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Method Development And Validation Of An Lc–Esi-Ms/Ms Technique For The Quantitation Of Fedratinib In Biological Matrices

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Abstract

An LC–ESI-MS/MS technique for the quantitation of anticancer agent fedratinib in plasma samples was developed and subjected for validation. Internal standard for the evaluation of drug was ledipasvir. After the extraction process from 200 µL plasma by liquid liquid extraction technique, samples were isolated on a Zorbax SB(250mm x 4.6mm, 5µm) C_{18} column with 0.1%V/V formic acid and acetonitrile in the ratio of 10:90 as mobile phase with 0.80 mL/min infusion flow rate. Total time for elution of analytes was 3.5 min. The technique was subjected for the validation in accordance with FDA standard guidelines in the concentration level of 1.5–5000.00 ng/mL for fedratinib with correlation coefficient value of 0.9994. The intra day and inter day precision findings were within 4.32% and the assay accuracy was 95.31–104.06 % of the nominal values. Matrix factor ranges from 94.25– 104.85 % with a %CV of 4.61 for analyte at LQC level and at HQC level, the matrix factor range was 94.62–103.88% with a %CV of 4.02. Stabilities revealed that the method has high degree of stability. The developed method can be applied successfully to routine analysis in quality control, bioavailability and bioequivalence studies of biological samples.

Keywords: Tyrosine kinase inhibitor, Fedratinib, FDA, Accuracy and Linearity.

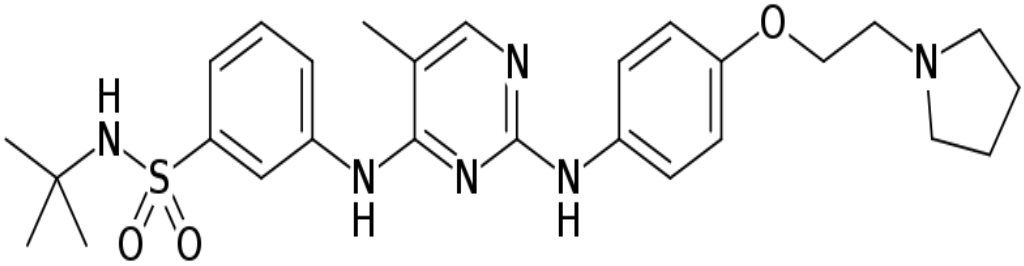
Introduction

Myelo proliferative neoplasms are clonal, BCRABL1 negative hematopoietic illnesses of myeloid propagation, and produced by irregular production of terminally distinguished functional blood cells. Myelo proliferative neoplasms are classified into 3 disease entities: essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) (Moulard et al, 2014; Vainchenker et al, 2017). Patients with ET and PV are characterized by an unusual increament in platelet and hematocrit/ hemoglobin number, correspondingly, and PMF is more advanced subtype of MPNs, accompanying with bone marrow fibrosis, release of profibrotic and proinflammatory cytokines and splenomegaly due to extramedullary hematopoiesis. Janus kinase (JAK)/signal transducer and activation of transcription (STAT) pathway is key to cytokine receptor signaling and plays a critical role in hematopoiesis and immune response. In human, the JAK family comprises four members: JAK-1, JAK-2, JAK-3 and tyrosine kinase-2 (TYK2), each of which relate with dissimilar cytokine receptors (Sangle et al, 2014; Pardanani et al, 2015).

Fedratinib acknowledged as TG101348 and SAR302503, is an inhibitor of tyrosine kinases utilized to treat high risk primary and secondary myelofibrosis and intermediate-2. It is an anilinopyrimidine derivative.

Fedratinib inhibits FMS-like tyrosine kinase 3 and JAK-2 (Jamieson et al, 2011; Zhang et al, 2015). JAK-2 is extremely active in myeloproliferative neoplasms like myelofibrosis. Fedratinib's inhibition of JAK-2 constrains phosphorylation of signal transducer and activator of transcription (STAT) 3 and 5, which avoids induces apoptosis and cell division. Fedratinib was metabolized by CYP2C19, CYP3A4, and flavin comprising monooxygenase 3 (Zhang et al, 2011; Pardanani et al, 2011). Beyond that, data concerning the metabolism of fedratinib is not freely obtainable. Patients utilizing fedratinib may get hepatic toxicity, thrombocytopenia, anemia, gastrointestinal toxicity, or higher lipase and amylase. These activities should be manage by decreasing dose, stoppage of medication temporarily, or giving transfusions on a case by case basis. Fedratinib designated as N-tert- Butyl-3. -{5-methyl-.2 -[4- (2- pyrrolidin -1- yl -ethoxy) –phenylamino.] –pyrimidin. -4-ylamino} -benzenesulfonamide (Figure 1) with molecular formula and weight of $C_{27}H_{36}N_6O_3S$ and 524.68 g·mol^{-1} respectively (Roskoski et al, 2016).

