Original Article

A Sensitive Bioanalytical Method Development And Validation Of Pazopanib In Human Plasma By LC-ESI-MS/MS

Annapurna M.[1], Shanthi Priya D. K.[2,3*]

[1]Professor, Department of Pharmaceutical Analysis, GITAM Institute of Pharmacy, GITAM (Deemed to be) University, Visakhapatnam, Andhra Pradesh, India-530045.
[2]Research Scholar, GITAM Institute of Pharmacy, GITAM (Deemed to be) University, Visakhapatnam, Andhra Pradesh, India-530045.
[3]Assistant Professor, Department of Pharmaceutical Analysis, Anurag University, Venkatapur(V), Ghatkesar, (M), Ranga Reddy (D), Hyderabad, Telangana-500088.
***Corresponding Author Email:** sdarga@gitam.in
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Abstract

A simple, sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of Pazopanib in human plasma using Verapamil as an internal standard (IS). Chromatographic separation was performed on Water X Bridge c18 2.1x100 column with an isocratic mobile phase composed of acetonitrile and 0.2% formic acid in2mM Ammonium formate in the ratio of (70:30 v/v), at a flow-rate of 0.250mL/min. Pazopanib and verapamil were detected with parent ions at m/z 438.15 to 357.14 and the daughter mass was found to be 353.55 to 342.13 in multiple reaction monitoring (MRM) positive mode respectively. The protein precipitation method was used to extract the drug and IS. The method was validated over a linear concentration range of 1.0-1000.0 ng/mL with a correlation coefficient (r2) ≥ 0.9994. This method demonstrated Intra and inter-day Precision within 0.5 to 3.0 and 0.6 to 3.3 % and Accuracy within 95.55 to 106.32 and 94.62 to 112.6%. Pazopanib was found to be stable throughout Long-term stability studies, benchtop, and postoperative stability studies.

Keywords: Pazopanib, Verapamil, Internal standard, Flow rate

1. Introduction

Pazopanib is a small molecule inhibitor of multiple protein tyrosine kinases with potential antineoplastic activity. It is developed by GlaxoSmithKline and was FDA approved on October 19, 2009. The chemical name for Pazopanib hydrochloride is 5-[[4-[(2, 3-dimethylindazol-6-yl)-methyl amino] pyrimidin-2-yl] amino]-2-methylbenzenesulfonamide. It has a molecular formula $C_{21}H_{23}N_7O_2S.HCl$ and a molecular weight of 473.991. Pazopanib hydrochloride is used to treat advanced Kidney cell Carcinoma, and it is used to treat advanced soft tissue sarcoma that has been treated with other anticancer drugs. Pazopanib hydrochloride works by decreasing the blood supply to the cancer tumor to reduce tumor growth. Structures of Pazopanib & Verapamil are represented in the Fig. 1.

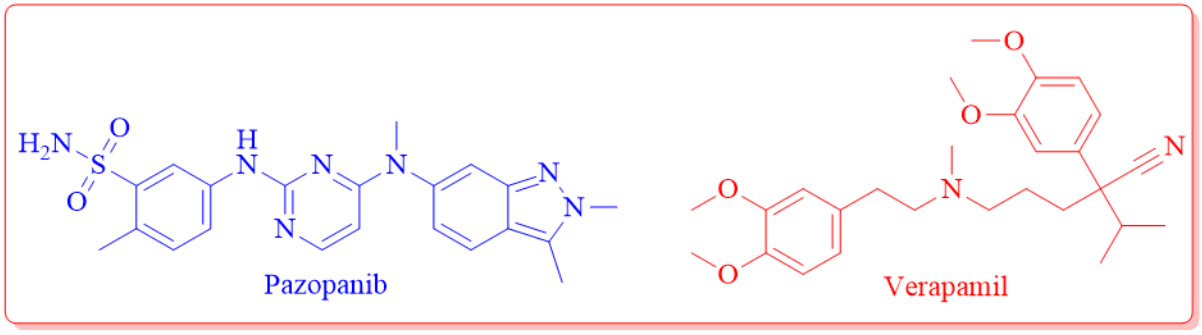

Fig. 1. Structures of Pazopanib and Verapamil

2. Materials and Methods

2.1 Materials and Reagents

Pazopanib and verapamil (Internal Standard) were procured from Fisher chemicals, Mumbai, India. Acetonitrile of HPLC grade was procured from Rankem Ltd., India. The water of HPLC grade was obtained from Merck Specialties Private Limited, Mumbai, India. In addition, ammonium formate and formic acid of HPLC grade were also procured from Merck Specialties Private Limited, Mumbai, India.

