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Research Article

HPLC–PDA ANALYSIS OF PAZOPANIB IN RABBIT PLASMA USING GEFITINIB AS INTERNAL STANDARD

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ABSTRACT
In the present investigation, a rapid, specific and sensitive isocratic HPLC method coupled with photodiode array detection (PDA) has been described for the assay of pazopanib in rabbit plasma using gefitinib as an internal standard. The pazopanib and internal standard gefitinib were extracted from rabbit plasma in a single step using acetonitrile. The analysis of pazopanib was performed on Hypersil ODS C18 (250 mm × 4.0 mm I.D., 5.0 µm particle size) column with a mobile phase, 0.01 M potassium dihydrogen orthophosphate (pH 3.6):acetonitrile (75:25, *v/v*) and UV detection set at 264 nm. The developed method was validated by evaluating system suitability, selectivity, sensitivity, linearity, precision, accuracy, ruggedness and stability in conformity with the guidelines of the United States Food and Drug Administration (FDA). The results of validation parameters were found to be within the acceptance limits. Hence, the developed and validated method can be utilized for the routine determination of pazopanib in plasma samples of rabbit.

Keywords: Pazopanib, Gefitinib, Plasma, HPLC and Analysis.

INTRODUCTION

Pazopanib (Fig. 1) is chemically described as 5-({4-[(2, 3-dimethyl-2*H*-indazol-6-yl) (methyl) amino] pyrimidin-2-yl} amino)-2-methylbenzene-1-sulfonamide. Pazopanib was approved by FDA for treating patients with advanced renal cell carcinoma and soft tissue sarcoma (who already received chemotherapy)[1,2]. Pazopanib exhibits antiangiogenic and antitumour effects through inhibiting multiple receptor tyrokinases[3,4]. Pazopanib is a potent and selective second-generation multi targeted tyrosine kinase inhibitor. Pazopanib inhibits key proteins responsible for tumor growth and angiogenesis such as vascular endothelial growth factor receptor -1, -2, -3, platelet-derived growth factor receptor -α, -β, cytokine receptor, fibroblast growth factor receptor -1, -3, interleukin-2 receptor inducible T-cell kinase, transmembrane glycoprotein receptor tyrosine kinase and leukocyte-specific protein tyrosine kinase.

Few analytical methods have been reported for the quantification of pazopanib. Chaitanya et al[5] and Susena et al[6] reported spectrophotometric methods for the assay of pazopanib in bulk and in tablet formulations. UPLC-MS/MS methods were proposed by Paludetto et al[7] and Qiu et al[8]. Paludetto et al[7] method was applied for the simultaneous quantification of pazopanib and its metabolites in plasma of patients treated with pazopanib. Qiu et al[8] method was applied to investigate the pharmacokinetics of pazopanib in rat plasma. Mukul et al[9] determined pazopanib in mouse plasma and brain tissue homogenate using LC-MS/MS. Verheijen et al[10] quantified pazopanib in a dried blood sample by LC-MS/MS.

HPLC technique is considered to be more suitable for analysis of drugs since it has advantages over the other techniques like minimal sample manipulation, rapid analysis and simultaneous quantification of multicomponent samples with excellent specificity, accuracy and precision. There are only five reports on the determination of pazopanib in pharmaceutical dosage forms using HPLC[11-15]. One HPLC method has also been presented for the assay of pazopanib in human plasma[16]. As per the knowledge of the authors, till now no HPLC method was reported for the determination of the pazopanib in rabbit plasma. Hence, in the present investigation simple, rapid, accurate, sensitive and precise HPLC method coupled photodiode array detector was developed for the determination of pazopanib in rabbit plasma using gefitinib as an internal standard. The developed method has been validated as per the guidelines of FDA[17].

EXPERIMENTAL

Chemicals and drugs

Reference drugs pazopanib and gefitinib are provided as gift samples by Spectrum Pharma Research Solutions, Hyderabad, India. HPLC grade acetonitrile and methanol, analytical reagent grade potassium dihydrogen phosphate and orthophosphoric acid were obtained from Merck Chemical Division, Mumbai, India. HPLC grade water obtained from Milli-Q water purification system, Bangalore, India was used throughout the study.

