

A Rapid Liquid Chromatography–Tandem Mass Spectrometry Bioanalytical Method For Quantification Of Gepirone In Rat Plasma Using Lurasidone As An Internal Standard: Method Development, Validation And Pharmacokinetic Application

Talluri Sarada Mrinalini^{1*}, Rajkumari Thagele², Naveen Babu Kilaru³

¹Research Scholar, Career Point School of Health Science and Pharmacy, Career Point University, Kota, Rajasthan, INDIA, 324005.

²Research supervisor, Career Point School of Health Science and Pharmacy, Career Point University, Kota, Rajasthan, INDIA, 324005.

³Research supervisor, KL University, Tadepalli, Vijayawada, Andhra Pradesh, INDIA, 522501

Corresponding author: Talluri sarada Mrinalini, **Email ID:** Mrinalini.talluri@gmail.com

Orcid Id: 0000-0001-8223-6444

Received Date: 28.09.2026 Revised Date: 05.10.2023 Accepted Date: 06.10.2026 Available Online: 10.10.2026

ABSTRACT

Gepirone is an azapirone antidepressant that acts primarily through partial agonism of serotonin 5-HT_{1A} receptors and has gained renewed attention following its approval for major depressive disorder. This study designed to create a simple and reliable Liquid Chromatography–Tandem Mass Spectrometry procedure for determining gepirone in rat plasma using lurasidone as the internal standard. Chromatographic separation was accomplished on a Zorbax SB C18 column (150 × 4.6 mm, 3.5 μm) under isocratic conditions with methanol and 0.1% aqueous formic acid (40:60, v/v) at a flow rate of 1.0 mL/min. Detection was carried out on a SCIEX QTRAP 5500 mass spectrometer operated in positive ESI and multiple-reaction-monitoring modes. Plasma samples were prepared by protein precipitation, providing a rapid and economical sample-processing approach. The assay was linear over 10–80 ng/mL, with 0.9998 determination coefficient. All validation results satisfied the predefined requirements for selectivity, precision, accuracy, recovery, matrix effect, ruggedness, carryover, dilution integrity, and stability. Gepirone recovery ranged from 96.05% to 97.16%, whereas lurasidone recovery was 97.31%. Mean accuracy during matrix-effect assessment was 96.05% at the low quality-control level and 97.11% at the high quality-control level. The RTs of gepirone and lurasidone were approximately 2.23 and 4.02 min, respectively, within a total analytical time of 7 min. The method was successfully applied to rat plasma samples and provided adequate performance for preclinical PK analysis. Its straightforward extraction, consistent recovery, and reproducible analytical response support its use for routine determination of gepirone in biological samples.

Keywords: Gepirone; LC–MS/MS; Rat plasma; Bioanalytical method validation; PK; Protein precipitation; Lurasidone; Recovery; Matrix effect; Azapirone.

How to cite this article: Talluri Sarada Mrinalini^{1*} Rajkumari Thagele² Naveen Babu Kilaru³. A Rapid Liquid Chromatography–Tandem Mass Spectrometry Bioanalytical Method For Quantification Of Gepirone In Rat Plasma Using Lurasidone As An Internal Standard: Method Development, Validation And Pharmacokinetic Application. Int J Drug Deliv Technol. 2026;16(85s):1124-1137. DOI: 10.25258/ijddt.16.85s.118.

1. INTRODUCTION

Major depressive disorder is a recurrent psychiatric illness associated with considerable personal, social, and economic burden. Although several antidepressant classes are available, incomplete response and treatment-related adverse effects remain common. These limitations have encouraged the development of agents with alternative mechanisms, including compounds that selectively modulate serotonin 5-HT_{1A} receptors [1,2].

Gepirone is an azapirone derivative and a selective partial agonist of the 5-HT_{1A} receptor. It is structurally related to buspirone and tandospirone but has minimal dopaminergic activity, with its antidepressant and anxiolytic effects arising mainly from serotonergic modulation. The extended-release formulation of gepirone, marketed as EXXUA®, approved in the year 2023 by USFDA for treating major depressive disorder in human adults. This approval has renewed interest in its PK behaviour

and quantitative determination in biological matrices [1,2].

Reliable measurement of gepirone in plasma is essential for PK, toxicokinetic, bioavailability, metabolism, and preclinical studies. Because plasma contains endogenous components that may influence extraction and ionization, the analytical method must provide adequate selectivity, sensitivity, precision, and reproducibility. LC–MS/MS is well suited for this purpose because it combines chromatographic separation with selective mass detection and allows quantification of drugs at low concentrations in complex biological samples. Bioanalytical liquid chromatography–tandem mass spectrometry methods had reported for related azapirones. Cho et al. quantified buspirone in human plasma using liquid–liquid extraction, whereas Joshi et al. developed a highly sensitive procedure based on solid-phase extraction and an isotope-labelled internal standard [3,4]. Hu et al. reported the

simultaneous determination of tandospirone and its active metabolite, 1-(2-pyrimidinyl) piperazine, in rat plasma for PK application [5]. Although these studies established the usefulness of LC–MS/MS for azapirone analysis, many reported procedures depend on solvent-intensive extraction, solid-phase cartridges, or expensive labelled standards. Moreover, detailed peer-reviewed methods specifically intended for routine quantification of gepirone in preclinical plasma samples remain limited.

Current FDA, EMA, and ICH M10 recommendations require bioanalytical methods to be assessed for selectivity, calibration performance, precision, accuracy, recovery, carryover, dilution integrity, matrix-related interference, and stability [6–8]. Accordingly, present study aimed to develop a simple, selective, and economical liquid chromatography–tandem mass spectrometry method for the estimation of gepirone in rat plasma using lurasidone as internal standard. The method employed methanol-based protein precipitation to reduce sample-processing complexity and was evaluated for routine application in preclinical PK analysis.

2. MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Gepirone and lurasidone reference standards (internal standard), acquired from Glenmark Life Sciences Ltd., Mumbai, India. HPLC-grade methanol and acetonitrile were procured from a reputable commercial supplier. Formic acid of analytical grade and Milli-Q water were used in the entire study. Blank rat plasma was procured from Bio Needs Pvt. Ltd., Bengaluru, India.

