Determination of pazopanib (GW-786034) in mouse plasma and brain tissue by liquid chromatography–tandem mass spectrometry (LC/MS–MS)

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ABSTRACT

A simple, rapid and sensitive liquid chromatography–tandem mass spectrometric (LC/MS–MS) method has been developed and validated for the quantitative determination of pazopanib in mouse plasma and brain tissue homogenate. Single liquid–liquid extraction step with ethyl acetate was employed for analysis of pazopanib and the internal standard (IS); vandetanib. HPLC separation was performed on an XTerra® MS C18 column 50 mm × 4.6 mm, 5.0 μm. The mobile phase consisted of 70% acetonitrile and 30% water with 0.1% formic acid, pumped at a flow rate of 0.25 ml/min. Analysis time was 3.5 min per run and both the analyte and IS eluted within 1.8–2.0 min. Multiple reactions monitoring (MRM) mode was utilized to detect the compounds of interest. The mass spectrometer was operated in the positive ion mode for detection. The precursor to product ions (Q1→Q3) selected for pazopanib and internal standard during quantitative optimization were (m/z) 438.1→357.2 and 475.0→112.2 respectively. The calibration curves were linear over the range of 3.9–1000 ng/ml in both biological matrices. Lower limit of quantification (LLOQ) for mouse plasma and brain tissue was 3.9 ng/ml. The values for inter and intra day precision and accuracy were well within the ranges acceptable for analytical assessment (<15%). This method was applied to determine brain to plasma concentration ratio and relevant pharmacokinetic parameters of pazopanib after a single intravenous dose of 5 mg/kg in FVB wild type mice.

1. Introduction

Angiogenesis plays a pivotal role in tumor growth and helps sustain metastasis from primary tumor cells [1]. Vascular endothelial growth factor receptor (VEGF) is considered to be a major determinant in pathogenesis of several tumor types [2–4]. Apart from VEGF, tumor cell proliferation is also dependent on other growth factors such as platelet derived growth factor (PDGF) [5] and epidermal growth factor (EGF) [6]. These growth factors are dysregulated in numerous cancer types and hence, targeting these receptors is an attractive strategy. Pazopanib (GW-786034, Votrient®) is an orally active multitargeted tyrosine kinase inhibitor (TKI) that targets VEGFR-1, -2, and -3, PDGFR-α, PDGFR-β, and c-Kit. It acts by competing with the binding of ATP to the intracellular tyrosine kinase domain of growth factor receptors, thereby inhibiting receptor autophosphorylation and blocking downstream signal transduction [7]. Numerous novel small molecule TKIs are under development and have already been approved by the United States

Food and Drug Administration (USFDA) for the treatment of solid tumors.

Preclinical studies suggest an IC_{50} value of pazopanib for human VEGF-2 inhibition in low nanomolar range [8]. Potent antiangiogenic and antitumor activity of pazopanib has been reported in several human tumor xenografts including colon, melanoma, prostate, renal, breast and lung [8]. Furthermore, the steady-state concentration of pazopanib determined from preclinical studies showed a strong correlation with the pharmacodynamic effects and antitumor activity in a Phase I clinical trial [8]. Recently, USFDA approved pazopanib for the treatment of advanced renal cell carcinoma [9]. Currently, many clinical trials are ongoing for testing the efficacy of pazopanib alone and in combination with other anti-cancer drugs for the treatment of metastatic cervical cancer, breast cancer, corneal neovasularization and recurrent brain tumors (www.clinicaltrials.gov). However, the tight endothelial junctions located in brain capillaries which are further fortified by the presence of ABC efflux transporters (blood–brain barrier, BBB) can restrict the brain penetration of pazopanib and may lead to its therapeutic inefficacy when used as an anti-tumor agent for recurrent brain tumors.

To systematically examine the preclinical plasma pharmacokinetics and brain accumulation of pazopanib in a reproducible and precise manner, a sensitive and validated assay is necessary. To the best of our knowledge, there is no published report in the

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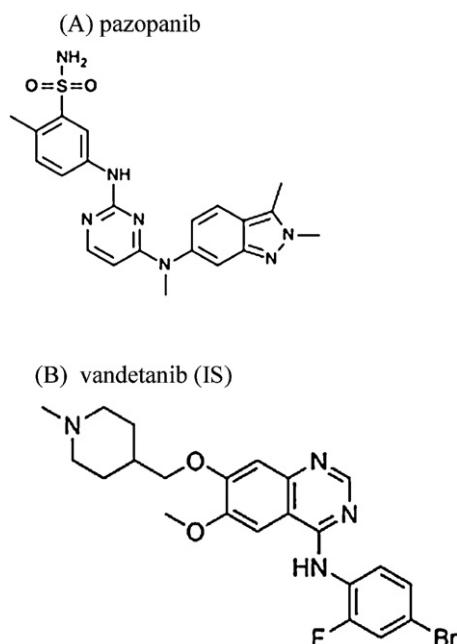E-mail address: mitraa@umkc.edu (A.K. Mitra).