Figure 1. Structure of Fedratinib.



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Literature on fedratinib revealed that only single analytical method was reported on LC-MS/MS (Ayesha et al, 2020). The development of specific method like LC-MS/MS is highly essential for the quantification of fedratinib in human plasma samples which can utilized for the bioavailability and bioequivalence studies.

Materials and Methods

Reagents and chemicals

Ledipasvir (99.52 % purity) was acquired from Hetero drugs, Hyderabad, India. Fedratinib obtained from Selleck chemicals, Houston, TX, United states. Drug free human plasma having K_2-EDTA anticoagulant was procured from Vivekananda blood bank, Hyderabad, India. MilliQ water purification system (Milli-pore, USA) was utilized for HPLC-water in the research work. HPLC grade Methanol and acetonitrile, ethyl acetate, ammonium acetate and HCOOH of the high grade purity were procured locally.

Processing of standards

Processing of individual analyte stock solution(1000µg/ml) was done in methyl alcohol. Resulting solution was subjected for serial dilutions to get concentration range of 1.5-5000.0 ng/mL with methanol.

Calibration standard solutions

For the processing of calibration standards, 20µl of fedratinib diluted sample was mixed with 960µl of K_2EDTA pooled plasma. To the resultant solution, 20µl of IS dilution was transferred to get final solution. The concentration range, 1.5-5000.0 ng/ml solutions were prepared and stored below −20 °C in a freezer.

Processing of quality controls

Quality control standard were processed at 3 dissimilar concentrations of high quality control(HQC) standards, median quality control(MQC) standards and low quality control(LQC) standards. These QC (quality control) solutions were processed as per the calibration standard solutions to get 3750.00, 2500.00 and 4.2 ng/mL for HQC, MQC and LQC correspondingly.

Preparation of internal standards

Ledipasvir was used as internal standard and 1mg/mL stock concentrations in acetonitrile were processed in a separate conical flask. Resulting stock was made dilution with acetonitrile to get 1µg/mL solution.

Sample preparation

Drug solution was executed by relocating 200µl of plasma and 50µL of ledipasvir (1µg/mL) in to a pre-labeled tube and sonicated for 10 min. Fedratinib and ledipasvir were isolated with 5.0mL of ethyl acetate and dichloromethane (3:2 ratio) solvent system and the solution was subjected for centrifugation at 3500rpm/min for 20min. Organic layer was isolated and dried with lyophiliser. Residue after the drying was made solubilize in 250µl of movable solvent and then translocated to LC-vials. These vials were placed and injected into LC-MS/MS instrument.

Chromatography

10µL of sample was injected on RP-Zorbax SB(250mm x 4.6mm, 5µm) C_{18} analytical column with an isocratic movable phase comprising 0.1%V/V HCOOH and ACN (acetonitrile) in proportion of 10:90, (%v/v) was utilized at a flowrate of 0.80ml/min. Analytical column was retained at 45°C and total chromatographic time was 3.5min. Chromatographic system was equipped with HPLC-Shimadzu combined with API-5000 Mass instrument of Applied Bio systems, America.

Mass instrument

Electro spray ionization technique was utilized and functioned in +ve ionization method for MRM. By injecting dilute stock solution of drug, the operating parameters were improvised as mentioned in Table 1. Auxiliary gas (GS2) and nebulizer gas (GS1) flows were 45 and 40 psi, respectively. Source temperature was set at 280 °C. Q3 and Q1 were monitored under unit resolution. Upon addition of HCOOH to the mobile phase, protonation of analytes were improvised and excellent peak intensities were obtained. MRM mode monitored at: m/z 525.26 → 57.1 for fedratinib and m/z 889.42 → 130.1 for ledipasvir. Concentrations of samples were estimated by regression line with the help of analyst software1.5.1. In this peak response ratio method was utilized.

Table 1. Optimized mass parameters for fedratinib and internal standard.