2.2 Instrumentation

An LC-MS/MS method was performed on a liquid chromatographic system consisting of a Waters Acquity UPLC system coupled with a Water Quattro Premier XE mass spectrometer with electrospray ionization (ESI) used for analysis and Mass Lynx 4.1 SCN 805 software for processing and data collecting. Agilent, Zorbx, and XDB C18 (2.1 x 50 mm ID, 5µm) are used as a stationary phase.

2.3 Standard solutions

Primary stock solutions of Pazopanib for preparation of standard calibration curve and quality control (QC) samples were prepared from separate weighing. The stock solution of Pazopanib (10 ng/mL) was prepared in acetonitrile and these stocks were stored at 2-8^{0}C. From these stock solutions, appropriate dilutions were made using acetonitrile, to produce working standard solutions of Pazopanib. A working concentration of the internal standard (10 ng/mL) solution was prepared in acetonitrile and refrigerated.

2.4 Preparation of calibration curve standards and quality control samples

A calibration curve consisting of a set of seven non-zero concentrations ranging from 1 to 1000 ng/mL of Pazopanib was prepared. The QC samples were prepared for Pazopanib 1.0 (LLOQ), 2.20 (LQC), 480 (MQC), and 780 ng/ mL (HQC). All the samples were incubated at 37^{0}C for subsequent use.

2.5 Sample processing

To 0.4 ml of plasma, add 20 ml of 10 mg/ml internal standard (Verapamil) and vortex for 30 sec, add 4 ml of Methanol and vortex for 3 min, by using a multi-pulse vortexer. Then 2 ml of the transparent organic layer from the supernatant is transferred into a 7.5 ml test tube and evaporated to dryness using Speed vapor at 400 C under a stream of nitrogen. Then the dried extract is reconstituted with 200 ml of diluent (Water: Acetonitrile 1:1) and a 20 ml aliquot is injected into the chromatographic system.

3. Method validation

The method was validated as per FDA guidelines. The fundamental parameters for this validation include (1) accuracy, (2) precision, (3) selectivity, (4) sensitivity, (5) reproducibility, and (6) stability

3.1 Selectivity

For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrices) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ)

3.2 Accuracy, Precision & Recovery

A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with un-extracted standards that represent 100% recovery.

3.3 Calibration Curve

A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen based on the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

3.4 Stability

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

3.5 Long-term stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

3.6 Stock solution stability

The stability of stock solutions of drugs and the internal standard should be evaluated at room temperature for at least 6 h. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

3.7 Benchtop Stability

It will be performed to evaluate the stability of the samples, which were kept on the bench during the extraction process. The anticipated time for the benchtop stability usually 4 to 24 hours should cover the duration of the time.

Table 1. Optimized method development parameters of Pazopanib

HPLC	Water Acquity UPLC
MASS	Water Quattro premier XE
ION SOURCE	Electrospray Ionization
COLUMN	Water X Bridge C18 2.1x100
COLUMN OVEN TEMPERATURE	30 ^{0}C

MOBILE PHASE	Acetonitrile:0.2% Formic acid in 2mM ammonium formate (70:30)
FLOW RATE	0.250 mL/min
VOLUME OF INJECTION	10 µL
RETENTION TIME	Pazopanib: 1.03 Verapamil:0.93

Table 2. Calibration curve samples of Pazopanib in human plasma

CC ID	**1 ng/mL**	**2 ng/mL**	**10 ng/mL**	**50 ng/mL**	**200 ng/ml**	**500 ng/mL**	**800 ng/mL**	**1000 ng/mL**
1	31	46	136	565	2166	5155	8016	10017
2	33	47	135	554	2158	5115	8020	10012
3	31	45	138	545	2145	5123	8058	10028
4	32	46	134	568	2114	5127	8064	10008
5	32	49	139	559	2122	5128	8011	10058
Mean	31.8	46.6	136.4	558.2	2141	5129.6	8033.8	10024
SD	0.837	1.15	2.07	9.14	22.47	15.093	25.12	20.11
%CV	2.63	3.25	1.52	1.64	1.05	0.29	0.31	0.2