Fig. 1. Chemical structures of pazopanib and internal standard, vandetanib.

literature that demonstrates validation of a sensitive assay for the determination of pazopanib in biological tissues. Hence, in this paper, we describe a simple liquid–liquid extraction method that utilizes reverse phase liquid chromatography coupled with mass spectrometry (LC/MS–MS) technique. This method requires just 100 μl aliquots of plasma and brain tissue homogenate to quantify pazopanib in preclinical pharmacokinetic and brain distribution studies by employing vandetanib (a TKI) as an internal standard (Fig. 1b)

2. Materials and methods

2.1. Chemicals

Pazopanib and internal standard (IS) vandetanib were purchased from LC labs (Woburn, MA). Drug free mouse plasma was obtained from Valley Biomedicals (Winchester, VA). Formic acid and HPLC grade water and acetonitrile were procured from Fisher Scientific (Pittsburgh, PA). All other reagents were HPLC or reagent grade and were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Stock solution, calibration standards and quality controls

Stock solution of pazopanib and IS were prepared in methanol and diluted to a final concentration of 100 μg/ml. All stock solutions were stored in $-80 \pm 5\,^\circ$C. A set of nine non-zero calibration standards ranging from 3.9 ng/ml to 1000 ng/ml were prepared by spiking the drug free mouse plasma and brain homogenate samples with appropriate amounts of pazopanib. The quality control (QC) samples 3.9 ng/ml (LLOQ), 7.81 ng/ml (LQC), 125 ng/ml (MQC) and 1000 ng/ml (HQC) were prepared in a similar manner and were included in non-zero calibration standards. All calibration standards were prepared in triplicate and quality control samples were prepared in six replicates.

2.3. Sample preparation

A 100 μl aliquot each of mouse plasma and brain homogenate (prepared by adding 3 vol. of 5% bovine serum albumin in water) were transferred into a 1.5 ml polypropylene microcentrifuge

tubes. To this aliquot, 40 ng of IS, vandetanib solution (40 μl from working solution of 1 μg/ml in methanol) was added and mixture was vortexed for 10 s. Next, 900 μl of ice cold ethyl acetate was added and the mixture was vortexed vigorously for 2 min followed by centrifugation at 10,000 rpm for 7 min at 4 °C to cause efficient separation of aqueous and organic layer. Seven hundred microliters of the organic layer was removed and transferred to freshly labeled microcentrifuge tubes and dried in vacuum (Gene-vac DD-4X). The dried residue was reconstituted in 100 μl of mobile phase (70:30:0.1, acetonitrile:water:formic acid, in %v/v) and 10 μl was injected into the LC/MS–MS for analysis.

2.4. Chromatographic and mass spectrometric conditions

The chromatographic system consisted of Shimadzu quaternary pump, vacuum degasser and autosampler (Shimadzu scientific instruments, Columbia MD, USA) coupled to QTrap® API-3200 mass spectrometer (Applied Biosystems, Foster City, CA, USA). HPLC separation was performed on an XTerra® MS C_{18} column 50 mm × 4.6 mm, 5.0 μm (Waters, Milford, MA). The mobile phase consisted of 70% acetonitrile and 30% water with 0.1% formic acid, pumped at a flow rate of 0.25 ml/min. Analysis time was 3.5 min per run.

API-3200 mass spectrometer (Applied Biosystems) equipped with ion spray source was employed for obtaining mass spectra. Data acquisition was carried out by analyst 1.2 software. Multiple reactions monitoring (MRM) mode was utilized to detect the compounds of interest. The mass spectrometer was operated in the positive ion mode for detection. The precursor to product ions (Q1→Q3) selected for pazopanib and vandetanib during quantitative optimization were (m/z) 438.2→357.2 and 475.0→112.2 respectively. The operational parameters for the tandem mass spectrum of each analyte were obtained after running them in quantitative optimization mode. The turbo ion spray setting and collision gas pressure were optimized (IS voltage: ±5500 V, temperature: 350 °C, nebulizer gas: 30 psi, curtain gas: 30 psi). Peak area ratios of pazopanib and IS were calculated for preparing calibration curves by employing least squares regression analysis and uniform weighting. Parameters obtained from these calibration curves were used for back-calculating pazopanib concentration in mouse plasma and brain homogenate QC samples.

2.5. Method validation

2.5.1. Inter-assay and intra assay variability

Method validation batches for pazopanib quantification in mouse plasma and brain homogenate were performed on three separate occasions. Precision and accuracy batches comprised of three replicates of nine non-zero concentrations of calibration standards and six replicates of QC samples at four different concentrations. Inter-assay and intra assay variability were determined by computing percentage relative error (%RE) and percentage coefficient of variation (%CV).

2.5.2. Limit of quantification

Lower limit of quantification (LLOQ, i.e., the lower calibration level) sample was designated based on the criteria that the variability in accuracy and precision (%CV) was less than 20% with corresponding signal/noise ratio greater than 10, as stated in the FDA guidance for bioanalytical method validation. Signal/noise ratio was calculated based on the peak areas of LLOQ samples verses that of the background noise in true blank samples in respective biological matrices.

