RESEARCH PAPER

Development And Validation Of A Sensitive Bioanalytical LC-MS/MS Method For Simultaneous Estimation Of Bisoprolol Fumarate And Cilnidipine In Human Plasma

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ABSTRACT

A highly sensitive, selective and robust LC-MS/MS bioanalytical technique was designed and tested to simultaneously determine Bisoprolol Fumarate (BISO) and Cilnidipine (CIL) in K_2EDTA human plasma. Chromatographic separation was achieved using a Sapphirus C_{18} HP Classic column (150 × 4.6 mm, 5 µm) with a mobile phase consisting of 10 mM ammonium acetate and methanol (10:90, v/v). The detection of each analyte was performed using turbo ion spray (TIS) in positive ion mode with Multiple Reaction Monitoring (MRM). Benidipine hydrochloride was taken as an internal standard (ISTD). Preparation of the sample was performed through liquid-liquid extraction (LLE). The method was validated according to ICH M10 guidelines over the concentration range of 0.2–40 ng/mL for Bisoprolol fumarate (correlation coefficients (R^2): 0.9993) and 0.1–20 ng/mL for Cilnidipine (correlation coefficients (R^2): 0.9999). The method met all regulatory acceptance criteria for precision, accuracy, recovery, matrix effect, dilution integrity, and stability. The validated method is suitable for application in pharmacokinetic studies, as well as bioavailability and bioequivalence assessments.

Keywords: Bisoprolol fumarate (BISO), Cilnidipine (CIL), LC-MS/MS, bioanalytical method development and validation, K_2EDTA human plasma

How To Cite This Article: Raval H, Kundu V, Patel D, Patel S, Parmar R, Thakkar V, Patel G. Development And Validation Of A Sensitive Bioanalytical Lc-Ms/Ms Method For Simultaneous Estimation Of Bisoprolol Fumarate And Cilnidipine In Human Plasma. Int J Drug Deliv Technol. 2026;16(27s):954-967. Doi: 10.25258/ijddt.16.27s.111

INTRODUCTION

Hypertension is a major contributor to cardiovascular morbidity worldwide. A combination of two β1-selective blockers, Bisoprolol fumarate and dual L/N-type calcium channel blocker cilnidipine enhances the therapeutic efficiency and cardiovascular safeguard.[1,2] The pharmacokinetic and bioequivalence studies require simultaneous quantification of the antihypertensive drugs in biological matrices. LC–MS/MS is the method of choice due to its high sensitivity and selectivity[3,4]; however, validated analytical procedures that comply with current regulatory requirements are essential to ensure reliability. The International Council for Harmonisation (ICH) M10 guideline provides harmonised requirements of bioanalytical method validation.[5] Despite the fact that individual bioanalytical methods have been reported on Bisoprolol and cilnidipine, only few reports have described a complete validated LC-MS/MS method of simultaneous determination of these two drugs in human plasma in accordance with the current regulatory requirements. The current research presents development and validation of rapid, sensitive and selective LC-MS/MS method for the simultaneous quantification of Bisoprolol fumarate and Cilnidipine in human plasma through liquid-liquid extraction using Benidipine hydrochloride as an internal standard.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical reference standards of Bisoprolol fumarate (100.7%; Batch No.: SLL/BF/0224002) were procured from Supriya Lifesciences Pvt. Ltd., India. Cilnidipine (99.79%; Batch No.: CLD 240709P11) and the internal standard, Benidipine hydrochloride (99.62%; Batch No.: BND 240703P11), were obtained from Purechem Pvt. Ltd., India. All standards were accompanied by certificates of analysis, used without further purification, and stored at 2–8 °C until use.

LC–MS grade methanol was sourced from JT Baker, while ammonium formate and ammonium acetate (LC–MS grade) were obtained from Carlo Erba Reagents. Formic acid (AR/ACS grade) was procured from Rankem, and HPLC-grade water from Kemix Fine Chem. All reagents were of analytical or LC–MS grade and used as received.

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Human plasma containing K_2EDTA (Salvus bioresearch solution, Ahmedabad, Gujarat, India) as an anticoagulant was obtained from a certified research laboratory. Plasma samples were handled in accordance with established bioanalytical and ethical guidelines and stored at −20 °C or lower until analysis to maintain analyte stability and integrity.

Instrumentation and Chromatographic Conditions

Chromatographic analysis was performed using a high-performance liquid chromatography system (Shimadzu SIL-HTC, Shimadzu Corporation, Japan) coupled with a triple quadrupole mass spectrometer (API 4000, SCIEX) equipped with an electrospray ionization (ESI) interface operated in positive ion mode.

Supporting laboratory equipment included a refrigerated system (MPR-S500H-PE) and deep freezer (MDF-MU549DH-PE) from PHC Corporation, centrifuge (ROTANTA 460R, Hettich), vortex mixer (Spinix), analytical balance (SECURA225D1OIN, Sartorius Secura), pH meter (PICO+, Lab India), ultrasonicator (PTM-U40L, Prochem), and solid-phase extraction (SPE, 144 PPP, Prochem). Liquid–liquid extraction (LLE) was performed using apparatus from Yashri Tradelink.

Chromatographic separation was achieved on a Sapphirus C_{18} HP classic analytical column (150 × 4.6 mm, 5 µm particle size). The mobile phase comprised 10 mM ammonium acetate and methanol (10:90, v/v), delivered at a flow rate of 1.0 mL/min. The injection volume was 10 µL, and the total run time was 7.0 min.

Under the optimized conditions, Bisoprolol fumarate, Cilnidipine, and the internal standard Benidipine hydrochloride were eluted at mean retention times of 2.63, 4.12, and 5.20 min, respectively, demonstrating efficient separation and suitability of the method for quantitative bioanalysis.[6]

Mass Spectrometric Conditions

Detection was carried out using multiple reaction monitoring (MRM) mode, with optimized precursor-to-product ion transitions for each analyte. The monitored transitions were m/z 326.2 → 222.2 for Bisoprolol, m/z 515.2 → 471.2 for Cilnidipine, and m/z 506.3 → 315.2 for Benidipine (internal standard). The electrospray ionization source was operated in positive ion mode with an ion spray voltage set at 5500 V and a source temperature maintained at 500 °C to ensure efficient ionization and stable signal response.

Preparation of Calibration Standards and Quality Control Samples

Calibration standards were prepared in human plasma using an eight-point calibration curve over the concentration ranges of 0.2-40 ng/mL for Bisoprolol and 0.1-20 ng/mL for Cilnidipine. Quality control (QC) samples were independently prepared at multiple concentration levels, including lower limit of quantification (LLOQ), low, middle, and high QC levels, to ensure accuracy and precision across the analytical range.

Sample Preparation

Aliquots of 300 µL plasma were combined with 50 µL of internal standard and 50 µL of 5% ammonia solution.[7] The liquid-liquid extraction was performed using 2000 µL of methyl tert-butyl ether. The organic phase was dried at 40ºC under nitrogen after centrifugation and reconstituted in 300 µL of mobile phase.

Method Validation

System Suitability testing

System suitability testing was performed by injecting six consecutive injections using MQC at least once in a day.[8,9] The % CV of the area ratio and retention time for the respective drug and ISTD should be within ≤ 4 %.

System Performance

System performance experiment was performed by injecting following sequence of injections at the beginning of analysis or before any re-injection. STD BLK*, ULOQ, STD BLK*, STD BLK*, LLOQ, STD BLK* (*Note: *Denotes the same sample with repeated acquisition).* Signal to noise ratio should be more than 5 for LLOQ samples. Interference observed in first STD BLK and carry over observed in STD BLK injected after ULOQ should be ≤5.0 % for ISTD response compared to analyte and ISTD response respectively of LLOQ sample.[10, 11]

Specificity and Selectivity

Five different plasma lots-three normal, one lipidemic, and one hemolyzed were analyzed at blank and LLOQ levels following the sample preparation protocol. To ensure method selectivity, no interfering peaks from endogenous substances or degradation products should be detected at the retention times of the analyte(s) or internal standard. If present, the peak area must not exceed 20% of the LLOQ response for the analyte and 5% for the internal standard.[12,13] This criterion must be met in at least 80% of the plasma lots to be considered compliant.

