Chinese Journal of Health Management
Volume 19 Issue 11, Year of Publication 2025, Page 1050-1058 | DoI-10.564220/1674-0815.2025.132

ISSN: 1674-0815

cjhmonline.com

DoI-10.564220/1674-0815

Chinese Journal of Health Management

Chinese Medical Association

Development and Validation of Rp-Hplc Method for Simultaneous Estimation of Bisoprolol Fumarate and Trimetazidine Hydrochloride in Synthetic Mixture

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Article Information
Received: 08-10-2025
Revised: 22-10-2025
Accepted: 04-11-2025
Published: 26-11-2025

Keywords
Bisoprolol fumarate, Trimetazidine hydrochloride, RP-HPLC, Synthetic mixture, Validation

ABSTRACT:
Objective: The present article describes simple, sensitive, accurate, precise and cost effective RP-HPLC method for the simultaneous estimation of Bisoprolol fumarate and Trimetazidine hydrochloride in Synthetic mixture.
Method and Results: The RP-HPLC using (Kromstar) Vertex C_{18} (250 × 4.6 mm, 5 µm) column in Isocratic mode, with Mobile Phase Acetonitrile: 10 mM Potassium dihydrogen Phosphate Buffer (pH 3.2) : Methanol (45:45:10 % v/v/v). The Flow Rate was 1.0 ml/min and effluents were monitored at 234 nm. The Retention Time of were found to be Bisoprolol fumarate and Trimetazidine hydrochloride 4.40min and 2.10 min, respectively. The Linearity for Bisoprolol fumarate and Trimetazidine hydrochloride were found to be 2-10 µg/ml and 7-35 µg/ml, respectively. The Recoveries of Bisoprolol fumarate and Trimetazidine hydrochloride were found to be 99.22% – 99.60% and 99.41% – 99.92% respectively. The suitability of these methods for the quantitative determination of Bisoprolol fumarate and Trimetazidine hydrochloride was proved by validation.
Conclusion: The proposed method has been validated as per ICH guideline and successfully applied to the simultaneous estimation of Bisoprolol fumarate and Trimetazidine hydrochloride in synthetic mixture.

INTRODUCTION:
Bisoprolol fumarate is chemically 1-[4-[[2-(1-methylethoxy) ethoxy] methyl]-phenoxy]-3-[(1-methylethyl) amino] -2-propanolethylene-1,2- dicarboxylic acid Bisoprolol fumarate is a α-blocker. Bisoprolol fumarate is a competitive, selective β1-adrenergic antagonist. Bisoprolol fumarate reduces cardiac workload by decreasing contractility and the need for oxygen through competitive inhibition of β1-adrenergic receptors. Bisoprolol fumarate is also thought to reduce the output of renin in the kidneys, which normally increases blood pressure. Bisoprolol fumarate may be used to treat high blood pressure and heart failure. Structure of Bisoprolol fumarate

showed in Figure 1.[1-3]

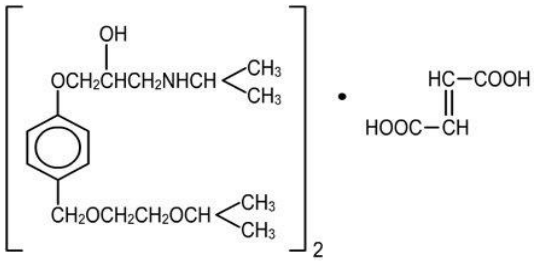

Figure 1: Structure of Bisoprolol Fumarate

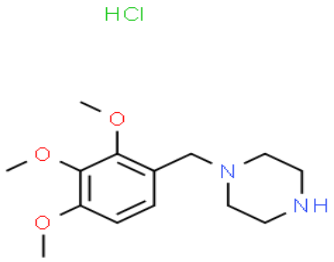

Figure 2: Structure of Trimetazidine HCl

Trimetazidine hydrochloride is chemically 1-[(2, 3, 4-trimethoxyphenyl) methyl] piperazine; hydrochloride. Trimetazidine hydrochloride is a Fatty Acid Oxidation inhibitor. Trimetazidine hydrochloride inhibits beta-oxidation of fatty acids by blocking long-chain 3-ketoacyl-CoA thiolase, which enhances glucose oxidation. Trimetazidine hydrochloride may be used to treat and prevent the symptoms of Angina (chest pain). Structure of Trimetazidine HCl showed in Figure 2.[1-3]