2.5.3. Evaluation of matrix effect

The effect of matrix on analyte ionization was determined post extraction in both the plasma and brain homogenate. Briefly, 100 µl aliquots of blank plasma or brain homogenate were extracted as described earlier employing liquid–liquid extraction method. Later, 100 µl aliquots from corresponding QC stock solutions (1000 ng/ml, 125 ng/ml and 7.8 ng/ml) and 40 µl IS (from 1 µg/ml stock) was added, vortexed and dried using speed vacuum at room temperature. The dried residue was then reconstituted in 100 µl of mobile phase and 10 µl was injected in mass spectrometer for analysis. The matrix effect was determined by calculating [the ratio of the peak area of the analyte in the post-extraction matrix versus the peak area of the non-extracted sample in the reconstituted solvent (mobile phase) − 1] × 100% [10]

2.5.4. Recovery

Plasma and brain homogenate samples were extracted ($n = 6$) according to the liquid–liquid extraction procedure as described earlier at three concentration levels: HQC, MQC and LQC. Extraction recovery was determined by [ratio of absolute peak area of drug extracted from biomatrices/absolute peak areas of non-extracted samples reconstituted in (mobile phase)] × 100. The extraction efficiency of the IS was assessed in an identical manner.

2.5.5. Stability

Stability of pazopanib was investigated using HQC, MQC and LQC plasma samples and MQC for brain homogenate samples. Analysis for six replicates was performed after storage at ambient temperature (4 h, short term stability), over three freeze–thaw cycles (thawing at room temperature and freezing again at −80 °C) and storage at −80 °C for 2 months. Furthermore, plasma and brain QC extracted samples were re-injected after additional storage of the extracts at 4 °C for 72 h. Stability was expressed as the percentage of drug concentration obtained in comparison to the freshly prepared QC samples.

2.6. Method application

Male FVB wild type mice were used as an animal model for in vivo experiments and for obtaining blank tissues. All mice were between 8 and 11 weeks of age at the time of experiment. Animals were obtained from Harlan laboratories, IN and were used in accordance with the protocols approved by the University of Missouri-Kansas City (UMKC), Institutional Animal Care and Use Committee and housed in Laboratory Animal Care accredited facilities at UMKC. Mice received pazopanib intravenously through tail vein injection at a dose of 5 mg/kg. Animals were euthanized using a CO_2 chamber at predetermined time points post dose (15, 30, 60, and 120 min, $n = 3$ for each time point). Blood was collected via cardiac puncture and immediately transferred to heparin coated microcentrifuge tubes. Plasma was isolated from blood by centrifugation at 10,000 rpm for 7 min at 4 °C. Whole brain was immediately removed, rinsed with ice-cold saline to remove extraneous blood and blot dried. All samples were stored at −80 °C until further analysis by LC/MS–MS. On the day of analysis, the brain samples were thawed, weighed and homogenized with 3 volumes of 5% bovine serum albumin in phosphate-buffered saline with use of a tissue homogenizer. Non-compartmental pharmacokinetic data analysis was performed using Phoenix WinNonlin 6.1®, Pharsight, CA.

3. Results and discussion

3.1. Mass spectrometric conditions and chromatography

Mass spectra of pazopanib and IS; vandetanib obtained during quantitative optimization showed a protonated molecular ion

Table 1
Back calculated pazopanib concentration of plasma calibration standards on three separate occasions.

Concentration added (ng/ml)	Concentration found (ng/ml)	%RE	%CV
1000	1066.0 ± 53.4	6.6	5.0
500	517.9 ± 34.4	3.6	6.6
250	249.9 ± 13.6	−0.1	5.5
125	125.9 ± 7.7	0.8	6.1
62.5	62.8 ± 2.6	0.5	4.1
31.3	31.8 ± 2.0	1.9	6.4
15.6	16.6 ± 0.9	6.1	5.2
7.8	7.6 ± 0.5	−3.2	6.7
3.9	3.5 ± 0.4	−11.5	12.7

$[M+H]^+$ at m/z of 438.1 and 475.0 respectively. Fragmentation of these precursor ions yielded strong product ions at m/z of 357.2 and 112.2 for pazopanib and vandetanib respectively (Fig. 2A and B). The temperature was optimized to 350 °C with spray ion voltage of 5500 V. Low retention times (1.8 min for pazopanib and 1.7 min for IS) aided in high throughput screening of tissue samples. Low background noise from the biological matrix showed good selectivity for both analyte and the IS as seen from the chromatograms for blank plasma and brain homogenate samples (Fig. 3). Typical chromatograms obtained using this assay for HQC and LLOQ in both matrices are also depicted in Figs. 4 and 5 respectively.

3.2. Limit of quantitation, linearity and precision

The LLOQ for pazopanib in mouse plasma and brain homogenate samples was found to be 3.9 ng/ml. A linear response in the peak area ratios was observed over a concentration range of 3.9–1000 ng/ml in both mouse plasma and brain tissue samples. The correlation co-efficients (R^2 values) obtained from plotting the peak area ratios against the nominal concentration were always greater than 0.99 ($n = 3$) in each run. Detailed summary of the calibration standards ran on 3 separate occasions in triplicate for both the matrices are provided in Tables 1 and 2 respectively.

