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Quantitative analysis of PD 0332991 in mouse plasma using automated micro-sample processing and microbore liquid chromatography coupled with tandem mass spectrometry

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ABSTRACT

In the oncology therapeutic area, the mouse is the primary animal model used for efficacy studies. Often with mouse pharmacokinetic (PK) and pharmacokinetic/pharmacodynamic (PK/PD) studies, less than 20 μL of total plasma sample volume is available for bioanalysis due to the small size of the animal and the need to split samples for other measurements such as biomarker analyses. The need to conduct automated "small volume" sample processing for quantitative bioanalysis has therefore increased. An automated fit for purpose protein precipitation (PPT) method using a Hamilton MicroLab Star (Reno, NV, USA) to support mouse PK and PK/PD studies for an oncology drug candidate PD 0332991, (a specific inhibitor of cyclin-dependent kinase 4 (CDK-4) currently in development) for processing "small volumes" was developed. The automated PPT method was achieved by extracting and processing 10 μL out of a minimum sample volume of 15 μL plasma utilizing the Hamilton MicroLab Star. A 96-conical shallow well plate by Agilent Technologies, Inc (Wilmington, DE, USA) was the labware of choice used in the automated Hamilton "small volume" method platform. Analyses of a 10 μL plasma aliquot from 15 μL of plasma study samples were conducted by both automated and manual PPT method. All plasma samples were quantitated using a Sciex API 4000 triple quadrupole mass spectrometer coupled with an Eksigent Express HT Ultra HPLC system. The chromatography was achieved using an Agilent microbore C_{18} Extend, 1.0 × 50 mm, 3.5 μm column at a flow rate of 0.150 mL/min with a total run time of 1.8 min. Accuracy and precision of standard and QC concentration levels were within 90–107% and <14%, respectively. Calibration curves were linear over the dynamic range of 1.0–1000 ng/mL. PK studies for PD 0332991 were conducted in female C3H mice following intravenous administration at 1 mg/kg and oral administration at 2 mg/kg. PK values such as area under curve (AUC), volume of distribution (Vd), clearance (Cl), half life ($T_{1/2}$) and bioavailability (*F*%) demonstrated less than 11% difference between the automated Hamilton and manual PPT methods. The results demonstrate that the automated Hamilton PPT method can accurately and precisely aliquot 10 μL of plasma from 15 μL or larger volume plasma samples. The fit for purpose Hamilton PPT method is suitable for routine analyses of plasma samples from micro-sampling PK and PK/PD samples to support discovery studies.

1. Introduction

In an attempt to reduce the attrition of development compounds, there is an increased emphasis and focus on target selection and validation to address efficacy earlier in drug discovery [1,2]. This has resulted in bioanalytical groups analyzing an increasing number of pharmacokinetic (PK) and pharmacokinetic/pharmacodynamic (PK/PD) samples, with the increased use of automated liquid handling robots to improve efficiency and throughput [3]. Liquid handling robots have been used for many years in the pharmaceutical industry for sample transfer and preparation at various stages of the drug discovery and development process. The 8-channel multiprobe design and/or the capability to perform direct liquid transfers utilizing a 96 core head platform are best used to minimize repetitive labor associated with manual sample preparation while ensuring robust data. A number of liquid handling systems from vendors such as Tecan, Beckman Coulter, Tomtec and Hamilton are available and are in use by various *in vivo* bioanalytical groups across Drug Metabolism and Pharmacokinetic (DMPK) groups throughout the industry [4–6]. The Hamilton MicroLab Star robot is a liquid handler used to support sample transfer and processing of biological matrices such