Combination of Bisoprolol fumarate and Trimetazidine hydrochloride was proved that the synergistic effect was observed by reducing the frequency of angina attacks and the use of short-acting nitrates in patients with stable angina.[4]

Bisoprolol fumarate and Trimetazidine hydrochloride are official available in IP 2022. From Literature Survey, various method (UV, HPLC, HPTLC, GC, LC-MS and LC/MS/MS)[5-14] were reported for the analysis of individual drug in combination with other drug but no method were reported for simultaneous estimation of Bisoprolol fumarate and Trimetazidine hydrochloride. Hence, the purpose of the present work was to develop and validate RP-HPLC method for simultaneous estimation of Bisoprolol fumarate and Trimetazidine hydrochloride in Synthetic mixture.

MATERIALS AND METHODS:

Chemicals and Reagents:

Intas Pharmaceutical provided the bulk drug Bisoprolol fumarate, while jigs Chemical Limited., provided Trimetazidine hydrochloride as a gift sample. Solvents were Solvents were procured Finar chemicals, Ahmedabad, while Astron Chemical Ltd. India provided AR grade potassium dihydrogen phosphate. Fresh solutions were prepared every day.

Equipment and Chromatographic Condition:

The present assay was carried out on a Systronics LC-138, Photodiode array detector, Manual injector and column (Kromstar) Vertex C_{18} (250 ×4.6 mm, 5µm) respectively.[15] The Clarify software was utilized to monitor and integrate the output data. Mobile phase (isocratic) like Acetonitrile: 10 mM Potassium dihydrogen Phosphate Buffer: Methanol (pH 3.2) (45:45:10 % v/v/v). were used. Analyte detected at a wavelength of 234 nm (10 min. run time).

The Retention Time of were found to be Bisoprolol fumarate and Trimetazidine hydrochloride 4.40 min and 2.10 min respectively. under the ideal chromatographic circumstances.

Method for Preparation of Analytical Solutions:

Stock and standard Solution:

Bisoprolol fumarate and Trimetazidine HCl should be accurately weighed (10 mg). Methanol should be added, and the mixture should be sonicated for 30 min to obtained 100 µg ml^{-1}. The Solution should be labelled as a Standard stock solution For further dilutions, pipetted out Bisoprolol fumarate (0.4 ml) and Trimetazidine hydrochloride (1.4 ml) was transferred to 10 ml volumetric flask and diluted up to the mark with Mobile phase and obtained concentration 4 µg/ml and 14 µg/ml by adding mobile phase, respectively.

Preparation of Sample Solution:

Accurately weighed equivalently weight of Bisoprolol fumarate (10 mg) and Trimetazidine hydrochloride (35 mg) which transferred in 100 ml volumetric flask and make up half mark with Water. This solution was sonicated till the drug dissolves and was made up to mark with Water. Then this solution was filtered through Whatmann filter paper. So, obtained concentration of Bisoprolol fumarate (100 □g/ml) and Trimetazidine hydrochloride (350 □g/ml). pipette out 0.4 ml and transferred into volumetric flask of 10 ml and make up the volume with optimized mobile phase, to get the concentration of 4 □g/ml and 14 □g/ml for Bisoprolol fumarate and Trimetazidine hydrochloride.

Preparation of Buffer (10 mM KH_2PO_4):

Accurately weighed quantity of 1.36g of Potassium Di-Hydrogen Phosphate (KH_2PO_4) was transferred in 1000 ml water, dissolved in HPLC grade water and sonicated for 10 min and diluted with HPLC grade water. It was filtered through 0.45□m membrane filter.[16]

Preparation of Mobile phase:

Mixtures of Acetonitrile, Buffer and Methanol in ratio of (45:45:10 % v/v/v) was mixed properly and adjust the pH 3.2 with 10% Orthophosphoric Acid.

Selection of Suitable Analytical Wavelength:

The blank solution was then tested for absorbance in the 200 – 400 nm range. Detection of the analytes occurred using a wavelength 234 nm. Both drugs yielded satisfactory results, as depicted in Figure 3.

