ORIGINAL ARTICLE

Development and Validation of a Stability-indicating Liquid Chromatography-Tandem Mass Spectrometry Method for Simultaneous Quantification of Pazopanib and Gemcitabine in Plasma: Overcoming Polarity and Stability Challenges

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Abstract

Background: Objectives Pazopanib and Gemcitabine are valuable anticancer drugs; when co-administered can be very difficult to quantitate from plasma due to differences in their polarities coupled with the inherent instability of Gemcitabine, and this necessitates a highly sensitive and robust method for simultaneous determination. **Aim:** This study developed and validated a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for simultaneous determination of Pazopanib and Gemcitabine in plasma that was stability-indicating, which considers polarity, matrix effects, and instability of Gemcitabine. **Methods:** The method was a gradient LC–MS/MS performed on a C18 column, with chilling the extraction solvent to −80°C before adding acetonitrile and using Zebularine for molecular stabilization, validated according to the International Council for Harmonisation (ICH) M10 and Food and Drug Administration (FDA) criteria. **Results:** The method displayed superb selectivity, sensitivity, linearity, reproducible recovery variability (RSD <0.09), as well as correlations of matrix effects with bias in the spiked samples; however, the stability was within standard limits, and accuracy/precision values were acceptable as well. **Discussion:** This method not only solved the issues of polarity and stability, but also gave reproducibility to pharmacokinetic use. **Conclusion:** The developed and validated LC–MS/MS method provides a simple, selective, sensitive, and stability-indicating solution to the simultaneous analysis of Pazopanib and Gemcitabine in plasma samples.

Key words: Bioanalytical validation, gemcitabine, liquid chromatography-tandem mass spectrometry, Pazopanib, plasma quantification, stability-indicating method

INTRODUCTION

Combination chemotherapy and targeted therapy are increasingly important in modern oncology, creating a need for robust analytical methods capable of simultaneously quantifying drugs with diverse physicochemical properties. Pazopanib, a multi-targeted tyrosine kinase inhibitor, is approved for renal cell carcinoma and soft tissue sarcoma,

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while Gemcitabine, a nucleoside analog, is widely used for pancreatic, lung, breast, and bladder cancers. Therefore, accurate pharmacokinetic evaluation and therapeutic drug monitoring of these agents are clinically important.[1-5] Simultaneous quantification of Pazopanib and Gemcitabine is challenging because of their contrasting polarity and stability characteristics. Pazopanib is lipophilic, whereas Gemcitabine is highly polar and rapidly degraded in plasma by cytidine deaminase. In addition, biological matrix effects may cause ion suppression or enhancement, affecting liquid chromatography-tandem mass spectrometry (LC–MS/MS) sensitivity and reproducibility.[6-10]

Although several analytical methods have been reported for individual determination of Pazopanib or Gemcitabine, few methods enable their simultaneous quantification.[11-15] Existing methods often suffer from limited sensitivity, lengthy run times, complex sample preparation, and inadequate stability-indicating capability, particularly for Gemcitabine.[16-20] The present study aimed to develop and validate a sensitive, selective, and robust LC–MS/MS method for simultaneous quantification of Pazopanib and Gemcitabine in spiked plasma. The method incorporated optimized chromatographic separation, rapid protein precipitation extraction, and Zebularine (ZEB)-mediated stabilization of Gemcitabine. Validation was performed according to the International Council for Harmonisation (ICH) M10 and Food and Drug Administration (FDA) bioanalytical guidelines.[21-26] The validated method demonstrated excellent sensitivity, selectivity, reproducibility, minimal matrix effects, and a short analysis time, supporting its application in pharmacokinetic, therapeutic drug monitoring, and clinical studies involving Pazopanib–Gemcitabine combination therapy.

MATERIALS AND METHODS

Chemicals and reagents

Pazopanib hydrochloride (purity ≥99.5%), Gemcitabine hydrochloride (purity ≥99.0%), and the internal standard Pazopanib-d4 were obtained from authorized commercial suppliers. High-performance liquid chromatography (HPLC)-grade methanol, acetonitrile, and water were purchased from Merck (Germany), while LC–MS-grade formic acid, ammonium formate, and ammonium acetate were obtained from Sigma-Aldrich (USA). ZEB, used as a stabilizer for Gemcitabine, met analytical quality specifications, including purity, residual solvent, heavy metal, and related substance limits in accordance with ICH requirements.[27-28]

Instrumentation and chromatographic conditions

Chromatographic analysis was performed using a HPLC system coupled with an electrospray ionization triple quadrupole tandem mass spectrometer.[29] Separation was achieved on an Agilent ZORBAX Eclipse Plus C18 RRHD column (50 × 2.1 mm, 1.8 µm) maintained at 40°C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile containing 0.1% formic acid and 2 mM ammonium formate). Gradient elution was optimized to accommodate differences in analyte polarity.[30-31] The flow rate was 1.0 mL/min with a 10 µL injection volume and a total run time of 5 min.

Mass spectrometric conditions

Detection was carried out in positive ESI mode.[32] Trace Ion (MSA) solution was used for the optimization of source conditions: Ion spray voltage 4500 V; source temperature 500°C; curtain gas 30 psi; nebulizer and auxiliary gases were set to 50 psi. Direct infusion was conducted to optimize the multiple reaction monitoring (MRM) transitions for Pazopanib, Gemcitabine, and Pazopanib-d4. Maximum sensitivity and selectivity were accomplished by varying collision energy, declustering potential, and dwell time.[33,34]

Preparation of standard and working solutions

Stock solutions of Pazopanib (30 mg/mL) and Gemcitabine (20 mg/mL) were prepared separately in methanol. Gemcitabine stock solutions were prepared in 0.1% formic acid and stored at −20°C to minimize degradation.[35] Working solutions were obtained by serial dilution using methanol–water (50:50, v/v), while internal standard solutions were prepared in acetonitrile.[36,37] Calibration standards and quality control (QC) samples were prepared in K_2EDTA plasma by serial dilution.[38-41] Calibration ranges were 1.1920–5006.9560 ng/mL for Pazopanib and 0.5000–2100.0680 ng/mL for Gemcitabine.[42-44] QC samples were prepared at the lower limit of quantification quality control (LLOQ), low, medium, intermediate, and high concentration levels. Pazopanib-d4 was selected as the internal standard because of its structural similarity and stable isotopic labeling.[45-47]

Plasma sample preparation

Protein precipitation was optimized to achieve high recovery and minimal matrix interference.[48] Briefly, 100 µL plasma was transferred to polypropylene tubes and mixed with 10 µL each of internal standard and ZEB solution for Gemcitabine stabilization. Samples were extracted with 300 µL chilled acetonitrile, vortex-mixed for 2 min, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was evaporated under nitrogen, reconstituted in water–acetonitrile (60:40, v/v) containing 5 mM ammonium formate, and analyzed by LC–MS/MS after addition of 0.1% formic acid.[49]

ZEB stabilization procedure

The primary ZEB stock solution was made in diluent and then diluted to a working solution for plasma stabilization.

