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ESTIMATION OF GILTERITINIB IN HUMAN PLASMA BY LC-ESI-MS/MS: METHOD DEVELOPMENT AND VALIDATION

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ABSTRACT

A simple, rapid and sensitive bioanalytical method developed to validate the quantity of Gilteritinib drug in human plasma. The development of method was done with the help of LC-ESI-MS/MS system. This method employs binary normal phase phase liquid chromatography-tandem mass spectroscopy operating in Multiple Reaction Monitoring (MRM) mode for detection. This method uses Electron spray ionization source and operates in positive mode. Solid phase extraction method was used to prepare samples, yielding high recovery rates for both Gilteritinib and its internal standard (ISTD). Chromatographic separation was achieved on column Kinetex, 2.6 µm Biphenyl (50mm × 4.6mm) with a mobile phase comprising Pump-A: 0.1% Formic acid in 10Mm Ammonium format and Pump B: Organic mixture (Methanol: Acetonitrile, 50:50 v/v) at yellow monochromatic light under Ambient temperatuare (25 ±5°C). The analyte and ISTD were eluted at approximately 2.7 minutes with total runtime of 5 minutes. Sample prepration was done by Solid phase extraction and this method yelds recover 75.91% for drug and 77.72% for ISTD respectively. The method demonstrated excellent linearity over a dynamic range of 0.200 ng/mL to 130.000 ng/mL, with correlation coefficients consistently within the acceptable range. The regression analysis were found in range of 0.9989. The accuracy of the method met regulatory criteria, within acceptance limit of ±20 at lower limit of quantification(LLOQ) and ±15% at other concentrations. The method was validated following ICH M10, ANVISA and USFDA guidelines, and confirming its reliability for bioanalytical application in pharmacokinetic and clinical studies.

KEYWORDS: Electron spray ionization, Bioanalytical method development, Gilteritinib, Solid-phase Extraction, Validation, LC-ESI-MS/MS.

1. INTRODUCTION

Gilteritinib received approval on 28 November 2018 by USFDA. This drug is marketed by Astellas Pharma under the brand name of Xospata® as a therapeutic option for acute myeloid leukaemia (AML). Additionally, Gilteritinib also been investigated for potential use in treatment of COVID 19 as an antiviral.[1]

Acute myeloid leukaemia (AML) treated by Gilteritinib, which is an innovative oral tyrosine kinase inhibitor. In order to improve therapeutic efficacy and patient safety, Gilteritinib medication levels must be monitored (therapeutic drug monitoring or TDM).[2] Primarily it is used for patients aged 18 and above it has relapsed or refractory acute myeloid leukaemia (AML). Although Gilteritinib offers substantial survival benefit over standard chemotherapy for AML patients. It is accompanied by side effects that could impair the daily functioning and comfort of patients also includes including hematologic toxicities such as anaemia, febrile

neutropenia, thrombocytopenia, and gastrointestinal disturbances like diarrhoea. Because of these toxicities, physicians may reduce in the dose or discontinuing treatment altogether.[3] Mode of action of Gilteritinib is an anti-cancer medication belonging to the kinase targeting agent class, for relapsed or refractory AML. Functions as selective FLT3 inhibitor, effectively blocking the point mutations and internal tandem duplications of the receptor.[1] It blocks FLT3 phosphorylation, it effectively downregulates associated signalling pathways, including AKT, STAT5, and ERK.[4]

FLT is member of type III tyrosine receptor, give its importance in proliferation, persistence, and maturation of multipotent stem cells into specialized linkages. Overexpression of FLT3 observed most in AML cases including internal tandem duplications (FLT3-ITD) within or near juxta membrane domain and tyrosine kinase domain which is result in the constitutive activation of FLT3. Inhibiting AXL disrupts its interaction with FLT3, thereby suppressing the augmentation of FLT3-wild-type AML cells and FLT3 mutant.[5,10]

The pharmacokinetics (PK) and pharmacodynamics (PD) of Gilteritinib studied among individual aged 18 and older with relapsed or refractory AML as part of an active phase 1/2 dose escalation trial and dose-extension trial. It is metabolized into inactive metabolites, by CYP3A4 and identified in vitro as a substrate of P-glycoprotein (P-gp).[6,7,8,9]

Gilteritinib dosages ranging from 20 to 450 mg given orally once day were examined in patient within initial clinicial trial for dosing. The dose ceiling for gilteritinib without causing any toxicity was found 300 milligram once daily. The appropriate phase 2 dose of gelderitinib was determined to be 120 mg once day. At a median Tmax of approximately 4 to 6 hours after oral gilteritinib tablet delivery, peak concentrations were noted. Gilteritinib had an estimated half-life (T1/2) of 113 hours and shown dose-proportional pharmacokinetics dosages spanning 20 to 450 mg given once every 24 hours. The main method of elimination was faeces. Gilteritinib exposure was same while fed and when fasting. The coadministration of gilteritinib with rifampicin—a potent inducer of P-glycoprotein and CYP3A—or with itraconazole—a strong inhibitor of both P-glycoprotein and CYP3A4—resulted in marked alterations in gilteritinib's pharmacokinetic profile. The primary metabolic pathway of Gilteritinib involves the cytochrome P450 (CYP) 3A4.[11]

Table 1: Drug Profile.

