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Bioanalytical Method Development and Validation for Quantification of an Anti-Neoplastic Agent - Glasdegib by using LC–MS/MS (ESI) in Human Plasma

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Abstract

Glasdegib is inhibitor of a Hedgehog signaling pathway used in the treatment of cancer associated with Sonic Hedgehog protein overexpression like breast, pancreatic, medulloblastoma, etc. Since this drug was recently approved by the food and drug administration the effectiveness of the treatment as well as the quality control of this drug need to be monitored. The highly sensitive and selective analytical technique like LC-MS/MS is necessary to monitor the quality and quantity of this drug in biological fluids. Hence this work aimed to develop an LC-MS/MS method to accurately quantify Glasdegib in biological fluids. Quantification of this drug was achieved by using a C_{18} symmetric column (150 mm x 4.6 mm, 3.5 µm) and isocratic elution, with a mobile phase containing Acetonitrile: 0.1% formic acid at a 30:70 ratios. The flow rate was set at 1 mL/min, and the mobile phase pH was adjusted to 4.0.The retention time for Glasdegib was found as 2.62 min, and a linear curve was established for concentrations between 6.00 and 120 ng/mL with regression coefficient of 0.999.Results showed that system suitability parameters, including theoretical plates, tailing factor, and resolution, within acceptable limits. Recovery testing indicated 99.94% extraction efficiency, while matrix effect studies revealed minimal interference (98.56%). Validation results of accuracy, linearity, and LOD/LOQ were found within acceptable ranges.The proposed LC-MS/MS method provides a sensitive, accurate, and reliable analytical approach for measuring Glasdegib in biological matrices, supporting its clinical applications in cancer treatment.

Keywords: Glasdegib, Development, Validation, LC-MS/MS, Biological Fluid

Introduction

Glasdegib, a novel smoothened inhibitor developed by Pfizer, has recently gained FDA approval for the treatment of cancers characterized by Sonic Hedgehog (SHH) protein overexpression. The SHH signaling pathway plays a critical role in cell proliferation and differentiation, and its dysregulation is implicated in various malignancies. By inhibiting the smoothened receptor, Glasdegib effectively blocks this pathway, offering a promising therapeutic strategy for patients with SHH-driven cancers. Currently, Glasdegib is being evaluated in ongoing Phase II clinical trials, including studies assessing its efficacy in myelofibrosis patients who have shown inadequate response to ruxolitinib, a standard treatment for this condition.

In 2018, the U.S. Food and Drug Administration (FDA) approved Glasdegib, a benzimidazole compound, for the treatment of AML(1,2). Glasdegib is characterized by its chemical structure, which includes a water-

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soluble group and a substituted piperidinyl group. The drug has a molecular formula of $C_{21}H_{22}N_6O$ and a molecular weight of 374.4 g/mol (3,4). It is administered orally, and its bioavailability is approximately 55%(5). In clinical trials, a dose of 50 mg of Glasdegib showed a peak plasma concentration (Cmax) of 542 ng/mL, achieved in about 4 hours (Tmax), with an area under the curve (AUC) of 9311 ng/mL(6,7). Glasdegib works by inhibiting the Hedgehog signalling pathway through its interaction with Smoothened (SMO) receptors, with an inhibitory concentration (IC_{50}) of 5 nm. This action not only targets cancer cells but also suppresses the growth of leukemia cells (8-11).

Glasdegib, marketed under the brand name Daurismo, is typically administered once a day for 28-day cycles, in conjunction with subcutaneous cytarabine injections on days 1-10 (12). The treatment regimen continues for a minimum of six cycles unless side effects become intolerable (13). It is important for patients to take Daurismo consistently, either with or without food, and avoid crushing the tablets (14). In the event of a missed dose, the patient should take it as soon as possible, but not within 12 hours of the next dose (15).

Despite the proven efficacy of Glasdegib in the treatment of AML, there remains a lack of comprehensive data on the quantification of Glasdegib levels in the bloodstream (16). Some studies have utilized RP-HPLC to measure the drug in pharmaceutical dosage forms, but these methods have not been fully validated for plasma quantification (17,18). To address this gap, this study introduces an LC-MS/MS assay, which offers high sensitivity and precision for measuring Glasdegib concentrations in human plasma (19). The internal standard for this method is Gilteritinib, another drug used in the treatment of AML (20). The validated LC-MS/MS technique can be applied for understanding the drug's behaviour in the body and potentially improve clinical management for AML patients. This new approach offers a more accurate and robust method for monitoring Glasdegib levels in human plasma by using LC-MS/MS Method (Figure 1).

