O$ and *m/z* 286 to $C_{18}H_{40}P$. Structure proposals for these ions are given in Fig. 1. The most abundant signal at *m/z* 369 was subsequently chosen for MRM after optimization of interface and collision cell conditions (see Experimental).

Determination of 25(OH)D_3 in different cell systems and uptake experiments

The chemical derivatization of 25(OH)D_3 with MDBP in the cellular samples was performed under native physiological conditions, directly in the cell samples, without any extraction and drying steps. Within cells, as much as 99% of 25(OH)D_3 is bound to the vitamin D binding protein (DBP). As no extraction with organic solvents was applied here, the present approach is likely to solely detect the free, non-DBP bound fraction of 25(OH)D_3. Therefore, in the basal state, no 25(OH)D_3 was detected, and only after incubation with 25(OH)D_3, the non-DBP bound 25(OH)D_3 became measurable in the cellular fraction. All cellular systems were analyzed in activated and non-activated forms; measurement of the controls (without added 25(OH)D_3) did not show significant signals for 25(OH)D_3, excluding any endogenous sources for 25(OH)D_3 in these samples (an example is shown as Fig. S3 in the ESM). Cellular systems are important to study because vitamin D plays a key role for immunological effector functions and vitamin D deficiency is associated with a range of diseases [29–31]. Furthermore, the immunological phenotype and the activation state of immune cells determine their vitamin D metabolism and, vice versa, vitamin D is an important determinant of cellular differentiation [32–36]. In particular, activated T cells, B cells, and macrophages stimulated with bacterial antigens display an increased uptake and turnover of 25(OH)D_3. Finally, the hydroxylated form of 25(OH)D_3 modulates cytokine production, thereby limiting T cell or myeloid differentiation. Therefore, the precise measurement of 25(OH)D_3 in resting and activated immune cells could be an important tool in the diagnosis and molecular elucidation of several diseases [29, 31]. As an example, the determination of 25(OH)D_3 in the set of 25(OH)D_3-supplemented T cells, helper T cells, B cells, and macrophages samples is shown in Fig. 2.

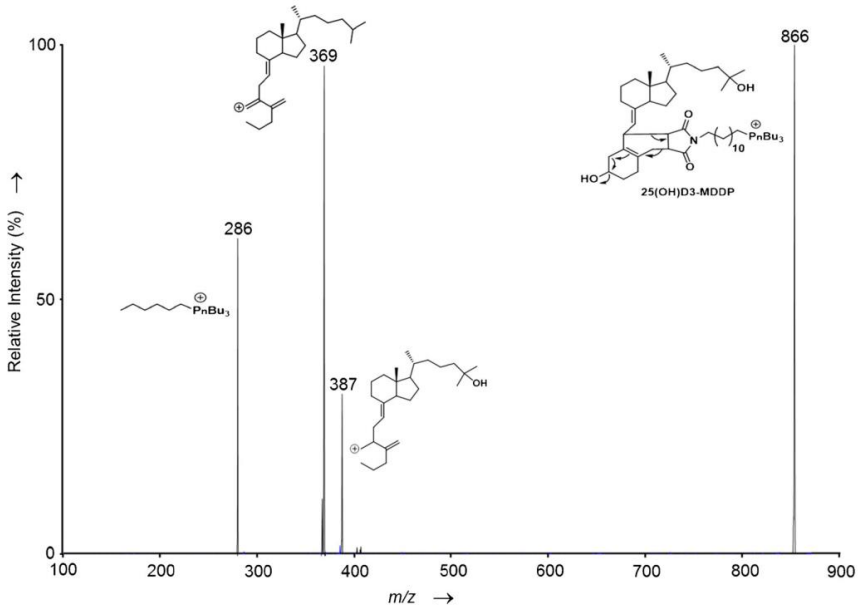


Fig. 1 Collision-induced dissociation spectrum of 25(OH)D_3-MDBP (M^+, *m/z* 866)

