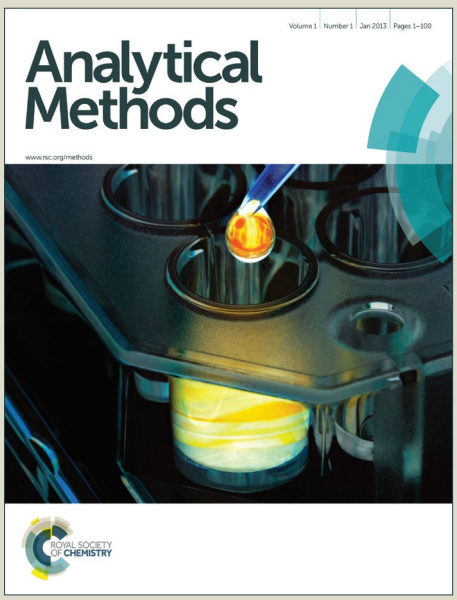
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Journal Name

ARTICLE

Simultaneous analysis of 25OHD$_3$ and 24,25(OH)$_2$D$_3$ both in human serum and cerebrospinal fluid by LC-MS/MS

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Xin He,†[ac] Pei Jiang, †[abc] Ying Xue,†[ac] Wen-Ye Zhu,[ac] Yang Deng,[a] Miao Yan[a], Huan-De Li,[*a] Rui-Li Dang,[abc] and Mi-Mi Tang[ac]

As a pleiotropic hormone, vitamin D (VD) is not only involved in bone health, but also associated with various disorders. Determination of circulating 25OHD$_3$ is routinely used to measure the status of VD, while previous reported methods exhibited various defects in accuracy, sensitivity, rapidness and simplicity in analysis of VD and its metabolites. We present here a validated method for the simultaneous analysis of 25OHD$_3$ and 24,25(OH)$_2$D$_3$ both in low volumes of serum and cerebrospinal fluid (CSF) with high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) using deuterated internal standard. Serum and CSF samples were simply processed by protein precipitation and derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) to enhance their responses and sensitivity. Separation was conducted using a gradient program on a Thermo Accucore C18 column. Mass spectrometry was operated using electrospray ionization (ESI) under positive ion and multiple reaction monitoring (MRM) mode. The method exhibited excellent linearity with correlation coefficients above 0.99. The lower limit of quantification (LLOQ) for 25OHD$_3$ and 24,25(OH)$_2$D$_3$ were 0.10 and 0.25 ng/mL, respectively. Intra-day precision ranged from 1.4% to 9.7% and inter-day precision ranged from 1.9% to 10.0% for both 25OHD$_3$ and 24,25(OH)$_2$D$_3$. This method is precise, sensitive and simple, which is practical for study on VD and its metabolites in human serum and CSF.

Introduction

Vitamin D (VD) is a fat-soluble nutrient and a steroid hormone related to various physiological functions, in addition to its classical role in calcium-phosphorus homeostasis and bone health [1]. It has also been connected with several disorders like diabetes, cardiovascular diseases, and cancers [2-4]. Interestingly, as a newly recognized neuro-active steroid, VD has been found to play an indispensable role in brain development and functions, such as regulation of the neuronal differentiation, neuro immunomodulation and actions of neurotransmitters and neurotrophic factors [5, 6]. Additionally, studies have progressively been reported that VD is associated with various central nervous system diseases including Alzheimer's disease (AD), multiple sclerosis (MS), schizophrenia and depression [7-10]. Therefore, accurately determining VD and its metabolites in biological specimens will be considerably essential for our understanding of their functions and importance.

VD$_3$ (Fig. 1), the main source of VD in human, is photosynthesized from 7-dehydrocholesterol in the skin with exposure to sunlight. In the liver, VD$_3$ is hydroxylated by 25-hydroxylase enzymes to produce 25-hydroxyvitamin D$_3$ (25OHD$_3$, Fig. 1), its major storage form, and then further hydroxylated by CYP27B1 in the kidney into 1α,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$, Fig. 1), the bioactive form of VD$_3$. 25OHD$_3$ is catabolized into 24,25-dihydroxyvitamin D$_3$ (24,25(OH)$_2$D$_3$, Fig. 1) by CYP24A1 at C-24 position [11]. Clinically, 25OHD$_3$ is considered as the best biomarker of VD$_3$ status due to its long half-life and high level.

A number of methods have been developed for quantification of circulating VD metabolites, including immunoassays, protein binding assays (PBA), high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) [12-15].