4. Results and Discussion

4.1 Mass spectrometry

Mass parameters were tuned in positive ionization modes for the analytes. Good response was achieved in positive ionization mode. Data from the MRM mode were considered to obtain better selectivity. Deprotonated form analyte and IS, [MeH] e ion have the m/z value of 438.15 and 357.14 respectively and the daughter mass was found to be 353.55 and 342.13 for the analyte and IS respectively.

4.2 Method development

A series of trials were conducted using Acetonitrile and ammonium formate having different pH to obtain the required separations. After reviewing the results, ammonium formate was selected as the buffer, and acetonitrile was employed as an organic solvent. Different ratios of the buffer and acetonitrile were tried and finally the acetonitrile: Ammonium formate at 70:30 ratio was selected as an optimized mobile phase as it eluted a peak with good characteristics for both pazopanib as well as verapamil (internal standard). (Figures 2 & 3). The developed method gave an asymmetric peak at a retention time of 1.03 min for Pazopanib and 0.93 min for verapamil and satisfied all the peak properties as per USP guidelines.

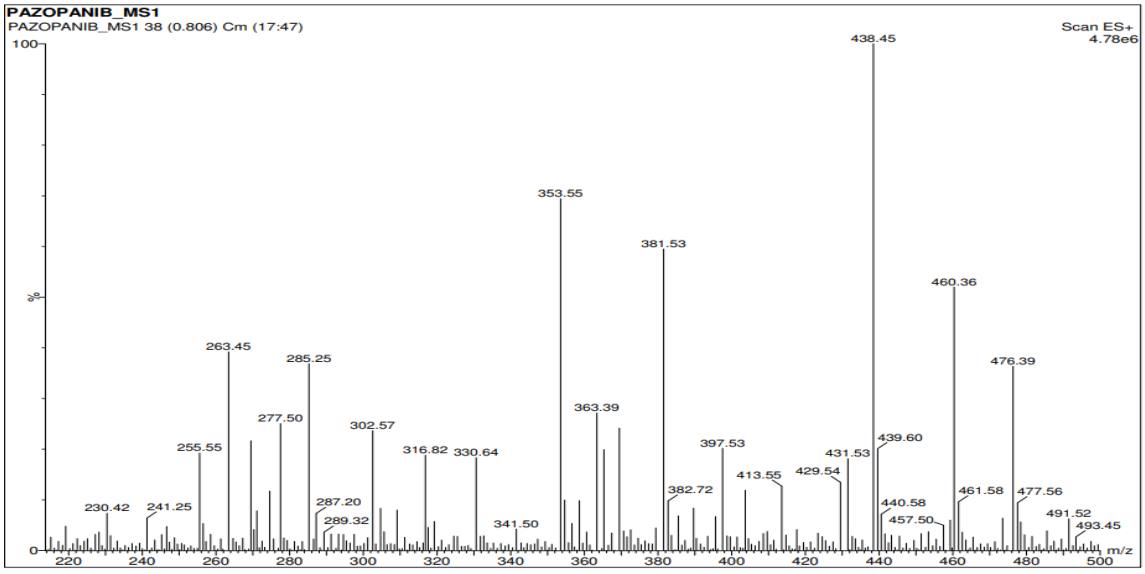

Figure 2. Mass spectra of Parent ion of Pazopanib

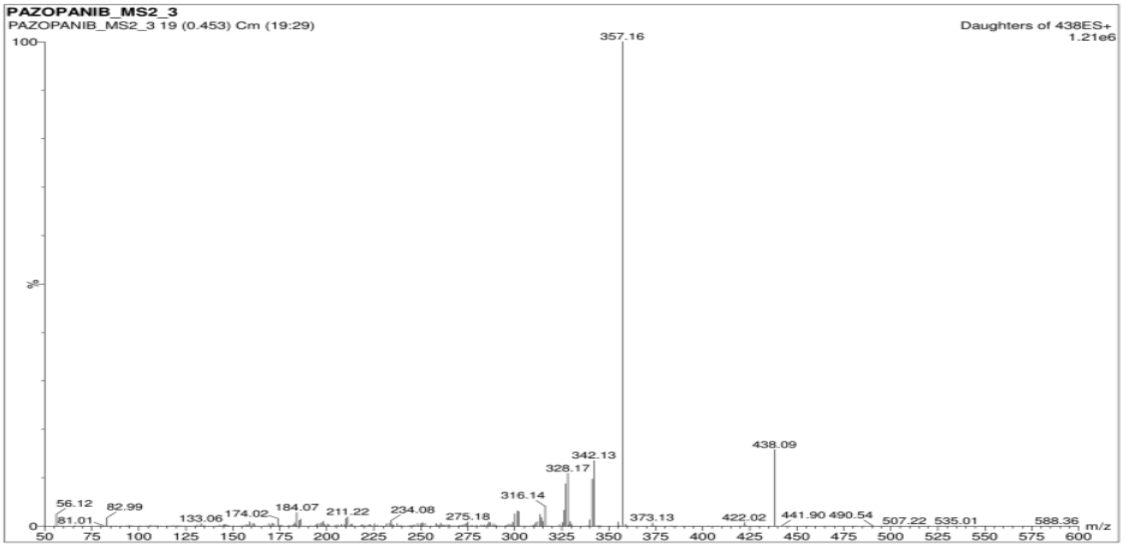

Figure 3. Mass spectra of Daughter ion of Pazopanib

4.3 Linearity

To establish the range of pazopanib concentrations that can be assayed by using the present method, eight different sets containing pazopanib concentrations from 1 to 1000 ng/ml were taken and analyzed. The area ratio obtained against each concentration is plotted against the amount of Pazopanib. A straight-line fit is made through the data points by the least square regression analysis and a constant proportionality is observed with minimal data scattering. The r2 value was consistently greater than 0.999 in all five cases. So, the Pazopanib can be successfully analyzed with the present system within this concentration.

