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Determination of unbound vismodegib (GDC-0449) concentration in human plasma using rapid equilibrium dialysis followed by solid phase extraction and high-performance liquid chromatography coupled to mass spectrometry

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ABSTRACT

A rapid equilibrium dialysis (RED) assay followed by a solid phase extraction (SPE) high-performance liquid chromatography tandem mass spectrometry (LC–MS/MS) assay for the quantitative determination of unbound vismodegib in human plasma was developed and validated. The equilibrium dialysis was carried out using 0.3 mL plasma samples in the single-use plate RED system at 37 °C for 6 h. The dialysis samples (0.1 mL) were extracted using a Strata-X-C 33u Polymeric Strong Cation SPE plate and the resulting extracts were analyzed using reverse-phase chromatography and positive electrospray ionization (ESI) mass spectrometry. The standard curve, which ranged from 0.100 to 100 ng/mL for vismodegib, was fitted to a $1/x^2$ weighted linear regression model. The lower limit of quantitation (LLOQ, 0.100 ng/mL) was sufficient to quantify unbound concentrations of vismodegib after dialysis. The intra-assay precision of the LC–MS/MS assay, based on the four analytical QC levels (LLOQ, low, medium and high), was within 7.7% CV and inter-assay precision was within 5.5% CV. The assay accuracy, expressed as %Bias, was within ±4.0% of the nominal concentration values. Extraction recovery of vismodegib was between 77.9 and 84.0%. The assay provides a means for accurate assessment of unbound vismodegib plasma concentrations in clinical studies.

1. Introduction

Vismodegib (GDC-0449) is a first-in-class small-molecule inhibitor of the Hedgehog signaling pathway and is currently in clinical development for treatment of various cancers [1–4]. In a previous phase 1 clinical trial in patients with solid tumors, vismodegib was well tolerated and promising efficacy in advanced basal cell carcinoma was observed [5,6]. Since vismodegib was highly bound to alpha-1-acid glycoprotein (AAG) and the pharmacokinetics (PKs) was nonlinear in patients, binding to AAG is perceived as an important determinant of total vismodegib plasma concentrations [7]. Given the potential for nonlinear PK as a result of plasma protein binding, it was important to develop and validate a bioanalytical assay to evaluate unbound vismodegib plasma concentration in clinical studies.

Equilibrium dialysis (ED) is one of the most commonly used methods for protein binding assessment and allows for quantitative determination of bound and unbound drug concentrations in plasma. It can minimize the effect of nonspecific binding and does not require large plasma volumes that impede other alternative techniques such as ultrafiltration and ultracentrifugation [8,9]. However, the classic equilibrium dialysis method usually is time-consuming and tedious with limited sample throughput capacity. To increase the throughput, the rapid equilibrium dialysis (RED) device has been introduced by Pierce Biotechnology (ThermoFisher Scientific, Waltham, MA, USA). This device utilizes a Teflon base plate and disposable dialysis cell which has a higher surface area-to-volume ration compared to the standard ED approaches. This design reduces the equilibration time and increases the assay throughput [10,11].

In the present study, a RED assay followed by a solid phase extraction high-performance liquid chromatography tandem mass spectrometry assay for the determination of unbound vismodegib concentration in human plasma was developed and validated. The RED assay procedure involved the use of a plasma:buffer mixed matrix. The described solid phase extraction high-performance liquid chromatography tandem mass spectrometry assay used to quantitate unbound vismodegib concentrations offered advantages

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over our previous reported plasma assay [12] in that it was compatible with our RED assay procedure and offered greater sensitivity required to measure low unbound concentrations. The validated assays were used to support multiple clinical trials, including a dose escalation study in cancer patients [5,7], a study to optimize the vismodegib dosing regimen (data on file) and a healthy volunteer PK study. The results from the healthy volunteer study are reported herein.

2. Experimental

2.1. Materials and reagents

Test compound vismodegib and internal standard D_5-vismodegib were provided by Genentech (South San Francisco, CA, USA). Acetonitrile, acetone, methanol, formic acid and ammonium hydroxide were purchased from EMD Chemicals (Gibbstown, NJ, USA). Deionized water (typically 18.2 MΩ cm) was purified with a Milli-Q water purification system purchased from Millipore (Bedford, MA, USA). N,N-Dimethylformamide (DMF) was purchased from Burdick and Jackson (Morristown, NJ, USA). Monobasic potassium phosphate and dibasic potassium phosphate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Human plasma with K_2EDTA anticoagulant, was purchased from Bioreclamation, Inc. (Westbury, NY, USA) and BioChemed Corp. (Charleston, SC, USA).

2.2. Equilibrium dialysis system

Equilibrium dialysis was conducted using the Single-Use Plate Rapid Equilibrium Dialysis device with dialysis membranes of molecular weight cut-off of approximately 8000 Da (Thermo Scientific, Rockford, IL, USA). The loaded dialysis plate was placed on an orbital shaker at approximately 500 rpm and incubated in a 5% CO_2 humidified incubator (Napco 5400) at 37 °C. Plasma samples (0.3 mL) were dialyzed against buffer (0.5 mL) at pH 7.4 for 6 h. A dialysis time of 6 h was chosen based upon previous time-to-equilibration experiments in pilot studies (data not shown).