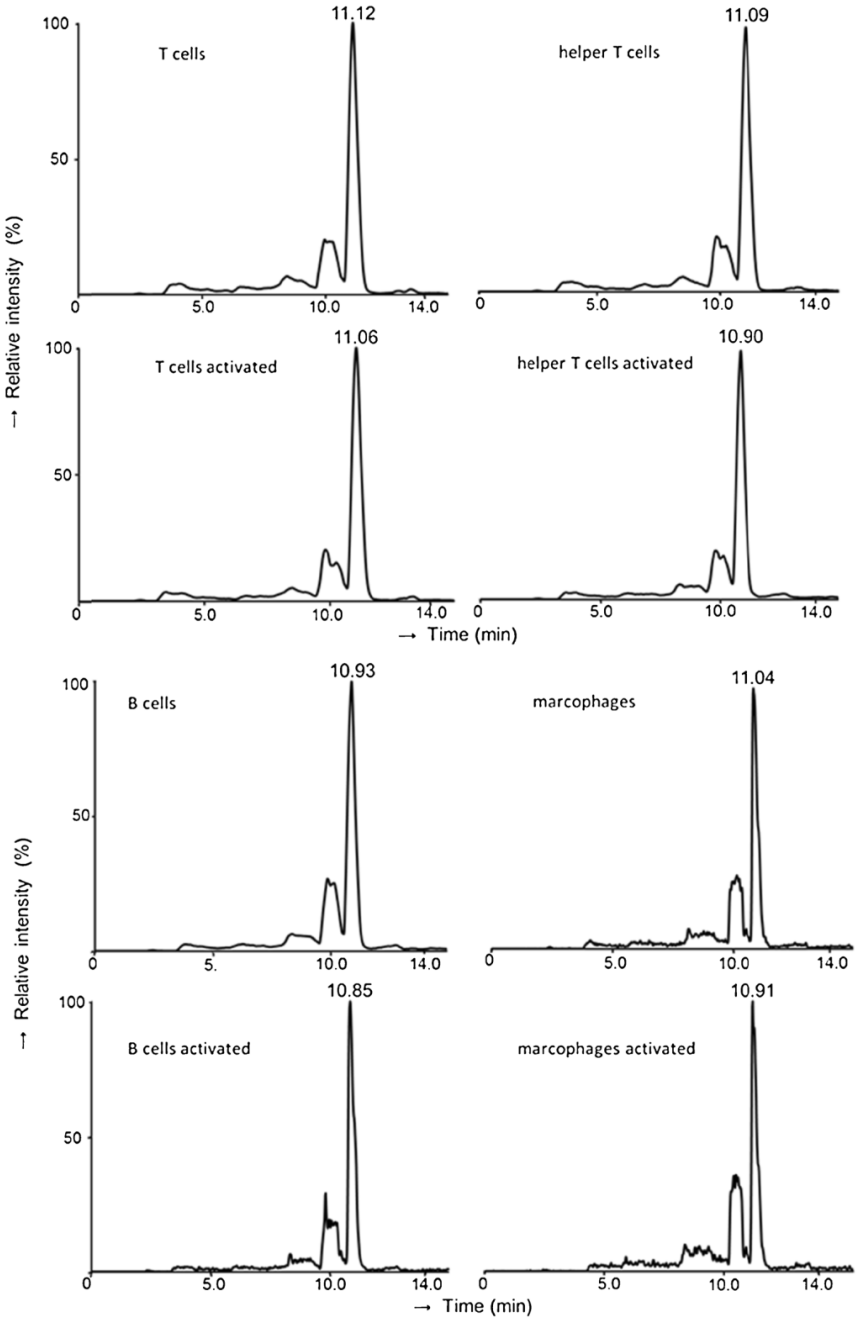


Fig. 2 MDBP derivatization of $25(OH)D_3$ in different cellular systems

These analyses were performed after MDBP derivatization in the aqueous cellular samples.