Sr.No.	Name	Gilteritinib
1.	Description	It is a oral tablet dosage form. Selective targets the FMS-like tyrosine kinase 3 (FLT3), including tyrosine kinase domain (TKD) mutations and FLT3 internal tandem duplication (ITD). It is also involve in AKT, STAT5, and ERK. Generally it is administered once daily.
2.	Structure	
3.	CAS No.	1254053-43-4
4.	Empirical Formula	$C_{29}H_{44}N_8O_3$
5.	Molecular Weight	552.72
6.	IUPAC Name	6-ethyl-3-[3-methoxy-4-[4-(4-methylpiperazin-1-yl)piperidin-1-yl]aphenyl]amino]-5-[(tetrahydro-2H-pyran-4-yl)amino]pyrazine-2-carboxamide
7.	Therapeutic Category	Antineoplastics, Tyrosine Kinase Inhibitors
8.	Colour	Yellow solid
9.	pKa	pKa (Strongest Acidic) =14.21 pKa (Strongest Basic) =8.47
10.	Storage	At ambient temperature in yellow monochromatic light
11.	Uses	Use as anti cancerous drug in relapse and refactory Acute myloid lukemia.

Table 2: Literature Survey.

Sr. No.	Refe. No.	Talking Point	Comprise
1.	12	In this, Analytical Assessment of Gilteritinib in Solid Oral Formulations via RP-HPLC is done. Tablets of Gilteritinib (40 mg) used as matrix	Column - C18 Column (250 x 4.6 mm; 5µm) M.P - Sodium Perchlorate, Acetonitrile Buffer – Sodium Perchlorate, water Flow rate - 1mL/min. Wavelength - 310nm
2.	13	Here, LC-MS-Based Analysis of Gilteritinib Fumarate and Structural Characterization of Its	Column - Acquity BEH C18 column (2.1 × 100 mm

		Major Degradants by Nmr are discussed. Bulk drugs	Mobile phase -Ammonium Acetate, Acetonitrile Flow rate - 0.3 mL min^{-1}
3	14.	Nanocarrier-Based Augmentation of Gilteritinib Using Hyaluronic Acid–EGCG Complexes. Bovine serum albumin	Column - HS C18 column Mobile Phase - Acetic acid, Methanol Flow rate - 1 mL/min Wavelength- 314 nm
4	15	Validated LC-MS/MS Method for the Estimation of Gilteritinib in Mouse Plasma for Preclinical Studies	Column - Accucore aQ column (50 mm × 2.1 mm Mobile Phase - Formic acid, Acetonitrile Flow rate - 0.4 mL/min, Run time - 2.5 min
5	16	Sensitive LC-MS/MS Assay for the Detection of Gilteritinib in Rat Plasma and Its Role in Pharmacokinetic Interaction Studies.	Column- 2.1 mm × 50 mm, 1.7 µm Mobile Phase- Acetonitrile, 0.1% formic acid in water Run time- 3.0 min
6.	17	Stress Degradation Analysis of Gilteritinib by LC-MS/MS and HPLC Method Establishment for Monitoring Synthetic Impurities.	Column - Waters Symmetry C18 (250 mm × 4.6 mm, 5µm) M.P - solvent A - pH 4.5 phosphate buffer and acetonitrile [25:75 (v/v)], solvent B - acetonitrile and methanol in [75: 25 (v/v)] Flow rate - Isocratic at 0.7 mL/min λ - 230 nm
7.	18	Integrated In Vitro and In Silico Estimation of Gilteritinib Metabolic Stability Using a UPLC–ESI–MS/MS Analytical Method. HLMs protein	Column - Eclipse plus-C18 (50 mm length, 1.8 µm particle size and 2.1 mm internal diameter). M.P - Organic part (50%; ACN) and an aqueous part (50%: 0.1% formic acid in H2O; pH: 3.2). Flow rate - 0.2 mL/min. Elution time - (2 min)
8.	19	Fast and Sensitive UPLC-MS/MS Method for the Pharmacokinetic Profiling of Duvelisib in Beagle Canines	Column- Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 µm) M.P- acetonitrile (solvent A) and 0.1 % formic acid in water (solvent B) Flow- Gradient elution Extraction Method- Protein precipitation

2. MATERIAL AND METHODS

Gilteritinib (98.2%purity) purchased from TLC Pharmaceutical and Gilteritinib D5 [97.34%purity, as Internal Standard (ISTD)] purchased from Simson pharma, Mumbai, India. Formic acid, Ammonium Formate, Sodium Hydroxide used grade of Emparta/AR/GR/Emplura grade, Methanol and Acetonitrile grade HPLC/ULCMS, Water fro Milli-Q water.

3. LC-MS/MS Instrumentation: This used in separation of analyte. This LC-ESI-MS/MS comprising of a pump, a column oven to control the temperature, a controller and a cooling autosampler. Using column Kinetix, 2.6µ Biphenyl 50mm× 4.6mm the chromatographic separation of analyte and their Gilteritinib D5 as an ISTD was done in minutes. It consist mobile phase which is mixture of Pump A: Mobile Phase Buffer (Formic Acid in 10 mM Ammonium Formate in Water, 0.1%V/V), Pump B: Organic Mixture (Methanol: Acetonitrile, 50:50 V/V), in binary mode maintaining a flow rate of 0.8000 mL/min. Autosampler temperature control was established at 5 ± 3^0 C and column were set at 40 ± 3^0 C. Positive ion mode was used to run a mass detector with an ESI interface. Quantification was done utilizing the Multiple Reaction Monitoring (MRM) mode of transition's m/z 553.35 →436.30 for Gilteritinib and m/z 558.35 →441.20 for Gilteritinib D5. The nebuliser, collision, auxiliary, and curtain gases were all nitrogen.

3.1 Preparation of Working solutions, Stock solutions, Standard solutions and Quality Control Solutions: Accurately weighted Gilteritinib and ISTD (Gilteritinib D5) dissolved in methanol, achieve the concentration of 1.000mg/mL to create separate stock solution and kept at ambient temperature before being used. Total ten working standard solutions are prepared from range of 0.200 to 130.000 ng/ mL for calibration curve. Serial dilutions were performed by taking methanol: water (50:50, v/v) as the diluent. Preparation for Calibration standard done by spiking 9.8ml blank plasma with 0.2 ml of working solution to get final concentration of 0.200, 0.400, 1.300, 3.250, 6.500, 13.000, 26.000, 52.000. 104.000, 130.000 ng/ mL. Quality control stock solution was prepared by same

process of working stock solution prepared. QC working standard solution were prepared from QC stock solution. Range of 0.200 for LLOQC was 0.200 ng/ mL, LOQ was 0.600 ng/ mL, MQC-2 was 9.000 ng/ mL, MQC-1 was 45.500 ng/ mL and HQC was 97.500 ng/ Ml.