APPARATUS

Chromatographic separation and analysis of pazopanib was performed with Waters 2695 HPLC system provided with high speed auto sampler, column, oven, degasser and 2996 photodiode array detector. Chromatographic data were processed with class Waters Empower 2 software.

Mobile phase and diluent solution

The mobile phase used was a mixture of 0.01 M potassium dihydrogen orthophosphate (pH 3.6) and acetonitrile (75:25, *v/v*). 0.01 M potassium dihydrogen orthophosphate solution was prepared by dissolving 1.36 gm of potassium dihydrogen phosphate in 1000 ml of HPLC grade water in a 100 ml volumetric flask. The pH was adjusted to 3.6 by using dilute orthophosphoric acid. The diluent solution was prepared through mixing water and acetonitrile in 50:50 (*v/v*) ratio.

Chromatographic conditions

Chromatographic separation and analysis was carried out at temperature 30°C on a Hypersil ODS C18 (250 mm × 4.0 mm I.D., 5.0 µm particle size) column. The auto sampler temperature was maintained 5°C. The mobile phase was pumped at a flow rate of 1.0 ml/min. The detector wavelength was set at 264 nm, with injection volume at 10 µl. The total run time was 7 min.

Standard solutions of pazopanib and gefitinib

The stock standard solution of pazopanib (100 mg/ml) was prepared by dissolving 1000 mg of pazopanib in diluent solution in a 10 ml volumetric flask. Working standard solutions of pazopanib (230 µg/ml, 1150 µg/ml, 2300 µg/ml, 4600 µg/ml, 9200 µg/ml, 13800 µg/ml, 18400 µg/ml and 23000 µg/ml) were prepared by duly diluting the pazopanib stock standard solution with diluent. The internal standard (gefitinib) stock standard solution (1 mg/ml) was prepared by dissolving 10 mg of gefitinib in diluent in a 10 ml volumetric flask. Working standard solution of gefitinib (460 µg /ml) was prepared by properly diluting the gefitinib stock standard solution with diluent. These standard solutions were stored at 2^0C-8^0C in a refrigerator until analysis.

Spiked plasma calibration curve standards and quality control samples

Pazopanib (1 µg/ml to 100 µg/ml) and gefitinib (10 µg/ml) spiking solutions were prepared from working standard solutions of drugs. Calibration curve standards were prepared by spiking appropriate volume of working standard solution in drug free rabbit plasma (2.3 ml) to obtain 1.0, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100 µg/ml of pazopanib and 10 µg/ml of gefitinib. Four quality control samples with concentrations 1 µg/ml (LLOQ), 10 µg/ml (LQC), 40 µg/ml (MQC) and 80 µg/ml (HQC) pazopanib were prepared by spiking drug free rabbit plasma with appropriate volume of pazopanib working solution. In all the quality control samples, gefitinib is spiked at a concentration of 10 µg/ml. Calibration curve standards and quality control samples were stored at -20^0C.

Plasma sample processing

Liquid-liquid extraction technique was applied to extract pazopanib and gefitinib from plasma samples. The frozen calibration curve standards and quality control samples were thawed at room temperature and then homogenized using a vortex shaker. To 250 µl of spiked plasma samples, 2 ml of acetonitrile was added. The sample was mixed for 15

seconds in a cyclomixer. The mixture was vortexed for 2 min. Finally the mixture was centrifuged at 3200 rpm for 5 min. The organic layer obtained after centrifugation was collected and transferred into auto sampler vials for injection (10 µl) into the HPLC system.

Calibration graph

Ten µl of calibration curve standard solutions (1-100 µg/ml pazopanib) were injected thrice onto the column. The corresponding chromatograms were recorded using the chromatography conditions described. Concentration of the unknown was computed from the calibration curve or regression equation derived using the peak area ratio and concentration data.

