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Quantitative analysis of PD 0332991 in xenograft mouse tumor tissue by a 96-well supported liquid extraction format and liquid chromatography/mass spectrometry

Leslie Nguyen, Wei-Zhu Zhong, Cory L. Painter, Cathy Zhang, Sadayappan V. Rahavendran, Zhongzhou Shen*

Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global Research & Development, 10724 Science Center Drive, San Diego, CA 92121, United States

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ABSTRACT

Phase II attrition of clinical candidates in the drug development cycle is currently a major issue facing the pharmaceutical industry. To decrease phase II attrition, there is an increased emphasis on validation of mechanism of action, development of efficacy models and measurement of drug levels at the site of action. PD 0332991, a highly specific inhibitor of cyclin-dependent kinase 4 (CDK-4) is currently in clinical development for the treatment of solid tumor. A clinical presurgical study will be required to better understand how PD 0332991 affects signaling pathways and how the intratumoral concentration of PD 0332991 correlates with plasma PK parameters and molecular alterations in breast cancer tissues after PD 0332991 treatment. Before conducting such a clinical study, it is important to evaluate PD 0332991 levels in tumor tissue samples from a xenograft mouse model for the determination of drug exposure at the site of action. Therefore, the objectives of this study were (1) to develop and validate a sensitive LC–MS/MS method to quantify PD 0332991 in mouse tumor tissues from MDA-MB-231-Luc human breast tumor xenografts in SCID-beige mice; (2) to quantify PD 0332991 levels in mouse tumor tissues after oral administration of PD 0332991 at 10 and 100 mg/kg using the validated LC–MS/MS method. Both liquid–liquid extraction (LLE) and supported liquid extraction (SLE) in a 96-well format were developed and evaluated to achieve optimal extraction recovery with minimal matrix effects. The newly developed SLE method is more efficient (speed and ease) and demonstrates comparable recovery (93.1–100% at three different concentrations) compared to the traditional LLE method. The validated LC–MS/MS for PD 032291 in mouse tumor tissue homogenate method exhibited a linear dynamic range of 0.1–100 ng/mL with inter-day accuracy and precision within 15%. The validated method was successfully applied to measure PD 0332991 levels in tumor tissues in MDA-MB-231-Luc human breast tumor xenografts in SCID beige mice. The mean tumor concentrations at 6 h post-oral PD 0332991 administration at 10 and 100 mg/kg were 1793 (±1008) and 25,163 (±3959) ng/g, respectively.

1. Introduction

Cyclin-dependent kinases (CDKs) are members of a family of serine-threonine protein kinase responsible for regulation of the eukaryotic cell cycle [1,2]. Inhibition of CDKs associated with cell cycle regulation is reported to provide an effective approach to the control of tumor growth [3,4]. PD 0332991 is currently in clinical development for the treatment of solid tumors and is a selective inhibitor of CDK-4 that targets specific oncogenic abnormalities in cancer [5,6]. It is well known that the use of preclinical studies in animals to derive predictive pharmacokinetic (PK)–pharmacodynamic (PD) models can aid in the prediction of human PK–PD relationships. Plasma concentrations are commonly used as a surrogate for drug concentrations in target tissues for PK–PD modeling [7]. Although the relationship between plasma concentrations and pharmacological effects has been successfully used in PK–PD modeling for many drugs, it is generally considered that pharmacological effects are related to the drug concentration in target tissues [8–10]. In certain cases, drug failure in cancer patients are attributed to development of drug resistance as a result of inadequate levels of drug in the tumor [11–13]. A clinical presurgical study will be required to better understand how PD 0332991 affects signaling pathways and how the intratumoral concentration of PD 0332991 correlates with plasma PK parameters and molecular alterations in breast cancer tissues after PD 0332991 treatment. Before conducting such a clinical study, it is important to evaluate PD 0332991 levels in tumor tissue samples from a xenograft mouse model for the determination of drug expo-

* Corresponding author. Tel.: +1 858 622 7935; fax: +1 858 622 5999.
E-mail address: Zhongzhou.Shen@pfizer.com (Z. Shen).

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sure at the site of action. Therefore, the objectives of this study were to develop and validate an LC–MS/MS method to quantify PD 0332991 in mice tumor tissues (obtained from MDA-MB-231-Luc human breast tumor xenografts in SCID-beige mice and quantify PD 0332991 levels in the xenograft mouse tumor tissues after a 10 or 100 mg/kg oral dose of PD 0332991 using the validated LC–MS/MS method.

Previous studies for the determination of drug concentration in mouse tissue were reported using liquid chromatography–tandem mass spectrometry (LC/MS) after tissue homogenization. Most of these reports used a one step protein precipitation with organic solvent such as acetonitrile or methanol [14–16]. Although protein precipitation is one of the simplest sample clean-up procedures employed in bioanalysis, there exists significant endogenous components such as phospholipids remaining in the samples that may result in matrix effects. In addition, rapid deterioration of chromatographic resolution and shortened analytical column lifetime and poorer figures of analytical merit may be observed. Liquid–liquid extraction (LLE) procedures were also reported for studies conducted in mouse tissue [17,18]. One of these studies concluded that there were no significant matrix effects observed between protein precipitation and LLE because a large dilution of the final protein precipitation sample extract was used for comparison [18]. Although LLE provides a cleaner sample extract, it is often difficult to automate successfully and may result in emulsion formation. Two-step extraction procedures were also utilized in certain studies [19]. A recent study reported using online solid phase extraction (SPE) for biological tissue samples. In this study, a protein precipitation procedure was initially applied to extract the tissue homogenates followed by injection of the supernatant into the online-SPE-LC–MS/MS system [20]. Extrelut columns are designed as solid phase replacements for traditional liquid–liquid extractions and contain specially processed wide-pore diatomaceous earth (chemically inert matrix). These columns have been used for toxicological analysis of drugs and poisons in organ tissues, blood and food products [21,22]. However, the Extrelut column is not amenable as a higher throughput sample clean up procedure since it is not available in a 96-well plate format. The newly developed supported liquid extraction (SLE) 96-well plate contains a similar modified form of diatomaceous earth (chemical and physical properties may not be exactly the same as the Extrelut column), is suitable for automation and serves as an alternative to traditional LLE. This technique has previously been reported primarily for extraction of plasma based samples [23–25].

