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# Single-Run RP-HPLC Method for Comprehensive Separation of Nine Bisoprolol Fumarate Impurities Aligned with USP and EP

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# Single-Run RP-HPLC Method for Comprehensive Separation of Nine Bisoprolol Fumarate Impurities Aligned with USP and EP

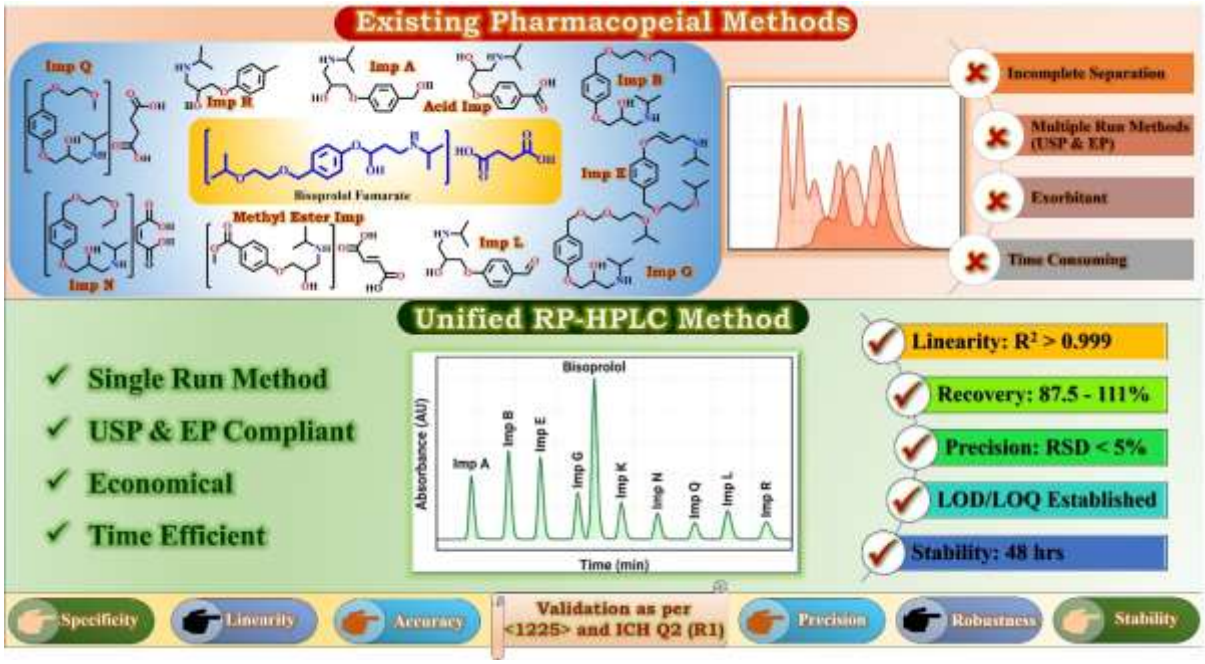
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## Graphical Abstract




## ABSTRACT

A novel, efficient reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the complete separation of nine impurities related to Bisoprolol fumarate active pharmaceutical ingredient. Since existing pharmacopeial methods have various analytical limitations, addressing only a subset of selected impurities, often require multiple approaches in order to meet regulatory expectations. Method validation was conducted in accordance with USP general chapter <1225> and ICH Q2 (R1) guidelines, ensuring satisfactory specificity, linearity, precision, accuracy, robustness and stability across a concentration range extending from the limit of quantification to 120% of the specified impurity level. Optimum chromatographic conditions including the use of appropriate stationary phase selection employing a monolithic silica rod with octyl silane chemically

bonded to superficially porous silica particles (3 µm), buffer composition, temperature, flow rate and gradient elution enabled successful baseline resolution of all targeted impurities within a single analytical run. A very successful and efficient impurity separations were carried out by optimising chromatographic conditions by making use of differences in polarities of impurities chosen. Besides that, the procedures showed some robustness, assuring reliable results, despite deliberate minor alterations in experimental parameters. The developed RP-HPLC method's ease of operation, its cost effectiveness, compliance with regulations and applicability to a wide variety of pharmaceutical products makes it an attractive technique in pharmaceutical analysis to meet the standards of regulatory acceptance that help in producing quality products.

***Keywords***: Bisoprolol fumarate, Method validation, HPLC, Related substances.

## 1. Introduction

Bisoprolol fumarate being a $\beta_1$-selective adrenergic receptor antagonist, is widely used API in the treatment of cardiovascular disorders, including hypertension, ischemic heart disease, and chronic heart failure. Owing to the higher cardioselectivity with good safety profile makes it a preferred therapeutic agent across global healthcare systems [1]. As an API, Bisoprolol fumarate must consistently comply with strict pharmacopeial quality and purity specifications to ensure safety, efficacy, and batch-to-batch reproducibility.

The Bisoprolol fumarate is chemically designated as (±)-1-[[α-(2-isopropoxyethoxy)-p-tolyl]oxy]-3-isopropylamino-2-propanol fumarate (2:1), corresponding to the molecular composition $(C_{18}H_{31}NO_4)_2 \cdot C_4H_4O_4$ and CAS number 104344-23-2. It is typically obtained as a crystalline solid with adequate solubility in aqueous as well as organic media, characteristics that facilitate both formulation development and analytical evaluation [2,3]. (Fig 1)

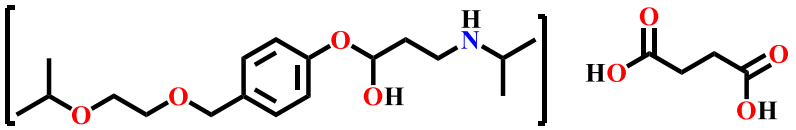



**FIGURE 1** Bisoprolol Fumarate.

The active pharmaceutical ingredient is manufactured by numerous suppliers worldwide, making effective impurity control a critical component of regulatory compliance and patient safety assurance [4]. Impurities associated with Bisoprolol fumarate may primarily arise from synthetic processes, raw materials, by-products and degradation products during storage, leading to the compromised drug efficacy along with toxicological risks due to rigorous regulatory oversight. Consequently, impurity profiling plays a pivotal role ensuring the desired purity of drug substances and hence in treatment and safety of patients with hypertension.

Pharmacopeial monographs provide regulatory guidance for impurity assessment; however, these approaches differ across regions. The United States Pharmacopeia (USP) recommends an RP-HPLC method based on isocratic elution using L7 stationary phase packing, with impurity quantification performed by area normalization [5]. In contrast, the European Pharmacopoeia (EP) describes a gradient RP-HPLC procedure employing octadecylsilyl silica gel columns, targeting a limited subset of related substances, namely Impurities A, E, and G [6]. Although these pharmacopeial approaches individually acceptable, the methods exhibit notable analytical constraints, particularly neither monograph provides complete chromatographic resolution of the full impurity spectrum associated with Bisoprolol fumarate. This urges to perform multiple analytical procedures to meet regulatory requirements when targeting both US and European markets, which impose huge analytical workload, solvent consumption, method transfer challenges, and the potential for variability in impurity reporting [7]. Lack of single analytical method is indeed a significant limitation, from a quality control and regulatory perspective.

Literature review reveals significant analytical effort towards estimation of Bisoprolol fumarate as a single API or in combination products using HPLC, HPTLC, and spectroscopic techniques [6-14]. For instance, RP-HPLC methods optimized for finished dosage forms, whereas alternative analytical techniques like TLC, fluorescence-based assays, or LC-MS are used for bioanalytical applications [14-19]. However, no single RP-HPLC method has been reported enabling simultaneous separation and quantification of all nine Bisoprolol fumarate impurities in accordance with combined USP and EP requirements. This underlines the lack and need of a unified, regulatory-oriented RP-HPLC method for separation and quantification of pharmacopeially relevant Bisoprolol fumarate impurities within a single chromatographic run.

Hence, it is pertinent to develop and validate a novel, regulatory-compliant RP-HPLC method capable of resolving and quantifying nine Bisoprolol fumarate related substances within a single chromatographic run, addressing the analytical gap in quality control processes effectively and support global regulatory submissions. The method is designed to be robust, sensitive, and selective, while simultaneously reducing analysis time and solvent consumption [20-27]. Notably, it bestows a harmonized analytical solution suitable for impurity profiling of Bisoprolol fumarate API intended for both US and European markets.

In the present study, for comprehensive impurity profiling, nine Bisoprolol fumarate related impurities were procured from commercial suppliers, and analysed by NMR spectroscopy to ensure analytical reliability prior to chromatographic evaluation. The molecular structures of

these impurities are illustrated in Fig. 2, while their systematic classification, based on origin and structural characteristics, is summarized in Table 1. $^{1}$H NMR spectra of representative samples are provided as supplementary information to support structural confirmation and reinforce the authenticity of the impurity standards employed in method development.