3.2 Sample Preparation: SPE was used to process sample of plasma. 0.200mLof each sample into prelabelled tube. Pipette out 50µL of ISTD dilution and mix. Add 50µL of Diluent to ISTD dilution to STD BL samples. Prepend 200µl of Extraction Buffer (0.1N Sodium Hydroxide in water, w/v) in all samples and stir. Centrifuge at 4000 rpm at 10±2°C for 2 minutes. Arrange the required number of pre labelled UCT, (30mg), 1mL HLB DVD extraction cartridges. 1.000mL of methanol followed by 1.000mL of water, used for condition the cartridges. Load the samples on conditioned cartridges. Flush cartridges using 1.000mL of Water subsequent 1.000mL Methanolic aqueous solution, 5%v/v. Reconstituent the contents of the cartridges by eluting with 1.000 mL of Methanol into pre-labelled tubes.

4. RESULT AND DISCUSSION

Above experiment was done on the LC-MS/MS system, followed the ICH M10 guidelines. This experiment estimates the drug in human plasma in which anti-coagulant was already present. Details of method development and validation are given.

Determination of Drug and ISTD Identity.

Table 3: Q1 and Q3 Mass for Drug and ISTD.

	Drug	ISTD
Molecular Weight	552.72	557.74
Q1 Mass	553.35	558.35
Q3 Mass	436.30	441.20

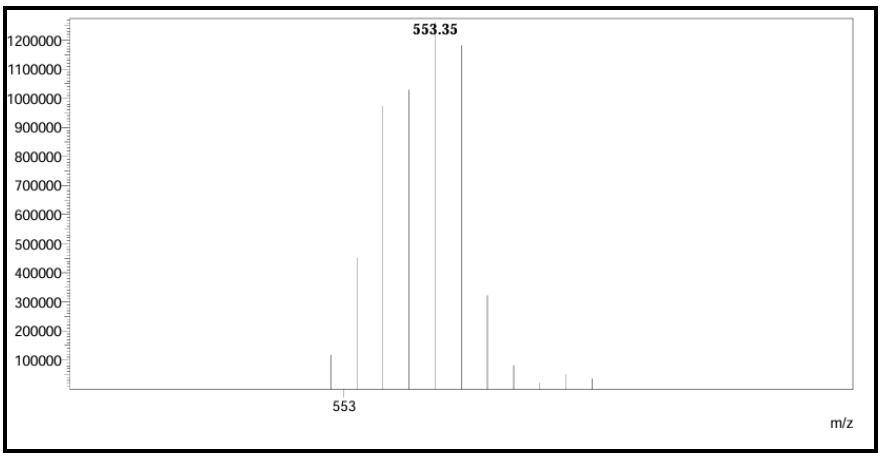

Figure 1. Gilteritinib Parent Ion Scan.

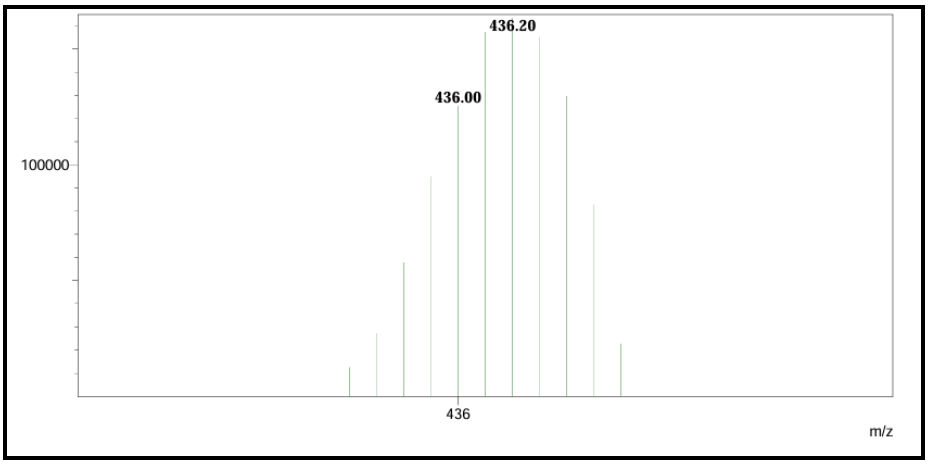

Figure 2. Gilteritinib Daughter Ion Scan.

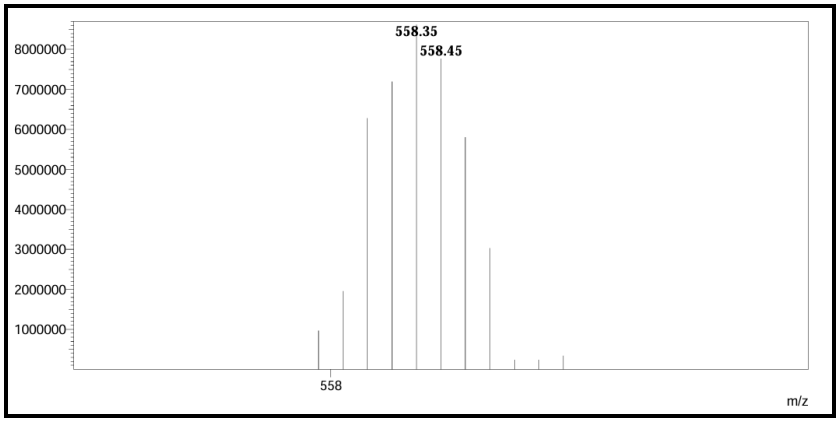

Figure 3. Gilteritinib D5 Parent Ion Scan.

