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Short communication

Determination of PF-04928473 in human plasma using liquid chromatography with tandem mass spectrometry☆

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ABSTRACT

A simple, rapid and sensitive liquid chromatography/tandem mass spectrometric (LC/MS/MS) analytical method was developed for quantification of Hsp90 inhibitor PF-04928473 in human plasma, following administration of its prodrug, PF-04929113. Sample processing involved protein precipitation by addition of 0.4 mL of methanol containing internal standard (PF-04972487) to 50 μL volume of plasma sample. Chromatographic separation of PF-04928473 and PF-04972487 was achieved on a Phenomenex® Luna C18(2) (2.0mm × 50 mm, 5 μm) column using a gradient elution method with mobile phase solvents: methanol containing 0.1% formic acid and 0.1% formic acid at a flow rate of 0.25 mL/min. Detection was performed in electrospray positive ionization mode, monitoring the ion transitions from m/z 465.1 → 350.1 (PF-04928473) and m/z 447.0 → 329.1 (PF-04972487). The retention times for PF-04928473 and PF-04972487 were 1.86 and 2.85 min, respectively. Calibration curves were generated in the range of 2–2000 ng/mL. The accuracy and precision ranged from 94.1 to 99.0% and 86.7 to 97.6%, respectively, which were calculated using quality control samples of three different concentrations analyzed in quintuplicate on four different days.

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1. Introduction

PF-04928473, also reported as SNX-2112, is a representative compound for a novel, small molecule class of inhibitors of the molecular chaperone heat shock protein 90 (Hsp90) [1]. It is delivered via its prodrug PF-04929113 (also reported as SNX-5422), which is rapidly absorbed and converted into PF-04928473 after oral administration, Fig. 1(a) [1,2].

PF-04928473 selectively binds to the NH_2-terminal of the ATP binding site of Hsp90 [1,2]. Hsp90 allows proper refolding of proteins that have been destabilized, as well as conformational maturation of a set of proteins involved in intracellular signaling [3], including growth factor receptors (EGFR, PDGFR, Her2), steroid hormone receptors, kinases (Raf-1, Src etc), transcription factors, and cell cycle regulators [4]. Disruption of Hsp90 results in degradation of these proteins, leading to inhibition of key signaling pathways and inhibition of tumor cell proliferation [1,2]. PF-04928473 has been shown to have activity against various cancers, *in vitro* and *in vivo* [2,5,6]. Three Phase I clinical trials of PF-04929113 are currently enrolling patients with refractory hematologic and solid tumor malignancies. To characterize the clinical pharmacokinetics of PF-04928473, a selective, reproducible, and accurate quantification method was necessary. Here, we describe the first analytical method for determination of PF-04928473 concentrations in human plasma in range of 2–2000 ng/mL. The reported method is rapid and sensitive, and is based on a simple protein precipitation approach.

2. Methods

2.1. Materials and reagents

PF-04928473 (Mol. Formula: $C_{23}H_{27}F_3N_4O_3$, Purity: 99.2% by weight) and internal standard PF-04972487 (also reported as SNX-2988, Mol. Formula: $C_{23}H_{25}F_3N_4O_2$, Purity: 95.8% by weight, see

