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Original Article

Bioanalytical Method Development and Validation of an LC-MS/MS Method for the Quantitation of Fruquintinib in Rabbit Plasma

Thota Manasa[1], Kumaraswamy Gandla[2,*]

[1]Department of Pharmaceutical Analysis, Chaitanya (Deemed to be University), Gandipet, Himayat Nagar, Moinabad, Hyderabad, Telangana, INDIA.
[2]Department of Pharmacy, Chaitanya (Deemed to be University), Gandipet, Himayat Nagar, Moinabad, Hyderabad, Telangana, INDIA.

ABSTRACT

Aim: An LC-MS/MS method is needed to accurately measure the concentration of Fruquintinib in a biological sample. This method should be both specific and sensitive. **Materials and Methods:** The processed materials were separated using a Hypersil ODS C18 column (50 mm × 4.6 mm) 3.5 µm with a mobile phase of acetonitrile, methyl alcohol, and 0.1% HCOOH in the proportion of 20:70:10. The moveable solvent system was measured using a column with 0.7 mL/min rate of flow. The drug and Internal Standard (IS), Dapagliflozin were assessed by monitoring the transitions of m/z -394.12/363.1 and 409.1/289.1 for Fruquintinib and IS, correspondingly, in multiple reaction monitoring mode. **Results:** The results of the linearity equation and the Correlation Coefficient (r^2) were as follows: y=0.0011x - 0.0022 and >0.99 correspondingly after the analysis. When compared to the reported methods in the rat plasma, those methods were in the linearity range of 93 (LLOQ) to 2710 (ULOQ) ng/mL in plasma with the %RSD values in between 2.4 to 4.51 which were poor as compared to the current method. It was discovered that the QC-samples (254, 1805 and 2160 ng/mL) had a relative standard deviation of between 2.49 and 4.24% for the intra and interday accuracy of the approach that was developed. The precision deviation values for intra and inter-batches were varied between 2.83 and 4.24 of relative standard deviations. At MQC, LQC, and HQC levels, an average Fruquintinib extraction recovery was 94.8%, 95.4%, and 94.7%, respectively. Average IS adjusted matrix factor for all the analytes was between 0.941 and 1.105, with a standard deviation of 4.99%. **Conclusion:** The new method was effectively used to the regular evaluation of Fruquintinib in biological materials, and it has been shown that Fruquintinib is more stable over a longer length of time.

Keywords: Bio-Analytical Method, Fruquintinib, ICH Validation, LC-MS/MS Method, Quantitative Determination, Rabbit Plasma, VEGFR.

Correspondence:
Prof. Kumaraswamy Gandla
Dean and BoS Chairperson, Department of Pharmacy, Chaitanya (Deemed to be University), Gandipet, Himayat Nagar, Moinabad, Hyderabad-500075, Telangana, INDIA.
Email: drkumaraswamygandla@gmail.com

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INTRODUCTION

Angiogenesis, the process of creating new blood vessels required for both normal tissue growth and solid tumor development, is mediated by a family of receptor tyrosine kinases known as Vascular Endothelial Growth Factor Receptors (VEGFRs). The extracellular ligand-binding domain of VEGFRs resembles Immunoglobulins (Ig), and the receptors also have a signal transduction tyrosine kinase domain and one transmembrane region. Fruquintinib is a recently developed small-molecule drug that is designed to inhibit the process of angiogenesis by targeting VEGFR-1, VEGFR-2, and VEGFR-3. Tumor angiogenesis is a critical biological process that improves the delivery of oxygen and nutrients to cancer cells. This phenomenon is significantly influenced by the VEGF/VEGFR pathway. It is widely recognized that the activation of oncogenes, the loss of tumour suppressor activity, and the presence of low oxygen levels (hypoxia), which are frequently caused by cancer cells, result in an increase in the expression of VEGF. Fruquintinib is a compound with 6-[(6,7-dimethoxyquinazolin-4-yl)oxy]-N,2-dimethyl-1-benzofuran-3-carboxamide as chemical name (Figure 1). It has a molecular weight and molecular formula of 393.39 g.mol^{-1} and $C_{21}H_{19}N_3O_5$.[1-3]