In this study, a Bessman tissue pulverizer was used to produce the best uniform homogenate for the mouse tumor tissues. Two simple extraction methods, LLE and SLE in a 96-well plate format were evaluated. The systematic evaluation of pH effect, extraction recovery, matrix effect, and carry-over during method development are described below. In addition assay sensitivity, linearity, precision, and accuracy of PD 0332991 in mouse tumor homogenates were assessed during method validation and reported as well. This study represents the first report of drug quantification in mouse tumor tissue using a 96-well plate SLE and LC–MS/MS.

2. Experiment

2.1. Chemicals, reagents, and materials

PD 332991 and the stable isotopically labeled internal standard (IS) were synthesized at Pfizer Global Research & Development. HPLC-grade water and all analytical organic solvents were purchased from EDM Chemicals, Inc. (Gibbstown, NJ, USA). Potassium bicarbonate and potassium hydroxide buffers were obtained from Sigma–Aldrich (St. Louis, MO, USA) and the pH adjusted with 1 N hydrochloric acid (HCl) solution (from Sigma–Aldrich) (refer to sample preparation section). 96-deep well collection plates, vials and tubes were purchased from VWR Scientific Products (West Chester, PA, USA). The Bessman tissue pulverizer was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA), and Isolute SLE+ supported liquid extraction plates (200 mg) were purchased from Biotage (Charlottesville, VA, USA).

2.2. Animal studies and sample collection

All animal husbandry and experimental procedures conducted in this study complied with the Guide for Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and were approved by Pfizer Global Research & Development Institutional Animal Care and Use Committee. SCID-beige female mice, 7–8 weeks, weighing 20–24 g were obtained from Charles River Laboratories (Sacramento, CA, USA) and acclimated for a 48-h period prior to use. The stable luciferase-expressing MDA-MB-231-Luc human breast cancer cell line (Caliper Life Sciences, Hopkinton, MA, USA), was selected for its robust tumor quality. Tumor cells (3×10^6) were suspended in 150 μL phosphate buffer saline (PBS) with 50% (v/v) Matrigel (BD Biosciences, Cambridge, MA, USA) and implanted subcutaneously into the right flank. Mice were randomized into three groups with the same mean tumor volume of 350 mm^3. Tumor volume was calculated using the formula $0.5 \times (\text{length} \times \text{width}^2)$.

PD 0332991 was administered via oral gavage at 10 and 100 mg/kg/day for 3 days prior to tumor collection. Tumors were harvested 6 h following final administration, and immediately transferred to a cryo-vial and frozen with liquid nitrogen after the wet weight was recorded.

2.3. Tumor tissue homogenization

Tumor samples were individually ground to a fine powder with a Bessman tissue pulverizer and cooled on dry ice. The powder was immediately transferred to a sample vial and vortex mixed with 4 volumes of HPLC grade water to obtain a homogenate ready for subsequent extraction. Tumor tissue homogenates were stored at −70 °C prior to sample extraction and LC–MS/MS analysis.

2.4. Sample preparation—LLE and SLE

All tumor samples were homogenized. The final concentration of PD 0332991 was determined in the tumor by normalization based upon the weight of mouse tumor tissue collected. Stock solutions of PD 0332991 (1 mg/mL) were prepared in acetonitrile. The IS stock solution was also prepared in acetonitrile. Two separate weighings of PD 0332991 were used to prepare stock solutions for standard curve and quality control (QC) samples. All stock solutions were stored at −20 °C. Standard curve samples were prepared by spiking 5 μL of PD 0332991 into 100 μL of pooled mouse tissue homogenate (pooled after confirming there were no interference peaks from each individual mouse tumor homogenate). The standard curves were prepared in triplicate and consisted of eight concentrations at 0.100, 0.250, 0.500, 2.50, 5.00, 12.5, 50.0, and 100 ng/mL. The QC samples were prepared at three concentrations (0.30, 10, and 75 ng/mL).

100 μL of mouse tumor homogenate samples, along with standards and QCs, were aliquoted into 1.2 mL mesh tubes in a 96-well plate for the LLE procedure. A 10 μL aliquot of IS working solution (60 ng/mL) was added to all samples except double blanks, followed by addition of 100 μL of potassium bicarbonate buffer (pH 10 or as indicated). PD 0332991 was extracted with 0.4 mL methyl tert-butyl ether (MTBE) following vortex mixing and centrifugation at 4 °C (3000 × *g* for 30 min). The organic layer was transferred to clean 0.65 mL mesh tubes in a 96-well plate and evaporated to dryness

at room temperature under a gentle stream of nitrogen gas with a SPE DRY-96 evaporator (Jones Chromatography, Lakewood, CO, USA). The samples were reconstituted in 100 μL of methanol, centrifuged for 5 min, and 10 μL aliquots of the reconstituted solution were injected onto the LC column for mass spectral analysis.

