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Determination of 1,25-dihydroxyvitamin D_2 in rat serum using liquid chromatography with tandem mass spectrometry

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ABSTRACT

Vitamin D therapy is widely used for the treatment of hyperparathyroidism associated with chronic renal failure in renal disease patients. The vitamin D prodrug, 1α-hydroxyvitamin D_2 (1α(OH)D_2), is used for the treatment of the end stage renal disease patients who as a result of impaired kidney function cannot convert the naturally occurring vitamin D to the active hormonal form namely 1,25-dihydroxyvitamin D_2 (1,25(OH)$_2D_2$). The systemic circulating levels of this active form are in the pg/mL range and represent a significant bioanalytical challenge for therapeutic monitoring. Liquid chromatography with tandem mass spectrometry (LC–MS/MS) is considered the gold standard for the selective and sensitive determination of small molecule therapeutics in biological matrices. However, the reported LC–MS/MS bioanalytical assays for 1,25(OH)$_2D_2$ suffer from extensive sample preparation procedures or derivatization protocols to achieve the requisite sensitivity and selectivity. In this paper, we describe an assay that employs 96-well plate solid phase extraction sample preparation combined with highly sensitive LC–MS/MS instrumentation. The utility of ultra high pressure liquid chromatography to reduce the analytical run time was also demonstrated. Employing this assay a lower limit of quantitation of 25.0 pg/mL using 300 μL sample aliquot of rat serum was achieved with linearity obtained over the range of 25.0–1000 pg/mL. Both intra-day and inter-day coefficients of variation were <15% and accuracy across the assay range was within 100 ± 7.24%. The application of the assay was demonstrated for the analysis of 1,25(OH)$_2D_2$ rat serum samples to support pharmacokinetic studies conducted at doses down to sub-microgram per kilogram of 1α(OH)D_2.

1. Introduction

Vitamin D exists in two forms, namely vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_3 can be obtained from dietary intake or exposure of skin to sunlight. The UV rays of sunlight induce the photolytic conversion of 7-dehydrocholesterol (a mammalian sterol) to pro-vitamin D_3 followed by thermal isomerization to vitamin D_3 [1–3]. Vitamin D_2 is synthesized in a similar manner from ergosterol through sunlight exposure to pro-vitamin D_2 which rapidly isomerizes to vitamin D_2 in plant and fungi [4,5]. Both vitamin D_2 and vitamin D_3 must be metabolized into their active forms before exerting their biological effects. Vitamin D_2 and vitamin D_3 undergo the same metabolic processes involving 25-hydroxlyation in the liver, followed by 1α-hydroxylation in the kidney, to produce the biologically active metabolites 1,25(OH)$_2D_2$ and 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$), respectively [6,7].

Several vitamin D_2 and vitamin D_3 analogs have been developed as therapeutic agents such as calcitriol (i.e., 1,25(OH)$_2D_3$), paricalcitol (i.e., 19-nor-1,25(OH)$_2D_2$), and doxercalciferol (i.e., 1α(OH)D_2) to treat hyperparathyroidism in chronic kidney disease (CKD) patients. In addition to the treatment of renal and bone diseases, therapeutic applications for vitamin D analogs in other disease areas including psoriasis, cancer, and autoimmune diseases have been explored in animal models and clinically [8,9].

The current methods for measuring 1,25(OH)$_2D_2$ in serum include competitive protein-binding assay (CPBA), radioimmunoassay (RIA), and radioreceptor assay (RRA) [10–14]. These assays require extensive sample pretreatment and purification [10,11], exhibit low precision (>15% CV) [12], or underestimate the level of 1,25(OH)$_2D_2$ [12]. Furthermore, in order to separate 1,25(OH)$_2D_2$ from 1,25(OH)$_2D_3$ and other structurally related metabolites of vitamin D_2, such as 1α,24-dihydroxyvitamin D_2 (1,24(OH)$_2D_2$), samples must be separated through multiple chromatographic approaches, followed by fractionation prior to the analytical assay [13–15]. Liquid chromatography with tandem mass spectrometry (LC–MS/MS) is considered the gold standard for the selective and sensitive determination of 1,25(OH)$_2D_2$ in serum