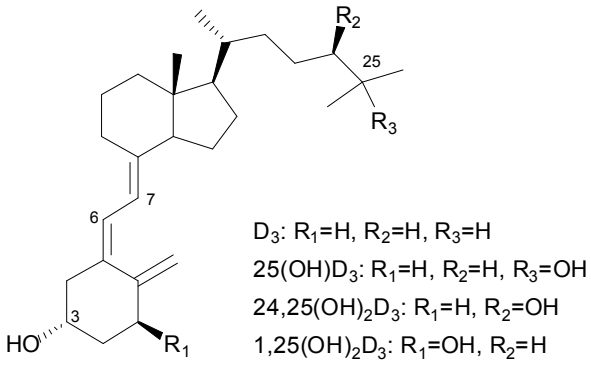


Fig. 1 Structures of VD$_3$ and its metabolites

[a] Institute of Clinical Pharmacy & Pharmacology, Second Xiangya Hospital, Central South University, Changsha, China.
[b] Department of Pharmacy, Jining First People's Hospital, Jining, China.
[c] School of Pharmaceutical Sciences, Central South University, Changsha, China
† These authors contributed equally to this work.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

However, these methods are confronted with prominent defects in accuracy, reproducibility, lower limit of quantification (LLOQ), sensitivity, selectivity and simplicity. Besides, simultaneous analysis of multiple metabolites often cannot be achieved by these methods. Currently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a promising alternative displaying better sensitivity and accuracy [16-19]. Moreover, derivatization of Diels-Alder reaction between VD metabolites and Cookson-type reagents (4-substituted-1,2,4-triazoline-3,5-dione) overcomes the challenge of poor ionization efficiency, and thereby increases the sensitivity [17-21].

Additionally, while VD is a novel neuro-active steroid affecting the development and functions of brain, there are little reports on methods of simultaneous determination of VD metabolites in cerebrospinal fluid (CSF) by LC-MS/MS. Accordingly, developing a simple and reliable method to measure the status of VD metabolites in CSF is in great demand. In this study, we developed a LC-MS/MS methodology for simultaneous quantification of $25OHD_3$ and $24,25(OH)_2D_3$ both in human serum and CSF using a simple procedure of sample preparation and derivatization with PTAD.

Experimental

Reagents and chemicals

The standards of $25(OH)D_3$ and $24,25(OH)_2D_3$ were purchased from ApexBio Technology LLC. (Boston, MA, USA). Deuterated internal standard (IS) d_6-$25(OH)D_3$ was obtained from Sigma-Aldrich (St. Louis, MO, USA). The 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan) and was used as derivatization reagent. HPLC-grade acetonitrile and methanol was purchased from Merck KGaA (Darmstadt, Germany). HPLC-grade formic acid was from ROE scientific INC. (St. Newark, DE, USA). Deionized water was produced by a series of compact, combination 12 VDC RO+DI reagent grade water purification systems from AQUA solutions, INC. (Jasper, Georgia, USA).

Preparation of stock and calibration solutions

Stock solutions of $25(OH)D_3$ and $24,25(OH)_2D_3$ were prepared in acetonitrile at concentrations of 1 mg/mL and 0.2 mg/mL, respectively. The standard of d_6-$25(OH)D_3$ (50 μg/mL in ethanol) was diluted to 10 μg/mL with acetonitrile as the IS stock solution, and then diluted to 100 ng/mL with acetonitrile to provide the IS working solution. All of the solutions were stored at -80 °C.

Calibration solutions of the two metabolites (0.10, 0.21, 1.04, 5.21, 20.0, 52.08 and 197.92 ng/mL for $25(OH)D_3$; 0.25, 0.50, 2.08, 7.92, 20.0, 50.0 and 100.0 ng/mL for $24,25(OH)_2D_3$) were prepared freshly with acetonitrile dilutions of each stock solutions.

Serum and CSF sampling

CSF from 6 adults was collected from the clinical laboratory and serum from healthy adults was collected from health care centre of the Second Xiangya Hospital of Central South University. A batch of serum was pooled for method validation, while serum from 63 healthy subjects was stored for successive determination. All samples were stored at -80 °C until assay. This study was approved by the Ethical Committee of the Second Xiangya Hospital of Central South University. Written informed consent was obtained from all participants.

Sample preparation

200 μL of thawed serum or CSF or calibration solution was spiked with 10 μL of IS working solution and equilibrated at room temperature for about 15 min. Proteins were precipitated by addition of 600 μL of acetonitrile, followed by vigorous vortex for 3 min at the maximum speed. After 10 min of centrifugation at 13,000g, 4 °C, a volume of 650 μL of the supernatant was transferred and evaporated to dryness by a vacuum drying system. Then 100 μL of derivatization reagent (1 mg/mL PTAD in acetonitrile) was added to the dry residue, followed by 1 min of mixing. The mixture was sequentially left without light at room temperature for 8 h to allow the derivatization reaction completing. 50 μL of the supernatant from 1 min of vortex and 5 min of centrifugation of the derivatized sample was transferred into vials and 5 μL of the solution was injected into LC-MS/MS for analysis.