For the SLE procedure, 100 μL mouse tumor homogenate samples, along with standards and QCs, were aliquoted into 0.65 mL mesh tubes into a 96-well plate. A 10 μL aliquot of IS working solution (60 ng/mL) was added to all samples except double blanks, followed by adding 100 μL of potassium bicarbonate buffer (pH 10 or as indicated). Samples were vortex mixed for approximately 5 min, and 200 μL of the aqueous mixture was then loaded onto a Isolute SLE+ (200 mg) plate. A slight vacuum was applied so that samples would only penetrate approximately three quarters of the bed height of the SLE cartridge. After samples were allowed to soak for 5 min, a total volume of 1.2 mL of MTBE (300 μL × 4 times) was applied to the cartridge and eluted by gravity flow. The collection tubes containing the organic eluant were placed in a SPE DRY-96 (Jones Chromatography Lakewood, CO, USA) and evaporated to dryness at room temperature under a gentle stream of nitrogen The samples were reconstituted in 100 μL of acetonitrile:water 75:25 (v/v), and 10 μL aliquots of the reconstituted solution were injected onto an LC/MS/MS for analyses.

2.5. LC–MS/MS

A Shimadzu LC system, equipped with a binary solvent delivery system LC-10 ADvp and a controller SLC-10Avp (Shimadzu, USA) were used. Chromatographic separation was performed using a 2.0 mm ID × 50 mm Polaris C8-A (5 μm particle size) column at a 0.6 mL/min flow rate. Mobile phase A contained water with 0.1% formic acid, and mobile phase B contained acetonitrile with 0.1% formic acid. A gradient elution program was used. The initial solvent composition was held at 5% B for 0.4 min and then changed linearly to 99% B in 1.8 min and held at 99% B for an additional 2.7 min. The solvent composition was then returned in a single step to the initial conditions for re-equilibration over an additional 1.5 min. The total run time was 6.4 min. The retention time for PD 0332991 and IS were approximately 1.2 min.

Quantitation was achieved using an MDS Sciex API 4000 triple quadrupole mass spectrometer (Toronto, Canada) equipped with a turbo ion source. The instrument was operated in the positive ESI mode using multiple reaction monitoring (MRM). The turbo-ion voltage was set to 4.5 kV and the auxiliary gas temperature was maintained at 525 °C. High purity nitrogen was used for GAS 1, GAS 2, curtain, and collision gases. The mass resolution was set to a peak width of 1 mass unit at half-height for both Q1 and Q3. The electron multiplier was set at 2100 V. Declustering potential (96 V), collision energy (37 V), entrance potential (10 V), and collision cell exit potential (12 V) were set as indicated. The dwell time of each MRM transition was 150 ms. PD 0332991 and the IS were monitored using specific precursor ion → product ion transitions of m/z 448 → 380 and m/z 451 → 383, respectively.

2.6. Calculations

MDS Sciex Analyst software (v. 1.4.1) was used for data acquisition and chromatographic peak integration. The peak area ratios of PD 0332991 and its stable isotopically labeled internal standard were plotted as a function of the nominal concentrations of the analytes. The standard calibration curve was constructed using weighted ($1/x^2$) linear regression. PD 0332991 concentrations in the mouse tumor homogenate samples were calculated from the constructed calibration curve and final tumor concentration was normalized to each individual mouse tumor weight and reported as ng/g.

2.7. Assay characterization and validation

The precision and accuracy of the method were evaluated using QC samples at three concentrations (low, medium, and high) in three replicates on three separate days of analyses. The precision and accuracy were expressed as the coefficient of variation (CV) and percentage of bias from the nominal concentrations of the QC samples, respectively. Dilution integrity was assessed by preparation of PD 0332991 at 200 and 2000 ng/mL concentrations in control tumor homogenate samples. A 100-fold dilution of the dilution integrity QC samples with control blank tumor homogenate was then performed prior to extraction.

The extraction recovery of PD 0332991 at 10 ng/mL was optimized by adjusting the buffer pH (adjusted by titrating potassium bicarbonate buffer with 1 N HCl solution). After pH optimization, extraction recovery for PD 0332991 was evaluated at three different concentrations (0.3, 10 and 75 ng/mL). The extraction recovery was determined in triplicate by comparing the mean chromatographic peak area of spiked-before-extraction samples with those of the corresponding spiked-after-extraction standards. To determine the bench-top stability of PD 0332991, three sets of QC samples (0.3, 10 and 75 ng/mL) in mouse tissue homogenate from the low, medium, and high range of the calibration curve were prepared in triplicate and left at room temperature for 3 h. The samples were then processed and analyzed against freshly prepared standard curves. The concentrations determined for the QC samples after storage were compared to their theoretical values. The freeze/thaw stability (3 cycles) was investigated in a similar manner. The freeze/thaw QC samples were analyzed after 3 cycles of frozen and thawed at room temperature using freshly prepared standard curves. The stability of PD 0332991 in mouse homogenate extracts was also determined. The extracted QC samples were kept in the 96-well injection plate over a 24-h period at 15 °C and then analyzed with freshly prepared calibration curves. In addition, the long-term stability of PD 0332991 was evaluated by re-analyzing the PD 0332991 in mouse tumor homogenates stored at −70 °C over a 5-month period.