Analyte/IS	Declustering potential (DP) (V)	Collision activated dissociation (CAD) (psi)	Dwell time (ms)	Entrance potential (EP) (V)	Curtain gas flow (CUR) (psi)	Collision cell exit potential (CXP) (V)	Collision energy (CE) (V)	Ion source voltage (V)
Fedratinib	30	8	400	10	30	17	18	4500
Ledipasvir	35	8	400	10	30	12	20	4500

Validation of analytical method

Developed LC–MS/MS work was subjected for validation according to USFDA-guidelines for sensitivity, inter and intraday precision, specificity, linearity, stability and accuracy. (FDA, 2001; EMA, 2011).

Results and Discussion

Development of method

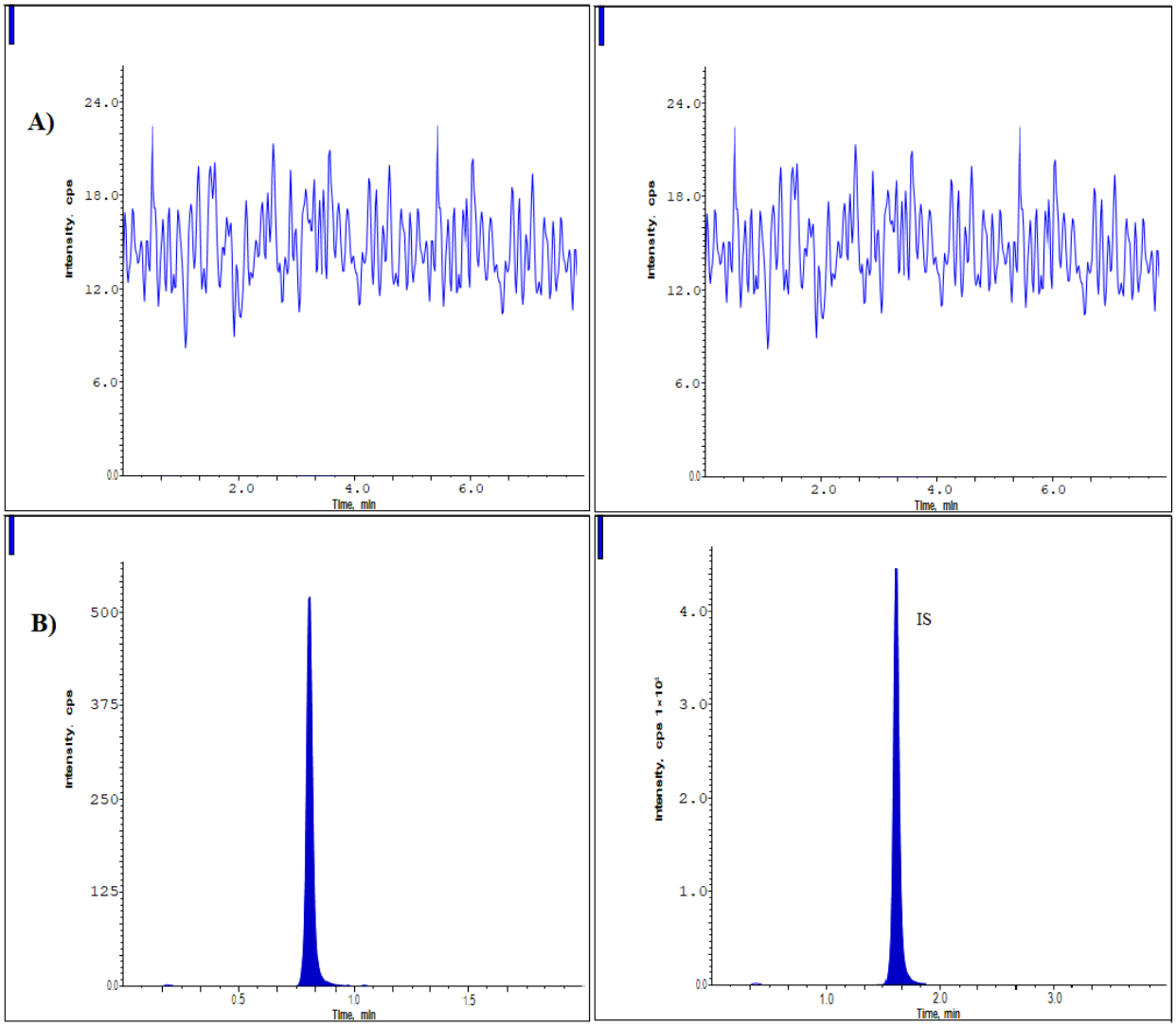
Effective and specific sample clean-up processes were required for selective and sensitive LC–MS/MS process for the quantification of very small level concentrations of pharmaceutical formulations existing in biological sample solutions (Henion et al, 1998; Chambers et al, 2014). Three methods, e.g., solid-phase extraction (SPE), protein precipitation (PPT) and liquid liquid extraction (LLE), were generally utilized for processing biological samples. Protein precipitation scheme utilizing organic segment was simple one but there are chance of matrix component interference. Next we tried with LLE with ethyl acetate and dichloromethane as solvents. Finally, good recovery was obtained with ethyl acetate and dichloromethane (3:2 ratio) as solvent for sample extraction.