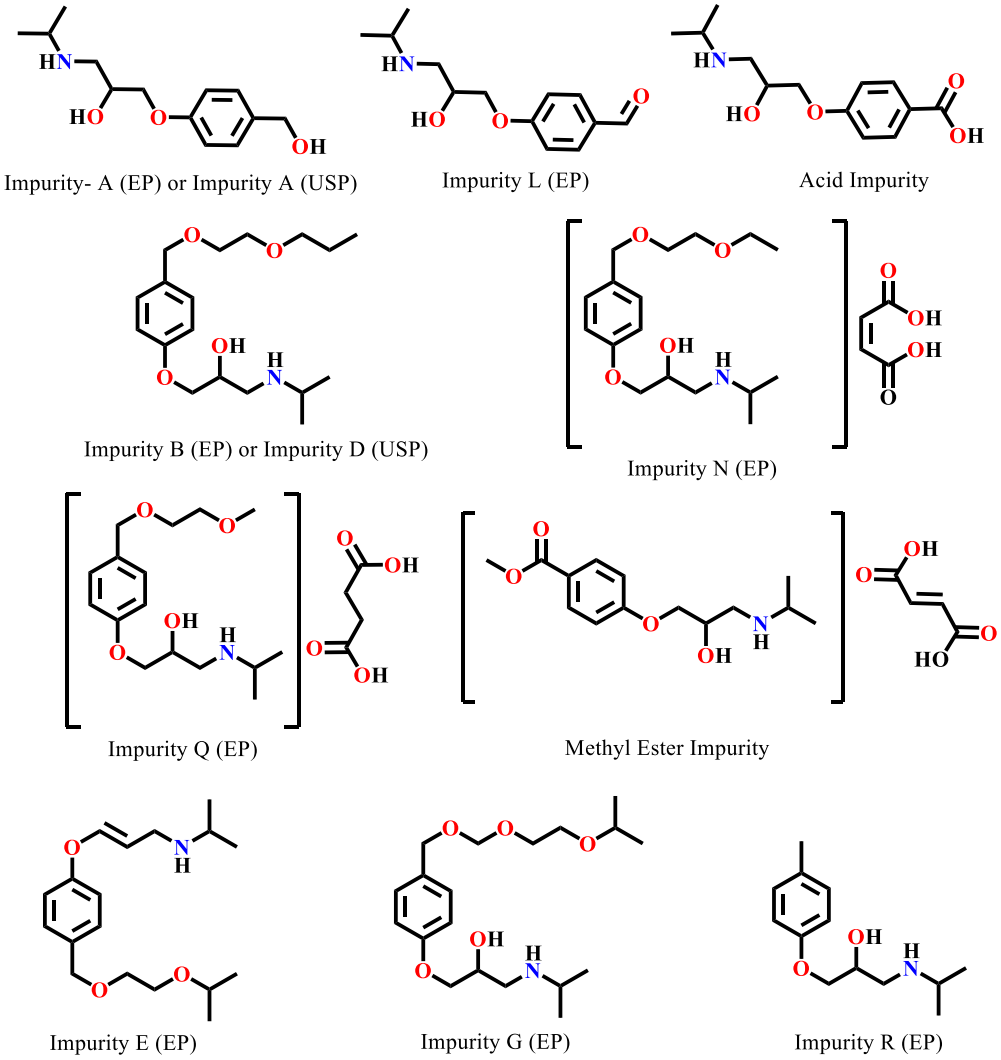



**FIGURE 2** Chemical structures and pharmacopoeial names of Bisoprolol impurities.

**TABLE 1** Classification of Bisoprolol impurities [5]

| Impurity | Impurity's Nature | Process related or degradant |
|---|---|---|
| Impurity A | Specified EP & USP Related compound A | degradant |
| Impurity B | Unspecified EP & USP Related compound D | Process related |
| Impurity E | Specified EP | degradant |
| Impurity G | Specified EP | degradant |
| Impurity K | Unspecified EP | Process related |
| Impurity L | Unspecified EP | degradant |
| Impurity N | Unspecified EP | Process related |
| Impurity Q | Unspecified EP | Process related |

| | | |
|---|---|---|
| Impurity R | Unspecified EP | Process related |
| Methyl Ester Impurity | Unspecified EP | Process related |
| Acid impurity | Unspecified EP | Process related |

## 2. Experimental

### 2.1 Methods and Materials

The analytical-grade chemicals, reagents and solvents used in the current study were procured from various commercial chemical suppliers. For instance, essential components of buffer system, namely Heptafluorobutyric acid, Diethyl amine (DEA) regulating the pH of buffer solution and Formic acid as an additive to the buffer for enhanced chromatographic separation were procured from Rurczynth, Labshemis and Rankem, respectively. HPLC solvents for mobile phase, including methanol and acetonitrile, were purchased from Merck and Finer Ltd. Reference standard and impurity reference standards of Bisoprolol fumarate (BF) was procured from CleanChem Laboratories, respectively. The suitable detector wavelength (λmax) for HPLC analysis was selected by analysing Bisoprolol impurities in 200-400 nm at a 1 cm pathlength on Agilent Cary 60 UV-vis spectrophotometer. The USP and EP monographs, recommends analysis of unknown impurities at 273 nm and 225 nm, respectively. Aligned to UV-vis analysis along with USP monograph recommendation, we opt detector wavelength (λmax: 273 nm) for efficient HPLC analysis of all the impurities under investigation. NMR ($^{1}H$ and $^{13}C$) data acquisition of compounds under investigation was done on Jeol 400 MHz (model: ECZ400S) in $CDCl_3$ or DMSO-$d_6$ solvent unless otherwise noted. The δ (chemical shift) and *J* (coupling constants) values are expressed *ppm* (as parts per million) and *Hz* (Hertz), respectively. Impurity compound's structure was confirmed with their NMR data from previous literature reports.

### *2.2* Instrumentation and Chromatographic Conditions

An integrated Shimadzu Prominence-1 and LC-2010 CHT (Shimadzu Incorporation) HPLC system equipped with an LC-2010 CHT quaternary pump, an auto-sampler, a column heater, and a SPD-M30A photodiode array (PDA) detector was used to perform chromatographic analysis. Data acquisition and analysis were performed using Chromelene software 6.8 (Dionex Corporation). pH meter (Pico Plus) procured from Lab India was used to measure pH of buffer solution prepared. Reference standards, impurities standards and Samples were precisely weighed on the Mettler Toledo Analytical balance (XS205 DR). An Avantor ACE C8 analytical column with dimensions 150 mm x 4.6 mm (I.D) and 3 µm practical size was used to achieve HPLC separation. Chromatographic separation was achieved using a linear gradient elution.

The mobile phase comprised of a buffer-acetonitrile-methanol mixture in the volumetric ratio of 75:15:10, respectively. The buffer solution (pH: 3.1) was prepared by dissolving 1-heptafluorobutyric acid (5 mL) as an ion-pairing reagent, diethyl amine (5 mL), and formic acid (2.5 mL) in 800 mL of milliQ-water in a volumetric flask, followed by dilution to 1 L. Acetonitrile was employed as mobile phase B. The HPLC separation was performed at a constant flow rate of 0.6 mL/min throughout run with the column temperature maintained at 30 °C. The mobile phase gradient was programmed as follows: 100 % A from 0-40 min, reduced to 95% A between 40-50 min, held isocratically at 95% A from 50-65 min, then decreased to 45% A from 65-110 min, followed by a return to 100% A from 110-115 min and maintained until 120 min for column re-equilibration. A 20 µL sample was injected for analysis and subsequent detection was carried out at a wavelength of 273 nm.

### 2.3 Standard and Sample Preparations

Reference stock solution of Bisoprolol fumarate was prepared by dissolving an appropriate amount in solvent and diluted to obtain 5 µg/mL concentration. An impurity standard stock solution of 100 µg/mL was prepared by dissolving appropriate amount of Impurity-G in the solvent and further dilution to volume. System suitability solution was prepared by adding appropriate amount of impurity standard stock solution to sample solution to attain 0.5 % impurity G concentration relative to the sample concentration. A working sample solution was prepared by dissolving an adequate amount of Bisoprolol fumarate API in the solvent and subsequent dilution to reach 5000 µg/mL concentration. All the standard and sample solutions were allowed to attain room temperature and then injected to the chromatographic system for analysis.

## 3. Results and Discussion

### 3.1 Method Development and Optimization

Several impurities are reported with Bisoprolol, three of them are specified as per EP monograph (impurities A, E and G) and rest are unspecified (impurities B, K, L, N, Q, R and S) as well as process related impurities (acid impurity and methyl ester impurity). Literature reports clearly highlight the importance of separation, identification and quantification of these impurities in Bisoprolol API.[26] Owing to the unavailability of a single HPLC method enabling separation of impurities, it is pertinent to develop a single, efficient, accurate and reproducible HPLC method capable to separate nine Bisoprolol impurities, useful for quality control analysis.

All the molecules under investigation were analysed by UV-vis spectrophotometer for the λmax values and subsequent characterization was done with NMR and results were consistent with the literature report.[27] (Supporting Information) Bisoprolol and its allied impurities showed significant absorbance at λmax values 223 nm as well as 274 nm. A common wavelength of 273 nm was opted for detection, as it offers the low detector noise and absence of any or minimal interference from the blank at the retention time of analytes under study.

The existing gradient method does not give the separation of nine impurities under investigation in a single method; hence it is modified by the use of acetonitrile as 100 % organic phase, along with an aqueous buffer and methanol. Difference in polarities is crucial factor for the elution of the impurities, all the impurities under investigation showed optimal solubility in the solvent system comprises 85:15 v/v water: acetonitrile solvents. Notably, use of acetonitrile gives the sharp and steady peaks of the impurities compared to methanol, hence preferred as diluent. Criteria for column selection was ability to separate nine selected impurities. Initial attempts with EP monograph gradient method could separate only few impurities were not adequate, hence method development was started utilising an L7 packing C8 column, and USP monograph isocratic method was studied.

For efficient separation of impurities, the premix buffer of USP was modified by addition of acetonitrile and methanol in 75:5:10 v/v/v respectively. Nevertheless, the Impurity B, Impurity G and Bisoprolol were close eluting, hence to improve the separation, acetonitrile was added to the premix buffer and tried gradient flow. This combination efficiently separated impurity B and impurity G, whereas impurity G is closely eluting with Bisoprolol. This was further resolved with reduced flow rate to 0.6 ml from 1ml/min which gave better separation in the all impurities however the run time was increase to 120 min. The optimum chromatographic conditions were achieved for the desired separation and noninterference of analyte with the diluent put forward this method for the validation.

**3.2 Method Validation**

Following the ICH Q2 (R1) guidelines, the optimized chromatographic method was thoroughly assessed for several parameters *viz*. specificity, linearity range, accuracy, precision, robustness, detection and quantification limits (LOD and LOQ), and overall system suitability [28].

**3.3 System Suitability**

To ensure efficiency of the chromatographic procedure, system suitability was gauged from chromatogram of reference solution. Less than 5 % relative standard deviation (% RSD) clearly reveals the acceptable repeatability. Moreover, separation of impurity G and Bisoprolol was confirm by peak-to-valley ratio, calculated using following equation.

$$\textbf{Peak to Valley Ratio} = \frac{Hp}{Hv} \geq 2.5 \qquad \text{Equation } \ldots\ldots\ldots(1)$$

Where, Hp (the height of the impurity G peak above the baseline), Hv (the height above the baseline of the lowest point between the impurity G peak and the Bisoprolol peak) retrieved from respective chromatogram. The obtained values are concomitant with the acceptance criteria, demonstrating adequate resolution between the desired peaks. Additionally, the percentage of impurity present in the sample was calculated as follows:

$$\%\ \textbf{Impurity}\ (\mathbf{w/w}) = \frac{Aspl}{Astd}\ X\ \frac{Cstd}{Cspl}\ X\ \frac{P}{100}\ X \frac{1}{RRF}\ X\ 100 \qquad \text{Equation } \ldots\ldots\ldots(2)$$

Where, $A_{spl}$, $A_{std}$, $C_{std}$, $C_{spl}$, P and RRF represents the peak area of the impurity in the sample, the peak area of the impurity standard, the concentration of the impurity standard (ppm), the concentration of the test sample (ppm), the % purity of the Bisoprolol standard and relative response factor, respectively. The results underline the accurate quantification of impurities in the tested samples.