3. Results and discussion

3.1. Chromatography and MS/MS detection

Positive turbo ion spray was chosen as the ionization technique. Mass transitions of m/z 448 → 380 for PD 0332991 and m/z 451 → 383 for IS were selected to monitor these analytes and the resulting product ion mass spectra for PD 0332991 and IS are shown in Fig. 1.

A Polaris C8, 5 μm, 2.0 × 50 mm (Varian, Palo Alto, CA, USA) with mobile phases A and B as water with 0.1% formic acid and acetonitrile with 0.1% formic acid, provided optimal results in terms of peak shape and sensitivity with acceptable retention time compared to other columns (data not shown).

3.2. Sample processing, extraction recovery, and matrix effects

There are several basic cleanup procedures, protein precipitation, SPE, LLE, and recently developed SLE, to achieve a rugged assay for biological samples. We initially developed a LLE procedure for PD 332991 from the mouse tumor homogenates. Based on the compound structure the pK_a values for PD 332991 were determined to be around 4 and 7. The extraction recovery for PD 0332991 was therefore evaluated at different extraction pH levels ranging between 8 and 13. The mean extraction recoveries for PD 0332991 were >93% from pH 9–11, while the extraction recovery decreased to 56% at pH 7 (data not shown). Therefore, the pH was adjusted to 10 prior to extraction with MTBE. Since the newly developed SLE could be further employed in a more automated

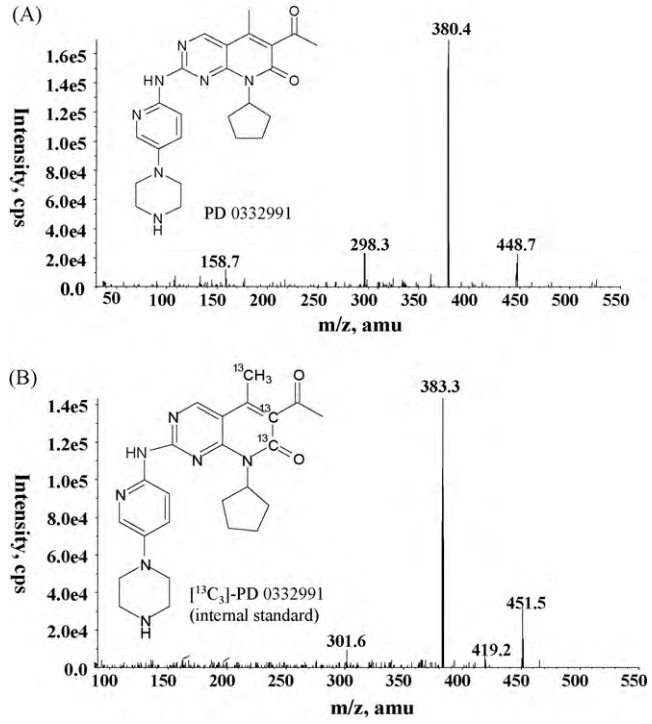

Fig. 1. MS/MS product ion mass spectra of PD 0332991 (A) and [$^{3}C_{3}$]-PD 0332991, the internal standard (IS) (B).

fashion, a simple procedure using the SLE plate was developed. With dilution of the homogenate no clogging was observed when applying the tumor homogenate onto the SLE plate. In addition, instead of adding 1.2 mL of organic solution (MTBE) to the cartridge at once for the elution, 0.3 mL of MTBE was eluted four times to maximize the extraction recovery. The mean extraction recoveries for PD 0332991 were 93.1–95.1% for SLE and 93.9–100% for LLE at 0.50, 7.5 and 75 ng/mL ($n = 3$ at each concentration level). Both the LLE and SLE procedures offer comparable extraction recoveries. The SLE procedure however, eliminates steps such as vortex mixing, centrifugation, and transferring organic solution and preventing emulsion formation. Therefore, compared to the traditional LLE method, the SLE procedure is easier, and faster, while

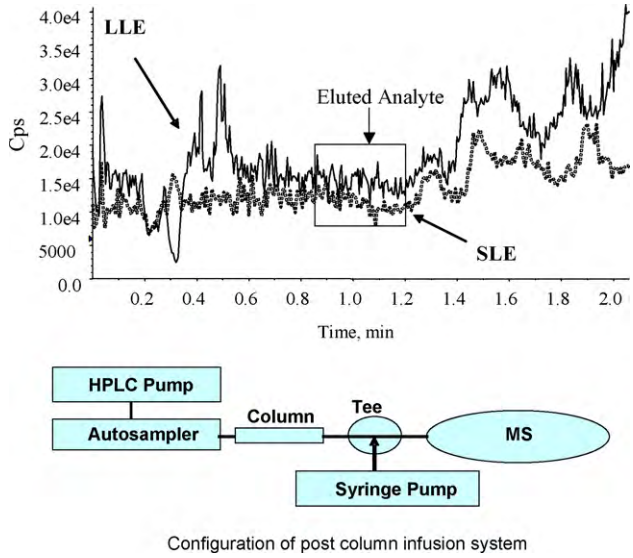

Fig. 2. Evaluation of matrix effect by T-infusing PD 0332991 in blank mouse tumor tissue extract utilization both LLE and SLE procedure. Configuration of post infusion system is included.

offering equivalent recoveries. Matrix enhancement/suppression of ionization was assessed by comparing the absolute peak areas of post-spiked extracted tumor homogenate analyte standards to those of corresponding neat standards. A post-column infusion experiment was also performed to further evaluate the matrix enhancement/suppression for sample extracts obtained from both the LLE and SLE procedures. As shown in Fig. 2, at the retention time of the eluted analyte, neither ion enhancement nor ion suppression was observed by both procedures. In addition, the average peak areas between post-extraction spiked tumor homogenate standards and the corresponding neat standards were very close (less than 6%). These results confirmed a lack of matrix effects for extracts from both the SLE and LLE methods.