Calibration standards and QC samples were prepared in plasma to which ZEB was added (10% v/v) before extraction, to stabilize Gemcitabine during handling and analytical procedures.[50-52]

Calibration standards and QC samples

For each analytical batch, new calibration standards and QC samples were obtained. Stored samples at −80°C until analysis. Calibration curves are constructed from analyte-to-internal standard peak area ratios plots against their respective concentrations within the validated concentration ranges.[53]

Method validation

Data are presented as mean ± SD. Precision was expressed as % coefficient of variation (%CV), and confidence intervals were calculated where appropriate. Acceptance criteria were evaluated according to FDA and ICH M10 bioanalytical method validation guidelines.[54]

Selectivity and specificity

Selectivity was assessed with normal, hemolyzed, lipemic, and heparinized plasma lots. Endogenous interference was evaluated through the analysis of LLOQ-spiked samples and blank samples. Interferences at analyte retention times were below acceptable regulatory thresholds, supporting method specificity and demonstrating reproducibility over three independent replicates.[54]

Linearity and sensitivity

Calibration curves were generated from weighted ($1/x^2$) linear regression of analyte to internal standard peak area ratios versus nominal concentrations. The LLOQ were acceptable for both precision and accuracy within regulatory thresholds.[55]

Accuracy and precision

QC samples were assessed for intra-day and inter-day accuracy and precision at different concentration levels. Accuracy and precision were determined as percentage nominal concentration and coefficient of variation (%CV), respectively. All results conformed to acceptance criteria of ±15% accuracy and ≤15% precision, except for correlation samples at the LLOQ (±20%).[56]

Data analysis

Data acquisition and processing were completed using validated instrument software. Peak integration was performed automatically and checked manually. Calibration curves were generated using weighted linear regression, and statistical analysis was performed with the appropriate analytical software.[57]

Freeze thaw stability FT3

Freeze–thaw stability was evaluated for Pazopanib and Gemcitabine after three complete freeze–thaw cycles (FT3). Plasma QC samples at low quality control (LQC) and high quality control (HQC) concentrations were frozen at −20°C and thawed unassisted at room temperature for each cycle. Following the third cycle, samples were processed and analyzed against freshly prepared calibration standards. Stability was considered acceptable when the measured concentrations remained within ±15% of nominal values with precision (%CV) ≤15%, in accordance with FDA and ICH M10 guidelines.

Long-term stability

Long-term stability was evaluated by storing LQC and HQC plasma samples at −20°C for 30 days. Following storage, samples were processed and analyzed against freshly prepared calibration standards. Stability was considered acceptable when measured concentrations remained within ±15% of nominal values with precision (%CV) ≤15%.

Autosampler stability

Autosampler stability was assessed by storing processed LQC and HQC samples of Pazopanib and Gemcitabine at 10°C for 24 h. Samples were reanalyzed against freshly prepared controls, and stability was considered acceptable when accuracy remained within ±15% of nominal concentrations and precision (%CV) was ≤15% according to FDA and ICH M10 guidelines.

RESULTS AND DISCUSSION

Method development and optimization

The present study developed and validated a stability-indicating LC–MS/MS method for simultaneous quantification of Pazopanib and Gemcitabine in spiked plasma. Due to their differing physicochemical properties, chromatographic and stabilization conditions were optimized. The Agilent ZORBAX Eclipse Plus C18 RRHD column with gradient elution provided optimal peak symmetry, resolution, reproducibility, and efficient retention of Gemcitabine.

As shown in Figure 1, Gemcitabine exhibited poor retention and broad peak shape under isocratic conditions. Gradient elution improved retention, peak symmetry, and resolution, enabling reliable simultaneous quantification. Chilled

acetonitrile protein precipitation provided high recovery with minimal matrix interference, while ZEB effectively stabilized Gemcitabine during sample processing.

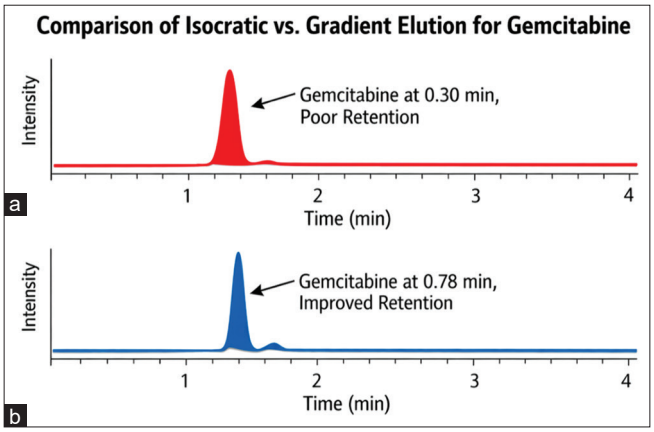

Figure 1: Isocratic versus gradient elution for Gemcitabine. Under isocratic conditions (a), Gemcitabine showed poor retention, early elution, and asymmetric peak shape. In contrast, gradient elution (b) improved retention (RT ≈ 1.60 min), peak symmetry, and chromatographic resolution, enabling efficient separation of analytes with differing polarities

Chromatographic separation and selectivity

Introduction the optimized chromatographic conditions displayed a perfect separation of the analytes and internal standards without striding interferences.

Analyte Pazopanib – Pazopanib D4

The specificity and reliability of the LC–MS/MS method were confirmed using MRM chromatograms of blank plasma, LLOQ, QC, and internal standard samples [Figure 2]. Pazopanib and Pazopanib-d4 exhibited well-resolved, symmetrical peaks at approximately 2.60 min without endogenous interference. No interference was observed in normal, hemolyzed, lipemic, or icteric plasma samples, and retention times remained consistent with acceptable sensitivity criteria.[9] Analyte response variation was within ±20%, while internal standard %CV values met regulatory acceptance limits, demonstrating excellent selectivity, reproducibility, and suitability for quantitative analysis of Pazopanib in spiked plasma.

Analyte Gemcitabine – Pazopanib D4

The specificity of the method was confirmed by evaluating multiple plasma matrices [Figure 3], with no significant