The system suitability results lie within the acceptance criteria, that confirmed that the chromatographic method developed is appropriate for analysis. The results are illustrated graphically in Fig.3

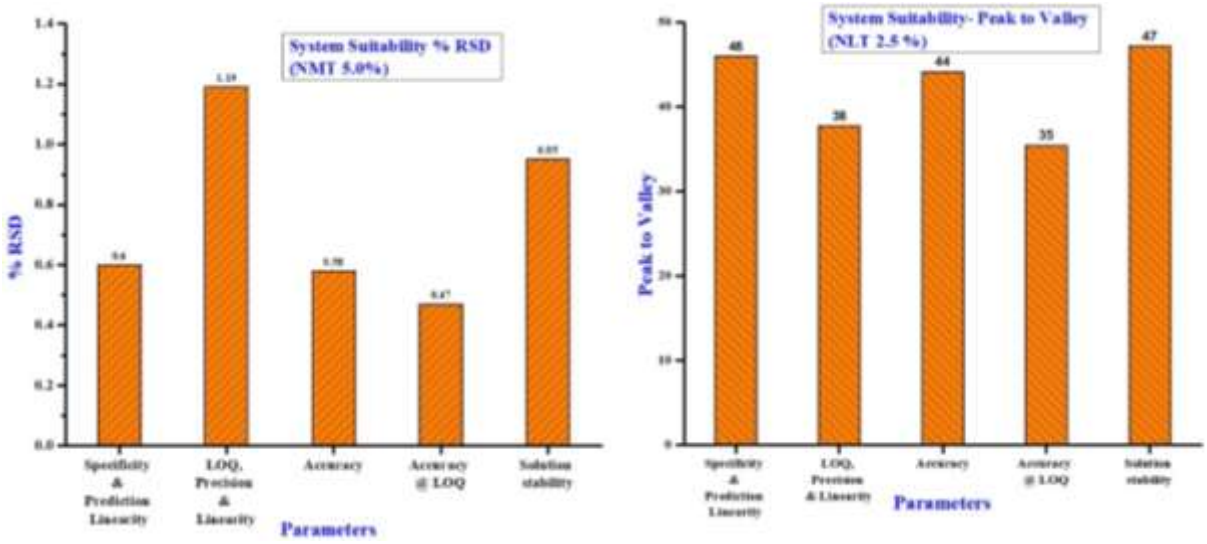


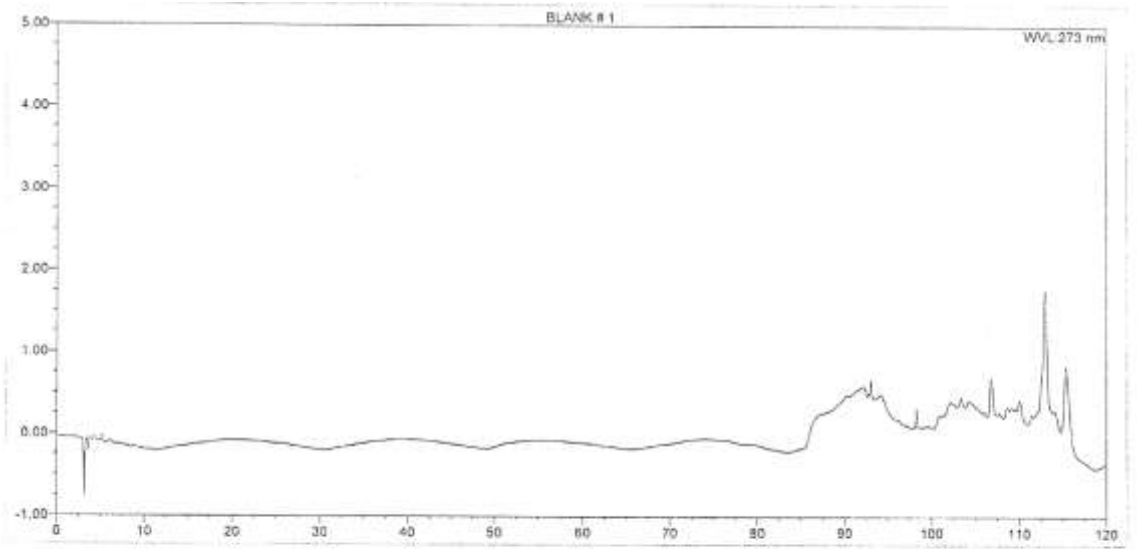

**FIGURE 3** System suitability results throughout validation study

### 3.4 Specificity

The specificity of the proposed analytical method was assessed evaluated to ensure its ability to specifically identify Bisoprolol fumarate and its related components in the presence of potential interferences. This is aim, a series of solutions were prepared and analyzed, including the diluent as a blank, reference solution, system suitability solution, individual impurity

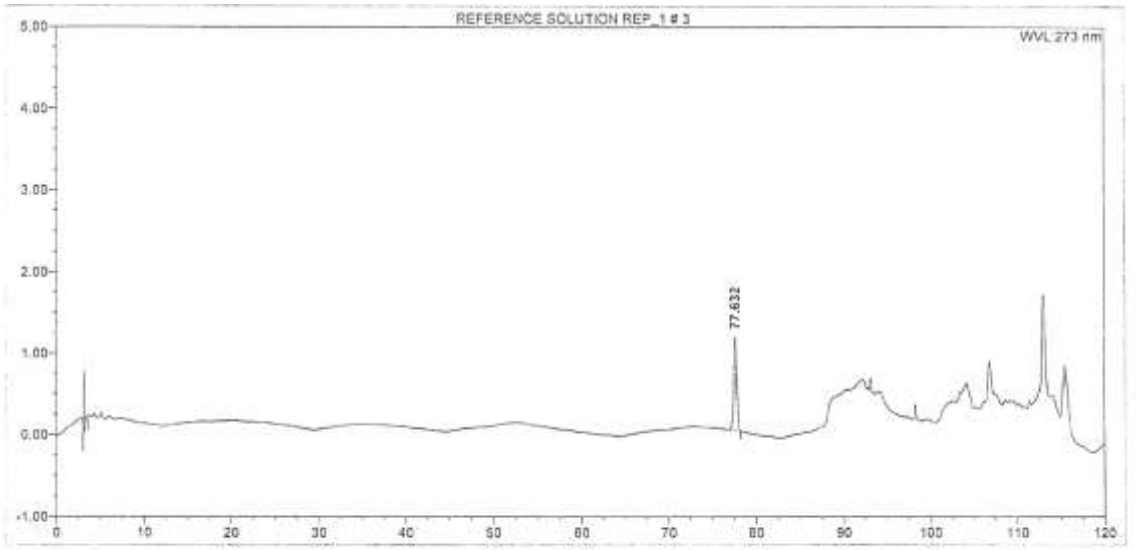
identification solutions, fumaric acid solution, and a combined specificity solution prepared in the diluent.

By injecting each solution separately, resultant chromatograms were thoroughly examined for any co-eluting peaks or interfering signals at the retention times of the analyte and its related impurities. Appearance of well resolved target peaks without interference from the blank or excipient components demonstrates the remarkable specificity of the method. The chromatograms of blank, reference, system suitability and spiked sample solution are presented in Fig.4



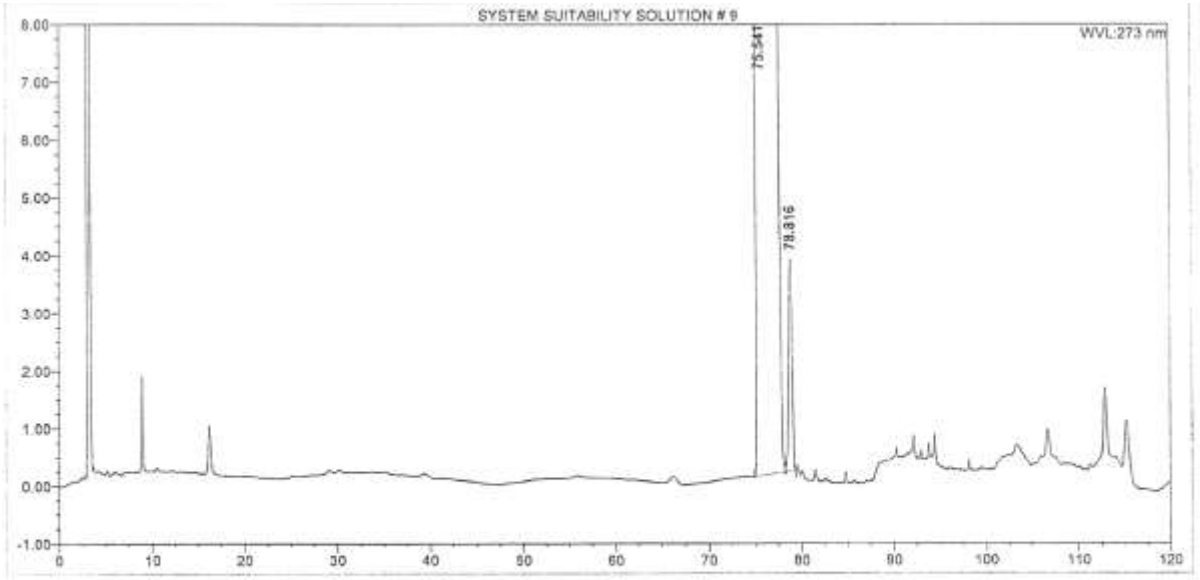

| Peak No. | Ret.Time (min) | Peak Name | Type | Area mAU*sec | Tailing Factor | Plates USP | Resolution USP | Match | Purity Limit | Results |
|---|---|---|---|---|---|---|---|---|---|---|
| Total | | | | 0.0000 | | | | | | |