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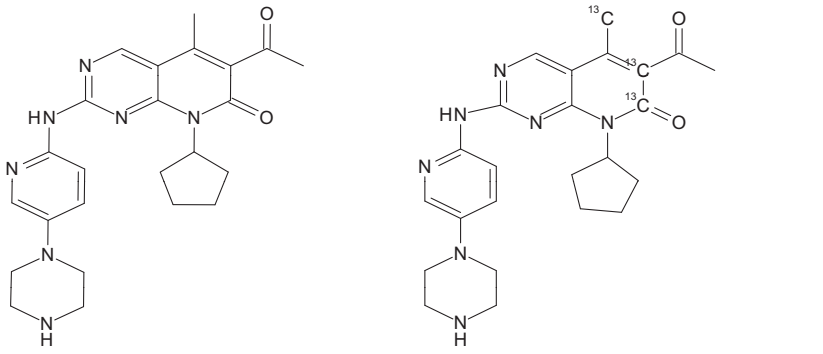

Fig. 1. Chemical structure of PD 0332991 and its isotopically labeled internal standard.

as plasma, serum, urine, tissue homogenates and cerebral spinal fluid (CSF) obtained from *in vivo* PK and PK/PD studies. It has been used successfully to handle multiple biological matrices along with incorporating different sample cleanup methods such as protein precipitation (PPT), liquid–liquid extraction (LLE), solid-phase extraction (SPE) and supported liquid extraction (SLE) followed by analyses using LC–MS/MS [7–11].

Microbore LC–MS/MS using a 0.5–1 mm id column has been shown to successfully support quantitative bioanalysis in *in vivo* PK studies [12]. The advantage of microbore LC–MS/MS includes shorter sample analyses time, reduced sample volume requirement, less matrix effect and reduced solvent usage, and comparable sensitivity to a conventional LC–MS/MS when using 10 fold less injection volume [13,14]. Microbore LC–MS/MS also provides new opportunities for designing bioanalytical studies with limited volumes. Often with mouse PK and PK/PD analysis and specifically with serial bleed sampling workflows [15,16], less than 20 μL of total plasma sample volume is collected for quantitative bioanalysis. The objective of this paper was to develop a sensitive and robust automated PPT method coupled with microbore LC–MS/MS that enables analyses of 10 μL aliquots of plasma samples from a minimum study sample volume (15 μL). PD 0332991, a cyclin-dependent kinase 4 (CDK-4) inhibitor [17,18], currently in clinical development for the treatment of solid tumors, was used as a model compound for this study.

2. Experimental

2.1. Chemicals and reagent

PD 0332991 and its stable isotopically labeled internal standard (IS) were synthesized at Pfizer Global Research & Development (Fig. 1). HPLC-grade water and formic acid were purchased from EDM Chemicals, Inc. (Gibbstown, NJ, USA). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Mouse control plasma with sodium EDTA as anti-coagulant was obtained from Bioreclamation, Inc. (Liverpool, NY, USA). A 96-conical shallow well plate (0.15 mL) was purchased from Agilent Technologies, Inc. (Wilmington, DE, USA). Other labware such as deep 96-well collection plates, vials and tubes were purchased from VWR Scientific Products (West Chester, PA, USA).

2.2. Automated liquid handling system

The Hamilton Microlab Star workstation (Reno, NV, USA) was equipped with the following: a 96 core pipetting head, an 8-channel pipettor head, a vacuum manifold, an internal swivel arm plate (iSWAP), CO-RE grippers, a five-plate shaker and an optional autoload/bar-code reader. The Hamilton Microlab Star workstation was operated using a Microlab Vector Software version 4.1.

2.3. In vivo animal studies and sample collections

All animal husbandry and in-life procedures conducted in this study complied with the Animal Welfare Acts Regulations (9 CFR parts 1–3) and the Guide for Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and approved by Pfizer's Institutional Animal Care and Use Committee (IACUC). C3H female mice that weighed between 20 and 24 g were purchased from Charles River Laboratories (Sacramento, CA, USA).

PD 0332991 was dosed to female C3H mice at 1 mg/kg intravenously and 2 mg/kg orally in water. To obtain adequate study sample volumes to be used for both the Hamilton PPT and manual methods, non-serial blood collection for the mouse PK study was employed. Blood was collected into sodium EDTA-containing tubes at predetermined intervals. Plasma samples were obtained after immediate centrifugation of blood at 4 °C and were stored at −80 °C until analyses.

