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Research Article

Simultaneous determination of vitamin D metabolites 25(OH)D_3 and 1α,25(OH)$_2D_3$ in human plasma using liquid chromatography tandem mass spectrometry

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A R T I C L E I N F O

Keywords:
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LC-MS/MS
Pharmacokinetics
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A B S T R A C T

Background: Although measurement of 25(OH)D_3 is a routine analytical method to determine plasma vitamin D status, 1α,25(OH)$_2D_3$ is the biologically active form. Hence, simultaneous measurement of 25(OH)D_3 and 1α,25(OH)$_2D_3$ could provide better insight into vitamin D status and pharmacokinetics. However, 1α,25(OH)$_2D_3$ has a low plasma concentration, making its quantification challenging for most analytical techniques. Here, we demonstrate use of liquid chromatography tandem mass spectrometry (LC-MSMS) for the development of a simple and rapid method for the simultaneous quantification of 25(OH)D_3 and 1α,25(OH)$_2D_3$.

Methods: Samples were purified from 250 μL human plasma. Chromatography was performed on an analytical column, under gradient conditions using a mobile phase consisting of methanol-lithium acetate. The mass detector was operated in positive multiple reaction monitoring mode. The established method was validated according to the guidance issued by ICH and FDA. Furthermore, a clinical study was performed using this method to detect the plasma concentrations of 1α,25(OH)$_2D_3$ after oral administration of calcitriol.

Results and conclusion: The method was acceptably linear over the concentration ranges of 20–1200 pg/mL for 1α,25(OH)$_2D_3$ and 1–60 ng/mL for 25(OH)D_3, respectively, with correlation coefficients of $r^2 > 0.993$. Both the inter-assay and intra-assay precision was < 15%, and the analytical recoveries were within 100% ± 10%, with no significant matrix effect or carryover. Thereby, we, provide a facile method for the simultaneous detection of vitamin D metabolites in plasma.

1. Introduction

Substantial clinical findings have demonstrated that vitamin D (VD) is related to various physiological processes and pathologies, such as cancer [1], asthma [2], and cardiovascular diseases [3,4]. After endogenous synthesis or intestinal absorption, cholecalciferol (vitamin D_3) is firstly metabolized in the liver by 25-hydroxylases producing 25-hydroxyvitamin D_3 (25(OH)D_3), which is used as a clinical biomarker for assessing vitamin D status [5–8]. It is generally agreed that a lower level 25(OH)D_3 is associated with an increased risk of fractures [7,9,10]. Then, 25(OH)D_3 is converted, primarily by the kidney, to its the most active form, 1α,25(OH)$_2D_3$, which is the ligand of the vitamin D receptor in target tissues [11]. However, recent studies suggest that VD status assessment based on concentrations of 25(OH)D_3 alone may be suboptimal [12]. Some populations have low 25(OH)D_3 concentrations without clinical manifestations of VD deficiency [13–16]. The VD Metabolite Ratio has been suggested as a superior indicator of VD status, where the 1α,25(OH)$_2D_3$:25(OH)D_3 ratio is a better predictor for the

Abbreviations: VD, vitamin D; 25(OH)D_3, 25-hydroxyvitamin D_3; 1α,25(OH)$_2D_3$, 1α,25-dihydroxy vitamin D_3; LC-MS/MS, liquid chromatography tandem mass spectrometry; ESI, electrospray ionization; MRM, multiple reaction monitoring; BSA, bovine serum albumin; ICH, International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; FDA, Food and Drug Administration; IS, internal standard; m/z, mass-to-charge ratios; PPT, protein precipitation; LLE, liquid liquid extraction; SPE, solid phase extraction.

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Table 1
MRM transitions, collision energies and cone voltages of vitamin D metabolites.

Compound	MRM transitions	Cone voltage (V)	Collision energy (eV)
25(OH)D_3	407.31 > 389.24	44	32
25(OH)D_3-d_3	410.50 > 392.26	77	22
1α,25(OH)$_2D_3$	423.26 > 369.25	49	22
1α,25(OH)$_2D_3$-d_6	429.36 > 374.36	60	25

development of diabetic and cardiovascular complications [12,17,18]. Therefore, measuring both analytes simultaneously could provide a valuable method for studying diseases caused by alterations in the VD pathway [19–22].

Partly because 1α,25(OH)$_2D_3$ circulates at low picomolar concentration ranges with highly lipophilic and plasma protein binding properties [23,24], measurement in the human body is challenging. Many assays for vitamin D_3 metabolites have been published, including enzyme-linked immunoassay, radioimmunoassay [25,26], high-performance liquid chromatography [27,28], and liquid chromatography coupled with mass spectrometry (LC-MS/MS), the latter of which is considered the "gold standard" for the determination of vitamin D_3 metabolite levels [29–33]. Currently, the majority of methods reported in the literature usually use derivatization to improve the ionization efficiency of 25(OH)D_3 and 1α,25(OH)$_2D_3$, which is financially costly and time consuming [31,33]. Therefore, development of an easy-to-operate and highly sensitive method to measure 25(OH)D_3 and 1α,25(OH)$_2D_3$, simultaneously, has high scientific and clinical value. Accordingly, in this study, we developed and validated a relatively simple and precise method for the simultaneous determination of plasma 25(OH)D_3 and 1α,25(OH)$_2D_3$. Furthermore, we performed proof-of-principle clinical research using this method to demonstrate its clinical applicability.

2. Materials and methods

2.1. Chemicals and reagents

In our study, 25(OH)D_3, 25(OH)D_3-d_3, 1α,25(OH)$_2D_3$, 1α,25(OH)$_2D_3$-d_6 (dissolved in 80% methanol) were provided by Toronto Research Chemicals (North York, Canada). LC-MS chromasolv-grade methanol (MeOH) and LC-MS chromasolv-grade *iso*-propyl alcohol (IPA) were purchased from Merk (Darmsrtadt, Germany). LC-MS Optima-grade water was purchased from Quchenshi (Shanghai, China). Further, 96-well SPE plates were purchased from Waters (Manchester, UK).

2.2. Instrumentation and bioanalytical conditions

Samples were analyzed using an Acquity UPLC ultra-high-performance liquid chromatography system coupled with Xevo TQ-S triple quadrupole mass spectrometer (Waters Crop, Manchester, UK). Ionization was performed in electrospray ionization (ESI) mode and the mass spectrometer was operated in the positive ion electrospray mode. The temperature of the electrospray source was maintained at 120 °C and a desolvation temperature of 500 °C. The capillary voltage was set 3.5 V. Multiple reaction monitoring (MRM) mode was used to monitor and quantify VD metabolites. The mass spectrometry conditions used for detecting the analytes are shown in Table 1.

Chromatographic separation was performed using a Waters Acquity UPLC BEH C_{18} (100 × 2.1 mm, 1.7 μm), which was maintained at 40 °C in the column oven. The mobile phase was composed of lithium acetate (0.378 mM) aqueous solution (solvent A) and methanol (solvent B), with a total flow rate of 0.25 mL/min. The integration of the peak area and concentration calculation were done by the workstation UNIFI software (UNIFY 1.7.1.022).

2.3. Preparation of standards and quality control samples

25(OH)D_3 and 1α,25(OH)$_2D_3$ were used to prepare standard curves and quality controls with internal standards (25(OH)D_3-d_3, 1α,25(OH)$_2D_3$-d_6 (100 ng/mL) were used to prepare working solutions.

These working solutions were further diluted with surrogate matrix (10% bovine serum albumin) to provide calibration standards in the range of 20–1200 pg/mL for 1α,25(OH)$_2D_3$ and 1–60 ng/mL for 25(OH)D_3. Surrogate calibration standards were prepared fresh daily from the working solutions. Quality control (QC) samples were independently prepared in the surrogate matrix at four different concentrations of 1, 2, 16, and 48 ng/mL (LLOQ, QCL, QCM, and QCH, respectively) for 25(OH)VD_3 and 20, 40, 320, and 960 pg/mL (LLOQ, QCL, QCM, and QCH, respectively) for 1α,25(OH)$_2D_3$. QC samples were stored at −70 °C until analysis.