RESULTS AND DISCUSSION

Method development

The present study was aimed at developing a rapid, selective and sensitive HPLC method coupled with photodiode array detection for the determination of pazopanib precisely and accurately at low concentrations in rabbit plasma. The chromatographic separation and analysis of pazopanib using gefitinib as internal standard was optimized after several trials using the Hypersil ODS C18 (250 mm × 4.0 mm I.D., 5.0 µm particle size) column. Mobile phase with different ratios of 0.01 M potassium dihydrogen orthophosphate and acetonitrile at various flow rates and different pH were tested in order to have suitable retention time, better resolution and sensitivity. The best results were achieved with 0.01 M potassium dihydrogen orthophosphate and acetonitrile in the ratio of 75:25 (*v/v*) with pH 3.6 and a flow rate of 1.0 ml/min. The sensitivity was good at a detection wavelength of 264 nm. Under the optimized conditions, pazopanib using gefitinib were eluted at 4.421 min and 3.852 min, respectively.

METHOD VALIDATION

The guidelines for bioanalytical method validation published by the United States Food and Drug Administration were followed for method validation.

System suitability

To assess system suitability, middle quality control (MQC) samples along with internal standard were injected into the HPLC system six times. The percent relative standard deviation (%RSD) for the peak area response and retention time of pazopanib and internal standard was calculated. The percent relative deviation value for peak area ratio was also determined. The results are summarized in Table 1. It was observed that the results were found to be within the acceptance criteria.

Auto sample carry over test

Auto sample carryover was performed to check whether the drug remains in system or not. The carryover test was estimated by injecting rabbit plasma blank sample after ULOQ sample (100 µg/ml) and LLOQ sample (1 µg/ml). Carryover in the blank sample after the ULOQ should not be >20% of the LLOQ for pazopanib and 5% for the internal standard. No response was found in blank plasma after injection of ULOQ and LLOQ, demonstrating no carryover of the pazopanib in subsequent runs. The chromatograms of blank plasma, ULOQ and LLOQ samples are shown in Fig. 2.

Specificity

Specificity was performed to demonstrate the absence of chromatographic interference from blank rabbit plasma components. Specificity was assessed using six blank plasma and LLOQ level (1 µg/ml) samples. The samples were checked for any interference of blank and sample response. The peak area of any interference peak should be ≤20% of the pazopanib peak area and ≤5% of the internal standard peak area. The peak area response in all the six blank plasma samples is zero. The results demonstrated the non interference from blank rabbit plasma components (Fig. 2). Hence the method is specific.

Matrix effect

Matrix effect was evaluated to quantify analyte with consistency. The matrix effect was checked in six different lots of plasma at LQC (10 µg/ml) and HQC (80 µg/ml) concentration levels. Each lot of plasma was analyzed three times. The overall mean percent accuracy was determined. As per acceptance criteria, at least 67% (2 out of 3) of samples should be within 80%-120%. Mean percent accuracy was 103.21% and 100.14% for LQC (10 µg/ml) and HQC (80 µg/ml) concentration levels, respectively. The results showed that the proposed method meet the acceptance limit.

Linearity

The method linearity was established by assaying calibration standards in rabbit plasma in triplicate. The calibration curve of pazopanib over the concentration range of 1–100 µg/ml was established by weighted ($1/x^2$) linear regression analysis. The typical equation of pazopanib calibration curve was: $y = 0.04915\ x - 0.01188$ ($R = 0.9987$) where y = ratio of the pazopanib peak area to that of

internal standard peak area, and x = concentration of pazopanib in plasma.

Sensitivity
To detect the lowest limit of detection of the method, sensitivity test was performed at LLOQ concentration (1 µg/ml) level. For this purpose, LLOQ samples were injected into the HPLC system six times. The mean accuracy and percent relative standard deviation was calculated. The mean accuracy of pazopanib at LLOQ level was 94.80% and % RSD was 4.72%. The values were found to be within the acceptance limits (mean accuracy - 80% to 120%; %RSD - ≤20%). Therefore, the proposed was sensitive.