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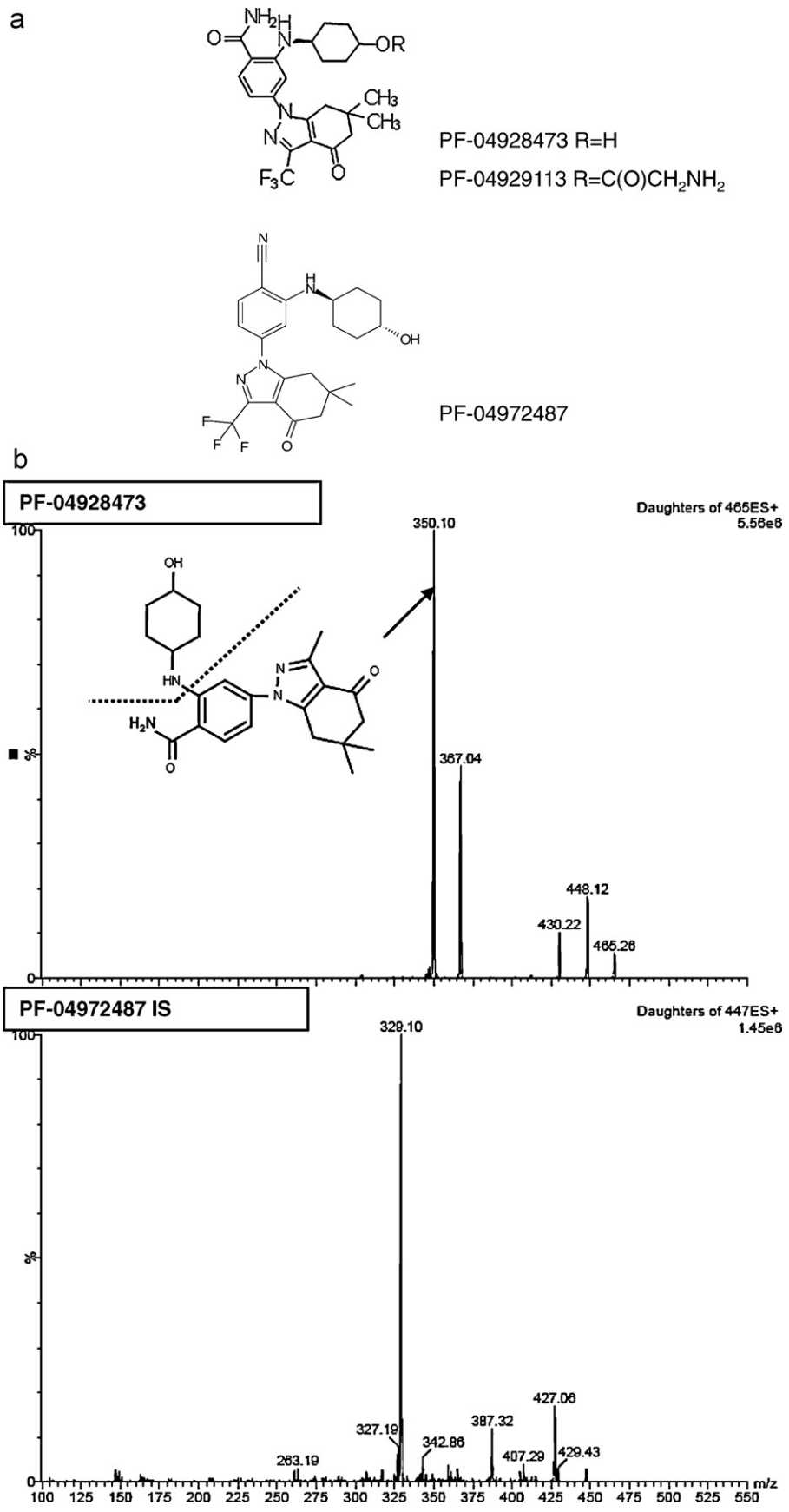

Fig. 1. (a) Chemical structures of PF-04929113 (pro-drug), PF-04928473 (active molecule), and PF-04972487 (internal standard). (b). Full scan product ion spectra of PF-04928473 and PF-04972487.

Fig. 1(a)) were provided by Serenex, Inc. (Durham, NC, USA). Methanol (Optima grade) and formic acid (purity ≥98%) were purchased from Fisher Scientific and Sigma–Aldrich, respectively. Deionized water was produced with a hydro-reverse osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Millipore, Billerica, MA, USA). Drug-free heparinized human plasma was obtained from the Clinical Center Blood Bank at the National Institutes of Health (Bethesda, MD, USA).

2.2. Stock solutions and standards

The primary stock solution of PF-04928473 was prepared at concentration of 100 μg/mL in methanol and stored at −20 °C. Serial dilutions were performed to prepare the secondary working solutions for calibration and quality control (QC) samples. Internal standard (IS) stock solution for PF-04972487 was prepared at concentration of 100 μg/mL in methanol and stored at −20 °C. The working IS solution was freshly prepared on each day of analysis by diluting the IS stock to 20 ng/mL concentration with methanol. QC samples were prepared in batch, by addition of working stocks to plasma for final concentrations of 7.5, 150 and 1500 ng/mL. The 300 μL aliquots of these QCs were stored at −20 °C, which were subdivided into five 50 μL aliquots on the day of analysis.