Experimental

Methods & Materials

Chemicals and reagents

The Biocon Pharma company (Bangalore, India) supplied Glasdegib at no cost. LC-MS grade solvents (acetonitrile and methanol) were supplied by Merck Chemical Division (Mumbai, India).Millipore water was obtained from the in house Millipore system. Human plasma was obtained from local blood bank and stored at -20°C for further analysis, Alliance Waters E2695 (High Performance Liquid Chromatography) apparatus, which includes a degasser, rapid automated sampling, column oven, and triple-quadrupole mass spectrometer model 5500 QTRAPoperated by SCIEX software was used for method development.

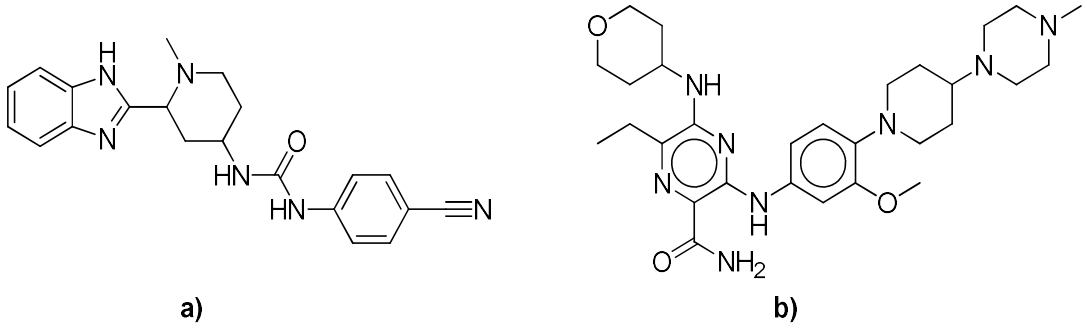

Figure 1: Chemical Structure of (A) Glasdegib & (B) Gilteritinib

LC–MS/MS (ESI) In Human Plasma

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Standard stock Solutions Preparation

It is necessary to measure out and add six milligrams of the Glasdegib working standard to a 100 millilitre volumetric flask that has already been filled up with diluent. liquid was used to increase the dilution from 1 mL to 10 mL. Adding diluents to a 10-milliliter volumetric flask will reduce the solution that was already stated, which is 0.4 mL. Making the Glasdegib standard solution (60 ng/mL): In a 2.0 mL centrifuge tube, 500 µL of the normal stock solution should be put in. Add 300 microliters of solvent, 500 microliters of IS, 500 microliters of acetonitrile, and 200 microliters of plasma to this and mix it well. The residual solution was put into the HPLC after the centrifuge had been running for 20 minutes. Prepared is the usual approach that works for Glasdegib. 500 µL of internal standard, mixed with 300 µL of acetonitrile, 200 µL of plasma, 500 µL of solvent, and 500 µL of standard stock solution should all be put into a 2 mL centrifuge tube. Once the sample was cool, it was spun at 4000 rpm for 20 minutes in a cooling centrifuge. The supernatant was carefully put into an HPLC bottle. Different portions of Glasdegib were made by centrifuging at 4000 rpm for 15 to 20 minutes. The amounts of these portions ranged from 6.0 ng/mL to 120.00 ng/mL. The supernatant that was introduced is used to fill the HPLC equipment withliquids.

The working standard for gilteritinib should weigh 6.0 mg. After that, transfer the determined amount into a 100 mL volumetric flask. The diluent was used to further dilute 1 to 10 mL. Transfer 0.4 millilitres of the solution that was previously described into a 10 mL volumetric flask, being sure to correct the concentration.

Sample solution preparation

The plasma samples that were used in the study were purchased straight from the local blood banks. Thawed stock solution (3.0 mL) was stored at -20. Following the separation and collection of the plasma in 200.00 µL eppendorf tubes, various concentrations of the solution containing the active pharmaceutical component were added and well mixed. To extract the plasma glasdegib from the plasma matrix, chilled acetonitrile was mixed using a PPE technique. After adding 500.00 µL of diluent, 200.00 µL of plasma, and 300.00 µL of Acetonitrile to the mixture to precipitate each protein, 2-minute centrifugation was carried out.

Method development

A LC-MS/MS system operated with optimised conditions was loaded with standard and sample solutions to optimise the method conditions trough several trial and error experiments by changing stationary, mobile phases, pH, column temperature, etc. The peak areas was used to determine the unknown concentration by comparing with standard solution peak area.

Validation of developed method

The primary stock solution was diluted to the required concentrations using the isocratic elution procedure, with gilteritinib acting as the internal standard (IS). According to USFDA guidelines, certain approval requirements for the enhanced bioanalytical method were verified.

System suitability

The HPLC system was injected with six injections of a commercial formulation of Gilteritinib (50.00 ng/mL) and Glasdegib (60.00 ng/mL) to evaluate the appropriateness of the suggested bioanalytical technique for the intended application and to set system suitability criteria. LLOQ values were used to assess Glasdegib's selectivity. Processing of the calibration curve and LLOQ was done three times in parallel.