We performed an additional set of experiments that directly followed the uptake of supplemented $25(OH)D_3$ from the extra- to the intracellular region. In these experiments, we prepared three different sets of the same cell system, which were varied in incubation times for $25(OH)D_3$. For this time course, it was important to keep the cellular environment intact during the uptake of $25(OH)D_3$ and probe the extra- and intracellular spaces. The first set of cells was directly separated from the supernatant after spiking with $25(OH)D_3$; cells showed no signal for 25(OH)D3, as no compound had diffused into the cells

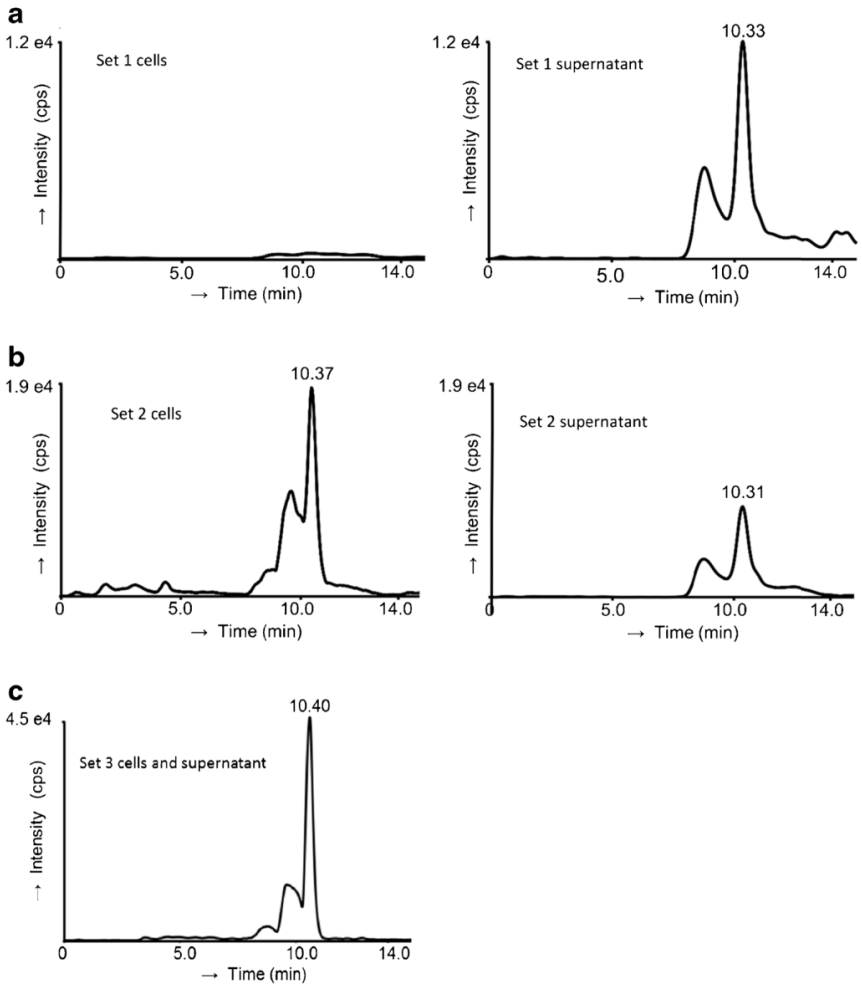


Fig. 3 $25(OH)D_3$ in the extra- and intracellular regions of three sets of PBMC cellular systems varying in incubation times over a 24-h time period, measured after derivatization with MDBP in the aqueous samples. (**a**) PBMC (peripheral blood mononuclear cell) set 1 was directly separated after supplementation with $25(OH)D_3$. (**b**) PBMC set 2 was measured after an incubation period of 24 h before separating supernatant and cells. (**c**) The third set of the same PBMC was spiked with $25(OH)D_3$ and incubated for 24 h, followed by derivatization and measuring the sum of $25(OH)D_3$ in the extra- and intracellular regions