Inter and intra assay variabilities were determined by analyzing QC samples at 4 different concentration levels (3.9, 7.81, 125 and 1000 ng/ml) for mouse plasma and brain tissue samples with six replicates each on three separate days. For plasma samples, intra-assay variabilities (%CV) ranged from 3.8 to 10.4% and inter-assay variability (%CV) ranged from 0.6 to 6.1%. In addition, for brain samples, intra-assay variabilities ranged from 2.5 to 10.3% and inter-assay variability ranged from 1.4 to 9.1%. Accuracy results for plasma and brain samples are summarized in Table 3. Overall, intra-assay and inter-assay variabilities were within ±15% for QC samples and within ±20% for LLOQ samples in both the matrices. Since these results met the acceptance limits set by the FDA guidance, this assay was suitable in terms of accuracy and precision.

Table 2
Back calculated pazopanib concentration of brain calibration standards on three separate occasions.

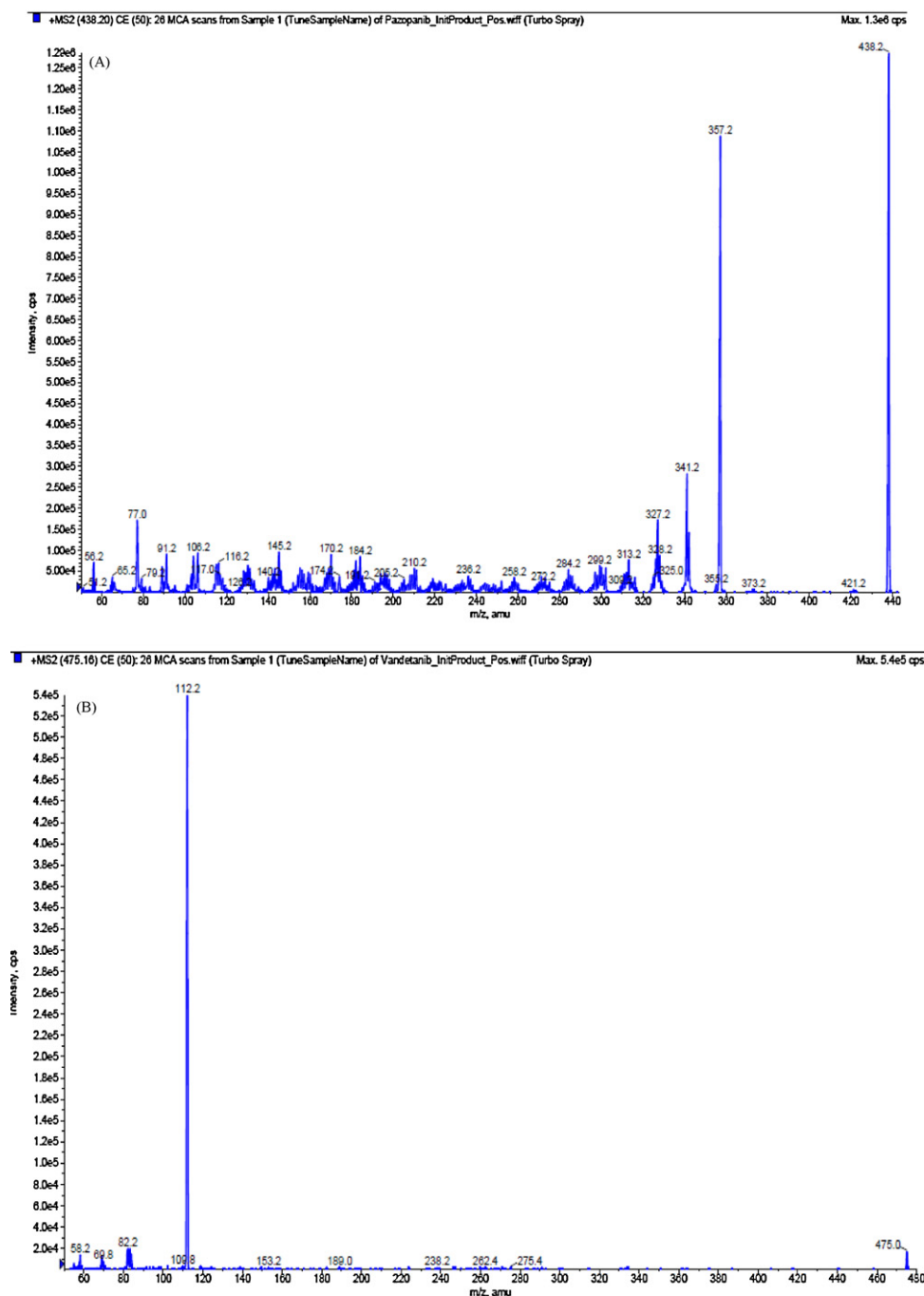| Concentration added (ng/ml)	Concentration found (ng/ml)	%RE	%CV
1000	1064.8 ± 77.8	6.5	7.3
500	528.4 ± 36.9	3.6	6.6
250	244.6 ± 15.8	−2.2	6.5
125	122.7 ± 6.6	−1.9	5.4
62.5	62.7 ± 5.9	0.3	9.4
31.3	29.9 ± 3.7	−4.4	12.5
15.6	15.2 ± 0.8	−3.0	5.6
7.8	7.3 ± 0.6	−6.1	8.6
3.9	3.8 ± 0.6	−2.9	17.1

Fig. 2. Product ion spectrum of pazopanib (A) and vandetanib (B) as obtained in positive ion mode.