2.4. Sample preparation

A portion of 250 μL plasma sample added with 2.5 μL internal standards was mixed with 250 μL of 0.2 mol/L zinc sulfate and vortex-mixed (10 s). Next, 900 μL MeOH was added to precipitate the proteins. The solution was then vortexed at high speed for 1 min before centrifugation (13000 rpm, 5 min). The supernatant was quickly transferred to a Waters C_{18} SPE cartridge (Waters Oasis HLB 96-Well plate), which was previously conditioned with 200 μL MeOH and 200 μL water. The solid phase was washed with 200 μL of a mixture of MeOH:water (5:95,v/v) twice and 200 μL of a mixture of MeOH:water (60:40, v/v) twice. Then, the targeted 25(OH)D_3 and 1α,25(OH)$_2D_3$ were eluted with 40 μL of a mixture of MeOH:IPA (95:5,v/v) twice and 20 μL of water was added. Lastly, 10 μL of the mixture was analyzed using the LC-MS/MS system.

2.5. Bioanalytical method validation

The method was validated according to the guidelines by the International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [34] and FDA [35]. Quantification was performed by calculating the peak-area ratios of 25(OH)D_3 to 25(OH)D_3-d_3 and 1α,25(OH)$_2D_3$ to 1α,25(OH)$_2D_3$-d_6, respectively.

Linearity was evaluated by analyzing a series of standard concentrations generated over the range of 20–1200 pg/mL for 1α,25(OH)$_2D_3$ and 1–60 ng/mL in 10% BSA. The curves were established by performing a linearly weighted ($1/X^2$) least squares regression obtained by plotting peak-area ratios of the analytes to IS against the nominal concentration of analytes. The ratio of response area for analytes to IS was used for regression analysis.

The intraday and interday accuracy and precision were assessed by replicate analysis of the four QC levels on three consecutive days. In each of the precision and accuracy sequences, five replicates at each QC level were analyzed. Recovery analysis of the extraction method was performed at three 25(OH)D_3 and 1α,25(OH)$_2D_3$ concentrations in five replicates each.

The stability of vitamin D_3 metabolism in 10% BSA during analysis and usual storage condition was inspected with the following parameters: freeze–thaw cycle stability, long-term stability, pre-extraction stability at room temperature (RT), post-extraction stability at 4 °C, and stability in the autosampler.

2.6. Method application

The validated method was applied for the simultaneous determination of 25(OH)D_3 and 1α,25(OH)$_2D_3$ in human plasma. A human pharmacokinetic study was performed in nine healthy Chinese volunteers (3 males, 29.2 ± 6.8 years old, and 6 females, 27.3 ± 6.8 years old; BMI range: 19.8–26 kg/m^2) after oral administration of Calcitriol Soft

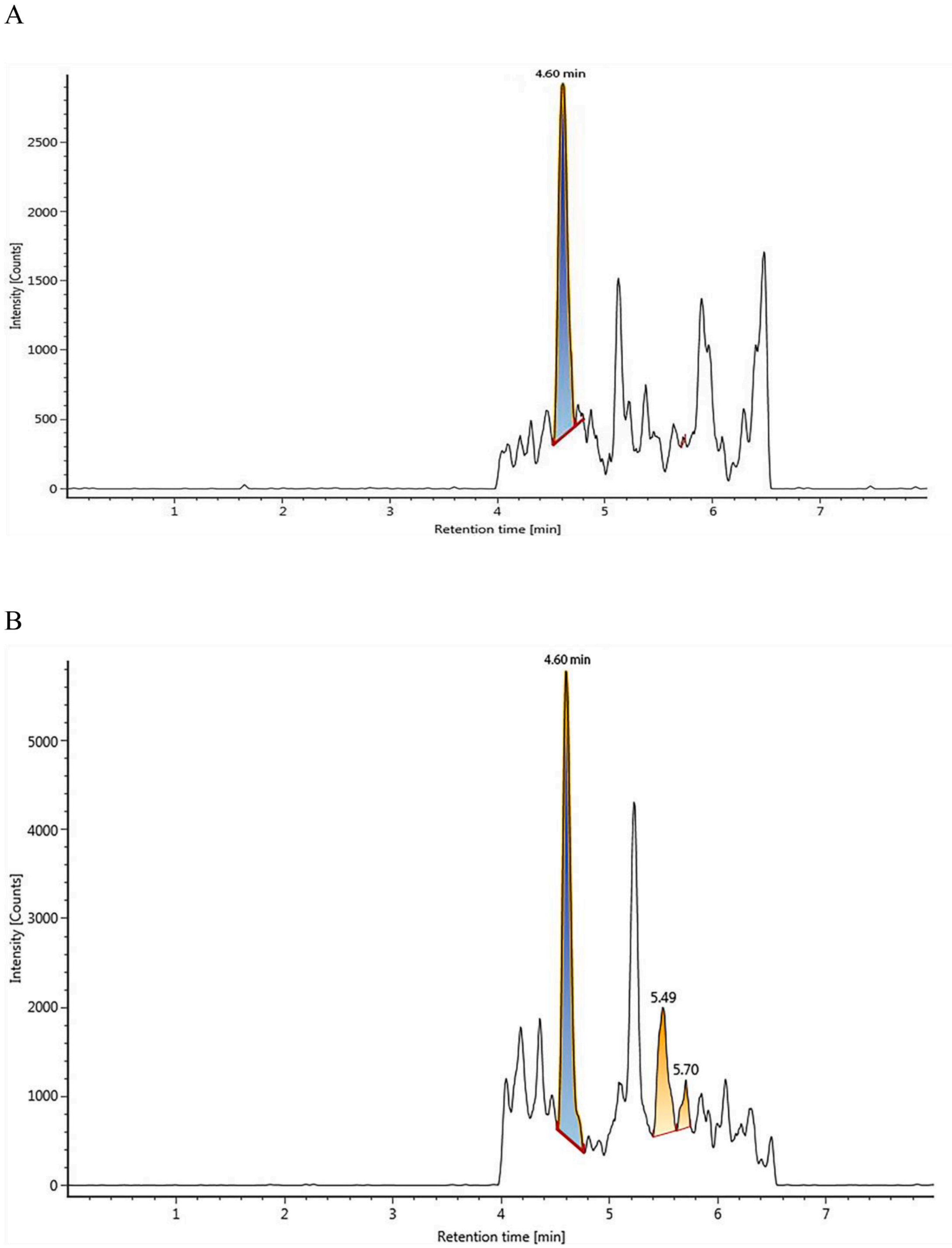

Fig. 1. Intensity of 1α,25$(OH)_2D_3$ at LLOQ with the lithium acetate mobile phase at various concentrations (A) 0.149 mM, (B) 0.378 mM, (C) 0.597 mM, and (D) 0.746 mM.

Capsules (CP Pharmaceutical Group, China) at a single dose of 4 μg. The clinical study was approved by the Medical Ethics Committee of the Xinxiang Central Hospital, Henan Province (Xinxiang, China). All volunteers provided written informed consent for their participation in the study, according to the principles of the Declaration of Helsinki and Good Clinical Practice. A total of 22 blood samples (1 mL each) were collected in heparin anticoagulant tubes at −18.00, −12.00, and −6.00 h (pre-dose) and then 0, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 6.00, 7.00, 8.00, 10.00, 12.00, 24.00, 36.00, 48.00, and 72.00 h (post-dose). The blood samples were centrifuged at 3000 g for 5 min at a temperature of 4 °C, and plasma samples were harvested, labeled, and stored at −70 °C before analysis.