Specificity

In order to assess whether the innovative bioanalytical investigation could effectively It was examined utilizing IS, in order of blank plasma, spiked plasma, and dilution agents in HPLC, To separate all analytes present in three distinct levels of the drug solution.

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Linearity

Regression was used to generate Glasdegib calibration curves from the ratio of analyte concentrations to substance maximum areas. The original stock solution (600.00 ng/mL) was diluted to get Glasdegib at the correct concentrations (6.0, 15.0, 30.0, 45.0, 60.0, 90.0, and 120.00 ng/mL). It was found that Glasdegib had an LLOQ of 6.0 ng/mL.

Precision

A detailed assessment was carried out to ascertain the repeatability of the developed bioanalytical methodology. Since Glasdegib was present in blank plasma matrix at established amounts, drug concentrations at various ranges, including 6.0, 15.0, 30.0, 45.0, 60.0, 90.0, and 120.00 ng/mL (LQC, MQC, and HQC), could be analysed. Six copies of every sample were subjected to three different quality controls in order to assess the accuracy both within and between days.

Accuracy

To verify the correctness of the created procedure, six duplicates of every sample were submitted to three QC levels (LQC, MQC, and HQC) using HPLC technology. Using linear equations, the ideal drug/IS peak area ratio was determined. After that, a backcalculation was performed on the actual concentrations. Accuracy was evaluated by comparing the actual and theoretical concentrations.

Recovery

The quantity of pharmaceutical recovery from the biological matrix was determined by comparing the reconstruction percentage of extracted and non-extracted samples. Six duplicates of spiked samples were subjected to LQC, MQC, and HQC level measurements using the HPLC. After injecting an unextracted material that met the identical Q.C. conditions, 100% recovery was likewise observed.

Results

Bioanalytical method development:

The bioanalytical approach was established by spiking the drug into the plasma and then extracting it from it by use of a protein precipitation procedure. The drug was extracted from the specimen's supernatant using centrifugal force, then the resulting solution was then put into an HPLC for drug measurement.

Validation of developed method:

System suitability

An analytical run that functions as a trial and error procedure is followed by an examination of a set of reference standards to gauge the instrument's performance. The mean peak area of six replications was found to be 385870, the retention length was found to be 2.605 minutes, and the CV was found to be 0.83 based on the Glasdegib and IS results (shown in Table 1). The system suitability chromatogram is shown in Figure 2. As a result, a connection emerged between the system's suitability metrics and the USFDA requirements.

Auto sampler carryover

There were no discernible reactions, according to the auto sampler carryover findings over many administrations. As a result, a relationship between the carryover parameter with US Food and Drug Administration guidelines was demonstrated.

Specificity and screening of biological matrix

Figure 3 illustrates that no interference peaks were formed at Glasdegib or ISTD elution times by six separate random blank human plasma samples. Consequently, the mean peak area of the biological matrix of the sample was found to be 35100. It was determined that there was a link between the USFDA standards and the specificity results (see Table 2).

Table 1: System Suitability results of Glasdegib

Parameter	AUC	Retention Time
(50 ng/mL)	355870	2.6
Std.Dev	0.02	0.004
%CV	0.83	0.17
*Mean of six replicates		

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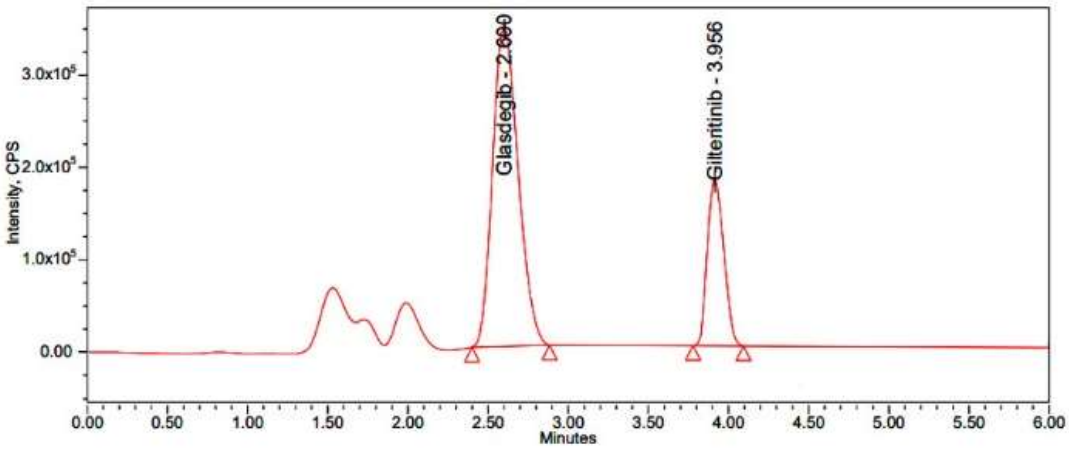