2.4. Preparation of calibrators, quality control and sample extractions

Calibration standards were prepared in mouse plasma by serial dilution of PD 0332991 from a 100 μg/mL acetonitrile stock solution to achieve a calibration curve in plasma between 1 ng/mL and 250 ng/mL using the Hamilton robot. The quality control (QC) samples were prepared using the Hamilton robot at four concentration levels (3, 20, 200, and 2000 ng/mL). In addition, manual-QC samples were prepared at 200 and 2000 ng/mL concentrations by manually spiking PD 322991 in mouse plasma. Approximately 15 μL aliquots ($n = 12$ replicates) of these manually prepared QCs were transferred into a 96-conical shallow well plate to test the accuracy of the Hamilton to aspirate 10 μL aliquots.

Analyses of mouse PK samples using 10 μL plasma sample aliquots were conducted in the following manner. For the Hamilton PPT method, 15 μL of the plasma samples were thawed and transferred into a 96-conical shallow well plate and 10 μL aliquots were processed by PPT using the Hamilton. Similarly, another 15 μL sample aliquot volumes were also used for the manual PPT and compared in parallel. A 50 μL volume of acetonitrile containing internal standard (20 ng/mL) was added to all tubes except the double blank, where acetonitrile was added, to precipitate plasma proteins. Samples were vortexed and centrifuged at 3000 rpm for 10 min. A 30 μL aliquot of supernatant was transferred to clean tubes and vortex mixed with 30 μL water containing 0.1% formic acid, which ensured consistent chromatography