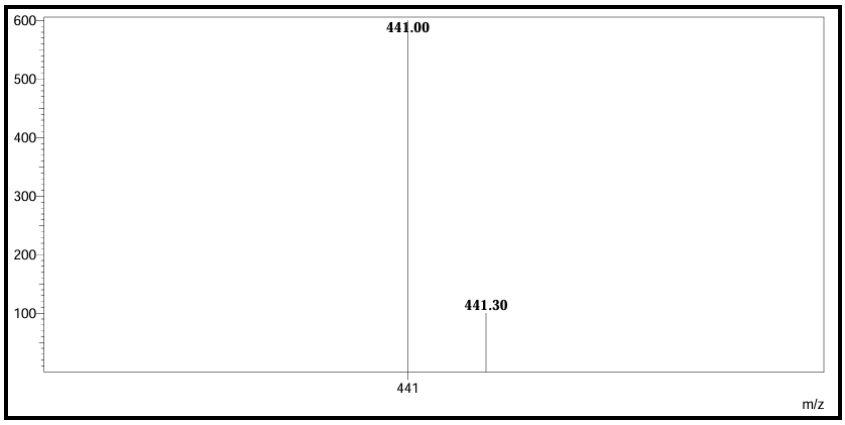

Figure 4. Gilteritinib D5 Daughter Ion Scan.

Table 4: Tuning of Drug and ISTD.

Parameter	Drug	ISTD
Scan Type	Multiple Reaction Monitoring (MRM)	Multiple Reaction Monitoring (MRM)
Polarity	Positive	Positive
Ion Source	Turbo Ion Spray (TIS)	Turbo Ion Spray (TIS)
Collision Energy (CE)	-33.0eV	-34.0eV
Q1 Pre Bias (V)	-24.0	-24.0
Q3 Pre Bias (V)	-30.0	-30.0

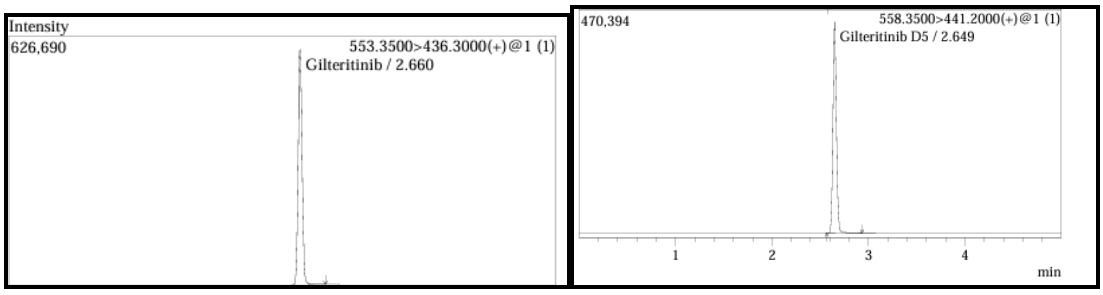

Figure 5. Good Peak Shape with appropriate response for both analyte and ISTD was observed.

Table 5: Optimized Chromatographic Condition.

Sr. No	Chromatographic Parameters	Condition
1	Mobile Phase	Pump A: Mobile Phase Buffer (Formic Acid in 10 mM Ammonium Formate in Water, 0.1%V/V) Pump B: Organic Mixture (Methanol : Acetonitrile, 50:50 V/V)
2	Column	Kinetix, 2.6µ Biphenyl 50mm× 4.6mm
3	Flow rate	0.8000 mL/min
4	Retention Time	2.700 min

5	Run time	5.00 min
6	Injection volume	10µL
7	Column Oven Temperature	40±3^0 C
8	Diluent	Water: Methanol, 50:50v/v
9	Autosampler Rinsing Volume	2000µL
10	Purgng Time	
11	Autosampler Temperature	5±3^0 C

Table 6: Optimized Extraction Process.

Sr. No	Parameter	Condition
1	Extraction Method	Solid Phase Extraction
2	Sample processed	STD BL, UL, LLOQ
3	Aliquoting Volume	0.200mL
4	Buffer	Formic Acid in 10 mM Ammonium Formate in Water, 0.1%V/V
5	Centrifugation	For 02 min at 4000 rpm & at 10±2°C
6	Washing	1.000ml of water followed by 1.000ml of methanol in water, 5%v/v
7	Elution	1.000ml Methanol
8	Drying	Evaporate the sample at 40°C under N2 gas
9	Reconstitution	Mobile Phase Buffer (Formic Acid in 10 mM Ammonium Formate in Water, 0.1%V/V) : Organic Mixture (Methanol: Acetonitrile, 50:50 V/V) [20: 80]

4.1 System suitability

Six injections are taken to check the performance of system before analysis, which ensures that whether system is working properly of not.

Table 7: System Suitability Parameters.

Sample Name	Gilteritinib RT (min)	Gilteritinib D5 RT (min)	Area Ratio
AQ SYS-1	2.952	2.940	2.61
AQ SYS-2	2.868	2.854	2.68
AQ SYS-3	2.868	2.855	2.69
AQ SYS-4	2.873	2.859	2.61
AQ SYS-5	2.868	2.855	2.63
AQ SYS-6	2.852	2.839	2.64
%CV	1.25	1.27	1.30
Accepted	Yes	Yes	Yes

4.2 System performance

Shows that how well system deliver accurate, precise and reliable result. The following sequence of injections: LLOQ (* indicates the same sample), ULOQ, STD BL*, STD BL*, and STD BL* with recurrent acquisition).

Table 8: System performance parameters.