The plasma samples were processed as described in Section "2.4. Sample preparation." In parallel with the actual plasma samples, QC samples at low, medium, and high concentrations were allocated in the analytical run, and analyzed in duplicates. The pharmacokinetic parameters, such as mean residence time, area under the concentration–time curve (AUC), maximum concentration (C_{max}), and half-life time ($T_{1/2}$), and time to reach maximum concentration (T_{max}) were calculated using the software Phoenix WinNonlin (7.0), and the plasma

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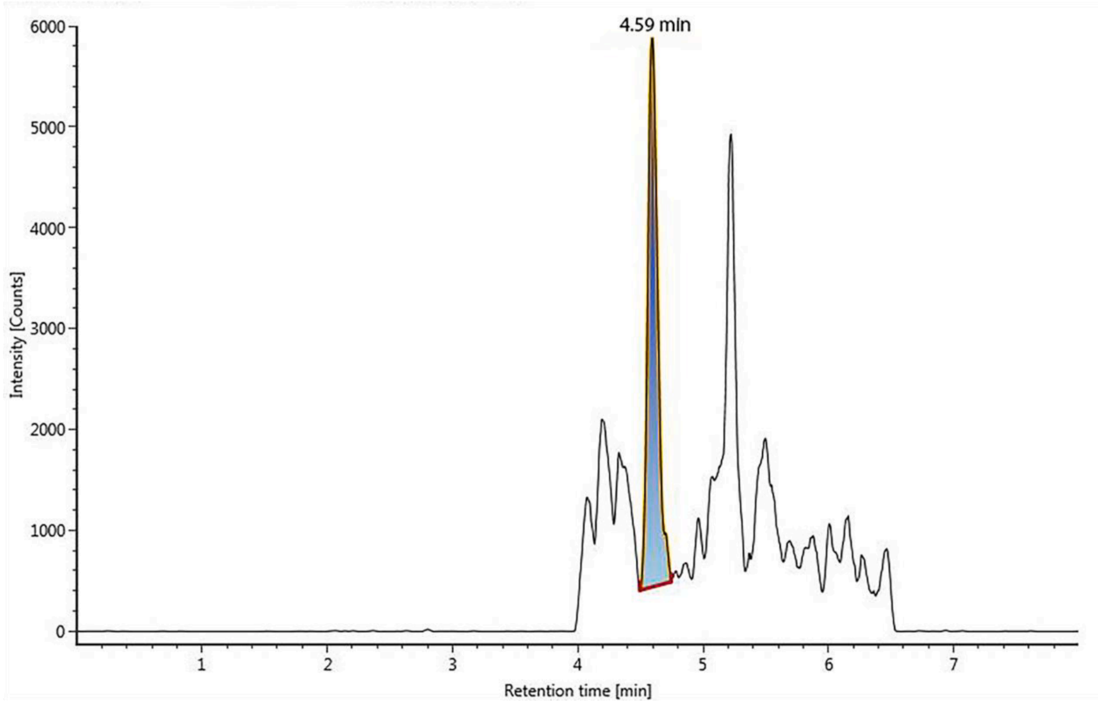

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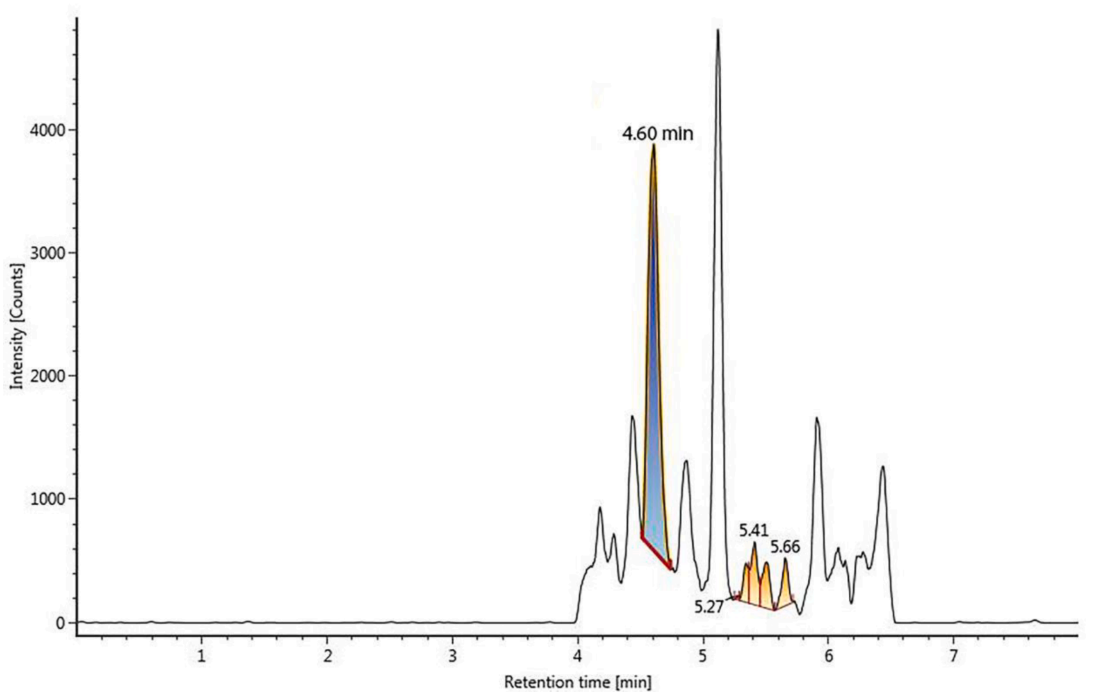

Fig. 1. (*continued*).

concentration–time curves of the volunteers drawn by Prism 7.0 (GraphPad Software, Inc, La Jolla, CA).

3. Results and discussion

3.1. Method development

Because 1α,25$(OH)_2D_3$ is tightly bound to plasma proteins and circulates at picomolar concentrations [23,24], the development of a rapid, simple method for the simultaneous determination of plasma 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ required considerable work, as described below.

3.1.1. Optimization of chromatography conditions and sample preparation

C18 and C8 columns designed for the analysis of polar compounds were tested. Although 25$(OH)D_3$ had similar results using both columns,

Table 2
LC gradient elution program detail.

Gradient elution program							
Time (min)	0	3.5	5	5.1	6.4	6.5	8
Mobile phase A (%)	28	8	8	0	0	28	28
Mobile phase B (%)	72	92	92	100	100	72	72

results for 1α,25$(OH)_2D_3$ suffered from poor selectivity and sensitivity. C18 columns, including BEH C_{18} (100 × 2.1 mm, 1.7 μm) and BEH C_{18} (50 × 2.1 mm, 1.7 μm) were then evaluated. BEH C_{18} (100 × 2.1 mm, 1.7 μm) was chosen for the method based on optimal retention time, selectivity, and peak shape. Different column temperatures were also tested from 20 °C to 45 °C, and the sensitivity improved under 40 °C column temperature.

Ammonium formate, ammonium acetate, formic acid, and lithium acetate were evaluated as aqueous mobile phase additives for sensitivity, selectivity, and chromatographic reproducibility. The addition of lithium acetate to 1α,25$(OH)_2D_3$ resulted in the formation of stable parent ions in the ESI mode, higher ion response, and compound cleavage into stable fragment ions. Therefore, lithium acetate was used in the mobile phase. Then, the concentration of the lithium acetate was optimized. The optimum concentration of lithium acetate was found to be 0.378 mM in water, which was used as the mobile phase A in this study, whereas methanol was used as mobile phase B (Fig. 1).