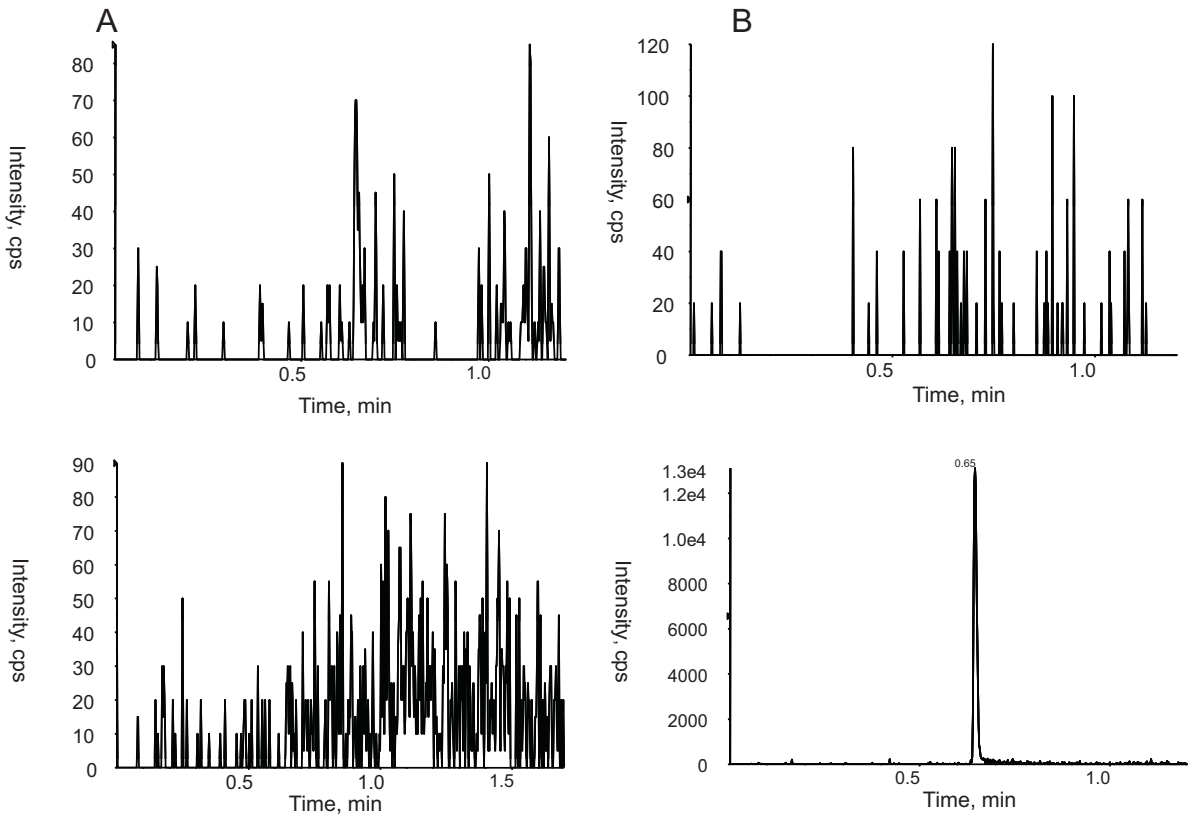

Fig. 2. Representative LC–MS/MS ion chromatograms of a blank plasma sample (A) and a blank plasma sample spiked with 20 ng/mL IS (B). Top panel is for PD 0332991 and bottom panel is for IS.

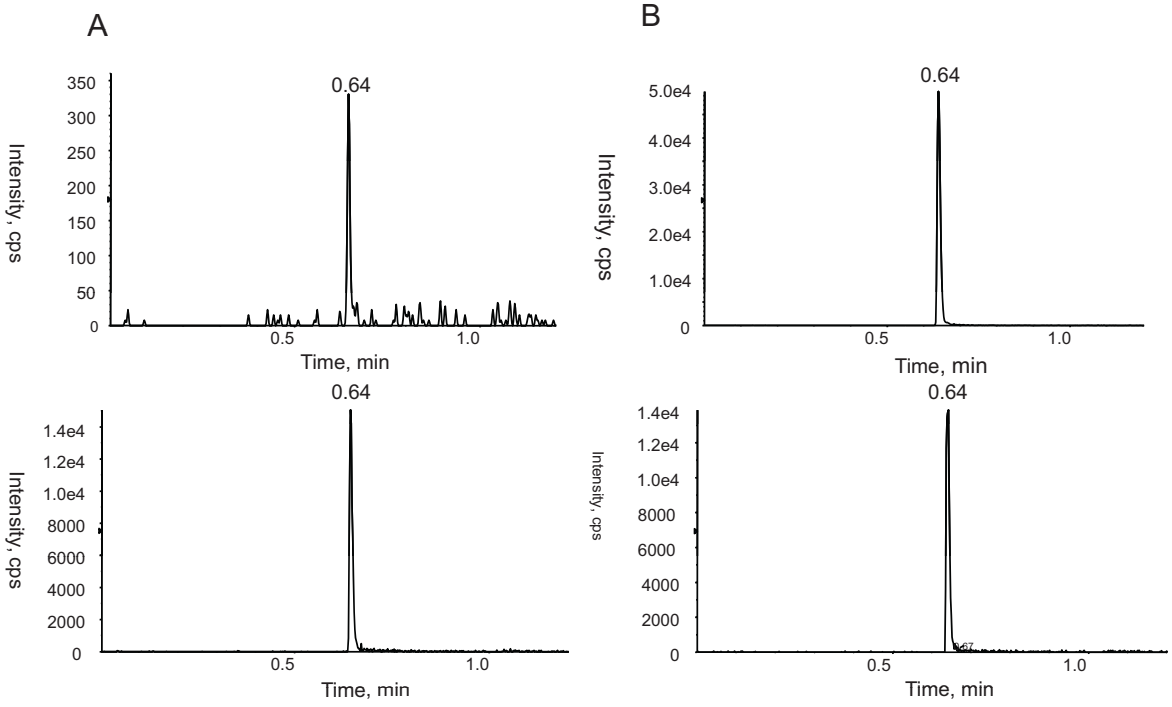

Fig. 3. Representative LC–MS/MS ion chromatogram of PD 00332991 at LLOQ (1 ng/mL) (A) or ULOQ at 250 ng/mL PD 00332991 (B). Top panel is for PD 0332991 and bottom panel is for IS.