Sample ID	Gilteritinib	Gilteritinib D5
STD BL (1st)	0	0
ULOQ	4525384	614341
STD BL (2nd)	0	0
STD BL (3rd)	395	0
LLOQ	8132	693034
% Interference (Compared to 1st STD BL)	0.00	0.00
% Carry Over (Compared to 2nd STD BL)	0.00	0.00
% Carry Over (Compared to 3rd STD BL)	4.86	0.00
Signal of Analyte in LLOQ (Compared to 1st STD BL)	8132.0	
Accepted	Yes	

4.3 Linearity graph

Has ability to test the result within given range which is proportional to the concentration of analyte present in sample. This plotted over concentration of 6 points: 130.0, 104.0, 52.0, 26.0, 13.0, 6.50, 3.250, 1.300, 0.400,

0.200 ng/mL. From given table correlation coefficient (R2) 0.9989 was obtained.

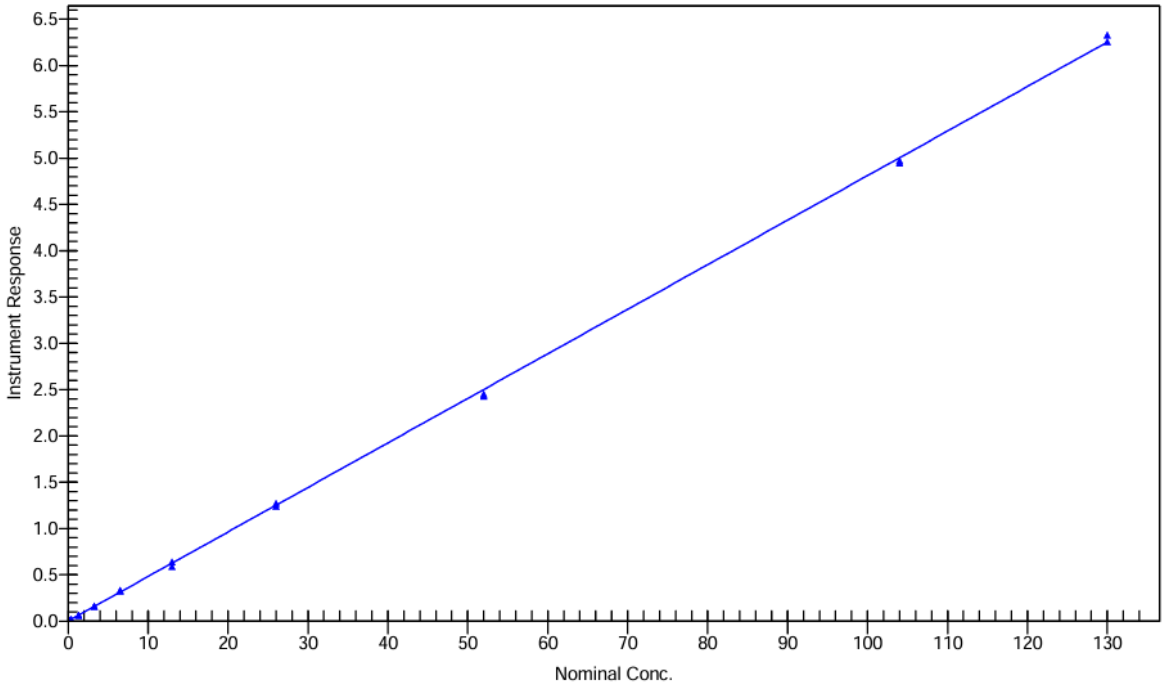

Figure 6. Result of linearity graph.

Table 9: Concentration of Precision and Accuracy.

STD ID	STD1	STD2	STD 3	STD4	STD5	STD6	STD7
nominal conc. (ng/ml)	130.000	104.000	52.000	26.000	13.000	6.500	3.250
1	130.012	102.82	50.517	26.413	13.237	6.838	3.357
	131.566	103.382	50.966	25.76	12.206	6.727	3.26
MEAN	130.789	103.101	50.7415	26.0865	12.7215	6.7825	3.3085
D	1.098	0.397	0.317	0.461	0.729	0.078	0.068
%CV	0.84	0.39	0.62	1.77	5.73	1.15	2.09
MEAN ACCURACY	100.61	99.14	97.58	100.33	97.86	104.35	101.82

STD ID	STD8	STD9	STD10	SLOPE	INTERCEPT	R2
nominal conc. (ng/ml)	1.300	0.400	0.200	0.0481	0	0.9989
1	1.31	0.391	0.211	REGRESSION FOOTNOTE Response = Slope * Conc + Intercept		
	1.276	0.392	0.193			
MEAN	1.293	0.3915	0.202			
D	0.024	0.0007	0.0127			
%CV	1.86	0.26	6.44			
MEAN ACCURACY	99.46	98	101			

4.4 Pression and accuracy

This conforms about reliable and reproducible the tested result is. Accuracy measures the value of true to checks the whether your method is correct or not. We get result after calculation of % Recovery. Result comes under acceptance range of ±15%. All LLOQ QC quality control samples had intra-run accuracy within the acceptable range of ±20%. Therefore, the outcome was deemed satisfactory.

Table 10: Result of the samples.

P&A	QC Sample	Mean	SD	%CV	%Mean Accuracy
P & A I	LLOQC	0.182	0.005	2.75	91.00
	LQC	0.580	0.019	3.28	96.67

	MQC2	8.712	0.122	1.40	96.81
	MQC1	44.119	0.784	1.78	96.97
	HQC	94.032	1.097	1.17	96.44
P & A II	LLOQC	0.206	0.005	2.43	103
	LQC	0.642	0.02	3.12	104
	MQC2	9.593	0.239	2.49	106.59
	MQC1	47.614	1.208	2.54	104.65
	HQC	98.46	1.207	1.23	100.98
P & A III	LLOQC	0.203	0.011	5.91	101.5
	LQC	0.637	0.023	3.61	106.33
	MQC2	9.626	0.175	1.82	106.97
	MQC1	47.681	1.233	2.59	104.8
	HQC	100.48	1.151	1.15	103.06

Table 11: Data of Inter-run Pression and Accuracy.