Table 3
Intra-assay and inter-assay variabilities and accuracy of the LC/MS–MS assay for pazopanib in mouse plasma and brain homogenate.

Matrix	Nominal (ng/ml)	Intra-assay		Inter-assay	
		%RE	%CV	%RE	%CV
Plasma	1000 (HQC)	−7.9	4.8	−7.4	0.6
	125 (MQC)	3.1	3.8	5.2	6.1
	7.8 (LQC)	1.4	10.4	4.3	2.8
	3.9 (LLOQ)	−8.9	8.9	5.1	11.5
Brain	1000 (HQC)	7.3	10.3	5.8	1.4
	125 (MQC)	4.4	2.5	4.7	1.7
	7.8 (LQC)	1.8	4.4	0.7	9.1
	3.9 (LLOQ)	17.1	4.3	−0.5	19.9

Table 4
Matrix effect on ionization suppression of pazopanib and IS.

Matrix effect	Conc. (ng/ml)	Plasma Mean (%) ± S.D (%)	Brain homogenate Mean (%) ± S.D (%)
Pazopanib	1000	−53.2 ± 2.4	−52.1 ± 3.8
	125	−13.8 ± 3.0	−40.1 ± 4.5
	7.8	−8.9 ± 2.4	−26.0 ± 3.7
IS	400	−5.8 ± 3.1	−30.5 ± 3.5

3.3. Matrix effect

Effect of biological matrix on ion suppression was evaluated at three concentration levels (HQC, MQC and LQC) for pazopanib and at a single concentration (400 ng/ml) for the IS. As shown in Table 4,
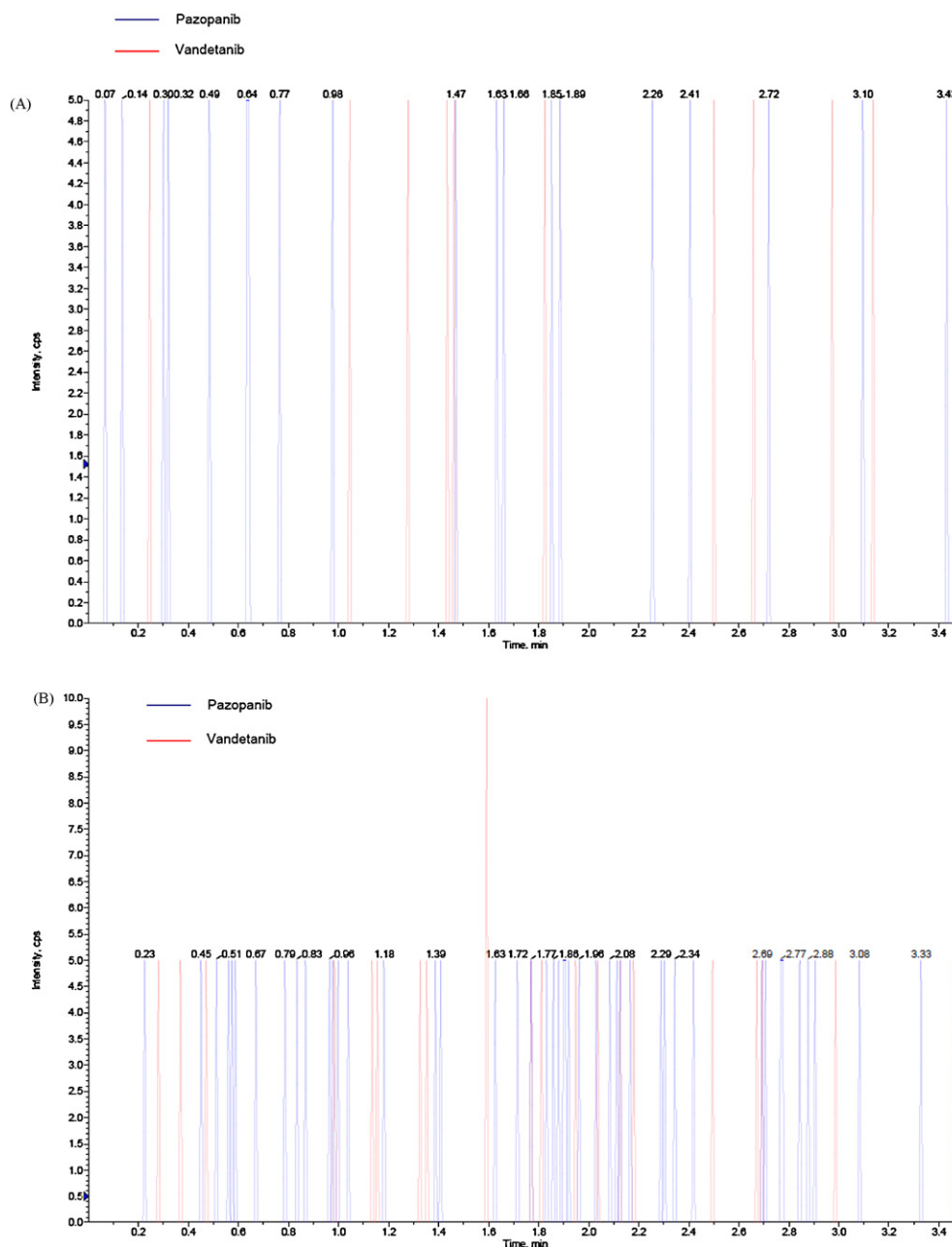
Fig. 3. Representative chromatograms of blank mouse plasma (A) and blank brain homogenate sample (B).