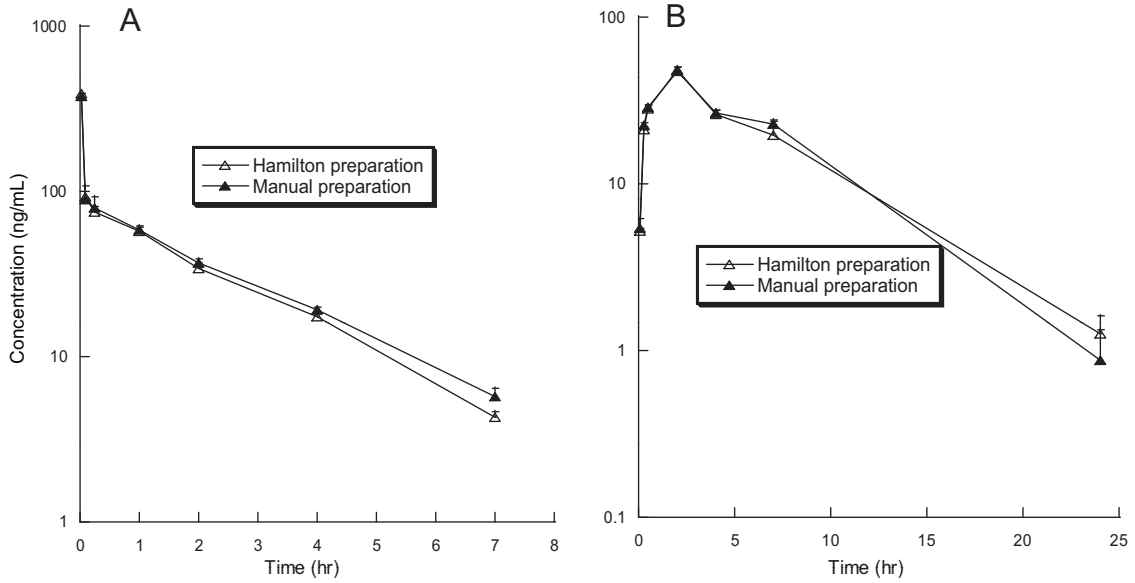

Fig. 4. Mean plasma concentration-versus-time curves for PD 0332991 in female C3H mice IV dosed at 1 mg/kg (A) and PO dosed at 2 mg/kg (B) by manual and Hamilton robot sample processing methods.

(peak shape and retention time) with the described microbore LC–MS/MS method. Approximately, 1 μL aliquot of the supernatant was injected onto the microbore LC–MS/MS system.

2.5. Microbore LC–MS/MS

Microbore LC–MS/MS was employed using an Eksigent Ultra HT system coupled to a Sciex API 4000 triple quadrupole mass spectrometer. Mobile phase A consisted of water containing 0.1% formic acid, and mobile phase B consisted of acetonitrile containing 0.1% formic acid. A microbore Agilent C_{18} Extend, 1.0 × 50 mm, 3.5 μm column at a flow rate of 0.150 mL/min was used to elute PD 0332991. A fast gradient elution program was utilized where the initial solvent composition was held at 2% B for 0.2 min and then changed linearly to 90% B over 0.7 min and held at 90% B for an additional 0.6 min. The column was then re-equilibrated to initial conditions of 2% B. The total run time was 1.8 min. A 25 μL syringe was used and a metered 1.0 μL sample volume was injected onto the column.

The mass spectrometer was operated in the positive electrospray ionization mode using multiple reaction monitoring (MRM). The turbo-ion spray voltage was set to 4.5 kV and the auxiliary gas temperature was maintained at 450 °C. High purity nitrogen was used for GAS 1, GAS 2, curtain, and CAD gases. The mass resolution was set to a peak width of 0.7 mass units at half-height for both Q1 and Q3. The electron multiplier was set at 2000 V. Declustering potential, collision energy, entrance potential, and collision cell exit potential were set for analysis of PD 0332991 and IS at their optimized MS conditions at 91, 41, 10 and 24 respectively. The dwell time of each MRM transition was 50 ms. PD 0332991 and IS were monitored using specific precursor ion → product ion transitions of m/z 448 → 380 and m/z 451 → 383, respectively.