2.3. Sample preparation

The standard calibrators were prepared by spiking 5 μL of appropriate PF-04928473 working solutions to 45 μL of blank human plasma in disposable glass centrifuge tubes (Kimble, Vineland, NJ). The QC samples and patient samples were thawed at room temperature, vortex mixed, and a 50 μL volume was transferred to glass centrifuge tubes. To these, a 400 μL volume of working internal standard solution (methanol containing IS) was added as a precipitation agent. These tubes were then vortex mixed for 30 s, followed by centrifugation at 1200 × *g* for 10 min. A 120 μL volume of clear supernatant was then mixed with 40 μL of deionized water in culture glass tubes and transferred to glass vials, of which 10 μL was injected onto the column.

2.4. HPLC-mass spectrometry apparatus and conditions

A Waters® Acquity UPLC system was used for chromatographic separation using a Phenomenex® Luna C18 (2) column (2.0mm × 50 mm, 5 μm). The autosampler and column were maintained at 4 and 35 °C, respectively. Samples were eluted using a gradient mobile phase at a flow rate of 0.25 mL/min. Mobile phases A and B were 0.1% formic acid and methanol with 0.1% formic acid, respectively. The gradient run increased linearly from 40% A and 60% B to 16.7% A and 83.3% B over 3.5 min, immediately followed by original conditions until the end of the 5.5 min run time to re-equilibrate the column to initial conditions.

The UPLC was attached to a Waters® Micromass Quattro micro API triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in positive mode. The MS analysis was carried out in multiple reaction monitoring (MRM) mode by monitoring the ion transitions from *m*/*z* 465.1 → 350.1 for PF-04928473 and *m*/*z* 447.0 → 329.1 for IS. The MS/MS conditions were as follows: capillary voltage, 3.5 kV; cone voltage, 30.0 V; source temperature, 130 °C; desolvation temperature, 410 °C; desolvation gas flow, 600 L/h; cone gas flow, 100 L/h, and collision energy, 23 eV. The instrumentation control and data acquisition were managed by MassLynx software (Waters®).

2.5. Validation procedures

Validation was performed on 4 different days following the guidelines published elsewhere [7]. On each day of analysis, two sets of calibration standards were prepared freshly at 2, 6, 12, 50, 200, 500, 1000, and 2000 ng/mL concentrations. One 300 μL aliquot for each QC concentration (7.5, 150 and 1500 ng/mL) was also thawed and sub-divided into five 50 μL volumes on each day of analysis. In addition, validation runs included two blank and two IS only samples.

The lower limit of detection (LOD) was the concentration for which the signal:noise ratio was at least three, across the retention window of PF-04928473. The lower limit of quantitation (LLOQ) for the assay was the lowest concentration at which PF-04928473 spiked in six different lots of plasma could be measured with accuracy greater than 80%. The selectivity was assessed by analyzing the blank plasma samples from six different sources. Five replicates were run for each QC concentration, every day, for four days. The accuracy was calculated as the percent deviation of mean predicted QC concentrations from the nominal concentrations. The between- and within-run precisions were determined by one-way analysis of variance (ANOVA) using measured QC concentrations [8]. The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$\mathrm{BRP} = \left(\frac{\sqrt{\left((\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n \right)}}{\mathrm{GM}} \right) \times 100$$

where $\mathrm{MS_{bet}}$ and $\mathrm{MS_{wit}}$ are between- and within-run mean square variances, respectively, which were obtained from ANOVA; *n* was the number of replicate observations within each run, and GM was the grand mean.For each QC concentration, the within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = \left(\frac{\sqrt{(\mathrm{MS_{wit}})}}{\mathrm{GM}} \right) \times 100$$

Sample dilution was validated with QC samples of concentration 10,000 ng/mL, which were diluted ten-fold with blank human plasma and processed as other QC samples. The recovery was determined as response ratio (i.e., response of analyte/IS) measured in pre-extraction spiked samples as a percentage of that measured from post-extraction spiked samples [9]. The matrix effect was determined as response ratio in post-extraction spiked samples as a percentage of response ratio observed in pure solvent [9].

2.6. Data evaluation

Data analysis was performed with QuanLynx application, a component of MassLynx. Calibration curves were generated by least-squares linear regression analysis of the peak area ratio of PF-04928473 and IS against the nominal drug concentration using an appropriate weighting factor. The regression line was not forced through the origin (0, 0). The calibration curve was then used to calculate the drug concentrations in QCs and unknown samples by using the calibration equation. Concentrations falling outside the range of calibration curve cannot be estimated by using this equation.