INTER RUN	LLOQC	LQC	MQC-2	MQC-1	HQC
Sr. No	0.200	0.600	9.000	45.500	97.500
Mean conc. Found (ng/mL)	0.198	0.619	9.311	46.47	97.11
Inter-run SD	0.012	0.018	0.121	0.784	1.096
inter-run% cv	6.288	3.049	1.306	1.687	1.129
inter run % mean accuracy	99.00	103.16	103.45	102.131	99.60
Number of samples	18				

4.5 Recovery

Refers to efficacy of analyte is extracted from biological matrix. In this result, at every QC level, the percentage CV was less than 15%. Therefor the outcome was deemed satisfactory.

Table 12: Recovery data.

Sr No.	HQC		MQC 1		LQC	
	Extracted Peak Area	Un-extracted Peak Area	Extracted Peak Area	Un-extracted Peak Area	Extracted Peak Area	Unextracted Peak Area
1	3830056	4779684	1697435	2433463	23958	34814
2	4004799	4960528	1842493	2397863	24835	32816
3	3874781	4940895	1854895	2452153	24116	33894
4	3804261	5049530	1901708	2292625	22251	33605
5	3613840	4894684	1743528	1992591	24940	31926
6	3843771	4943066	1868092	2322021	23120	33174
Mean	382858.7	4928064.5	1818025.1	2315119.3	23870	33371.5
SD	126590.2	88688.71	79447.31	169828.22	1032.28	984.41774
%CV	3.3	1.8	4.36	7.34	4.32	2.95
% Mean Recovery	77.689		78.528		71.528	
CF	1.00					
% Mean Recovery with CF	77.689		78.528		71.528	
% Overall Recovery	75.915					
% Overall Recovery with CF	75.915					
% Overall CV	3.96875					

Table 13: ISTD Recovery.

Sr No.	HQC		MQC1		LQC	
	Extracted Peak Area	Un-extracted Peak Area	Extracted Peak Area	Un-extracted Peak Area	Extracted Peak Area	Un-extracted Peak Area
1	908914	1275196	1021565	1356481	1055403	1442256
2	112250	1376641	1083613	1376988	1091361	1366811
3	105820	1325337	1086227	1380851	1033726	1442712
4	101981	1363066	1123906	1304992	1031889	1451579
5	969108	1285984	1019646	1146169	1076781	1327609
6	105756	1355630	1103366	1347526	1066622	1450501
Mean	1022697	1330309	1073054	1318835	1059297	1413578
SD	75195.41	4216.8	43112.17	88851.93	23696.77	53021.55
%CV	7.35	3.16	4.01	6.73	2.23	3.75
% Mean Recovery	76.87		81.36		74.93	
CF	1.00					
% Mean Recovery with CF	76.87		81.36		74.93	
% Overall Recovery	77.72					
% overall Recovery with CF	77.72					
% Overall CV	4.538333					

4.6 Dilution integrity

For a 1/10 dilution, DI Spiking Solution (DISS) was made with a concentration that was around 5-6 multiple grow of CC spiking solution. K3EDTA was used as an anticoagulant in the sample dilution process using veil human plasma. The dilution quality control percentage CV is 1.63%, falling within the 15% acceptable range.

Table 14: Dilution Integrity.

Sr. No	DQC 650.000 ng/mL
1	613.694
2	588.796
3	600.965
4	603.380
5	587.402
6	598.149
Mean	598.731
SD	9.776
% CV	1.63
% Accuracy	92.11

4.7 Matrix effect

This term describes the effect of elements other than analyte which may affect the measuring of analyte of interest. It measured by using three replicates of LQC and three replicates of HQC, which is prepared from 8 different matrix (which include one female matrix, one Haemolytic matrix, one Lipidemic matrix). The %CV at HQC and LQC level were found 1.51% and 2.60% respectively which is not more than 15 %.

Table 15: Matrix Effect at LQC Level.

Sample	Nominal Conc.(ng/mL)			Mean of Nominal conc. found	%Accuracy
	Replicate 1	Replicate 2	Replicate 3		
1	0.558	0.584	0.574	0.572	95.33
2	0.586	0.569	0.592	0.582	97
3	0.587	0.594	0.571	0.584	97.33
4	0.614	0.556	0.577	0.582	97

5	0.564	0.567	0.597	0.576	96
6	0.592	0.595	0.571	0.586	97.66
LIP (Lipidemic)	0.621	0.605	0.580	0.602	100.33
H (Haemolytic)	0.595	0.604	0.582	0.593	98.83
	Over all mean			0.584	-
	Over all SD			0.008	-
	% Over all CV			1.517	-
	% Over all mean accuracy				97.435

Table 16: Matrix Effect at HQC Level.

Sample	Nominal Conc.(ng/mL)			Mean of Nominal conc. found	%Accuracy
	Replicate 1	Replicate 2	Replicate 3		
1	100.848	100.228	100.977	100.684	103.26
2	99.262	100.942	101.053	100.419	102.99
3	100.178	96.794	95.434	97.468	99.96
4	98.154	95.855	95.069	96.359	98.82
5	100.347	94.944	99.839	98.3766	100.89
6	97.559	95.956	94.336	95.950	98.41
LIP (Lipidemic)	93.487	93.487	93.446	93.473	95.87
H (Haemolytic)	93.474	93.555	94.547	93.858	96.25
	Over all mean			97.073	-
	Over all SD			2.528	-
	% Over all CV			2.604	-
	% Over all mean accuracy				99.556

4.8 Ruggedness

Several pieces of equipment, columns, and analysts were used to assess the method's robustness. All high, moderate, and low-quality control samples had inter-run accuracy within the 15% acceptable range. For every LLOQ QC quality control sample, inter-run accuracy was shown to be within 20% is the acceptable limit. The ruggedness experiment satisfies the acceptance requirements for linearity as previously stated, as well as within-run (intra-run) accuracy and precision as previously stated. Therefore, the outcome was deemed satisfactory.