2.6. Data analysis

Analyst® software, version 1.4.1 was used for data acquisition and chromatographic peak integration. The peak area ratios of PD 0332991 and IS were plotted as a function of the nominal concentrations of the analytes. Quantitation was performed by

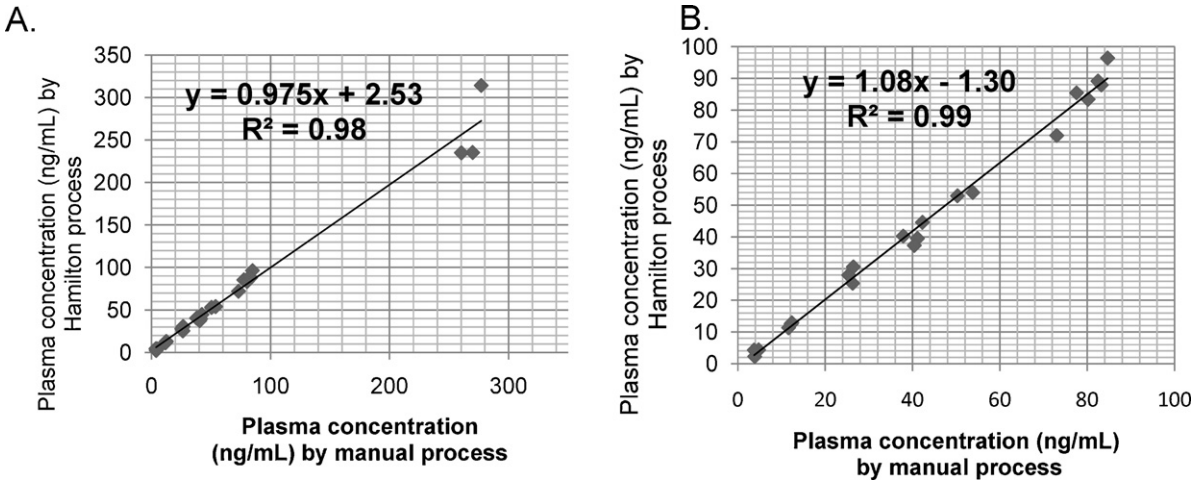

Fig. 5. Correlation of individual plasma concentrations between Hamilton and manual process (A) and plasma concentration up to 100 ng/mL (B).

Table 1
Inter-day accuracy and precision of calibration standard of PD 00332991 in mouse plasma.

Norminal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy (%)	CV (%)
Inter-day (n = 3)			
1.00	0.98	98.5	2.95
5.00	5.22	104	10.1
10.0	10.2	102	7.89
25.0	27.3	109	3.24
50.0	52.8	105	0.82
100	101	101	3.32
250	247	98.6	1.19

%CV calculated as Standard deviation/Mean × 100.

linear regression with a $1/x^2$ weighting. Pharmacokinetic parameters were calculated using Watson™ Bioanalytical LIMS software version. 7.2.0.03.

3. Results and discussion

3.1. Optimization of automated liquid handling system

The successful development of micro-sample analyses using 10 μL plasma extractions with a Hamilton robot required optimization of key parameters such as dead volume, appropriate labware and specific software programming. Although the MicroLab Star liquid level detection (LLD) technology has been widely used by the Hamilton liquid handler, we determined that accurate pipetting and transfer of 10 μL mouse plasma samples from a 15 μL plasma volume, required the utilization of the fixed position setting. The dead volume was determined to be 5 μL when the minimum volume was set to 15 μL utilizing the 96-conical shallow well plate at a fixed dispensing height (3 mm) from the bottom of the plate well. The 50 μL volume Hamilton tips were used for this process. Precise pipetting of samples were achieved by ensuring that the pipette tip was immersed in the plasma sample at all times and did not touch the sides or bottom of the 96 well plate.