3. Results and discussion

3.1. Optimization of UPLC/MS parameters

The MS parameters were optimized in electrospray positive ionization mode. The steps involved were: (a) Identification of

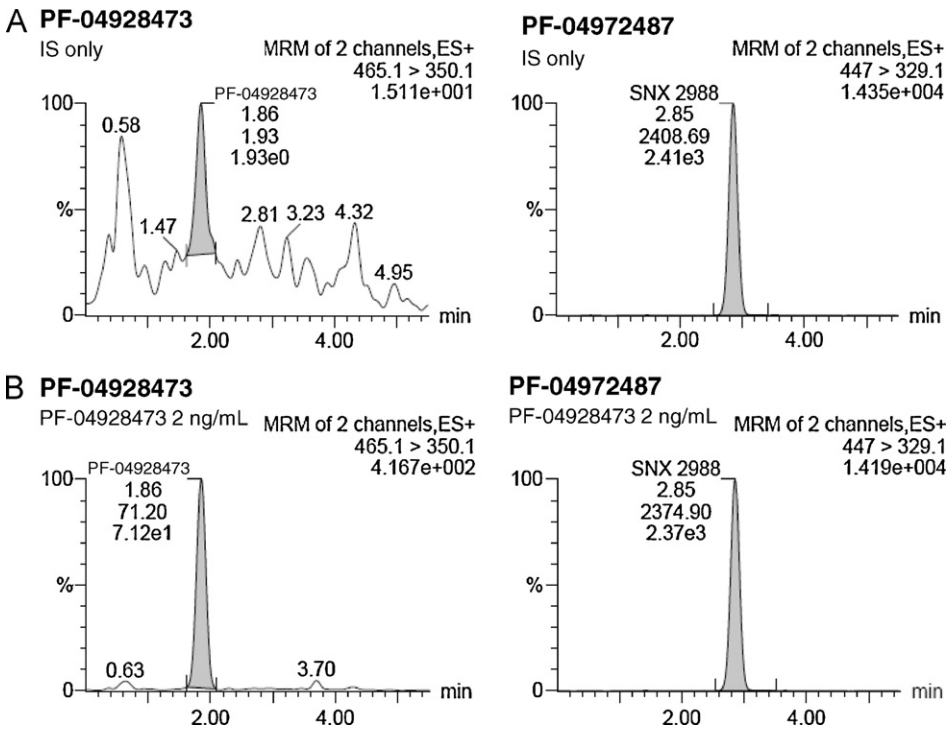

Fig. 2. Typical chromatograms showing the m/z 465.1 → 350.1 (PF- 04928473) and m/z 447.0 → 329.1 (PF-04972487, IS) transitions for (A) a blank human plasma sample with IS, and (B) an LLOQ (2 ng/mL) calibrator sample with IS.

parent ions for PF-04928473 and PF-04972487 in MS scan mode, determined based on maximum signal, by syringe infusion of drug solutions, (b) Tuning of MS parameters to maximize the signal for PF-04928473, the compound of interest, in MS scans. This included the optimization of cone voltage, capillary voltage, extractor voltage, desolvation temperature, desolvation gas flow, cone gas flow, resolutions, ion energies, and entrance and exit widths, (c) Identification of daughter ion for PF-04928473 by fixing the results of steps (a) and (b), i.e., m/z value for parent ion and MS parameters, and by only adjusting the collision energy to the largest signal for daughter ion in daughter ion scan mode, (d) selection of daughter ion for internal standard PF-04972487, by keeping all the MS parameters same as for PF-04928473, except cone voltage and collision energy, which are determined separately for PF-04972487. The daughter ion scans for PF-04928473 and PF-04972487 are shown in Fig. 1(b).

LC parameters were initially optimized on Waters Alliance 2695 HPLC. Isocratic and gradient methods were tested for selection of optimum LC separation conditions. Protein precipitation method was optimized for extraction of drug from plasma samples. The optimum method on Waters Alliance System was based on a gradient separation with a run time of 13 min. Mobile phase A and B were 0.1% formic acid and methanol with 0.1% formic acid, respectively. The gradient increased linearly from 50% A and 50% B to 10% A and 90% B over 6 min, and was held at that level up to 8 min. Immediately following that, it was brought to the original conditions of 50%A and 50%B until the end of the 13 min run time, to re-equilibrate the column to initial conditions. The peak shapes after simple protein precipitation were spread out and asymmetric. To 3 parts of supernatant, 1 part of water was added, which improved the peak shapes to a narrow and symmetric one. It was thought that addition of water would increase the polarity of sample, resulting in narrow width of elution band in lipophillic C-18 column and a narrower chromatographic peak. Parallel to selection of mobile phases and extraction method, injection volume, needle wash and seal wash solvents were also optimized.