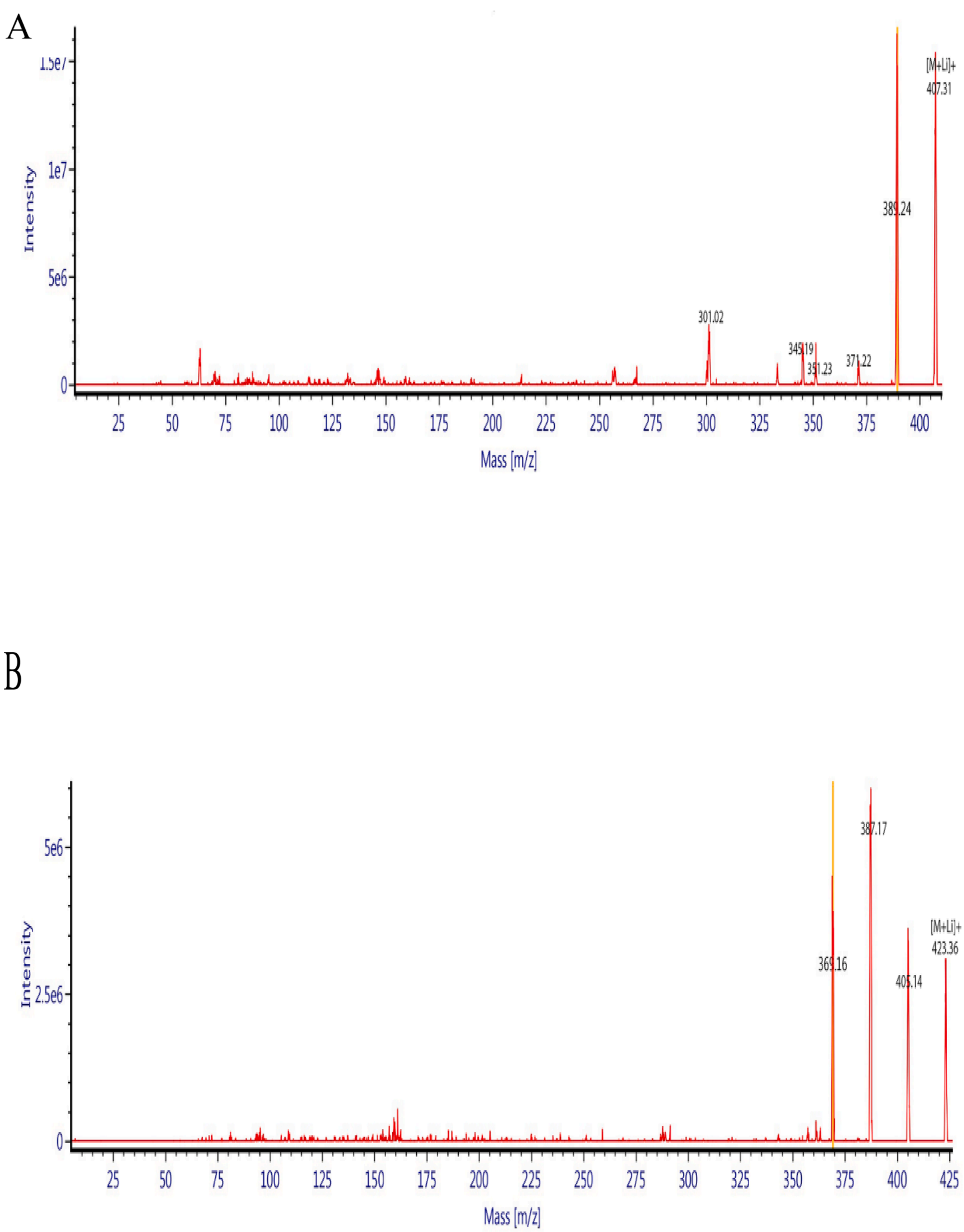

Fig. 2. Fragmentation pattern for (A) 25$(OH)D_3$ and (B)1α,25$(OH)_2D_3$.

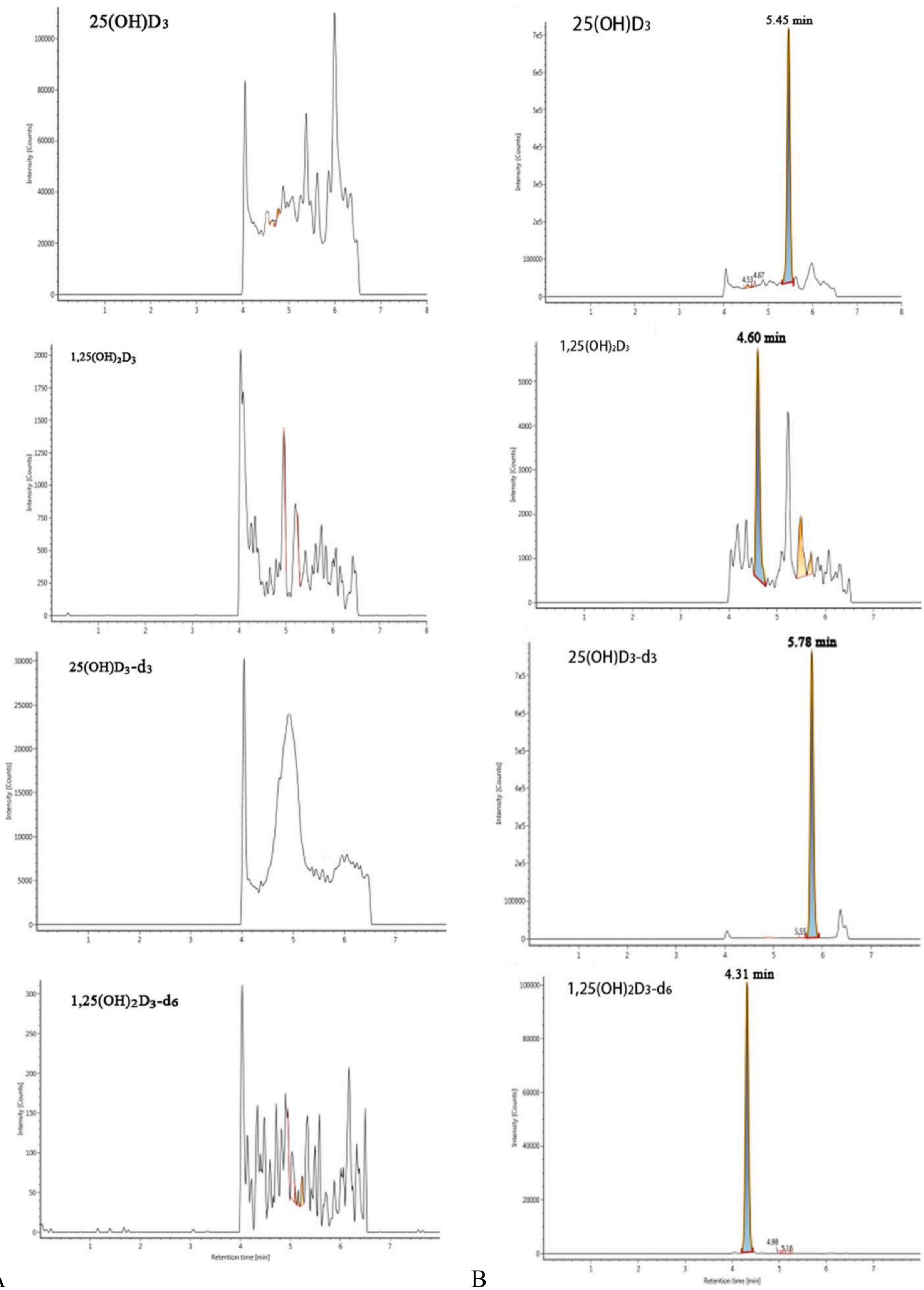

Fig. 3. Representative chromatograms from 25(OH)D_3 and 1α,25$(OH)_2D_3$ analyses. (A) blank 10% BSA, (B) analytes at LLOQs and ISs, and (C) plasma sample collected at 3 h after oral administration of Calcitriol Soft Capsules at a single dose of 4 μg.