Tumor angiogenesis can be addressed through the use of monoclonal antibodies to neutralize VEGF/VEGFR activity or the use of small-molecule inhibitors to disrupt VEGFR kinase activity. These are the two primary methods. The first method is illustrated by the utilization of bevacizumab, an antibody that functions as a VEGF-A snare. Although bevacizumab effectively maintains target inhibition, its clinical applicability is impeded by

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the need for intravenous administration, immunogenicity, and the potential to induce autoimmune disorders. Previous iterations of VEGFR inhibitors, such as sunitinib, sorafenib, regorafenib and pazopanib, have exhibited limited selectivity in the small-molecule approach, thereby increasing the probability of off-target damage. Therefore, the viability of the use of small-molecule inhibitors has been demonstrated by the introduction of Fruquintinib, a novel class of VEGFR inhibitors that specifically target a wide spectrum of kinases.[4-6]

According to the literature on Fruquintinib, an analytical technique was documented using LC-MS/MS.[7] The precise measurement of Fruquintinib in a biological sample necessitates the utilization of specialized techniques, such as LC-MSMS.

MATERIALS AND METHODS

Chemicals and reagents

The acquisition of Fruquintinib from Jinan Million Pharmaceutical Co., Ltd., in India was successful. An HPLC-water purification system located in Bedford, United States of America and manufactured by Millipore was employed during the investigation. MSN Labs in India was acquired to obtain the internal standard for Dapagliflozin (IS). The reagent grades of acetonitrile, methylalcohol and formic acid were obtained from A.B. Enterprise in Mumbai, India. The methanol was of HPLC quality.

Instrument

The current investigation made use of the Shimadzu LC20AD prominence liquid chromatography system with SIL/HTC autosampler, DGU20A3 prominence degassing system, and Applied Biosystems MDS/SCIEX API 6500 mass spectrometric system, located in Japan. Chromatograms from the chromatographic system were acquired using the 1.4.2 version of the Analyst program.

Processing of quality control and calibration standards

The stock solutions of Fruquintinib and IS were executed by solubilizing the reference standards in diluent methyl alcohol and ACN (1:1%v/v) to produce concentrations of 0.2 g/mL. Resulting solutions were carefully monitored at temperatures below - 20°C in a refrigerator. The analyte and internal standard were diluted in the diluent to create working solutions. The solutions were put away at the temperature of room and processed on a daily basis. The calibration standards underwent processing by adding Fruquintinib to blank plasma, resulting in concentrations of 93, 182, 315, 615, 1085, 1625, 2165, and 2710 ng/mL. The samples for quality control were processed at various concentrations to ensure accuracy and precision in the analysis of Fruquintinib. These concentrations included a LLOQ QC, a LQC, a MQC, and a HQC. Separate portions of the bulk spiked samples were placed in polypropylene tubes and kept at a temperature of -70°C. After thawing to room temperature, all frozen calibration standards and QC samples were processed in preparation for the analysis.

Chromatographic parameters

Processed samples were separated chromatographically using a HypersilODS C18 column (50 mm × 4.6 mm) 3.5 µm with a mobile phase of acetonitrile, methyl alcohol, and 0.1% HCOOH in the proportion of 20:70:10. The moveable solvent system was measured using a column with 0.7 mL/min rate of flow. The autosampler was retained at 5°C with a 10 µL infusion volume. At a temperature of 35°C, the analytical column was seen in the oven. The drug and IS were separated in 6.0 min total.

Conditions for mass detection

While the transitions of m/z findings of 394.12/363.1 for Fruquintinib and 409.1/289.1 for Dapagliflozin were analysed using an MRM mode, mass quantitation was performed on the transitions. Mass instrument was run in the positive ionizing method, with the DP (Declustering Potentials) values being 30 V and 50 V, and the CE (Collision Energies) findings being 35 and 18 V for Fruquintinib and Dapagliflozin, respectively. Pressures of the nebulizer gas (GS1), collision gas, curtain gas, and auxiliary gas were retained at 30 psi, 20 psi, 50 psi, and 55 psi, correspondingly during the duration of the experiment. The temperature of ion-spray was maintained at 450°C, while the voltage of ion-spray was maintained at 4500 V.