Matrix Effect

Matrix factor (MF) is calculated as the ratio of the analyte response in post-extraction spiked samples to the response in neat solutions at equivalent low and high QC levels.[14-16] Neat solutions of analyte and ISTD were prepared at matching concentrations (n=4 per level). Five matrix lots (including haemolytic and lipidemic) were processed, then spiked post-extraction with analyte and ISTD. Mean peak areas in the presence and absence of matrix ions were determined, and %CV was calculated. Matrix factor was then derived using the formula:

$$\text{Matrix Factor} = \frac{Mean\,peak\,response\,in\,the\,presence\,of\,matrix\,ions}{Mean\,peak\,response\,in\,the\,absence\,of\,matrix\,ions}$$

$$\text{ISTD normalized matrix factor} = \frac{Matrix\,factor\,of\,analyte}{Matrix\,factor\,of\,istd}$$

The back-calculated concentrations of LQC and HQC must fall within 85.00–115.00% of their theoretical values. For each plasma lot, at least 67% of LQC and HQC samples must meet this requirement. Additionally, a minimum of 75% of buffered plasma samples, including lipidemic and hemolyzed types, must comply with the acceptance criteria. If these conditions are not met, the experiment should be repeated or the method should be revised.[17-19]

Linearity

Chromatographic data were processed using Analyst® software (SCIEX). Calibration curves were constructed by plotting the peak area ratio of analyte to internal standard versus nominal concentration using weighted ($1/x^2$) linear regression ($y = ax + b$).

An eight-point calibration curve was established for Bisoprolol fumarate (0.2–40 ng/mL) and Cilnidipine (0.1–20 ng/mL). Quality control samples were prepared at LLOQ, LQC, MQC, and HQC levels.

Linearity was evaluated based on correlation coefficient ($r^2 \geq 0.9900$), back-calculated concentrations, and residual distribution. The LLOQ was required to be within ±20% of nominal concentration, while other standards were within ±15%. The selected model met all acceptance criteria, demonstrating reliable linearity across the calibration range.

Precision

Through intra- and inter-batch precision tests, the precision of Cilnidipine (at HQC: 17 ng/mL, MQC: 9 ng/mL, INT QC: 0.9 ng/mL, LQC: 0.3 ng/mL, and LOQ QC: 0.1 ng/mL) and Bisoprolol (at HQC: 34 ng/mL, MQC: 18 ng/mL, INT QC: 1.8 ng/mL, LQC: 0.6 ng/mL, and LOQ QC: 0.2 ng/mL) were assessed. Six replicates of each QC level were analyzed in a single analytical run to determine intra-batch precision, whereas six replicates from at least three distinct analytical batches carried out on the same or different days were analyzed to determine inter-batch precision.[20-22] With the exception of LOQ QC, where ≤20% was permissible, precision was expressed as %CV and had to be ≤15% for all QC levels. Furthermore, in order to verify adequate precision, at least 67% of the QC samples at each level required to satisfy these requirements.

Accuracy

By calculating mean back-calculated concentrations as a percentage of nominal values, the accuracy of Bisoprolol (HQC: 34 ng/mL, MQC: 18 ng/mL, INT QC: 1.8 ng/mL, LQC: 0.6 ng/mL, LOQ QC: 0.2 ng/mL) and Cilnidipine (HQC: 17 ng/mL, MQC: 9 ng/mL, INT QC: 0.9 ng/mL, LQC: 0.3 ng/mL, LOQ QC: 0.1 ng/mL) were calculated. Six replicates of each QC level were analyzed within a single batch to determine intra-batch accuracy, whereas three to five analytical batches completed on the same day were used to determine inter-batch accuracy. With the exception of LOQ QC, which was permitted to fall between 80.00 and 120.00% of nominal values, all QC levels must fall within 85.00 to 115.00% of acceptable accuracy. To verify the accuracy of the approach, at least 67% of QC samples at each level required to satisfy these requirements.[23]

Recovery

By comparing the mean peak areas of extracted samples to those of unextracted standards, which indicate 100% recovery, the recovery of Bisoprolol, Cilnidipine, and the internal standard (ISTD) was evaluated. While ISTD recovery was measured at the concentration used during validation, analyte recovery was studied at HQC, MQC, INT QC, LQC, and LOQ QC levels.[8] Six replicates at each level were used to compute the percentage mean recovery, and in order to maintain consistency, the percentage CV at all QC levels had to be within 15.00%. The same methodology was used to compute recovery for ISTD at the mid-QC level. Additionally, the %CV had to be ≤15.00% to confirm satisfactory and repeatable extraction efficiency.[24]

Dilution Integrity

The dilution integrity was evaluated by diluting the stock solution prepared as spiked QC (AUL QC (4000 ng/mL)-above Upper Limit of QC) at concentration 1.5-2 times of the concentration of the highest standard in the calibration curve in the screened biological matrix. DI (Dilution integrity) was evaluated by using six replicates each of DQC (1/2) and DOC (1/10) samples. The DQC (Dilution Quality Control) samples were processed and analysed along with freshly spiked CC (Calibration Curve) standards and at least two sets of batch qualifying QCs (at lower and higher). The concentration of the DQC samples was calculated against the CC. The DI was evaluated by ensuring the precision and mean accuracy of DQC samples as per acceptance criteria The precision of DQC samples should be within 15.00% and the % mean accuracy.[25]

Stability Study

Under a variety of circumstances, the stability of Bisoprolol Fumarate, Cilnidipine, and the internal standard Benidipine was thoroughly assessed. Analyte responses to freshly made solutions were comparable for both short-term stock solution stability at room temperature (≥6 hours) and long-term stability at -20 ± 5 °C (≥4 days), demonstrating stability. Under comparable circumstances, working solution stability was also achieved at ULOQ, LLOQ, and ISTD concentrations, with mean recoveries falling between 90.00 and 110.00% and %CV ≤15.00%. The LQC and HQC levels of both analytes showed bench-top stability, with ≥67% of samples falling between 85.00 to 115.00% of nominal values and the mean stability percentage falling within an acceptable range. Consistent analyte integrity was confirmed by freeze-thaw stability for 1 and 5 cycles at -20 ± 5 °C and -78 ± 8 °C, satisfying acceptance criteria. Furthermore, analyte stability under pre-analytical settings was established by evaluating whole blood stability using LQC and HQC levels at ambient temperature and on wet ice (<10 °C).

RESULTS AND DISCUSSION

Optimization of Mass Parameter

The mass spectrometry optimization for Bisoprolol Fumarate, Cilnidipine, and the internal standard Benidipine involved tuning three categories of parameters: common, source-dependent, and compound-dependent. The method used an ESI (Turbo Spray) ion source operating in positive polarity and a Multiple Reaction Monitoring (MRM) scan mode. Source-dependent settings included curtain gas (CUR) at 40.00, collision gas (CAD) at 4.00, ion spray voltage at 5500 mV, temperature at 500 °C, and both ion source gases (GS1 and GS2) set to 50. Compound-specific optimization involved identifying parent and daughter ions and adjusting key voltages: Bisoprolol had a parent mass of 326.2 Da and daughter mass of 222.2 Da with a declustering potential (DP) of 65, entrance potential (EP) of 5, collision energy (CE) of 18 eV, and collision cell exit potential (CXP) of 5. Cilnidipine showed a parent mass of 515.2 Da and daughter mass of 471.2 Da, with DP 75, EP 11, CE 24 eV, and CXP 15. For Benidipine, the parent/daughter masses were 506.3/315.2 Da, with DP 55, EP 12, CE 35 eV, and CXP 15. These parameters ensured optimal ionization, fragmentation, and detection of the analytes and ISTD during LC-MS/MS analysis. These optimized parameters ensured efficient ionization, fragmentation, and reproducible detection. The detailed mass parameters are summarized in Table 1.