Switching to Acquity UPLC system, which has a significantly smaller system volume, while maintaining the gradient change rate the same, enabled us to decrease run time, including re-equilibration, to 5.5 min, a significant decrease from the initial 13 min run time.

3.2. Selectivity

No interference was observable across the retention windows for PF-04928473 and IS in blank plasma samples from six different lots of human plasma. The chromatograms for IS only and LLOQ samples are shown in Fig. 2. For all blank samples, peak area was less than 5% of the area for LLOQ.

3.3. Calibration curve

For the PF-04928473 concentration range of 2–2000 ng/mL, the ratio of PF-04928473 to IS peak areas was fit against the nominal concentrations with a quadratic equation employing a weighting factor of $1/x^2$, where x is the nominal concentration. The mean correlation coefficient for regression equations from four days was: 0.9969 (SD: ±0.0005; range 0.9964–0.9976). The intercept for these quadratic equations was approximately close to zero with negative or positive values of 0.0006 to 0.0023. The % deviations for calibrators from nominal concentrations were less than 2% (Supplementary Table 1). This suggests that weighted quadratic equation was adequate in describing the relationship between response ratios and nominal concentrations.

3.4. Limits of detection and quantitation

The LOD, based on estimated peak-to-peak noise, was determined to be 0.2 ng/mL. The LOQ was estimated as 2 ng/mL. For six 2 ng/mL samples prepared using plasma from different sources, percent deviation of mean measured concentration from nominal was 5% and the percent relative standard deviation (RSD) was 3%.

Table 1
Intra-run and inter-run accuracy and precision from quality control samples.

	PF- 04928473 nominal concentration (ng/mL)			
	7.5 (N = 20)	150 (N = 20)	1500 (N = 20)	10,000 (dilution 10×) (N = 15)
Mean	7.4	141.1	1476.4	10380
RSD (%)	5.6	5.8	5.0	5.8
DEV (%)	−1.0	−5.9	−1.6	3.9
Imprecision				
Within-run	2.7	2.9	2.4	2.7
Between-run	12.4	13.3	11.2	11.2

RSD: relative standard deviation, DEV: percent deviation from nominal value.

Table 2
Freeze–thaw and autosampler stability.

Nominal concentration (ng/mL)	Freeze–thaw stability				Autosampler stability		
	Calculated QC concentration, (ng/mL) (percent with respect to cycle zero concentration)				Calculated QC concentration, (ng/mL) (percent with respect to zero hr concentration)		
	Fresh	After 1 cycle[a]	After 2 cycles[a]	After 3 cycles[a]	Zero hour	24th hour	% Change
7.5	7.1 (100)	7.8 (109)	7.5 (105)	7.7 (108)	8.0	7.8 (97.5)	−2.5
150	135.1 (100)	146.1 (108)	147.2 (109)	145.4 (108)	152.2	146.1 (99.2)	−0.8
1500	1398.9 (100)	1519.9 (109)	1521.4 (109)	1522.9 (109)	1565.1	1519.9 (99.0)	1.0

[a] Number of freeze–thaw cycles.

The mean deviation for 2 ng/mL calibrators from nominal concentration was 0.0% (RSD, 7.1%, Supplementary Table 1). Thus, 2 ng/mL concentration exceeds the limits of accuracy and precision, and concentrations above it can be quantified with acceptable accuracy and precision.

3.5. Accuracy and precision

The % deviation of mean calculated QC concentrations from nominal concentrations ranged from −1 to −6% (Table 1) at three concentrations of 7.5, 150 and 1500 ng/mL, which were well within the acceptable limits of ±15%, suggesting that assay method accurately estimated PF-04928473 plasma concentrations. The between-run imprecision ranged from 11.2 to 13.3% and within-run imprecision was between 2.4 and 3%, which were also within the acceptable limits of ±15% and indicates that assay predictions were precise. The inaccuracy and between- and within-run imprecisions for dilution analysis were 3.9%, 11.2% and 2.7%, respectively, all below the acceptable limits of ±15%. This confirms that samples with concentrations above the upper limit of quantification can be analyzed using ten-fold dilution.