4.4 Sensitivity

The lower limit of quantification is found to be 1 ng/ml for Pazopanib. The between run accuracy and precision for Pazopanib at 1 ng/ml were 93.5 % respectively.

4.5 Precision

Precision was checked by three concentrations of LQC, MQC, and HQC (2.25, 480,780 ng/ml) against a single linearity curve, and the assay values to the actual value expressed in percentage. It can be observed from these tables that the intra-day or with run accuracy ranged from 95.55 to 106.32%. The between run or inter-day or total accuracy ranged between 94.62 and 112.6%.

4.6 Recovery

The percentage recoveries were determined by measuring the peak area of prepared plasma validation samples at a concentration of 2.25, 480, and 780 ng/ml respectively. The peak areas of the validation samples were compared to the peak area of the extracted blank plasma spiked with standards containing the same area concentrations of the Pazopanib. Recovery for Pazopanib ranged between 99.26 and 102.32%.

4.7 Stability

Benchtop stability is measured since the plasma is taken out from the freezer and thawed. This study is necessary to avoid repeated access to deep freezers within shorter intervals. The thawed plasma samples are triplicated at each QC concentration are processed at 3-6 h and Pazopanib concentration is measured. From tables, 2 & 3 it can be seen that Pazopanib is highly stable in the present matrix. Since nearly 100.21, 101.25, and 100.05% concentrations are observed even after 6 hrs. In addition, the long-term stability of Pazopanib in QC samples after 105 days of storage at -30oC was also evaluated. The concentrations ranged from 94.6 to 101.8% of the theoretical values. These results confirmed the stability of pazopanib in human plasma for at least 105 days at -30 ^{0}C.