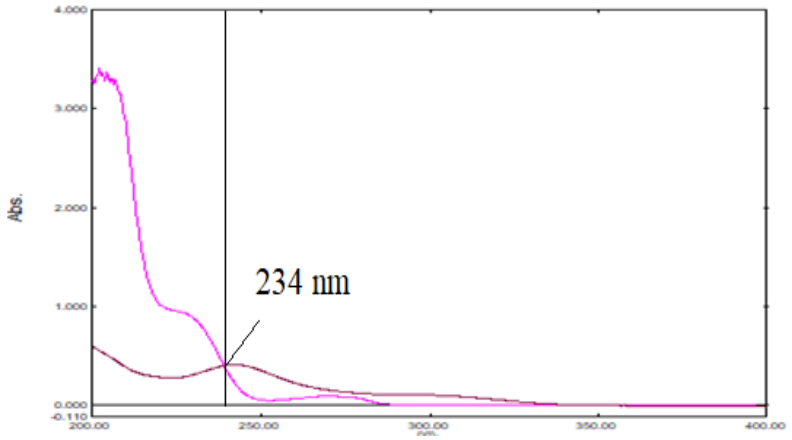

Figure 3: Overlain Zero order spectra of Bisoprolol Fumarate (4 µg/ml) and Trimetazidine HCl (14 µg/ml) in Methanol

RP-HPLC METHOD DEVELOPMENT AND VALIDATION:

The Purpose of this research was to develop new, trustworthy, practical, and affordable technique for the simultaneous estimation of both Drugs using RP-HPLC in Synthetic Mixture. The method that was established underwent validation for various factor, including system suitability, linearity, precision, detection and quantitation limit, accuracy, assay and robustness.

System Suitability:

Six replicates of freshly prepared standard solutions of Bisoprolol fumarate and Trimetazidine Hydrochloride were injected to conduct system suitability tests. Parameters like the theoretical plate, retention time, and tailing factor were assessed by the standard chromatogram and the detailed results are summarized in Table 1.

Table 1: System Suitability Parameter

Name of drugs	Area	Retention time	Tailing factor	No. of Theoretical Plates
Bisoprolol fumarate	407.52	4.40	1.60	23733.47
Trimetazidine HCl	980.53	2.10	1.85	65180.78

Specificity:

To check for degradation and interferences, sample solutions of Bisoprolol fumarate (4 µg/ml) and Trimetazidine HCl (14 µg/ml) were prepared and injected. The analysis of the drugs was verified by checking for interference from Bisoprolol fumarate and Trimetazidine HCl using a blank chromatogram and results as depicted in Figure 4 as well as single and Combined Chromatogram of both drugs depicted in Figure 5-7.[17]

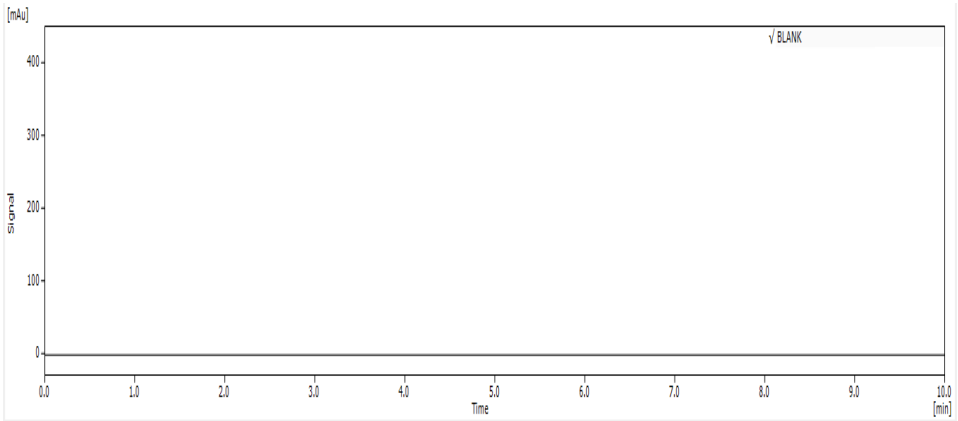

Figure 4: RP-HPLC Chromatogram of Blank in (ACN: Buffer: Methanol) (pH 3.2) (45:45:10 % v/v/v) Flow rate: 1 ml/min at 234 nm