LC and MS conditions

LC-MS/MS analysis was carried out on a Shimadzu LC-20AD chromatograph (Shimadzu Corporation, Kyoto, Japan) connected to a 4000 QTRAP triple quadrupole-linear ion trap mass spectrometer with a Turbolon-Spray interface (AB Sciex, Concord, Ontario, Canada). Separation was performed using a Thermo Accucore C18 column (2.6 μm, 100×4.6 mm; Thermo Fisher Scientific INC. Waltham, MA, USA) with column temperature at 40 °C. The mobile phase was established with solvent A (water containing 0.1% formic acid) and solvent B (methanol) at flow rate of 0.3 mL/min. The gradient elution program was started with 39% A /61% B from 0 to 1 min, reaching 14% A /86% B at 2 min and maintaining for 5.5 min , then returned to 39% A /61% B at 8 min and retained 3.5 min for equilibration. Samples in vials were kept at 4 °C and the injection volume was 5 μL. The ion source was operated under ESI-positive ionization mode and mass spectrometry conditions were set as follows: curtain gas, 25 psi; ion spray voltage, 5000 V; source temperature, 600 °C; ion source gas 1, 70 psi; ion source gas 2, 70 psi; decluttering potential, 80 V; entrance potential, 10 V; collision cell exit potential, 10 V. The multiple reaction monitoring (MRM) mode was utilized for quantification and the monitored transitions were *m/z* 558/298, 574/298 and 564/298 for $25(OH)D_3$, $24,25(OH)_2D_3$ and d_6-$25(OH)D_3$, respectively. Other compound-specific parameters were listed in Table 1.

Method validation

Calibration curves were calculated by plotting the peak area ratios of analyte/IS versus corresponding concentrations of each analyte. Using least square regression, the calibration curves were constructed by fitting the data to the equation: $y= bx+ a$, where y referred to the peak area ratio and x represented the concentration of analyte. The linearity of the calibration curves was determined by correlation coefficient (r^2).

The quality control (QC) samples for serum were prepared by spiking 10 µL of mixed solutions containing certain amounts of standards at three levels into pooled human serum (n=5) in which the levels of endogenous analytes were measured (The added concentrations in QC samples were 5 ng/mL, 10 ng/mL and 12.5 ng/mL for 25(OH)D_3 and 0.5 ng/mL, 0.75 ng/mL and 1 ng/mL for 24,25$(OH)_2D_3$, respectively). Then the QC samples for CSF were prepared similarly as serum, except that the pooled CSF was diluted 2:1 (v/v) by phosphate buffered solution (PBS) for the inadequacy of CSF samples (The added concentrations in QC samples were 2.5 ng/mL, 3 ng/mL and 5 ng/mL for 25(OH)D_3 and 0.25 ng/mL, 0.3 ng/mL and 0.5 ng/mL for 24,25$(OH)_2D_3$, respectively). QC samples were extracted and measured as described in the "sample preparation" section. The intra-day accuracy and precision of the method was evaluated by determining the QC samples in replicates of five on one day. The inter-day accuracy and precision of the method was assessed by measuring these samples in replicates of five over three successive days. Defined as recovery of each analyte in QC samples at three levels, the accuracy was calculated as follows: the difference of the concentrations between analytes measured in QC samples and in the blank samples was divided by the theoretically added concentrations. The precision was estimated by the relative standard deviation (RSD) of these determinations. The lower limit of quantification (LLOQ) was defined as the lowest concentrations in the calibration curves with a signal/noise (S/N) of 10 and with acceptable precision (<20%) and accuracy (80-120%) [22], which was validated based on the added concentrations of standards in pooled serum.

Table 1 MRM transition parameters of analytes and internal standard.

Analyte	Dwell (ms)	MRM transition (m/z)	CE (V)
25(OH)D_3-PTAD	100	558/298	25
24,25$(OH)_2D_3$-PTAD	100	574/298	30
d_6-25(OH)D_3-PTAD	100	564/298	25

For evaluation of freeze-thaw stability, three aliquots of pooled serum added with 10 µL of low (5 ng/mL for 25(OH)D_3 and 0.5 ng/mL for 24,25$(OH)_2D_3$) or high (12.5 ng/mL for 25(OH)D_3 and 1 ng/mL for 24,25$(OH)_2D_3$) concentration of analytes were prepared and underwent three freeze-thaw cycles consisting of 1 h of thaw at room temperature followed by 12 h of freeze at -80 °C. After the final cycle was completed, samples were stored at -80 °C until assay. These samples were extracted and measured replicatedly as described in the "sample preparation" section.

For evaluation of stability of derivatized analytes in the autosampler tray at 4 °C, another batch of QC samples were made as mentioned above and prepared as described in the "sample preparation" section. The derivatized samples were stored in the autosampler tray at 4 °C for 96 h and then measured.

The stability after freeze-thaw cycles and in the autosampler tray at 4 °C was determined by the percentage of the measured concentrations of analytes in QC samples to the endogenous and theoretically added concentrations of analytes.

For evaluation of matrix effect, experiment was conducted according to our previous work [23]. The spiked samples were prepared by spiking known amounts of standards to the supernatant of extracted blank serum/CSF samples. The added concentrations of analytes were same as the QC samples. And calibrator solutions in acetonitrile with same levels of standards as the QC samples were also prepared (n=5). The matrix effect, estimated by ME, was calculated as follows:

$$\text{ME (\%)} = \left[1 - \frac{\text{(peak area ratio in spiked sample - peak area ratio in blank sample)}}{\text{peak area ratio in calibrator solution}}\right] \times 100$$

Results and discussion

Sample preparation and derivatization

In this method, a simple method of sample pretreatment was developed which made it feasible for fast determination of large scale of samples. Only small volumes of sample (200 µL) and a two-step of sample preparation including protein precipitation and extraction by acetonitrile were required. Target samples can be prepared without complicated procedures such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE) which are time-consuming.