Processing of sample solution

A small portion of 300 µL plasma sample was relocated to the 5 mL polypropylene tube. After that, 10 µL of IS (100 ng/mL) solution was added and the tube was vortexed. Then, 500 µL of acetonitrile was added to the solution, and it was subjected for centrifugation at 15,000 rpm for 25 min at 5°C. The organic layer was then dried with an evaporating unit, and 300 µL of mobile solvent system was added to the dry material that was left over. The solution was then put into autosampler tubes so that it could be infused into the chromatographic machine.

Analytical method validation

The developed technique for measuring Fruquintinib was accepted by the USFDA following their guidelines for bioanalytical method evaluation.[8-12] It was necessary to show that the parameters were stable, accurate, linear, selective, residual, precise, recovered, and had a matrix effect.

RESULTS AND DISCUSSION

Method optimization

Mass spectrum conditions

We used positive ionizing method in MRM with Fruquintinib to figure out the mass, which gave us more sensitivity and good

precision. The standard solutions were pumped into a mass system through the syringe pump to find precursor and product ionic components. The mass spectrum of the IS and Fruquintinib product ions were found to be 289.1 and 363.1, which were chosen as a measurement ionic component. At the same time, the mass spectrum settings factors were fine-tuned to get better mass reaction. These included voltage of capillary, voltage of ionic-spray, temperature of ionic-spray and gases related to heating, nebulizing, colliding, curtain and more.[13-15]

Chromatography

The column temperature, organic phase, and moveable phase (varying concentrations ammonium acetate and of HCOOH) were tuned for liquid chromatography to provide superior peak shape, increased sensitivity, and no matrix impact. Furthermore, the methyl alcohol-ACN organic phase was chosen as mobile solvent system over water because of its strong elution impact and minimal background noise. Various trials were executed with HCOOH, methyl alcohol, ACN, and 10mM ammonium acetate, and finally with a HypersilODS C18 column (50 mm × 4.6 mm) 3.5 µm with a mobile phase of acetonitrile, methyl alcohol, and 0.1% HCOOH in the proportion of 20:70:10. The moveable solvent system was measured using a column with 0.7 mL/min rate of flow and with 35°C oven temperature.

Selection of internal standard

In a current investigation, IS was chosen as an internal standard because it exhibited comparable chromatographic properties, extraction efficiency, ionization, and retaining times to Fruquintinib. According to the technique validation findings, no evident interferences were identified at analyte and IS retention periods.[16-19]

Validation of the method

Specificity

Plasma samples from 6 different batches of rabbit plasma were spiked with Fruquintinib at the LLOQ and Internal Standard (IS) to estimate specificity. According to Figure 2, the retaining times of Fruquintinib and IS were 2.53 and 3.0 min, correspondingly.[20] There were no apparent interferences from any natural substances or internal standards on the analytical results of Fruquintinib. Additionally, the responses of all nosy peaks were found to be <20% of a sample with the LLOQ. Meanwhile, the LLOQ was measured with accuracy that met acceptable standards, demonstrating precision below 20% and a signal-to-noise ratio (S/N) above 5. In the analytical run, the highest concentration of calibration standard was immediately infused into LC-MSMS after blank rabbit plasma for carry-over effects assessment. Upon further observation, no discernible carry-over effect was detected.

Sensitivity and calibration curve

The established method for determining Fruquintinib exhibited excellent linearity, covering a range of 93 to 2710 ng/mL. The linearity curves were made using peak response ratios of Fruquintinib to IS against the concentration.[21] The calibration curves' regression line equation is y=0.0011x - 0.0022, indicating a strong correlation coefficient >0.99 (Figure 3 and Table 1). LLOQ of Fruquintinib was determined to be 93 ng/mL, with a S/N ratio

Table 1: Fruquintinib data for calibration controls.

LS-ID	Concentrations (ng/mL)	Mean (ng/mL)	% RSD	% RE
LS1	93	91.86	3.64	1.22
LS2	182	177.76	4.51	2.31
LS3	315	303.42	3.5	3.65
LS4	615	626.49	2.4	-1.86
LS5	1085	1149.25	3.29	-3.89
LS6	1625	1722.68	4.17	-4.13
LS7	2165	2095.29	4.36	3.31
LS8	2710	2831.08	2.5	-4.15

RE- Relative Error; LS- Linearity Standard; RSD- Relative Standard Deviations.