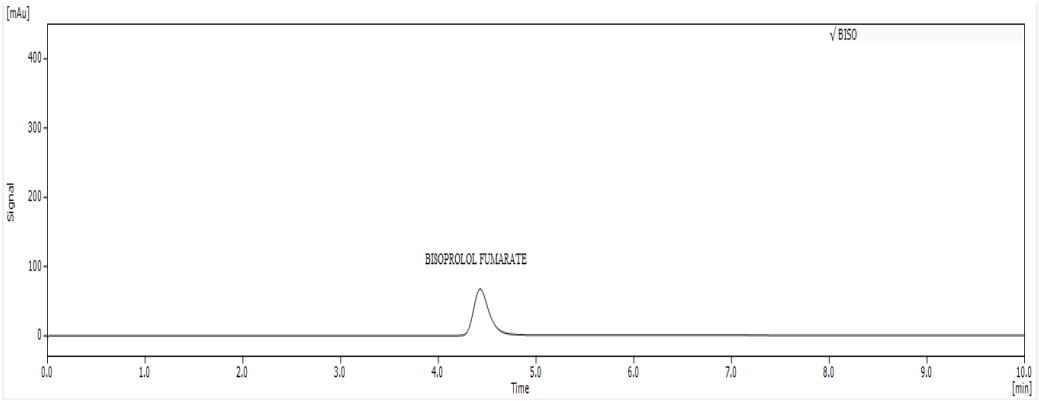

Figure 5: RP-HPLC Chromatogram of Bisoprolol fumarate (4 □g/ml) in (ACN: Buffer: Methanol) (pH 3.2) (45:45:10 % v/v/v) Flow rate: 1 ml/min at 234 nm

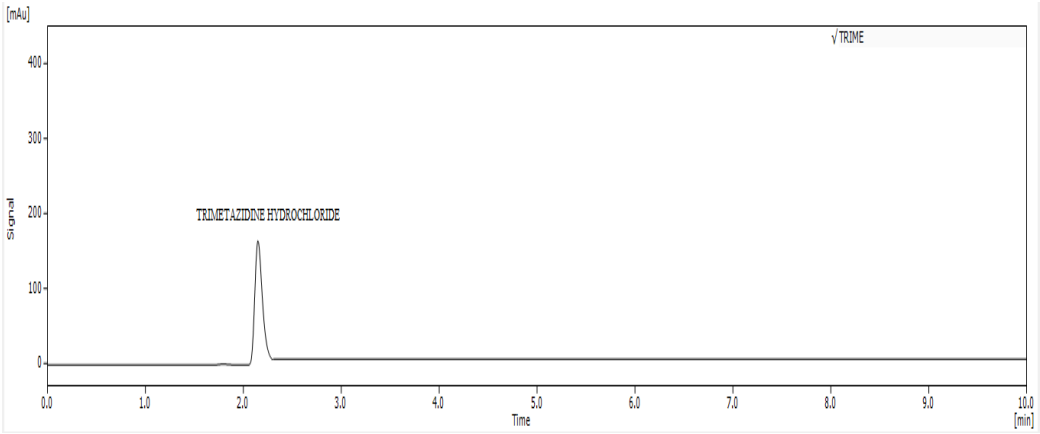

Figure 6: RP-HPLC Chromatogram of Trimetazidine hydrochloride (14 □g/ml) in (ACN: Buffer: Methanol) (pH 3.2) (45:45:10 % v/v/v) Flow rate: 1 ml/min at 234 nm