The PBS buffer used in this study, 0.133 M potassium phosphate buffer containing 0.9% sodium chloride, was prepared by combining 190 mL of 0.133 M monobasic potassium phosphate (KH_2PO_4) with 810 mL of 0.133 M dibasic potassium phosphate (K_2HPO_4) and 9.0 g sodium chloride in 1.0 L of phosphate buffer. The pH was adjusted to 7.4 and stored at 4 °C until use.

Frozen blank human plasma, pooled from 4 males and 4 females, with K_2EDTA as an anticoagulant, was completely thawed at room temperature before use. This blank human plasma was used to prepare plasma/buffer mixed matrix for buffer samples at the end of dialysis.

2.3. Human plasma samples for dialysis

Spiked vismodegib human plasma samples at four concentrations (126, 1260, 10,500, and 31,500 ng/mL) were used for the validation study. These plasma samples were stored at approximately −70 °C until the day of the protein binding experiment.

2.4. Equilibrium dialysis method

On the day of dialysis, human plasma samples were removed from storage and allowed to come to room temperature. Aliquots of plasma samples (0.3 mL) and buffer (0.5 mL) were added to the corresponding RED dialysis chambers. The loaded dialysis plate was covered with sealing tape and put on an orbital shaker at approximately 500 rpm and incubated at 37 °C for 6 h. At the end of dialysis, a 200 μL plasma aliquot was collected from the plasma chamber and transferred to cryovials containing 200 μL of blank buffer. A 200 μL buffer sample aliquot was collected from the buffer chamber and transferred to cryovials containing 200 μL of blank human plasma. Samples with less than 200 μL volume were collected and transferred into a cryovial containing an equal volume of buffer or plasma. The remaining post-dialysis plasma and buffer samples were collected and transferred into pre-weighed polypropylene vials to measure the weights. Total sample weights were calculated by adding the remaining post-dialysis sample weights plus 200 μL sample weights (assuming 0.2 g). These weights were used to calculate the volume change and percent recovery during the dialysis. All plasma/buffer mixed matrix samples were stored at approximately −70 °C until analysis.

2.5. RED method validation

The method validation included the determination of intra-day and inter-day variability of vismodegib percent unbound in human plasma from determinations made on three separate days. Protein binding was conducted at 37 °C by a RED method using the Single-Use Plate RED system as described above. Human plasma samples containing vismodegib at concentrations of 126, 1260, 10,500, and 31,500 ng/mL, respectively were used for protein binding determination on three separate days using a plasma volume of 0.3 mL dialyzed against 0.5 mL buffer at 37 °C for 6 h. Protein binding samples in plasma/buffer mixed matrix were analyzed for vismodegib concentrations using the validated LC–MS/MS method.

2.6. Sample preparation for LC–MS/MS analysis

Vismodegib stock solutions for standard (STD) and quality control (QC), at a nominal concentration of 0.791 and 1.01 mg/mL, respectively, were prepared in DMF. Subsequent working solutions were prepared by serial dilution of the stock solutions with DMF. Internal standard stock solution and working solution of D_5-vismodegib were prepared at 0.598 mg/mL and 100 ng/mL in DMF, respectively. The calibration standards at nominal concentrations of 0.1, 0.2, 0.5, 2.0, 10, 50, 90 and 100 ng/mL were prepared by spiking corresponding 20X of vismodegib working solutions into an appropriate amount of blank matrix [human plasma: 0.133 M PBS (50:50, v/v)]. QC samples at nominal concentrations of 0.1 (LLOQ QC), 0.3 (low QC), 15 (medium QC), 80 (high QC) and 8,000 (dilution QC) ng/mL were also prepared by spiking corresponding 20X working solutions into an appropriate amount of blank human plasma: 0.133 M PBS (50:50, v/v).

Aliquots of 100 μL of plasma:PBS samples, STD and QC were transferred to a 96-well plate. Subsequently, 50 μL of internal standard working solution and 100 μL of 5% formic acid in water were added to each well. Following vortexing and centrifugation, the samples were loaded onto a Strata-X-C 33u Polymeric Strong Cation 96-well SPE plate which was pre-conditioned by 500 μL of methanol followed by 500 μL of 5% formic acid in water. After sample loading, the SPE plate was then washed by 500 μL of 5% formic acid in water followed by 750 μL of methanol. The analytes were eluted by 300 μL of 5% NH_4OH in methanol. The eluent was evaporated to dryness at 50 °C and reconstituted with 200 μL of 0.1% formic acid in acetonitrile:water (50:50, v/v). Injection volume was 10 μL for the LC–MS/MS sample analysis.