Table 17: Ruggedness of Quality Samples.

Different analyst Ruggedness.					
Sr. No.	LLOQC 0.200 ng/mL	LQC 0.600 ng/mL	MQC-2 9.00 ng/mL	MQC-1 45.500 ng/mL	HQC 97.500 ng/mL
1	0.211	0.606	8.906	44.272	93.089
2	0.199	0.632	8.732	43.946	94.003
3	0.207	0.616	8.631	42.542	93.741
4	0.211	0.618	8.4	43.275	93.05
5	0.195	0.581	8.951	42.595	51.738
6	0.2	0.592	8.5	42.78	94.04
Mean	0.204	0.608	8.687	43.235	93.2777
S.D	0.007	0.019	0.219	0.732	0.869
%CV	3.43	3.13	2.52	1.69	0.93
%Accuracy	102	101.33	96.52	95.02	95.67
N	6				

Table 18: In different day ruggedness.

Different day Ruggedness.					
Sr. No.	LLOQC 0.200 ng/mL	LQC 0.600 ng/mL	MQC-2 9.00 ng/mL	MQC-1 45.500 ng/mL	HQC 97.500 ng/mL
1	0.226	0.633	9.644	48.637	100.039
2	0.211	0.639	9.457	48.706	97.623

3	0.211	0.645	9.623	46.813	101.859
4	0.21	0.645	9.536	45.873	99.946
5	0.202	0.654	9.376	46.059	98.995
6	0.21	0.636	9.383	47.135	97.343
Mean	0.211	0.642	9.503	47.203	99.300
S.D	0.007	0.007	0.116	1.228	1.688
%CV	3.68	1.16	1.22	2.60	1.69
%Accuracy	105.8	107	105.588	103.742	101.846

5. STABILITY

This indicate that, how a drug remains stable through its shelf life. It has to remain stable in different temperature and conditions.

5.1 Stability of analyte in blood

Assessed for both HQC and LQC levels. 9.800 mL of whole blood was spiked with 0.200 mL of SS HQC and SS LQC in duplicate (three repetitions), yielding six aliquots of 1 mL of each. For 2 hours samples, the mean at ambient degree is 90.10% owing to HQC and 90.70% for LQC. For sample in ice bath less than 10°C. 92.08% for HQC and 90.80% for LQC, which is within acceptance criteria of 85 - 115%.

Table 19: Performing the stability in blood at different temperature.

Sr. No	HQC		LQC	
	Analyte Peak Area Ratio		Analyte Peak Area Ratio	
	Comparison Sample (0 hr)	Stability Sample (02 hr)	Comparison Sample (0 hr)	Stability Sample (02 hr)
1	2.062	1.924	0.012	0.011
2	2.107	1.985	0.011	0.01
3	2.048	1.838	0.012	0.011
4	1.998	1.844	0.011	0.01
5	1.98	1.828	0.012	0.011
6	1.966	1.778	0.012	0.01
Mean	2.0267	1.8663	0.0116	0.0105
% CV	2.69	4	2.04	3.08
% Mean Stability		92.08	-	90.8

Table 20: At Ambient Temperature.

Sr. No	HQC		LQC	
	Analyte Peak Area Ratio		Analyte Peak Area Ratio	
	Comparison Sample (0 hr)	Stability Sample(2 hr)	Comparison Sample (0 hr)	Stability Sample (2 hr)
1	2.062	1.794	0.012	0.011
2	2.107	1.777	0.011	0.01
3	2.048	1.841	0.012	0.011
4	1.998	1.842	0.011	0.011
5	1.98	1.864	0.012	0.011
6	1.966	1.84	0.012	0.011
Mean	2.0267	1.8261	0.0116	0.0106
% CV	2.69	1.83	2.04	1.37
% Mean Stability		90.1	-	91.7

5.2 Bench top stability

This parameter determines how long an analyte remains stable in biological matrix at room temperature or ambient condition. The results were deemed acceptable as the mean bench top stability percentages for the drug's LQC and HQC samples were 96.50% and 93.46%, respectively, falling between the 85 and 115% acceptance thresholds.

Table 21: BT at HQC - LQC Stage.

Sr. No.	BT LQC 0.600 NG/ML	BT HQC 97.500NG/ML
1	0.586	89.576
2	0.585	89.918
3	0.575	92.219

4	0.588	95.097
5	0.561	88.807
Mean	0.579	91.123
S.D	0.011	2.559
%CV	1.9	2.81
%Accuracy	96.5	93.46
N	5	5

Table 22: Bench Top Stability for Quality Control Samples.

Sr. No.	LQC (0.600ng/mL)	MQC 2 (9.000ng/mL)	MQC 1 (45.500ng/mL)	HQC (97.500 ng/mL)
1.	0.626	9.312	46.229	103.694
2.	0.613	9.405	47.751	105.749
Mean	0.6195	9.3585	46.99	104.7215
SD	0.0065	0.0465	0.761	1.0275
% CV	1.05	0.496	1.619	0.98
% Accuracy	103.166	103.97	103.27	107.405

5.3 Freeze thaw stability

Refers that evaluating whether an analyte remains stable in biological matrix after being long frozen and multiple thawed. The % Accuracy of freeze thaw stability at -20±5°C for LQC 91.33% while for HQC 88.75%. And at -78±8°C, LQC 87.83% and for HQC 87.28% respectively which is within acceptance criteria.