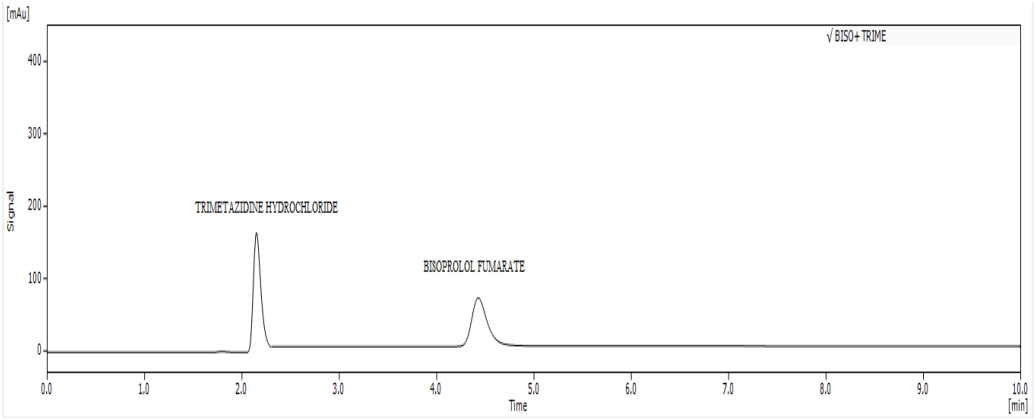

Figure 7: RP-HPLC Chromatogram of Bisoprolol fumarate (4 □g/ml) and Trimetazidine hydrochloride (14 □g/ml) in (ACN: Buffer: Methanol) (pH 3.2) (45:45:10 % v/v/v) Flow rate: 1 ml/min at 234 nm

Linearity:

Accurately measured aliquots of the stock solutions of Bisoprolol fumarate (100 µg/mL), i.e., 0.2, 0.4, 0.6, 0.8, and 1.0 mL, and Trimetazidine hydrochloride (100 µg/mL), i.e., 0.7, 1.4, 2.1, 2.8, and 3.5 mL, were transferred into five separate 10 mL volumetric flasks. The volumes were made up with the mobile phase consisting of ACN: buffer: methanol (pH 3.2) in the ratio 45:45:10 (% v/v/v) to obtain final concentrations of 2, 4, 6, 8, and 10 µg/mL for Bisoprolol fumarate and 7, 14, 21, 28, and 35 µg/mL for Trimetazidine hydrochloride and results as depicted in Figure 8.[18]

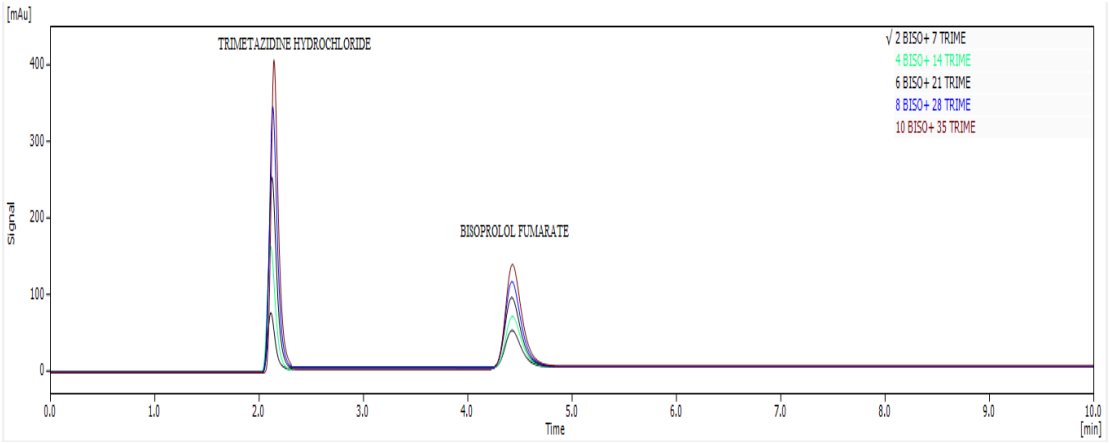

Figure 8: Overlay RP-HPLC Chromatogram of Bisoprolol Fumarate (2 -□10□g/ml) and Trimetazidine Hydrochloride (7 -□35 □g/ml) in ACN: Buffer: Methanol) (pH 3.2) (45:45:10 % v/v/v) Flow rate: 1ml/min at 234 nm

Precision:

Intraday, Interday, and Repeatability study has been performed. For Intraday, on same day, Bisoprolol fumarate solution (2, 4, and 6 µg ml^{-1}) and Trimetazidine HCl solution (7, 14, and 21 µg ml^{-1}) were analyzed in triplicate. For Interday, three different days, Bisoprolol fumarate solution (2, 4, and 6 µg ml^{-1}) and Trimetazidine HCl solution (7, 14, and 21 µg ml^{-1}) were analyzed. For Repeatability, Bisoprolol fumarate (4 µg ml^{-1}) and Trimetazidine HCl (14 µg ml^{-1}) were analyzed for six times. The results were expressed as % RSD.[19]

Accuracy:

The pre-analyzed solution was spiked with known amounts of Bisoprolol fumarate and Trimetazidine HCl at three concentration levels 50%, 100%, and 150%. Each level was injected in triplicate into the HPLC system, and the mean percentage recovery for both drugs was calculated.[20]

Detection Limit and Quantification Limit:

According to ICH guidelines, the Detection Limit and Quantification Limit are calculated using standardized equations.[21]

Robustness:
Robustness was assessed by ensuring that all variations met the established system suitability criteria. Parameters such as detection wavelength and flow rate were deliberately varied to evaluate the method's robustness.

Method durability was assessed three times by subjecting the sample to altered analytical conditions, specifically adjustments in flow rate and detection wavelength.

RESULTS :

An isocratic RP-HPLC method was successfully developed and validated for the simultaneous estimation of Bisoprolol fumarate and Trimetazidine HCl in a synthetic mixture. The method is simple, rapid, accurate, and precise. Both analytes exhibited an absorption maximum at 234 nm, which was selected as the detection wavelength for the RP-HPLC analysis. Method performance was further enhanced by optimizing chromatographic parameters such as flow rate and detection wavelength. Efficient separation of Bisoprolol fumarate and Trimetazidine HCl with well-resolved peaks was achieved using a mobile phase consisting of ACN: buffer: methanol (pH 3.2) in the ratio 45:45:10 (% v/v/v) at a flow rate of 1 mL/min. A (Kromstar) Vertex C_{18} column (250 × 4.6 mm, 5 µm) was employed as the stationary phase under ambient temperature conditions with an injection volume of 20 µL to ensure reproducibility and repeatability.[22-23]

Linearity:
The method demonstrated excellent linearity over the concentration ranges of 2 – 10 µg/mL for Bisoprolol fumarate and 7–35 µg/mL for Trimetazidine HCl. The correlation coefficients were 0.997 for Bisoprolol fumarate and 0.998 for Trimetazidine HCl. The corresponding calibration curves are showed in Figures 8 and 9, and the detailed results are summarized in Table 2.