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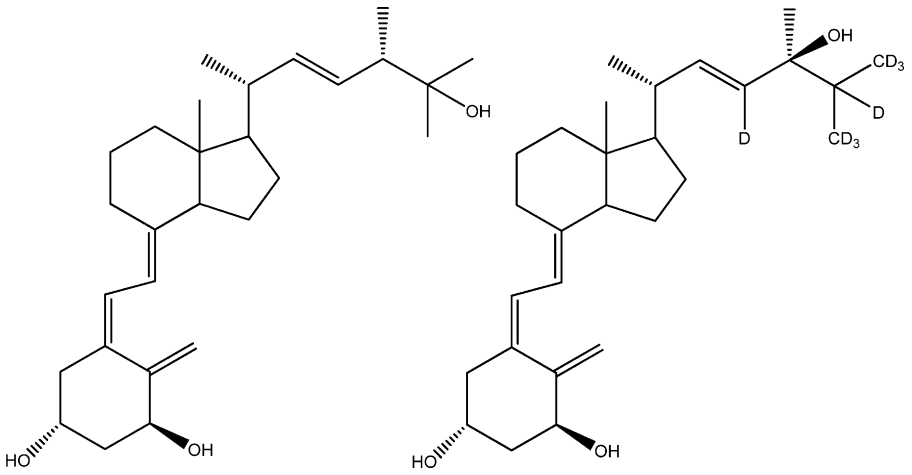

Fig. 1. Structure of $1,25(OH)_2D_2$ (left) and (23,25,26,26,26,27,27,27-$^2H_8$) $1,24(OH)_2D_2$ (internal standard) (right).

or plasma. However the reported bioanalytical assays again suffer from extensive sample preparation procedures or derivatization protocols to achieve the requisite sensitivity and selectivity [16,17].

The goal of this study was to develop and validate an assay using LC–MS/MS on the highly sensitive mass spectrometer API5000 platform that would improve the speed, simplicity, and sensitivity of reported methods for the determination of $1,25(OH)_2D_2$ in serum. The developed assay was successfully implemented for the analysis of $1,25(OH)_2D_2$ in serum samples to support rat pharmacokinetic studies conducted at doses down to sub-microgram per kilogram of $1\alpha(OH)D_2$. To the best of our knowledge, this reported assay is the most sensitive and selective method to date for the direct quantitation of $1,25(OH)_2D_2$ in a biological matrix using LC–MS/MS.

2. Experimental

2.1. Materials and reagents

$1\alpha(OH)D_2$, $1,25(OH)_2D_2$, and octadeuterated 1α,24-dihydroxyvitamin D_2 (internal standard (I.S.)) (Fig. 1) were synthesized by Genzyme. All solvents employed in this study were HPLC-grade. HPLC water was purchased from JT Baker (Phillipsburg, NJ, USA). All other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Male Sprague–Dawley rat serum was purchased from Bioreclamation Inc. (Hicksville, NY, USA).

2.2. Instrumentation

Liquid chromatography with tandem mass spectrometry detection was performed on an Applied Biosystems Sciex API 5000 triple quadruple mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) probe (Applied Biosystems, Foster City, CA, USA) interfaced to a Shimadzu Prominence LC system coupled with a SIL-HTc autosampler (Shimadzu, Columbia, MD, USA).

2.3. Chromatographic conditions

The analytical column employed was a Phenomenex Luna C8 (100 mm × 2.1 mm I.D., 3 μm, Phenomenex, Torrance, CA, USA). Eluent A was HPLC-grade water and eluent B was acetonitrile. The mobile phase was a linear gradient from 35%B to 98%B over 7.6 min at a flow rate of 0.5 mL/min. The column temperature was maintained at 40 °C and the injection volume was 35 μL. The column was washed and equilibrated between each injection. The retention time of $1,25(OH)_2D_2$ was 5.4 min and total run time per sample was 16 min.

2.4. Mass spectrometric conditions

Analytes were ionized in the positive ion mode using an APCI probe set at 315 °C. The curtain, auxiliary, and collision gases were nitrogen and set at 16, 90, and 11, respectively. The declustering potential (DP), collision energy (CE), collision exit potential (CXP), entrance potential (EP), channel electron multiplier (CEM), and nebulizer current (NC) were set at 90, 32, 14, 10, 3100, and 5, respectively. Detection of $1,25(OH)_2D_2$ and I.S. was performed in the multiple reaction monitoring (MRM) mode using the mass-to-charge transitions of m/z 411.5 → 151.0 and 419.3 → 151.0, respectively. The acquisition dwell time for $1,25(OH)_2D_2$ and I.S was 150 ms and 100 ms, respectively. Quadrupoles Q1 and Q3 were set at unit and low resolution respectively. The LC–MS/MS system was controlled and the analytical data were collected and processed using Sciex Analyst software (Version 1.4.2).