2.2 INSTRUMENTATION

Liquid chromatography of make Waters Alliance and model e2695 was used for analysis. It was connected to mass spectrometer of model SCIEX QTRAP 5500 fitted with an ESI interface. Data acquisition and chromatographic interpretation were executed using SCIEX software.

2.3 CHROMATOGRAPHIC CONDITIONS:

Table 1: LC-MS/MS chromatographic system

Analytical Column	ZORBAX SB-C18 9150mm x 4.6mm, 3.5µm)
Injection Volume	10µL
Column Oven Temperature	Ambient
Mobile phase Flow rate	1.0 ml/min
Autosampler Temperature	Ambient
Total analytical Run time	7min

Mass spectrometer conditions:

Mass-spectrometric detection was performed using a SCIEX QTRAP 5500 mass spectrometer equipped with a Turbo V ESI source[24]. The instrument was operated in positive-ion electrospray mode, and gepirone was quantified using multiple-reaction monitoring. Source-dependent and compound-dependent parameters were optimized experimentally to obtain maximum and reproducible analyte response[25]. The final operating conditions are presented in Table 2.

Table 2: Optimized Mass spectrometric conditions

Collision energy	15 V
Ion spray voltage (ISV)	5500 V
Source temperature (TEM)	550°C
Drying gas temperature	120-250°C
Collision gas	nitrogen
Drying gas flow stream	5 mL/min
Declustering potential (DP)	40 V
Entrance potential (EP)	10V
Collision cell Exit Potential (CSV)	7 V
Dwell time	1sec

2.5 OPTIMIZATION OF MRM CONDITIONS

MRM conditions were optimized under positive ESI to achieve sensitive and selective detection of Gepirone and Lurasidone. The protonated precursor ions of Gepirone (m/z 360.4740) and Lurasidone (m/z 494.6837), and the corresponding product ions exhibiting the highest signal intensity (m/z 108.2934 and m/z 340.2517, respectively) were chosen for quantitative analysis. The optimized MRM transitions provided in table 3 selective and reproducible detection throughout the study.

Table 3: Selected Precursor and Product Ion Transitions for Gepirone and Lurasidone

Compound	Ionization Mode	Precursor Ion (m/z)	Product Ion (m/z)	Purpose
Gepirone	Positive ESI	360.4740	108.2934	Quantifier
Lurasidone (IS)	Positive ESI	494.6837	340.2517	Internal Standard

2.6 FRAGMENTATION PATTERN OF GEPIRONE AND LURASIDONE

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Under positive ESI conditions, gepirone and lurasidone generated protonated molecular ions at m/z 360.4740 and 494.6837, respectively shown in figure 1 and figure 2. Product-ion scanning produced the most intense and reproducible fragment ions at m/z 108.2934 for gepirone and m/z 340.2517 for lurasidone. Accordingly, MRM transitions $360.4740 \rightarrow 108.2934$ and $494.6837 \rightarrow 340.2517$ were selected for quantitative detection.

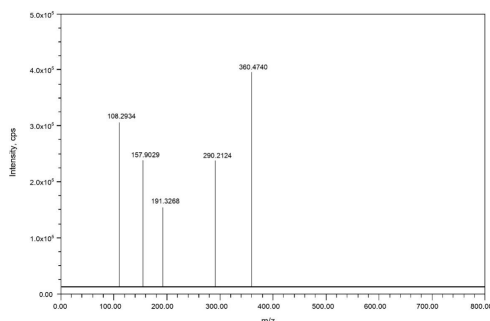


Figure 1: Product Ion Mass Spectrum of Gepirone Acquired in Positive Electrospray Ionization (ESI+) Mode.

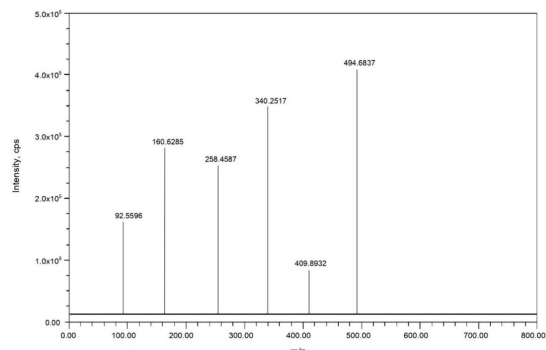


Figure 2: Product Ion Mass Spectrum of Lurasidone Acquired in Positive Electrospray Ionization (ESI+) Mode

2.7 PREPARATION OF SOLUTIONS

Buffer: A 0.1% (v/v) solution of formic acid was generated by diluting 1.0 mL of formic acid to 1 litre with water (HPLC Grade).

Mobile Phase: Methanol and 0.1% formic acid solution was mixed in the ratio of 40:60 (v/v) and filtered through a 0.45 μ m membrane filter prior to use.

Diluent: water (HPLC Grade) and ACN were combined in the ratio 50:50, v/v and utilized as the diluent throughout the investigation.

Gepirone Stock Solution (160ng/ml): A volumetric flask (100 mL) was filled with 8 mg of the Gepirone working standard after it had been weighed. To get a concentration of 80 μ g/mL, the volume was adjusted using diluent. 0.2 mL of the stock solution was diluted to 10mL volume using diluent. The final working solution was then obtained by diluting 1.0 mL of this solution into a 10 mL with diluent.

Internal Standard Stock Solution (100ng/ml): precisely weighed 5 mg of lurasidone, which served as the internal standard was taken in 100 mL volumetric flask. The diluent was used to make up the volume, resulting in a 50 μ g/mL primary stock

solution. An intermediate solution of 1 μ g/mL was created by diluting an aliquot of 0.2 mL to 10 mL with the diluent. The final internal standard working solution of 100 ng/mL was then obtained by further diluting 1.0 mL of the intermediate solution to 10 mL with the diluent.

Gepirone Standard Solution (40ng/ml): A 2 mL centrifuge tube was filled with an aliquot of 500 μ L of the gepirone working solution (160 ng/mL). A total volume of 2 mL and a final gepirone concentration of 40 ng/mL were obtained by adding 200 μ L of blank rat plasma, 500 μ L of internal standard working solution, 300 μ L of methanol, and 500 μ L of diluent. After vortexing the mixture, it was centrifuged for 20 minutes at 4000 rpm. After filtering, the clean supernatant was put into an LC vial for LC–MS/MS analysis.