3.3. Assay selectivity, sensitivity, linearity, precision, and accuracy

The assay selectivity was assessed using 6 individual blank mouse tumor homogenates. The chromatographic conditions

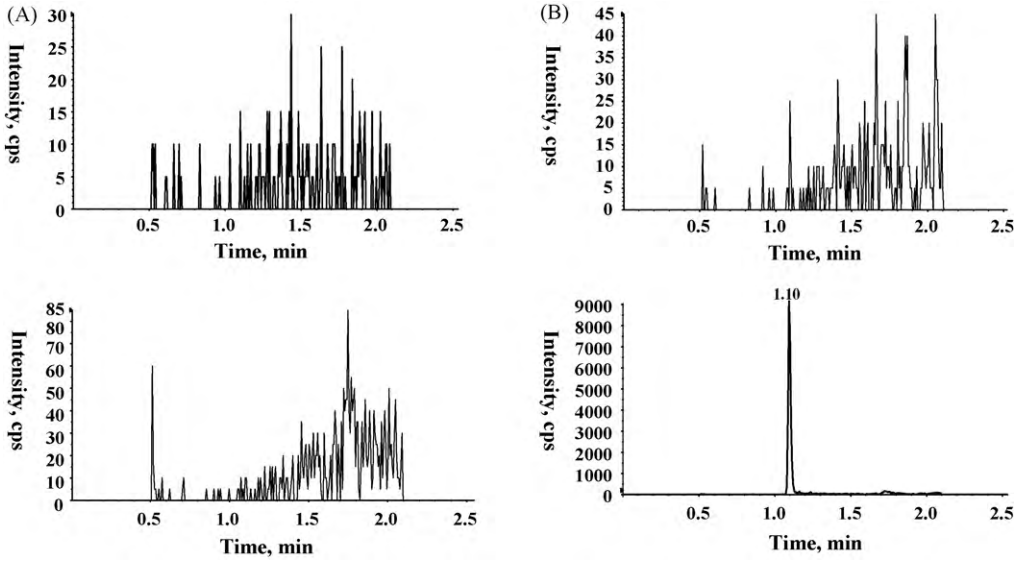

Fig. 3. Representative LC–MS/MS ion chromatograms of a blank plasma sample (A) and a blank plasma sample spiked with 6 ng/mL IS (B). Top panel is for PD 0332991 and bottom panel is for IS.

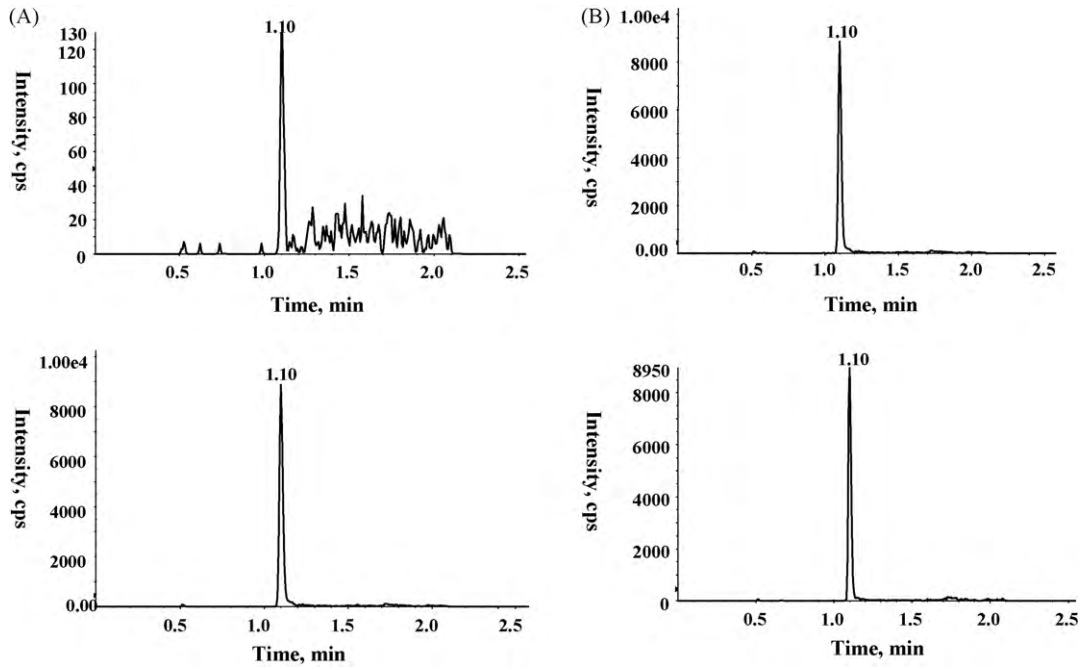

Fig. 4. Representative LC–MS/MS ion chromatogram of PD 0332991 at LLOQ (0.1 ng/mL) (A) or ULOQ at 100 ng/mL PD 0332991 (B). Top panel is for PD 0332991 and bottom panel is for IS.