**FIGURE 4** (A) Chromatogram of blank solution



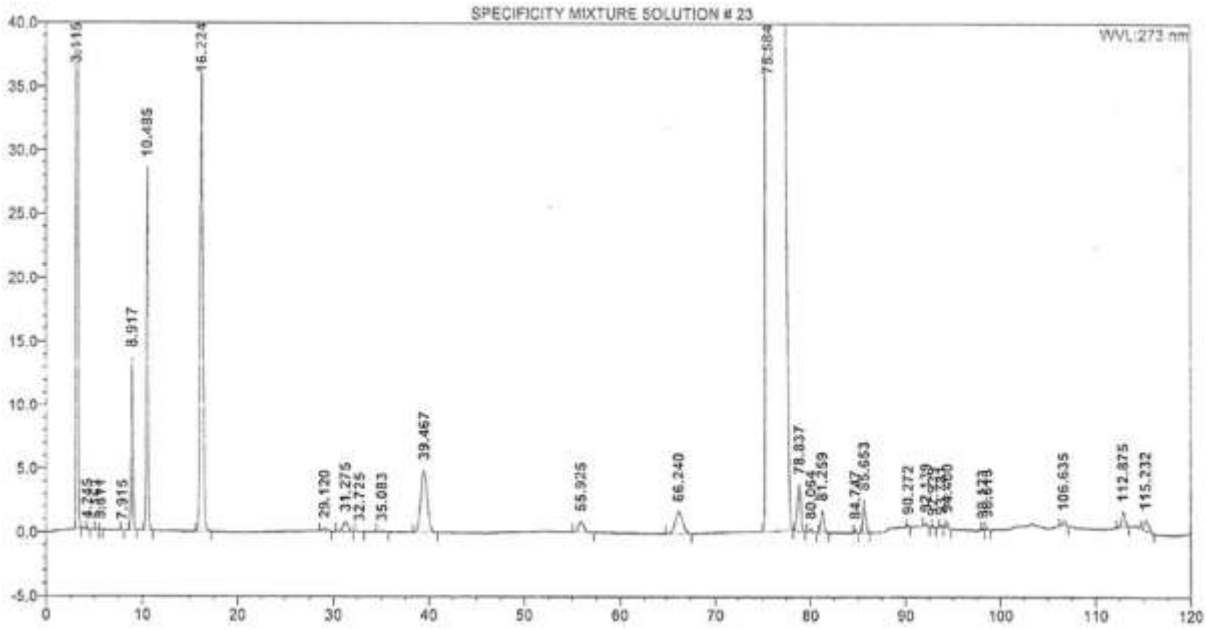

| Peak No. | Ret.Time (min) | Peak Name | Type | Area mAU*sec | Tailing Factor | Plates USP | Resolution USP | Match | Purity Limit | Results |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 77.632 | BISOPROLOL | BMB | 27.2538 | 1.02 | 246106 | n.a. | 998 | 900 | Peak Pure |
| Total | | | | 27.2538 | | | | | | |

**FIGURE 4** (B) Chromatogram of reference (standard) solution




| Peak No. | Ret.Time (min) | Peak Name | Type | Area mAU*sec | Tailing Factor | Plates USP | Resolution USP | Match | Purity Limit | Results |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 75.541 | BISOPROLOL | BM | 27234.9775 | 3.32 | 15448 | n.a. | 949 | 900 | Peak Pure |
| 2 | 78.816 | IMPURITY G | MB | 98.3616 | 0.91 | 195328 | 2.08 | 942 | 900 | Peak Pure |
| Total | | | | 27333.3391 | | | | | | |

**FIGURE 4** (C) Chromatogram of system suitability solution




| Peak No. | Ret. Time (min) | Peak Name | Type | Area mAU*sec | Tailing Factor | Plates USP | Resolution USP | Match | Purity Limit | Results |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3.115 | Fumaric Acid | BMB | 4509.3080 | 1.05 | 3492 | n.a. | 1000 | 900 | Peak Pure |
| 2 | 4.245 | Blank | BMB | 0.5808 | 2.10 | n.a. | n.a. | 694 | 900 | Peak Impure |
| 3 | 5.163 | Blank | BMB | 0.9462 | 1.56 | n.a. | n.a. | 999 | 900 | Peak Pure |
| 4 | 5.611 | Unknown | BMB | 0.6126 | 1.40 | 10328 | n.a. | 999 | 900 | Peak Pure |
| 5 | 7.915 | Unknown | BMB | 0.3967 | 1.06 | n.a. | n.a. | 638 | 900 | Peak Impure |
| 6 | 8.917 | Impurity A | BMB | 163.5375 | 1.18 | 13001 | n.a. | 1000 | 900 | Peak Pure |
| 7 | 10.485 | Acid Impurity | BMB | 386.4006 | 1.16 | 14238 | 4.72 | 1000 | 900 | Peak Pure |
| 8 | 16.224 | Impurity L | BMB | 716.0451 | 1.14 | 16118 | 13.30 | 1000 | 900 | Peak Pure |
| 9 | 29.120 | Unknown | BMB | 7.1034 | 1.15 | 19236 | 19.09 | 984 | 900 | Peak Pure |
| 10 | 31.275 | Impurity Q | BMB | 28.1318 | 1.01 | 16730 | 2.38 | 997 | 900 | Peak Pure |
| 11 | 32.725 | Unknown | BMB | 1.2298 | 0.88 | 39516 | 1.78 | 837 | 900 | Peak Impure |
| 12 | 35.083 | Unknown | BMB | 2.4970 | 1.02 | 45231 | 3.58 | 789 | 900 | Peak Impure |
| 13 | 39.467 | Methyl Ester Impurity | BMB | 231.2909 | 1.05 | 16061 | 4.60 | 1000 | 900 | Peak Pure |
| 14 | 55.925 | Impurity N | BMB | 33.9263 | 1.11 | 49414 | 14.62 | 904 | 900 | Peak Pure |
| 15 | 66.240 | Impurity R | BMB | 93.7707 | 1.04 | 34188 | 8.46 | 999 | 900 | Peak Pure |
| 16 | 75.584 | Bisoprolol | BMB | 26878.8574 | 3.30 | 16173 | 4.90 | 1000 | 900 | Peak Pure |
| 17 | 78.837 | Impurity G | BMB | 98.3335 | 0.94 | 197088 | 2.11 | 1000 | 900 | Peak Pure |
| 18 | 80.064 | Unknown | BMB | 5.0286 | 1.00 | 421989 | 2.04 | 993 | 900 | Peak Pure |
| 19 | 81.259 | Impurity B | BMB | 35.3604 | 1.15 | 376814 | 2.34 | 999 | 900 | Peak Pure |
| 20 | 84.747 | Unknown | BMB | 3.2152 | 1.14 | 1804746 | 8.92 | 976 | 900 | Peak Pure |
| 21 | 85.653 | Impurity E | BMB | 49.8090 | 1.37 | 515907 | 2.49 | 998 | 900 | Peak Pure |

**FIGURE 4** (D) Spiked sample chromatogram and its details

### 3.5 LOD and LOQ

The limits of detection (LOD) and quantitation (LOQ) of the individual known impurities were established by injecting diluted solutions of impurities with progressively higher concentrations in duplicates. In accordance with ICH guidelines, the limits of detection (LOD) and quantification (LOQ) were determined using the standard deviation of the response and the slope of the calibration curve, applying the predictive linearity approach. The results are illustrated graphically in Fig.5, with the corresponding numerical values provided in Table 2.

$$\text{LOD} = \frac{STEYX}{Slope} X\ 3.3 \qquad Equation \ldots\ldots\ldots\ldots (3)$$

$$\text{LOQ} = \frac{STEYX}{Slope} X\ 10 \qquad Equation \ldots\ldots\ldots\ldots (4)$$

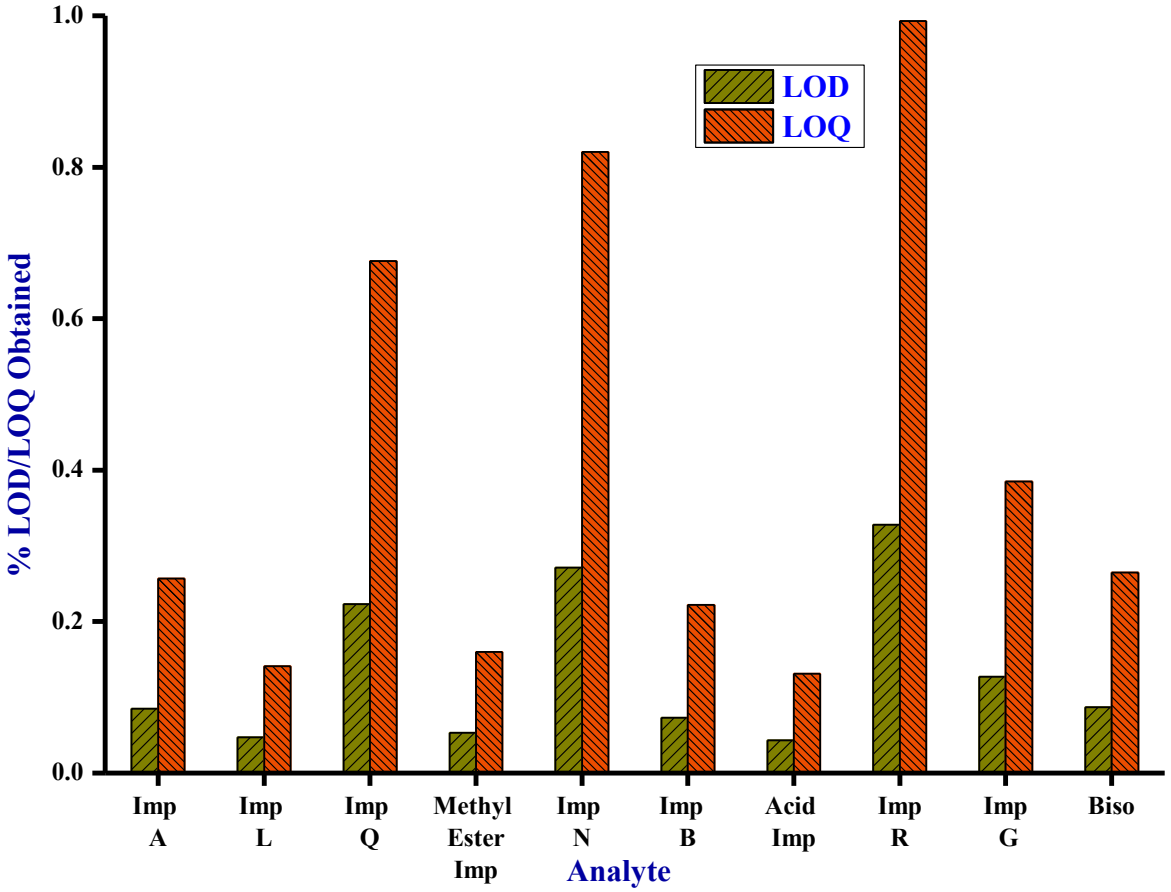



**FIGURE 5** LOD and LOQ

### *3.5 Linearity and range*

The linearity of the detector response was gauged by injecting five different concentration levels of Bisoprolol fumarate and its related impurities, prepared ranging from the limit of quantification (LOQ) up to 120% of the nominal working concentration (LOQ, 50%, 80% 100%, and 120%). For each concentration level, chromatographic responses were recorded and linearity graphs were plotted between the detector response (absorbance) against concentration (ppm). Analysing calibration curves, regression parameters like slope, intercept, and correlation coefficient were calculated. The linearity profiles are illustrated in Fig.6, while the corresponding numerical results are summarized in Table 2. The 0.9999-0.9997 correlation coefficient value clearly reveals that the developed method demonstrated a consistent linear response throughout the tested concentration range. Based on the outcomes of the linearity

assessment, together with accuracy and precision analysis, the validated analytical range of the method was established from the LOQ to 120% of the target concentration.