Analytical method validation

Selectivity

Method selectivity was determined in 8 individual lots of K_2 EDTA human plasma along with 1- lipemic and 1-hemolytic lot. No interference constituents were detected at the retaining timings of drug and IS when peak areas in blanks were matched against the areas of spiked LLOQ standard comprising IS mixtures (Patel et al, 2011). Respective peaks were given in Figure 2.

Figure 2. (A) Blank and (B) LLOQ sample chromatograms.



Recovery

This procedure was revealed to be a robust analytical method and produced decent and reproducible recovery values of fedratinib and IS. The extraction recoveries of analytes were assessed by associating responses of peaks from samples of spiked plasma (n = 6) before extraction with aqueous sample solutions (Murphy et al, 1995; Shankar and Bhikshapthi, 2021). The average overall recovery findings across QC standards (with precision) were 95.57%. The recovery of IS was found to be 97.75 % and the findings were shown in Table 2 (Figure 3 to 5).

Table 2. Extraction Recovery Rates of Analytes

Concentration	A	B	% Recovery	% Mean recovery	%RSD
LQC	1485	1412	95.61	95.57	0.84
MQC	875354	846467	96.42		

HQC	1324862	1257294	94.93
IS	452161	440857	97.75

A, mean recovery of un-extracted sample; B, mean recovery of extracted sample.

Figure 3. Chromatographic peaks of Fedratinib at LQC standard.

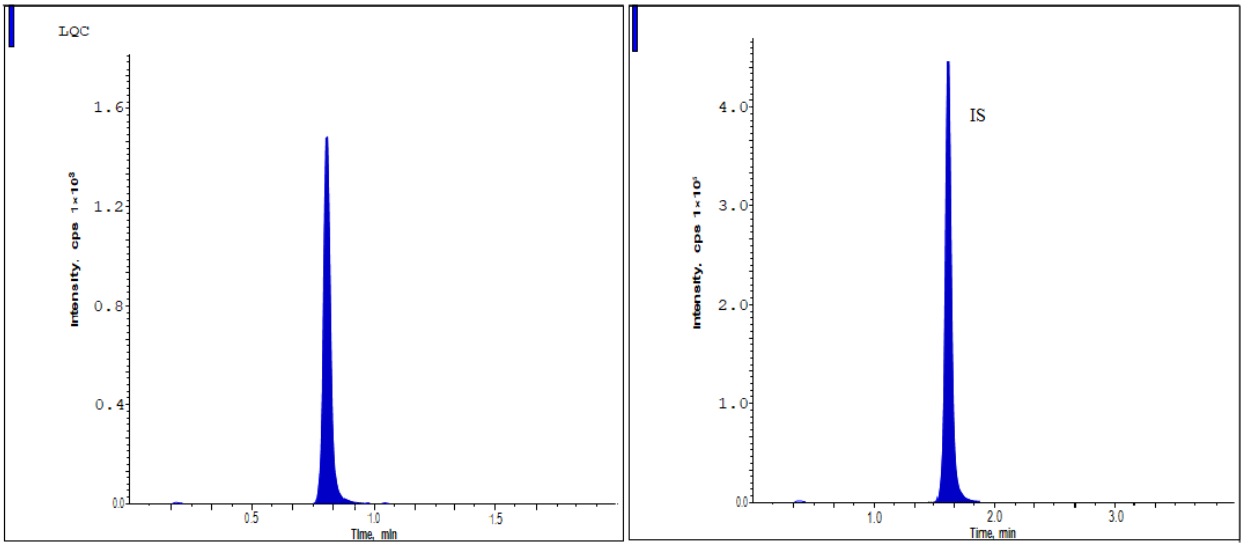


Figure 4. Chromatographic peaks of Fedratinib at MQC standard.