during the very short time period between supplementation and separation. The full amount of supplemented $25(OH)D_3$ was therefore visible, as expected, in the supernatant (Fig. 3a). Any endogenous levels of $25(OH)D_3$ within the cells could also be excluded at the same time. The second sample set after incubation for 24 h showed the reversed situation: The supplemented $25(OH)D_3$ was nearly completely located in the cells and the supernatant showed only minor (residual) levels of $25(OH)D_3$ (Fig. 3b). We demonstrated a third set, which enabled the direct comparison of separated cells and supernatant $25(OH)D_3$ values, as compared to summed $25(OH)D_3$ values of cells and supernatant in one sample. The latter measurement was used as control indicator for detectable $25(OH)D_3$ values in PBMCs (peripheral blood mononuclear cells) (Fig. 3c). The physiological environment within the cellular systems was maintained during the uptake experiments and regions were individually probed.

Conclusions

In this study, we have implemented a new MDBP label for chemical derivatization of $25(OH)D_3$ in aqueous environments and developed an LC-MS/MS assay for quantification of $25(OH)D_3$ based on the reaction with MDBP. The assay exhibited comparable performance to established assays using chemical derivatization. Importantly, solutions of the new reagent were shown to be very stable in comparison to established derivatization reagents for vitamin D, with excellent stability of weeks versus a few hours for PTAD or Amplifex. To demonstrate the proof-of-principle, we studied the cellular uptake of $25(OH)D_3$ from extracellular regions into the cells. As the reagents utilizes the *cis*-diene moiety in the A/B region of the secosteroidal vitamin D compounds, it will equally convert all vitamin D analogs, independent of metabolic transformation, as previously shown for established derivatizations

reagents. Thus, MDBP assays for other metabolites such as 24,25-dihydroxyvitamin D_3 or 1,25-dihydroxyvitamin D_3 will exhibit virtually identical performance as the method shown here. We are aware that the required reaction time for MDBP is very long (24 h versus <1 h for Amplifex or PTAD), but we do not propagate MDBP as a replacement for the established reagents, but rather, as a specialized technique for investigating vitamin D metabolites in aqueous environments, in particular where the sample integrity cannot be disturbed. These could be cellular systems, in particular vitamin D metabolic footprinting versus fingerprinting applications for multiple metabolites. In addition, the assay has the potential to be utilized as a direct screening method for 25(OH)D_3 in matrices such as saliva, where 25(OH)D_3 is mostly non-protein bound. We also envision another important area, viz., imaging mass spectrometry (MSI). In matrix-assisted laser desorption/ionization (MALDI)-MSI, the on-tissue concentrations of vitamin D metabolites are very low [7], necessitating the use of derivatization techniques if low abundant species are to be analyzed. As tissue surfaces are not dry and the sample integrity must be fully maintained, established vitamin D reagents for derivatization cannot be used. A label such as MDBP, on the other hand, might function if the reagent is applied with an appropriate liquid dispensing technique.

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Compliance with ethical standards

Conflicts of interest The authors declare no competing financial and non-financial interests.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Direct aqueous measurement of 25-hydroxyvitamin D levels in a cellular environment by LC-MS/MS using the novel chemical derivatization reagent MDBP