2.7. LC–MS/MS method

LC–MS/MS data were acquired by Analyst® software (version 1.4) on a Sciex API 5000 tandem mass spectrometer (AB Sciex, Foster City, CA) in positive electrospray ionization (ESI) mode. The separation was achieved on a Gemini C18 column (2 mm × 50 mm, 5 μm) (Phenomenex, Torrance, CA, USA) with a 0.5 μm frit with isocratic elution using 50:50 mobile phase A of 0.1% formic acid

in water and mobile phase B of acetonitrile. The column temperature was kept at 30 °C by a Cera column oven. Shimadzu (Tokyo, Japan) LC-10AD pumps and a Leap autosampler (LEAP Technologies, Chapel Hill, NC, USA) were used as the delivery pumps and autosampler, respectively. The total run time was 4 min including a back-flush program with 90:10 acetonitrile:water at a flow rate of 1.00 mL/min to clean up any phospholipids which may stay on column and cannot be removed by the 50:50 acetonitrile:water isocratic run. For quantitation, the mass spectrometer was operated in the positive selected reaction monitoring mode (SRM) for vismodegib. The following SRM transitions were monitored: m/z 421.1 → 139.1 for vismodegib, m/z 200 → 100 for dummy ion, m/z 426.1 → 139.1 for D_5-vismodegib and m/z 800 → 200 for dummy ion. The dwell times were 100 ms for vismodegib and D_5-vismodegib and 50 ms for the dummy ions, respectively. The non-specific dummy transitions used here reduced instrument "cross-talk" between analyte and internal standard SRM transitions. The ionspray voltage was +4500 V, the declustering potentials were 100 V and the collision energies were 38 eV for vismodegib and D_5-vismodegib, respectively. The source temperature was set at 400 °C.

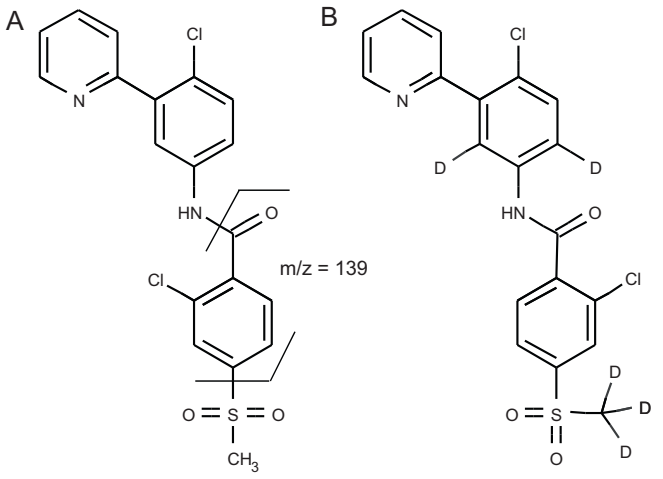

Fig. 1. Chemical structures of vismodegib (A) and internal standard D_5-vismodegib (B).

2.8. SPE LC–MS/MS method validation

For the human plasma:PBS assay, linearity, accuracy and precision, selectivity, recovery, matrix effect, stability and re-injection reproducibility were evaluated following the FDA Guidance for Industry – Bioanalytical Method Validation (2001).

Accuracy and precision were evaluated using QC samples prepared at five different levels covering the STD curve ranges. Three accuracy and precision runs were conducted for full validations in human plasma:PBS assay. The interferences from endogenous materials and IS were tested by extracting six different lots of blank matrices with and without IS, and six LLOQ samples prepared in the corresponding six different lots of blank matrix. The interference was evaluated by comparing the SRM chromatograms among the analyte only and internal standard only samples. The extraction recovery for vismodegib was evaluated by comparing the responses of the pre-extraction spiked samples to those of post-extraction spiked samples. The reinjection reproducibility was evaluated by re-injecting previously acceptable standards and QCs that have been stored under specified conditions for a pre-determined length of time.

2.9. Stability

Quality control samples (n = 6 at low and high QC concentrations) were subjected to four freeze–thaw cycles with thawing at room temperature and refreezing at −70 °C for at least 12 h. After four freeze–thaw cycles the samples were analyzed using freshly prepared calibration STD and analytical QCs. The bench-top stability at room temperature in stock solution and in matrix (n = 6 at low and high QC concentrations) was evaluated for over 6 h periods. Reproducibility of sample reinjection was evaluated by reinjection of an acceptable accuracy and precision run after STD and QC samples being stored at room temperature for 101 h. Long-term matrix stability was evaluated using pre-qualified QC samples (n = 6 at low and high QC concentrations) that were stored at a temperature between −60 and −80 °C for 396 days.

2.10. Pharmacokinetic assessments of vismodegib in healthy volunteers

PK of vismodegib was studied in an open-label, single dose phase I study in three healthy females of non-childbearing potential following Institutional Review Board approval of the protocol. Upon obtaining informed consent, meeting institutional and federal requirements and meeting study entry criteria, vismodegib was administered orally as a single 150 mg dose with 240 mL room temperature tap water after a minimum of an 8-h fast. After drug administration, 22 serial blood samples were obtained from each subject and plasma harvested during an 8-week sampling period for the determination of vismodegib plasma concentration over time.