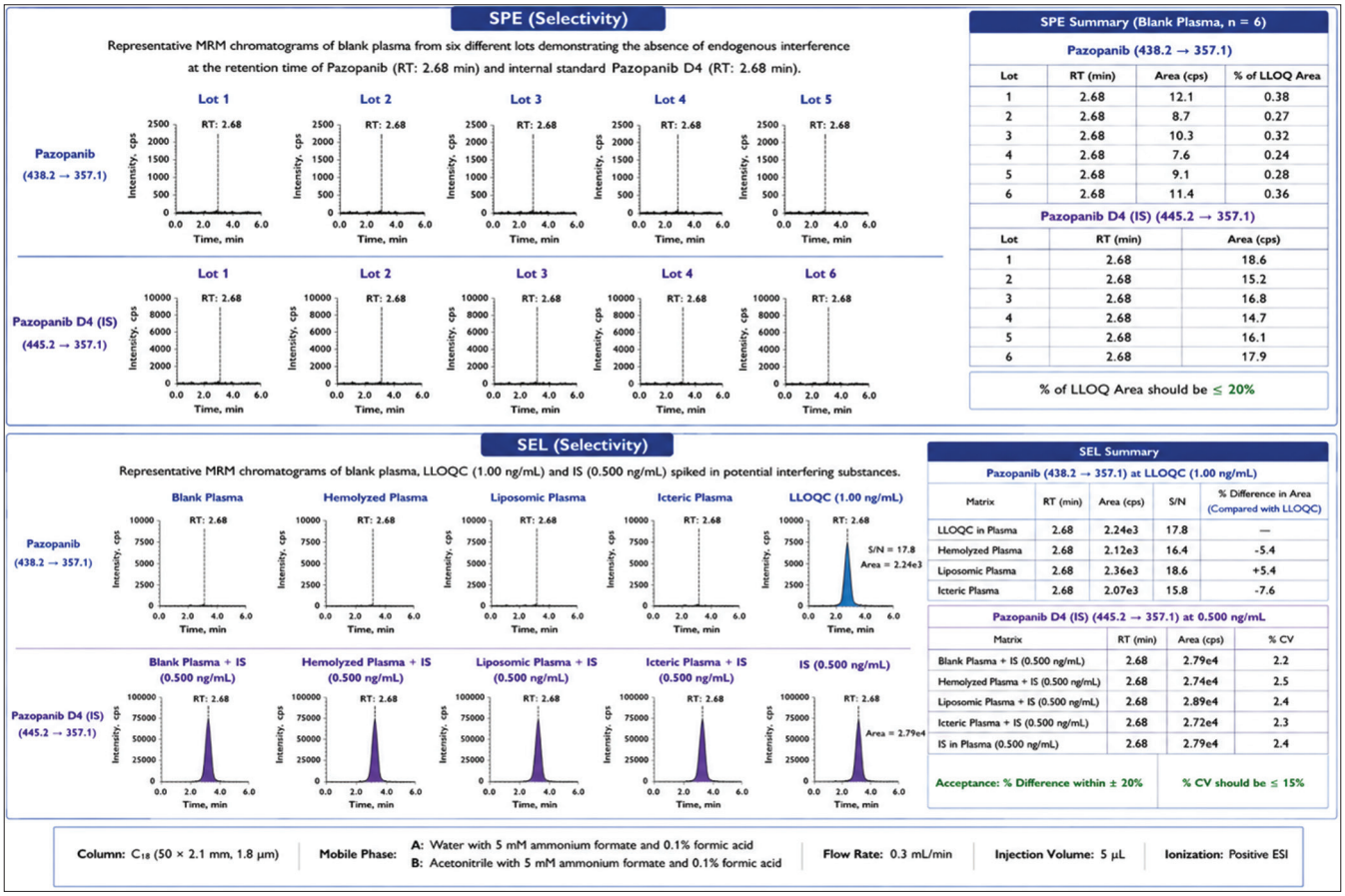

Figure 2: Representative multiple reaction monitoring chromatograms of Pazopanib (438.2→357.1) and Pazopanib-d4 (445.2→357.1) in spiked plasma. No significant endogenous interference was observed at the retention time (~2.68 min) in blank, hemolyzed, lipemic, icteric, or control plasma samples (n = 7), nor in lower limit of quantitation with quality control (n = 6) and Internal standard-spiked plasma samples (1000 ng/mL, n = 6), confirming method selectivity

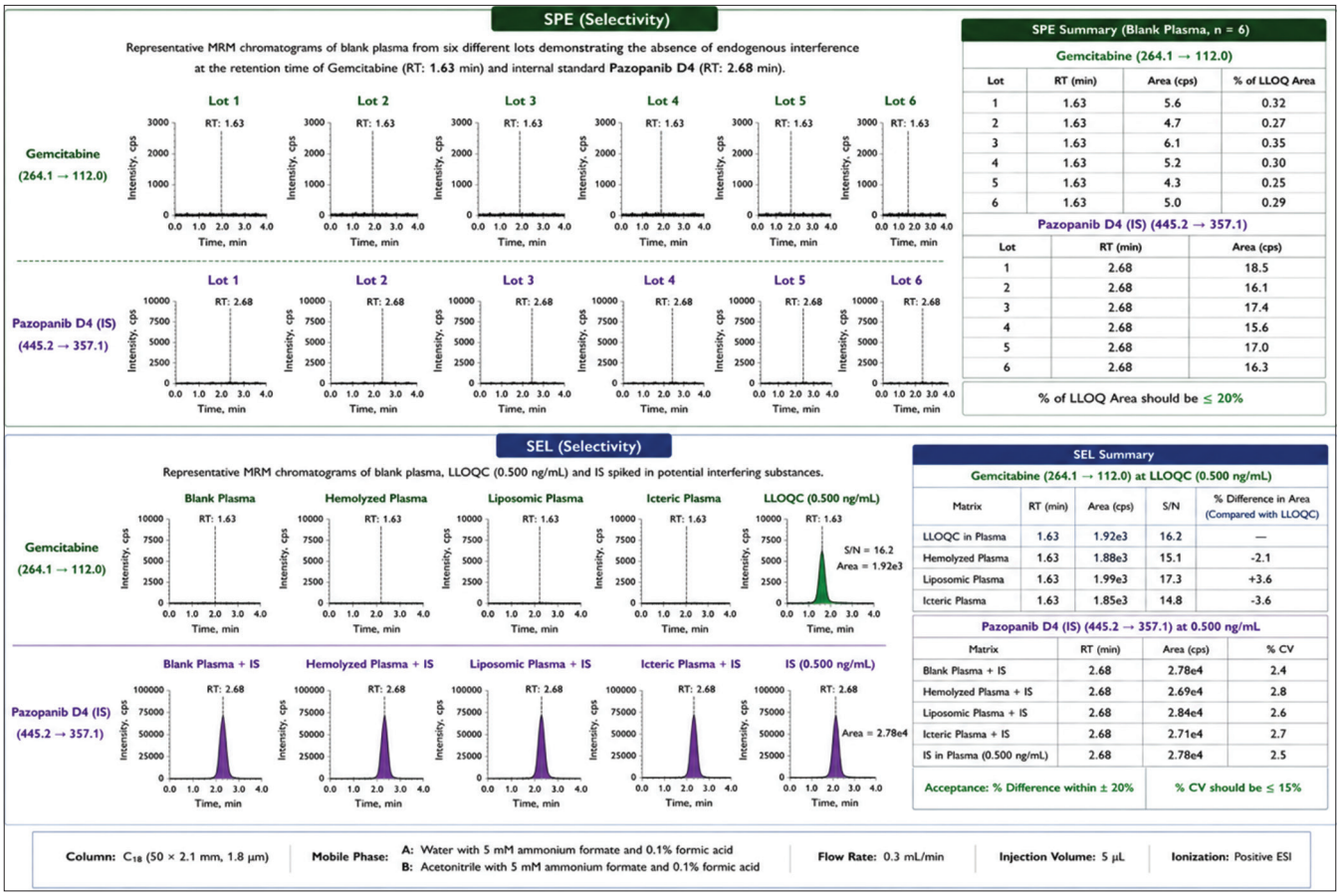

Figure 3: Representative multiple reaction monitoring chromatograms of Gemcitabine (264.1→112.0) and Pazopanib-d4 (445.2→357.1) in spiked plasma. No endogenous interference was observed at the retention times of Gemcitabine (RT 1.63 min) and Pazopanib-d4 (RT 2.68 min) in blank, hemolyzed, lipemic, icteric, lower limit of quantitation with quality control, and IS-spiked plasma samples. The developed liquid chromatography-tandem mass spectrometry method demonstrated excellent selectivity, reproducibility, and minimal matrix effects across all plasma matrices tested

interference at the retention times of Gemcitabine or Pazopanib-d4. Consistent retention times, peak shapes, and signal responses were observed in normal, hemolyzed, lipemic, and icteric plasma samples. Analyte response variation remained within ±20%, while internal standard %CV values were below 15%, meeting bioanalytical validation criteria. These results demonstrate that the developed LC–MS/MS method is selective, robust, and reliable for quantification of Gemcitabine in spiked plasma.

A summary of the optimized chromatographic and mass spectrometric conditions for simultaneous determination of Pazopanib and Gemcitabine is presented in Table 1. Analytes were optimized using MRM in positive electrospray ionization mode. The observed retention times for Pazopanib (2.6 min) and Gemcitabine (1.6 min) were consistent with reported literature values, confirming effective chromatographic separation. The optimized 5-min run time enabled rapid, high-throughput analysis while providing excellent sensitivity, peak symmetry, and reproducibility for bioanalytical applications.

Table 1: Chromatographic parameters

Parameter	Pazopanib	Gemcitabine	Pazopanib D4
Retention time (min)	2.6±0.5	1.6±0.5	2.6±0.5
MRM transition	438.2→357.1	264.1→112.0	445.2→357.1
Run time	5 min	5 min	5 min

MRM: Multiple reaction monitoring

Figure 4 Full-scan pre-cursor ion (Q1) and product ion (Q3) mass spectra of Pazopanib, Pazopanib-d4, and Gemcitabine used for optimization of MRM transitions in the LC–MS/MS method. (a) Q1 spectrum of Pazopanib showing the pre-cursor ion selected for fragmentation; (b) Q3 spectrum of Pazopanib showing the major product ions generated after collision-induced dissociation; (c) Q1 spectrum of Pazopanib-d4 internal standard; (d) Q3 spectrum of Pazopanib-d4 showing characteristic fragment ions; (e) Q1 spectrum of Gemcitabine displaying the pre-cursor ion used for MRM analysis; and (f) Q3 spectrum of Gemcitabine showing the corresponding product ions. The selected pre-cursor-to-product ion transitions provided high sensitivity, selectivity, and reproducibility for simultaneous quantification of Pazopanib and Gemcitabine in plasma samples.