3.2. Hamilton PPT method for a 10 μL out of 15 μL sample volume

The assay selectivity for PD 00332991 was assessed using 6 different lots of blank mouse plasma. The chromatographic conditions reported were found to be selective for PD 00332991 and IS. Comparison of the chromatograms for the control blank with IS and the double blank indicated no significant interference at the expected retention time for PD 00332991 and IS from endogenous material in plasma (shown in Fig. 2). Fig. 3 displays a typical chromatogram of PD 032991 and IS extracted from mouse plasma at a concentration of 1.0 ng/mL low limit of quantitation (LLOQ) and 250 ng/mL upper limit of quantitation (ULOQ). Matrix effects (i.e. enhancement or suppression of ionization) was assessed by comparing the absolute peak areas of post-spiked extracted plasma analyte standards to those of corresponding neat standards in mobile phase. The average peak areas between the post-extraction spiked plasma standards and the corresponding neat standards were within ±3% of each other, confirming a lack of matrix effects following the injection of a 1 μL plasma extract containing PD 00332991 into the microbore LC–MS/MS system.

To assess the accuracy and precision of the Hamilton PPT method, intra- and inter-day performances were conducted over three days of analysis. Calibration curves were prepared daily for PD 00332991. The linear dynamic range for PD 0332991 was from 1 ng/mL to 250 ng/mL. The accuracy and precision for the inter-day calibration standards extracted using 10 μL aliquots of mouse plasma were within ±15% of the nominal concentration and less than 10% CV (Table 1). Table 2 shows the results of intra- and inter-day accuracy and precision for three different levels of QCs at 3, 20, and 200 ng/mL with CV% of less than 13% and accuracy from 90% to 103%, which met the in house acceptance criteria (At least 2/3 of the standards and QC samples should have their values within 15% of the nominal concentrations and CV% should not exceed 15% at all concentrations except LLOQ at 20%). In addition, the accuracy and precision from 15 μL manual QC samples, prepared at 200 ng/mL to evaluate the ability to extract 10 μL from the 96-shallow conical well plate were within the acceptance criteria (<15%).

Sample dilution integrity was assessed by performing triplicate analyses of dilution QC samples manually. The dilution step was performed to evaluate whether the Hamilton robot could accurately aspirate a fixed 5 or 10 μL aliquot of plasma from the 96-shallow conical well plate and transfer the appropriate volume to the dilution plate containing various amounts of blank plasma depending on final concentrations (Table 3). The next step involved aspirating 10 μL aliquot of diluted plasma and dispensing to a second (target) plate for the PPT step. As shown in Table 3, the inter- and intra-day accuracy and %CV for the dilution QCs using 4 different dilution factors was between 86% and 103% of the nominal concentration, while the %CV was <13%, and met the acceptance criteria. Long term stability of PD 0332991 was not evaluated in this study since it was reported previously [17].

3.3. Application to mouse PK studies

The pharmacokinetics of PD 0332991 was studied in female C3H mice following intravenous administration at 1 mg/kg and oral administration at 2 mg/kg. 10 μL aliquots of mouse plasma samples from animals utilizing non-serial bleed were processed both

Table 2
Intra- and inter-day accuracy and precision of QC samples (both Hamilton prepared and manually prepared) of PD 0332991 in mouse plasma.

Type of samples	Norminal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy (%)	CV (%)
QC, Intra-day (n = 3)	3	3.01	100	11.2
	20	20.0	99.7	3.52
	200	205	103	1.97
Manual QC[a], Intra-day (n = 12)	200	212	106	4.13
QC, Inter-day (n = 9)	3	2.75	91.7	13.2
	20	18.5	92.2	9.25
	200	192	96.2	7.22
Manual QC[a], Inter-day (n = 36)	200	195	97.7	8.39

[a] 15 μL at 200 ng/mL plasma QCs were manually prepared and transferred to a 96-shallow conical well plate to test the accuracy and precision of extracting 10 μL out of 15 μL across multiple replicates.