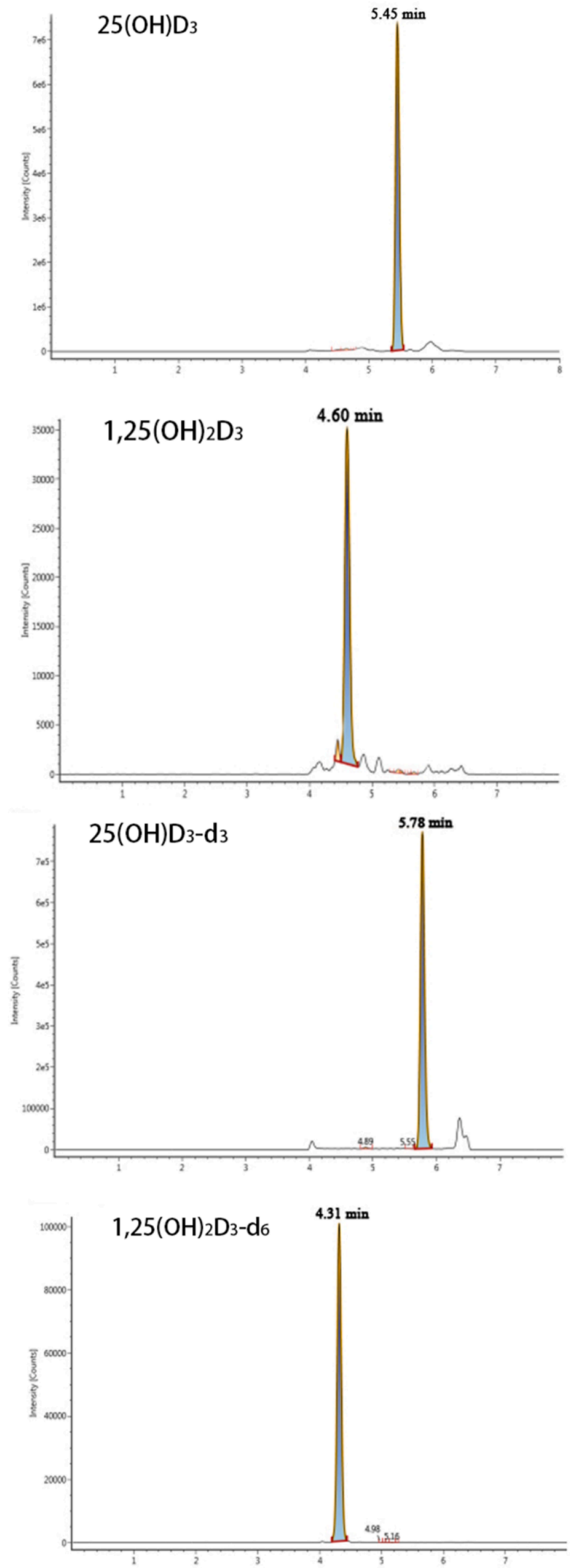

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Fig. 3. (*continued*).

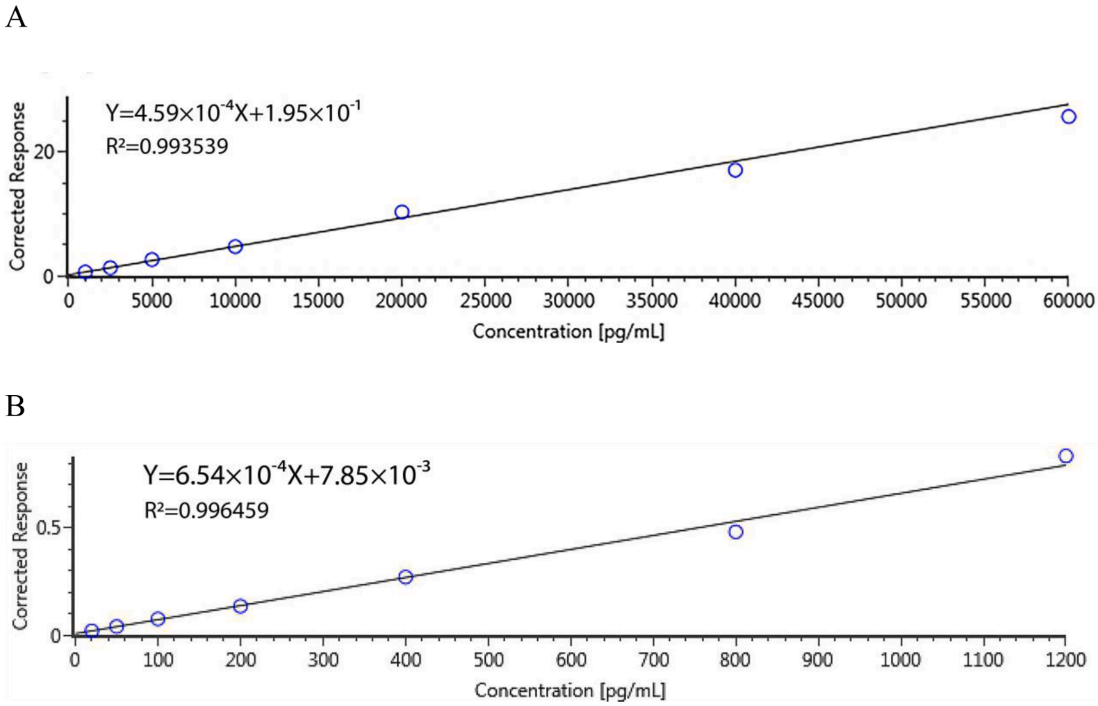


Fig. 4. Plasma calibration curves for (A) $25(OH)D_3$ and (B) $1\alpha,25(OH)_2D_3$.

Deuterated internal standards were used to minimize any analytical variation due to solvent evaporation, integrity of the column, and ionization efficiency of the analytes.

The gradient program (Table 2) was initiated with a step increase in the proportion of mobile phase B from 72% to 92% to delay the elution of the more polar derivative of $1\alpha,25(OH)_2D_{3,}$ to isolate it from any possible early eluting matrix contaminants, or from the remnants of the derivatization reaction solution. Moreover, a step increase in the composition of mobile phase B from 92% in 5 min to 100% at 5.1 min was necessary to elute the less polar derivative of $25(OH)D_3$. The ratio was maintained for 1.3 min at 100% B to guarantee a completed elution. Then an equilibration stage of 1.5 min at 72% B again was necessary for the column to obtain reproducible chromatography.

Different extraction methods, such as protein precipitation and liquid–liquid extraction (LLE), were explored to achieve acceptable reproducibility and recovery. Both methanol and acetonitrile were used for protein precipitation, but these could not achieve the required reproducibility. The recovery and reproducibility of LLE were also poor for $1\alpha,25(OH)_2D_3$ due to ion suppression in the solvent systems. Solid phase extraction (SPE) showed consistent recovery and reproducibility with Waters Oasis HLB SPE cartridge without any immunoextraction, derivatization or drying under nitrogen. The entire process lasted approximately 45 min including 8 min for the LC-MS/MS run time. Samples were prepared in an ice bath due to instability of analytes in the matrix.

3.1.2. *Optimization of mass spectrometric conditions*

Carl Jenkinson et al. reported the protonated molecule mass-to-charge ratios (*m/z*) 383.2 and *m/z* 399.2 as the precursor ions of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ [36], respectively. In our study, a Q1 scan of $25(OH)D_3$ and $1\alpha,25(OH)2D_3$ with electrospray ionization (ESI) mode revealed a high abundance of lithium adducts ($[M + Li]^+$), with $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ showing the highest signals at *m/z* of 407.31 and 423.26, respectively. The product ion scan of $25(OH)D_3$ resulted in high-intensity peaks of fragment ions at *m/z* 389.24, whereas $1\alpha,25(OH)_2D_3$ resulted in fragment ions at *m/z* 387.17 and 369.0 (Fig. 2). Fragment ions with *m/z* of 389.24 and 369.25 exhibited higher ion responses and more stable signals; therefore, they were selected as the MRM quantitative detection ions of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$, respectively. Finally, the quantitative MRM channels for $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ were determined to be *m/z* 407.31 → 389.24 and *m/z* 423.26 → 369.25, respectively. The same method was used to determine the MRM ion channel of the isotope internal standards $25(OH)VD_3$-d_3 and $1\alpha,25(OH)_2D_3$-d_6, which was *m/z* 410.50 > 392.26 and 429.36 > 374.36, respectively.

Table 3
Inter-day and Intra-day precision and accuracy of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ in human plasma.

QC samples	Target value (pg/mL)	Accuracy%		Precision%	
		Inter-day[a]	Intra-day[b]	Inter-day[a]	Intra-day[b]
$25(OH)D_3$					
QC-LLOQ	1000	95.25	97.22	2.94	2
QCL	2000	105.27	102.68	4.55	5.31
QCM	16,000	102.58	106.21	8.4	9.7
QCH	48,000	94.17	92.79	8.27	6.08
$1\alpha,25(OH)_2D_3$					
QC-LLOQ	20	94.59	100.95	9.5	7.2
QCL	40	101.42	93.66	11.2	14.2
QCM	320	99.8	92.07	7.4	3.3
QCH	960	110.19	102.86	5.7	4.5

[a] (n = 5), expressed as (found concentration /target value) × 100.
[b] Values obtained from all three runs (n = 15).

3.2. Bioanalytical method validation

3.2.1. Selectivity and carryover

An LC-MS/MS workflow was developed for accurate and precise quantification of 25(OH)D_3 and 1α,25$(OH)_2D_3$. Being endogenous compounds, it is challenging to define the selectivity of these analytes. Contrarily, the challenge could be minimized by using 10% BSA as a blank. Furthermore, the LLOQ was determined to coincide with the standard, which was a 1/100 dilution of the working solution. Fig. 3 illustrates the representative chromatograms. As expected, there were no peaks corresponding to each analyte and IS in the blank 10% BSA run, indicating that BSA, as a better matrix, can be applied for this analytical method.