reported were found to be selective for PD 0332991 and IS. As shown in Fig. 3, there was no interference in the blank tumor tissue homogenate chromatograms from endogenous substances or from the IS contributing to the PD 0332991 MRM channel. To evaluate the assay sensitivity, LLOQ ($n = 6$) was established at 0.1 ng/mL based upon a signal-to-noise (S/N) ratio (based upon peak to peak) of 19.1 ± 2.06. The linearity of the standard curve was assessed based on a plot of the peak area ratio of PD 0332991 to IS versus drug concentration. A $1/x^2$ weighted linear regression model was used. Calibration curves were found to be linear over a concentration range of 0.1–100 ng/mL for PD 0332991, with correlation coefficients greater than 0.99. Fig. 4 illustrates representative ion chromatograms for the LLOQ (0.1 ng/mL) and ULOQ (100 ng/mL) of the calibration curve. Fig. 5 shows the representative ion chromatographs of PD 0332991 from a 6 h post-dose mouse tumor homogenate samples after an oral dose of 10 mg/kg. A strong wash solvent (acetone:isopropanol:acetonitrile, 20:40:40, v/v/v) coupled with an increased wash/equilibrium cycle (described in the LC–MS/MS section), ensured that no significant carry-over (less than 20% of LLOQ) was observed (data not shown).

Validation of the intra- and inter-day accuracy and precision of the method over 3 days of analysis was assessed. Calibration curves were prepared daily in triplicate for PD 0332991. In addition, three replicates at each of three QC concentration levels were analyzed on each validation day. Table 1 summarizes the validation data for accuracy and precision of each calibration standard concentration of PD 0332991. Table 2 presents the inter- and intra-assay precision for each of the QC samples. The intra- and inter-day accuracy and precision (% CV) acceptance criteria for each QC and calibration standard was ≤15% (≤20% for standard at the LLOQ of 0.1 ng/mL) and the validated method met these criteria. In order to measure tumor concentration levels higher than the upper limit of the standard curve, dilution integrity samples were assessed in triplicate. These samples were analyzed following 1:100 dilutions with control tumor homogenates. The mean calculated concentrations were within 111% of nominal concentration, with %CV values within ±5.03%, demonstrating that the dilution process met the acceptance criteria (Table 2).

Table 1
Intra- and inter-day accuracy and precision of standards of PD 0332991 in mouse tumor homogenates.

Nominal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy (%)	CV (%)
Intra-day ($n = 3$)			
0.100	0.098	98.5	8.2
0.250	0.260	104	6.7
0.500	0.503	101	6.9
2.50	2.33	93.4	2.4
5.00	4.96	99.2	1.8
12.5	12.5	101	5.6
50.0	52.4	105	2.3
100	98.7	98.7	7.4
Inter-day ($n = 9$)			
0.100	0.104	103	12.0
0.250	0.238	95.0	9.3
0.500	0.449	89.9	2.1
2.50	2.65	106	0.7
5.00	5.16	103	3.4
12.5	13.4	107	3.3
50.0	51.6	103	5.3
100	91.7	91.7	5.5

Table 2
Intra- and inter-day accuracy and precision of qc samples of PD 0332991 in mouse tumor homogenates.

	Nominal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy (%)	CV (%)
Intra-day ($n = 3$)	0.300	0.317	106	8.2
	10.0	9.43	94.3	4.7
	75.0	76.9	103	3.7
Inter-day ($n = 9$)	0.300	0.271	90.4	10.4
	10.0	10.7	107	5.3
	75.0	69.9	93.1	6.6
Dilution QC ($n = 3$)	200	222	111	2.0
100-fold dilution	2000	2180	109	5.3

Table 3
Assessment of stability of PD 0332991 in mouse tumor homogenates.

	Nominal concentration (ng/mL)					
	0.3 ng/mL		10.0 ng/mL		75.0 ng/mL	
	Mean conc. ($n = 3$)	Accuracy (%)	Mean conc. ($n = 3$)	Accuracy (%)	Mean conc. ($n = 3$)	Accuracy (%)
Bench-top room temperature (3 h)	0.323 ± 0.021	108	10.1 ± 0.3	101	73.1 ± 0.7	97.4
Freeze–thaw (3 cycles)	0.338 ± 0.042	113	9.21 ± 0.66	92.1	73.7 ± 1.5	98.3
Auto sampler 15 °C (24 h)	0.311 ± 0.032	104	10.8 ± 0.4	108	72.9 ± 2.3	97.2

Results are presented as mean ± standard deviation.

3.4. Stability

The bench-top stability was investigated to determine whether the samples were adequately stable under these conditions over a sufficient period of time to cover the sample preparation process. The resulting data as shown in Table 3 indicated that PD 0332991 was stable in the mouse tumor tissue homogenate for at least 3 h at room temperature. Freeze–thaw stability was evaluated after three cycles of freezing and thawing of three sets of mouse tumor homogenate QC samples at low, mid, and high concentrations. The data listed in Table 3 demonstrated that PD 0332991 was stable after three freeze–thaw cycles. Extracts of PD 0332991 reconstituted in acetonitrile:water (75:25, v/v) were also found to be stable in the autosampler for at least 24 h at 15 °C prior to injection onto the LC/MS/MS system. In addition, long-term stability was evaluated by re-extracting and re-analyzing aliquots of the same study samples after the mouse tumor homogenates was stored at −70 °C for a period of 5 months. Similar PD 0332991 concentrations in mouse tumor homogenates were obtained (<15% from the original sample analysis, data now shown) suggesting that PD 0332991 was stable in mouse tumor homogenates for at least 5 months when stored at −70 °C.