The process of derivatization was optimized as well. Duration of reaction, temperatures and concentration of PTAD were taken into examinations to determine the derivatization conditions. In accordance with previous reports [17, 19], derivatization with 1 mg/mL PTAD in acetonitrile exhibited satisfactory response in LC-MS/MS among several investigated PTAD solutions with different concentrations. Meanwhile, both analytes and IS showed the highest responses after 8 h of incubation at room temperature so that derivatization was conducted under this condition in the present method.

Intact standards of 25(OH)D_3 and 24,25$(OH)_2D_3$ displayed dissatisfied responses, sensitivities and shapes of chromatographic peaks in LC-MS/MS system which was mainly due to the low ionization efficiency of these VD compounds. However, conjugated diene groups in both of the analytes make them perfect substrates of Diels-Alder reactions with Cookson-type reagents (4-substituted-1,2,4-triazoline -3,5-dione). Actually, this kind of reagent has been reported in several studies concerning determinations of VD metabolites, such as 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4--triazoline-3,5-dione (DMEQTAD), 4-[4-(6-methoxy-2-2benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MBOTAD), 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NPTAD) and 4-(4'-dimethylaminopheyl)-1,2,4-triazoline-3,5-dione (DAPTAD) [17, 19-21, 24-27]. However, none of them except PTAD are commercially available.

So we chose PTAD as the derivatization reagent which can add polar groups to the compounds of analytes. Thus, ionization efficiency was enhanced and sensitivity was thereby elevated sharply compared with underivatized counterparts. In addition, molecular weights of the analytes were increased after derivatization resulting in raised *m/z* ratios and highly specific ion fragments so that interfering responses at low *m/z* region were avoided.

Optimization of LC and MS conditions

Parameters of mass spectrography were optimized by injecting 1 μg/mL of derivatized standards into LC-MS/MS system. Several chromatographic columns and different mobile phase gradients were explored to get satisfying separation. The optimized LC-MS/MS conditions were listed in "experimental" section.

PTAD derivatized VD metabolites consisted of two epimers, *6S* and *6R*, because the dienophile attacked the *s-cis*-diene of the target compounds from the *α*- and *β*-sides. Therefore, 25(OH)D_3-PTAD and 24,25$(OH)_2D_3$-PTAD eluted two peaks (see Fig. 2 and 3). The major peak produced by *6S*-isomer was selected for quantification.

As shown in Fig. 4 and 5, derivative from PTAD provided the base ion at *m/z* 558 and 574 for 25(OH)D_3 and 24,25$(OH)_2D_3$, respectively, which were derived from losing one molecule of water from the C-25 position of the protonated compound ($[M+H-H_2O]^+$). Thus, the $[M+H-H_2O]^+$ ions were acquired as precursor ions. Furthermore, we also observed a dominant fragment ion for both PTAD modified analytes at *m/z* 298, which was obtained from cleavage between C-6 and C-7 and was acquired as product ion.