3.2.2. Linearity

The results showed acceptable linearity for both 25(OH)D_3 and 1α,25$(OH)_2D_3$, with $R^2 > 0.993$ for 25(OH)D_3 and $R^2 > 0.996$ for 1α,25$(OH)_2D_3$(Fig. 4).

3.2.3. Precision and accuracy

The precision, accuracy and LLOQ We have accepted the revision.are displayed in Table 3. Satisfactory repeatability and precision were achieved as CV values ranged from 2% to 14.2 %. Accuracy was also well within the acceptance range for both intraday and interday measurements, with the acquired bias value from 92.07% to 110.19% for calibrators.

3.2.4. Recovery

Recovery analysis was performed at three concentrations independently to assess the quality and applicability of the developed method. Results revealed that the spiked recovery of 25(OH)D_3 ranged from 91.22% ± 2.65% to 99.56% ± 10.26%, and the spiked recovery of 1α,25$(OH)_2D_3$ ranged from 94.13% ± 6.25% to 97.98% ± 9.20%. All the recovery rates were within ± 15% bias, which indicates that the processes of the study led to low loss of both 25(OH)D_3 and 1α,25$(OH)_2D_3$ in the test samples.

3.2.5. Stability

The stability of 25(OH)D_3 and 1α,25$(OH)_2D_3$ is summarized in Table 4. Evaluation of the freeze–thaw cycle stability demonstrated the analytes were stable for at least two freeze and thaw cycles. The results demonstrated that the analytes was stable in human plasma at room temperature for at least 4 h. Both 25(OH)D_3 and 1α,25$(OH)_2D_3$ were stable in a processed form (extracted) throughout the residence time of 24 h in the autosampler. The stability results demonstrated that the analytes were stable under refrigeration (−70 °C) for 1 month.

3.3. Method application

The fully validated LC-MS/MS method was successfully implemented in a clinical pharmacokinetic study of 1α,25$(OH)_2D_3$ in human plasma, as well as of 25(OH)D_3. Figs. 5 and 6 described the plasma concentration–time profiles of 1α,25$(OH)_2D_3$ and 25(OH)D_3 in human plasma after oral administration of Calcitriol Soft Capsules at a single dose of 4 μg. The main pharmacokinetic parameters calculated by Phoenix WinNonlin (7.0) are summarized in Table 5. After oral administration, plasma concentrations of 25(OH)D_3 were essentially unchanged. A statistical analysis was conducted to study the change of the 25(OH)D_3 concentrations. Statistical analysis was conducted using one-way repeated measures ANOVA (using the Shapiro–Wilk test and Mauchly's test of sphericity), which was performed using IBM SPSS®Statistics 26. There was no significant difference in 25(OH)D_3 concentration before and after an oral dose of 1α,25$(OH)_2D_3$; after correction, $F(4.881,39.047) = 2.018$, $p = 0.099 > 0.05$. The precision and accuracy for calibration and QC samples along with subject samples were analyzed during a period of 3 days, and the precision and accuracy

Table 4
Stability of 25(OH)D_3 and 1α,25$(OH)_2D_3$ under various conditions (n = 5).

QC samples	Target value (pg/mL)	Accuracy%					RSD%				
		Autosampler stability (10°C, 24H)	Bench top stability (room temperature, 4H)	Freeze-Thaw stability(2 cycles, −70°C)	Processed samples stability (4°C, 4H)	Long term stability (-70°C,30 D)	Autosampler stability (10°C, 24H)	Bench top stability (room temperature, 4H)	Freeze-Thaw stability(2 cycles, −70°C)	Processed samples stability (4°C, 4H)	Long term stability (-70°C, 30 D)
25(OH)D_3											
QCL	2000	105.49	93.51	100.32	107.26	104.42	3.23	13.96	7.73	13.86	6.35
QCH	48,000	94.49	86.19	96.76	95.88	95.43	5.48	5.8	3.25	13.89	8.79
1α,25$(OH)_2D_3$											
QCL	40	94.23	95.58	105.8	91.34	107.62	6.42	5.83	7	9.19	7.24
QCH	960	93.59	95.23	105.8	89.5	92.49	7.71	6.93	3.3	0.84	7.01

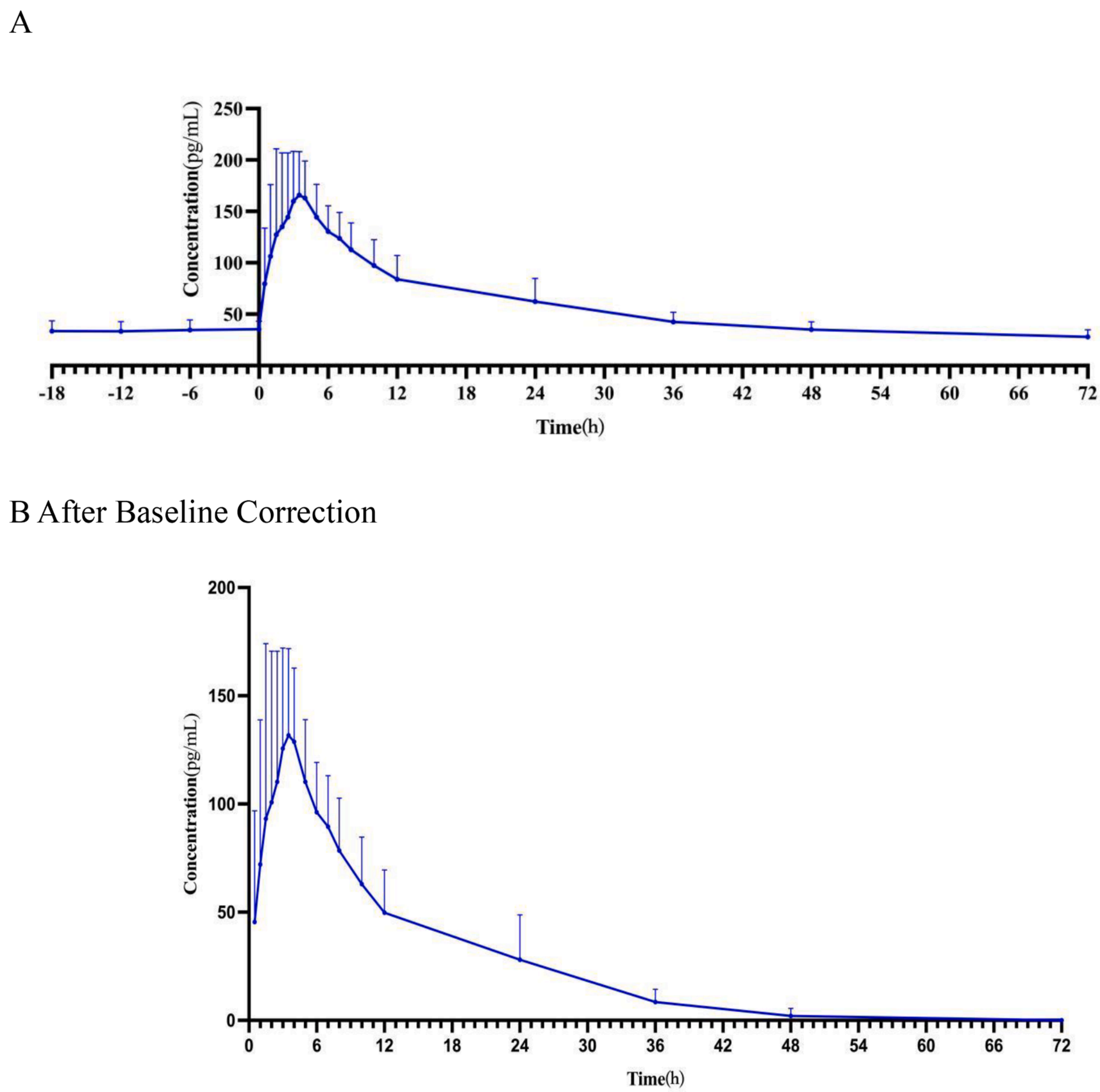


Fig. 5. Mean plasma concentration–time curves of 1α,25$(OH)_2D_3$ before and after the administration of Calcitriol Soft Capsules at a single dose of 4 μg (n = 9).

for calibration and QC samples were well within the acceptable limits. This study is limited by its relatively small population. Therefore further studies on the clinical pharmacokinetic study of 1α,25$(OH)_2D_3$ in human plasma as well as 25$(OH)D_3$ based on a larger population in China are needed.