Linearity solution: In order to achieve final concentrations ranging from 10 to 80 ng/mL, calibration standards were created by spiking blank rat plasma with the proper amounts of the gepirone working solution. The improved protein-precipitation method was applied to each calibration sample. After vortex-mixing the samples and centrifuging them for 20 minutes at 4000 rpm, the clean supernatants were transferred into LC vials for analysis utilizing the newly devised LC–MS/MS technique, shown in table 4.

Table 4: Preparations of Linearity Solutions

Name	Vol. of Plasma (μ L)	Vol. of internal standard solution (μ L)	Vol. of Stock solution (μ L)	Methanol (μ L)	Dilute to (μ L)	Concentration (ng/ml)
Blank	0	0	0	2000	2000	0
Plasma	200	500	0	1300	2000	0
Linearity-1	200	500	125	1175	2000	10.00
Linearity-2	200	500	250	1050	2000	20.00

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Linearity -3	200	500	375	925	2000	30.00
Linearity -4	200	500	500	800	2000	40.00
Linearity -5	200	500	625	675	2000	50.00
Linearity -6	200	500	750	550	2000	60.00
Linearity -7	200	500	1000	300	2000	80.00

Preparation of Quality Control Samples:

In order to generate final plasma concentrations of 4, 20, 40, and 60 ng/mL, which correspond to LLOQ, QC, LQC, MQC, and HQC levels, respectively, suitable amounts of gepirone working solutions were spiked into blank rat plasma to create quality control samples. While LQC, MQC, and HQC samples were created at 20, 40, and 60 ng/mL, respectively, to assess precision, accuracy, recovery, matrix effect, ruggedness, and stability, the LLOQ QC sample was prepared at 4 ng/mL to test the method's sensitivity.

The improved protein precipitation process was applied to every QC sample. In short, according to the sample preparation protocol, 200 µL of spiked rat plasma was put into a 2 mL centrifuge tube, and then internal standard solution, methanol, and diluent were added. After centrifuging the samples for 20 minutes at 4000 rpm and vortexing them to guarantee thorough mixing and protein precipitation, the clear supernatant was filtered and put into HPLC vials for LC–MS/MS analysis.

All QC samples were freshly prepared or stored under validated stability conditions, as applicable, and were analyzed along with calibration standards and study samples during method validation.

Extraction procedure:

Plasma samples collected at the specified time points were labelled appropriately. After transferring a 200 µL aliquot of each plasma sample into a 2 mL centrifuge tube, 500 µL of internal standard working solution, 300 µL of methanol, and 1000 µL of diluent were added. To guarantee full protein precipitation, the mixture was vortexed and centrifuged for 20 minutes at 4000 rpm. After being moved into an LC vial, the clear supernatant was injected into the LC–MS/MS apparatus.

2.8 METHOD DEVELOPMENT AND OPTIMIZATION

The chromatographic conditions were systematically optimized to achieve satisfactory analyte retention, symmetrical peak shape, acceptable column efficiency, and adequate resolution for both gepirone and the internal standard. Different stationary phases and mobile-phase compositions were evaluated during method development shown in table 5.

Table 5: Method development and optimization

Trial	Column	Mobile Phase (v/v)	RT 1 (min)	RT 2 (min)	Plate Count -1	Plate Count -2	Observation	Decision
T1	Symmetry C18	ACN:0.1% FA (80:20)	1.059	1.586	426	925	Very low plate count	Rejected
T2	Symmetry C18	ACN:0.1% FA (70:30)	1.090	2.014	481	1537	Insufficient retention	Rejected
T3	Symmetry C18	MeOH:0.1% FA (70:30)	1.145	3.063	16376	4595	First peak plate count inadequate	Rejected
T4	Zorbax	MeOH:	1.4	3.0	1020	4208	Improved	Further

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Rationale for Final Method Selection

Acetonitrile-based mobile phases were found to provide inadequate retention and poor chromatographic efficiency, resulting in early elution and low theoretical plate counts. In contrast, methanol-based mobile phases improved analyte retention and peak shape. The Zorbax SB C18 column demonstrated superior chromatographic performance compared with the Symmetry C18 column, producing higher plate counts, improved peak response, and better overall separation. Among the evaluated mobile-phase compositions, methanol and 0.1% formic acid (40:60, v/v) provided the best balance between retention, peak symmetry, resolution, baseline stability, and chromatographic efficiency. Therefore, these conditions were selected

as the optimized chromatographic method for subsequent validation and application studies.

3.0 METHOD VALIDATION

3.1 SYSTEM SUITABILITY: System appropriateness was assessed after six replicate injections of MQC samples. To evaluate the repeatability of the system, the percentage CV of peak area, RT, and area ratio was computed shown in table 6.

Table 6. System Suitability Parameters for Gepirone and Lurasidone (IS) (n = 6)

Parameter	Mean ± SD	%CV
Gepirone Area (cps)	(2.186 ± 0.0027) × 10 ⁵	0.13
Gepirone RT (min)	2.219 ± 0.0080	0.36
IS Area (cps)	(2.109 ± 0.0029) × 10 ⁵	0.14
IS RT (min)	4.030 ± 0.0185	0.46
Area Ratio	1.0364 ± 0.0019	0.19

Result: The %CV values for Gepirone area, internal standard area, RTs, and area ratio were found to be less than 2.0%, indicating satisfactory system performance and reproducibility. The %CV of the area ratio was 0.19%, confirming acceptable system suitability for the analysis.

3.2 AUTO SAMPLER CARRYOVER

In order to predict carryover, blank samples were injected just after the upper limit quality control (HQC) sample, and the analyte and internal standard responses were tracked at the appropriate retention durations shown in table 7.

Table 7. Carryover Results of Gepirone

Parameter	Result
Carryover in Blank after HQC (Analyte)	0.00 %
Carryover in Blank after HQC (IS)	0.00 %
Acceptance Criteria (Analyte)	≤ 20 % of LLOQ response
Acceptance Criteria (IS)	≤ 5 % of IS response
Conclusion	Pass

Result: No detectable carryover was observed in subsequent blank injections following HQC samples. The carryover response was well within the

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acceptance criteria, confirming the absence of significant autosampler carryover.