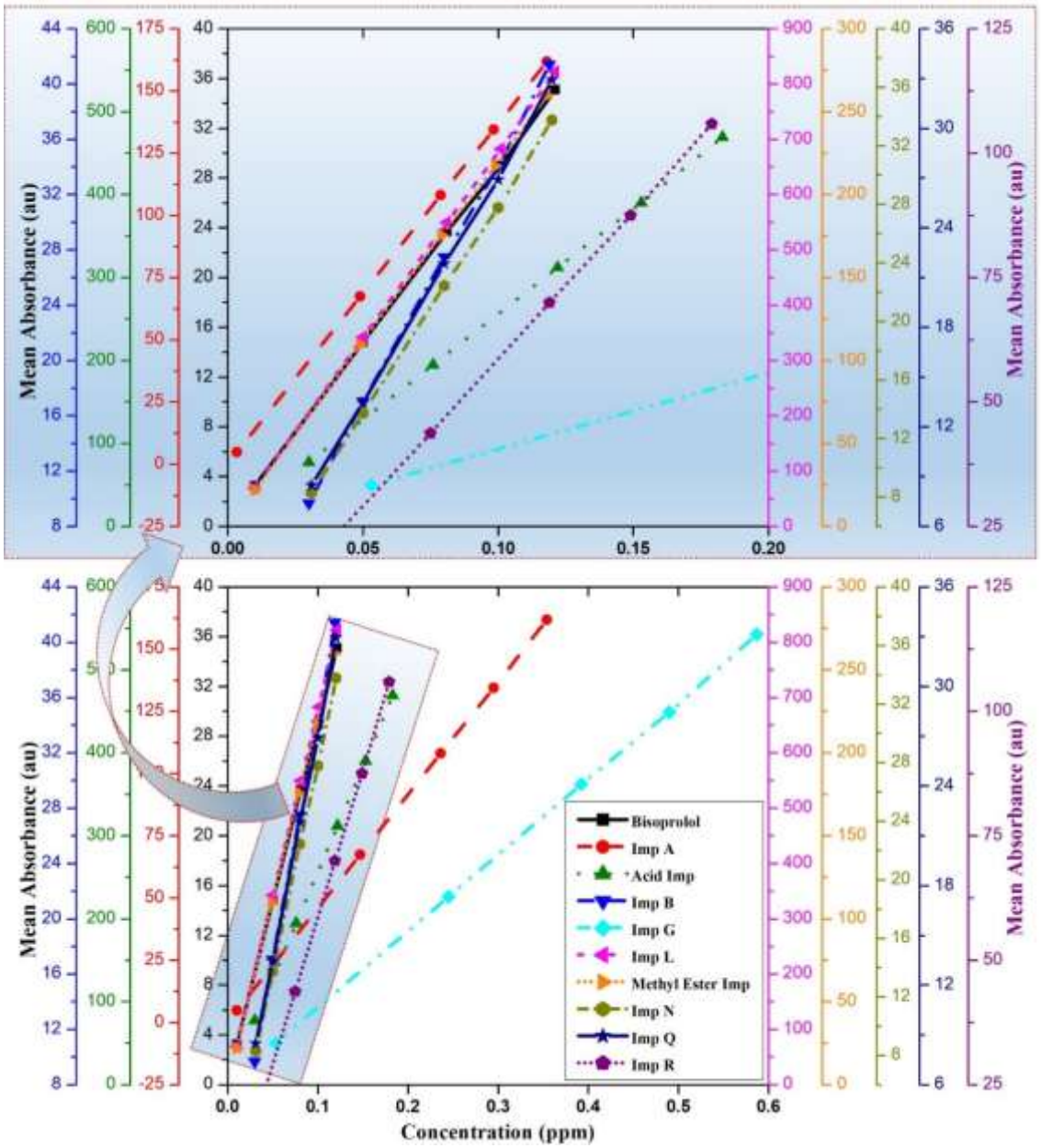



**FIGURE 6** Impurity linearity test graph

### 3.6 Accuracy

The accuracy of the analytical method was evaluated through recovery studies using known impurities of Bisoprolol fumarate. To assess the method's ability to accurately quantify impurities, a total of twelve prepared sample solutions containing known amounts of each impurity were spiked in triplicate at four distinct concentration levels corresponding to the limit of quantification (LOQ), 50 %, 100 %, and 120 % of the specified limit. The recovery (%) at each concentration level was calculated as:

% Recovery = (Amount detected in sample / Amount added in sample) × 100

The recovery of nine impurities is observed from 87.5 % to 111.1 %, whereas the % RSD of the impurities ranging from 0.1 % to 4.56 %. The detailed results of the accuracy study are summarized in Table 2, while a graphical representation is shown in the Fig.7.

The excellent recovery values obtained across all concentration levels demonstrated satisfactory accuracy and adds merit to the developed method.

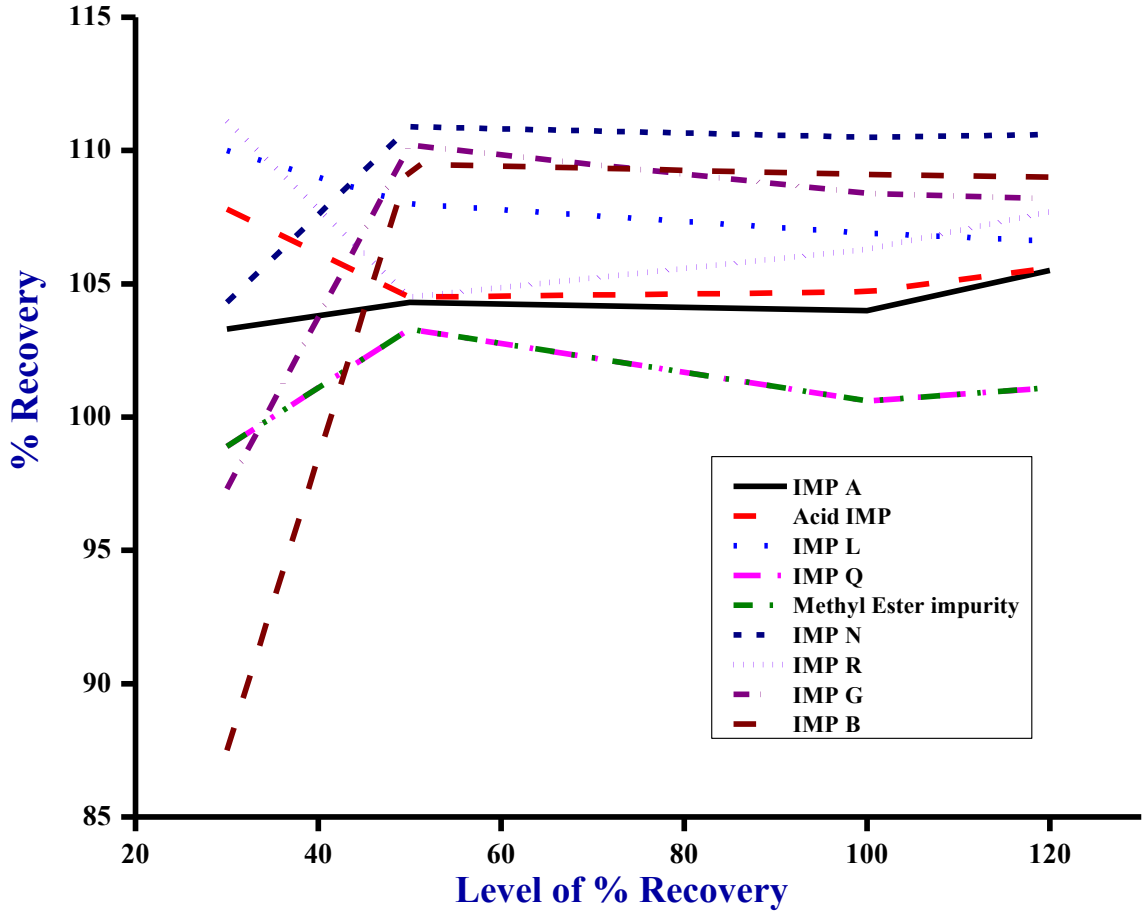



**FIGURE 7** % Recovery of individual impurities

**TABLE 2** Method validation result summary

| Parameter | Imp A | Acid Imp | Imp B | Imp G | Imp L | Methyl Ester | Imp N | Imp Q | Imp R | Biso |
|---|---|---|---|---|---|---|---|---|---|---|
| **Accuracy** | | | | | | | | | | |
| LOQ | 103.3 | 107.8 | 87.5 | 97.3 | 110.0 | 110.0% | 104.3 | 98.9 | 111.1 | - |
| 50% | 104.3 | 104.5 | 109.5 | 110.2 | 108.0 | 106.0% | 110.9 | 103.3 | 104.5 | - |
| 100% | 104.0 | 105.7 | 109.1 | 108.4 | 106.9 | 105.9% | 110.5 | 100.6 | 106.3 | - |
| 120% | 105.5 | 105.6 | 109.0 | 108.2 | 106.6 | 105.5% | 110.6 | 101.1 | 107.7 | - |
| LOD (ppm) | 0.085 | 0.043 | 0.073 | 0.127 | 0.047 | 0.053 | 0.271 | 0.223 | 0.328 | 0.087 |
| LOQ (ppm) | 0.257 | 0.131 | 0.222 | 0.385 | 0.141 | 0.160 | 0.820 | 0.676 | 0.993 | 0.265 |
| Precision at LOQ (% RSD) | 1.59 | 0.10 | 0.88 | 0.60 | 0.32 | 2.37 | 2.25 | 2.09 | 3.49 | 4.56 |
| **Regression equation (y)** | | | | | | | | | | |
| Regression ($R^2$) | 1 | 1 | 0.9995 | 0.9999 | 1 | 0.9999 | 0.9998 | 0.9995 | 0.9999 | 0.9998 |
| Slope | 455.57 | 2554.59 | 352.688 | 268.31 | 6760.98 | 2194.837 | 285.092 | 273.254 | 592.483 | 285.383 |
| Intercept | 0.3862 | 0.6441 | -0.7983 | 0.2642 | 2.3503 | 0.9852 | -0.5136 | -0.0964 | -0.4948 | 0.4526 |
| Correlation Coefficient (r) | 0.9999 | 0.9999 | 0.9997 | 0.9999 | 0.9999 | 0.9999 | 0.9999 | 0.9997 | 0.9999 | 0.9999 |
| Method Precision (% RSD) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 5.20 | 2.28 | 0.02 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Intermediate Precision (% RSD) Accuracy | 1.49 | 0.00 | 0.00 | 0.82 | 0.00 | 0.00 | 0.00 | 4.73 | 0.00 | 0.02 |
| Precision (pooled % RSD) | 1.11 | 0.00 | 0.00 | 1.96 | 0.00 | 0.00 | 0.00 | 6.55 | 1.61 | |
| Solution stability % Analyte diff. (Initial-48hrs) | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | - |

### 3.7 Precision

The precision of the developed method was evaluated by repeated injections of the reference solution, and the consistency of the chromatographic performance was assessed based on peak area and retention time of Bisoprolol. The percentage relative standard deviation (% RSD) calculated peak area and retention time from six replicate injections were 0.58 %, and 0.02 % respectively, indicating excellent reproducibility and system precision. Fig.8 Furthermore, the peak-to-valley ratio for the system suitability solution was found to be 44.170, demonstrating adequate peak resolution confirmed system suitability. The % RSD of the impurity responses was calculated across these six independent sample solutions spiked with impurities at the specified limit concentration, confirming the repeatability of the method under the established analytical conditions.

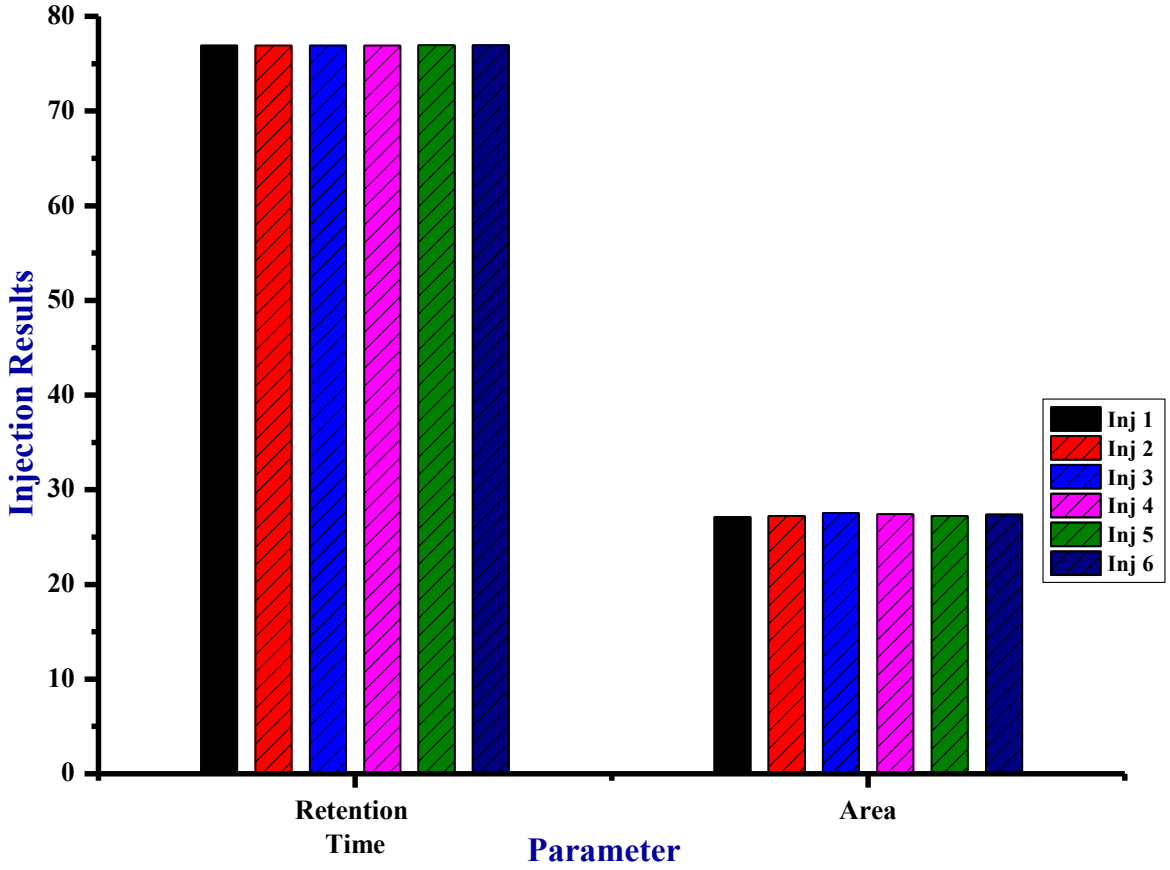



**FIGURE 8** System precision

To ensure the method's robustness, precision studies were repeated on a different day with six freshly prepared sample solutions spiked with impurities at the limit level and subsequent results of this intermediate precision study are summarized in Table 2 and shown in Fig.9. The results demonstrate that the method remains precise and reliable even under normal variations in analytical conditions.

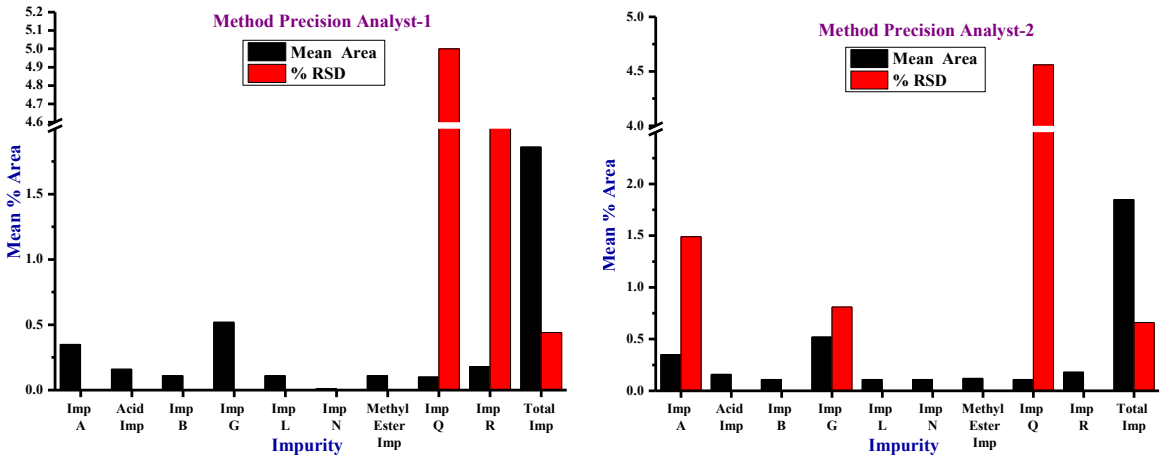



**FIGURE 9** Intermediate precision

## 3.8 Robustness

The efforts have been made to check the performance of the analytical method under deliberately varied chromatographic conditions to confirm its robustness. Analysing the reference solution, system suitability solution, and spiked sample solutions for diverse parameters, including peak-to-valley ratio, percentage relative standard deviation (% RSD) of the Bisoprolol peak area, and the quantitative content of Bisoprolol related impurities. Elegant alterations in chromatographic parameters were made to find their significance on method performance. For instance, column temperature (28 °C, 30 °C, and 32 °C), the flow rate (0.5, 0.6, and 0.7 mL $min^{-1}$) changed, also different columns with same specification but separate serial number were tried. (Fig. 10) All various tested conditions, showed consistency in results for analysis of Bisoprolol and its impurities; clearly highlighting experimental robustness. The key findings of robustness experiment, particularly the reference solution, system suitability solution, and spiked sample solutions are illustrated in Figures 11(A), 11(B), and 11(C), respectively.