Table 2: Inter and Intra-batch Precision and Accuracy.

QC levels	Nominal concentrations (ng/mL)	Intrabatch			Interbatch		
		Amount found (ng/mL)	% RSD	% RE	Amount found (ng/mL)	% RSD	% RE
LLOQ	93	91.2	4.13	-2.27	91.21	2.83	-4.42
LQC	254	269.16	3.14	3.13	262.76	4.24	3.64
MQC	1805	1881.2	2.96	3.97	1859.35	3.77	2.75
HQC	2160	2292	3.37	3.29	2177.29	2.49	0.56

Table 3: Fruquintinib matrix effects data.

Fruquintinib	LQC			HQC		
	MF for analyte	MF for IS	IS normalized MF	MF for analyte	MF for IS	IS normalized MF
B1	1.02	1.11	0.961	1.09	1.11	0.986
B2	1.08	1.09	0.943	1.16	1.09	1.062
B3	1.11	1.12	1.011	1.03	1.1	0.941
B4	1.12	1.07	1.03	1.08	1.11	0.977
B5[a]	1.13	1.08	1.061	1.12	1.02	1.105
B6[a]	1.05	1.13	0.963	1.12	1.08	1.0343
B7[b]	1.09	1.03	0.962	1.09	1.1	0.991
B-8[b]	1.11	1.09	1.084	1.07	1.02	1.057
Mean	0.99			1.02		
SD	0.05			0.05		
%RSD	4.75			4.99		

RSD: Relative standard Deviation; B: Batch; a, Hemolyzed lot; b, Lipemic lot; MF: Matrix Factor.

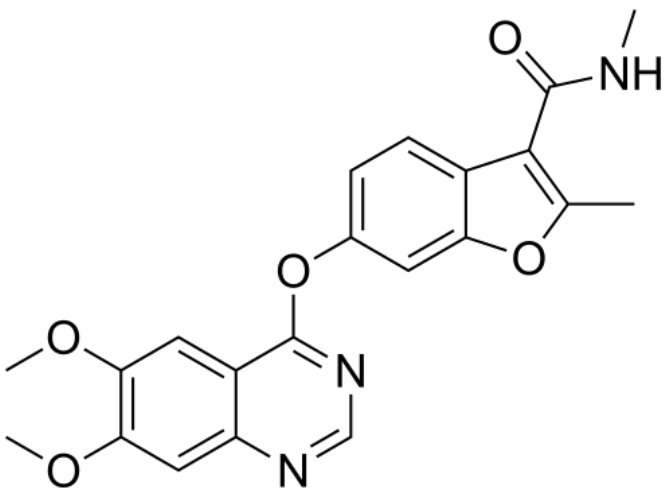

Figure 1: Fruquintinib chemical structure.

>5. This analysis was conducted using five replicates, ensuring the reliable quantitation of Fruquintinib in plasma samples studied.

Precision and accuracy

The precision and accuracies of intra and inter-batches was assessed by analyzing six spiked plasma samples of Fruquintinib at the lower limit of quantification and 3 quality control levels in single batch and in 3 successive batches, correspondingly. The precision and accuracy findings for quantification of Fruquintinib can be found in Table 2. The precision deviation values for intra and inter-batches were varied between 2.83 and 4.24 of relative standard deviations. The accuracy deviation findings for intra and interbatches varied from -4.42 to 3.97 of relative error.[22] The findings clearly indicate that the precision and accuracies of determining Fruquintinib in plasma were consistently reliable and reproducible.

Extraction recoveries

Proper pretreatment was performed on the biosamples prior to detection. A peak area ratio between the extracted spiked samples and 3 QC standard samples (n=6) at matching concentrations was used to assess the Fruquintinib extraction recoveries (Figure 4). In a similar vein, the peak responses ratio of spiked samples of plasma at matching concentration levels to QC plasma sample solutions (n=6) was used to assess the extraction recovery of IS.[23] At MQC, LQC, and HQC levels, an average Fruquintinib extraction recovery was 94.8%, 95.4%, and 94.7%, respectively. The average recovery rate of IS extraction at a concentration of 100 ng/l was 97.6 percent.