3.3 SELECTIVITY

Selectivity was assessed using six separate lots of blank rat plasma. To ascertain whether endogenous plasma components interfered at the corresponding retention durations of the analyte and internal standard, chromatograms from the blank matrices were compared with those of LLOQ samples spiked with gepirone and lurasidone shown in table 8.

Table 8. Selectivity Results of Gepirone in Rat Plasma (n = 6)

Parameter	Gepirone	Internal Standard
Mean LLOQ Response (cps)	0.211×10^5	2.103×10^5
Blank Response (cps)	0	0
% Interference	0.00	0.00
Acceptance Criteria	$\leq 20\%$ of LLOQ response	$\leq 5\%$ of IS response
Result	Pass	Pass

Result: None of the six blank rat plasma batches showed interfering peaks at either the internal standard or the Gepirone retention durations. Blank samples showed 0.00% interference, which fell within the predetermined acceptable range.

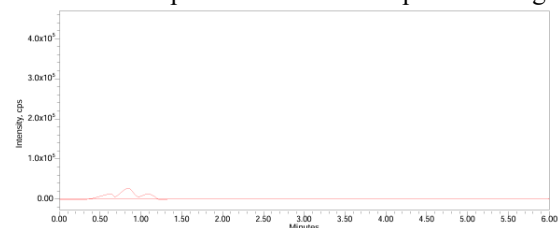


Figure 3: Representative Chromatogram of Blank Rat Plasma

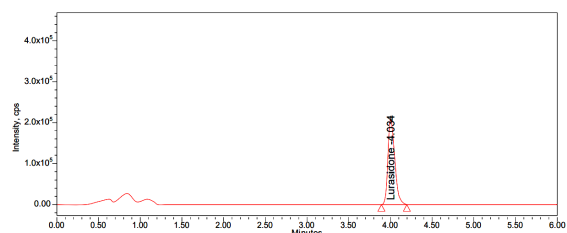


Figure 4: Specificity Representative Chromatogram of Internal Standard

3.4 SENSITIVITY

Six duplicate plasma samples that had been spiked with gepirone at a concentration of 4 ng/mL were analyzed to determine the method's sensitivity. In accordance with the relevant bioanalytical validation requirements, the precision and accuracy of the measured analyte concentrations, reproducibility and proximity to the nominal concentration were assessed and shown in table 9.

Table 9. Sensitivity (LLOQ) Results of Gepirone

Parameter	Result
LLOQ Concentration (ng/mL)	4
Mean Peak Area (cps)	0.213×10^5
SD	0.00286×10^5
%CV	1.34
Mean Accuracy (%)	94.54
Result	Pass

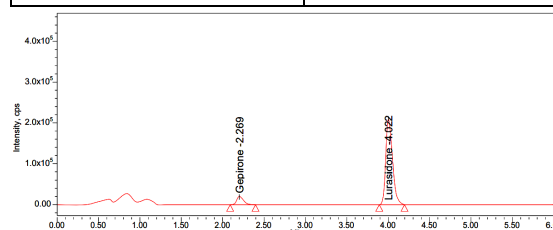


Figure 5: Sensitivity Chromatogram of LLOQ Result: The method's ability to detect and quantify low concentrations under the testing conditions was demonstrated by a separate 4 ng/mL sample, which showed satisfactory responsiveness, precision, and accuracy. However, because 4 ng/mL was not included as the lowest calibration standard, it was treated as a sensitivity verification level rather than as the validated LLOQ.”

3.5 MATRIX EFFECT

To assess the matrix effect Six separate lots of rat plasma at LQC (20 ng/mL) and HQC (60 ng/mL) levels were used. The impact of endogenous plasma components was evaluated by comparing the responses of post-extraction spiking samples with those of corresponding plain standard solutions and shown in table 10.

Table 10: Matrix Effect Results of Gepirone in Rat Plasma

Parameter	HQC (60 ng/mL)	LQC (20 ng/mL)
n	18	18
Mean Peak Area (cps)	3.282×10^5	1.082×10^5
SD	0.00471×10^5	0.00536×10^5
%CV	0.14	0.50
Mean Accuracy (%)	97.11	96.05
QC Failures	0	0
Result	Pass	Pass

Result: Six rat plasma lots were used to evaluate the matrix effect at both LQC and HQC levels. Mean accuracies ranged from 96.05% to 97.11%, with %CV values below 1%. No QC failures were

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observed, indicating no significant matrix effect on Gepirone ionization

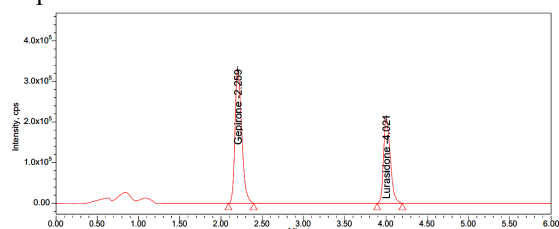


Figure 6: Matrix Effect Chromatogram of HQC

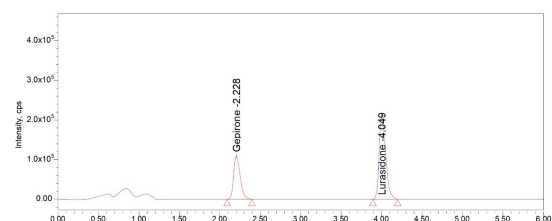


Figure 7: Matrix Effect Chromatogram of LQC

3.6 LINEARITY

Rat plasma was used to create calibration standards with concentrations ranging from 10 to 80 ng/mL. Plotting the analyte-to-internal standard peak area ratio vs nominal concentration allowed for the construction of calibration curves, which were then subjected to linear regression analysis shown in table 11 and figure 8.