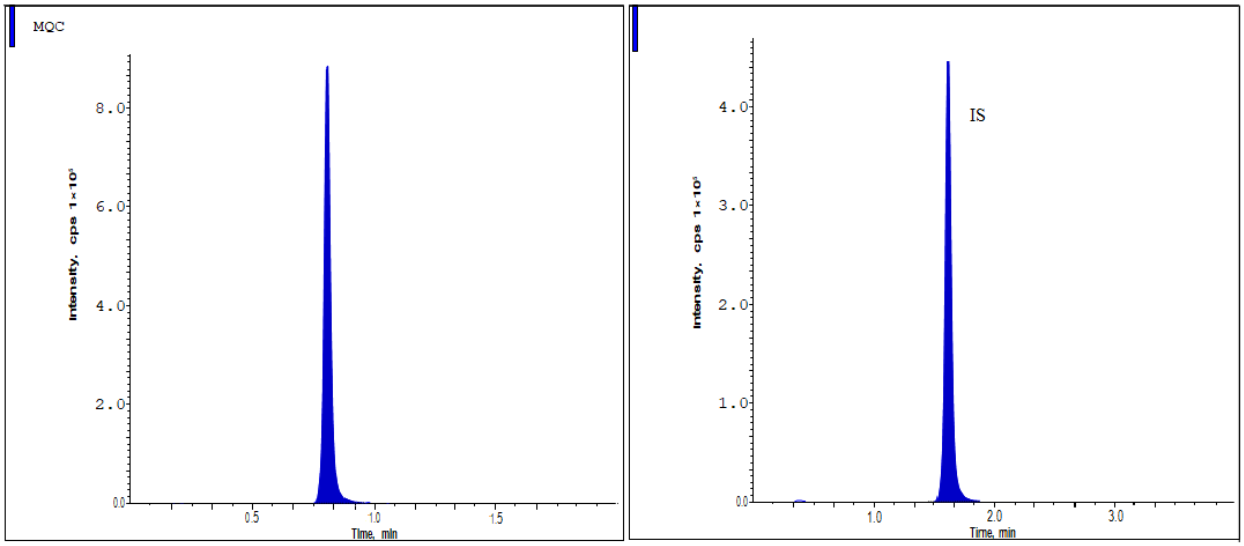
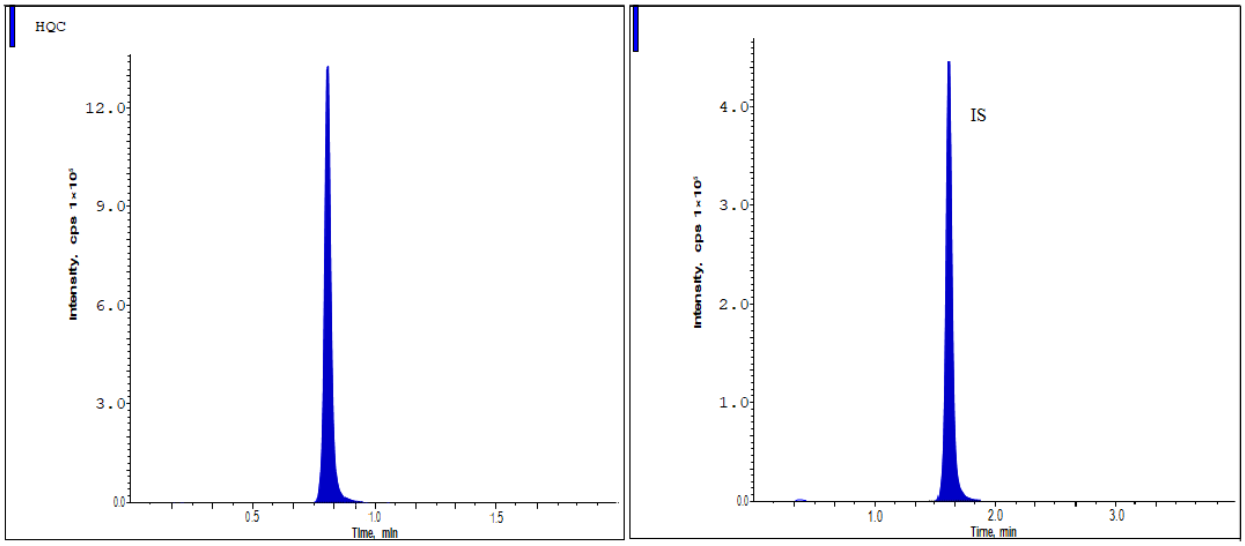


Figure 5. Chromatographic peaks of Fedratinib at HQC standard.