Individual subject PK parameter values for unbound vismodegib plasma concentration–time data were derived using non-compartmental methods (WinNonlin version 5.2.1, Pharsight Corp., Mountain View, CA). All AUC values were calculated using the linear trapezoidal method when the concentrations were rising and the logarithmic trapezoidal method when the concentrations were declining (Linear up/Log Down rule in WinNonlin®). Below the limit of quantitation (BLQ) values were considered as zero for PK analysis. Actual blood collection time was used to calculate PK parameters. PK parameters were reported as their means and standard deviations (SDs). Fraction unbound was calculated as unbound divided by total vismodegib concentration; total vismodegib concentrations were reported previously [12]. The fraction unbound was time invariant, thus the average fraction unbound was calculated across all time points from each of the three subjects.

3. Results and discussion

3.1. LC–MS/MS sensitivity

A LC–MS/MS assay with a range of 5–5000 ng/mL had already been validated to measure the total concentration of vismodegib in human plasma [12]. The chemical structures of vismodegib and D_5-vismodegib are shown in Fig. 1. One aim of the present study, was to validate an mixed plasma:buffer assay which with better sensitivity to measure the unbound concentration of vismodegib in human plasma after RED. Six replicates of LLOQ QC samples at 0.1 ng/mL were extracted and analyzed in three accuracy and precision (A&P) runs. The representative chromatograms of vismodegib at LLOQ (0.1 ng/mL) and IS D_5-vismodegib at 100 ng/mL are shown in Fig. 2. The signal to noise ratio of the LLOQ QC was approximately 7 which was sufficient for the reliable detection of vismodegib in the dialysis samples.

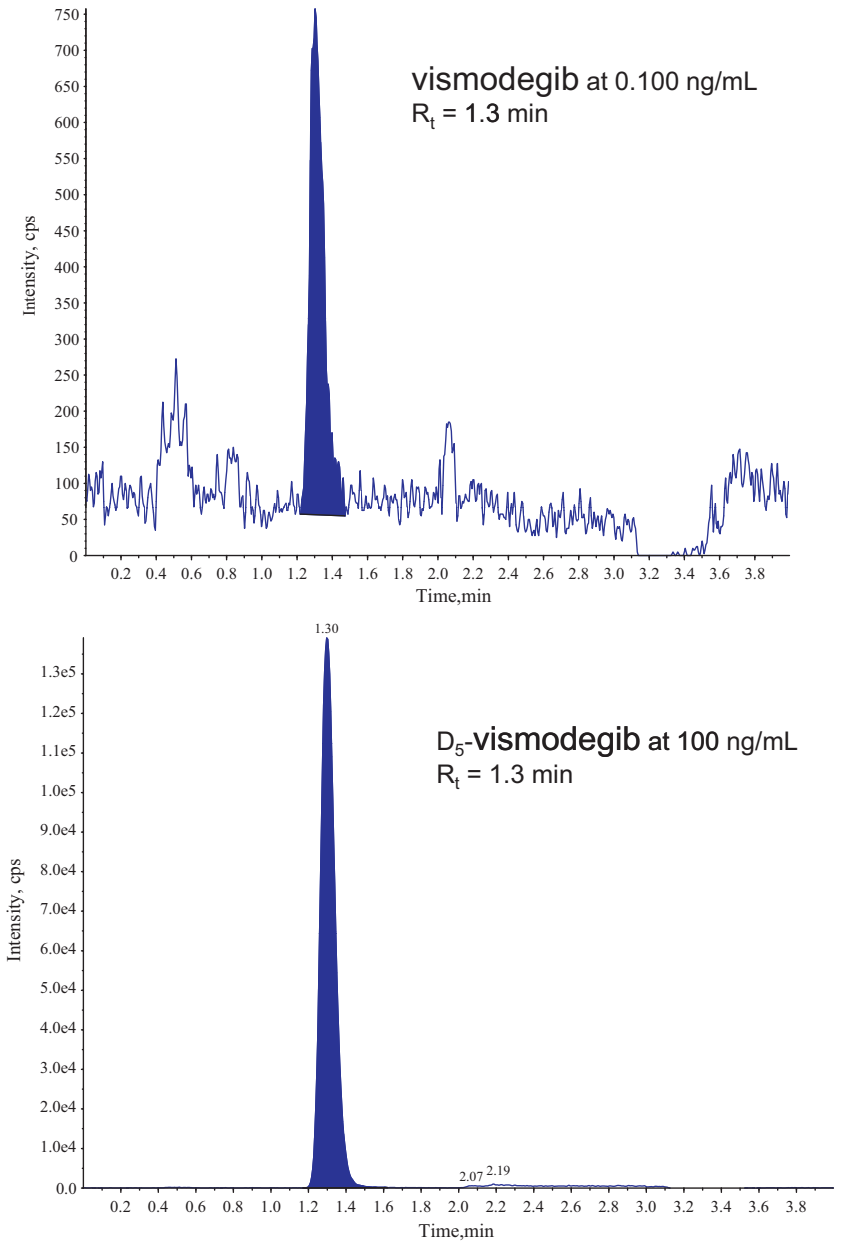

Fig. 2. Chromatograms of vismodegib at LLOQ 0.100 ng/mL and internal standard D_5-vismodegib at 100 ng/mL.