Table 11: Linearity Results of Gepirone in Rat Plasma

Parameter	Result
Concentration Range (ng/mL)	10–80
Regression Equation	$y = 0.0260x + 0.001938$
Correlation Coefficient (R^2)	0.99982
Linearity	Linear
Result	Pass

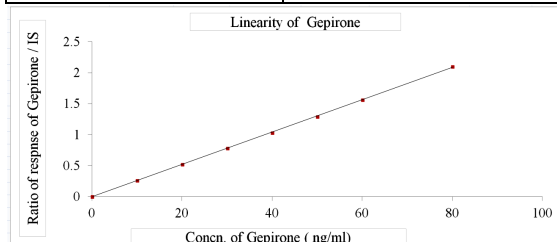


Figure 8: Calibration representing concentration v/s Area of Gepirone

Result: R^2 value of 0.99982 and a regression equation of $y = 0.0260x + 0.001938$, showed the technique proved good linearity within the concentration range of 10–80 ng/mL. Every calibration standard satisfied the requirements for acceptance.

3.7 PRECISION AND ACCURACY

Six duplicates of LLQC, LQC, MQC, and HQC samples were analyzed in a single analytical run to

assess intra-day precision and accuracy. Accuracy was obtained as the proportion of the nominal concentration recovered, while precision was represented as %CV shown in table 12

Table 12: Intra-day results of Precision and Accuracy of Gepirone

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)	LLQ C (4 ng/mL)
Mean Peak Area (cps)	3.313 $\times 10^5$	2.187 $\times 10^5$	1.088 $\times 10^5$	0.214 $\times 10^5$
SD	0.0030 $\times 10^5$	0.0032 $\times 10^5$	0.0021 $\times 10^5$	0.0036 $\times 10^5$
%CV	0.09	0.14	0.20	1.70
Mean Accuracy (%)	98.03	97.07	96.58	94.98
Result	Pass	Pass	Pass	Pass

Result: Accuracy varied from 94.98% to 98.03%, while intra-day precision (%CV) varied from 0.09% to 1.70%. The precision and accuracy of the LC–MS/MS technique for gepirone in rat plasma were confirmed by all values meeting the acceptance criteria.

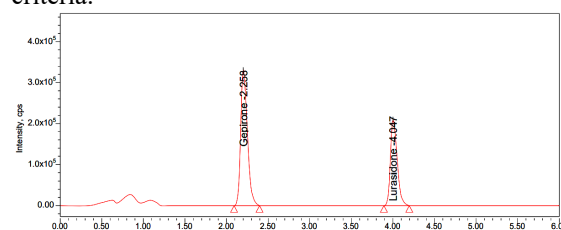


Figure 9: Chromatogram of Precision and accuracy of HQC

3.8 RECOVERY

Gepirone recovery was assessed at LQC (20 ng/mL), MQC (40 ng/mL), and HQC (60 ng/mL) levels by contrasting extracted sample responses with equivalent unextracted sample responses. Lurasidone (25 ng/mL) was used to measure internal standard recovery under the same circumstances, recovery results are shown in table 13.

Table 13: Recovery of Gepirone and Internal Standard from Rat Plasma

QC Level	Mean Recovery (%)	%CV
LQC (20 ng/mL)	96.05	0.29

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MQC (40 ng/mL)	97.16	0.15
HQC (60 ng/mL)	97.09	0.14
Internal Standard (25 ng/mL)	97.31	0.12
Result	Pass	Pass

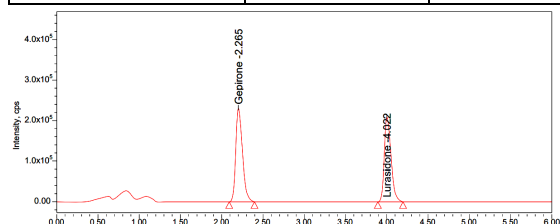


Figure 10: Chromatogram of MQC

3.9 RUGGEDNESS

LQC, MQC, and HQC samples were analyzed using a separate chromatographic column to assess robustness. To ascertain the method's reproducibility under altered analytical conditions, precision and accuracy were evaluated as shown in table 14.

Table 14: Ruggedness Results of Gepirone Using a Different Column (n = 6)

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)
Mean Peak Area (cps)	3.279×10^5	2.208×10^5	1.091×10^5
SD	0.00286×10^5	0.00216×10^5	0.00286×10^5
%CV	0.09	0.10	0.26
Mean Accuracy (%)	97.03	98.00	96.94
Result	Pass	Pass	Pass

Result: A separate chromatographic column was used to evaluate the method's robustness. Across all QC levels, the mean accuracy varied from 96.94% to 98.00%, and the %CV values varied from 0.09% to 0.26%. The method's robustness and reproducibility under various analytical settings were demonstrated by the fact that every result fell within the predetermined acceptance criteria.

3.10 DILUTION INTEGRITY

Six duplicate samples were used to measure dilution integrity at MQC (40 ng/mL) and ULQC (80 ng/mL). Samples were processed using the validated extraction method and analyzed by LC–MS/MS. Precision (%CV), accuracy (%), and bias (%) were evaluated, shown in table 15.

Table 15: Dilution Integrity Results of Gepirone (n = 6)

QC Level	Nominal Concentration (ng/mL)	Mean Peak Area $\pm$ SD (cps)	% CV	Mean Calculated Concentration (ng/mL)	Accuracy (%)	Bias (%)
MQC	40.0	$(2.2375 \pm 0.00274) \times 10^5$	0.12	39.47	98.68	-1.32
ULQC	80.0	$(4.5572 \pm 0.00286) \times 10^5$	0.06	80.66	100.83	+0.83

Result: Dilution integrity was demonstrated at MQC and ULQC levels. The mean accuracy ranged from 98.68% to 100.83%, with precision between 0.06% and 0.12% CV, confirming that diluted samples could be quantified reliably within the validated calibration range.

3.11 STABILITY STUDIES

3.11.1 BENCH-TOP STABILITY

Gepirone's benchtop stability was assessed at LQC (20 ng/mL), MQC (40 ng/mL), and HQC (60 ng/mL) levels by allowing the samples to remain at room temperature for the predetermined stability period before analysis. Stability was assessed in terms of precision (%CV) and accuracy (%) shown in table 16.