Linearity and calibration curve

Figure 5 shows the linearity of the developed LC–MS/MS method was evaluated using calibration standards prepared in spiked plasma over the validated concentration ranges. As shown in Figure 5, excellent linear relationships were observed between nominal and calculated concentrations for both analytes. Pazopanib demonstrated linearity over the range of 1.1920–5006.9560 ng/mL (a), while Gemcitabine showed linearity from 0.5000 to 2100.0680 ng/mL (b). The calibration curves exhibited excellent correlation coefficients, confirming the suitability of the method for accurate and

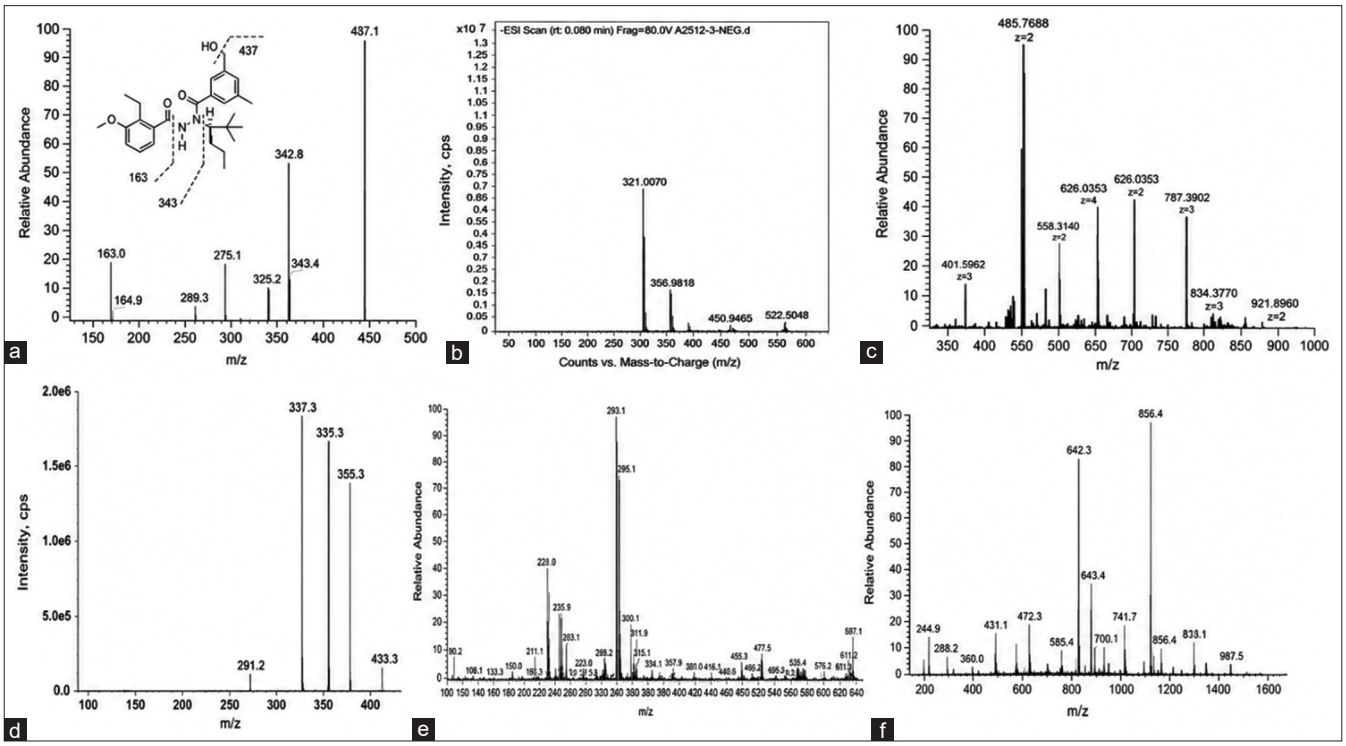

Figure 4: Full-scan and product ion mass spectra used for multiple reaction monitoring optimization of Pazopanib, Pazopanib-d4, and Gemcitabine: (a) Pazopanib Q1 spectrum, (b) Pazopanib Q3 spectrum, (c) Pazopanib-d4 Q1 spectrum, (d) Pazopanib-d4 Q3 spectrum, (e) Gemcitabine Q1 spectrum, and (f) Gemcitabine Q3 spectrum. The selected precursor and product ions were used for sensitive and selective liquid chromatography-tandem mass spectrometry quantification

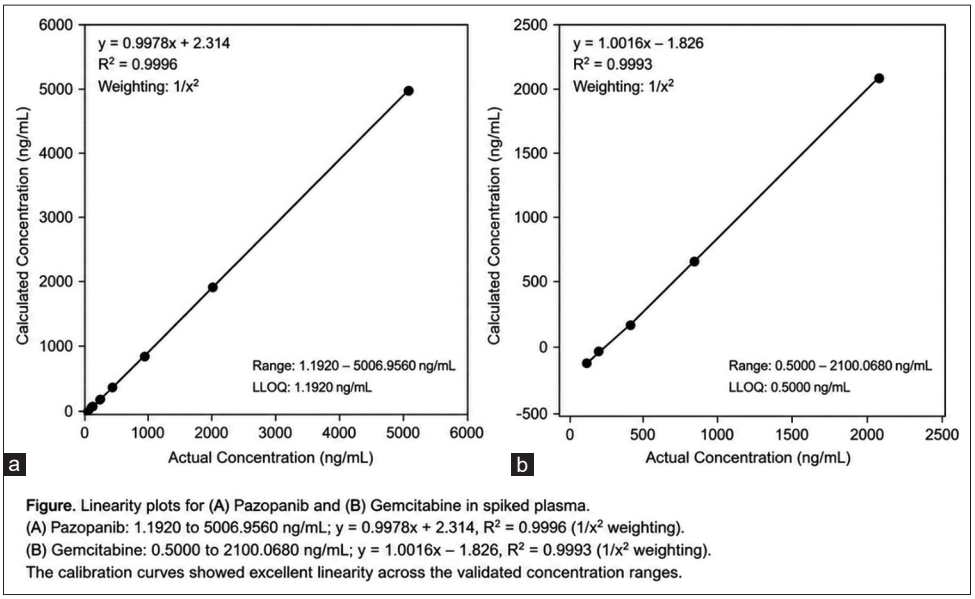

Figure. Linearity plots for (A) Pazopanib and (B) Gemcitabine in spiked plasma.
(A) Pazopanib: 1.1920 to 5006.9560 ng/mL; y = 0.9978x + 2.314, R^2 = 0.9996 ($1/x^2$ weighting).
(B) Gemcitabine: 0.5000 to 2100.0680 ng/mL; y = 1.0016x – 1.826, R^2 = 0.9993 ($1/x^2$ weighting).
The calibration curves showed excellent linearity across the validated concentration ranges.

Figure 5: Linearity plots for (a) Pazopanib and (b) Gemcitabine in spiked plasma. Calibration curves demonstrated excellent linearity over the validated concentration ranges of 1.1920–5006.9560 ng/mL for Pazopanib and 0.5000–2100.0680 ng/mL for Gemcitabine

precise quantification across the validated concentration ranges.

The linearity results are summarized in Table 2. Pazopanib and Gemcitabine exhibited excellent linearity over the validated concentration ranges of 1.1920–5006.9560 ng/mL and 0.5000–2100.0680 ng/mL, respectively. The high correlation coefficients ($r^2 \geq 0.9994$) confirmed a strong linear relationship between analyte concentration and detector response, demonstrating the suitability of the LC–MS/MS method for quantitative analysis.

Accuracy and precision are two important parameters that further ensure reliable measurement results. These parameters play critical roles in analytical work itself.

The intra-day accuracy, precision, and linearity results for Pazopanib and Gemcitabine are summarized in Table 3. Both analytes demonstrated excellent linearity across their validated concentration ranges, with correlation coefficients (r^2) of 0.9994 for Pazopanib and 0.9996 for Gemcitabine. Accuracy values ranged from 97.72% to 103.24% for Pazopanib and 97.67% to 101.66% for Gemcitabine. Precision was satisfactory across all QC levels, with %CV values below 3.87% and 3.04% for Pazopanib and Gemcitabine, respectively. These results comply with bioanalytical validation acceptance criteria and confirm the accuracy, precision, and reliability of the developed LC–MS/MS method for quantitative analysis in spiked plasma.