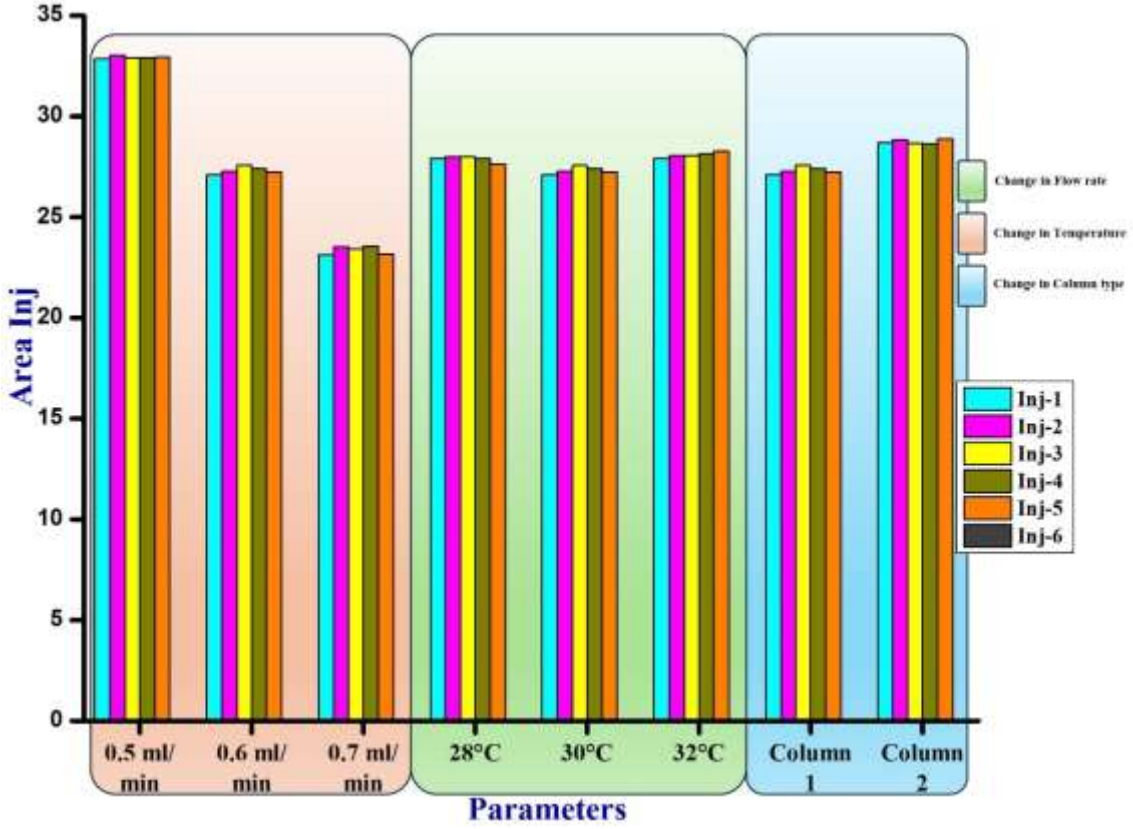



**FIGURE 10** Robustness reference solution system suitability

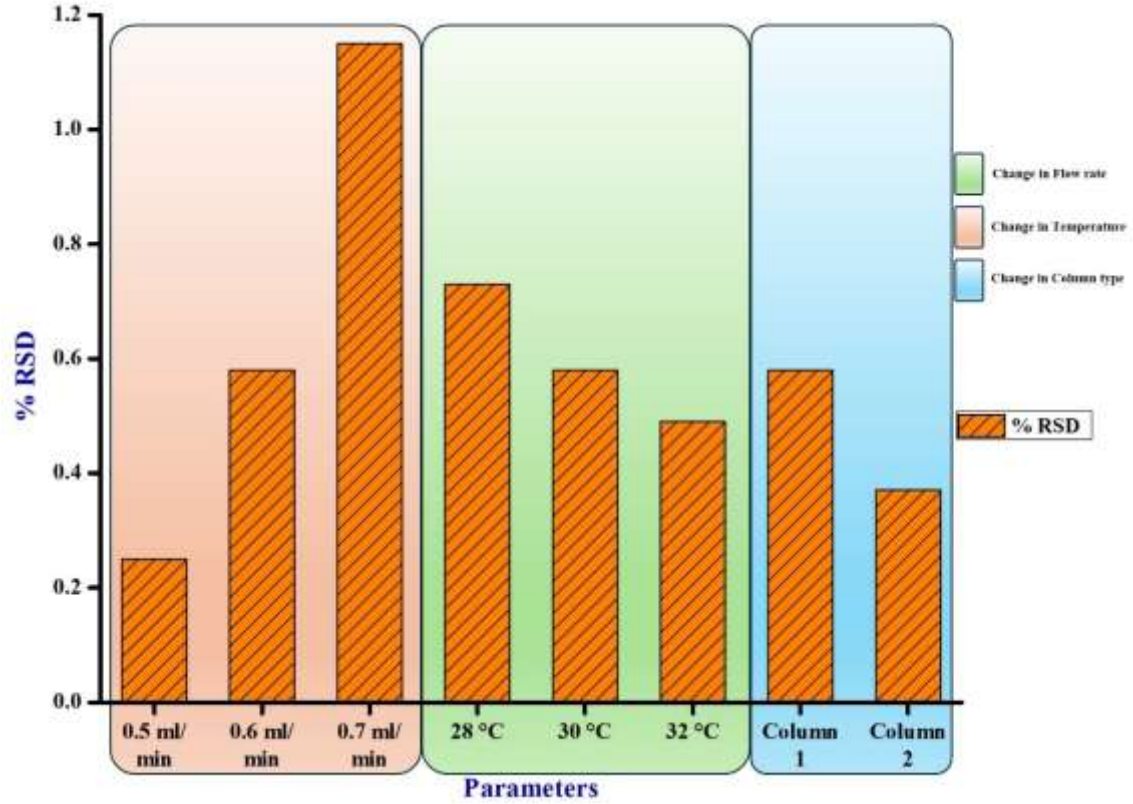
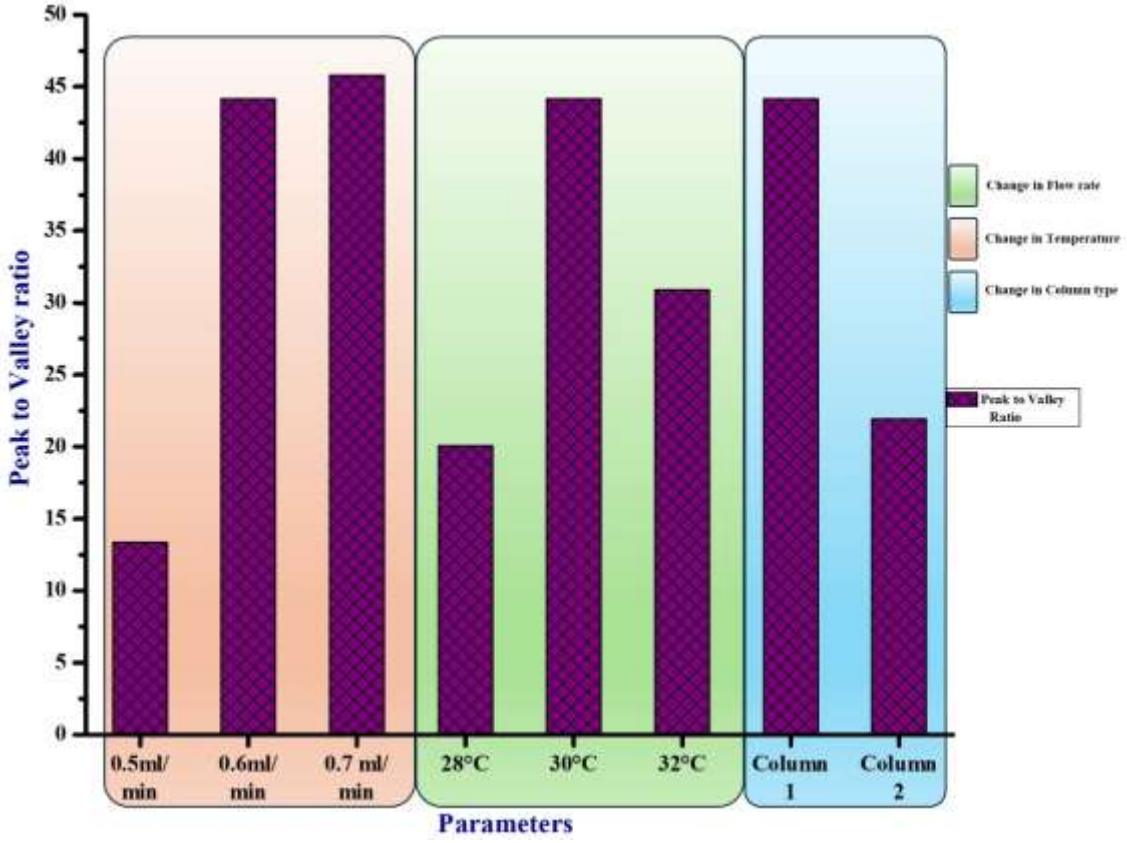
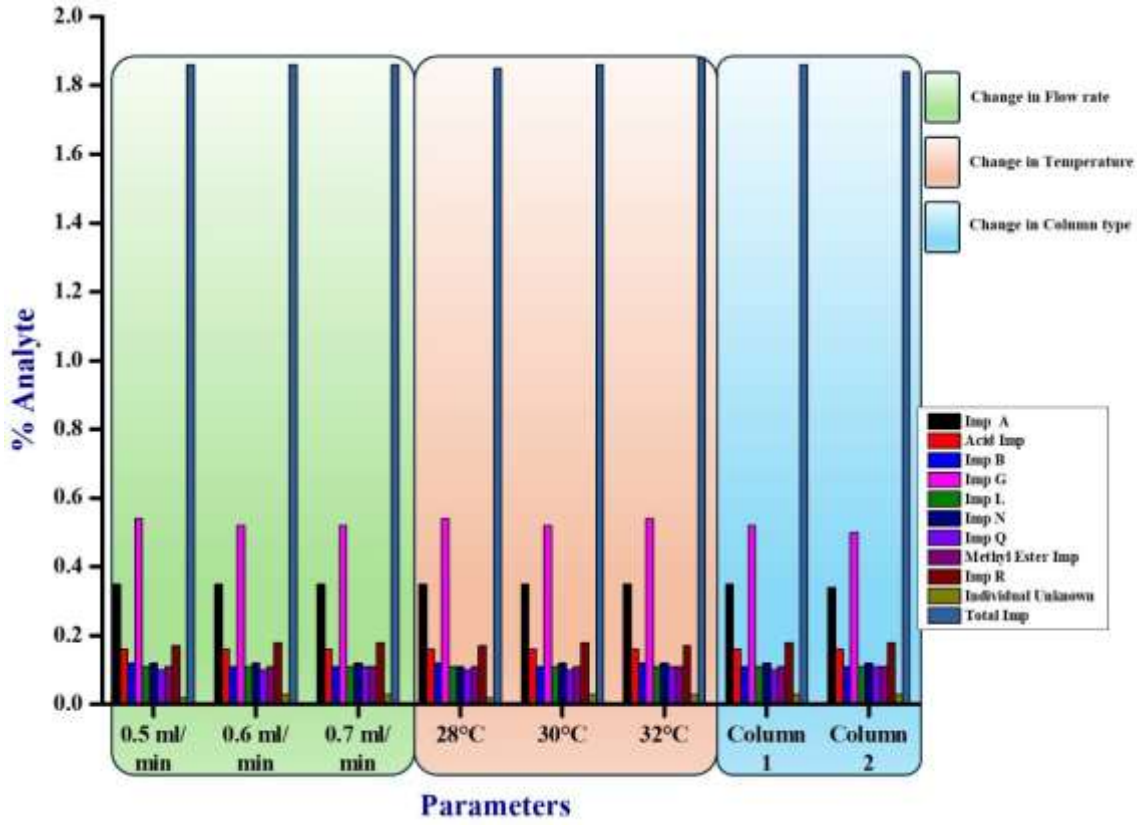



**FIGURE 11** (A)Robustness results for reference solution




312

313 **FIGURE 11** (B) Robustness reference solution system suitability Peak to Valley ratio




314

315 **FIGURE 11** (C) Robustness results for spiked sample solution

316

### 3.9 Stability of Analytical Solution

The results of spiked samples stored for 24 and 48 hours were compared with a freshly prepared reference solutions and the impurity levels were calculated for each time interval. The results for the samples, particularly freshly prepared and stored for 48 hours samples did not exceed 0.02%. (Fig.12, Table 3) This minimal variation in results over the study period, clearly revealed the significant stability of the analytical sample solution for at least 48 hours under the evaluated conditions.