2.5. Standards and quality control samples

All solutions were prepared in amber containers to protect $1,25(OH)_2D_2$ from light. The primary stock solution of $1,25(OH)_2D_2$ was prepared at 100 ng/mL in methanol. Calibration curves were prepared by spiking the $1,25(OH)_2D_2$ primary stock solution into rat serum and then serially diluting with rat serum to obtain calibration standards of 25.0 pg/mL, 50.0 pg/mL, 100 pg/mL, 200 pg/mL, 500 pg/mL, 750 pg/mL and 1000 pg/mL. Similarly, quality control (QC) samples were prepared separately to give concentrations of 25.0 pg/mL, 200 pg/mL and 1000 pg/mL for HPLC–MS/MS and 25.0 pg/mL, 200 pg/mL and 750 pg/mL for UPLC–MS/MS.

2.6. Sample preparation

After thawing at room temperature, 300 μL of blanks, standards, QC samples, and study serum samples were prepared by precipitation with 500 μL acetonitrile containing 30.0 pg/mL of the internal standard. The samples were then vortexed and centrifuged at approximately 1000 *g* for 10 min at 4 °C. Oasis HLB LP 96-well plate with 60 μm sorbent particle size (60 mg) (Waters, Milford, MA, USA) was used for the solid phase extraction (SPE) sample preparation of $1,25(OH)_2D_2$ from serum. The SPE plate was conditioned using 1.0 mL methanol, followed by 1.0 mL water. After conditioning, the SPE plate was loaded with 600 μL of water followed by the supernatant from the protein-precipitated serum samples. Fol-

lowing filtration under vacuum the SPE plate was then washed in sequence with 800 μL of water, 600 μL of water:methanol (40:60, v/v), and then 500 μL of hexane. After washing, the analyte was eluted with 600 μL of tert-butylmethyl ether (TBME) followed by a second elution with 400 μL of TBME. The eluent was evaporated to dryness under a nitrogen gas stream at 30 °C. The sample was then reconstituted with 50 μL of water:acetonitrile (50:50, v/v) prior to analysis by LC–MS/MS.

2.7. Assay validation

The analytical methodology was validated according to the guidelines outlined in the US Food and Administration guidance for industry on bioanalytical method validation [18]. As part of the validation, assay precision, accuracy, recovery, linearity, specificity and stability were assessed.

2.7.1. Specificity

Specificity was defined as no signal greater than or equal to 20% of the signal achieved at the lower limit of quantitation (25.0 pg/mL) at the retention times of $1,25(OH)_2D_2$ in the blank matrix. Specificity for the internal standard was established as no signal greater than or equal to 5% of the signal of the internal standard. Six different lots of pooled rat serum were evaluated to establish the assay specificity.

2.7.2. Linearity of calibration curve

Calibration curves were generated by plotting the peak area ratios (analyte/internal standard) versus the theoretical concentration. The calibration curves were run in singlet and the linearity of the calibration curve was evaluated by a linear regression analysis using a $1/(\text{concentration})^2$ weighting.

2.7.3. Precision and accuracy

The intra-run assay accuracy and precision were established through the performance of six replicates of the QC samples at the three concentrations described. The inter-run assay accuracy and precision were established through the performance of three consecutive intra-day runs. The assay accuracies were evaluated by the deviation of the mean concentration measurement of the replicates versus the theoretical concentration value expressed as a percentage (%Bias). The assay precisions were evaluated from the relative standard deviation (RSD) of the concentration measurements and expressed as the percent coefficient of variation (%CV) from the mean concentration of the replicates. Intra- and inter-run accuracies and precisions at QC concentrations of less than or equal to ±15% were deemed to be acceptable.

2.7.4. Recovery

Recovery samples were prepared in rat serum at 25.0 pg/mL, 200 pg/mL and 1000 pg/mL and extracted according to the sample extraction procedure. The recovery samples were analyzed against the solvent standards prepared in water:acetonitrile (50:50, v/v).

2.7.5. Bench-top and freeze–thaw cycle stability

Three sets of bench-top stability samples were prepared in rat serum at 25.0 pg/mL, 200 pg/mL and 1000 pg/mL. These were stored in a light-protected area at room temperature for 72 h. Samples were then extracted and analyzed in comparison to freshly prepared and extracted calibration standards ($t = 0$ h). The freeze–thaw cycle stability was determined from three sets of samples prepared in rat serum at concentrations of 25.0, 200 and 1000 pg/mL. The freeze–thaw cycle stability samples were stored at −80 °C for 24 h and thawed at room temperature. This process was repeated two more times. After three freeze–thaw cycles, all samples were extracted and analyzed in comparison to freshly prepared and extracted calibration standards.