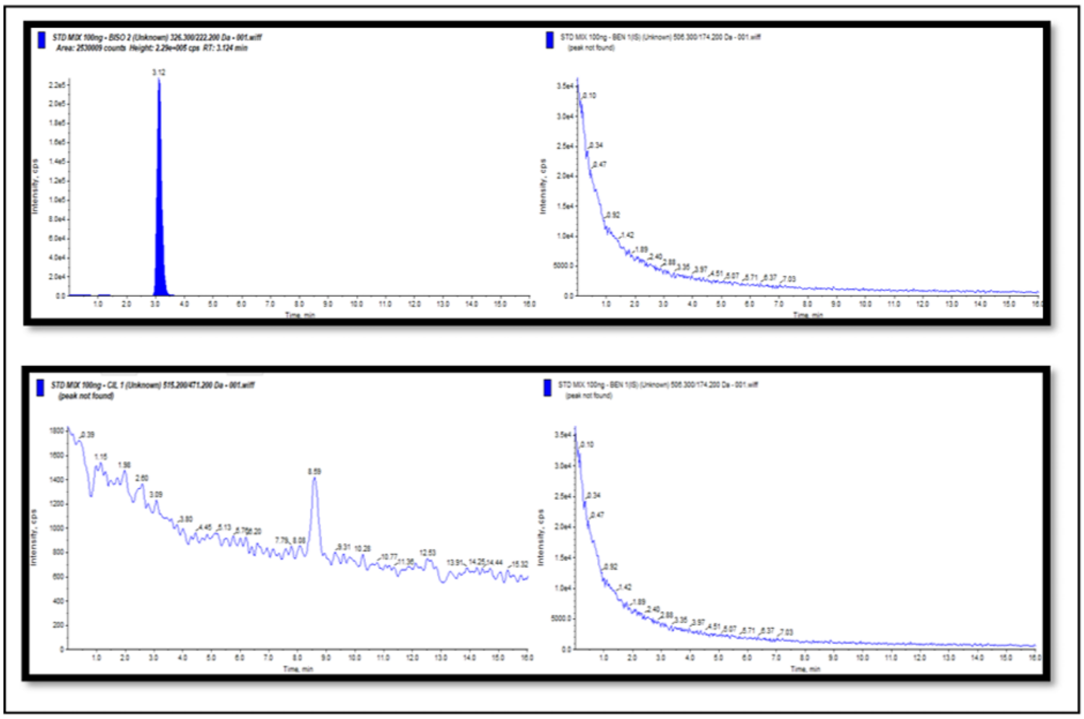
Table 1. Mass Parameters

Common Mass Parameters				
Scan Type	MRM (Multiple reaction monitoring)			
Ion Source	Turbo Spray (ESI)			
Ion Polarity	Positive			
Source Dependent Parameters				
Curtain Gas (CUR) (PSI)	40			
Collision Gas (CAD) (PSI)	4			
Ion Spray (v)	5500 mV			
Temperature (TEM) °C	500°C			
Nebulizer Gas (PSI)	50			
Heater Gas (PSI)	50			
Compound Dependent Parameters				
Analyte	DP (v)	EP (v)	CE (ev)	CXP (v)
Bisoprolol fumarate	65	5	18	5
Cilnidipine	75	11	24	15
Benidipine	55	12	35	15

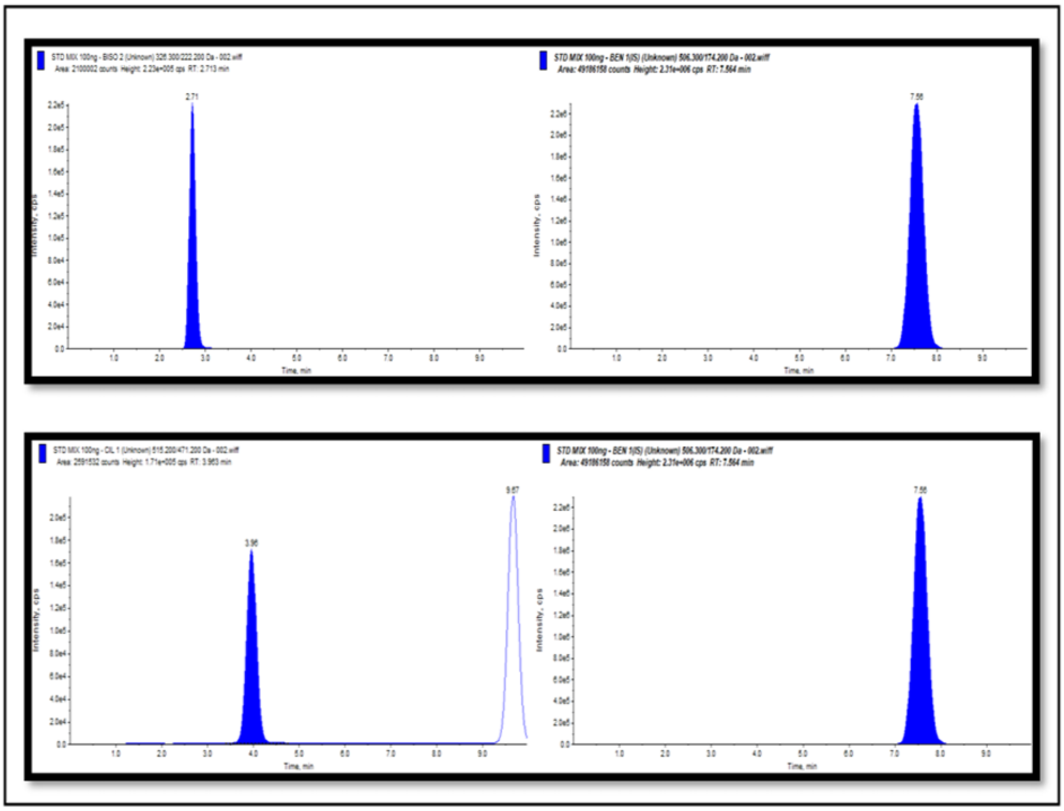
Development and Optimization of Chromatographic Conditions

Chromatographic conditions were optimized through six trials by varying column type and mobile phase composition. Trials 1 and 2, performed on a Zorbax SB C_8 column using 0.1% formic acid with acetonitrile and methanol (30:70, v/v), showed significant carryover. Trial 3, using a Sapphirus C_{18} column with 0.1% formic acid and methanol (30:70), improved performance. Further optimization in Trials 4–6 with 10 mM ammonium acetate and methanol showed acceptable peak shape in Trial 4 (30:70), reduced run time in Trial 5 (20:80), and optimal performance in Trial 6 (10:90), which was selected as the final condition. The results of all optimization trials are summarized in Figure 1.