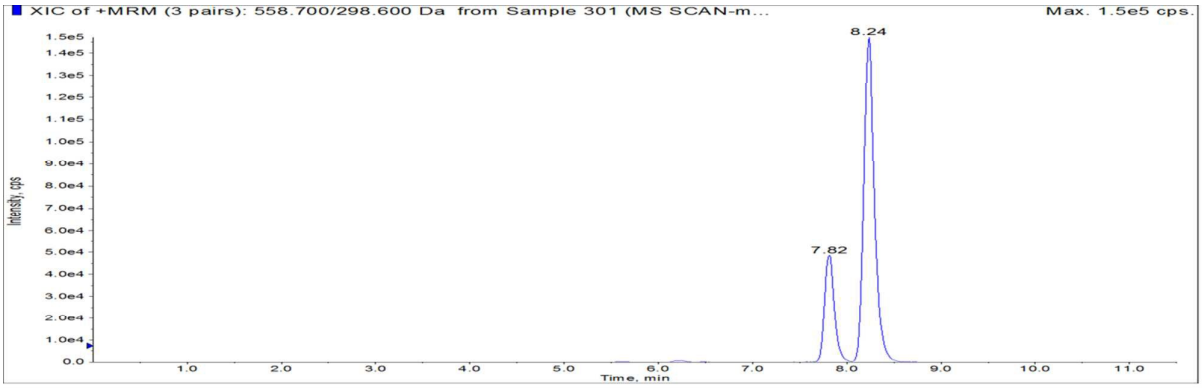


Fig. 2 Typical chromatographs of 25(OH)D_3

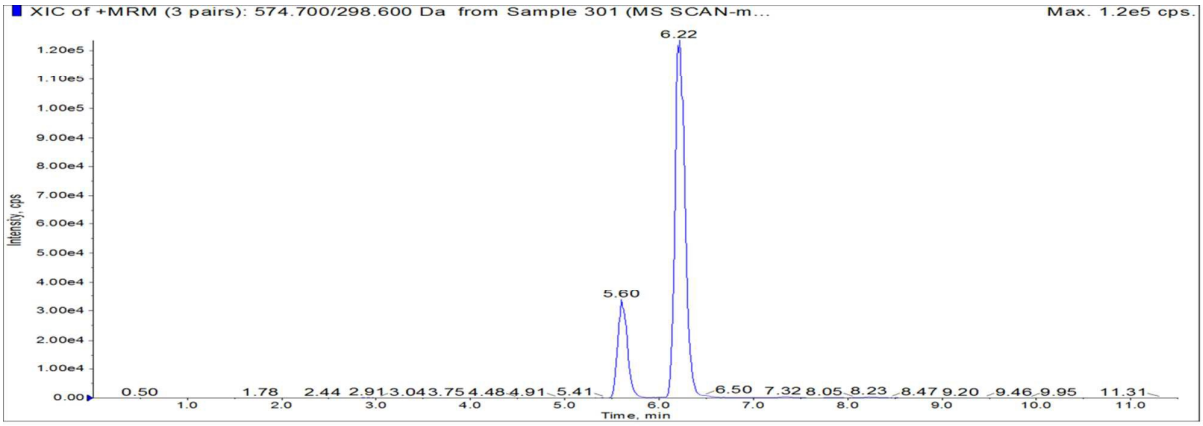


Fig. 3 Typical chromatographs of 24,25$(OH)_2D_3$

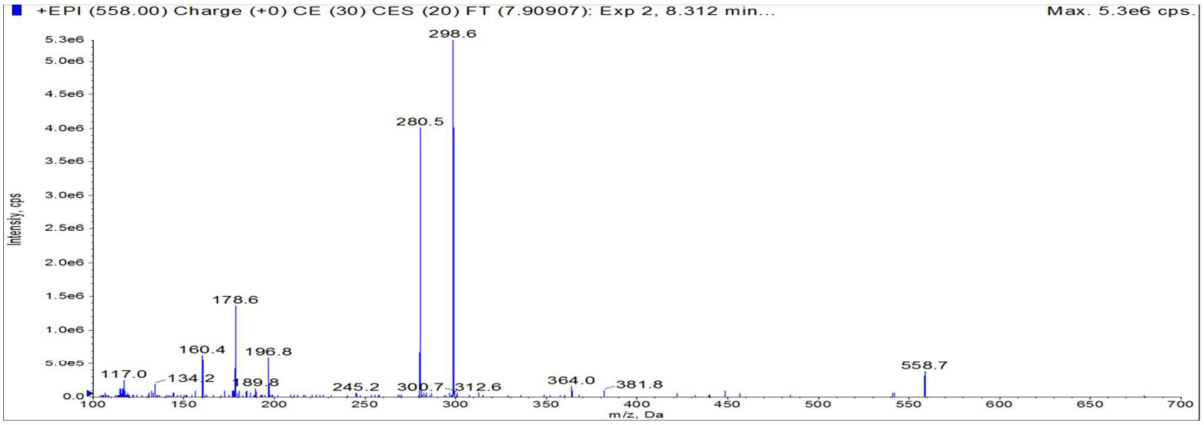


Fig. 4 Product ion mass spectra of $25(OH)D_3$

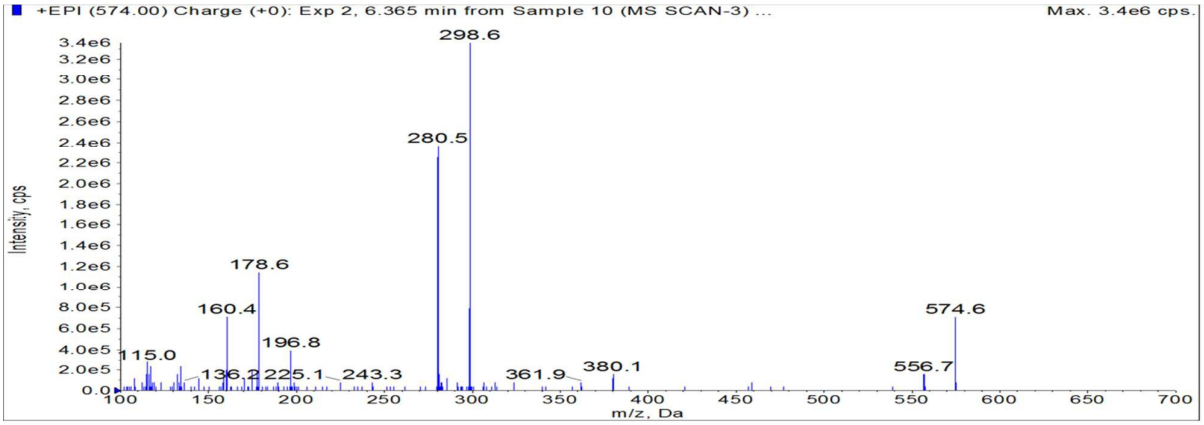


Fig. 5 Product ion mass spectra of $24,25(OH)_2D_3$

Method validation

Equations of calibration curves, linear ranges, correlation coefficients (r^2) and LLOQ for both standards are illustrated in Table 2. The calibration curves exhibited excellent linearity with correlation coefficients above 0.99.