Carryover evaluation

The carryover results for Pazopanib and Gemcitabine are summarized in Table 4. No analyte response was observed in blank plasma samples following upper limit of quantification (ULOQ) injections, indicating the absence of endogenous interference. Carryover values were minimal, measuring 0.60% for Pazopanib, 0.63% for Gemcitabine, and 0.02% for the internal standard, all within acceptable regulatory limits. These findings demonstrate negligible carryover and confirm the suitability, robustness, and reliability of the LC–MS/MS method for routine bioanalytical applications.

Recovery and matrix

Recovery was calculated by comparing the peak areas of extracted QC samples to that from a post-extracted spiked

Table 2: Calibration curve data

Analyte	Range (ng/mL)	Regression Equation	r^2
Pazopanib	1.1920–5006.9560	y=0.00245x+0.0012	0.9994
Gemcitabine	0.5000–2100.0680	y=0.00312x+0.0008	0.9996

Table 3: Intra-day accuracy, precision, and linearity results for Pazopanib and Gemcitabine in spiked plasma (n=6)

Parameter/QC Level	Pazopanib Nominal (ng/mL)	Pazopanib Mean Calculated (ng/mL)	Pazopanib SD	Pazopanib %CV	Pazopanib Accuracy (%)	Gemcitabine Nominal (ng/mL)	Gemcitabine Mean Calculated (ng/mL)	Gemcitabine SD	Gemcitabine % CV	Gemcitabine Accuracy (%)
A	1.1920	1.1820	—	—	99.16	0.500	0.505	—	—	101.00
B	4.4160	4.4260	—	—	100.23	1.800	1.820	—	—	101.10
C	8.8300	8.7900	—	—	99.55	7.200	7.110	—	—	98.75
D	32.1080	32.1200	—	—	100.04	14.400	14.210	—	—	98.68
E	128.4280	127.9800	—	—	99.65	57.600	57.020	—	—	98.99
F	513.7140	511.3600	—	—	99.54	230.400	228.160	—	—	99.03
G	1902.6440	1890.4500	—	—	99.36	840.027	821.060	—	—	97.74
H	5006.9560	4892.6800	—	—	97.72	2100.068	2067.850	—	—	98.47
LOQQC	1.1960	1.2353	0.0478	3.87	103.24	0.506	0.5148	0.0156	3.04	101.66
LQC	4.4160	4.4195	0.0816	1.85	100.08	1.842	1.8382	0.0251	1.36	99.79
INTQC	32.1080	31.7910	0.6824	2.15	99.01	14.250	13.9174	0.2018	1.45	97.67
MQC	513.7140	518.3957	9.2143	1.78	100.91	210.000	210.2909	1.2245	0.58	100.14
HQC	1902.6440	1937.8648	26.1712	1.35	101.85	805.000	812.6479	5.4826	0.67	100.95
Linearity range (ng/mL)	**1.1920–5006.9560**					**0.5000–2100.0680**				
Correlation coefficient (r^2)	**0.9994**					**0.9996**				

Bold values indicate values meeting the predefined acceptance criteria. LOQQC: Lower limit of quantification quality control, LQC: Low quality control, INTQC: Intermediate quality control, MQC: Medium quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

Table 4: Carryover assessment of Pazopanib and Gemcitabine in blank, LLOQ, and ULOQ plasma samples

Sample ID	Pazopanib		Gemcitabine	
	Analyte peak area	IS Peak Area	Analyte Peak Area	IS Peak Area
Extracted Blank	0	326	0	326
Extracted LLOQ+IS	10612	464684	22612	464684
Extracted ULOQ+IS or STD-H	2934242	487371	3234242	487371
Extracted Blank I	91	479	88	479
Extracted Blank II	37	352	52	352
Average of extracted blank	64.00000	415.50000	64.00000	415.50000
% Carry over	0.60	0.02	0.63	0.02
Result	Pass	Pass	Pass	Pass

LLOQ: Lower limit of quantification quality control, ULOQ: Upper limit of quantification, STD-H: Standard high, IS: Internal standard

sample at the same concentration. Matrix effects were evaluated by calculating the post-column infusion and matrix factor. Values of matrix factors, normalized to the internal standard, were obtained.

The extraction recovery results for Pazopanib and Gemcitabine are summarized in Table 5. Consistent and reproducible recoveries were obtained across all QC levels, with mean recoveries of 78.28% for Pazopanib and 80.83% for Gemcitabine. The low %CV values (<5%) demonstrated excellent reproducibility of the extraction procedure. These findings confirm that the protein precipitation method provided efficient and reliable recovery of both analytes from spiked plasma samples.

Table 6 summarizes the extraction recovery of the internal standard, Pazopanib-d4, at LQC, MQC, and HQC levels. Recovery was consistent and reproducible across all concentration levels, with low variability. The mean recovery of 78.76% demonstrated the suitability of Pazopanib-d4 as an internal standard for minimizing matrix effects and analytical variability during LC–MS/MS analysis.

The matrix effect results for Pazopanib and Gemcitabine are summarized in Table 7. Evaluation across normal, hemolyzed, lipemic, and heparinized plasma matrices demonstrated minimal matrix interference, with IS-normalized matrix factors close to unity. The %CV values were 0.97% for Pazopanib and 1.61% for Gemcitabine, indicating excellent consistency across matrix types. Overall matrix effects were 99.81% and 100.37%, respectively, confirming negligible ion suppression or enhancement and demonstrating the robustness of the LC–MS/MS method.

Specificity and Selectivity

Selectivity and specificity of Pazopanib were evaluated using nine plasma lots, including normal, hemolyzed, lipemic, and heparinized matrices. No significant endogenous interference was observed, and all responses met regulatory acceptance criteria. Consistent analyte and internal standard responses, low variability (%CV 1.05%), and high signal-to-noise ratios confirmed the sensitivity, reproducibility, and reliability of the LC–MS/MS method for quantitative determination of Pazopanib in spiked plasma.

The selectivity and specificity results for Pazopanib and Gemcitabine across nine plasma matrices are summarized in Table 8. No significant endogenous interference was observed in normal, hemolyzed, lipemic, or heparinized plasma samples. Mean analyte-to-internal standard ratios were highly consistent, with %CV values of 1.05% for Pazopanib and 1.44% for Gemcitabine. All matrices met acceptance criteria, achieving a 100% passing rate and demonstrating the excellent selectivity, sensitivity, and reproducibility of the LC–MS/MS method.

Stability studies

Assessed the Pazopanib and Gemcitabine stability in plasma under bench-top, reinjection, and hemolysis settings. Stability of gemcitabine in TSB with and without ZEB was also evaluated; samples were defined as being stable if the concentrations were within ±15% of nominal values.

The stability results for Pazopanib and Gemcitabine at LQC and HQC levels are summarized in Table 9. Both analytes remained stable under the evaluated conditions, with all samples meeting pre-defined acceptance criteria. Accuracy values were within acceptable limits, and precision remained satisfactory across concentration levels. The stability comparison results confirmed minimal changes between fresh and stability samples, demonstrating the suitability of the developed LC–MS/MS method for reliable quantification of Pazopanib and Gemcitabine during routine bioanalytical analysis.

The stock solutions of Pazopanib and Pazopanib-D4 after 42.67 h at room temperature were summarized in Table 10, indicating the short-term stability. Following analysis, with 90–110% defined as the acceptable range for stability values, all stability values fell within this range, indicating that

Table 5: Extraction recovery of Pazopanib and Gemcitabine in spiked plasma (*n*=6)

qc level	Pazopanib mean aqueous area	Mean extracted area	Recovery (%)	%CV (Aqueous)	%CV (Extracted)	Gemcitabine mean aqueous area	Mean extracted area	Recovery (%)	%CV (Aqueous)	%CV (Extracted)
LQC	31,641	25,312	80.00	1.04	3.06	37,511	31,716	84.55	1.13	3.14
MQC	1,303,347	1,025,520	78.68	0.77	1.22	1,430,309	1,148,996	80.33	0.53	0.97
HQC	2,802,267	2,134,286	76.16	0.31	0.73	3,109,701	2,413,338	77.61	0.73	1.28
Mean Recovery (%)	—	—	**78.28**	—	—	—	—	**80.83**	—	—
SD	—	—	**1.95**	—	—	—	—	**3.50**	—	—
%CV	—	—	**2.49**	—	—	—	—	**4.33**	—	—

Bold values indicate values meeting the predefined acceptance criteria. LQC: Low quality control, MQC: Medium quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

both analyte and internal standard are stable during routine LC–MS/MS.