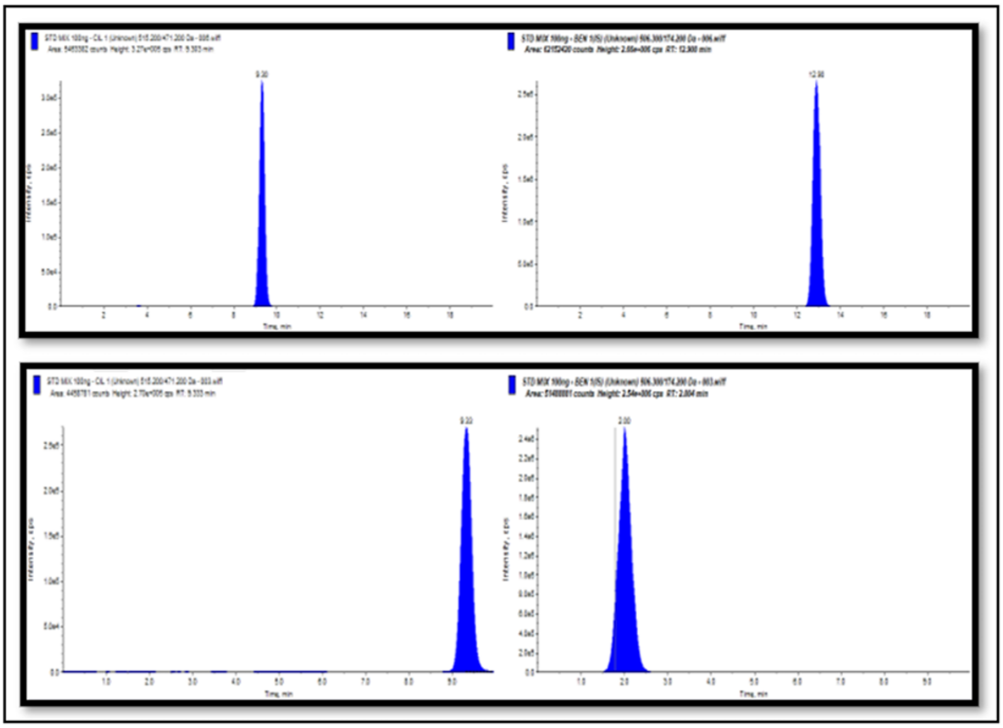
Chromatographic separation was achieved on a Sapphirus C_{18} HP Classic column (150 × 4.6 mm, 5 µm) using 10 mM ammonium acetate: methanol (10:90, v/v) at 1.0 mL/min. The column oven and autosampler temperatures were maintained at 40 ± 5 °C and 5 ± 3 °C, respectively, with a 10 µL injection volume and 7 min run time. Bisoprolol fumarate, Cilnidipine, and Benidipine hydrochloride were eluted at approximately 2.63, 4.12, and 5.20 min, respectively.



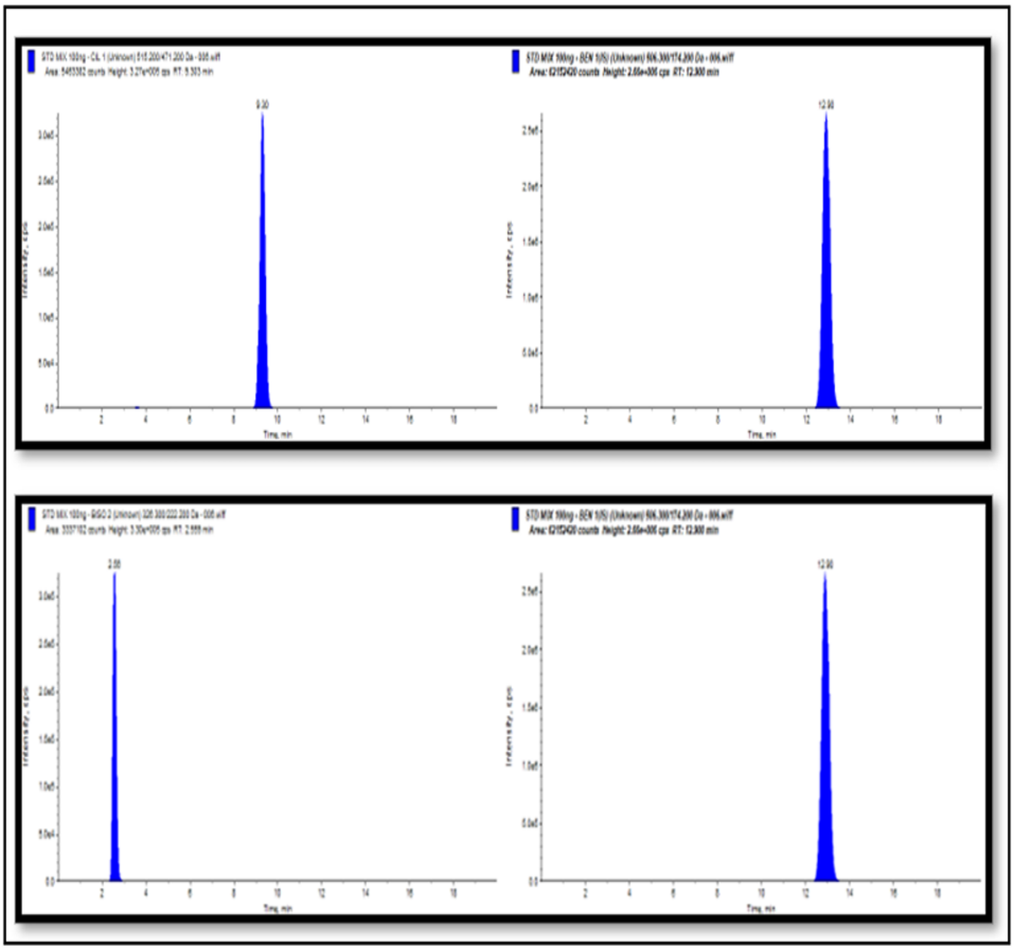
Trial 1: 0.1% formic acid in water: ACN (30:70 %v/v)



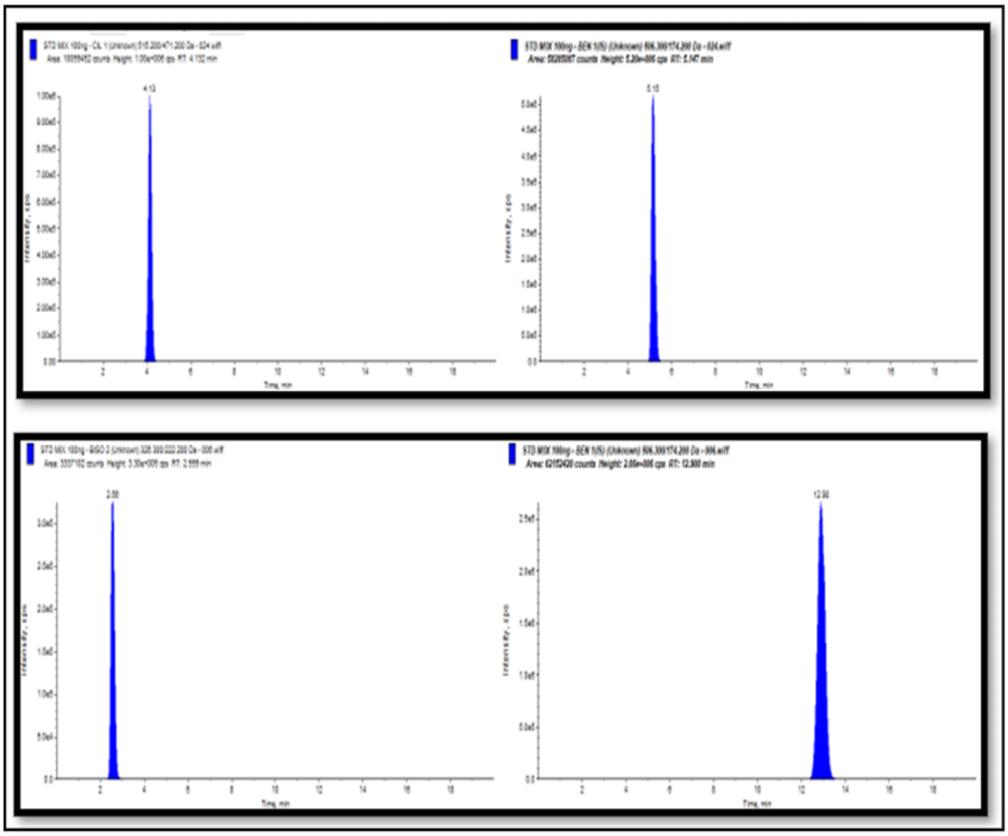
Trial 2: 0.1% formic acid in water: MeOH (30:70 % v/v)