Table 23: Freeze Thaw Samples for HQC and LQC Level at 20±5°C and -78±8°C.

Sr No.	FT LQC (-20±5^0 C) 0.600 ng/mL	FT LQC (78±8^0 C) 0.600 ng/mL	FT HQC (20±5^0 C) 97.500ng/mL	FT HQC (-78±8^0 C) 97.500 ng/mL
1	0.55	0.515	86.878	86.299
2	0.536	0.539	89.272	84.32
3	0.55	0.54	85.48	85.983
4	0.567	0.52	86.264	84.39
5	0.538	0.523	84.77	84.501
Mean	0.548	0.527	86.533	85.099
SD	0.012	0.011	1.726	0.96
%CV	2.19	2.09	1.99	1.13
% Accuracy	91.33	87.83	88.75	87.28
%Bias	-8.67	-12.17	-11.25	-12.72
N	5	5	5	5

Table 24: Freeze Thaw Quality Samples.

Sr. No.	LQC (0.600 ng/mL)	MQC 2 (9.000 ng/mL)	MQC1 (45.500ng/mL)	HQC (97.500ng/mL)
1.	0.549	8.501	41.755	96.175
2.	0.545	8.285	42.884	94.100
Mean	0.547	8.393	42.3195	95.1375
SD	0.002	0.108	0.5645	1.0375
% CV	0.365	1.286	1.332	1.09
% Accuracy	91.166	93.25	93	97.57

5.4 Long term stability (LTS)

Ability of drug remains stable in biological matrix for extended period under (-20±5^0 C) and (-78±8^0 C) storage conditions. Samples are stored in refrigerator for long duration before analyses. The %Accuracy of Long-Term Stability at (-20±5^0 C) for LQC and HQC was found to be 97.67% and 94.50% and stability at (-78±8^0 C) for LQC and HQC was found to be 97% and 96.23%.

Table 25: Long Term stability at (-20±5^0 C).

Sr No.	LQC (-20±5^0 C) 0.600 ng/mL	HQC (-20±5^0 C) 97.500 ng/mL
1	0.593	90.534
2	0.588	92.345

3	0.592	91.69
4	0.566	93.499
5	0.592	94.637
Mean	0.586	92.541
S.D	0.011	1.589
%CV	1.88	1.72
%Accuracy	97.67	94.91
N	5	5

Table 26: Long Term Stability at (-78±8^0 C).

Sr. No.	LQC (-78±8^0 C) 0.600 ng/mL	HQC (-78±8^0 C) 97.500ng/mL
1	0.591	95.426
2	0.522	93.848
3	0.565	92.675
4	0.617	93.682
5	0.583	93.493
Mean	0.582	93.825
S.D	0.025	1.002
%CV	4.3	1.07
%Accuracy	97	96.23
%Bias	-3	-3.77
N	5	5

5.5 First day stability (FDS)

This stability refers to first day of analysis. Show that how the analyte remains stable after sample preparation before injection into LCMS/MS. The %Accuracy of FDS, LQC 94.5% while HQC 96.35%.

Table 27: First Day Stability at LQC and HQC level.

Sr. No.	FDSA LQC 0.600ng/mL	FDSA HQC 97.500 ng/mL
1	0.562	95.119
2	0.59	92.274
3	0.575	94.038
4	0.561	92.443
5	0.545	95.829
Mean	0.567	93.941
S.D	0.017	1.58
%CV	3	1.68
%Accuracy	94.5	96.35
N	5	5

5.6 Stability of extract (SE)

This refers to stability of analyte after sample is extracted but done before analysis. This stability shows how analyte remains stable in final extract under autosampler condition. The % Accuracy of Stability of Extract at (5±3°C) for HQC and LQC was 103.3% and 103.3. Stability at ambient temperature foe HQC and LQC was 98.65% and 100.033 respectively.

Table 28: Stability of Extract at (5±3°C) and at Ambient Temperature for HQC and LQC level.

Sr. No	HQC (5±3°C)	LQC (5±3°C)	HQC (AT)	LQC (AT)
1.	100.821	0.631	97.275	0.607
2.	99.901	0.621	96.482	0.596
3.	102.164	0.638	96.854	0.598
4.	100.538	0.643	95.024	0.598
5.	100.210	0.627	95.317	0.602
Mean	100.7268	0.632	96.1904	0.6002
S.D	0.78218806	0.007797	0.874651	0.003919
%CV	0.776	0.776	0.908	0.65
%Accuracy	103.3	103.3	98.656	100.033

5.7 Dry extract (DE) stability

HQC and LQC were prepared by using 5 sets. Sample were evaporated and stored in deep refrigerator for 7 days. The %Accuracy of stability in Dry extract for HQC and LQC was found to be 93.7% and 92.13% respectively.

Table 29: Dry Extract (DE) Stability at HQC and LQC level.

Sr No.	HQC	LQC
1	90.358	0.559
2	91.669	0.552
3	92.398	0.570
4	92.060	0.547
5	90.348	0.536
Mean	91.366	0.552
S.D	0.859	0.011
%CV	0.94	2.06
%Accuracy	93.7	92.13

6. CONCLUSION

- Using HPLC-MS/MS, a bio-analytical approach for estimating the amount of Gilteritinib in human plasma is created that is accurate, precise, and reproducible.
- The novel tandem Mass Spectrometry-Liquid Chromatographic technique was created for Gilteritinib levels in human plasma are measured.
- The created approach was straight forward, quick, sensitive, economical, accurate, and reliable.
- Gilteritinib in K3EDTA human samples was extracted and analysed using the established technique, which was deemed legitimate. The technique was verified in accordance with the USFDA, ICH M10, and ANVISA regulatory criteria.
- BABE investigations, which are necessary for IND, NDA, and ANDA, will benefit from the established and validated bioanalytical approach.

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