Linearity and sensitivity

Eight point calibration curve was processed with analyte concentration ranging from 1.5–5000.00 ng/ml. The ratios of peak responses (y) of drug to IS were plotted in contrast to the concentration (x) of drug to evaluate the linear response of calibration curve. The linearity equation was found to be y= 0.003216 x+ 0.00428 with correlation coefficient value more than 0.999 (0.9994). The concentration findings of calibration standards were back measured to get accurate values of each calibration standard (Richard et al, 1997). The calibration points accuracy ranges for analyte were 95.41–103.93. Precision and accuracy of analyte at the LLOQ were found to be ≤ 4.19 and 96.82 % respectively. The LLOQ concentration of the technique was 1.50 ng/ml for fedratinib. This designates that the developed technique was more sensible for the bioavailability studies.

Accuracy and precision

Accuracy and precision for inter and intraday groups for all analytical concentrations were evaluated by analyzing the 6 replicate solutions at QC levels (n = 6) at 4 variable standards: high quality control 3750.00 ng/ml (HQC) middle quality control 2500.00 ng/ml (MQC), low quality control 4.20 ng/ml (LQC) and lower limit of quantification 1.50 ng/ml (LLOQ). Findings of accuracy and precision were shown in Table 3. The intraday and interday precision were within 4.32% and the assay accuracy was 95.31–104.06 % of the nominal values.

Table 3. Intra-batch and Inter-batch Precision and Accuracy

		Intra-batch			Inter-batch		
Concentration level	Concentration (ng/ml)	Amount found[a] (ng/ml)	% Accuracy	%RSD	Amount found[a] (ng/ml)	% Accuracy	%RSD
LLOQ	1.50	1.46	97.52	4.32	1.43	95.31	3.15

LQC	4.20	4.04	96.24	2.89	4.31	102.55	2.54
MQC	2500.00	2593.75	103.75	3.21	2466.75	98.67	3.91
HQC	3750.00	3613.50	96.36	1.75	3902.25	104.06	4.11

a:Average of 6 replicates; RSD, Relative standard deviation.

Matrix effect

This parameter was assessed by extraction of blank plasma samples from 8 dissimilar lots, including lipemic-1 and hemolytic-1 lot. 100 µl blank plasma was collected from every batch and subjected for processing as per the sample preparation procedure. Aqueous solutions and post-extracted solutions were processed at LQC level or HQC (Zhon et al, 2005; Murphy et al, 1995). Matrix effect was estimated with help of the formula: % Matrix effect= $A_2/A_1 \times 100$, where A_1 is peak response of aqueous sample and A_2 is peak response of post-extraction samples. Mean matrix factor (n = 6)present in between 94.25–104.85 % with a %CV of 4.61 for analyte at LQC level and at HQC level, the matrix factor range was 94.62–103.88% with a %CV of 4.02 (Table 4).

Table 4. Matrix factor for analyte at LQC and HQC levels.

S.No	LQC			HQC		
	Concentration in absence of matrix	**Concentration in presence of matrix**	**Matrix factor**	**Concentration in absence of matrix**	**Concentration in presence of matrix**	**Matrix factor**
1	4.01	3.81	94.89	2510.48	2390.98	95.24
2	3.96	3.86	97.52	2481.88	2374.41	95.67
3	4.12	3.97	96.34	2472.69	2530.55	102.34
4	4.51	4.24	94.25	2326.15	2416.4	103.88
5	4.00	4.19	104.85	2306.75	2248.62	97.48
6	4.16	4.30	103.65	2556.97	2419.4	94.62
Mean			98.58			98.21
± SD			4.55			3.95
% CV			4.61			4.02

Dilution integrity

Six duplicates of the sample solutions were prepared and estimated against a set of freshly spike calibration solutions. The drug higher concentration limit was shown to be extend up to 5000 ng/mL by diluting with plasma blank. The average back calculated concentrations for 2-fold and 4-fold diluted sample solutions were present in between 96.43–103.81 % with a % coefficient of variance of less than 3.50 for the analyte (Patel et al, 2011).

Carryover effect

This parameter was processed by employing sample solutions in the order of LLOQQC of drug, plasma blank, and ULOQ (upper limit of quantitation) of drug and plasma blank. Carryover of any drug or IS was identified during the analysis.

Stability studies

Stability studies were evaluated in both matrix-based and aqueous samples. For aqueous samples, both long and short term stability studies were evaluated as :(Kumar et al, 2015)

Aqueous solution stability

Short term stability was executed by processing MQC standard solutions and was stored for 24h at 25.0 °C. Six replicates of these standards were injected and estimated for any differences with freshly processed MQC standard sample solution at 25.0 °C. The average stability was 95.64%. Long term stability was executed by processing MQC standard solutions and was stored for 40 days at 2–8 °C. Six replicates of these standards were injected and estimated for any differences with freshly processed MQC standard sample solution. The average stability was 95.23%.