higher suppression of ionization of ∼−53% was observed for HQC sample in plasma whereas a moderate effect of −8.9, −13.8% and −5.8% was observed for low, mid QC samples and IS respectively. Absolute brain homogenate effect was −26 to −52% for pazopanib and −30.5% for IS. These results indicated that plasma and brain extract caused significant ionization suppression of both analyte and IS.

3.4. Recovery

Recovery of pazopanib was determined by dividing the absolute peak area of extracted QC samples (7.8, 125 and 1000 ng/ml) in six replicates to that of non-extracted reference standards prepared at same concentration for both plasma and brain homogenate. Similar procedure was followed for assessing recovery of IS at 400 ng/ml in both matrices. Average recovery for pazopanib at all concentration levels in both plasma and brain homogenate ranged from 50 to 57%.

Whereas, an average recovery of 30–34% was observed for IS in both matrices at 400 ng/ml. The results are summarized in Table 5. Despite low recovery values, this method allowed quantitation of pazopanib to up to 3.9 ng/ml in both plasma and brain homogenate with sufficient precision and accuracy.

3.5. Stability

Stability of pazopanib was investigated using HQC, MQC and LQC plasma samples and MQC for brain homogenate samples. Excellent recoveries were obtained at different storage conditions as summarized in Table 6. No significant degradation of QC samples of pazopanib in either mouse plasma or brain samples was detected after storing the samples for 4 h at room temperature (bench top stability) or after 2 months of storage at −80 °C. Three freeze thaw cycles also did not show any significant degradation in both biological matrices.
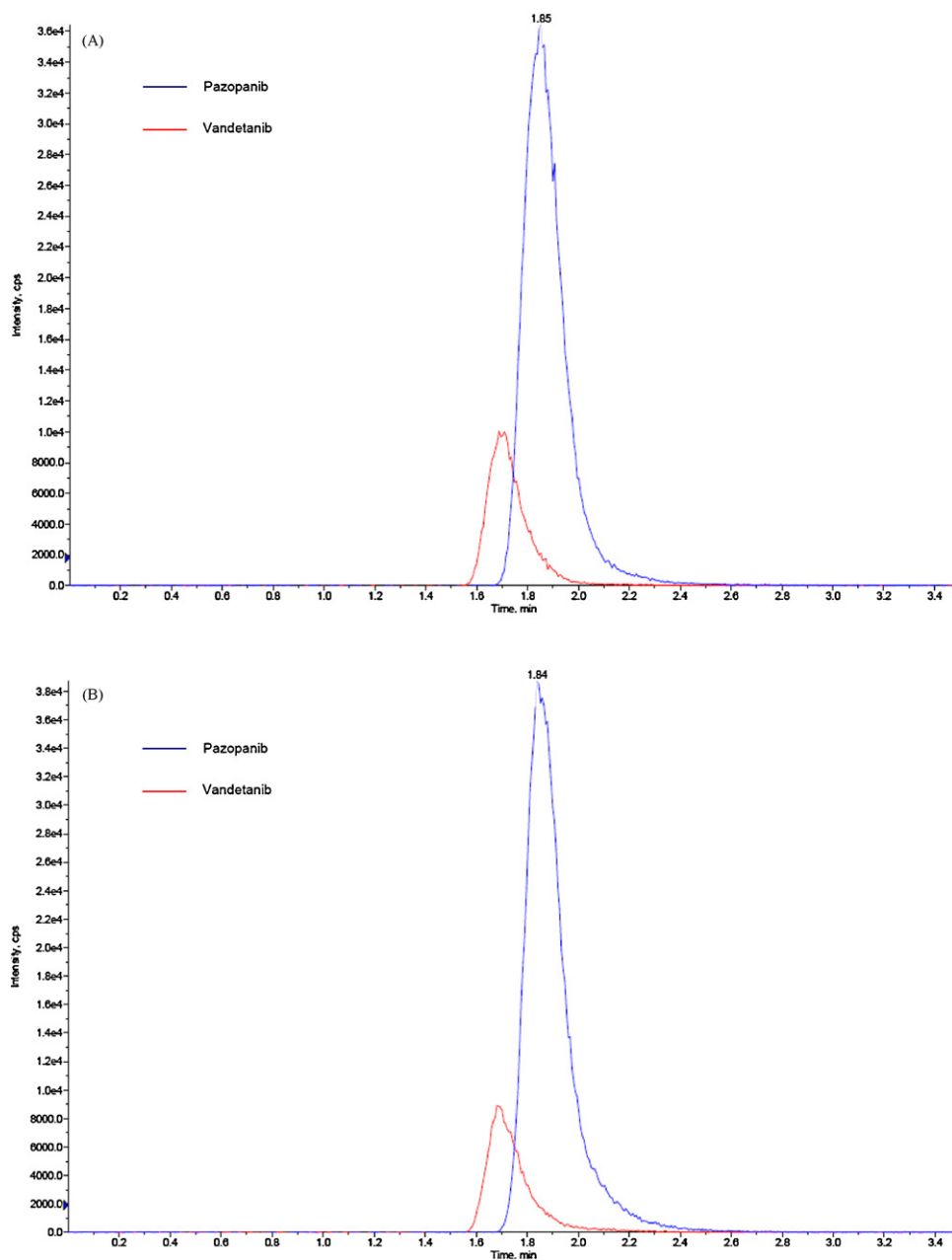
Fig. 4. Representative chromatograms of extracted HQC samples spiked in mouse plasma (A) and blank brain homogenate sample (B).