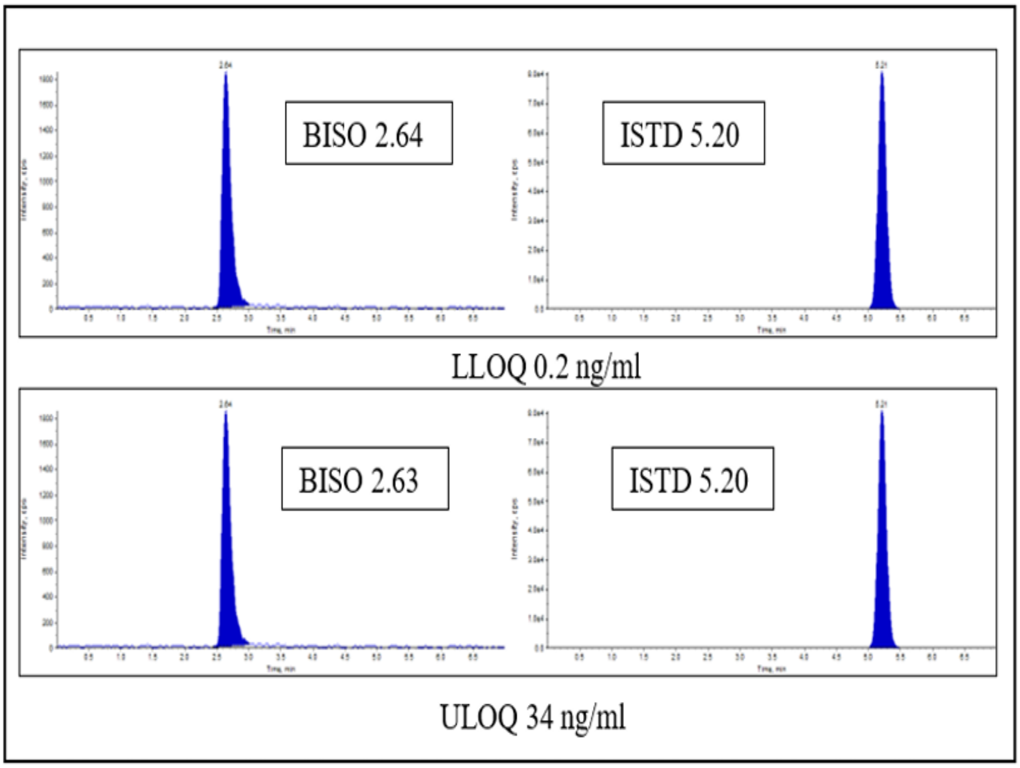
Trial 3: 0.1% formic acid in water: MeOH (30:70 % v/v)



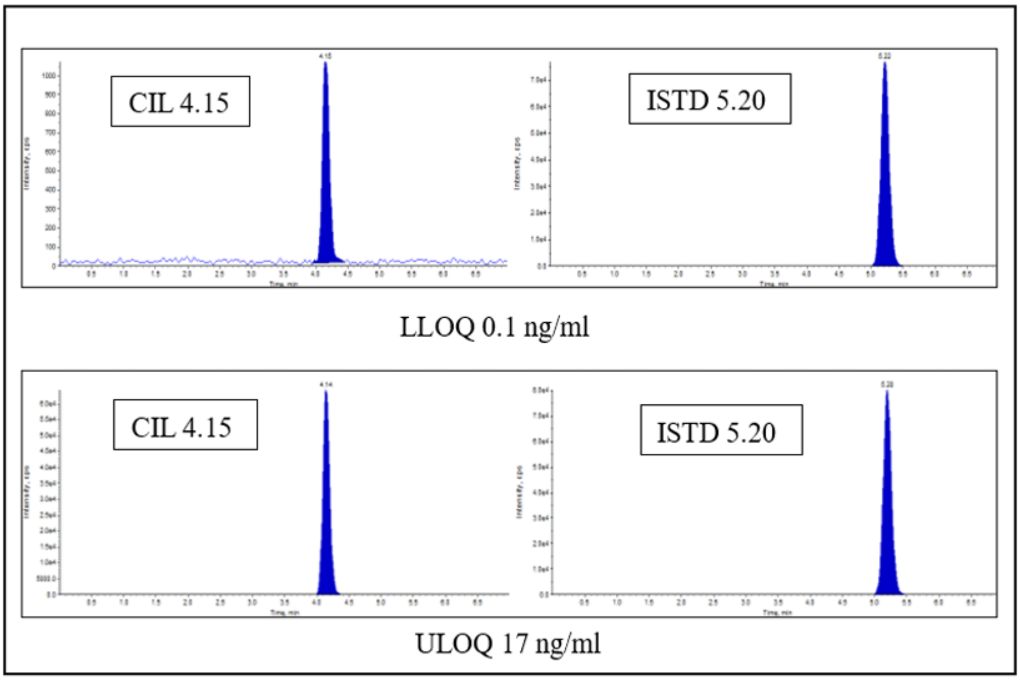
Trial 4: 10mM Ammonium Acetate in water: MeOH (30:70 % v/v)



Trial 5: 10mM Ammonium Acetate in water: MeOH (20:80 % v/v)



Trial 6 (a) : 10mM Ammonium Acetate in water: MeOH (10:90 % v/v) of BISO (optimized mobile phase)



Trial 6(b) : 10mM Ammonium Acetate in water: MeOH (10:90 % v/v) of CIL (optimized mobile phase)

Figure:1 Chromatographic trial towards mobile phase optimization for BISO and CIL

Validation of Bioanalytical Method

System suitability study

System suitability was assessed using six replicate injections, demonstrating consistent chromatographic performance for Bisoprolol fumarate, Cilnidipine, and the internal standard Benidipine hydrochloride. Mean retention times were 2.64, 4.16, and 5.23 min, respectively. The %CV of peak area was 2.9% (Bisoprolol), 1.0% (Cilnidipine), and 1.2% (IS), with area ratio %CV of 2.9%. All parameters were within acceptable limits (%CV ≤ 4%), confirming system reliability.