Figure 2: Chromatogram for System Suitability

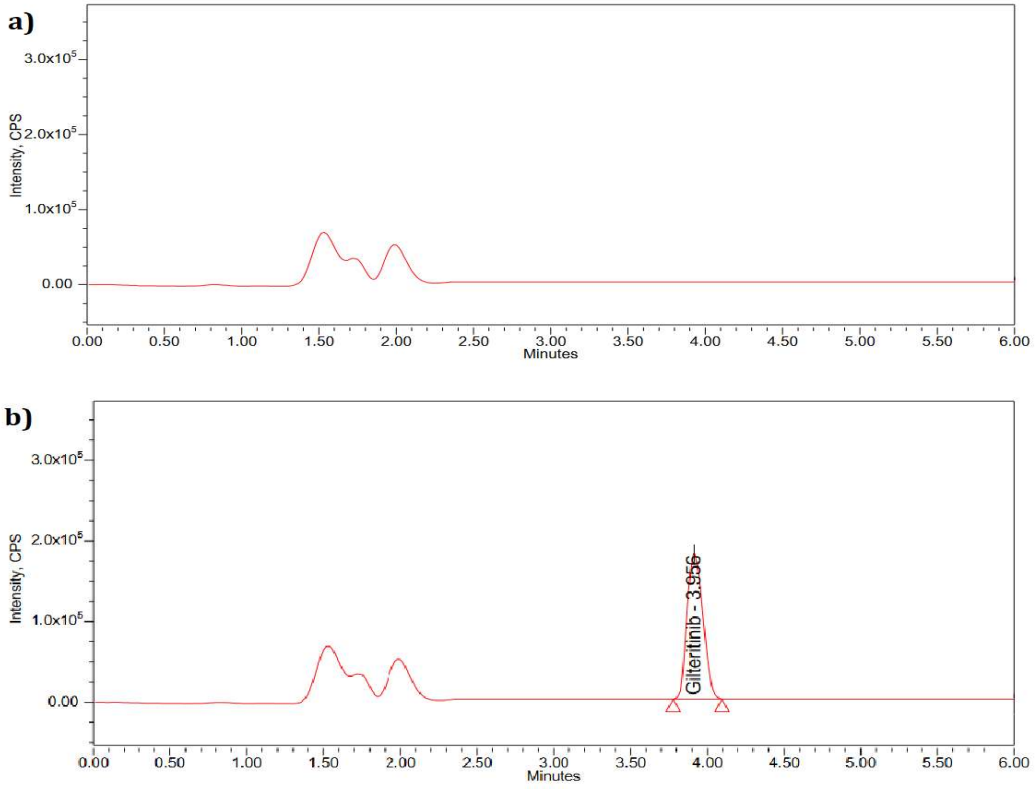

Figure 3: Specificity Chromatogram of (A) Placebo & (B) Blank & IS

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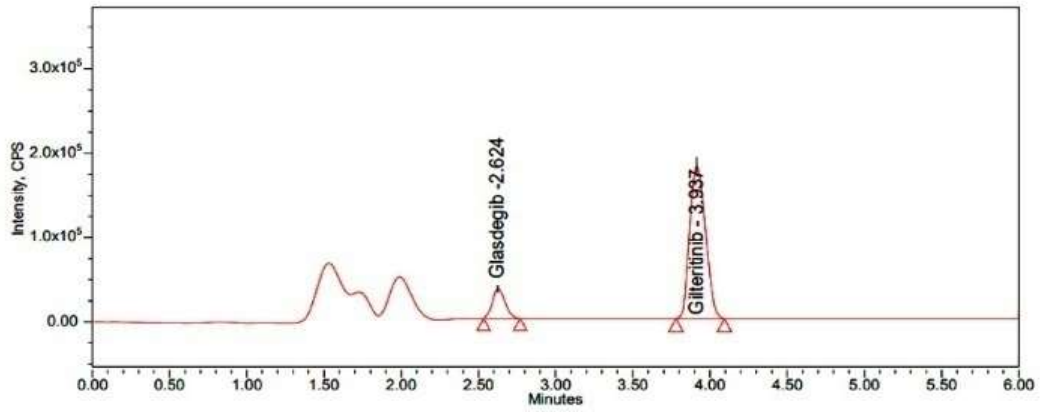

Figure 4: Sensitivity Chromatogram of LLOQ

Table 2: Specificity results of Glasdegib			
Parameter	AUC (cps)*	% Interference	
		Drug	Pass/ Fail
LLOQC (6ng/ml)	35100	0	Pass
SD	0.84		
%CV	1.14		
*Mean of six replicates			

Table 3: Sensitivity Results of Glasdegib	
Parameter	AUC (cps)*
LLOQ (6ng/ml)	35400
SD	0.00505
%CV	1.43
% Mean	99.41%
*Mean of six replicates	

Table 4: Matrix effect of Glasdegib		
Parameter	AUC (cps)*	
	(90 ng/mL)	(30 ng/mL)
Mean	525900	176100
SD	0.12	0.63
%CV	0.54	1.94
% Mean	98.55	98.46
*Mean of twelve replicates		

Sensitivity

Figure 4 showed the chromatogram, and the findings (included in Table 3) were reviewed. Within the US FDA-approved tolerance range, the percent CV for six replicates of the Glasdegib mean peak area was found to be 35400.