Because VD metabolites are endogenous substances and analyte-free serum and CSF is hard to obtain, method accuracy and precision was estimated by addition of different concentrations of standards to pooled human serum and CSF samples with known levels of analytes and prepared and measured as true samples. Precision was expressed as RSD (%) and accuracy was defined as recovery (%). As shown in Table 3 and 4, the intra-day precision ranged from 3.7% to 8.1% and 1.4% to 9.7% for serum and CSF, respectively, and the inter-day precision ranged from 3.9% to 10.0% and 1.9% to 7.6% for serum and CSF, respectively. The LLOQ of $25(OH)D_3$ and $24,25(OH)_2D_3$ was validated in standard addition samples of serum and was listed in Table 3.

Table 2 Equations of calibration curves with correlation coefficients (r^2), linear range and LLOQ.

Analyte	Regression equation	r^2	Linear range (ng/mL)	LLOQ (ng/mL)
$25(OH)D_3$	$y = 0.3329x + 0.2161$	0.9999	0.10-200.0	0.1
$24,25(OH)_2D_3$	$y = 0.3772x - 0.9973$	0.9989	0.25-100.0	0.25

Table 3 Intra-day and inter-day accuracy and precision in serum.

Analyte	Added (ng/mL)	Intra-day		Inter-day	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
$25(OH)D_3$	0.1 (LLOQ)	7.8	106.9	10.0	105.4
	5	8.1	100.3	8.6	103.1
	10	6.6	104.5	5.3	103.6
	12.5	4.3	98.6	6.1	103.0
$24,25(OH)_2D_3$	0.25 (LLOQ)	4.3	105.5	3.9	112.8
	0.5	3.7	99.6	5.6	99.8
	0.75	5.3	102.3	4.8	103.3
	1	4.1	98.1	4.7	104.7

Table 4 Intra-day and inter-day accuracy and precision in CSF.

Analyte	Added (ng/mL)	Intra-day		Inter-day	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
$25(OH)D_3$	2.5	8.9	103.1	7.6	106.8
	3	6.7	98.8	5.2	104.4
	5	5.7	107.0	6.1	10.4
$24,25(OH)_2D_3$	0.25	1.4	106.3	1.9	107.4
	0.3	9.7	109.5	7.0	103.3
	0.5	2.3	99.7	2.0	111.2

Previous studies have shown that derivatized VD metabolites were stable within five freeze-thaw cycles and were also stable in autosampler tray at 4 °C for 96 h [19, 28]. In this method, we observed that derivatized analytes were stable for at least three freeze-thaw cycles and can be stably stored in autosampler tray at 4 °C at least for 96 h. Data were shown in Table 5 and 6, respectively.

The matrix effect ranged from -13.5% to -7.3% in serum and ranged from -14.2% to -3.0% in CSF, suggesting insignificant matrix effects in the present method.

Analysis of $25(OH)D_3$ and $24,25(OH)_2D_3$ in human serum and CSF

Using the established method, serum samples from 63 healthy individuals recruited from health care centre and CSF samples from 6 subjects collected from the clinical laboratory of the Second Xiangya Hospital of Central South University were determined. The concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ in serum were 35.3 ± 5.25 (15.0-66.4) ng/mL and 3.72 ± 0.243 (2.71-4.96) ng/mL (mean ± SD), respectively. The mean levels of $25(OH)D_3$ and $24,25(OH)_2D_3$ in CSF were 2.76 ± 1.02 (1.67-4.07) ng/mL and 0.881 ± 0.0856 (0.795-1.06) ng/mL, respectively (mean ± SD).

Researches on quantitation of VD metabolites in CSF were sparse. The first study on this issue, conducted by Balabanova et al using PBA, reported the median concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ in CSF from patients with lumbar disc protrusion were 8.3 ng/mL and 1.8 ng/mL, respectively [29]. Whereas, recently published works reported lower but relatively indefinite $25(OH)D_3$ levels in CSF without measuring $24,25(OH)_2D_3$ in CSF. They reported that the median levels of $25(OH)D_3$ in CSF was 4.4 ng/mL in patients with AD by immunoassay [30]. While two groups reported the median concentrations of $25(OH)D_3$ in CSF were both below 0.4 ng/mL in patients with MS by UPLC-MS and ELISA [31-33]. Diversity in

Table 5 Freeze-thaw stability of analytes (%).