3.5. Determination of PD 0332991 concentrations in mouse tumor

The validated method was successfully applied to determine PD 0332991 concentrations in mouse tumor in a MDA-MB-231-Luc human breast xenograft model. The determined PD 0332991 tumor concentrations from this experiment are shown in Table 4. After normalizing the weight of mouse tumors, the mean tumor concentrations of PD 0332991 at 6 h post-dose at 10 and 100 mg/kg were 1793 and 25,163 ng/g, respectively. The tumor concentrations of PD 0332991 increased dose-proportionally by 10-fold as the dose was increased from 10 to 100 mg/kg. Compared to the plasma concentrations from a previous mouse PK–PD study at similar doses (data not shown), tumor concentrations of PD 0332991 appeared to be higher than plasma concentrations at these two tested doses, suggesting that this compound was well distributed to tumor tissues in mice based on the assumption that extent of protein binding was equivalent in both plasma and tumor.

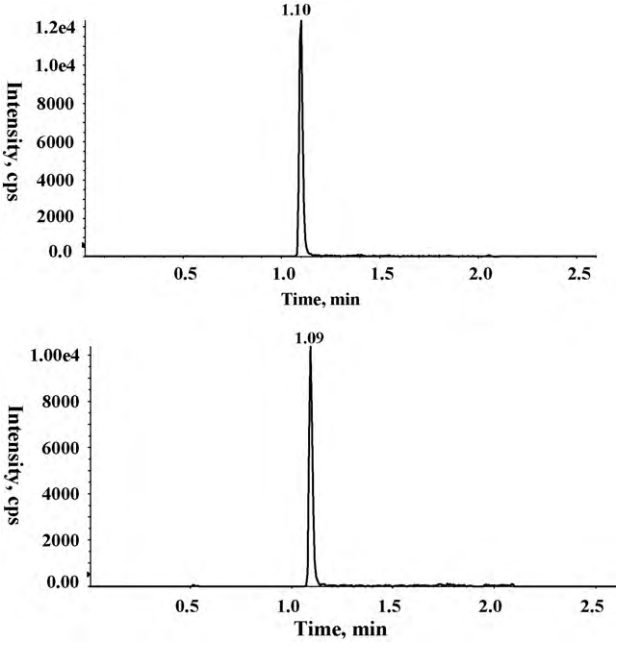

Fig. 5. Representative LC–MS/MS ion chromatogram of PD 0332991 from a 6 h post-dose mouse tumor homogenate samples after the oral dose of 10 mg/kg. Top panel is for PD 0332991 and bottom panel is for IS.

Table 4
PD 0332991 concentration in mouse tumor at 6 h.[a].

Dose (mg/kg)	Concentration (ng/g)					
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mean	SD
10	3230	1145	1045	1750	1793	1008
100	25, 350	27,250	28,500	19,550	25,163	3959

[a] Mouse tumor concentration was normalized by mouse tumor weight.

4. Conclusions

We have successfully developed and validated an assay for measuring PD 0332991 in mouse tumor using SLE in a 96-well format as the extraction procedure. Compared to traditional LLE, this SLE procedure is simpler, easier, and faster, while offering good extraction recovery. The validated method exhibited good intra- and inter-day accuracy and precision over a dynamic range of 0.1–100 ng/mL for PD 0332991 in the mouse tumor homogenate. This method has been successfully applied to quantify tumor concentrations of PD 0332991 in mice in a MDA-MB-231-Luc human breast xenograft model. The currently developed assay for mouse tumor tissues could be easily adopted and validated to support future clinical studies. The use of SLE in a 96-well format as the sample extraction procedure provides the potential for this method to be fully automated.

References

[1] A.J. Obaya, J.M. Sedivy, Regulation of cyclin-CDK activity in mammalian cells, Cell. Mol. Life Sci. 59 (2002) 126–142.
[2] D.O. Morgan, R.P. Fisher, F.H. Espinoza, Control of eukaryotic cell cycle progression by phosphorylation of cyclin-dependent kinases, Cancer J. Sci. Am. 4 (1998) S77–S83.
[3] T.M. Sielecki, J.F. Boylan, P.A. Benfield, G.L. Trainor, Cyclin-dependent kinase inhibitors: useful targets in cell cycle regulation, J. Med. Chem. 43 (2000) 1–18.
[4] M.D. Garrett, A. Fattaey, CDK inhibition and cancer therapy, Curr. Opin. Genet. Dev. J. Med. Chem. 9 (1999) 104–111.
[5] R.S. Finn, J. Dering, D. Conklin, O. Kalous, D.J. Cohen, A.J. Desai, C. Ginther, M. Atefi, I. Chen, C. Fowst, G. Los, D.J. Slamon, PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro, Breast Cancer Res. 11 (2009) R77, http://breast-cancer-research.com/content/pdf/bcr2419.pdf.
[6] D.W. Fry, P.J. Harvey, P.R. Keller, W.L. Elliott, M. Meade, E. Trachet, M. Albassam, X. Zheng, W.R. Leopold, N.K. Pryer, P.L. Toogood, Specific inhibition of

cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts, Mol. Cancer Ther. 3 (2004) 1427–1438.
[7] E. Masson, W.C. Zamboni, Pharmacokinetic optimisation of cancer chemotherapy. Effect on outcomes, Clin. Pharmacokinet. 32 (1997) 324–343.
[8] J.H. Lin, Tissue distribution and pharmacodynamics: a complicated relationship, Curr. Drug Metab. 7 (2006) 39–65.
[9] H.G. Eichler, M. Mueller, Drug distribution. The forgotten relative in clinical pharmacokinetics, Clin. Pharmacokinet. 34 (1998) 95–99.
[10] W.E. Stumpf, Memo to the FDA and ICH: appeal for in vivo drug target identification and target pharmacokinetics. Recommendations for improved procedures and requirements, Drug Discov. Today 12 (2007) 594–598.
[11] R.K. Jain, Delivery of molecular and cellular medicine to solid tumors, Adv. Drug Deliv. Rev. 46 (2001) 149–168.
[12] O. Langer, M. Mueller, Methods to assess tissue-specific distribution and metabolism of drugs, Curr. Drug Metab. 5 (2004) 463–481.
[13] C.M. Galmarini, F.C. Galmarini, Multidrug resistance in cancer therapy: role of the microenvironment, Curr. Opin. Investig. Drugs 4 (2003) 1416–1421.
[14] M. Zhao, C. Hartke, A. Jimeno, J. Li, P. He, Y. Zabelina, M. Hidalgo, S.D. Baker, Specific method for determination of gefitinib in human plasma, mouse plasma and tissues using high performance liquid chromatography coupled to tandem mass spectrometry, J. Chromatogr. B 819 (2005) 73–80.
[15] R.E. Savage, T. Hall, K. Bresciano, J. Bailey, M. Starace, T.C.K. Chan, Development and validation of a liquid chromatography-tandem mass spectrometry method for the determination of ARQ 501 (β-lapachone) in plasma and tumors from nu/nu mouse xenografts, J. Chromatogr. B 872 (2008) 148–153.
[16] K. Patel, D. Lweiston, Y. Gu, K.O. Hicks, W.R. Wilson, Analysis of the hypoxia-activated dinitrobenzamide mustard phosphate pre-prodrug PR-104 and its alcohol metabolite PR-104A in plasma and tissues by liquid chromatography-mass spectrometry, J. Chromatogr. B 856 (2007) 302–311.
[17] D. Otaegui, A. Rodriguez-Gascon, A. Zubia, F.P. Cossio, J.L. Pedraz, Development and validation of a liquid chromatography-tandem mass spectrometry for the determination of Kendine 91, a novel histone deacetylase inhibitor, in mice plasma and tissues: application to a pharmacokinetic study, J. Chromatogr. B 870 (2008) 109–116.
[18] J.A. Zirrolli, E.L. Bradshaw, M.E. Long, D.L. Gustafson, Rapid and sensitive LC/MS/MS analysis of the novel tyrosine kinase inhibitor ZD6474 in mouse plasma and tissues, J. Pharm. Biomed. Anal. Pharmacol. 39 (2005) 705–711.
[19] M.M. Warnke, E. Wanigasekara, S.S. Singhal, J. Singhal, S. Awasthi, D.W. Armstrong, The determination of glutathione-4-hydroxynonenal (GSHNE), E-4-hydroxynonenal (HNE), and E-1-hydroxynon-2-en-4-one (HNO) in mouse liver tissue by LC-ESI-MS, Anal. Bioanal. Chem. 392 (2008) 1325–1333.
[20] C. Magnes, M. Suppan, T.R. Pieber, T. Moustafa, M. Trauner, G. Haemmerle, F.M. Sinner, Validated comprehensive analytical method for quantification of coenzyme A activated compounds in biological tissues by online solid-phase extraction LC/MS/MS, Anal. Chem. 80 (2008) 5736–5742.
[21] S. Ito, K. Kudo, T. Imamura, T. Suzuki, N. Ikeda, Sensitive determination of methomyl in blood using gas chromatography–mass spectrometry as its oxime tert.-butyldimethylsilyl derivative, J. Chromatogr. B 713 (1998) 323–330.
[22] A. Kaufmann, P. Butcher, Quantitative liquid chromatography/tandem mass spectrometry determination of chloramphenicol residues in food suing sub-2 μm particulate high-performance liquid chromatography columns for sensitivity and speed, Rapid Commun. Mass Spectrom. 19 (2005) 3694–3700.
[23] L. Williams, M. Cleeve, S. Merriman, H. Lodder, S. Jordan, R. Calverley, J. Smith, The isolation of three β-blockers from human plasma using Isolute SLE+ supported liquid extraction 96-well plate, LC GC Europe (2007) 26–27.
[24] G. O'Maille, S.M. Pai, X. Tao, G.T. Douglas Jr., R.G. Jenkins, An improved LC-ESI-MS-MS method for simultaneous quantitation of rosiglitazone and N-desmethyl rosiglitazone in human plasma, J. Pharm. Biomed. Anal. 48 (2008) 934–939.
[25] H. Jiang, X. Jiang, Q.C. Ji, Enantioselective determination of alprenolol in human plasma by liquid chromatography with tandem mass spectrometry using cellubiohydrolase chiral stationary phases, J. Chromatogr. B 872 (2008) 121–127.