3.2. Accuracy and precision

In order to determine the within-run and between-run accuracy and precision, three A&P runs were performed on three different days which included duplicated standard curves and 6 replicates of QC samples at 4 different concentrations. The accuracy and precision information, based on the four QC levels, obtained using one-way ANOVA in Watson are listed in Table 1. The within-run precision for the four levels of QC samples was within 7.7% and the between-run precision was within 5.5%. The assay accuracy,

Table 1
Summary of precision and accuracy from four different QC level samples by one-way ANOVA from Watson.

Nominal conc.	LLOQ QC, 0.100 ng/mL	Low QC, 0.300 ng/mL	Medium QC, 15.0 ng/mL	High QC, 80.0 ng/mL
Mean observed conc.	0.0960	0.296	14.6	77.1
%Bias	−4.0	−1.3	−2.7	−3.6
Between run precision (%CV)	5.5	3.9	1.2	3.5
Within run precision (%CV)	7.7	3.7	0.9	0.8
Total variation (%CV)	9.5	5.3	1.5	3.6
n	18	18	18	18
Number of runs	3	3	3	3

%Bias expressed as [(mean − nominal)/nominal] × 100.
%CV expressed as (standard deviation/mean) × 100.

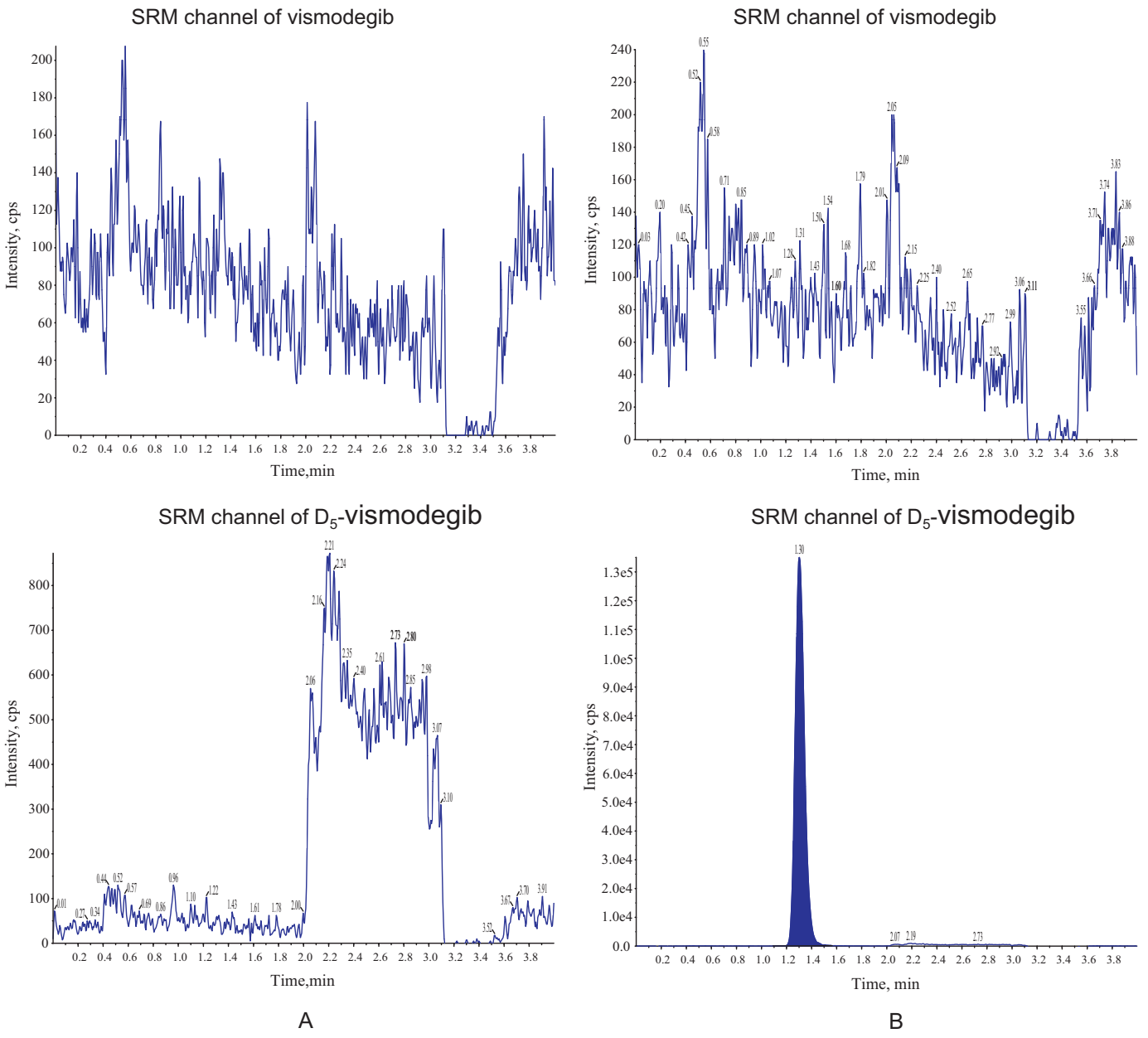

Fig. 3. Chromatograms of blank plasma:PBS (A) and blank plasma:PBS with IS at 100 ng/mL (B).