Miriam J. Müller, Heiko Bruns, Dietrich A. Volmer

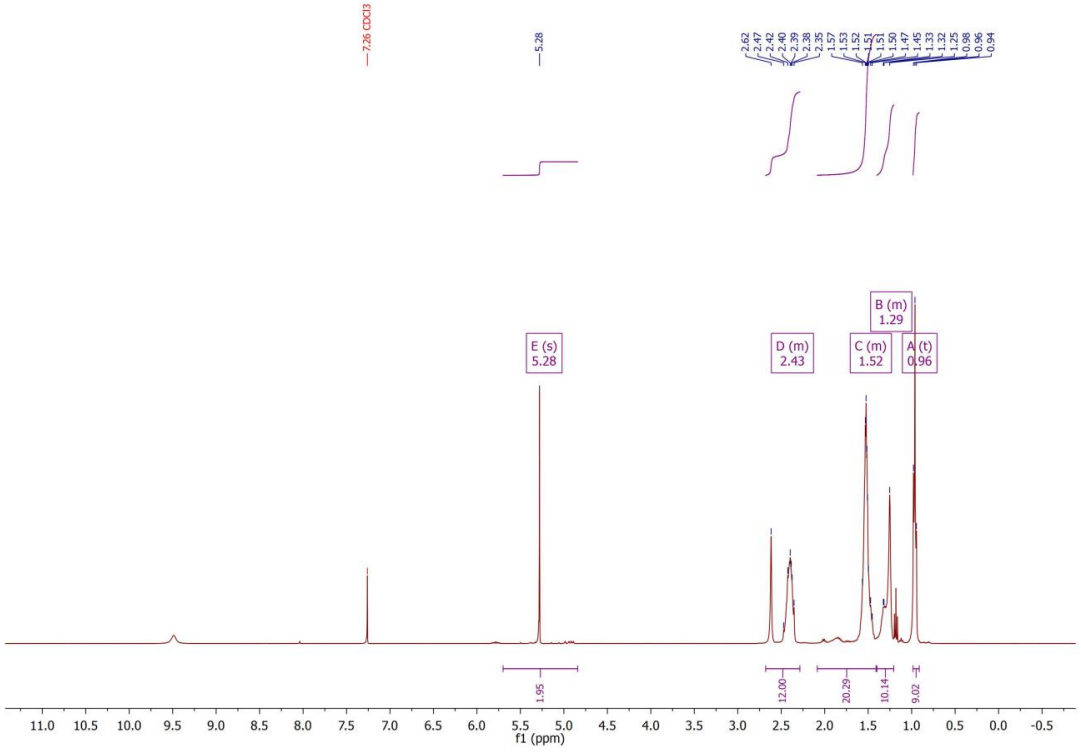

Fig. S1 ^{1}H NMR spectrum of 12-(maleimidyl)dodecyl-tri-*n*-butylphosphonium bromide (MDBP)

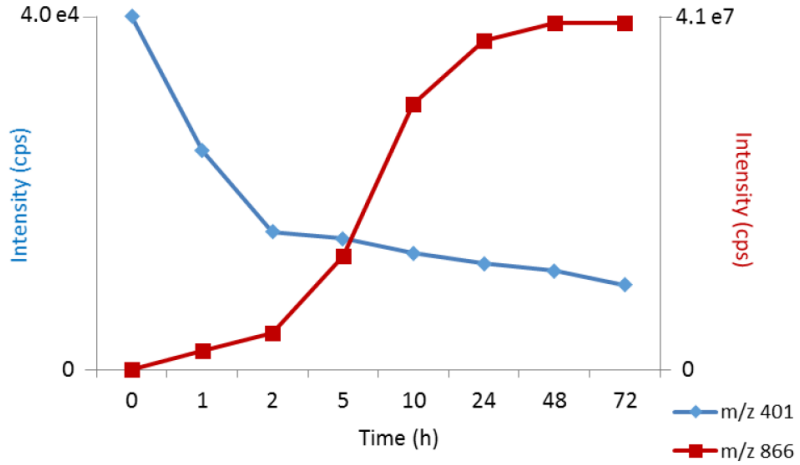

Fig. S2 Time course of the derivatization reaction of 25(OH)D_3 and MDBP, showing intensity of underivatized precursor ion (*m/z* 401) and transformation product at *m/z* 866. *NB*: experiments were conducted by flow injection analysis without prior chromatography separation. The *m/z* 401 and 866 ion signals were measured in selected ion monitoring (SIM) mode. Note that the ionization efficiency of precursor and product differed by approx. three orders of magnitude and therefore different y-scales are used in the diagram