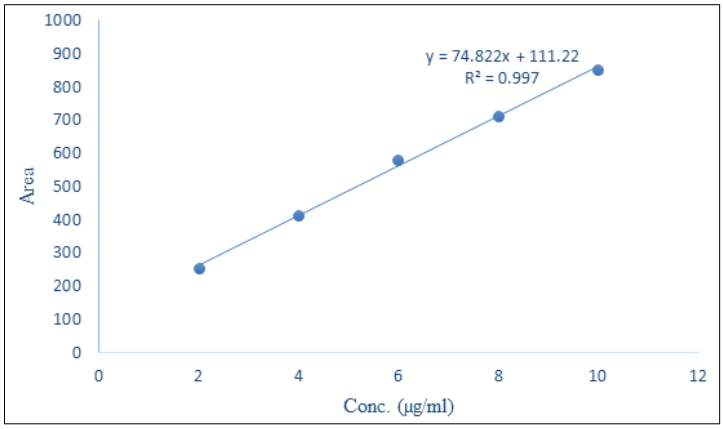

Figure 9: Calibration Curve of Bisoprolol fumarate (2−10 µg/ml) at 234 nm

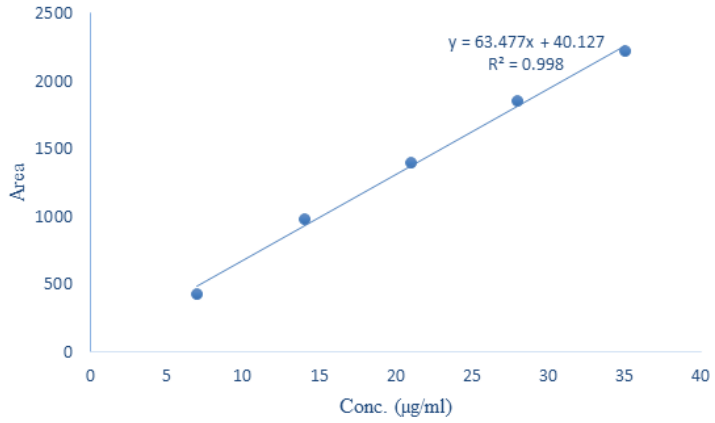

Figure 10: Calibration Curve of Trimetazidine hydrochloride (7−35 µg/ml) at 234 nm

Table 2: Linearity of Bisoprolol Fumarate and Trimetazidine HCl

Concentration (µg/ml)		Area ± SD (n=6)		% RSD	
Bisoprolol Fumarate	**Trimetazidine HCl**	**Bisoprolol Fumarate**	**Trimetazidine HCl**	**Bisoprolol Fumarate**	**Trimetazidine HCl**
2	7	250.9932± 2.80431	428.2798± 5.18644	1.11	1.21
4	14	407.5263± 3.73851	980.5335± 9.89859	0.91	1.00
6	21	626.7145± 4.38706	1441.3150± 11.66198	0.70	0.80
8	28	709.9467± 4.41920	1915.7467± 12.06973	0.62	0.63
10	35	848.7028± 4.57464	2214.8800± 12.61912	0.53	0.56

Precision:

Precision refers to the degree of agreement among repeated measurements of the same sample. To evaluate method interday, intraday, and repeatability studies were performed. The %RSD values for system precision are reported in Table 3 and 4 for Bisoprolol fumarate and Trimetazidine HCl, respectively. As all %RSD values were below 2%, the method was confirmed to be precise, reproducible, and repeatable.

Table 3: Precision Study of Bisoprolol Fumarate

Intraday Precision of Bisoprolol Fumarate		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
2	252.0127±2.29939	0.91
4	409.7527±2.88865	0.70
6	627.8537±3.54828	0.56
Interday Precision of Bisoprolol Fumarate		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
2	251.8647±2.54429	1.01
4	409.4877±3.35637	0.81
6	628.1873±4.00858	0.63
Repeatability of Bisoprolol Fumarate		
Conc. (µg/ml)	**Mean Area ± SD (n=6)**	**% RSD**
2	408.2468±3.55628	0.87

Table 4: Precision Study of Trimetazidine HCl

Intraday Precision of Trimetazidine HCl		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
7	427.8303±4.56690	1.06
14	979.7103±8.50072	0.86
21	1439.9800±9.44673	0.65
Interday Precision of Trimetazidine HCl		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
7	428.0613±4.88203	1.14
14	978.7107±9.07506	0.92
21	1439.7233±10.17780	0.70
Repeatability of Trimetazidine HCl		
Conc. (µg/ml)	**Mean Area ± SD (n=6)**	**% RSD**
14	979.6847±8.90587	0.90

Accuracy:

Recovery studies were performed at concentration levels of 50%, 100%, and 150%. Three samples at each level were injected, and the mean percentage recoveries were calculated. As showed in Table 5, the recoveries for Bisoprolol fumarate and Trimetazidine HCl ranged from 99.22% – 99.60% and 99.41% – 99.92%, respectively. Since all values fell within the acceptable range of 98.0% – 102%, the method was considered accurate. The favorable recovery results further indicate the method's suitability for routine quality control analysis.

Table 5: Recovery of Bisoprolol Fumarate and Trimetazidine HCl

Name of Drug	**%Level Of Recovery**	**Test Amount (µg/ml)**	**Amount of drug taken (µg/ml)**	**Spiked Std Amount (µg/ml)**	**Total amount Recovered (µg/ml)**	**% Recovery ±S.D (n=3)**
	50	2	4	6	5.95	99.22±0.2553

Bisoprolol Fumarate	100	4	4	8	7.95	99.45±0.3172
	150	6	4	10	9.96	99.60±0.3464
Trimetazidine HCl	50	7	14	21	20.87	99.41±0.3835
	100	14	14	28	27.89	99.61±0.4142
	150	21	14	35	34.97	99.92±0.6450

Detection Limit and Quantitation Limit:

The LOD serves as a qualitative parameter used to determine whether the analyte concentration is within the specified limit, whereas the LOQ defines the minimum analyte concentration that can be accurately quantified, making it particularly useful for detecting impurities or degradation products. Table 6 presents the LOD and LOQ values, which were 0.288 µg/mL and 0.950 µg/mL for Bisoprolol fumarate, and 0.459 µg/mL and 1.514 µg/mL for Trimetazidine HCl, respectively.