Matrix effect

The post-extracted sample's response was compared to the conventional high quality control and Low quality control samples (90.00ng/mL, 30.00 ng/mL of Glasdegib), permitting the determination of the matrix impact of biological fluid quantities on the analytes' ionization. In Figure 5, a chromatogram is shown, and Table 4 provides analytical samples at identical amounts of chemicals.

Linearity

It was discovered that the linearity of Glasdegib's typical curves ranged from 06.00 to 120.00 ng/mL.The proportion of the analyte to the IS peak regions had to be determined in order to analyse these analytical samples. The linearity results for the chromatogram (Figure 6) with plasma concentrations were given in Table 5.

Precision &Accuracy

The precision and repeatability of the findings were verified using six duplicates at four distinct QC levels. In order to evaluate reproducibility, four distinct specimens from various trails were analysed. The various concentrations of HQC, MQC, LQC,

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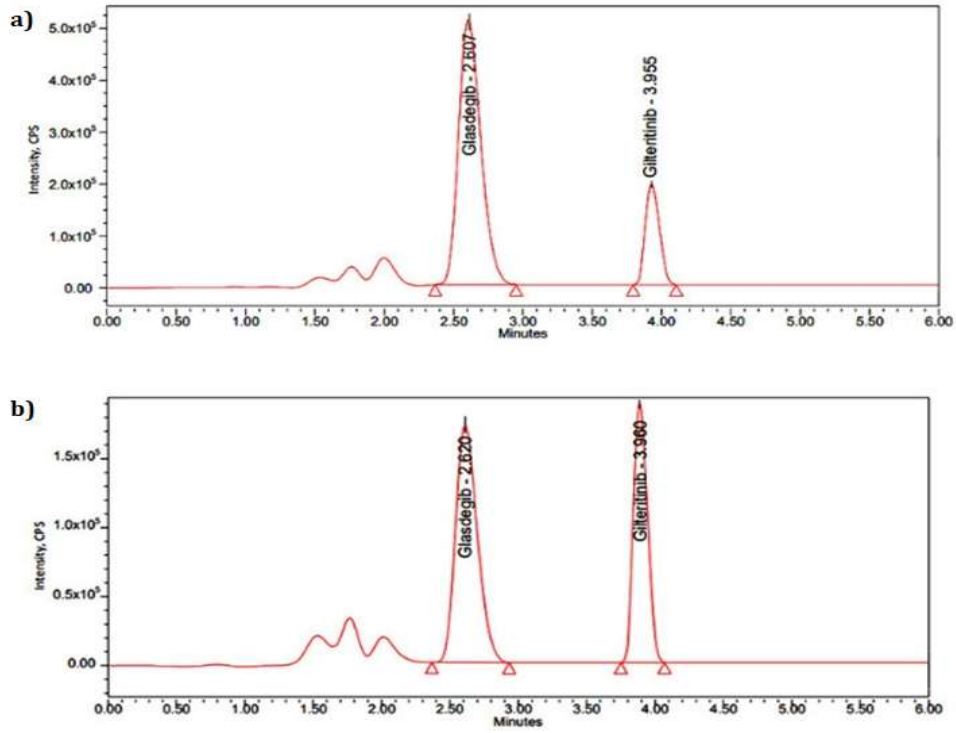

Figure 5: Matrix Effect of Chromatograms (A) HQC & (B) LQC

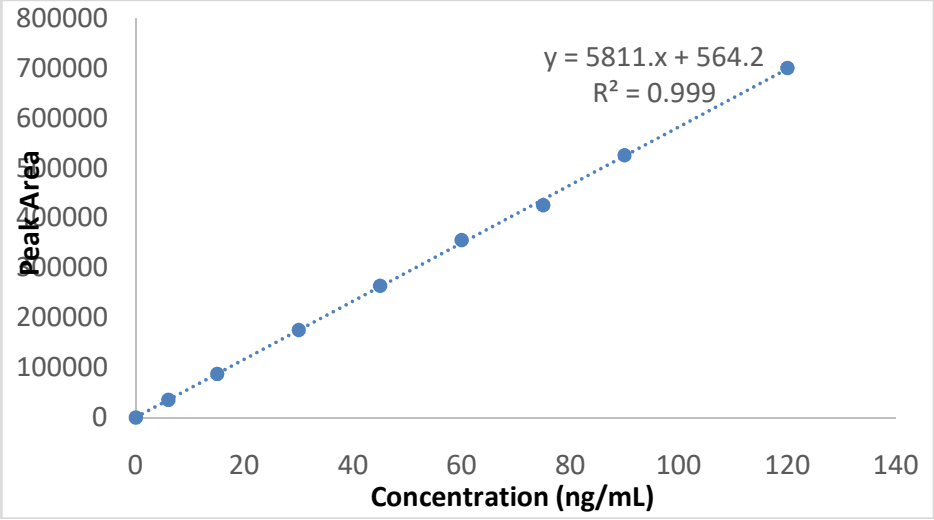

Figure 6: Calibration Curve of Glasdegib

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and LLQC were found to have mean values of 525300, 356000, 176900, and 035100. As shown in Tables 6 and 7, these results are compliant with the USFDA regulations' validation requirements. The chromatograms of accuracy and precision shown in Figure 7.