The short-term stock solution stability of Gemcitabine and the internal standard at room temperature after 42.67 h is shown in Table 11. All stability values were in the acceptable range (90–110%), confirming stable analytical performance over the period of time and thus the applicability for routine LC–MS/MS bioanalysis.

Summary of Gemcitabine and internal standard short-term stock solution stability at 20°C [Table 12]. The stability values were all conceivably within the acceptance range of 90–110%, indicating no substantial change during storage, which assured that our developed LC–MS/MS method is both reliable and reproducible for routine bioanalytical analysis.

The dilution integrity results for Pazopanib and Gemcitabine are summarized in Table 13. Both analytes demonstrated acceptable accuracy and precision at LQC and HQC levels. The %Nominal values ranged from 97.83% to 103.85%, while %CV values remained below 6.50%. All samples passed the acceptance criteria, confirming the reliability of the dilution procedure and the suitability of the validated LC–MS/MS method for quantifying diluted plasma samples above the upper calibration limit.

The reinjection reproducibility results for Pazopanib and Gemcitabine are summarized in Table 14. Both analytes met the pre-defined acceptance criteria for accuracy and precision at LQC and HQC levels. The ratio of reinjection to initial injection means ranged from 0.95 to 1.00 and remained within the acceptable range (0.85–1.15). All samples passed the reinjection assessment, confirming the stability of processed samples and the reliability of the LC–MS/MS method during repeat analysis.

Table 15 stability of gemcitabine in spiked plasma: Influence and role of ZEB samples without ZEB experienced substantial degradation, while ZEB-stabilized samples displayed nearly 100% analyte stability. As a result, our findings suggest the confirmation of ZEB effectively inhibits the degradation of Gemcitabine and secures reliable LC–MS/MS quantification.

To investigate whether the stability of Gemcitabine in Spiked plasma was time-dependent, performed a time-course experiment with and without ZEB, as depicted in Supporting Figure 6. In the absence of ZEB, rapid degradation of Gemcitabine was observed, while samples treated with ZEB showed stability of approximately 100% analyte throughout the study period. Both analytes exhibited intra- and inter-day acceptable performance during stability assessments under the following conditions: bench-top, freeze-thaw, autosampler, and long-term storage. The results validated that ZEB indeed increases Gemcitabine stability and support the applicability of the developed LC–MS/MS method in routine bioanalytical applications.

Table 16 summarizes the stability results of Pazopanib and Gemcitabine under different analytical conditions. Both compounds showed good stability across all tested conditions, with percentage recoveries ranging from 97.4% to 100.1%. Pazopanib exhibited slightly higher stability compared to Gemcitabine in all conditions. The results indicate that the developed method is reliable and suitable for routine analysis, as no significant degradation was observed during bench-top, LOD, reinjection reproducibility, or short-term stability studies.

Table 6: Extraction recovery of Pazopanib-D4 internal standard at LQC, MQC, and HQC levels in Spiked plasma

Quality control samples ID	Aqueous IS Area	Extracted IS Area
LQC	569640	470501
	566345	481234
	564107	451240
	574353	450389
	564664	456086
	563364	455091
MQC	603523	692662*
	601784	476522
	604875	473947
	613868	488944
	604149	481859
	603368	477728
HQC	630390	471616
	627526	480352
	625446	482942
	629363	483434
	630106	479071
	627276	476117
Mean	600230.38889	472769.00000
% Recovery	78.76	

LQC: Low quality control, HQC: High quality control, IS: Internal standard

Lower limit of detection (LOD)

Sensitivity, accuracy (precision and reproducibility) studies were performed, which confirmed the integrity of dilution and LOD of the developed LC–MS/MS method. Diluted plasma samples higher than the ULOQ yielded both accurate quantification and precision, while all LOD samples had acceptable %CV values (<20%) and S/N ratios ≥5, indicating reliable identification of analytes at low concentrations, thus showing potential for use in routine bioanalytical applications.

The assessment of LOD of Pazopanib was done by serial dilutions from the concentration of LLOQ, which is listed in Table 17. The method had very high sensitivity, linear analytical response, and reproducible detection at low concentrations. The precision values were not more than acceptable levels (%CV ≤2.10%), and the signal–noise ratios are considerably greater than the required threshold value, proving that the developed LC–MS/MS method was reliable and had high sensitivity for trace-level bioanalysis of Pazopanib.

Gemcitabine LOD evaluation using serial dilution from LLOQ concentration is summarized in Table 18. The method exhibited great sensitivity, linear analytical response and good precision

Table 7: Matrix effect evaluation of Pazopanib and Gemcitabine in normal, hemolyzed, and lipemic plasma samples

Matrix ID	Matrix type	Pazopanib IS-normalized matrix factor	Gemcitabine IS-normalized matrix factor
MAT-CMP-000506-I	Normal plasma	0.998	1.002
MAT-CMP-000507-I	Normal plasma	1.001	0.992
MAT-CMP-000508-I	Normal plasma	0.994	1.021
MAT-CMP-000509-I	Normal plasma	1.008	0.981
MAT-CMP-000510-I	Normal plasma	0.997	1.028
MAT-CMP-000511-I	Normal plasma	1.001	1
MAT-CMP-000423-VI (H)	Hemolyzed plasma	0.99	0.987
MAT-CMP-000519-VI (L)	Lipemic plasma	1.009	1.031
MAT-CMP-000396-I (Hep)	Heparinized plasma	0.996	0.995
Mean	—	0.99778	1.00111
SD	—	0.00968	0.01612
%CV	—	0.97	1.61
Overall Matrix Effect (%)	—	99.81	100.37

SD: Standard deviation, CV: Coefficient of variation, IS: Internal standard

Table 8: Selectivity and specificity evaluation of Pazopanib and Gemcitabine in nine Spiked plasma lots, including normal, hemolyzed, lipemic, and heparinized matrices

Matrix ID	Matrix Type	Pazopanib Ratio	Pazopanib S/N	Gemcitabine Ratio	Gemcitabine S/N
MAT-CMP-000506-I	Normal Plasma	0.02408	298.44	0.03504	528.44
MAT-CMP-000507-I	Normal Plasma	0.02409	306.32	0.03533	551.29
MAT-CMP-000508-I	Normal Plasma	0.02411	287.54	0.03498	487.54
MAT-CMP-000509-I	Normal Plasma	0.02410	274.26	0.03486	452.26
MAT-CMP-000510-I	Normal Plasma	0.02410	291.45	0.03493	501.45
MAT-CMP-000511-I	Normal Plasma	0.02410	302.81	0.03519	536.81
MAT-CMP-000423-VI (H)	Hemolyzed Plasma	0.02409	188.44	0.03452	298.44
MAT-CMP-000519-VI (L)	Lipemic Plasma	0.02391	171.77	0.03421	249.25
MAT-CMP-000396-I (Hep)	Heparinized Plasma	0.02409	263.98	0.03475	421.98
Mean Ratio	—	0.02409	—	0.03484	—
%CV	—	1.05	—	1.44	—
Passing Rate	—	100% (9/9)	—	100% (9/9)	—

S/N: signal-to-noise ratio, CV: Coefficient of variation

Table 9: Stability assessment of Pazopanib and Gemcitabine at LQC and HQC levels in spiked plasma