Freeze-thaw cycle	$25(OH)D_3$		$24,25(OH)_2D_3$	
	5 ng/mL	12.5 ng/mL	0.5 ng/mL	1 ng/mL
1	104.6	104.7	98.6	100.5
2	101.2	100.6	105.1	101.3
3	98.8	101.6	103.0	99.2

Table 6 Stability of derivatized analytes in autosampler tray at 4 °C for 96 h.

Analyte	Added (ng/mL)	Percentage of theoretical concentration in spiked sample (%)
$25(OH)D_3$	5	102.9
	10	98.8
	12.5	104.4
$24,25(OH)_2D_3$	0.5	99.4
	0.75	100.5
	1	103.4

participants and methods of analysis may contribute to these inconsistent results. Hence, larger population of subjects should be enrolled to analyse the exact concentration of $25(OH)D_3$ in CSF, as well as $24,25(OH)_2D_3$. Furthermore, it is necessary to determine VD metabolites in serum and CSF from identical subjects in order to explore the relationship of concentrations of VD metabolites between circulating and brain, which will be essential in facilitating the investigations of the functions, transports and metabolism of VD.

Conclusions

In conclusion, we have developed a reliable and sensitive method for simultaneous determination of $25(OH)D_3$ and $24,25(OH)_2D_3$ in human serum and CSF. A simple procedure of sample preparation further makes it satisfactory in fast and large-scale analysis of these VD metabolites. Since VD and its metabolites are of extensive activities, and various diseases are proved to be relevant to VD deficiency, the present method will be practical to promote our understanding of the physiological roleds of VD and its metabolites

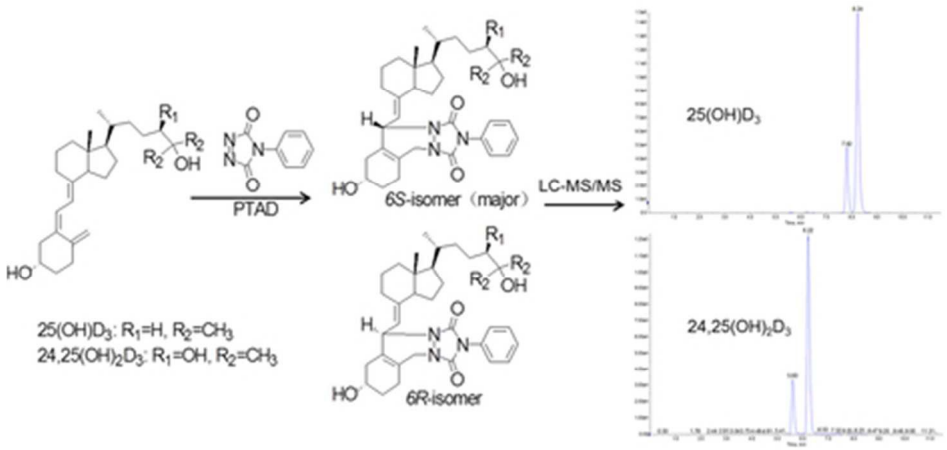
Acknowledgements

This research was supported by National Natural Science Foundation of China (No. 81401113) and Hunan Provincial Innovation Foundation for Postgraduate (no.CX2013B099).

Notes and references

1. I. R. Reid, M. J. Bolland and A. Grey, *Lancet*, 2014, **383**, 146-155.
2. A. G. Pittas, B. Dawson-Hughes, P. R. Sheehan, C. J. Rosen, J. H. Ware, W. C. Knowler, M. A. Staten and D. d. R. Group, *Diabetes care*, 2014, **37**, 3227-3234.
3. A. Zittermann, J. Kuhn, J. Dreier, C. Knabbe, J. F. Gummert and J. Borgermann, *European heart journal*, 2013, **34**, 1358-1364.
4. Q. Zhang, B. Kanterewicz, S. Buch, M. Petkovich, R. Parise, J. Beumer, Y. Lin, B. Diergaarde and P. A. Hershberger, *Molecular and cellular endocrinology*, 2012, **355**, 153-161.
5. D. W. Eyles, T. H. Burne and J. J. McGrath, *Front Neuroendocrinol*, 2013, **34**, 47-64.
6. U. Bang, L. Kolte, M. Hitz, S. Dam Nielsen, L. L. Schierbeck, O. Andersen, S. B. Haugaard, L. Mathiesen, T. Benfield and J. E. Jensen, *HIV clinical trials*, 2012, **13**, 162-170.