4. Conclusions

In this study, we describe a novel and validated high throughput LC-MS/MS method for the simultaneous separation and concentration determination analysis of 25$(OH)D_3$ and 1α,25$(OH)_2D_3$, which meets the requirements proposed by the US Food and Drug Administration. The lowest detected concentrations of 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ were 1 ng/mL and 20 pg/mL, respectively, both satisfying the clinical determination thresholds. The novel validated LC-MS/MS method has been successfully implemented in a clinical pharmacokinetic study of 1α,25$(OH)_2D_3$ and 25$(OH)D_3$ in human plasma after oral administration of Calcitriol Soft Capsules at a single dose of 4 μg. The results revealed that plasma concentrations of 25$(OH)D_3$ were effectively unchanged after oral administration of Calcitriol Soft Capsules.

A normal level of vitamin D is usually defined as a 25$(OH)D_3$ concentration higher than 30 ng/mL (75 nmol/L) [37]. Vitamin D insufficiency and deficiency are usually defined as a 25$(OH)D_3$ concentration of 20–30 ng/mL and $<$ 20 ng/mL, respectively. In this study, all volunteers showed a high incidence of vitamin D deficiency, diagnosed by 25$(OH)D_3$ concentrations lower than 20 ng/mL. Lyra et al. have shown that a low 1α,25$(OH)_2D_3$ serum concentration is associated with cancer, in spite of normal levels of 25$(OH)D_3$, however, mechanisms explaining a lower 1α,25$(OH)_2D_3$ plasma concentration in breast cancer patients is currently unclear [19]. The study by Irwinda et al reports that lower placental 25$(OH)D_3$ status and a higher placental CYP27B1 and 25(OH) D_3 ratio is more likely to be found in subjects with preterm than with term births [21]. These studies highlight the importance of proper VD levels in humans, and consequently for analytical methods to measure VD in human plasma. Our study provides a rapid and accurate method to simultaneously investigate 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ levels in

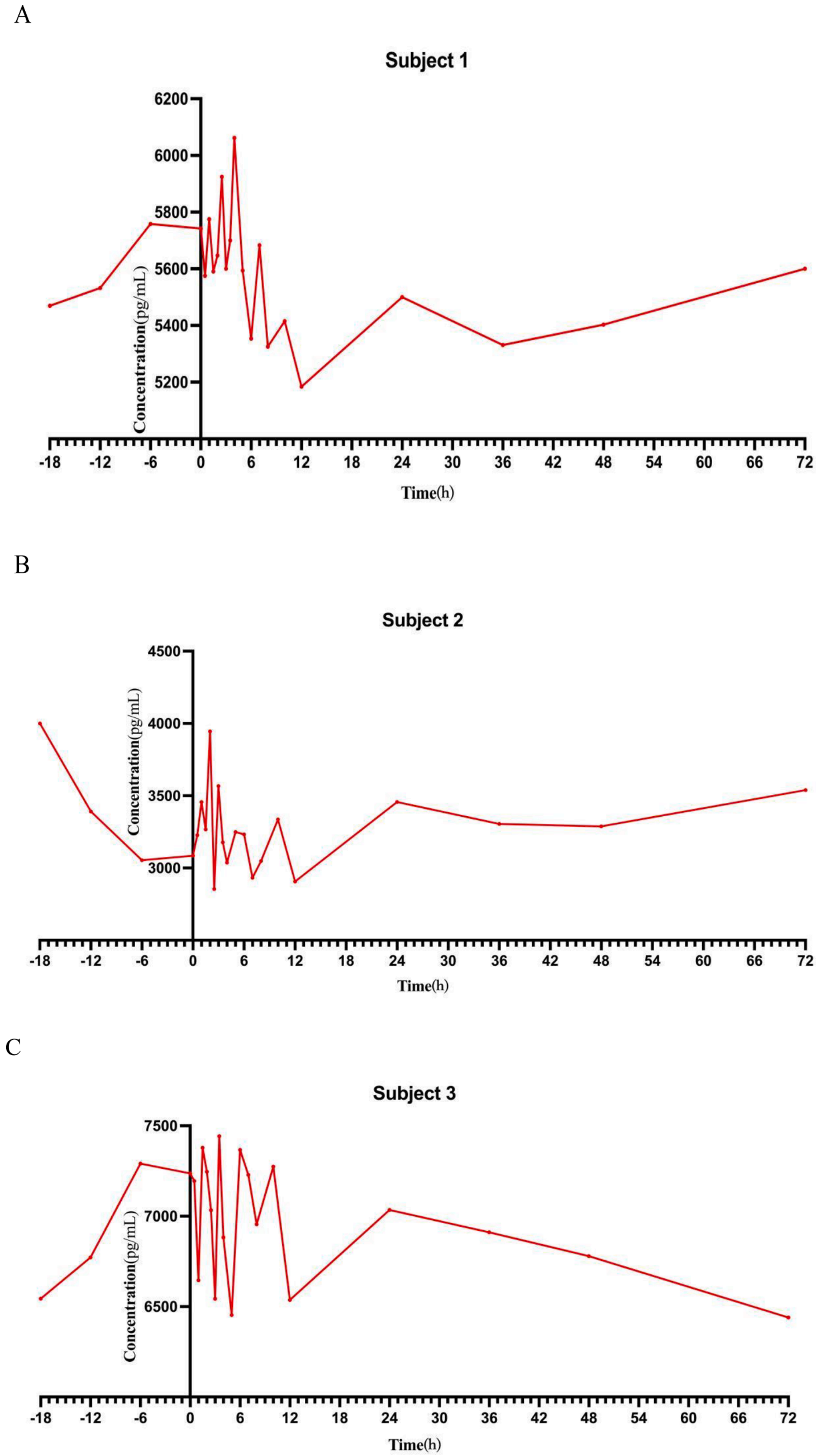


Fig. 6. Plasma 25(OH)D_3 concentration (A) subject 1, (B) subject 2, (C) subject 3, (D) subject 4, (E) subject 5, (F) subject 6, (G) subject 7, (H) subject 8, (I) subject 9 before and after administration of Calcitriol Soft Capsules at a single dose of 4 μg.