2.8. UPLC conditions

The analytical column was an Acquity HSS T3, 100 mm × 2.1 mm I.D., 1.8 μm (Waters, Milford, MA, USA). Eluent A was HPLC-grade water and eluent B was acetonitrile. The mobile phase was maintained at 55%B for 1.3 min followed by a gradient elution from 55%B to 67%B over 3 min at a flow rate of 0.75 mL/min. The column was washed and equilibrated before each injection. Total run time per sample was 8 min. The column temperature was set at 55 °C and the injection volume was 40 μL. The UPLC for the analysis of $1,25(OH)_2D_2$ was performed on an Acquity UPLC system (Waters, Milford, MA, USA).

2.9. Pharmacokinetic studies

Male Sprague–Dawley (SD) rats weighing approximately 300–350 g rats (Charles River Laboratory, Montreal, Canada, and Taconic, NY, USA) were used to evaluate the pharmacokinetics of $1,25(OH)_2D_2$ following administration of oral (PO) and intravenous (IV) doses of $1\alpha(OH)D_2$.

In the IV study, each rat received a single bolus IV injection of $1\alpha(OH)D_2$ at 0.1 μg/kg ($n = 15$ rats) or 1.0 μg/kg ($n = 20$ rats). The dosing vehicle contained 0.1 μg/mL or 1.0 μg/mL $1\alpha(OH)D_2$ in 0.4% polysorbate, 0.15% NaCl and 0.11% disodium EDTA in phosphate buffer pH 7. The dose volume was 1.0 mL/kg of body weight. For the 0.1 μg/kg dose group, 1.25 mL of whole blood was collected at 0, 2, 10, 30, 60, 120, 240, 480, and 1380 min post-dose. Blood was drawn from each animal three times including one terminal bleeding. For the 1.0 μg/kg dose group, 1.25 mL of whole blood was collected at 2, 10, 30, 60, 240, 360, 480, and 1440 min post-dose. The blood was allowed to clot at room temperature, then centrifuged at approximately 2000 × g for 15 min and the supernant was transferred into 1.5 mL Eppendorf tubes. Serum samples were stored at −70 °C until sample analysis.

In the oral study, each rat ($n = 16$) received a singe dose 0.5 μg/kg $1\alpha(OH)D_2$ via oral gavage. The dosing vehicle contained 0.5 μg/mL $1\alpha(OH)D_2$ in 0.4% polysorbate, 0.15% NaCl and 0.11% disodium EDTA in phosphate buffer pH 7. The dose volume was 1.0 mL/kg of body weight. 1.25 mL of whole blood was collected through the cannula from rats at 0, 30, 60, 120, 240, 480, 1080, 1440, and 2880 min post-dose. Blood was drawn from each animal two times. Four samples were collected at each time point. The serum sample processing was performed as described above.

Pharmacokinetic analysis was performed using non-compartmental analysis.

3. Results and discussion

3.1. LC–MS/MS method development

The ionization and fragmentation of $1,25(OH)_2D_2$ was obtained from infusion of 100 ng/mL $1,25(OH)_2D_2$ at a flow rate of 90 μL/min using atmospheric pressure chemical ionization tandem mass spectrometry. The parent ion at m/z 429.6 lost one molecule of water producing the most intense ion at m/z 411.5 (Fig. 2A) which was selected as the precursor ion. The collision-induced dissociation of the precursor ion (i.e., m/z 411.5) produced the most intense fragmentation ion at m/z 151.0 under optimum collision energy at 32 (Fig. 2B). The multiple reaction monitoring, m/z 411.5 → 151.0 transition, was the same for both $1,25(OH)_2D_2$ and $1,24(OH)_2D_2$.