Table 5
Absolute recovery for pazopanib and IS in mouse plasma and brain homogenate.

Absolute recovery	Conc. (ng/ml)	Recovery in plasma Mean (%) ± S.D (%)	Recovery in brain homogenate Mean (%) ± S.D (%)
Pazopanib	1000	52.2 ± 8.5	54.2 ± 4.9
	125	53.0 ± 7.5	57.3 ± 2.6
	7.8	55.3 ± 7.1	50.8 ± 10.8
IS	400	30.0 ± 3.6	34.4 ± 4.1

Table 6
Stability of pazopanib and IS in plasma and brain homogenate.

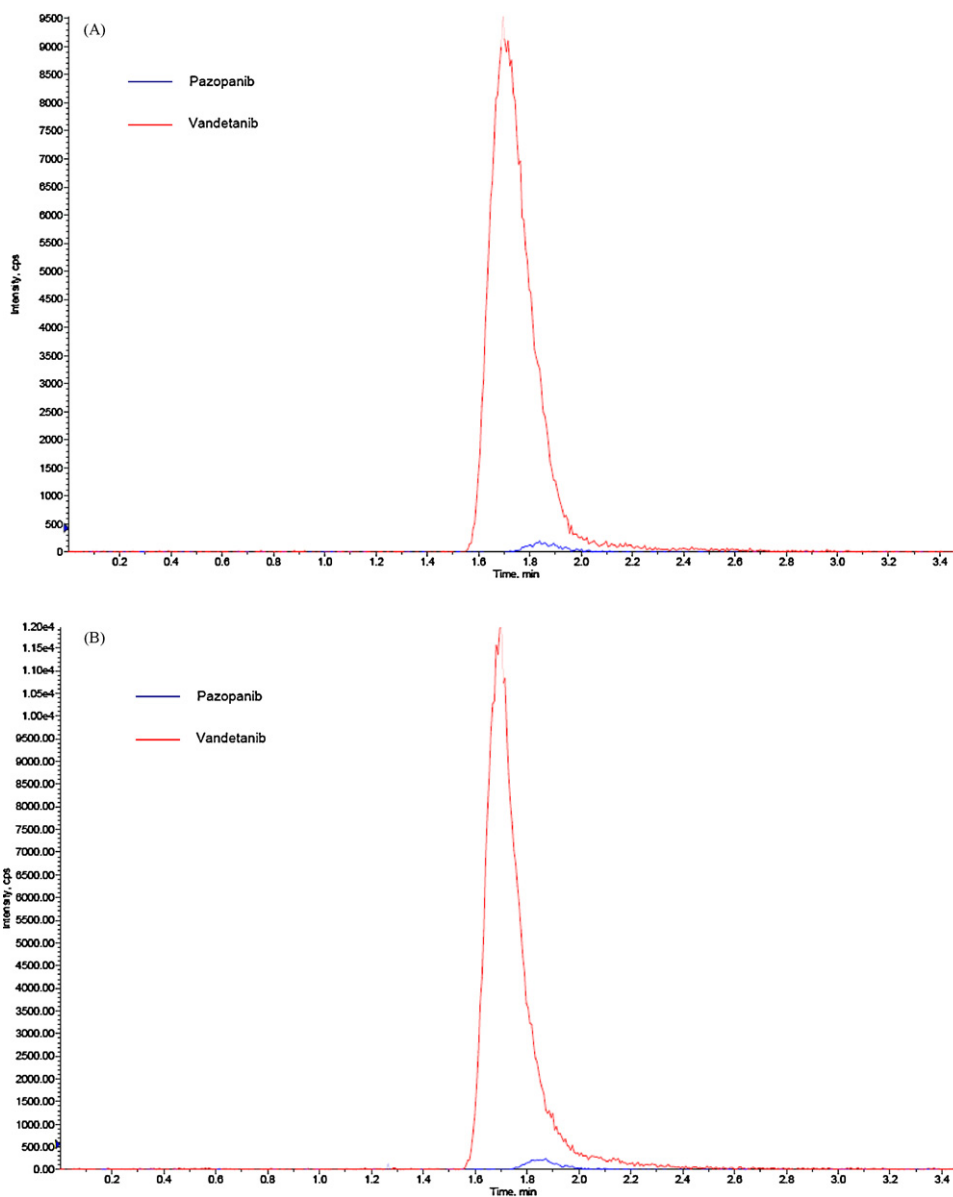| Matrix	Nominal (ng/ml)	Short term stability (4 h at room temp.) % recovery Mean ± S.D	Long term stability (60 days at −80 °C) % recovery Mean ± S.D	Stability after 3 freeze–thaw cycles % recovery Mean ± S.D
Plasma	1000 (HQC)	92.3 ± 3.9	121.9 ± 22.3	95.1 ± 6.7
	125 (MQC)	112.1 ± 4.2	95.6 ± 0.3	96.8 ± 2.1
	7.8 (LQC)	106.0 ± 6.6	91.4 ± 4.2	93.1 ± 0.8
Brain	125 (MQC)	103.2 ± 7.7	93.0 ± 1.9	110.8 ± 2.2