Dilution integrity

The dilution integrity test was done with all three analytes at 2 times the ULOQ (upper limit of quantification) concentration. We found that the percentage of the dilution QC sample solutions was in a range of 85.0 to 115.0% of original figure after a 1:4 dilution, with a %RSD of ≤5.2. In the same way, LQC samples that were spiked with a drug at the same time were measured within 15% of the standard value, with a %RSD of 4.6.

Matrix effects

Because the test is so accurate, co-eluting matrix constituents can either stop or boost ionization, but blank matrixes might not show any response.[23] So, the possibility of varying matrix relating ion destruction was tested in eight 8 sources of rabbit plasma. This was done by finding the IS normalized matrix factor. Table 3 shows that an average IS adjusted matrix factor for all the analytes was between 0.941 and 1.105, with a standard deviation of 4.99%.

Stability studies

For the purpose of determining stability, both matrix-based and aqueous sample solutions were subjected to tests. Analyte and IS

Table 4: Fruquintinib stability data.

Parameter	QC level	X	Y	%RSD	%Stability
Refrigerator stability (at 1 to 10°C for 48 hr)	LQC	254	239.395	4.2	94.25
	HQC	2160	2089.584	2.9	96.74
Freeze and thaw stabilities (6 cycles after)	LQC	254	235.8136	3.4	92.84
	HQC	2160	2231.064	1.9	103.29
Long term stabilities (for 63 days at -20°C)	LQC	254	237.5154	2.5	93.51
	HQC	2160	2222.856	3.7	102.91
Benchtop stabilities (17 hr at <10°C)	LQC	254	239.1918	5.8	94.17
	HQC	2160	2241.432	2.7	103.77
Long-term stabilities (for 63 days at -70°C)	LQC	254	239.4458	6.2	94.27
	HQC	2160	1971.432	1.61	91.27
In-injector stabilities (47 hr at 10°C)	LQC	254	244.2972	2.4	96.18
	HQC	2160	2217.024	4.6	102.64

Y, average drug concentration; X, nominal concentration of drug.

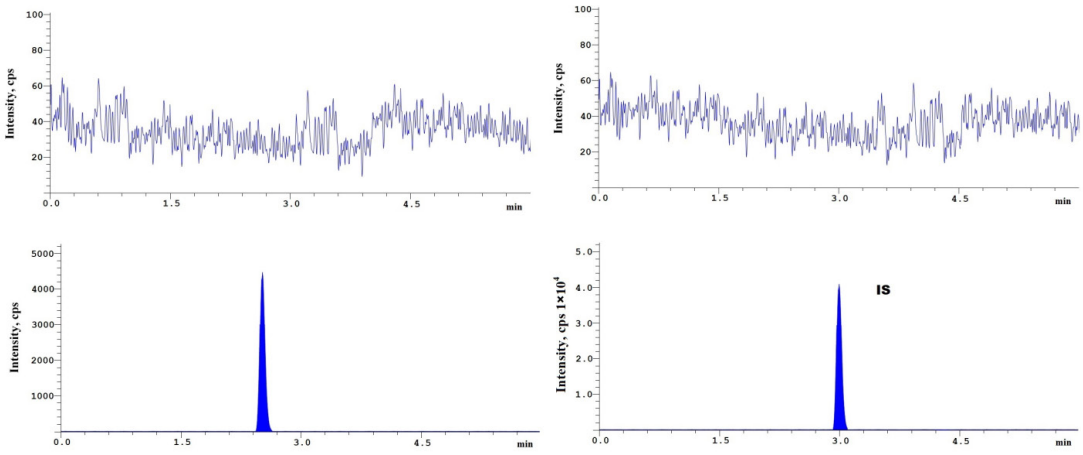

Figure 2: (A) Blank plasma, (B) Spiked LLOQ-sample chromatograms of Fruquintinib.