Accuracy and precision
Accuracy and precision were established by analyzing quality control samples at four concentration levels (LLOQ, LQC, MQC and HQC) in six replicates on three validation runs. The percent recovery was used to assess accuracy and relative standard deviation was used to assess precision. The acceptable criterion of the inter-day and intra-day precision was ≤15 % for LQC, MQC and HQC, and ≤20% for LLOQ samples. The acceptable criterion for method accuracy was within ±15% for LQC, MQC and HQC, and ± 20% for LLOQ samples. The inter- and intra-day precision and accuracy results of four quality control samples were depicted in Table 2. The chromatograms of four quality control samples are shown in Fig. 3. The data obtained indicated the acceptable accuracy and precision for both intra-day and inter-day samples at all the four concentration levels assayed.

Analyte recovery
The analyte recovery of pazopanib was performed by assaying two sets of samples at three concentration levels (LQC, MQC and HQC). Pazopanib and internal standard were spiked into plasma before extraction (set A) and spiked into plasma after extraction (set B). Analyte recovery was determined by comparing the pazopanib and internal standard peak area obtained from set A samples with that from set B samples. The acceptance criterion was that the relative standard deviation of recovery at each quality control concentration level and for internal standard should be ≤15%. The results are summarized in Table 3. The values are within the acceptance limits. The method provided good extraction efficiency for pazopanib and internal standard gefitinib in rabbit plasma.

Ruggedness
Method ruggedness is evaluated by analyzing pazopanib for precision and accuracy at four quality control concentration (ULOQ, LQC, MQC and HQC) levels under a variety of test conditions, such as different analysts and different columns. The acceptable criterion of precision was ≤15% for LQC, MQC and HQC, and ≤20% for LLOQ samples. The acceptable criterion for accuracy was within ±15% for LQC, MQC and HQC, and ± 20% for LLOQ samples. Acceptable relative standard deviation and percent recovery values were acquired for different analysts and different columns Table 4. The results indicated the ruggedness of the method.

Stability of the sample
The stability of the pazopanib in rabbit plasma was evaluated under different study conditions; i.e. standing at room temperature over 24 h, storing at −28°C for one month (long-term stability) and storing at −80°C for one month (long-term stability). The results of the stability of pazopanib in rabbit plasma at diverse storage conditions were expressed as percentage recoveries and relative standard deviation. The percent stability of pazopanib stored -28°C and -80°C were assessed by comparing stability sample with freshly spiked samples. The stability studies were performed with LQC (10 µg/ml) and HQC (80 µg/ml) samples. Percent recovery should be within the range 85%-115% and percent relative standard deviation should be ≤15%. As shown in Table 5, the results are within the acceptance limits. The results indicated that pazopanib was stable for the complete period of analysis.

CONCLUSION
The developed HPLC method is an appropriate technique for the determination of pazopanib in rabbit plasma using gefitinib as internal standard. The method is simple and utilizes acetonitrile to precipitate the proteins as the only sample preparation step prior to analysis. The rapid (runtime 7 min), single step plasma preparation coupled with the HPLC-PDA isocratic chromatographic apparatus makes the method cost-effective and apt for analysis of a large number of samples. The developed method was proved to be sensitive, selective, rugged, precise and accurate in harmony with the FDA guidelines. The pazopanib was stable in the rabbit plasma placed at room temperature for 1 day and for 30 days when stored at −28°C and −80°C. As a result, the developed and validated HPLC coupled with PDA method can be used for routine analysis of pazopanib in rabbit plasma.

Further study on pharmacokinetics after oral administration pazopanib to rabbit will be carried out with this developed and validated method.

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Table 1: System suitability data for the analysis of pazopanib

Sample	Pazopanib		Internal standard		Peak area Ratio
	Peak area (mAU)	Retention time (min)	Peak area (mAU)	Retention time (min)	
MQC	201675	4.42	102365	3.85	1.9702
	201876	4.43	103265	3.85	1.9549
	203659	4.43	102879	3.86	1.9796
	202145	4.41	103458	3.84	1.9539
	201568	4.42	102398	3.86	1.9685
	203659	4.43	102478	3.87	1.9873
Mean*	**202430**	**4.424**	**102807**	**3.856**	**1.969**
% RSD	**0.480**	**0.184**	**0.458**	**0.257**	**0.673**
Acceptance criteria	**% RSD = ≤2.0%**	**% RSD = ≤2.0%**	**% RSD = ≤2.0%**	**% RSD = ≤2.0%**	**% RSD = ≤5.0%**