To accurately measure systemic levels of $1,25(OH)_2D_2$, the bioanalytical method must be selective to separate 1,25(OH)2D2 from $1,24(OH)_2D_2$, the closest structural vitamin D_2 metabolite

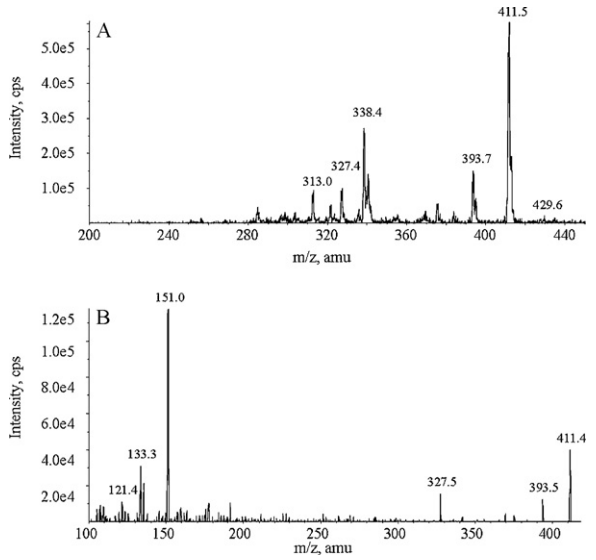

Fig. 2. (A) Mass spectrum (Q1) of 1,25$(OH)_2D_2$ obtained by positive atmospheric pressure chemical ionization. (B) Product ion scan of the $[M-18+H]^+$ precursor ion.

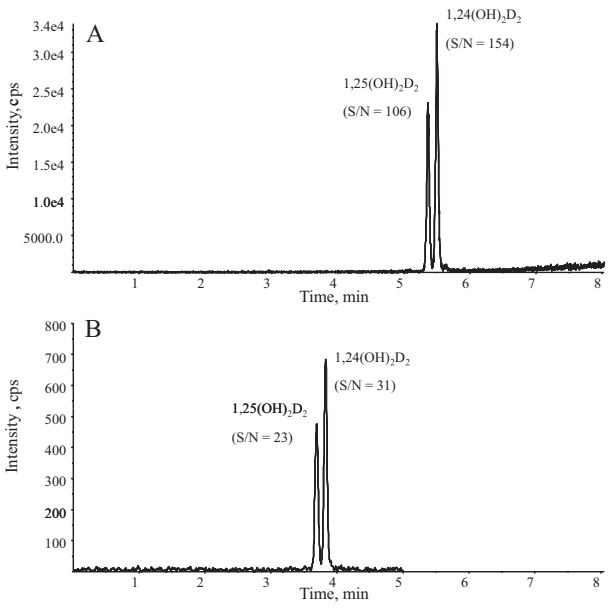

Fig. 3. (A) Multiple reaction monitoring (MRM) ion chromatogram (transition 411.5 → 151.0) for a mixture of 1000 pg/mL 1,25$(OH)_2D_2$ (t_R = 5.4 min) and 1,24$(OH)_2D_2$ (t_R = 5.5 min) using the HPLC–MS/MS method. (B) Multiple reaction monitoring (MRM) ion chromatogram (transition 411.5 → 151.0) for a mixture of 200 pg/mL 1,25$(OH)_2D_2$ (t_R = 3.7 min) and 1α,24$(OH)_2D_2$ (t_R = 3.8 min) using the UPLC–MS/MS method.

[19,20]. Various chromatographic conditions with different column chemistries and mobile phase compositions were investigated. Satisfactory HPLC conditions were obtained using a Phenomenex Luna C8 column and a linear gradient of mobile phase from 35% to 98% acetonitrile in water over 7.6 min. The peaks of 1,25$(OH)_2D_2$ and 1,24$(OH)_2D_2$ were effectively separated with a resolution (R_s) of 1.67 and retention times of 5.4 min and 5.5 min, respectively (Fig. 3).

3.2. Selectivity and LLOQ

Six different lots of rat serum were extracted and analyzed to determine if endogenous components could interfere with the analysis of 1,25$(OH)_2D_2$ in rat serum samples. None of the blank lots of serum evaluated showed any interference at the retention time of 1,25$(OH)_2D_2$ with either method. The LLOQ achieved was 25.0 pg/mL for both HPLC–MS/MS (Fig. 4B) and UPLC–MS/MS methods (Fig. 4D).

3.3. Linearity of 1,25$(OH)_2D_2$

Calibration curves were plotted with concentration ratio of analyte and internal standard versus peak area ratio of analyte and internal standard. The slope, intercept, and correlation of determination for the HPLC–MS/MS method are shown in Table 1. The calibration curves were linear over the range 25.0–1000 pg/mL for both methods. The accuracy and precision of each standard level are reported in Table 2.