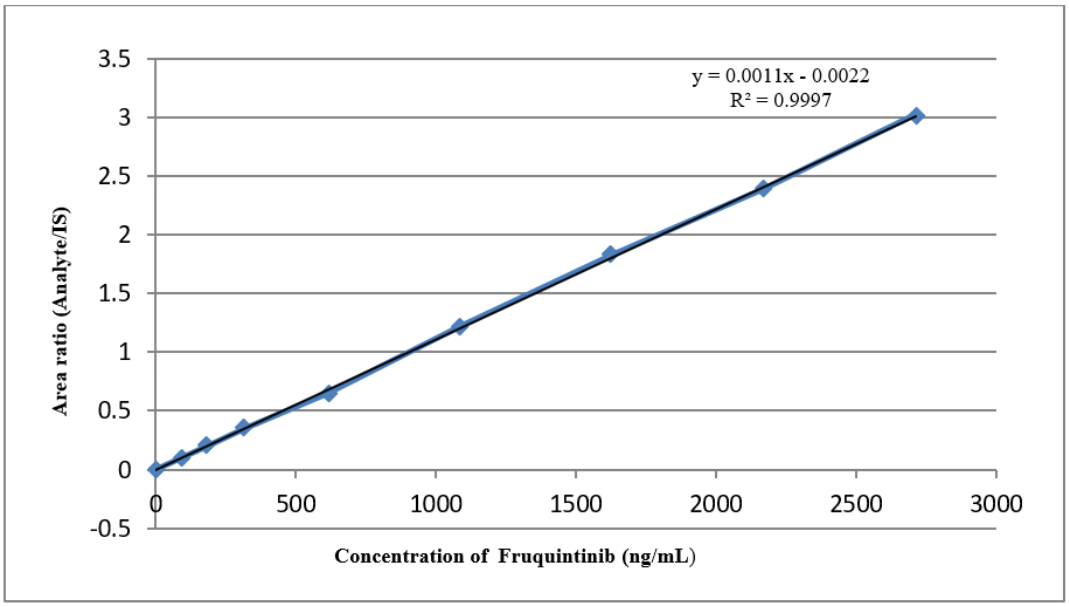

Figure 3: Linearity graph of Fruquintinib.

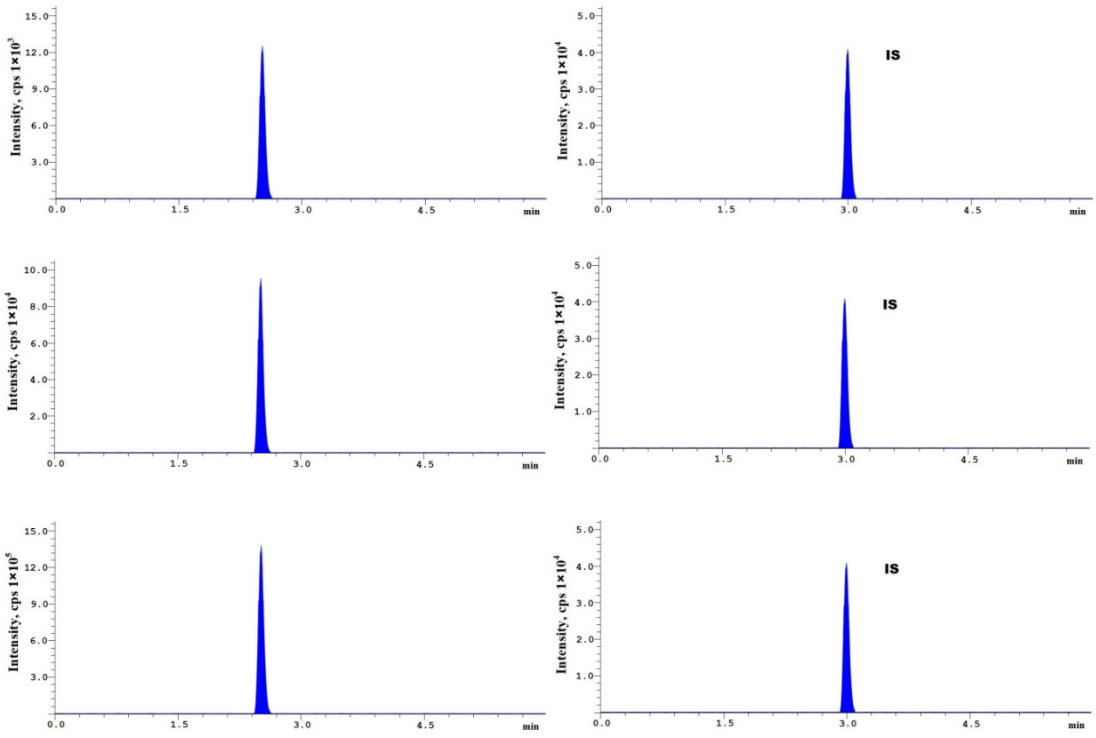

Figure 4: (A) LQC, (B) MQC, and (C) HQC sample Chromatograms of Fruquintinib.