*Average of six determinations

Table 2: Precision and accuracy data for pazopanib in rabbit plasma

QCS* / Values	HQC (80 µg/ml)	MQC (40 µg/ml)	LQC (10 µg/ml)	LLOQ (1 µg/ml)
Intra-day precision and accuracy (Day 1, n=6)				
Mean calculated (µg/ml)**	78.736	40.011	9.480	1.010
RSD (%)	8.68	8.91	2.73	5.67
Recovery (%)	98.42	100.03	94.80	101.02
Intra-day precision and accuracy (Day 2, n=6)				
Mean calculated (µg/ml)**	82.136	41.051	10.042	1.015
RSD (%)	8.45	8.23	7.53	12.42
Recovery (%)	102.67	102.63	100.42	101.58
Intra-day precision and accuracy (Day 3, n=6)				
Mean calculated (µg/ml)**	81.279	41.619	10.082	0.970
RSD (%)	9.58	9.49	6.27	3.68
Recovery (%)	101.60	104.05	100.82	97.08
Inter-day precision and accuracy (n=18)				
Mean calculated (µg/ml)**	80.717	40.894	9.868	0.998
RSD (%)	8.58	8.52	6.29	8.04
Recovery (%)	100.90	102.24	98.68	99.89

* QCS – Quality control samples
** Mean of six determinations

Table 3: Recovery of pazopanib and internal standard in rabbit plasma

Quality control sample	Sample set	Pazopanib		Internal standard	
		Mean peak area (mAU)*	RSD (%)	Mean peak area (mAU)*	RSD (%)
HQC	Set A	413750.2	1.66	103247.5	1.60
	Set B	510808.8	1.15	135074.0	0.47
MQC	Set A	209881.7	2.22	104004.8	1.83
	Set B	314419.5	0.84	134346.2	1.68
LQC	Set A	51255.2	2.00	107175.5	2.41
	Set B	64061.8	2.89	134371.7	0.53

*Average of six determinations

Table 4: Ruggedness data on precision and accuracy for pazopanib in rabbit plasma

QCS* / Values	HQC (80 µg/ml)	MQC (40 µg/ml)	LQC (10 µg/ml)	LLOQ (1 µg/ml)
Different column				
Mean calculated (µg/ml)**	80.526	39.111	9.381	1.025
RSD (%)	7.12	4.13	6.46	9.19
Recovery (%)	100.66	97.78	93.82	102.55
Different analyst				
Mean calculated (µg/ml)**	83.959	40.372	9.642	0.974
RSD (%)	10.19	5.71	6.16	5.76
Recovery (%)	104.95	100.93	96.43	97.47

* QCS – Quality control samples
** Mean of six determinations

Table 5: Summary of stability of pazopanib in rabbit plasma under different storage conditions

Quality control sample	Concentration of pazopanib (µg/ml)		RSD (%)
	Nominal	Mean calculated*	
Stability at day zero			
HQC	80	79.978	11.15
LQC	10	9.887	10.13
Long term storage at -28°C			
HQC	80	81.622	9.94
LQC	10	9.579	7.51
Long term storage at -80°C			
HQC	80	80.725	9.43
LQC	10	9.205	5.93