**TABLE 3** Stability of analytical solution (Spiked Sample solution)

| Duration / Sample | Initial | | After 24 hr | | Difference (%) | After 48 hr | | Difference (%) |
|---|---|---|---|---|---|---|---|---|
| | Area | Result (%) | Area | Result (%) | | Area | Result (%) | |
| **Imp A** | 150.79 | 0.35 | 150.52 | 0.34 | **0.01** | 151.86 | 0.35 | **0** |
| **Acid Impurity** | 384.88 | 0.16 | 386.37 | 0.16 | **0** | 388.07 | 0.16 | **0** |
| **Imp B** | 33.95 | 0.1 | 34.22 | 0.1 | **0** | 34.21 | 0.1 | **0** |
| **Imp G** | 128.39 | 0.5 | 129.92 | 0.51 | **0.01** | 130.27 | 0.51 | **0.01** |
| **Imp L** | 699.82 | 0.11 | 702.9 | 0.11 | **0** | 706.6 | 0.11 | **0** |
| **Methyl Ester** | 225.74 | 0.11 | 225.25 | 0.11 | **0** | 227.6 | 0.11 | **0** |
| **Imp N** | 29.71 | 0.11 | 29.46 | 0.11 | **0** | 30.47 | 0.12 | **0.01** |
| **Imp Q** | 27.77 | 0.1 | 27.76 | 0.1 | **0** | 27.49 | 0.1 | **0** |
| **Imp R** | 91.97 | 0.16 | 92.12 | 0.16 | **0** | 92.62 | 0.16 | **0** |

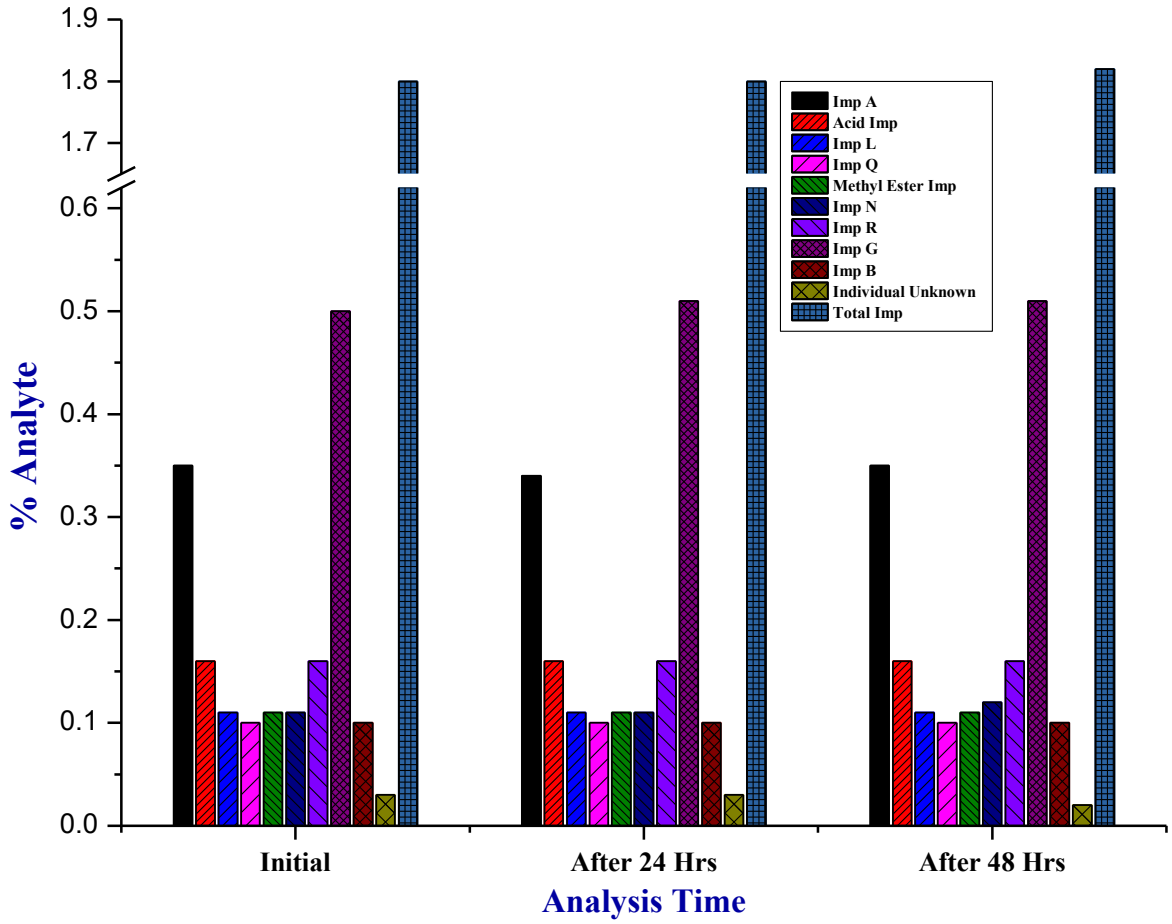
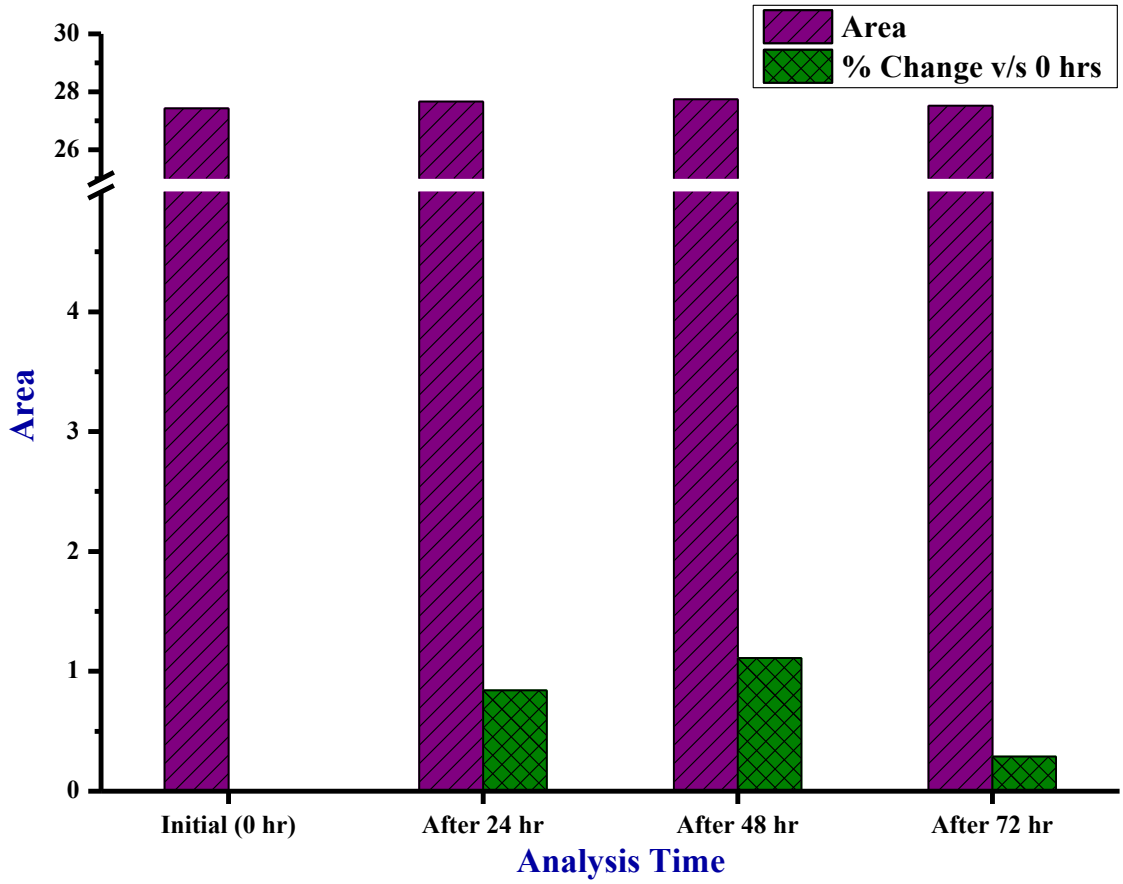



**FIGURE 12** Stability of spiked sample solution

Furthermore, less than 1.11% change was observed, when analysed peak area over 0, 24, 48, and 72 hours, the reference solution was found to be stable for up to 72 hours. (Fig 11)




**FIGURES 13** Stability of Bisoprolol in reference solution

## 4. Conclusion

This study introduces a robust and efficient HPLC method for identification and quantification of Bisoprolol and its related impurities, that has been successfully developed and validated in accordance with ICH regulatory requirements. The method demonstrated satisfactory linearity, specificity, accuracy, and precision across a well-defined analytical range extending from the limit of quantification (LOQ) to 120% of the target concentration. The method was shown to be capable of reliably measuring chromatographic purity and effectively separating Bisoprolol from its related impurities, *viz*. Imp A, Acid Imp, Imp L, Imp Q, Methyl Ester Imp, Imp N, Imp R, Imp G, and Imp B. The LODs and LOQs for all specified impurities were successfully established. In addition, stability studies confirmed that the sample solution remained stable for up to 48 hours, while both the reference solution and mobile phase were stable for at least 72 hours. Furthermore, robustness studies demonstrated that minor, deliberate variations in chromatographic conditions did not significantly affect system suitability or analytical performance, confirming the reliability and reproducibility of the method. The validated HPLC procedure described can be effectively used for the testing of Bisoprolol fumarate API marketed

for US market as well as European market. This newly developed method significantly reduces analysis time and solvent consumption enhancing efficiency within the quality control setting while providing accurate determination of Bisoprolol API purity. Its suitability for routine application supports consistent product quality and patient safety. Furthermore, its compliance with regulatory acceptance criteria and adaptability to pharmaceutical analysis make it a valuable tool for both research and manufacturing applications.

***Future perspectives:***

Bisoprolol and its related impurities in pharmaceutical formulations can be routinely analysed using this validated HPLC method. Its suitability for quality control applications supports the continuous monitoring of drug purity and quality, thereby contributing to improved regulatory compliance and the development of high-quality pharmaceutical products.

**Author Contributions**

**Nitin Patil:** Conceptualization, Methodology, Resources, Data Curation, Formal Analysis, Investigation, Writing – Original Draft. **Prajjwal Bhapkar:** resources, Writing – Original Draft, review and editing. **Monika Trivedi:** Project Administration, Supervision, Writing – Original Draft. **Rahul Kadu:** Conceptualization, Methodology, Resources, Supervision, Writing – Original Draft.

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- Funding Declaration: *No external funding was received to carry out this research*

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**Supporting Information:** Additional supporting information can be found online in the Supporting Information section.

**Supporting File 1:** JOSS_ESI_NP_10 Mar 26.docx