in the stock solution were stable for 66 days between 1 to 10°C, while stock dilutions in diluent were stable for up to 48 hr at any temperature between 1 and 10°C. Both -70 and -20°C were used to achieve stability in the matrix for a period of 63 days. Table 4 presents the findings of the stability assessments that were conducted.

An examination of the matrix's stability was carried out in comparison to recently spiked calibration standards.[21-23] At a temperature of less than 10°C and after six cycles of freezing and thawing, the drug remained stable for 16 hr on the bench. Up to 47 hr in the autosampler at 10°C, the processed samples remained stable. Over the course of the stability length and circumstances, there was no identification of any substantial degradation or inter-conversion of the analyte. The average responses ratio of stability solutions was compared to the response ratio of reference samples in order to assess the stability of whole rabbit blood at both low- and high-quality control levels. Above 10°C, the analyte remained stable in whole rabbit blood for up to 2.5 hr.

CONCLUSION

In this study, a linear and specific LC-MS/MS technique was developed and validated for the purpose of effectively determining the presence of Fruquintinib in rabbit plasma. In addition to displaying great specificity and linearity, the created technique also shown accuracy, precision, and stability. This approach, on the other hand, was uncomplicated and it saved time. The results of the linearity equation and the correlation coefficient (r2) were as follows: y=0.0011x - 0.0022 and >0.99 correspondingly after the analysis. When compared to the reported methods in the rat plasma, those methods were in the linearity range of 93 to 2710 ng/mL in plasma with the % RSD values in between 2.4 to 4.51 which were poor as compared to the current method. It was discovered that the QC-samples (254, 1805, and 2160 ng/mL) had a relative standard deviation of between 2.49 and 4.24% for the intra and interday accuracy of the approach that was developed. The new method was effectively used to the regular evaluation of Fruquintinib in biological materials, and it has been shown that Fruquintinib is more stable over a longer length of time.

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ABBREVIATIONS

LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; **ICH:** International Conference on Harmonization; **VEGFR:** Vascular Endothelial Growth Factor Receptor; **WHO:** World Health Organization; **Tf:** Tailing factor; **NTP:** Number of theoretical plates; **RT:** Retention time; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; RSD: Relative Standard Deviation; **CV:** Coefficient of Variation; **SD:** Standard Deviation; **MRT:** Mean Residence Time.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Fruquintinib is a recently developed small-molecule drug that is designed to inhibit the process of angiogenesis by targeting VEGFR-1, VEGFR-2, and VEGFR-3. Tumor angiogenesis is a critical biological process that improves the delivery of oxygen and nutrients to cancer cells. This phenomenon is significantly influenced by the VEGF/VEGFR pathway. The precise measurement of Fruquintinib in a biological sample necessitates the utilization of specialized techniques, such as LC-MSMS. A linear and specific LC-MS/MS technique was developed and validated for the purpose of effectively determining the presence of Fruquintinib in rabbit plasma. In addition to displaying great specificity and linearity, the created technique also shown accuracy, precision, and stability. This approach, on the other hand, was uncomplicated and it saved time. The results of the linearity equation and the Correlation Coefficient (r2) were as follows: y=0.0011x - 0.0022 and >0.99 correspondingly after the analysis. When compared to the reported methods in the rat plasma, those methods were in the linearity range of 93 to 2710 ng/mL in plasma with the % RSD values in between 2.4 to 4.51 which were poor as compared to the current method. It was discovered that the QC-samples (254, 1805, and 2160 ng/mL) had a relative standard deviation of between 2.49 and 4.24% for the intra and interday accuracy of the approach that was developed.

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