LOD, and LOQ

The readings of 2.04 and 6 in Table 8 were found to be within acceptable limits and to be in line with USFDA standards. Both the LOD and LOQ chromatograms were shown in Figure 8.

Recovery of Analyte

The medication and IS recovery was determined by comparing the responses from six duplicate specimens with the response from standard solutions. The quantity of material injected and the amount anticipated from the matrix were compared using the overall quantitative reactivity of the analyte extracted from the specimen's matrix.Data for three concentration levels of six replicates are generated using the mean peak area ratio. The formulation concentrations and standards are as follows: HQC (99.48, 99.59%), MQC (98.31, 98.95%), and LQC (97.10, 97.14%). The extraction process used a solvent as the mobile phase; Table 9 displays the results.

Reproducibility of ruggedness on reinjection

Ruggedness on reinjection reproducibility was passed by Glasdegib's %CV. The repeatability results for the mean recovery percentages at three different concentrationsHQC (99.52%), MQC (98.63%), and LQC (99.07%) with six replicates are

Table 5: Linearity of Glasdegib

Conc. (ng/mL)	AUC (cps)*
0	0
6.00	35600
15.00	87500
30.00	175400
45.00	263700
60.00	355400
75.00	425100
90.00	525300
120.00	700000
Slope*	5811.6
Intercept*	564.21
r^2*	0.9996

*Mean of three replicates

Table 8: LOD and LOQ Results

Drug	LOD (ng/mL)	LOQ (ng/mL)
Glasdegib	02.05	06.01

Table 6: Precision Results of Glasdegib

Parameters	AUC (cps)*			
	(90 ng/mL)	(60 ng/mL)	(30 ng/mL)	(6ng/ml)
Mean	525300	356000	176900	35100
SD	0.21	0. 31	0.26	0.38
%CV	0.44	0.91	1.64	4.86
% Mean Accuracy	99.15	98.93	99.61	98.42
*Mean of three replicates				

Table7: Ruggedness on precision accuracy Results of Glasdegib

Parameters	AUC (cps)*		
	(90 ng/mL)	(60 ng/mL)	(30 ng/mL)
Mean	525900	356100	177400
SD	0.21	0. 32	0.26
%CV	0.41	0.89	1.49
% Mean Accuracy	99.52	98.63	99.07
*Mean of six replicates			

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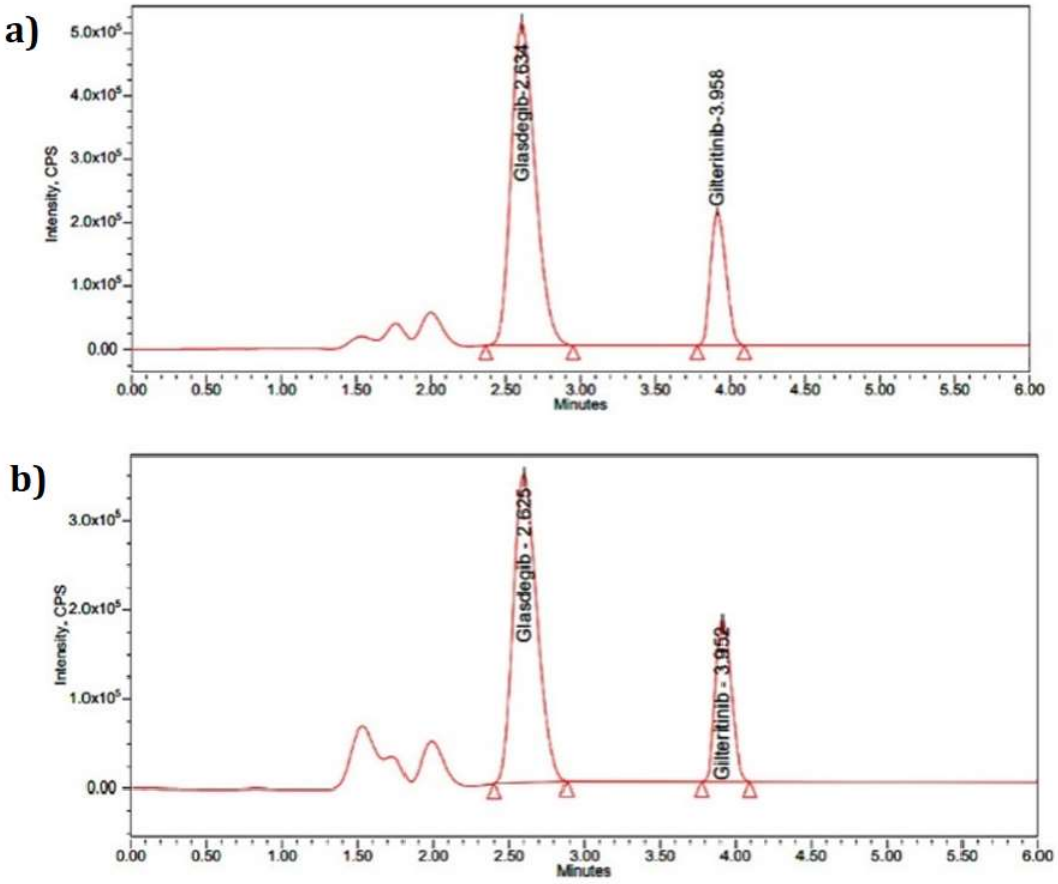