7. Y. Slinin, M. L. Paudel, B. C. Taylor, H. A. Fink, A. Ishani, M. T. Canales, K. Yaffe, E. Barrett-Connor, E. S. Orwoll, J. M. Shikany, E. S. Leblanc, J. A. Cauley, K. E. Ensrud and G. Osteoporotic Fractures in Men Study Research, *Neurology*, 2010, **74**, 33-41.
8. E. M. Mowry, E. Waubant, C. E. McCulloch, D. T. Okuda, A. A. Evangelista, R. R. Lincoln, P. A. Gourraud, D. Brenneman, M. C. Owen, P. Qualley, M. Bucci, S. L. Hauser and D. Pelletier, *Annals of neurology*, 2012, **72**, 234-240.
9. M. Crews, J. Lally, P. Gardner-Sood, O. Howes, S. Bonaccorso, S. Smith, R. M. Murray, M. Di Forti and F. Gaughran, *Schizophrenia research*, 2013, **150**, 533-537.
10. P. Jiang, W. Y. Zhang, H. D. Li, H. L. Cai, Y. P. Liu and L. Y. Chen, *Psychoneuroendocrinology*, 2013, **38**, 2091-2098.
11. M. F. Holick, *The New England journal of medicine*, 2007, **357**, 266-281.
12. H. J. Roth, H. Schmidt-Gayk, H. Weber and C. Niederau, *Annals of clinical biochemistry*, 2008, **45**, 153-159.
13. G. D. Carter, J. C. Jones and J. L. Berry, *The Journal of steroid biochemistry and molecular biology*, 2007, **103**, 480-482.
14. A. de la Hunty, A. M. Wallace, S. Gibson, H. Viljakainen, C. Lamberg-Allardt and M. Ashwell, *The British journal of nutrition*, 2010, **104**, 612-619.
15. U. Holler, A. P. Quintana, R. Gossl, K. Olszewski, G. Riss, A. Schattner and C. S. Nunes, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2010, **878**, 963-968.
16. J. M. van den Ouweland, M. Vogeser and S. Bacher, *Reviews in endocrine & metabolic disorders*, 2013, **14**, 159-184.
17. P. A. Aronov, L. M. Hall, K. Dettmer, C. B. Stephensen and B. D. Hammock, *Analytical and bioanalytical chemistry*, 2008, **391**, 1917-1930.
18. S. Ding, I. Schoenmakers, K. Jones, A. Koulman, A. Prentice and D. A. Volmer, *Analytical and bioanalytical chemistry*, 2010, **398**, 779-789.
19. X. Duan, B. Weinstock-Guttman, H. Wang, E. Bang, J. Li, M. Ramanathan and J. Qu, *Analytical chemistry*, 2010, **82**, 2488-2497.
20. T. Higashi, D. Awada and K. Shimada, *Biological & pharmaceutical bulletin*, 2001, **24**, 738-743.
21. T. Higashi, Y. Shibayama, M. Fuji and K. Shimada, *Analytical and bioanalytical chemistry*, 2008, **391**, 229-238.
22. F. D. Administration, *Federal Register*, 2001, **66**, 206-207.
23. H. L. Cai, R. H. Zhu and H. D. Li, *Anal Biochem*, 2010, **396**, 103-111.
24. M. Kaufmann, J. C. Gallagher, M. Peacock, K. P. Schlingmann, M. Konrad, H. F. DeLuca, R. Sigueiro, B. Lopez, A. Mourino, M. Maestro, R. St-Arnaud, J. S. Finkelstein, D. P. Cooper and G. Jones, *The Journal of clinical endocrinology and metabolism*, 2014, **99**, 2567-2574.
25. T. Higashi, D. Awada and K. Shimada, *Biomedical chromatography : BMC*, 2001, **15**, 133-140.
26. T. Higashi, A. Yamauchi and K. Shimada, *Analytical sciences : the international journal of the Japan Society for Analytical Chemistry*, 2003, **19**, 941-943.
27. S. Ogawa, S. Ooki, K. Shinoda and T. Higashi, *Analytical and bioanalytical chemistry*, 2014, **406**, 6647-6654.
28. Z. Wang, T. Senn, T. Kalhorn, X. E. Zheng, S. Zheng, C. L. Davis, M. F. Hebert, Y. S. Lin and K. E. Thummel, *Anal Biochem*, 2011, **418**, 126-133.
29. S. Balabanova, H. P. Richter, G. Antoniadis, J. Homoki, N. Kremmer, J. Hanle and W. M. Teller, *Klinische Wochenschrift*, 1984, **62**, 1086-1090.
30. P. Johansson, E. G. Almqvist, J. O. Johansson, N. Mattsson, U. Andreasson, O. Hansson, A. Wallin, K. Blennow, H. Zetterberg and J. Svensson, *PloS one*, 2013, **8**, e81989.
31. T. Holmoy, S. M. Moen, T. A. Gundersen, M. F. Holick, E. Fainardi, M. Castellazzi and I. Casetta, *Multiple sclerosis*, 2009, **15**, 1280-1285.
32. T. Holmoy, A. Lossius, T. E. Gundersen, S. M. Moen, M. Castellazzi, E. Fainardi and I. Casetta, *Acta Neurol Scand*, 2012, **125**, e28-31.
33. A. Moghtaderi, G. H. Tamadon and F. Haghighi, *Prague Med Rep*, 2013, **114**, 162-171.



Derivatization reaction between analytes, 25(OH)D_3 and 24,25(OH)$_2D_3$, and PTAD highly increased their ionization efficiency and molecular weight, thereby the lower limit of quantification was reduced and the sensitivity of determination was considerably enhanced.
39x19mm (300 x 300 DPI)