3.4. Accuracy and precision

The accuracy and precision were determined by the analysis of QC samples at three concentration levels. Six replicates at each level were extracted and analyzed on two different days and three replicates at each level were extracted and analyzed on another day for the third batch to assess intra-day and inter-day accuracy and precision. The accuracy and precision of HPLC–MS/MS method were within 15% for both intra-day (n = 6) and inter-day (n = 15) experiments as shown in Table 3.

Table 2
Summary of the back-calculated 1,25$(OH)_2D_2$ calibration standards (n = 6) in rat serum.

Method	Nominal concentration (pg/mL)	Concentration found (pg/mL)	Accuracy (%Bias)	Precision (%CV)
HPLC–MS/MS	25.0	25.8	3.00	12.7
	50.0	49.8	−0.433	7.2
	100	98.8	−1.23	5.6
	200	198	−1.25	3.8
	500	493	−1.40	2.5
	750	760	1.29	3.9
	1000	1000	0.117	2.3
UPLC–MS/MS	25.0	24.7	−1.13	9.0
	50.0	53.9	7.77	14.8
	100	99.0	−0.983	5.9
	200	185	−7.33	8.1
	500	513	2.67	6.3
	750	719	−4.13	2.9
	1000	1040	4.30	2.5

3.5. Recovery of 1,25$(OH)_2D_2$

The recovery experiment in this study evaluated the combined effects of extraction efficiency and matrix effect. The recovery of 1,25$(OH)_2D_2$ was determined by analyzing the extracted rat serum samples versus standard solution prepared in water:acetonitrile

Table 1
Calibration statistics.

Chromatography interface	Concentration range (pg/mL)	Slope (n = 6) Mean ± SD	Intercept (n = 6) Mean ± SD	Coefficient of determination
HPLC–MS/MS	25.0–1000	0.00958 ± 0.00087	0.212 ± 0.082	0.9993
UPLC–MS/MS	25.0–1000	0.01010 ± 0.00133	−0.013 ± 0.031	0.9976

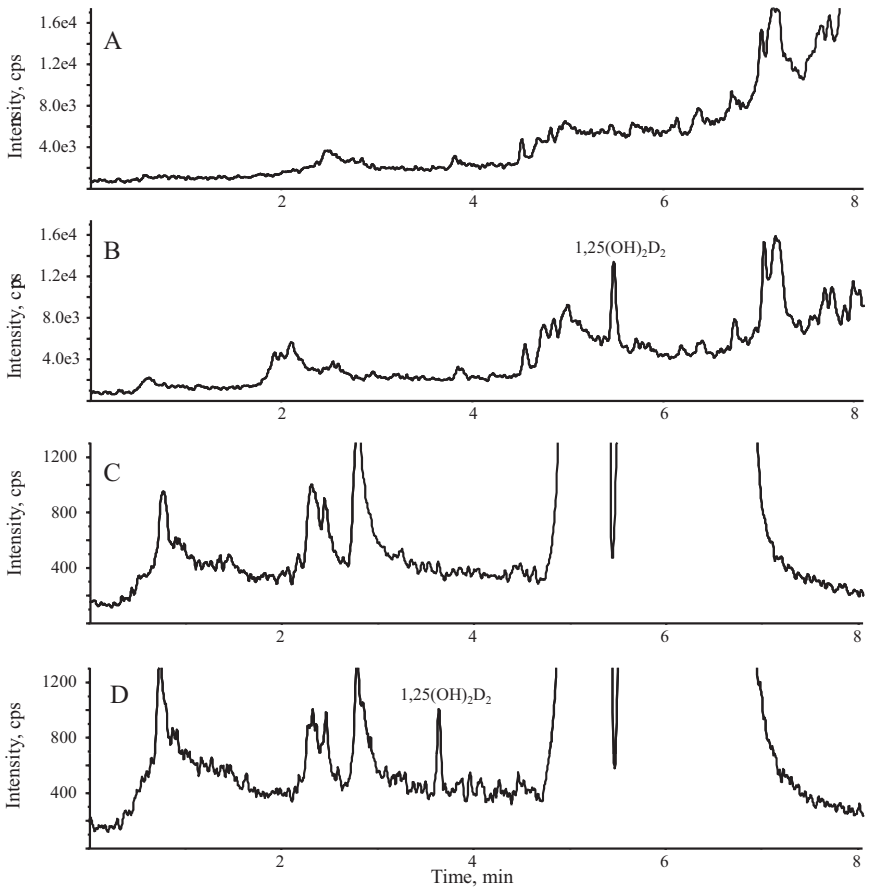

Fig. 4. Ion chromatogram of (A) blank rat serum and (B) blank rat serum spiked with 25.0 pg/mL $1,25(OH)_2D_2$ using the HPLC–MS/MS method; (C) blank rat serum and (D) blank rat serum spiked with 25.0 pg/mL $1,25(OH)_2D_2$ using the UPLC–MS/MS method.