expressed as %Bias, was within ±4.0% of the nominal concentration values.

3.3. Selectivity

Six different lots of blank plasma and the analyte (vismodegib) and IS (D_5-vismodegib) only samples were used to test the selectivity of the assay. No significant interference was found at the retention times of vismodegib and D_5-vismodegib in all six lots of blank plasma. Any interference peaks observed in all six lots of blank plasma at the retention time of vismodegib and D_5-vismodegib were less than 20% of the LLOQ and 5% of the internal standard response, respectively. There was also no significant interference found at the retention time of interest for analyte and IS only samples. The representative chromatograms of the extracted blank plasma and Q0 (blank plasma plus IS) are shown in Fig. 3.

3.4. Extraction recovery

Extraction recovery of vismodegib was evaluated at three (low, medium and high) QC levels. Relative extraction recovery of vismodegib was determined by comparing the peak area ratio of analyte/internal standard of samples with both analyte and internal standard added before extraction and the peak area ratio of the analyte/internal standard of samples with analyte added after extraction and internal standard added before extraction. Extraction recovery of IS (D_5-vismodegib) was evaluated at the medium QC levels. Extraction recovery of D_5-vismodegib was determined by comparing the peak area ratio of the analyte/internal standard of samples and the peak area ratio of analyte/internal standard of samples with analyte added before extraction and internal standard added after extraction. Extraction recoveries for vismodegib at low, medium and high QC levels were 77.9, 84.0 and 80.6%. Extraction recovery for D_5-vismodegib at medium QC level was 89.2% (data not shown).

Table 2
Matrix factors of vismodegib and D_5-vismodegib.

Replicate	MF extract Analyte	MF neat Analyte	MF extract IS	MF neat IS
1	290,294	324,773	172,790	192,004
2	293,994	309,069	174,836	180,335
3	303,906	303,914	181,752	179,059
4	302,891	329,055	177,778	195,535
5	303,447	351,479	178,151	208,696
6	336,416	365,675	201,817	216,700
Mean	305,158	330,661	181,187	195,388
SD	16,323	23,993	10,560	15,073
%CV	5.3	7.3	5.8	7.7
n	6	6	6	6
Matrix factor	0.9		0.9	

SD: standard deviation.
Matrix factor expressed as (mean area of extract)/(mean area of neat).

Table 3
Summary of precision and accuracy from the dilution QC samples of vismodegib in human plasma:PBS.

Nominal conc. (ng/mL)	DQC1	DQC2	DQC3	DQC4	DQC5	DQC6	Mean	Accuracy (%)	RSD (%)
8000	7930	8010	7900	7720	8020	7900	7910	98.9	1.4

Accuracy (%) expressed as (mean observed concentration/nominal concentration) × 100.
RSD: relative standard deviation.

3.5. Matrix effect

Matrix factor (MF) test was used to evaluate the suppression or enhancement of analyte ionization by the presence of matrix components in the biological samples. Six different lots of blank human plasma were extracted. After evaporation, each extracted blank sample was reconstituted with 200 μL of 20.0/25.0 ng/mL vismodegib/D_5-vismodegib in 0.1% FA in [50:50 water:MeCN (v/v)]. The LC–MS/MS responses of the extracted blank samples were compared to six neat solution with 20.0/25.0 ng/mL vismodegib/D_5-vismodegib in 0.1% FA in [50:50 water:MeCN (v/v)]. The MF were 0.9 for vismodegib and 0.9 for D_5-vismodegib which indicated that there was no significant matrix suppression observed for both vismodegib and D_5-vismodegib (Table 2).

3.6. Dilution capability

In order to measure the concentration of vismodegib on the plasma side of the dialysis samples, a 200-fold dilution was evaluated in this validation. Dilution QC was made at the 8000 ng/mL level. The accuracy of the dilution QCs was within 98.9% of nominal and the precision was within 1.4% (Table 3).

3.7. Stability

Freeze–thaw stability, bench-top stability, long-term stability and reinjection reproducibility of vismodegib in human plasma:PBS were assessed in this validation and the results are summarized in Table 4. The solution stabilities of vismodegib were validated in the previous validation [12] and are not shown here. Vismodegib was shown to be stable in human plasma:PBS solution after four freeze–thaw cycles and stable on the bench-top at room temperature for 6 h prior to extraction. Vismodegib was also shown to be stable in human plasma:PBS solution after storage between −60 °C and −80 °C for 396 days. The reinjection reproducibility test showed the acceptable run could be repeated after 101 h storage at room temperature.