Table 6: LOD and LOQ for Bisoprolol Fumarate and Trimetazidine HCl

Parameter	Bisoprolol Fumarate	Trimetazidine HCl
LOD(µg/ml)	0.288	0.459
LOQ(µg/ml)	0.950	1.514

Robustness

Variations in flow rate and detection wavelength were introduced, and the results are summarized in Table 7. The findings showed that these minor changes did not significantly impact the analysis, confirming the robustness of the method.

Table 7: Robustness data for Bisoprolol Fumarate and Trimetazidine HCl

SR NO.	Parameter	Variation	Area ± S.D (n=3)		% RSD	
			Bisoprolol Fumarate	Trimetazidine HCl	Bisoprolol Fumarate	Trimetazidine HCl
1	Flow rate (1 ml/min) (±0.2 ml/min)	0.8 ml/min	408.578±3.446	974.009±7.444	0.84	0.76
2		**1.0 ml/min**	410.501±2.587	974.596±5.227	**0.63**	**0.53**
3		1.2 ml/min	411.754±2.978	979.352±6.065	0.72	0.61
1	Detection Wavelength (234 nm) (± 2 nm)	232 nm	410.594±2.863	970.276±7.751	0.69	0.79
2		**234 nm**	411.136±1.714	975.407±6.634	**0.41**	**0.68**
3		236 nm	412.899±2.276	979.886±8.618	0.55	0.87

Assay:

Three separate injections of the same sample solution were analyzed, and the resulting chromatograms were recorded. Bisoprolol fumarate and Trimetazidine HCl exhibited recoveries of 99.25% and 99.14%, respectively, as showed in Table 8.

Table 8: Analysis of Pharmaceutical Dosage form

Name of Drug	Amount taken (µg/ml)	amount Found(µg/ml)	%Assay± S.D (n=3)	% RSD
Bisoprolol Fumarate	4	3.97	99.25±0.6614	0.66
Trimetazidine HCl	14	13.88	99.14±0.5144	0.51

DISCUSSION:

The method was systematically optimized to enhance sensitivity and specificity. Validation, performed as per ICH guidelines, confirmed satisfactory linearity, precision, accuracy, and robustness. Future investigations should explore its suitability for different pharmaceutical formulations and dosage forms.

CONCLUSION:

The findings of the study confirm that the proposed RP-HPLC method is simple, rapid, accurate, and cost-effective for the simultaneous estimation of Bisoprolol fumarate and Trimetazidine hydrochloride in a synthetic mixture. Statistical evaluation demonstrated excellent repeatability, precision, and selectivity, meeting all ICH validation criteria. The method showed robust performance even under deliberate variations in chromatographic conditions, indicating strong reliability for routine laboratory use.

The high recovery values and low %RSD further validate that the method is suitable for quality control testing and can effectively detect small variations in drug concentration. Its sensitivity, reflected by acceptable LOD and LOQ values, also highlights its capability for impurity monitoring and stability-indicating applications.

ACKNOWLEDGEMENT:
The author thankful to Smt. N. M. Padalia pharmacy college, Ahmedabad for providing facilities to carry out the research.

CONFLICT OF INTEREST:
The authors declare that there is no conflict of interest.

ABBREVIATIONS:
ICH: International Council for Harmonization; **RP-HPLC:** Reverse phase High Performance liquid chromatography; **API:** Active Pharmaceutical Ingredient; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **RSD:** Relative Standard deviation; **KH_2PO_4:** Potassium Dihydrogen ortho phosphate buffer; **BISO:** Bisoprolol Fumarate; **TRIE:** Trimetazidine HCl.

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