(50:50, v/v) at each concentration. The recovery was tested at three different concentrations in triplicate. The recovery was calculated as a percentage of the peak area of $1,25(OH)_2D_2$ detected in the extracted serum samples compared with that detected in the standard solution. The recovery results were consistently greater than 80% from 25.0 pg/mL to 1000 pg/mL (Table 4).

3.6. Stability of $1,25(OH)_2D_2$

The bench-top stability of $1,25(OH)_2D_2$ in rat serum was determined after storage of the stability samples under light protected conditions at room temperature for 72 h. Samples were extracted and analyzed against the freshly prepared standard curve. The bench-top stability results demonstrated that $1,25(OH)_2D_2$ was stable in rat serum at room temperature for 72 h (Table 5). For freeze–thaw stability study, the freeze–thaw samples were extracted and analyzed after three freeze–thaw cycles against a freshly prepared standard curve. The freeze–thaw cycle stability results demonstrated that $1,25(OH)_2D_2$ was stable after three freeze–thaw cycles (Table 5).

Table 3
Intra-day and inter-day precision and accuracy data for the determination of $1,25(OH)_2D_2$ in rat serum.

Chromatography interface	Concentration (pg/mL)	Intra-day precision (%CV, $n=6$)	Inter-day precision[a] (%CV)	Accuracy[a] (%Bias)
HPLC–MS/MS	25.0	15.0	12.8	1.92
	200	2.6	6.6	−2.83
	1000	1.6	5.6	−1.30
UPLC–MS/MS	25.0	14.1	14.6	5.93
	200	11.6	8.8	−5.88
	750	6.0	7.1	−7.24

[a] $n=15$ for HPLC–MS/MS and $n=12$ for UPLC–MS/MS.

Table 4
Recovery results of $1,25(OH)_2D_2$ in rat serum.

Chromatography interface	Concentration (pg/mL)	Recovery (%)[a] ± SD (%)
HPLC–MS/MS ($n=3$)	25.0	81.8 ± 3.2
	200	91.2 ± 4.6
	1000	96.3 ± 5.4
UPLC–MS/MS ($n=6$)	25.0	99.0 ± 9.4
	200	89.8 ± 9.7
	750	84.8 ± 3.3

[a] Recovery values reported here took into account of the matrix effect.

3.7. Pharmacokinetic studies

Employing the validated assay, the serum concentration versus time profile of $1,25(OH)_2D_2$ was determined after a single IV admin-

Table 5
Stability of $1,25(OH)_2D_2$ in rat serum presented as the percentage relative to the measured concentrations obtained from freshly prepared QC samples using the HPLC–MS/MS method.

Concentration (pg/mL)	72 h at room temperature ($n=3$)	3 × freeze/thaw cycles at −80 °C ($n=3$)
25.0	112	101
200	99.2	96.7
1000	99.5	96.6

Table 6
Estimated pharmacokinetic parameters of $1,25(OH)_2D_2$ after a single IV bolus administration at 0.1 μg/kg or 1.0 μg/kg, and a single oral administration of 0.5 μg/kg $1\alpha(OH)D_2$ to male SD rats.

Route	Dose (μg/kg)	C_{max} (pg/mL) Mean ± SE	T_{max} (h) Mean ± SD	C_{last} (pg/mL) Mean ± SD	$t_{1/2}$ (h)	AUC_{0-t} (pg h/mL) Mean ± SE
IV	0.1	116 ± 14.0	4.00 ± 0.00	61.0 ± 9.92	17.2	2121 ± 95.9
IV	1.0	334 ± 32.0	4.00 ± 0.00	286 ± 33.2	–[a]	6971 ± 194
PO	0.5	235 ± 21.0	8.00 ± 0.00	62.3 ± 21.3	27.0	6236 ± 407

[a] No terminal phase was observed.