System performance

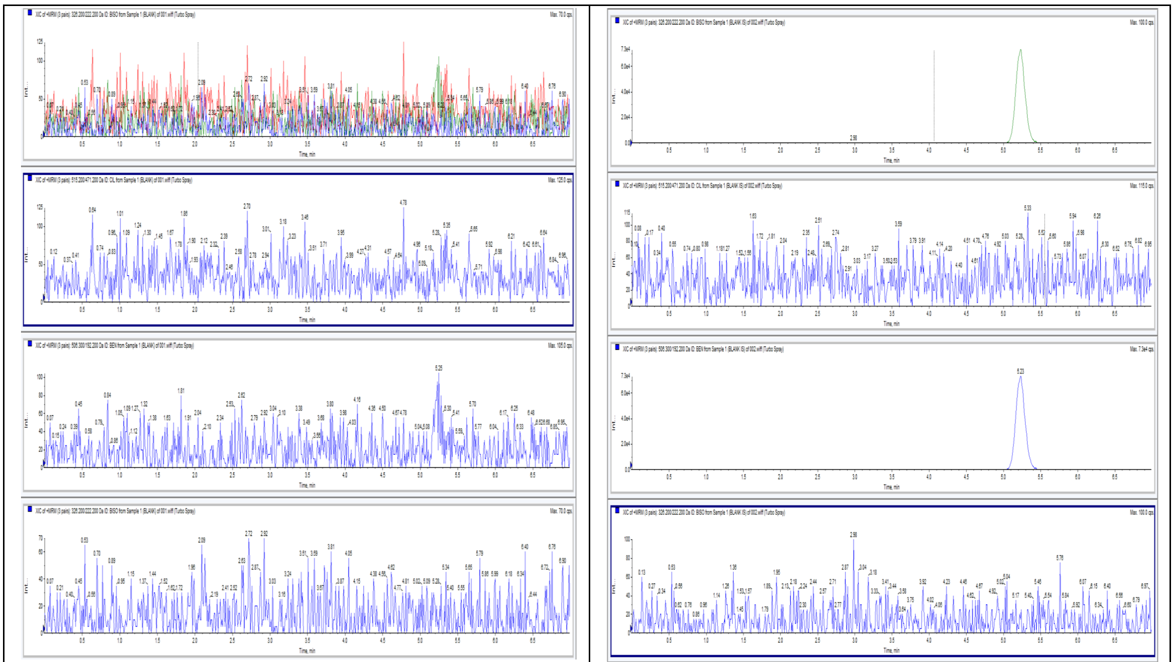
System performance was assessed in terms of signal-to-noise ratio, carryover, and interference. The signal-to-noise ratio at LLOQ exceeded 5 for both Bisoprolol fumarate and Cilnidipine, indicating sufficient sensitivity. No notable interference was detected at the retention times of the analytes or the internal standard Benidipine hydrochloride in blank samples.

Carryover in post-ULOQ blank injections remained within acceptable limits (≤20% for analytes and ≤5% for internal standard relative to LLOQ response), confirming the absence of significant carryover and ensuring reliable system performance.

Selectivity

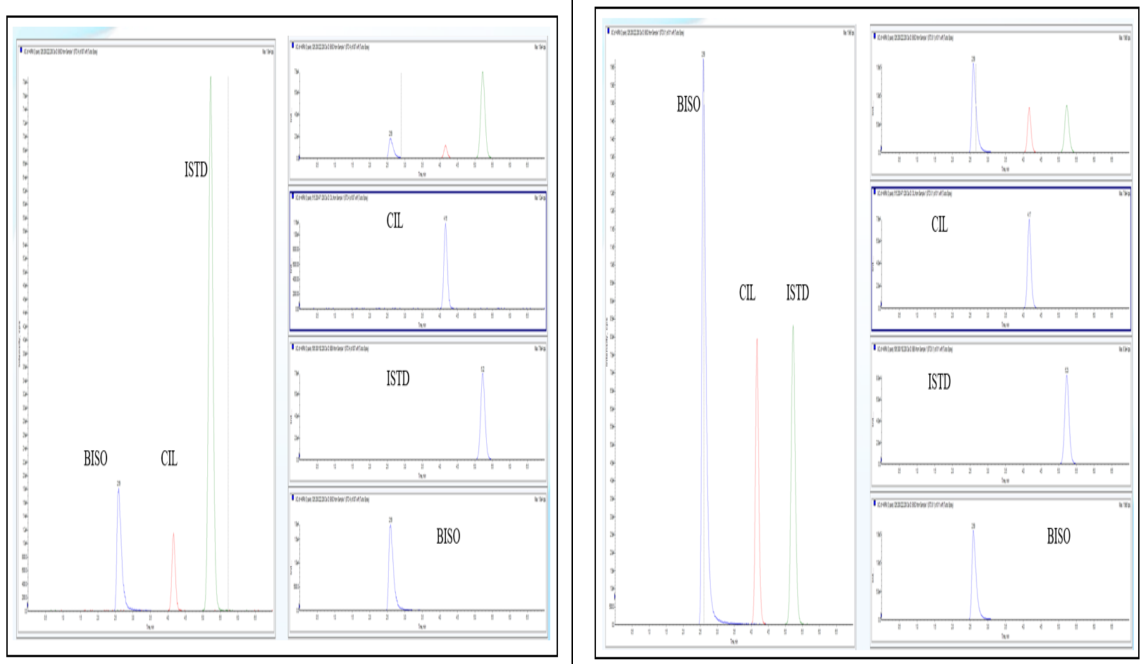
Selectivity was evaluated across six plasma lots, including normal, lipidemic, and hemolyzed samples, to assess interference at the retention times of Bisoprolol fumarate, Cilnidipine, and the internal standard Benidipine hydrochloride. No significant endogenous interference was observed in blank samples. At LLOQ, interference was 0.00% for both analytes and ≤0.1% for the internal standard (Figure 2).

The %CV across plasma lots was 2.4% for Bisoprolol and 9.8% for Cilnidipine, demonstrating acceptable variability. The method met selectivity acceptance criteria (<20% for analytes and <5% for internal standard).



(A) blank plasma

(B) blank + IS



(C) LLOQ

(D) ULOQ

Figure 2: Representative MRM chromatograms of (A) blank plasma, (B) blank + IS, (C) LLOQ, and (D) ULOQ.

Matrix effect

Matrix effect was evaluated across multiple plasma lots, including lipidemic and hemolyzed samples, at LQC and HQC levels for Bisoprolol fumarate and Cilnidipine. The %CV of matrix factor ranged from 5.4% (LQC) to 1.4% (HQC) for Bisoprolol and 6.1% (LQC) to 1.7% (HQC) for Cilnidipine, indicating low variability across different matrices. Mean accuracy was within acceptable limits, ranging from 95.9% to 103.5% for Bisoprolol and 103.7% to 103.8% for Cilnidipine (Table 3). These results demonstrate minimal matrix interference and confirm that the method is robust and reproducible across different plasma conditions, meeting acceptance criteria.

Table 3. Matrix Effect of Analyte

Matrix ID	BISO LQC	BISO HQC	CIL LQC	CIL HQC
SBS2024-0949	0.585	34.669	0.287	17.356
	0.539	34.685	0.134	17.392
	0.595	34.989	0.295	17.342
	0.524	34.585	0.287	17.318
SBS2024-0953	0.561	35.835	0.313	17.147
	0.625	35.670	0.337	17.526
	0.566	34.680	0.286	17.548
	0.561	35.625	0.322	17.824
SBS2024-0952	0.562	34.465	0.302	17.852
	0.572	34.786	0.298	17.762
	0.527	35.811	0.333	17.370
	0.606	35.091	0.345	17.522
SBS2024-0951	0.582	35.798	0.305	17.933
	0.595	35.882	0.314	18.152
	0.552	34.932	0.302	17.848
	0.596	35.411	0.334	17.810
SBS2024-0950	0.582	35.490	0.309	17.719
	0.649	35.376	0.345	17.632
	0.548	35.244	0.302	18.313
	0.584	34.496	0.293	17.700
MEAN	0.576	35.176	0.311	17.653
SD	0.03	0.50	0.02	0.30
%CV	5.4	1.4	6.1	1.7
% ACCURACY	95.9	103.5	103.7	103.8

Linearity Study

The method demonstrated acceptable linearity for Bisoprolol fumarate (0.2–40 ng/mL) (Figure 3) and Cilnidipine (0.1–20 ng/mL) (Figure 4) with correlation coefficients (r^2) ≥ 0.99. A weighted ($1/x^2$) regression model ensured minimal bias across the range. The %CV ranged from 0.824–6.266% and 0.454–4.444%, while mean accuracy was within 92.08–106.15% and 97.39–101.79% for Bisoprolol and Cilnidipine, respectively. All standards met acceptance criteria (±15%, LLOQ ±20%).