Fig. 5. Representative chromatograms of extracted LLOQ samples spiked in mouse plasma (A) and blank brain homogenate sample (B).

Re-injection of QC samples after additional storage at 4 °C for 3 days resulted again in acceptable results; these results indicate that reconstituted extracts could be stored for a prolonged period of time before final analysis.

3.6. Method application

Plasma and brain pharmacokinetics of pazopanib was investigated following a single i.v dose of 5 mg/kg (3:4:3, DMSO:propylene-glycol:0.9% saline, v/v/v) to male FVB wild-type mice, to demonstrate the applicability of this assay. 15, 30, 60 and 120 min post-dose; plasma and brain samples were collected and analyzed using this assay. Non-compartmental analysis of pazopanib concentration time profiles (Fig. 6) yielded terminal half lives of 1.4 h and 1.9 h in plasma and brain tissue respectively. Total body clearance was 2.38 ml/(min kg), volume of distribution was 285.86 ml/kg. As expected, brain delivery of pazopanib was severely restricted. Plasma area under the curve from time zero to the last time point (AUC$_t$) was 1.35 ± 0.04 min mg/ml, as compared to 0.020 ± 0.001 min mg/ml in the brain, giving a ratio of 0.015. This
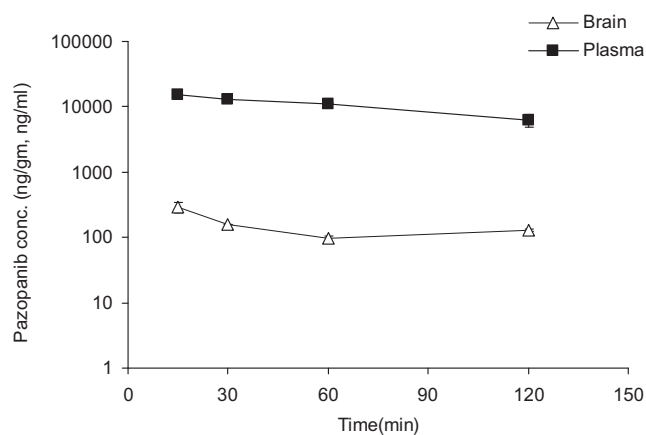

Fig. 6. Method application: semi-log plot for plasma and brain concentration time profile obtained in FVB wild type mice after 5 mg/kg tail vein injection of pazopanib. Values are presented as mean $\pm$ SE ($n = 3$).

Table 7
Dilution validation of pazopanib in plasma.

Nominal conc. (μg/ml)	Dilution factor	Conc. obtained (μg/ml) Mean ± SD	%RE	%CV
40	40	40.5 ± 1.7	1.2	4.2
20	40	20.9 ± 2.4	4.7	11.4
10	40	9.2 ± 0.8	−8.0	8.2

suggested that only 1.5% of the concentration in plasma was able to reach the brain. We have shown in our laboratory (manuscript under revision with *Int J Pharm*) that one probable reason for limited brain penetration of pazopanib is due to ATP binding cassette efflux proteins; P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) present at the BBB.

As most of the plasma samples were outside the range of the standard curve, plasma samples were diluted accordingly and hence a dilution validation study was performed at three concentration levels i.e., 40, 20 and 10 μg/ml. Freshly spiked samples were diluted 40-fold with blank mouse plasma to reach a nominal concentration of 1000, 500 and 250 ng/ml respectively which fell within the range of the standard curve ranging from 3.9 to 1000 ng/ml. Later 100 μl aliquots of respective diluted samples were processed in the same manner along with freshly spiked samples of corresponding concentrations. A 40-fold correction factor was applied to the back calculated concentrations obtained from the standard curve as shown in Table 7, these concentrations were well within ±10% of the nominal concentration values.

4. Conclusion

In conclusion we have developed and validated an assay for quantitatively determining pazopanib in mouse plasma and brain tissue. This is the first report for a validated method development of pazopanib that has been successfully applied for pre-clinical pharmacokinetic studies. The assay reported in this paper requires just 100 μl of sample and a simple liquid–liquid extraction step, thus eliminating the need for protein precipitation. Taken together, these characteristics combined with a short chromatographic run time of 3.5 min, allows this assay to be easily applied for the determination of pazopanib in a large number of plasma and brain tissue samples. The described method is linear over the concentration range of 3.9–1000 ng/ml which is sufficient to allow plasma and brain pharmacokinetic monitoring of pazopanib in preclinical investigations.

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