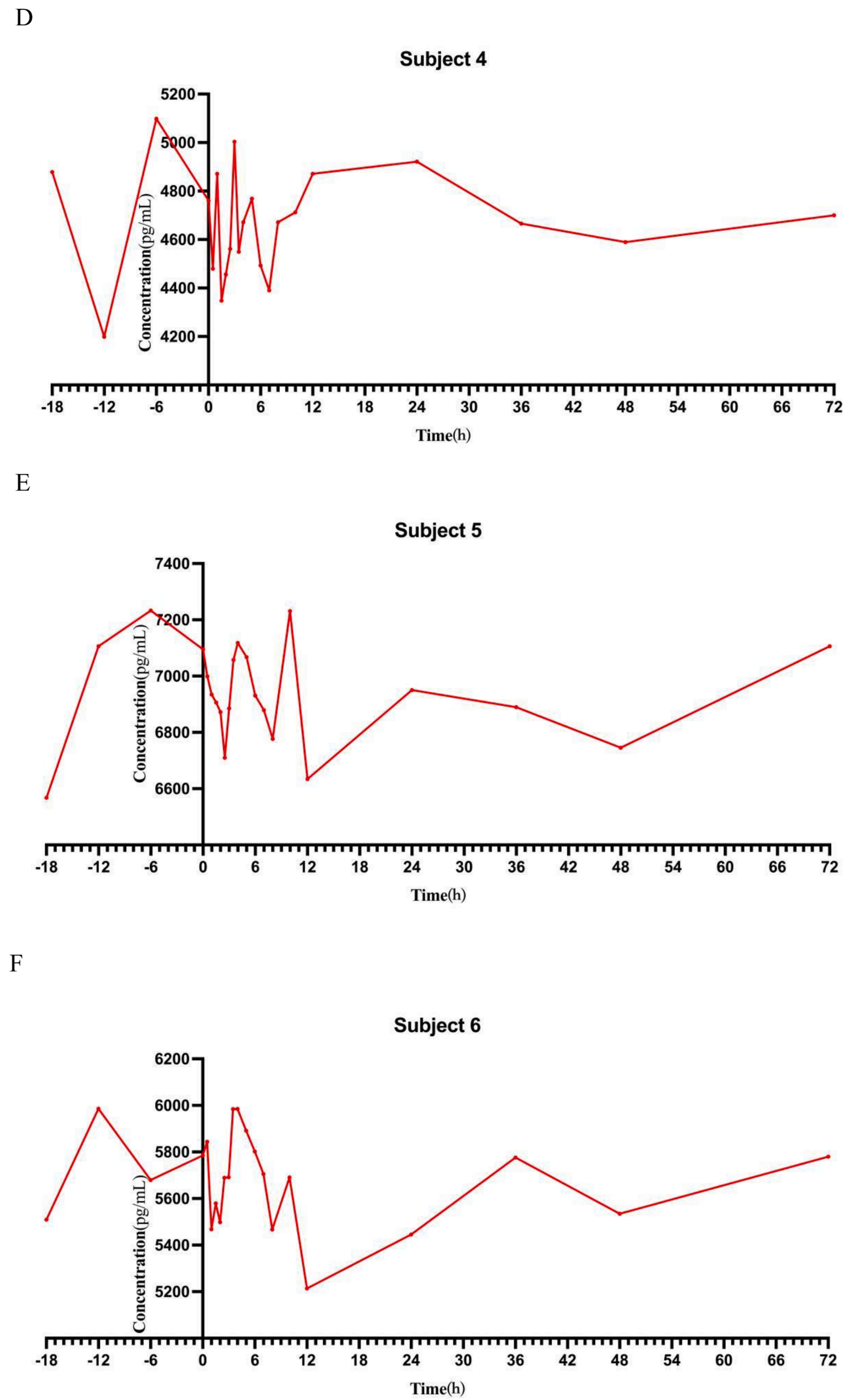
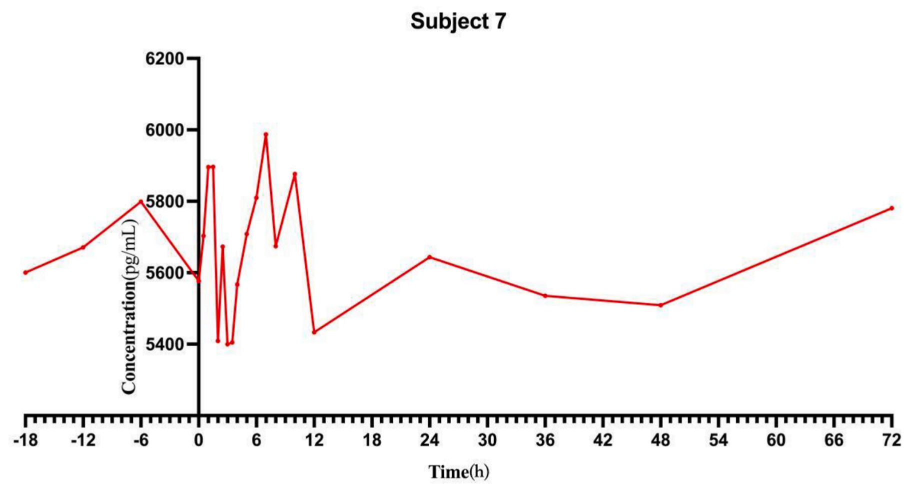


Fig. 6. (*continued*).

G



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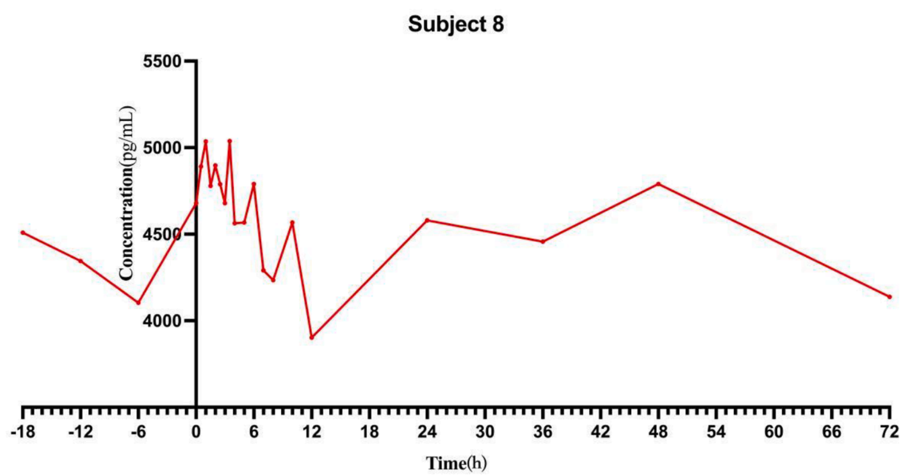

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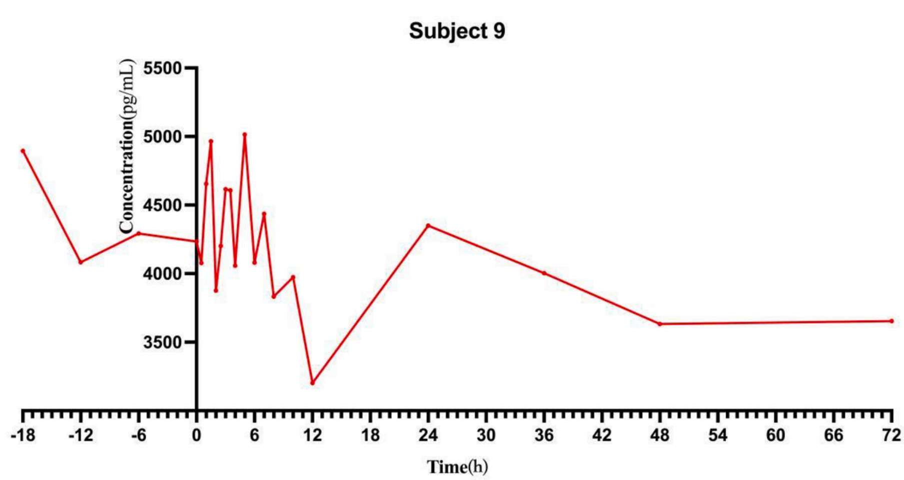

Fig. 6. (*continued*).

Table 5
Mean pharmacokinetic parameters obtained from 9 volunteers after administration of 4 μg Calcitriol Soft Capsules to each.

Parameters	1α,25(OH)$_2$D$_3$
$T_{1/2}$(h)	9.14 ± 5.33
C_{max}(ng/L)	177.88 ± 47.94
T_{max}(h)	3.06 ± 1.01
$AUC_{(0-t)}$(ng/L*h)	4168.20 ± 1017.16
$AUC_{(0-\infty)}$ (ng/L*h)	5589.32 ± 1384.10
After Baseline Correction **Parameters**	**1α,25(OH)$_2$D$_3$**
$T_{1/2}$(h)	9.25±5.13
C_{max}(ng/L)	137.46±38.77
T_{max}(h)	2.94±1.01
$AUC_{(0-t)}$ (ng/L*h)	1745.52±874.53
$AUC_{(0-\infty)}$ (ng/L*h)	1821.80±892.01

clinical samples, and presents a potentially valuable analytical technique for low concentration measurement of VD.

Declaration of Competing Interests

The authors declare they have no known competing financial interests or personal relationships that could affect the work described in this article.

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IRB and Ethics statement

The study was approved by the Medical Ethics Committee of the Xinxiang Central Hospital, Henan Province (Xinxiang, China) and the IRB number is 2019–001. All volunteers provided written informed consent for their participation in the study, according to the principles of the Declaration of Helsinki and Good Clinical Practice.

We certify that this manuscript is original and has not been published and will not be submitted elsewhere for publication while being considered by Journal of Mass Spectrometry and Advances in the Clinical Lab. And the study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time. No data have been fabricated or manipulated (including images) to support your conclusions. No data, text, or theories by others are presented as if they were our own. The submission has been received explicitly from all co-authors. And authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

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