Table 3
Intra- and inter-day accuracy and precision of QC samples of PD 0332991 at 2000 ng/mL in mouse plasma with different dilution factors.

Sample volume (μL)	Volume of control plasma to dilution plate (μL)	Dilution factors	Mean determined concentration (ng/mL)	Accuracy (%)	CV (%)
Intra-day (n = 3)					
5	295	60	1730	86.3	6.19
5	145	30	1890	94.6	3.75
10	90	10	2070	103	7.50
10	40	5	1890	94.5	3.82
Inter-day (n = 9)					
5	295	60	1910	95.3	12.9
5	145	30	1920	96.0	5.49
10	90	10	1890	94.3	10.3
10	90	5	1870	93.3	7.20

Table 4
Summary of IV and PO pharmacokinetic parameters for PD 0332991 in female C3H mice by manual and Hamilton robot sample processing methods.[a]

Process	$AUC_{0-\infty}$ (ng h/mL)	Cl_p (mL/min/kg)	Vd_{ss} (L/kg)	$T_{1/2}$ (h)
I. Intravenous (1 mg/kg)				
Hamilton (10 μL out of 15 μL)	239	69.7	10.0	1.9
Manual (10 μL out of 15 μL)	228	73.1	9.18	1.7
Difference from manual process[a]	4.8%	4.7%	8.9%	11%

Process	$AUC_{0-\infty}$ (ng h/mL)	C_{max} (ng/mL)	t_{max} (h)	F[b] (%)
II. Oral (2 mg/kg)				
Hamilton (10 μL out of 15 μL)	384	48.7	2.0	80
Manual (10 μL out of 15 μL)	411	47.7	2.0	90
Difference from manual process	6.6%	2.1%	0%	11%

[a] Difference from manual process was calculated using the different plasma concentration between Hamilton and manual process relative to plasma concentration from manual process.
[b] Bioavailability (F%) was calculated using the mean $AUC_{0-\infty}$ values at 2 mg/kg PO, relative to the mean $AUC_{0-\infty}$ at 1 mg/kg IV.

manually and using the Hamilton robot. Pharmacokinetic parameters (area under curve (AUC), volume of distribution (Vd), clearance (Cl), half life ($T_{1/2}$) and bioavailability (F%)) for PD 0332991 were determined by both the manual and the Hamilton sample preparation approaches. Overall, the *in vivo* PK values obtained after intravenous and oral administration were comparable and there were less than 11% difference between the automated Hamilton and manual PPT methods (Table 4). The mean plasma concentration-versus-time curves for PD 0332991 in mice processed by both methods were similar as well (Fig. 4). Each individual concentration from both methods showed good linear correlation with $R^2 > 0.98$ (Fig. 5). In addition, a mouse PK study using one additional in house compound was also conducted using the same approach. Similar PK results by both the manual PPT and Hamilton PPT methods were obtained (AUC, Vd, Cl, $T_{1/2}$, C_{max}, t_{max} and F values were all within 10%). Collectively, these results show that the Hamilton PPT method can accurately and precisely aliquot 10 μL out of a 15 μL sample volume and the results were similar to the manual PPT process.

4. Conclusion

A fit for purpose Hamilton robot based protein precipitation method for processing 10 μL plasma sample from a minimum 15 μL total volume of plasma has been successfully developed and validated. This method is suitable for analyses of plasma samples from micro-sampling discovery PK and PK/PD studies where the plasma sample (minimum 15 μL) from the *in vivo* mouse serial micro-sampling can routinely be collected directly into the 96-conical shallow well plate. Micro-scale mouse plasma samples can be stored in a 96-conical shallow well plate and 10 μL aliquots processed by the Hamilton robot directly. The implementation of this "small volume" Hamilton method, will enable automated sample processing of low volume samples using the Hamilton robotic system.

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