Human plasma stability

Benchtop stability was executed by processing LQC and MQC standards in plasma solutions and were collected from -20.0°C and kept at 25.0 °C (room temperature) for seven hours. Six replicates of these standards were injected and estimated for any differences with freshly processed LQC and MQC standard sample solutions. The average stability was present in between 94.36–97.56%. In-injector stability was executed by processing LQC and MQC standards in plasma solutions at 10.0°C in an autosampler for 22 h and was found to be 96.83–99.45 %. Freeze and thaw stability was executed at LQC and MQC standards in plasma solutions. After completion of 4 freezethaw cycles, percentage recovery was estimated and was found to be 94.76 to 101.69 % . Wet extract stability was executed by processing LQC and MQC standards in plasma solutions at 25.0°C for 7 h and was found to be 95.98–103.27 % (Table 5).

Table 5. Stability Results of Fedratinib

Parameter	QC level	X	Y	%RSD	%Stability
Freeze–thaw stability	LQC	4.20	3.92	3.90	94.76
	HQC	2500.00	2542.40	4.01	101.69

In-injector stability	LQC	4.20	4.15	2.85	96.83
	HQC	2500.00	2446.69	2.43	99.45
Benchtop stability	LQC	4.20	4.09	3.75	97.56
	HQC	2500.00	2359.20	4.16	94.36
Wet extract stability	LQC	4.20	4.29	4.21	103.27
	HQC	2500.00	2477.92	3.73	95.98

X, nominal concentration (ng/mL); Y, mean concentrations (ng/mL) of analytes

Extended accuracy and precision run

Extended accuracy and precision run was executed by preparing and estimating forty sets of HQC and LQC and one batch of calibration curve standards. Resultant findings related to extended accuracy and precision were represented in the Table 6. The precision and stability for fedratinib was 4.2 and 98.87% at LQC level and 1.61 and 102.81% at HQC level.

Table 6. Extended precision and accuracy analysis

Analyte	QC level	A	B	%RSD	%Stability
Fedratinib	LQC	4.20	4.15	4.20	98.87
	HQC	2500.00	2570.31	1.61	102.81

A, Original concentration (ng/mL); B, mean average concentrations (ng/mL)

Conclusion

A rapid, specific, accurate and sensitive LC–MS/MS approach for the estimation of fedratinib in human plasma was developed and validated. In the developed method LLE extraction technique was executed for the reproducible recovery findings for the drug and IS. The technique was undergone for validation in accordance with FDA guidelines over the concentration range of 1.5–5000.00 ng/mL for fedratinib with correlation coefficient value of 0.9994. The intraday and interday precision were within 4.32% and the assay accuracy was 95.31–104.06 % of the nominal values. Matrix factor ranges from 94.25– 104.85 % with a %CV of 4.61 for analyte at LQC level and at HQC level, the matrix factor range was 94.62–103.88% with a %CV of 4.02. Stabilities revealed that the method has high degree of stability. The developed method can be utilized in the bioavailability and bioequivalence studies for the quantification of fedratinib in biological matrices.

References

1. Ayesha Begum, K., Gubbiyappa, Shiva, Kumar., Pamu, Sandhya., & Bhikshapathi, D.V.R.N. (2020) A Highly Sensitive LC–MS/MS Method Development and Validation of Fedratinib in Human Plasma and Pharmacokinetic Evaluation in Healthy Rabbits. Cur Pharm Ana, 17(6), 782 – 791.