Table 16: Bench-Top Stability Results of Gepirone (n=6)

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)
Mean Peak Area (cps)	3.311×10^5	2.188×10^5	1.082×10^5
SD	0.00237×10^5	0.00216×10^5	0.00232×10^5

%CV	0.07	0.10	0.21
Mean			
Accuracy (%)	97.97	97.11	96.05
Result	Stable	Stable	Stable

Result: Bench-top stability studies confirmed that Gepirone was stable under the tested conditions, with %CV values of 0.07–0.21% and mean accuracy of 96.05–97.97%, meeting all acceptance criteria.

3.11.2 AUTOSAMPLER STABILITY

Gepirone autosampler stability was assessed at LQC (20 ng/mL), MQC (40 ng/mL), and HQC (60 ng/mL) by reanalyzing processed samples following autosampler storage. Accuracy (%) and precision (%CV) were used to evaluate stability shown in table 17.

Table 17: Autosampler Stability Results of Gepirone (n = 24)

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)
Mean Peak Area (cps)	3.278 × 10 ⁵	2.208 × 10 ⁵	1.093 × 10 ⁵
SD	0.00635 × 10 ⁵	0.00616 × 10 ⁵	0.00679 × 10 ⁵
%CV	0.19	0.28	0.62
Mean Accuracy (%)	97.00	98.00	97.03
Result	Stable	Stable	Stable

Result: Gepirone remained stable during the autosampler storage period, with %CV values of 0.19–0.62% and mean accuracy of 97.00–98.00%. All results met the acceptance criteria, confirming autosampler stability in rat plasma.

3.11.3 FREEZE–THAW STABILITY

Gepirone's freeze-thaw stability was assessed at LQC (20 ng/mL), MQC (40 ng/mL), and HQC (60 ng/mL) levels during freeze-thaw cycles. Accuracy (%) and precision (%CV) were used to evaluate stability shown in table 18.

Table 18: Freeze–Thaw Stability Results of Gepirone (n = 6)

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)
Mean Peak Area (cps)	3.312 × 10 ⁵	2.189 × 10 ⁵	1.083 × 10 ⁵

SD	0.00216 × 10 ⁵	0.00288 × 10 ⁵	0.00283 × 10 ⁵
%CV	0.07	0.13	0.26
Mean			
Accuracy (%)	98.00	97.16	96.14
Result	Stable	Stable	Stable

Result: Freeze–thaw stability studies confirmed that Gepirone remained stable in rat plasma, with %CV values of 0.07–0.26% and mean accuracy of 96.14–98.00%, meeting all acceptance criteria.

3.11.4 WET EXTRACT STABILITY

Gepirone's wet extract stability was assessed at LQC (20 ng/mL), MQC (40 ng/mL), and HQC (60 ng/mL) levels by keeping processed samples in wet extract conditions for 12 and 18 hours before analysis. Accuracy (%) and precision (%CV) were used to evaluate stability shown in table 19.

Table 19: Stability Evaluation of Wet Extract Samples Containing Gepirone

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)
12 h			
Mean Peak Area (cps)	3.305 × 10 ⁵	2.208 × 10 ⁵	1.093 × 10 ⁵
%CV	0.10	0.18	0.24
Mean Accuracy (%)	97.80	98.00	97.03
18 h			
Mean Peak Area (cps)	3.281 × 10 ⁵	2.195 × 10 ⁵	1.085 × 10 ⁵
%CV	0.08	0.14	0.26
Mean Accuracy (%)	97.09	97.43	96.32
Result	Stable	Stable	Stable

Result: Wet extract stability studies confirmed that Gepirone remained stable for up to 18 h, with %CV values of 0.08–0.26% and mean accuracy of 96.32–98.00%. All results met the acceptance criteria, demonstrating stability in rat plasma.

3.11.5 DRY EXTRACT STABILITY

Gepirone's dry extract stability was assessed at lqc (20 ng/ml), mqc (40 ng/ml), and hqc (60 ng/ml) levels by keeping dried extracts for 12 and 18 hours before reconstitution and analysis. Stability was assessed using precision (%cv) and accuracy (%).

Table 20: Dry Extract Stability Results of Gepirone

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)
12 h			
Mean Peak Area (cps)	3.312 × 10 ⁵	2.206 × 10 ⁵	1.092 × 10 ⁵
%CV	0.07	0.13	0.29
Mean Accuracy (%)	98.00	97.91	96.94
18 h			
Mean Peak Area (cps)	3.293 × 10 ⁵	2.188 × 10 ⁵	1.082 × 10 ⁵
%CV	0.09	0.14	0.32
Mean Accuracy (%)	97.44	97.11	96.05
Result	Stable	Stable	Stable

Result: Dry extract stability studies confirmed that Gepirone remained stable for up to 18 h. The %CV values (0.07–0.32%) and mean accuracy values (96.05–98.00%) met the acceptance criteria, demonstrating the stability of Gepirone in rat plasma shown in table 20.

3.11.6 SHORT-TERM STABILITY

Gepirone's short-term stability was assessed under the designated storage conditions at LQC (20 ng/mL), MQC (40 ng/mL), and HQC (60 ng/mL) levels. Stability was assessed using precision (%CV) and accuracy (%).

Table 21: Short-Term Stability Results of Gepirone (n = 6)

Parameter	HQC (60 ng/mL)	MQC (40 ng/mL)	LQC (20 ng/mL)
Mean Peak Area (cps)	3.139 × 10 ⁵	2.073 × 10 ⁵	1.028 × 10 ⁵
SD	0.00301 × 10 ⁵	0.00374 × 10 ⁵	0.00351 × 10 ⁵
%CV	0.10	0.18	0.34
Mean Accuracy (%)	92.88	92.01	91.26
Result	Stable	Stable	Stable

Result: Short-term stability studies confirmed that Gepirone remained stable under the tested conditions. %CV values ranged from 0.10% to 0.34%, and mean accuracy ranged from 91.26% to 92.88%, meeting all acceptance criteria shown in table 21.

3.11.7 LONG-TERM STABILITY

Long-term stability of Gepirone was evaluated at LQC (20 ng/mL), MQC (40 ng/mL), and HQC (60 ng/mL) levels by storing samples under long-term storage conditions and analyzing them on Days 1, 7, 14, 21, and 28. Stability was assessed using precision (%CV) and accuracy (%).