*Average of six determinations

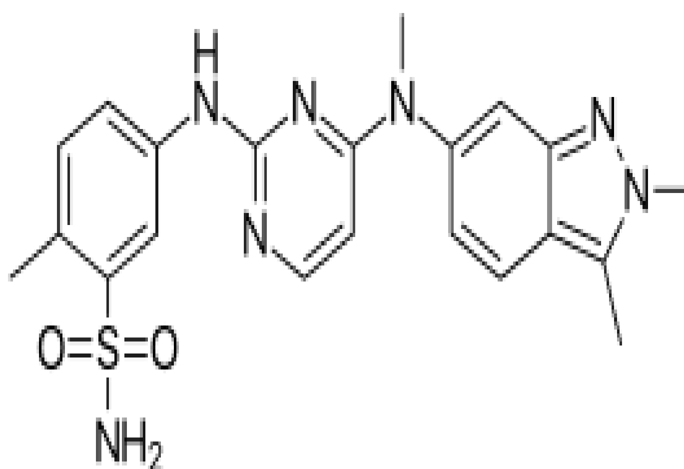

Fig. 1: Chemical structure of Pazopanib

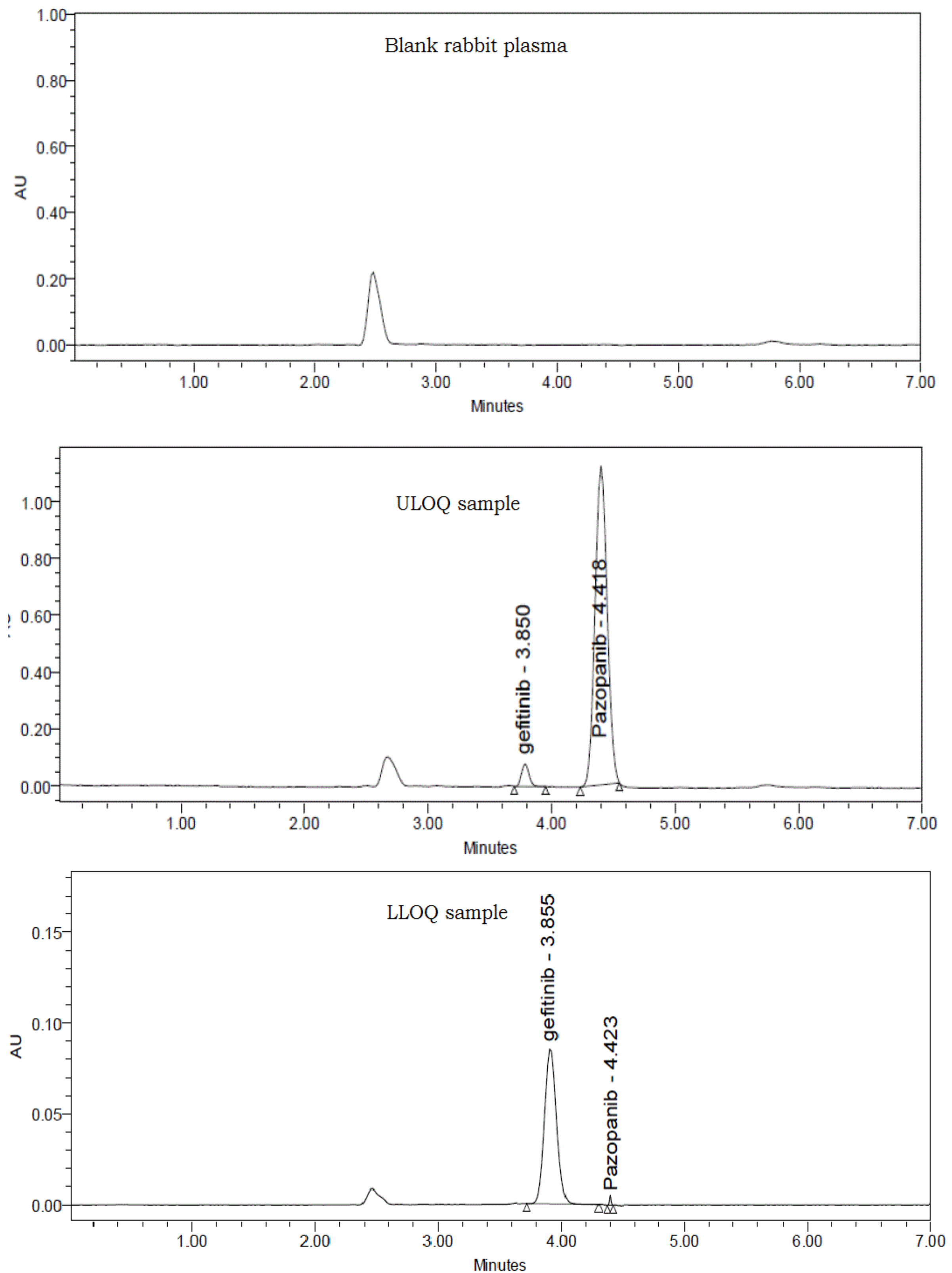