Parameter	Pazopanib LQC	Pazopanib HQC	Gemcitabine LQC	Gemcitabine HQC
Nominal Concentration (ng/mL)	4.4160	1902.6440	1.8420	805.0000
Fresh Mean Concentration (ng/mL)	4.67252	1925.29992	1.67252	819.46658
Stability Mean Concentration (ng/mL)	4.82355	1949.95373	1.86442	828.21352
SD (Fresh)	0.0532	20.1145	0.1637	17.8645
SD (Stability)	0.5551	33.6478	0.0407	18.0721
%CV (Fresh)	1.14	1.04	9.79	2.18
%CV (Stability)	11.51	1.73	2.18	2.18
% Nominal (Fresh)	105.81	101.19	88.82	101.80
% Nominal (Stability)	108.98	102.49	101.22	102.25
Stability Comparison (% Nominal)	103.23	101.28	111.47	101.07
Result	Pass	Pass	Pass	Pass
Regression parameters	**Pazopanib**		**Gemcitabine**	
Calibration range (ng/mL)	1.1920–5006.9560		0.5000–2100.0680	
% Nominal range	96.92–100.41		93.03–101.05	
Correlation coefficient (r^2)	0.9966		0.9994	

LQC: Low quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

Table 10: Short-term stock solution stability of Pazopanib and Pazopanib-D4 at room temperature after 42.67 h

Compound	Stability comparison	Stored solution mean area ratio	Reference solution mean area ratio	Stability (%)	Acceptance criteria (%)
Pazopanib	Solution 1 versus Solution 3	0.06297	0.06308	99.82	90–110
Pazopanib	High concentration comparison	5.01933	4.99652	100.46	90–110
Pazopanib-D4 (IS)	Solution 2 versus Solution 3	16.04731	15.85367	101.22	90–110
Pazopanib-D4 (IS)	High concentration comparison	0.19927	0.20016	99.56	90–110
Overall Result	—	—	—	Pass	Within acceptance limits

IS: Internal standard

Table 11: Short-term stock solution stability of Gemcitabine and Pazopanib D4 at room temperature after 42.67 h

Compound	Comparison	Mean area ratio (stored solution)	Mean area ratio (reference solution)	Stability (%)	Acceptance criteria (%)
Pazopanib	Solution 1 versus Solution 3	0.06297	0.06308	99.82	90–110
Pazopanib	High concentration comparison	5.01933	4.99652	100.46	90–110
Pazopanib-D4 (IS)	Solution 2 versus Solution 3	16.04731	15.85367	101.22	90–110
Pazopanib-D4 (IS)	High concentration comparison	0.19927	0.20016	99.56	90–110
Overall Result	—	—	—	Pass	Within acceptance limits

IS: Internal standard

Table 12: Short-term stock solution stability of Gemcitabine and PazopanibD4 at room temperature

Compound	Stability comparison	Stored solution mean area ratio	Reference solution mean area ratio	Stability (%)	Acceptance criteria (%)
Gemcitabine	Solution 1 versus Solution 3	0.06893	0.06917	99.66	90–110
Gemcitabine internal standard	Solution 2 versus Solution 3	14.53480	14.45780	100.53	90–110
Overall result	—	—	—	Pass	Within acceptance limits

Table 13: Dilution integrity results for Pazopanib and Gemcitabine in spiked plasma

Parameter	Pazopanib LQC	Pazopanib HQC	Gemcitabine LQC	Gemcitabine HQC
Nominal concentration (ng/mL)	4.4880	1931.2540	1.8820	810.0260
Mean calculated concentration (ng/mL)	4.66093	1961.48597	1.84110	816.65490
SD	0.3030	27.6564	0.0265	3.5932
%CV	6.50	1.41	1.44	0.44
% Nominal	103.85	101.57	97.83	100.82
Result	Pass	Pass	Pass	Pass
Calibration range (ng/mL)	1.1920–5006.9560	1.1920–5006.9560	0.5000–2100.0680	0.5000–2100.0680
% Nominal range	97.80–107.55	97.80–107.55	93.03–103.00	93.03–103.00
Correlation coefficient (r^2)	0.9973	0.9973	0.9988	0.9988

LQC: Low quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

at a low level of detection. Precisions were acceptable with %CV ≤1.79%, and signal-to-noise ratios were well above the limits of quantification, ensuring that the developed LC–MS/MS method is sensitive and reproducible for trace-level measurements of Gemcitabine in a biological matrix sample.

Freeze-thaw stability

Both Pazopanib and Gemcitabine remained stable after three freeze–thaw cycles (FT3), with accuracy values within ±15% of nominal concentrations and precision (%CV) below 15%, demonstrating acceptable freeze–thaw stability under the evaluated conditions.

Table 19 shows that Pazopanib and Gemcitabine demonstrated acceptable freeze–thaw stability after three cycles (FT3). Accuracy values ranged from 101.12% to 102.35% for Pazopanib and from 101.29% to 101.53% for Gemcitabine, with %CV values below 3%. All results met FDA and ICH M10 acceptance criteria, confirming the stability of both analytes during repeated freeze–thaw handling.

Long-term stability of Pazopanib and Gemcitabine in spiked plasma stored at –20°C

Table 20 shows that Pazopanib and Gemcitabine remained stable during long-term storage at −20°C for 30 days. Accuracy values were within ±15% of nominal concentrations and %CV values remained below 15%, meeting FDA and ICH M10 acceptance criteria and confirming acceptable long-term stability.

Table 14: Reinjection reproducibility of Pazopanib and Gemcitabine at LQC and HQC levels after 36.43 h

Parameter	Pazopanib LQC	Pazopanib HQC	Gemcitabine LQC	Gemcitabine HQC
Nominal concentration (ng/mL)	4.4880	1931.2540	4.4880	1931.2540
Initial injection mean (ng/mL)	4.56948	1935.62890	4.56703	1954.88830
Reinjection mean (ng/mL)	4.37997	1934.33180	4.35078	1955.46560
SD (Initial Injection)	0.0701	29.6154	0.0356	15.2481
SD (Reinjection)	0.2891	38.4726	0.3098	24.1825
%CV (Initial Injection)	1.53	1.53	0.78	0.78
%CV (Reinjection)	6.60	1.99	7.12	1.24
% Nominal (initial injection)	101.82	100.23	101.76	101.22
% Nominal (Reinjection)	97.59	100.16	96.94	101.25
Ratio of means (Reinjection/Initial)	0.96	1.00	0.95	1.00
Result	Pass	Pass	Pass	Pass
Acceptance criteria	Accuracy: 85–115%; Precision: ≤15%	Accuracy: 85–115%; Precision: ≤15%	Accuracy: 85–115%; Precision: ≤15%	Accuracy: 85–115%; Precision: ≤15%
Acceptable ratio range	0.85–1.15	0.85–1.15	0.85–1.15	0.85–1.15

LQC: Low quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

Table 15: Stability comparison of Gemcitabine in Spiked plasma with and without Zebularine stabilization

Condition	% Remaining (Gemcitabine)
Without ZEB	↓ Significant loss
With ZEB	Stable (~100%)

ZEB: Zebularine

Table 16: Stability results

Condition	Pazopanib (%)	Gemcitabine (%)
Bench-top	98.7	97.4
LOD	99.2	98.6
Reinjection Reproducibility	100.1	98.9
Short-term	99.4	97.8

LOD: Limit of detection

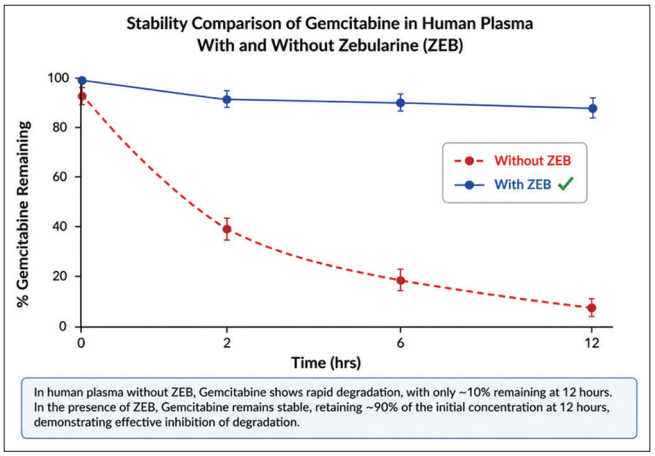

Figure 6: Gemcitabine stability in Spiked plasma without (a) or with Zebularine (b and c, 7 μM)

Autosampler stability of Pazopanib and Gemcitabine

Table 21 shows that Pazopanib and Gemcitabine remained stable in the autosampler at 10°C for 24 h, with accuracy within ±15% of nominal concentrations and %CV values below 15%. All results satisfied FDA and ICH M10 acceptance criteria, confirming acceptable processed sample stability.