Table 22: Long-Term Stability Results of Gepirone

Storage Period	HQC Accuracy (%)	MQC Accuracy (%)	LQC Accuracy (%)	%CV Range
Day 1	98.00	97.47	96.32	0.08–0.28
Day 7	92.00	92.99	91.97	0.09–0.30
Day 14	90.01	90.99	89.48	0.11–0.56
Day 21	87.97	87.00	86.55	0.14–0.32
Day 28	84.01	84.95	83.53	0.09–0.28

Result: Long-term stability studies demonstrated a gradual decline in the measured response of Gepirone with increasing storage duration. Samples remained within the acceptance criteria up to Day 21, with mean accuracy values ranging from 86.55% to 98.00% and %CV values below 1%. On Day 28, the mean accuracy values decreased to 83.53–84.95%, which fell below the predefined acceptance range of 85–115%, indicating that Gepirone was stable in rat plasma for up to 21 days under the evaluated storage conditions shown in table 22.

4. RESULTS AND DISCUSSION

4.1 METHOD DEVELOPMENT AND OPTIMIZATION

The method was optimized to obtain good retention, stable baseline, and reproducible peak response for gepirone and lurasidone using a simple isocratic LC-MS/MS method. Initial trials were executed on a C18 Symmetric column using acetonitrile and 0.1% formic acid. However, these conditions produced very early elution of gepirone and poor chromatographic efficiency. At acetonitrile:0.1% formic acid ratios of 80:20 and 70:30, gepirone eluted at about 1.06–1.09 min, with plate counts below 500, indicating inadequate retention.

Replacing acetonitrile with methanol improved retention and peak efficiency. Using methanol:0.1%

formic acid at 70:30, the RTs of gepirone and lurasidone increased to 1.145 and 3.633 min, respectively. The plate counts also improved to 1,676 for gepirone and 4,595 for lurasidone, although the gepirone peak still required further improvement.

Better chromatographic performance was obtained after changing the column to Zorbax SB C18. With methanol:0.1% formic acid at 50:50, the plate counts improved markedly; however, this condition showed baseline instability. Therefore, it was not selected. The final optimized mobile phase was methanol:0.1% formic acid in water at 40:60 v/v. This condition gave suitable retention, stable baseline, and good efficiency. Gepirone and lurasidone eluted at 2.246 and 4.054 min during method development, with plate counts of 3,895 and 7,841, respectively. During validation, the RTs were 2.230 and 4.017 min, confirming good reproducibility of the optimized method.

The acidic mobile phase helped improve ionization of gepirone in positive electrospray mode. Lurasidone showed adequate retention, separation from gepirone, and reproducible response, supporting its use as the internal standard for this method.

4.2 CHROMATOGRAPHIC PERFORMANCE

The optimized method provided clear separation of gepirone and lurasidone within a 7-min isocratic run. In six system-suitability injections, the mean RTs were 2.219 ± 0.008 min for gepirone and 4.030 ± 0.019 min for lurasidone, with CV values of 0.36% and 0.46%, respectively. Mean peak areas were 2.186×10^5 for gepirone and 2.109×10^5 for lurasidone, with CV values below 0.15%. The analyte/internal-standard area ratio was also consistent, with a CV of 0.19%.

No carryover was observed after injection of the high-concentration sample, and no interfering peaks were detected in six blank rat-plasma lots. These results confirm good retention reproducibility, stable detector response, acceptable selectivity, and absence of significant carryover.

4.3 VALIDATION

The method showed linear response over 10–80 ng/mL, with the equation $y = 0.0260x + 0.001938$ and $R^2 = 0.99982$. A separate 4 ng/mL sample showed acceptable response, precision, and accuracy (CV 1.34%, accuracy 94.54%), but since it was below the calibration range, it should be reported as a sensitivity verification level, not as the validated LLOQ.

Within-run precision and accuracy were satisfactory across the tested QC levels. Precision ranged from 0.09% to 1.70% CV, and accuracy ranged from 94.98% to 98.03%, confirming good repeatability. However, between-run precision and accuracy should be added before describing the method as fully validated according to ICH M10.

Matrix-effect results showed acceptable accuracy at 20 ng/mL and 60 ng/mL, with accuracies of 96.05% and 97.11% and CVs below 0.50%. Recovery was

consistent, with gepirone recovery of 96.05–97.16% across QC levels and lurasidone recovery of 97.31%, indicating efficient and reproducible extraction.

Ruggedness results were acceptable, with accuracy of 96.94–98.00% and CVs of 0.09–0.26%. The alternative column details should be reported for reproducibility. Dilution precision was good at 40 and 80 ng/mL, with CVs of 0.32% and 0.09%; dilution accuracy, bias, and dilution factor should also be stated.

Stability results supported acceptable stability during bench-top, autosampler, freeze-thaw, wet-extract, dry-extract, and short-term testing, with accuracy generally within 91.26–98.00% and low CVs. Long-term stability was acceptable up to 21 days, but by Day 28 accuracy decreased to 83.53–84.95%, below the 85–115% acceptance range. Therefore, long-term stability should be claimed only up to 21 days, and exact storage temperatures, durations, freeze-thaw cycles, and reference-sample preparation should be clearly stated.

4.4 Comparison with Previously Reported Azapirone Methods

The developed LC–MS/MS method for Gepirone was compared with reported methods for azapirones such as Buspirone and Tandoospirone. Unlike methods using liquid–liquid or solid-phase extraction, this method employs simple methanol-based protein precipitation. It showed good recovery, minimal matrix interference, adequate sensitivity, and was successfully applied to rat PK studies, making it suitable for routine bioanalysis.