Table 3. Bench Top stability of Pazopanib

Sr. No.	**Concentration ng/mL**					
	LQC [2.00 ng/mL]		**MQC [480 ng/mL]**		**HQC [780 ng/mL]**	
	Comparison	**Stability**	**Comparison**	**Stability**	**Comparison**	**stability**
1	2.023	2.321	480.21	490.02	780.04	781.42
2	2.541	2.358	480.42	490.28	780.52	781.23
3	2.481	2.369	480.02	490.87	780.48	782.43
4	2.874	2.321	480.85	490.02	780.28	781.24
5	2.024	2.389	480.52	491.26	780.35	780.02
6	2.256	2.302	480.25	490.09	780.28	780.55
SD	0.0245	0.0248	0.1725	0.44523	0.0522	0.6212
% CV	1.29	2.87	0.04	0.09	0.01	0.08
% Difference	1.28		0.04		0.07	

Table: 4 Long-term stability of pazopanib

Sr. No.	**Concentration ng/mL**					
	LQC [2.00 ng/mL]		**MQC [480 ng/mL]**		**HQC [780 ng/mL]**	
	Comparison	**Stability**	**Comparison**	**Stability**	**Comparison**	**stability**
1	2.420	2.650	480.21	490.12	780.12	781.42
2	2.422	2.621	480.42	491.23	780.52	780.23
3	2.420	2.620	480.28	490.52	780.43	781.43
4	2.425	2.603	480.26	491.53	780.23	781.24
5	2.428	2.602	480.52	491.53	780.42	780.57
6	2.420	2.602	480.16	492.21	780.25	780.08
SD	0.02420	0.1121	0.1625	0.6023	0.06259	0.6325
% CV	1.50	4.62	0.04	0.14	0.01	0.385
% Difference	3.33		0.1		0.375	

5. Conclusion

Based on the data presented in this report, it can be included that the present method is validated for the estimation of Pazopanib in human plasma over a concentration range of 1.0-1000 ng/ml. The precision and accuracy are very much within the prescribed limits in this concentration range. Expected recoveries are observed in the present processing technique for LQC, MQC, and HQC. The drug is found to be very stable to the effect up to 6 h delay on the benchtop.

References

1. Lacy, Charles F, Armstrong, Lora L, Goldman, Morton P, Lance, Leonard L. Lexi-Comp's Drug Information Handbook (12th Edition). Lexi-Comp Inc. ISBN 1-59195-083-X, 2004.
2. http://www.drugbank.ca/system/fda_labels/DB0061 9.pdf?1265922812
3. http://en.wikipedia.org/wiki/Pazopanib hydrochloride nib
4. Gorja Ashok, Sumanta Mondal, S. Ganapaty and Jahnavi Bandla. Development and validation of stability indicating method for the Estimation of pazopanib hydrochloride in pharmaceutical dosage forms By RP-HPLC. Der Pharmacia Lettre, 2015; 7 (12): 234-241. IAJPS 2017
5. K. Vinodh Kumar, K. Puspha and D. Gowri Sankar. Development and validation of stability indicating RP-HPLC method for the determination of pazopanib hydrochloride in bulk drug and its pharmaceutical dosage form. Journal of Chemical and Pharmaceutical Research, 2015; 7(8):114-120.
6. Kishore Kumar Reddy Y., J. Raveendra Reddy, and N. Devanna. Development and validation of a stability-indicating RP-HPLC method for the determination of Pazopanib in bulk drug and its pharmaceutical dosage form. Journal of Chemical and Pharmaceutical Research, 2015; 7(9):127-132.
7. Ravi Pratap Pulla, Asadulla Khan, J. Venkateswara Rao, Suresh Kumar Sudam; K. Sujana. International Journal of Advances in Pharmaceutical Analysis. 2013; 3, 1, 24-29.
8. ICH, Q1A(R2) Stability Testing of New Drug Substances and Products (Geneva, Feb. 2003). 9. ICH, Q1B Stability Testing: Photostability Testing of New Drug Substances and Products (Geneva, Nov. 1996).
9. ICH, Q6A: Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (Geneva, Oct. 1999).
10. ICH, Q3A(R2) Impurities in New Drug Substances (Geneva, Oct. 2006).
11. ICH, Q3B(R2) Impurities in New Drug Products (Geneva, June 2006).
12. ICH, Validation of Analytical Procedures: Text and methodology Q2 (R1): International Conference on Harmonization, IFPMA, Geneva (2005).
13. A United States Pharmacopoeia (USP), XXVI. Validation of Compendial methods. United States Pharmacopeial Convention Inc.; Rockville, MD, USA: 2003.