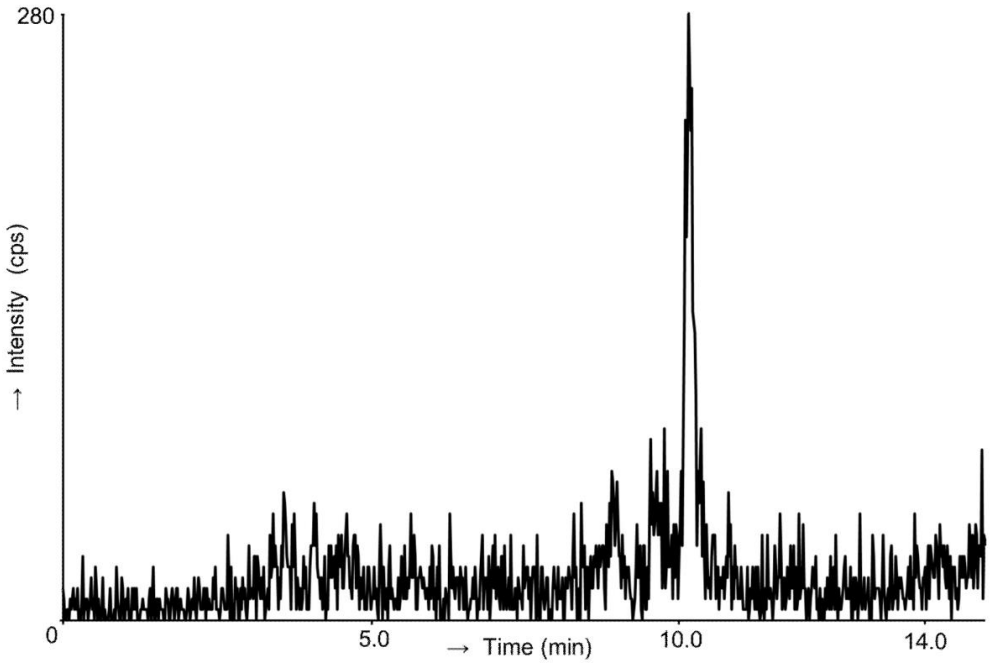

Fig. S3 LC-MS/MS chromatogram of a native cell sample measured as control to determine endogenous background levels of 25(OH)D_3 (MRM using *m/z* 401→383 ($[MH]^+$→$[M+H-H_2O]^+$) transition; the MRM chromatogram of a supplemented cell sample after derivatization with Amplifex (without drying and extraction steps) using *m/z* 732 → 673 ($[M]^+$→$[M+H-N(CH_3)_3]^+$) gave very similar traces)

VIII. CURRICULUM VITAE

Education

10/2014 – 09/2017 Saarland University (UdS), Saarbrücken, Germany
PhD at the Institute of Bioanalytical Chemistry

Topic of the doctoral thesis: "*Development of new methods for determination of vitamin D metabolites using advanced analytical mass spectrometry techniques*." Supervisor: Prof. Dr. Dietrich A. Volmer

04/2010 – 07/2014 Technical University Dortmund (TU), Dortmund, Germany
Degree: *Master of Science – Chemistry*.

Focus: Analytical Chemistry
Topic Master Thesis: *Application of MALDI-Imaging of metabolic interactions of microorganism.*

Technical University Dortmund (TU), Dortmund, Germany
Degree: *Bachelor of Science – Chemistry*.

Focus: Technical Chemistry
Topic Bachelor Thesis: *Optimizing aqueous two-phase system for the selective synthesis of primary amines from renewable raw materials.*

08/2008-06/2010 Vocational Training : Chemical laboratory assistant
Landesamt für Soziales, Gesundheit und Verbraucherschutz Saarbrücken, Germany

Focus :

- Residual analysis of food and consumer goods
- Application / Compliance with QM standards

04/2006-05/2008 Study Pharmacy

06/2005 *Allgemeine Hochschulreife* (equivalent to High School Diploma) received from the Berufsbildendes Gymnasium f. Soziales u. Gesundheit Trier, Germany

Training

04/2017-07/2017	Holding lecture ”Interpretation of mass spectra”
04/2016	Training „ *Leadership*“
02/2016	Training „*Project management*“
12/2015	Training „ *Communication in industry*“
since 2015	*Exzellenzprogramm for Scientist III*
10/2012	*Extension of knowledge additional testing for the placing on the market of biocidal products and plant protection products according to § 3 Absatz 1 ChemVerbotsV*
08/2012	*Expertise according to § 5 ChemVerbotsV vom 14.10.1993*