Table 23: Comparison of the Proposed Method with Reported Azapirone LC–MS/MS Methods

Met hod	Matrix and prepar ation	Intern al standa rd	Rang e / LLO Q	Run time and interpreta tion
Cho et al. [9]	Human plasma ; MTBE liquid- liquid extracti on	Amlod ipine	Buspi rone; LLO Q 0.02 ng/m L	Highly sensitive; run time not stated in abstract
Joshi et al. [10]	Human plasma ; solid- phase extracti on	Buspir one-d8	10.4- 6690. 4 pg/m L; LLO	3.0 min; highest sensitivity, greater consumabl e cost

			Q 10.4 pg/m L	
Hu et al. [11]	Rat plasma ; acetone precipitation	Compound-specific ISs	TDS 1-500 ng/m L; LLO Q 1 ng/m L	3.0 min; simultaneous analyte/meabolite assay
Proposed method	Rat plasma ; methanol precipitation	Lurasidone	Validated range 10-80 ng/m L; 4 ng/m L sensitivity QC	7.0 min; simple preparation and high recovery

4.5 Advantages of the Developed LC–MS/MS Method

The main advantage of the proposed method is its simplicity and suitability for routine preclinical analysis. The sample preparation is based on protein precipitation, which avoids time-consuming steps such as phase separation, evaporation, and cartridge conditioning required in liquid-liquid or solid-phase extraction methods. The method also uses commonly available solvents and a non-isotopically labelled internal standard, which may reduce routine analytical cost.

Despite the simple extraction procedure, the method showed consistent performance. Gepirone recovery was above 96% at all QC levels, and lurasidone also showed reproducible recovery as the internal standard. The method demonstrated stable retention, low response variability, absence of measurable carryover, and no interference in six blank rat-plasma lots.

The validated calibration range of 10–80 ng/mL was suitable for the reported rat PK application, including the observed C_{max} of 39.366 ng/mL. However, samples below 10 ng/mL should be treated as below the validated quantification range unless the calibration curve is extended and revalidated.

Overall, the proposed method provides a practical balance of simple sample preparation, reproducible recovery, acceptable selectivity, and adequate sensitivity for routine rat-plasma analysis of gepirone. Future strengthening of the method should include between-run validation, formal matrix-factor evaluation, complete dilution-integrity reporting, and confirmation of a calibration-supported LLOQ.

5. APPLICATION TO PK STUDY

Rats were given a single oral dosage of Gepirone in accordance with the authorized study protocol. Blood was drawn into K2-EDTA tubes at pre-dose, 0.5, 1, 2, 3, 4, 5, and 6 hours after the dose. Centrifugation was used to separate the plasma, which was then kept at -70 °C until analysis. The developed LC–MS/MS technology was used to evaluate the plasma samples after they were processed using the validated extraction procedure. WinNonlin® software was used to calculate PK parameters shown in table 24.

Table 24: Mean Plasma Concentration–Time Profile of Gepirone

Time (h)	Concentration (ng/mL)
0.5	11.765
1.0	24.855
2.0	39.366
3.0	28.473
4.0	19.068
5.0	8.245
6.0	0.000

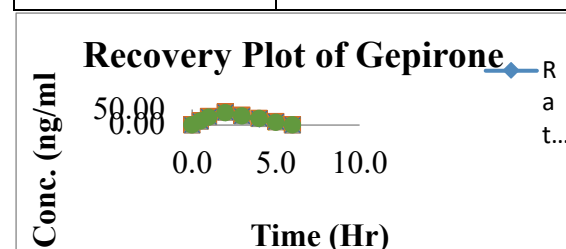


Figure 11: Plasma concentration–time profile of Gepirone following oral administration in rats.

5.3 Pharmacokinetic Parameters

Table 25: PK parameters were estimated from the mean concentration-time profile

Parameter	Value
C _{max}	39.366 ng/mL
T _{max}	2 h
AUC _{0–6}	119.68 ng·h/mL
K _{el}	0.620 h ⁻¹
t _{1/2}	1.12 h
AUC _{0–∞}	128.86 ng·h/mL

A Rapid Liquid Chromatography–Tandem Mass Spectrometry Bioanalytical Method For Quantification Of Gepirone In Rat Plasma Using Lurasidone As An Internal Standard: Method Development, Validation And Pharmacokinetic Application

Result: Following oral administration, Gepirone was rapidly absorbed and reached a maximum plasma concentration (C_{max}) of 39.366 ng/mL at 2.0 h (T_{max}). The elimination rate constant (K_{el}) was found to be 0.620 h⁻¹, corresponding to an elimination half-life (t_{1/2}) of 1.12 h. The area under the plasma concentration–time curve was 119.68 ng·h/mL (AUC_{0–6}) and 128.86 ng·h/mL (AUC_{0–∞}), indicating adequate systemic exposure of Gepirone in rats following oral administration.

6.0 CONCLUSION

In order to quantify gepirone in rat plasma using lurasidone as the internal standard, a straightforward and repeatable LC-MS/MS method was developed. Various chromatographic conditions were assessed during method development, and the best results were obtained using a Zorbax SB C18 column with methanol and 0.1% formic acid in water at 40:60 v/v under isocratic conditions. The optimized method provided adequate retention, stable baseline response, and clear separation of gepirone and the internal standard within a 7-min run.

The method showed linear response over the validated concentration range of 10–80 ng/mL, with a correlation coefficient of 0.99982. The 4 ng/mL level showed acceptable precision and accuracy and therefore demonstrated the sensitivity capability of the method; however, it was treated as a sensitivity verification level because it was below the routine calibration range. Validation results showed acceptable selectivity, no measurable carryover, good within-run precision and accuracy, consistent recovery above 96%, acceptable matrix performance, and satisfactory stability under the evaluated conditions. Long-term stability was supported up to 21 days, while Day 28 results were below the acceptance range.

Instead of using liquid-liquid extraction or solid-phase extraction, the suggested method uses simple protein precipitation, which makes it realistically applicable. This preserves consistent recovery and dependable chromatographic performance while lowering the complexity of sample preparation. Consequently, within the defined calibration range, the approach can be used for routine preclinical analysis of gepirone in rat plasma.

The technique was effectively used in a PK research involving rats. Based on the mean concentration–time profile, gepirone had an elimination half-life of 1.12 hours, an AUC_{0–6} of 119.68 ng·h/mL, and a mean C_{max} of 39.366 ng/mL at 2 hours. These results confirm that the assay is appropriate for monitoring gepirone exposure in the tested preclinical plasma samples. All things considered, the new method supports the use of gepirone bioanalysis in PK studies and offers a straightforward, affordable, selective, and repeatable analytical approach.

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