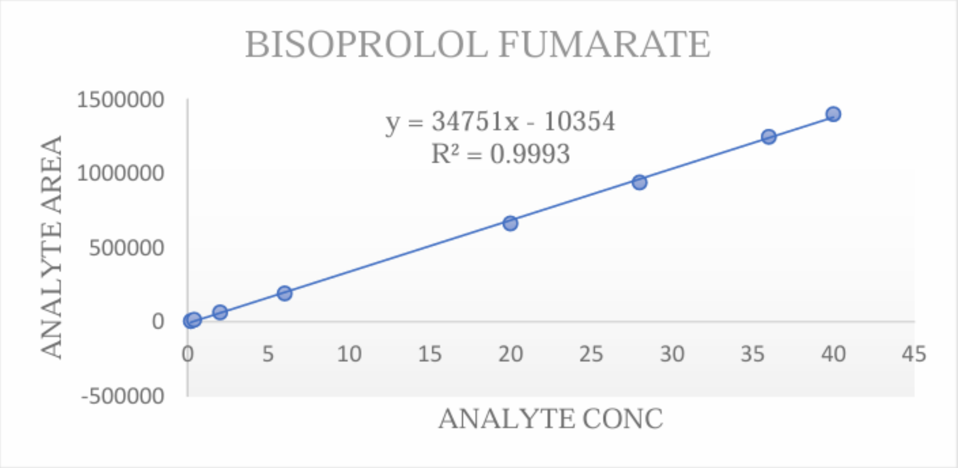


Figure 3: Calibration curve of Bisoprolol fumarate (0.2–40 ng/mL)

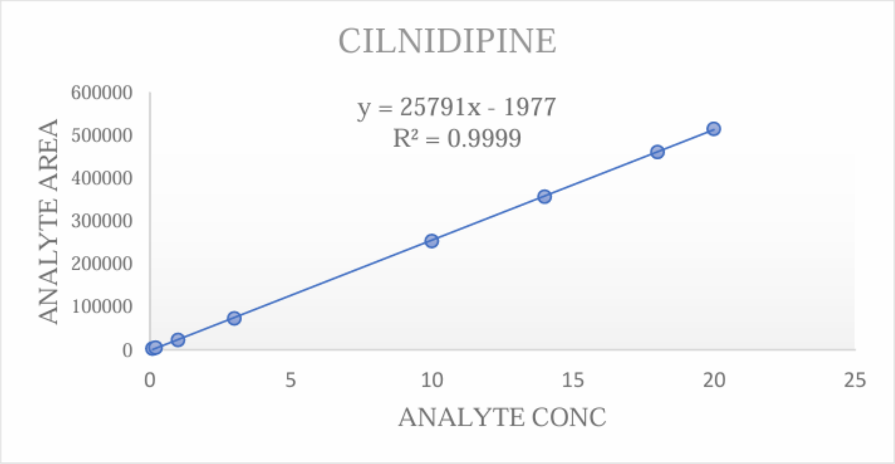


Figure 4: Calibration curve of Cilnidipine (0.1–20 ng/mL)

Precision and Accuracy Study

The developed LC–MS/MS method demonstrated acceptable precision and accuracy for both Bisoprolol fumarate and Cilnidipine across all QC levels. Intra- and inter-day precision (%CV) values were within acceptable limits (≤15%), indicating high reproducibility of the method. Accuracy values ranged within 96.51–102.21% for Bisoprolol and 101.83–113.00% for Cilnidipine (Table 4), meeting regulatory acceptance criteria. The results confirm minimal variability and consistent performance across different analytical runs. Overall, the method is precise, accurate, and suitable for routine bioanalytical applications.

Table 4. Interday and Intraday Precision of Bisoprolol Fumarate and Cilnidipine

Drug	Study Type	QC Level	Nominal (ng/mL)	Mean (ng/mL)	SD	%CV	% Accuracy
Bisoprolol Fumarate	Inter-day	LLOQ	0.2	0.195	0.02	8.4	97.72
		LQC	0.6	0.579	0.02	3.5	96.51
		MQC	18	17.534	0.32	1.8	97.41
		HQC	34	34.470	0.92	2.7	101.38
Bisoprolol Fumarate	Intra-day	LLOQ	0.2	0.202	0.02	10.4	100.92
		LQC	0.6	0.603	0.04	6.6	100.56
		MQC	18	17.722	0.21	1.2	98.46
		HQC	34	34.753	0.53	1.5	102.21
Cilnidipine	Inter-day	LLOQ	0.100	0.113	0.01	5.7	113.00
		LQC	0.300	0.316	0.02	6.1	105.39
		MQC	9.000	9.345	0.14	1.5	103.83
		HQC	17.000	17.406	0.34	2.0	102.39
Cilnidipine	Intra-day	LLOQ	0.100	0.104	0.00	4.6	104.00
		LQC	0.300	0.306	0.01	3.4	101.83
		MQC	9.000	9.350	0.15	1.6	103.89
		HQC	17.000	17.560	0.37	2.1	103.29

Recovery study

The extraction recovery of Bisoprolol fumarate, Cilnidipine, and the internal standard Benidipine hydrochloride was consistent across HQC, MQC, and LQC levels. Mean recovery ranged from 94.41–98.21% for Bisoprolol, 94.08–97.64% for Cilnidipine, and 96.45–97.80% for the internal standard. The %CV values were within 1.00–3.84% for Bisoprolol, 1.03–6.41% for cilnidipine, and ≤1.94% for the internal standard, indicating good precision (Table 5).

The recovery was reproducible and concentration-independent across QC levels. These results demonstrate efficient and consistent extraction with minimal variability, confirming the suitability of the method for bioanalytical quantification.

Table 5. Recovery for Bisoprolol Fumarate, Cilnidipine and Benidipine (ISTD)

Drug Name	**QC Level**	**Mean Extracted Area**	**Mean Unextracted Area**	**Mean % Recovery**	**% CV (Precision)**
Bisoprolol fumarate	**HQC**	13,887.83	14,713.50	94.41	3.84
	MQC	476,344.50	492,990.00	96.63	1.49
	LQC	920,824.00	937,665.83	98.21	1.00
Cilnidipine	**HQC**	7,122.17	7,590.67	94.08	6.41
	MQC	224,469.33	231,283.83	97.06	1.65
	LQC	404,959.50	414,752.67	97.64	1.03
Benidipine (IS)	**HQC**	629,890.33	653,066.83	96.45	0.96
	MQC	659,093.00	673,991.17	97.80	1.94
	LQC	641,973.50	658,141.00	97.55	0.81

Stock solution stability study

Stability of Bisoprolol fumarate, Cilnidipine, and the internal standard Benidipine hydrochloride was evaluated under various conditions, including stock, working, bench-top, freeze–thaw, and whole blood stability.

Stock and working solution stability showed %stability within 90.0–110.0% with %CV ≤6.6%, indicating no significant degradation. Bench-top stability results demonstrated %accuracy within 93.4–112.2% with acceptable precision, confirming analyte stability during sample handling. Freeze–thaw stability across cycles showed %accuracy within 103.2–106.4% for Bisoprolol and 104.2–105.0% for Cilnidipine, with %CV ≤3.13%, indicating robustness under repeated freezing and thawing.

Whole blood stability at room temperature showed %accuracy within 102.27–104.38% for Bisoprolol and 102.27–103.20% for Cilnidipine, confirming pre-analytical stability. Additionally, short-term stability at ambient conditions demonstrated %accuracy within 100.42–103.99% with %CV ≤4.97%.

Overall, all stability results met acceptance criteria (±15%), confirming that the developed method is stable, reliable, and suitable for routine bioanalytical applications (Table 6).