3.8. RED experiment and data analysis

Vismodegib has been shown to be highly bound to human plasma proteins [13]. The unbound concentration of vismodegib was therefore expected to be in the sub-nanogram per milliliter range. From our experience, when compound concentrations are low, the impact of adsorption to sample tubes can be significant even if only a small amount of compound is lost, especially in neat solutions or buffers. In order to minimize any potential adsorption issues with vismodegib, the post-dialysis buffer sample aliquot was transferred from the buffer chamber to cryovials containing equal volume of blank human plasma. The post-dialysis plasma sample aliquot was also transferred from the plasma chamber to cryovials containing equal volume of blank buffer. Mixing post-dialysis plasma with buffer allowed one bioanalytical assay validation and assay run in the mixed plasma:buffer matrix instead of two separate assay validations and assay runs for plasma and neat buffer samples.

Table 4
Stability assessments for vismodegib in human plasma:PBS.

Nominal conc. (ng/mL)	Mean observed conc. (ng/mL, *n* = 6)	Accuracy (%) (*n* = 6)	RSD (%) (*n* = 6)
Stability after 4 freeze–thaw cycles			
0.300	0.303	101.0	2.3
80.0	74.8	93.5	1.1
Bench-top stability at room temperature for 6 h			
0.300	0.288	96.0	5.0
80.0	77.6	97.0	0.7
Storage at −60 °C to −80 °C for 396 days			
0.300	0.319	106.3	3.2
80.0	77.2	96.5	0.7
Storage in autosampler at room temperature for 101 h			
0.300	0.303	101.0	2.5
15.0	15.0	100.0	1.3
80.0	78.5	98.1	1.2

Table 5
Vismodegib percent unbound in human plasma samples at four concentrations.

Day	Percent unbound (mean ± SD)[a]			
	126 ng/mL	1260 ng/mL	10,500 ng/mL	31,500 ng/mL
Day 1	1.76 ± 0.31	1.77 ± 0.93	3.05 ± 0.55	5.20 ± 0.25
Day 2	1.60 ± 0.44	1.18 ± 0.08	2.87 ± 0.22	5.73 ± 0.12
Day 3	1.45 ± 0.08	1.49 ± 0.32	3.00 ± 0.51	5.04 ± 0.39
Overall	1.59 ± 0.32	1.48 ± 0.56	2.96 ± 0.39	5.30 ± 0.40
n	14	13	12	13

[a] Mean and SD exclude the outliers (greater than 3SD from the mean value for each concentration).

The percent unbound and the percent recovery were calculated using the following equations:

$$\%\text{Unbound} = 100 \times \left(\frac{C_u}{C_t}\right)$$

$$\%\text{Recovery} = 100 \times \frac{C_u \times V_{bi} + C_t \times V_{pi}}{C_i \times V_{pi}}$$

where C_u and C_t are unbound and total (bound and unbound) concentrations determined as the post-dialysis buffer and plasma concentrations, respectively. V_{pi} and V_{bi} represent the loading plasma and buffer volume, respectively. C_i represents the spiked plasma concentration at time zero. Since the use of RED device does not involve presoaking the membrane, a certain volume of plasma and buffer remains on the membrane or dialysis plate when collecting post-dialysis plasma or buffer samples. Therefore loading volume of the plasma and buffer was used for recovery calculation instead of post-dialysis plasma and buffer volume, which would have resulted in underestimated recovery.

To validate the process, the percent unbound of vismodegib and percent recovery of the RED system were evaluated on three separate days at four different human plasma concentration levels. The results are summarized in Tables 5 and 6.

Over the three day test period, percent unbound of vismodegib in human plasma at 126 ng/mL varied from 1.34% to 2.37% with one value over 5%. At 1260 ng/mL, the percent unbound varied from 1.06% to 3.14% with two values over 5%. At 10,500 ng/mL, the percent unbound varied from 2.48% to 3.56% with three values over 4.9%. At 31,500 ng/mL, the percent unbound values were relatively consistent varying from 4.67% to 5.82%. The extent of protein binding of vismodegib in human plasma was similar at the lower concentrations of 126 and 1260 ng/mL and decreased at higher concentrations. The percent recovery was over 95% except for two values, one at 77% and one at 88% (data not shown).

In some instances, a high variability was observed in percent unbound of vismodegib in replicates of plasma samples. Investigation of the single-use rapid equilibrium dialysis plates used for these experiments was performed. It was confirmed that the abnormally high percent unbound observed in a small number samples was due to leakage from the plasma chamber to the buffer chamber in the RED system. Based on the outlier exclusion criteria, any value outside of ±3 standard deviation (SD) from the overall mean determined after excluding the suspected outlier value was considered an outlier, a total of 8 outlier values out of 64 were excluded from further calculations (one at 126 ng/mL, two at 1260 ng/mL, three at 10,500 ng/mL and two at 31,500 ng/mL).