Figure 7: Chromatogram of Precision and Accuracy (A) HQC & (B) MQC

Table 9: Recovery Studies of Glasdegib

Replicate Number	(90 ng/mL)			(60 ng/mL)			(30 ng/mL)		
	Formulation	Standard	M.F	Formulation	Standard	M.F	Formulation	Standard	M.F
Mean*	52600.00	527100.00	0.9980	353600.00	355900.00	0.9937	175400.00	176500.00	0.9939
SD	0.02983 ± 0.58	0.026 ± 0.48	0.002± 0.15	0.028 ± 0.78	0.04012 ± 1.14	0.005 ± 0.45	0.02634± 1.50	0.028 ± 1.52	0.003 ± 0.19
%Mean	99.48%	99.59%	-	98.31%	98.95%	-	97.10%	97.14%	-
*Mean of six replicates									

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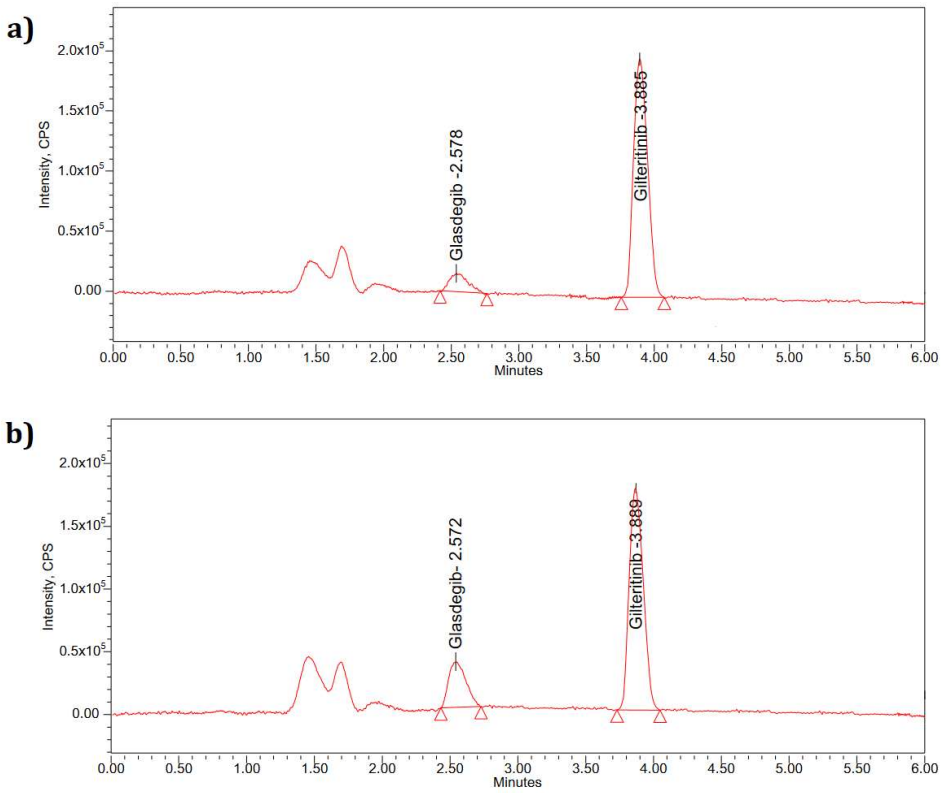

Figure 8: Chromatogram of (A) LOD & (B) LOQ

Table 10: Ruggedness on reinjection Results of Glasdegib			
Parameters	AUC (cps)*		
	(90 ng/mL)	(60 ng/mL)	(30 ng/mL)
Mean*	525900	355700	176100
SD	0.28	0.25	0.19
%CV	0.54	0.71	1.13
% Mean Accuracy	99.84	99.04	99.49
*Mean of six replicates			

shown in Table 10. The findings show that the USFDA approval criteria for repeatability of toughness on reinjection were satisfied.