3.6. Recovery and matrix effect

Recovery and matrix effects were checked at three concentrations, 6, 200 and 2000 ng/mL. Recovery was determined by comparison of response ratio for the samples with pre-extraction and post-extraction addition of PF-04928473. Recovery ranged from 99% to 113% for studied concentrations (Supplementary Table 2). Matrix effect was calculated by comparison of response ratio for sample with post-extraction addition of PF-04928473 to that of response for PF-04928473 spiked in water. Matrix effect was less than 11% for the studied concentrations (Supplementary Table 2).

3.7. Freeze–thaw stability

Three sets of QC samples at were subjected to one, two and three freeze–and-thaw cycles, respectively. Each freeze cycle lasted for more than 15 h. All these samples were analyzed on the same day along with freshly prepared QC samples. The % change in measured concentrations after each freeze–thaw cycles was less than 10% (Table 2), indicating that repeated exposure to freeze–thaw cycles does not degrade PF-04928473 in human plasma through at least three cycles.

3.8. Autosampler, short-term and long-term stability

An entire set of samples was left in autosampler at 4 °C for 24 h after initial run, and was subsequently reanalyzed. Comparison of measured concentrations after 24 h with initial concentrations showed less than 3% deviation (Table 2). This suggests that when necessary, samples can be reanalyzed the following day without compromising on the measurements, if stored properly.

The short-term or “bench-top” stability of PF-04928473 working stock solutions were assessed at three concentrations: 6, 200 and 2000 ng/mL. Three samples at each concentration were left on bench-top at room temperature and a set of similar samples was stored at −20 °C. After 10 h these samples were analyzed in the same run. The % change at studied concentrations was less than 2%, suggesting that integrity of PF-04928473 in methanol solvent was not affected for at least 10 h when stored at room temperature. For long-term stability, the QC samples stored at −20 °C for 51 days were compared with freshly prepared QCs. The percentage deviation at 7.5, 150 and 1500 ng/mL concentrations was less than 6%, suggesting that samples integrity is not compromised when stored at −20 °C for at least 51 days.

4. Application

The validated method was used to determine the plasma concentrations of PF-04928473 in a phase I dose escalation study, enrolling patients with refractory solid tumor malignancies and lymphomas. In this trial, blood samples were collected up to 48 h, in 7 mL sodium heparin tubes, and plasma was separated by centrifugation for 5 min at 1200 × g and 4 °C. The plasma samples were stored at −80 °C prior to analysis. A typical plasma concentration–time profile for a patient receiving 33 mg/m^2 PF-04929113 is shown in Fig. 3.

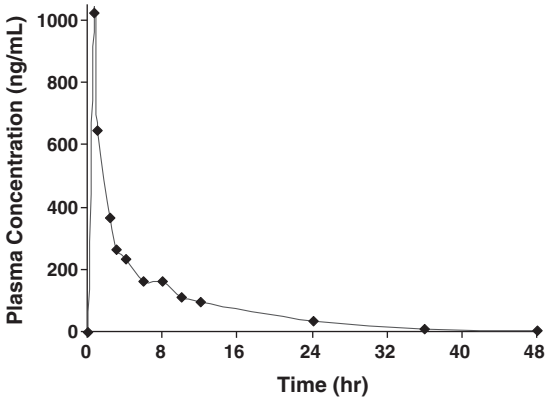

Fig. 3. Typical plasma concentration–time profile for PF- 04928473 from a patient with cancer following treated with a single oral dose of 33 mg/m^2 PF-04929113.

5. Conclusion

In conclusion, a novel bioanalysis method was developed and validated for determination of PF-04928473 in human plasma using tandem mass spectrometric detection. The described method is simple, rapid, selective and sensitive and can be easily implemented into routine practice. The method gives accurate and precise measurements of PF-04928473 plasma concentrations with a small plasma volume of 50 μL and in a short run time of 5.5 min. The results for validation parameters such as recovery, matrix effect and stability following freeze–thaw cycles, at room temperature and after long-term were within the acceptable limits. This method is currently being used for analysis of plasma samples from a dose escalation trial in patients with solid tumors and lymphomas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.09.017.

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