Table 6: Summary of Stability Results for Bioanalytical Method

Stability Type	**Analyte**	**QC Level**	**% Accuracy / % Stability**	**% CV**	**Conclusion**
Stock & Working	Bisoprolol	Std1/Std8	95.8-107.6%	≤6.2	Stable
	Cilnidipine	Std1/Std8	94.5-109.6%	≤6.6	Stable
	ISTD	—	90.1-107.7%	≤0.8	Stable
Bench-top	Bisoprolol	LQC/HQC	93.4–112.2%	≤6.3	Stable
	Cilnidipine	LQC/HQC	~100-104%	≤1.7	Stable
Freeze–thaw	Bisoprolol	LQC/HQC	103.3-106.4%	≤3.13	Stable
	Cilnidipine	LQC/HQC	104.2-105.0%	≤2.21	Stable
Blood Stability	Bisoprolol	LQC/HQC	102.27-104.38%	≤2.37	Stable
	Cilnidipine	LQC/HQC	102.27-103.20%	≤1.70	Stable
Ambient Stability	Bisoprolol	LQC/HQC	101.38-102.78%	≤4.97	Stable
	Cilnidipine	LQC/HQC	100.42-103.99%	≤3.80	Stable

CONCLUSIONS

A robust, sensitive, and selective LC–MS/MS method was successfully developed and validated for the simultaneous quantification of Bisoprolol fumarate and Cilnidipine in human plasma using Benidipine hydrochloride as an internal standard. The method demonstrated acceptable linearity, precision, and accuracy across the entire calibration range, with all validation parameters complying with regulatory acceptance criteria.

Efficient and reproducible extraction was achieved with high recovery and negligible matrix effect, ensuring reliable quantification in complex biological matrices. The method exhibited no significant interference or carryover, confirming high selectivity and system performance. Comprehensive stability studies under various conditions, including bench-top, freeze–thaw, stock, and whole blood stability, established the robustness and reliability of the analytes during sample handling and analysis.

Overall, the developed method is simple, rapid, and highly reproducible, making it well-suited for routine bioanalytical applications, including pharmacokinetic, bioavailability, and therapeutic drug monitoring studies. The method's compliance with ICH M10 guidelines further supports its applicability for regulatory submissions and clinical research.

REFERENCES

1. T. Saxena, A.O. Ali, M. Saxena, Pathophysiology of essential hypertension: An update, Expert Rev. Cardiovasc. Ther. 2018;16:879–887.
2. Bakheit A, Ali R, Alshahrani A, El-Azab S. Bisoprolol: a comprehensive profile of drug substances, excipients and related methodology. In: Brittain HG, editor. Profiles of Drug Substances, Excipients and Related Methodology. Vol. 46. Amsterdam: Academic Press; 2021. p. 51–89.
3. Uneyama H, Uchida H, Konda T, Yoshimoto R. Cilnidipine: preclinical profile and clinical evaluation. Cardiovasc Drug Rev. 1999;17(4):341–357.
4. Kadian R, Nanda A. A comprehensive insight on pharmacological properties of Cilnidipine: A fourth-generation calcium channel blocker. Cardiovasc Hematol Agents Med Chem. 2024;22(1):40–50.
5. Zimmer D. Introduction to quantitative liquid chromatography–tandem mass spectrometry (LC–MS/MS). Chromatographia. 2003;57(Suppl 1):S325–S332.
6. Rossi DT, Sinz MW, editors. Mass spectrometry in drug discovery. New York: Marcel Dekker; 2002.
7. Bhatt J, Subbaiah G, Kambli S, Shah B, Patel M, Saxena A, et al. A high-throughput and sensitive LC–MS/MS method for determination of bisoprolol in human plasma using multiplexing technique. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;852(1–2):374–381.
8. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). ICH guideline M10 on bioanalytical method validation and study sample analysis. Geneva: ICH; 2022. Updated 2024. Available from: https://www.ich.org/page/multidisciplinary-guidelines
9. Lee S, Kim H, Park J, Lee Y. Development and validation of a sensitive LC–MS/MS method for simultaneous quantification of amoxicillin and clavulanate in human plasma. Biomed Chromatogr. 2024;38(1):e5964.
10. De Palma R, Matta MK, Patel V, Florian J, Strauss DG, Rouse R. Development and validation of a high-throughput LC–MS/MS bioanalytical method for quantification of cannabidiol and metabolites in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2025;1263:124694.
11. Thomann J, Nguyen T, Bizec B, et al. Rapid LC–MS/MS method for simultaneous quantification of diamorphine and metabolites in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2024;1234:123–130.
12. Cheruku S, Darna B, Medipalli V, Nekkalapudi AR, Sadasivam RK. Bioanalytical method development and validation for the quantitation of larotrectinib in human plasma: application to pharmacokinetics in healthy rabbits. J Appl Pharm Sci. 2023;13(11):111–118.
13. Kantipudi R, Pavuluri SK. Bioanalytical method development and validation for the simultaneous estimation of Olanzapine and Samidorphan in rabbit plasma by using HPLC–MS/MS and application to pharmacokinetic study. Future J Pharm Sci. 2024;10(1):1-14.
14. Gandu S, Gandla K, Repudi L. Development and validation of an LC–MS/MS method for the simultaneous estimation of tadalafil and macitentan in rat plasma: greenness assessment and design of experiment approach. Green Anal Chem. 2025;12:100211.
15. Mancini A, Simeoli R, Cristiani L, Cairoli S, Ciciriello F, Boni A, et al. Development and validation of a new LC–MS/MS method for simultaneous quantification of ivacaftor, tezacaftor and elexacaftor plasma levels in pediatric cystic fibrosis patients. Pharmaceuticals (Basel). 2025;18(7):1028.
16. Al-Tannak NF, Hafez HM, Hemdan A, Aldasem A, Khadra I. LC–MS/MS method development and validation for clinical pharmacokinetics and therapeutic drug monitoring of potassium-competitive acid blocker vonoprazan-based triple therapy for H. pylori in human plasma. Pharmaceuticals (Basel). 2025;18(10):1509.
17. Kaewkhao N, Tarning J, Blessborn D. LC–MS/MS method validation for quantification of nirmatrelvir in human plasma. Int J Anal Chem. 2025;2025:6625833.
18. Lee S, Kim DH, Shin S, Min JS, Kim DY, Jo SJ, et al. Development and validation of bioanalytical LC–MS/MS method for pharmacokinetic assessment of amoxicillin and clavulanate in human plasma. Pharmaceuticals (Basel). 2025;18(7):998.
19. Li J, Lan J, Li Y, Xue Y, Wang X, Xu W, et al. Development and validation of an LC–MS/MS method for quantitative determination of LXT-101 sustained-release suspension, a novel drug in treating prostate cancer, in beagle plasma. Sci Rep. 2025;15:41868.

20. Bernard R, Maputla SP, Zuma P, Joubert A, Castel S, van der Merwe M, et al. Development and validation of an LC–MS/MS method for quantifying total and unbound doravirine in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2025;1252:124439.
21. Zamlynny L, Morris HM, Giddings SD, Kollatz J, Niedermeyer THJ, Jamieson RC, et al. Development and validation of a multiclass LC–MS/MS method for the analysis of cyanotoxins. Anal Bioanal Chem. 2026;418:667–678.
22. Chernonosov A, Aksenova L, Koval V. The development of a liquid chromatography high-resolution mass spectrometric method for apixaban quantification in dried plasma spots in parallel reaction monitoring mode. Processes. 2021;9(3):450.
23. Anusha K, Sowjanya G. Development of an LC–MS/MS technique and its validation for the determination of infigratinib in human K2EDTA plasma; pharmacokinetics in healthy rabbits. J Appl Pharm Sci. 2024;14(9): 148-155
24. Atmakuri LR, Peeriga R, Begum S, Gaddamedi N, Vallamkonda B, Baratam A. Novel bioanalytical LC–MS/MS method for determination of metoprolol in human plasma. J Appl Pharm Sci. 2024;14(12):131–138.
25. Kallem RR, Chan R, Neal K, Yeager M, Fletcher K, Srinivas N. Development and validation of an LC–MS/MS method for the quantitative estimation of bexicaserin in human cerebrospinal fluid. Biomed Chromatogr. 2026;40(3):e70371.