Practical Experience

10/2014 - 10/2017 Bioanalytical Chemistry, Saarland University, Saarbrücken
Scientific Associate (wissenschaftliche Mitarbeiterin) at DFG Project *“New methods for improved determination of vitamin D metabolic markers using advanced analytical mass spectrometry techniques”*

- Project management
- Interdisciplinary cooperation
- Performance of analytical experiments
- Supervision of Bachelor/Master- students

03/2014 - 06/2014 INFU (Institut für Umwelt), TU Dortmund
Scientific Associate (wissenschaftliche Hilfskraft)

- MALDI - Imaging of Metalaxyl on lupine roots
- Time-dependent MALDI-Imaging experiments of Nectria rigidiuscula funghi (Ardesia)

IX. SCIENTIFIC CONTRIBUTIONS

Publications

M.J. Müller, H. Bruns, D.A. Volmer, Direct aqueous measurement of 25-hydroxyvitamin D levels in a cellular environment by LC-MS/MS using the novel chemical derivatization reagent MDBP, Analytical and Bioanalytical Chemistry, **2017**, 409 (10), 2705-2717.

M.J. Müller, C.S. Stokes, D.A. Volmer, Quantification of the 3a and 3ß epimers of 25-hydroxyvitamin D3 in dried blood spots by LC-MS/MS using artificial whole blood calibration and chemical derivatization, Talanta, **2017**, 167, 398-404.

M.J. Müller, C.S. Stokes, F. Lammert, D.A. Volmer, Chemotyping the distribution of vitamin D metabolites in human serum, *Sci Rep.*, **2016**, 6, 21080.

M.J. Müller, D.A. Volmer, Mass Spectrometric Profiling of Vitamin D Metabolites beyond 25-Hydroxyvitamin D, Clin Chem., **2015**, 61 (8), 1033-48.

A. Behr, A. Wintzer, C. Lübke, M. Müller, Synthesis of primary amines from the renewable compound citronellal via biphasic reductive amination, J Mole Catalysis A Chemical, **2015**, 404, 74-82.

Oral presentation

- 50th Annual Conference of the German Society of Mass Spectrometry (DGMS), **03/2017**, Kiel, Germany.
  "Direct aqueous measurement of 25-hydroxyvitamin D in cellular environments by LC-MS/MS using the novel chemical derivatization reagent MDBP"

- 65th Annual Conference of the American Society of Mass Spectrometry (ASMS), **06/2017**, Indianapolis, USA.
  "Determination of 25-hydroxyvitamin D in aqueous cellular environments by LC-MS/MS using the novel chemical derivatization reagent MDBP"

Poster presentations

- 65th Annual Conference of the American Society of Mass Spectrometry (ASMS), **06/2017**, Indianapolis, USA.
  "Quantification of 3α and 3β epimers of 25-hydroxyvitamin D3 in DBS using artificial whole blood calibration and chemical derivatization"

- 21st International Mass Spectrometry Conference (IMSC), **08/2016,** Toronto, Canada.
  "Metabolic distributions of vitamin D metabolites in dried blood spots after chemical derivatization and LC-MS/MS"

- 49th Annual Conference of the German Society of Mass Spectrometry (DGMS), **03/2016**, Hamburg, Germany.
  "Measurement of metabolic distributions of Vitamin D metabolites in human serum after chemical derivatization"

- 3rd Doctoral Students Day of Faculty 8, Saarland University, **11/2015**, Saarbrücken, Germany.

  "Quantitative measurement of Vitamin D metabolic distributions in human serum after chemical derivatization"

- 63rd Annual Conference of the American Society of Mass Spectrometry (ASMS), **06/2015**, St. Louis, Missouri, USA.

  "Quantitative measurement of Vitamin D metabolic distributions in human serum after chemical derivatization"