Based on the intra-day and inter-day validation results, acceptance criteria were established for subsequent *ex vivo* protein binding determinations for clinical study samples. The acceptance criteria for *ex vivo* protein binding determination using 126 ng/mL and 10,500 ng/mL as quality control plasma samples were as follows: For each RED plate, 2/3 of the quality control plasma samples must have a percent unbound within the range of 1.0–3.0% if the nominal concentration is 126 ng/mL, or within the range of 2.0–5.5% if the nominal concentration is 10,500 ng/mL. The percent unbound ranges of these two control samples did not represent the percent unbound range of the *ex vivo* protein binding samples. The percent unbound ranges of these two controls were used here to ensure the dialysis process was correct and reproducible in day-to-day operation.

3.9. Pharmacokinetics

Validation of the LC–MS/MS assay was performed over the calibration curve range 0.100–100 ng/mL, which was sufficient to detect unbound vismodegib plasma concentrations throughout the course of the 56-day study in healthy human volunteers ($n = 3$). Unbound vismodegib concentrations were calculated from the equation $y = ax + b$, by weighted ($1/x^2$) linear regression of the calibration line constructed from peak area ratios of vismodegib to internal standard versus nominal vismodegib concentration. Fig. 4 shows the unbound vismodegib concentration versus time profiles for each of the three healthy volunteers that were administered a single 150 mg oral dose. Unbound vismodegib plasma concentrations were below the limit of detection in only two of 66 samples (one each, subject 2 and 3) and ranged from 0.210 to 8.35 ng/mL, with C_{max} ranging from 5.27 to 8.35 ng/mL and C_{last} ranging from 0.250 to 0.410. Fraction unbound was low and consistent over the entire time course in all three subjects and on average, was similar to the fraction unbound reported after a single 150 mg dose of vismodegib to cancer patients [7]. Over the 56 day PK sampling period, the area under the concentration–time curve and terminal elimination rate constant were adequately determined with less than 6.00% of the AUC

Table 6
Protein binding recovery of vismodegib in plasma samples during the dialysis.

Day	Percent recovery (mean ± SD)[a]			
	126 ng/mL	1260 ng/mL	10,500 ng/mL	31,500 ng/mL
Day 1	103.8 ± 3.8	98.8 ± 3.0	103.8 ± 3.8	101.0 ± 1.2
Day 2	101.6 ± 1.8	98.2 ± 3.5	100.6 ± 2.7	98.7 ± 1.9
Day 3	103.4 ± 1.1	90.8 ± 8.5	103.6 ± 3.2	101.2 ± 1.1
Overall	102.9 ± 2.5	95.9 ± 6.4	102.7 ± 3.4	100.4 ± 1.7
n	15	15	15	13

[a] Mean and SD exclude the outliers (greater than 3SD from the mean value for each concentration).

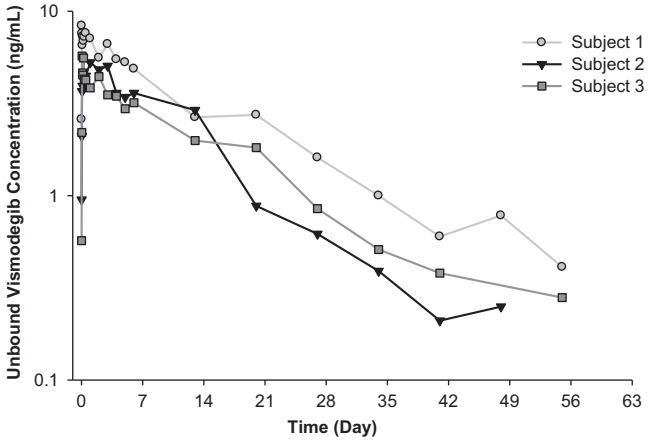

Fig. 4. Individual unbound concentration versus time profiles for 3 healthy volunteers following a single 150 mg oral dose of vismodegib in a Phase I clinical trial.

extrapolated from T_{last} to infinity. Mean (SD) pharmacokinetic parameters for the three subjects were: C_{max} = 6.44 (1.67) ng/mL, AUC_{0-last} = 2160 (632) ng/mL h, AUC_{0-inf} = 2290 (681) ng/mL h, fraction unbound = 0.00285 (0.000400), and $t_{1/2}$ = 283 (48.4) h.

4. Conclusions

A rapid equilibrium dialysis assay was developed and validated for measurement of unbound vismodegib in human plasma. The dialysis assay using small plasma volumes was proven to be reproducible in day-to-day operation. A SPE LC–MS/MS assay for the determination of unbound concentration of vismodegib in human plasma was also developed and validated. This validated assay was proven to be rugged, accurate and sensitive and it was applied successfully determine the contribution of plasma protein binding to the nonlinear PK of vismodegib. The assay has subsequently been utilized for the analysis of human plasma samples generated from multiple clinical studies.

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