Fig. 2: Chromatograms of auto sample carry over test

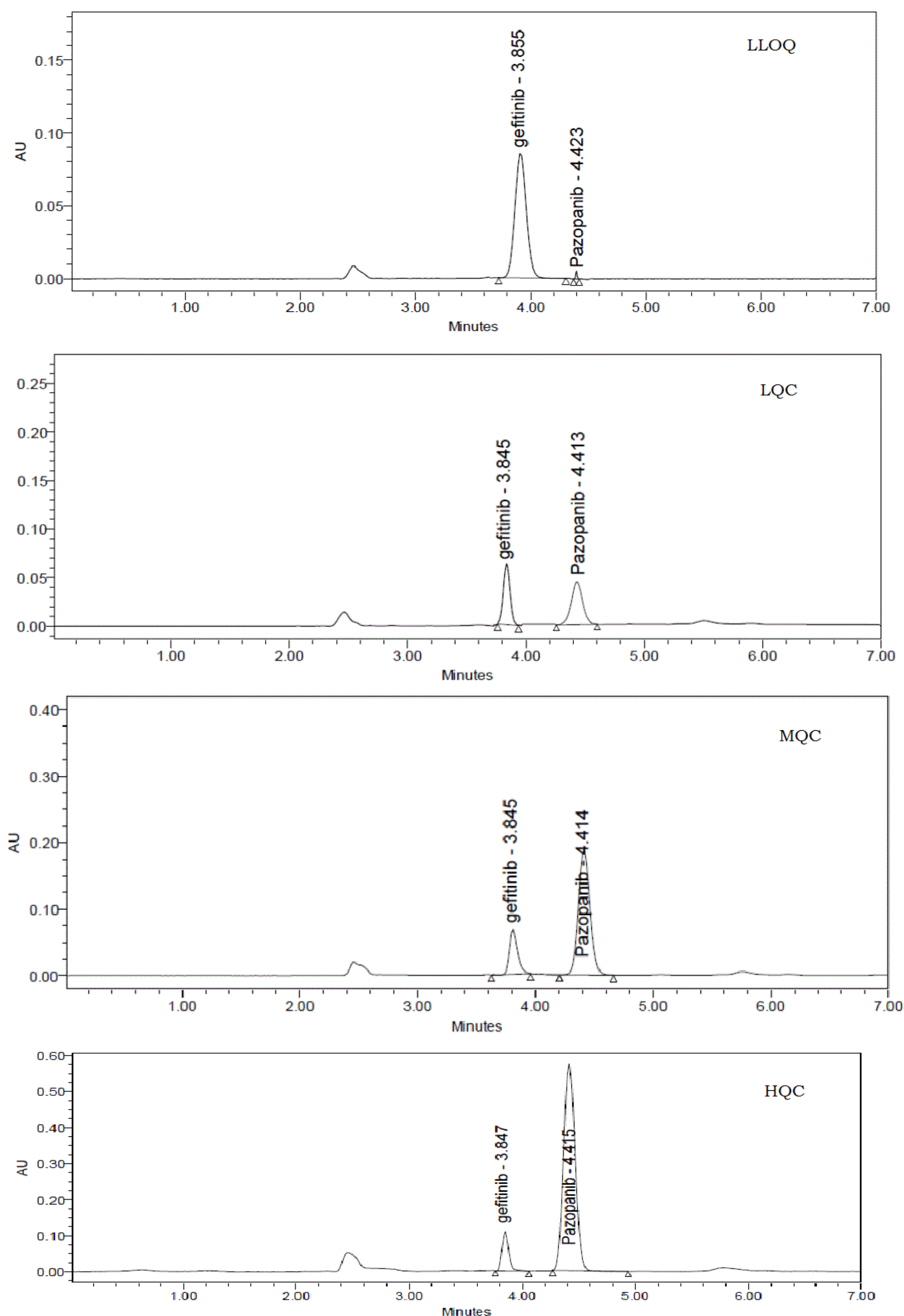

Fig. 3: Chromatograms of quality control samples

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