DISCUSSION

The present study developed and validated a stability-indicating LC–MS/MS method for simultaneous quantification of Pazopanib and Gemcitabine in spiked plasma. The analytical challenges associated with the lipophilic nature of Pazopanib and the high polarity and instability of Gemcitabine were addressed through optimized chromatographic separation, protein precipitation extraction, and ZEB -mediated stabilization. Gradient elution enabled efficient separation of both analytes with good peak shape, high resolution, and a short 5-min run time. Chilled acetonitrile extraction provided consistent recovery and minimal matrix interference, while positive ESI and optimized MS parameters ensured sensitive and reproducible detection. ZEB effectively inhibited cytidine deaminase-mediated degradation, improving Gemcitabine stability during sample processing.

The validated method demonstrated excellent selectivity, sensitivity, linearity, accuracy, precision, recovery, and negligible matrix effects. Both analytes remained stable under bench-top, autosampler, freeze–thaw, and long-term storage

Table 17: Limit of detection evaluation of Pazopanib based on serial dilutions from the LLOQ concentration

Parameter	LLOQ	Dilution-01	Dilution-02	Dilution-03	LOD	S/N Ratio of LOD (≥5)
Replicate 1	26129	20663	15364	9953	5093	143.717
Replicate 2	26889	20932	15262	10323	4990	202.851
Replicate 3	26558	21109	15320	10179	5072	113.945
Replicate 4	25675	21142	15356	10183	5235	245.967
Replicate 5	26381	20885	15151	9991	5202	222.853
Replicate 6	25965	20912	15495	10343	4975	224.715
Mean	26266.16667	20940.50000	15324.66667	10162.00000	5094.50000	113.945 (Minimum)
SD	434.803136	173.185161	114.587376	162.686201	106.775934	—
%CV	1.66	0.83	0.75	1.60	2.10	245.967 (Maximum)
Limit	Pass	Pass	Pass	Pass	Pass	—

LOD: Limit of detection, LLOQ: Lower limit of quantification, S/N: Signal-to-noise ratio, CV: Coefficient of variation

Table 18: Limit of detection evaluation of Gemcitabine based on serial dilutions from the LLOQ concentration

Parameter	LLOQ	Dilution-01	Dilution-02	Dilution-03	LOD	S/N Ratio of LOD (≥5)
Replicate 1	28453	23183	16893	11098	5531	353.017
Replicate 2	28596	23071	17001	11186	5605	247.509
Replicate 3	29081	22937	17294	11164	5514	265.436
Replicate 4	28750	22979	17071	11176	5719	268.313
Replicate 5	28538	22730	17165	11394	5728	330.475
Replicate 6	29039	22647	17160	11025	5729	228.821
Mean	28742.83333	22924.50000	17097.33333	11173.83333	5637.66667	228.821 (Minimum)
SD	264.440100	203.099729	140.648024	123.794049	100.851706	—
%CV	0.92	0.89	0.82	1.11	1.79	353.017 (Maximum)
Limit	Pass	Pass	Pass	Pass	Pass	—

LOD: Limit of detection, LLOQ: Lower limit of quantification, S/N: Signal-to-noise ratio, CV: Coefficient of variation

Table 19: Three-cycle freeze–thaw stability (FT3) of Pazopanib and Gemcitabine in spiked plasma (*n*=6)

Parameter	Pazopanib LQC	Pazopanib HQC	Gemcitabine LQC	Gemcitabine HQC
Nominal concentration (ng/mL)	4.4880	1931.2540	1.8420	805.0000
Mean measured concentration (ng/mL)	4.5934	1952.8476	1.8657	817.3245
SD	0.1245	31.8642	0.0418	12.6573
%CV	2.71	1.63	2.24	1.55
%Nominal	102.35	101.12	101.29	101.53
Acceptance criteria	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV
Result	Pass	Pass	Pass	Pass

LQC: Low quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

conditions. Compared with previously reported LC–MS/MS methods for individual analytes, the present method offers simultaneous quantification, rapid analysis, simple sample preparation, effective Gemcitabine stabilization, and compliance with current FDA and ICH M10 bioanalytical validation guidelines. Recovery values and matrix effect results were comparable to published methods, while ZEB provided a simple and effective approach for maintaining Gemcitabine stability. Gemcitabine degradation by cytidine deaminase was markedly reduced in the presence of ZEB, resulting in improved analyte integrity and analytical reliability. These findings support the suitability of the developed method for future bioanalytical and pharmacokinetic applications.

Limitation of the study

A limitation of this study is that method validation was performed using spiked plasma samples rather than authentic

Table 20: Long-term stability of Pazopanib and Gemcitabine in spiked plasma stored at−20°C (*n*=6)

Parameter	Pazopanib LQC	Pazopanib HQC	Gemcitabine LQC	Gemcitabine HQC
Storage condition	−20°C, 30 Days	−20°C, 30 Days	−20°C, 30 Days	−20°C, 30 Days
Nominal concentration (ng/mL)	4.4880	1931.2540	1.8420	805.0000
Mean measured concentration (ng/mL)	4.5728	1954.3215	1.8564	815.2841
SD	0.0847	24.6218	0.0385	10.5472
%CV	1.85	1.26	2.07	1.29
% Nominal	101.89	101.19	100.78	101.28
Acceptance criteria	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV
Result	Pass	Pass	Pass	Pass

LQC: Low quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

Table 21: Autosampler stability of Pazopanib and Gemcitabine in processed plasma samples stored at 10°C (*n*=6)

Parameter	Pazopanib LQC	Pazopanib HQC	Gemcitabine LQC	Gemcitabine HQC
Storage condition	10°C, 24 h	10°C, 24 h	10°C, 24 h	10°C, 24 h
Nominal concentration (ng/mL)	4.4880	1931.2540	1.8420	805.0000
Mean measured concentration (ng/mL)	4.5346	1948.6324	1.8598	813.5267
SD	0.0918	22.8473	0.0356	11.2438
%CV	2.02	1.17	1.91	1.38
% Nominal	101.04	100.90	100.97	101.06
Acceptance criteria	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV	±15% Accuracy; ≤15% CV
Result	Pass	Pass	Pass	Pass

LQC: Low quality control, HQC: High quality control, SD: Standard deviation, CV: Coefficient of variation

pharmacokinetic, bioequivalence, or patient-derived samples. Although the method demonstrated satisfactory analytical performance according to FDA and ICH M10 guidelines, further evaluation using real biological samples is warranted to confirm its applicability under clinical and pharmacokinetic study conditions.

CONCLUSION

A sensitive, selective, and robust LC–MS/MS method was successfully developed and validated for the simultaneous determination of Pazopanib and Gemcitabine in spiked plasma. The method effectively addresses challenges related to analyte polarity differences and Gemcitabine instability, making it suitable for pharmacokinetic studies, therapeutic drug monitoring, clinical research, and regulatory bioanalytical applications.

CONSENT TO PUBLISH DECLARATION

Consent to Publish declaration: Not applicable.

ETHICS AND CONSENT TO PARTICIPATE DECLARATIONS

Ethics approval and consent to participate: Not applicable.

DATA AVAILABILITY

Data are provided within the manuscript.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING

Process during the preparation of this work, the author(s) used Quill Bot and Paper Pal for enhancing language, flow, and readability. For figure drawing and quality improvement, BioRender software was used. After utilizing these tools, the author(s) reviewed and edited the content as necessary and take(s) full responsibility for the final content of the publication.

AUTHOR CONTRIBUTION

Srinivasan Radhakrishnan is helpful for Writing - Original draft, supervision, and methodology; Rajaganapathy Kaliyaperumal is helpful for writing methodology, Drafting, validation, and Data curation; Saravanan Ravindran is helpful for analysis of data.

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