Stability study

Bench Top Stability

Glasdegib achieved a mean accuracy and % CV passing the Bench-Top Stability test of HQC (98.99), MQC (100.17), and LQC (99.40) the results are shown in Table 11.

Auto Sampler Stability

Table 12 shows the results of Glasdegib's auto sampler stability tests, which included the mean accuracy %CV, HQC (99.65), MQC (98.13), and LQC (99.76).

Freeze Thaw Stability

Glasdegib's freeze-thaw stability showed that the HQC (99.00), MQC (98.99), LQC (97.98), and mean accuracy %CV tests passed. The results are shown in Table 13.

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Table 11: Bench Top Stability Studies of Glasdegib			
Parameters	AUC (cps)*		
	(90 ng/mL)	(60 ng/mL)	(30 ng/mL)
Mean*	523400	356300	356300
SD	0.20	0.33	3.25
%CV	0.39	0.93	1.58
% Mean Accuracy	98.99	100.17	99.40
*Mean of six replicates			

Table 12: Auto Sampler Stability of Glasdegib			
Parameters	AUC (cps)*		
	(90 ng/mL)	(60 ng/mL)	(30 ng/mL)
Mean*	525400	355000	175600
SD	0.32	0.30	0.25
%CV	0.62	0.88	1.44
% Mean Accuracy	99.65	98.13	99.76
*Mean of twenty-four replicates			

Table 13: Freeze Thaw Stability of Glasdegib			
Parameters	AUC (cps)*		
	(90 ng/mL)	(60 ng/mL)	(30 ng/mL)
Mean	524500	356400	176100
SD	0.23	0.29	0.24
%CV	0.44	0.83	1.52
% Mean Accuracy	99.00	98.99	97.98%
*Mean of six replicates			

Discussion

The present study successfully developed and validated an LC-MS/MS method for the quantification of Glasdegib in human plasma. This bioanalytical approach demonstrated high sensitivity, specificity, accuracy, and precision, ensuring reliable drug measurement in plasma samples. The developed method met all regulatory requirements set by the USFDA, making it a robust tool for pharmacokinetic and therapeutic drug monitoring applications.

The method was optimized through systematic trials involving variations in chromatographic conditions, including mobile phase composition, pH, and column temperature. The final method utilized an optimized LC-MS/MS system, which provided high specificity and sensitivity for Glasdegib detection. The use of Gilteritinib as an internal standard ensured consistency and accuracy in analyte quantification.

The method validation was conducted in accordance with USFDA guidelines, confirming its suitability for bioanalytical applications. System suitability tests demonstrated a consistent and reproducible peak area with a CV of 0.83%, ensuring reliable instrument performance. Auto sampler carryover tests confirmed the absence of residual analytes between injections, minimizing potential contamination risks.

The specificity assessment confirmed that there were no interfering peaks from the biological matrix at Glasdegib's retention time, ensuring accurate analyte detection. The sensitivity study established a lower limit of quantification (LLOQ) of 6.0 ng/mL, which is suitable for detecting Glasdegib at clinically relevant concentrations.

A strong linear relationship was observed within the concentration range of 6.0 to 120.0 ng/mL, with correlation coefficients (r) consistently exceeding 0.99.

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This confirms the method's reliability in quantifying Glasdegib across a broad concentration range. The accuracy assessment demonstrated that the calculated concentrations closely matched the theoretical values, with deviations well within the acceptable limits set by regulatory authorities.

Inter-day and intra-day precision studies showed minimal variability, with CV values within the acceptable range. The recovery studies indicated that Glasdegib could be effectively extracted from plasma with high consistency, with mean recovery rates exceeding 97% across all QC levels.

The stability assessments, including bench-top, auto sampler, and freeze-thaw stability, demonstrated that Glasdegib remained stable under various storage and handling conditions. These findings confirm the method's reliability for routine bioanalytical applications, ensuring accurate drug measurement throughout different phases of sample processing.

This validated LC-MS/MS method provides a valuable tool for pharmacokinetic studies and therapeutic drug monitoring of Glasdegib in clinical settings. The ability to accurately quantify Glasdegib levels in plasma will enhance understanding of its pharmacokinetics, optimize dosing regimens, and improve therapeutic outcomes in patients with AML and other SHH-driven malignancies.

Conclusion

The developed LC-MS/MS method for Glasdegib quantification in human plasma is a highly sensitive, precise, and accurate technique that meets USFDA validation standards. This method can be effectively applied in clinical and pharmacokinetic studies, facilitating better therapeutic management and individualized treatment strategies for patients receiving Glasdegib therapy.

Acknowledgement

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Conflict of interest

A conflict of interest does not exist.

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