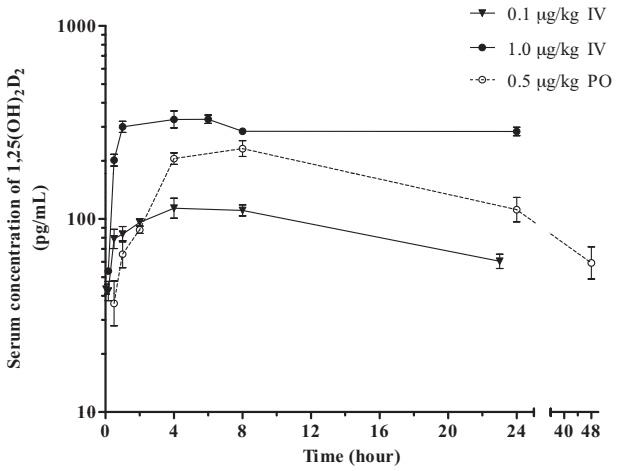

Fig. 5. Serum concentration of $1,25(OH)_2D_2$ vs. time profiles obtained after IV administration of 0.1 and 1.0 μg/kg, and oral administration of 0.5 μg/kg $1\alpha(OH)D_2$ to male SD rats.

istration of 0.1 μg/kg and 1.0 μg/kg, and a single oral administration of 0.5 μg/kg of $1\alpha(OH)D_2$ to male SD rats. A number of study samples at the earlier timepoints from the low dose IV and PO groups were close to the LLOQ as shown in Fig. 5. A chromatogram (Fig. 6) from a rat PK sample following IV administration of 1.0 μg/kg illustrates the separation achieved for $1,25(OH)_2D_2$ from $1,24(OH)_2D_2$, the structurally similar metabolite, generated by 24-hydroxylation of $1\alpha(OH)D_2$. This metabolite was detected at an appreciable levels, emphasizing the importance of the chromatographic separation required for $1,25(OH)_2D_2$ and $1,24(OH)_2D_2$ in the developed assay. The pharmacokinetic curves generated for $1,25(OH)_2D_2$ in serum is shown in Fig. 6 and the corresponding pharmacokinetic parameters calculated using non-compartmental model in WinNonlin®, version 5.1 (Pharsight, Cary, NC, USA) are summarized in Table 6.

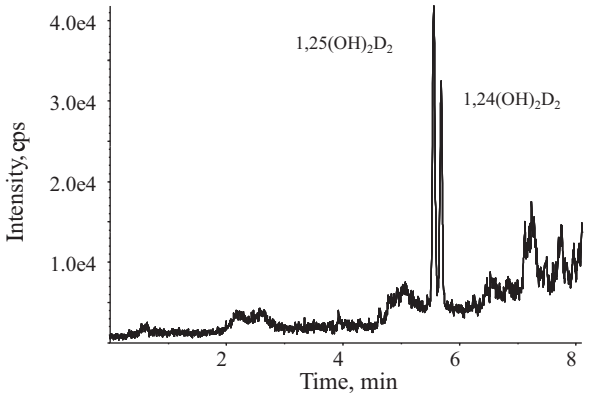

Fig. 6. Ion chromatogram using the HPLC–MS/MS method of a rat serum sample at 24 h following IV administration of 1.0 μg/kg of $1\alpha(OH)D_2$.

3.8. UPLC–MS/MS

To assess the applicability of UPLC to reduce the chromatographic run time, a UPLC–MS/MS method was also developed. Similar assay validation performance was achieved as shown in Tables 1–4. The method employed an Acquity HSS T3 column and an initial mobile phase consisting of water and acetonitrile (45:55, v/v) for 1.3 min followed by a linear gradient from 55% to 67% acetonitrile over 3 min. The retention times of $1,25(OH)_2D_2$ and $1,24(OH)_2D_2$ were 3.65 min and 3.78 min, respectively (Fig. 3). The peaks of $1,25(OH)_2D_2$ and $1,24(OH)_2D_2$ were effectively separated with a resolution (R_s) of 1.67. No difference in assay sensitivity was observed using UPLC versus HPLC. The UPLC–MS/MS method maintained the same selectivity as HLPC–MS/MS but with half the run time (i.e., 8 min), enabling improved sample throughput.

4. Conclusion

A simple and highly sensitive HPLC–MS/MS assay was successfully developed and validated for the determination of $1,25(OH)_2D_2$ in rat serum. Previously reported assays for the analysis of $1,25(OH)_2D_2$ in biological matrices suffered from extensive sample pretreatment and purification, low precision, and metabolite interference. To demonstrate the utility of the assay, the pharmacokinetics of $1,25(OH)_2D_2$ in rats at microgram and sub-microgram doses was determined using the validated assay. Additionally, the application of UPLC–MS/MS was explored to reduce the chromatographic run time and was found to provide a 2-fold reduction in cycle time without any compromise of assay performance.

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