2. Chambers, E.E., Woodcock, M.J., Wheaton, J.P. (2014) Systematic development of an UPLC–MS/MS method for the determination of tricyclic antidepressants in human urine. J Pharm Biomed Anal, 88, 660–665.
3. European Medicines Agency, Guideline on bioanalytical method validation 2011.
4. FDA Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) May 2001.
5. Henion, J., Brewer, E., Rule, G. (1998) Sample Preparation for LC/MS/MS: Knowing the Basic Requirements and the Big Picture of an LC/MS System can Ensure Success in Most Instances. Anal Chem, 70, 650A-656A.
6. Jamieson, C., Hasserjian, R., Gotlib, J., Cortes, J., Stone, R., Talpaz, M., Thiele, J., Rodig, S., Pozdnyakova, O. (2015) Effect of treatment with a JAK2-selective inhibitor, fedratinib, on bone marrow fibrosis in patients with myelofibrosis. J Transl Med, 10(13), 294.
7. Kumar, P.P., Murthy, T.E., Basaveswara, Rao, M.V. (2015) Development, validation of liquid chromatography–tandem mass spectrometry method for simultaneous determination of rosuvastatin and metformin in human plasma and its application to a pharmacokinetic study. J Adv Pharm Technol Res, 6, 118–124 32.
8. Moulard, O., Mehta, J., Fryzek, J., Olivares, R., Iqbal, U., Mesa, R.A. (2014) Epidemiology of myelofibrosis, essential thrombocythemia, and polycythemia vera in the European Union. Eur J Haematol, 92(4), 289–297.
9. Murphy, A.T., Kasper, S.C., Gillespie, T.A., DeLong, A.F. (1995) Determination of Xanomeline and Active Metabolite, N-Desmethylxanomeline, in Human Plasma by Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry. J Chromatogr B Biomed Appl, 668, 273-280.
10. Pardanani, A., Gotlib, J.R., Jamieson, C., Cortes, J.E., Talpaz, M., Stone, R.M., Silverman, M.H., Gilliland, D.G., Shorr, J., Tefferi, A. (2011) Safety and efficacy of TG101348, a selective JAK2 inhibitor, in myelofibrosis. J Clin Oncol, 29(7), 789-96.
11. Pardanani, A., Harrison, C., Cortes, J.E., Cervantes, F., Mesa, R.A., Milligan, D., Masszi, T., Mishchenko, E., Jourdan, E., Vannucchi, A.M., Drummond, M.W., Jurgutis, M., Kuliczkowski, K., Gheorghita, E., Passamonti, F., Neumann, F., Patki, A., Gao, G., Tefferi, A. (2015) Safety and Efficacy of Fedratinib in Patients With Primary or Secondary Myelofibrosis: A Randomized Clinical Trial. JAMA Oncol, 1(5), 643-51.
12. Patel, D.S., Sharma, N., Patel, M.C. (2011) Development and validation of a selective and sensitive LC–MS/MS method for determination of cycloserine in human plasma: application to bioequivalence study. J Chromatogr B, 879, 2265–2273.
13. Richard, Hoetelmans, M.W., Marjolijn, Van, Essenberg., Pieter, Meenhorst, L., Jan, Mulder, W., Jos, Beijnen, H. (1997) Determination of saquinavir in human plasma, saliva, and cerebrospinal fluid by ion-pair high-performance liquid chromatography with ultraviolet detection. Journal of Chromatography B, 698, 235-241.
14. Roskoski, R. (2016) Janus kinase (JAK) inhibitors in the treatment of inflammatory and neoplastic diseases. Pharmacol Res, 111, 784-803.

15. Sangle, N., Cook, J., Perkins, S., Teman, C.J., Bahler, D., Hickman, K., Wilson, A., Prchal, J., Salama, M.E. (2014) Myelofibrotic transformations of polycythemia vera and essential thrombocythemia are morphologically, biologically, and prognostically indistinguishable from primary myelofibrosis. Appl Immunohistochem Mol Morphol, 22(9), 663–668.
16. Shankar, Cheruku., Bhikshapathi, D.V.R.N. (2021) Method development and validation for the quantification of pexidartinib in biological samples by LC-MS/MS. IJPR, 13(1), 6522-30.
17. Vainchenker, W., Kralovics, R. (2017) Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. Blood, 129(6), 667–679.
18. Zhang, M., Xu, C., Ma, L., Shamiyeh, E., Yin, J., von, Moltke, L.L., & Smith, W.B. (2015) Effect of food on the bioavailability and tolerability of the JAK2-selective inhibitor fedratinib (SAR302503): Results from two phase I studies in healthy volunteers. Clin Pharmacol Drug Dev, 4(4), 315-21.
19. Zhang, M., Xu, C.R., Shamiyeh, E., Liu, F., Yin, J.Y., von, Moltke, L.L., Smith, W.B. (2014) A randomized, placebo-controlled study of the pharmacokinetics, pharmacodynamics, and tolerability of the oral JAK2 inhibitor fedratinib (SAR302503) in healthy volunteers. J Clin Pharmacol, 54(4), 415-21.
20. Zhong, G., Bi, H., Zhou, S., Chen, X., Huang, M. (2005) Simultaneous determination of metformin and gliclazide in human plasma by liquid chromatography–tandem mass spectrometry: application to a bioequivalence study of two formulations in healthy volunteers. J Mass Spectrom, 40, 1462–1471.