Original Article

Spectroscopic Substantiation for the Identification of Degradants by Q-TOF Micromass (ESI-MS) in Bisoprolol Fumarate with an Inventive Validation Approach for Stability Indicating HPLC Method

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ABSTRACT

Background: Stability studies, stress testing of drug substances and finished drug product is obligatory due to the guidelines by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and other Regulatory Authorities. **Materials and Methods:** In the present work the selective Beta-1 receptor blocker, Bisoprolol fumarate was subjected to the forced degradation studies which includes hydrolysis (acidic, basic and neutral), oxidative, photolytic and thermal degradation. The drug was stable under light and basic and neutral medium. **Results:** The major findings in the presented work are the degradation products formed in the course of thermal degradation. The complete thermal degradation products were identified by High Performance Liquid Chromatography, Quadrupole-Time of Flight micromass (Electronspray Ionization-Mass Spectroscopy). The chromatographic conditions opted for the study includes Cosmosil C_{18} column (4.6ID*250mm) with mobile phase consisting of methanol: phosphate buffer (pH 3.5 adjusted with orthophosphoric acid): Acetonitrile (45:35:20). **Conclusion:** The stability indicating method was validated as per the International Conference on Harmonization guidelines. The use of superior statistical tools for validation ensures the efficiency, quality and reproducibility of the presented method. Results obtained were agreeable and ensured the quality and reproducibility of the method.

Key words: Bisoprolol fumarate, Forced degradation study, Impurity profiling, International council for Harmonization Q3B.

INTRODUCTION

Bisoprolol fumarate (BF) approved by FDA was introduced in the year 1992 as a potent beta-blocker that selectively binds to the beta 1 receptor and competitively antagonizes the action of the adrenergic receptors. It is effectively used in the prophylaxis of angina pectoris and hypertension.[1,2] Maintenance of the quality, safety and efficacy is of paramount importance for delivering a dosage form with its full potency to the patients. As we consider Bisoprolol and other drugs belonging to this category that are given as a daily dose for the sustaining of hypertension, maintaining the safety becomes much more desirable.

As stated by the International Conference on Harmonization ICH Q3B (R2) guidelines, monitoring of the identification level, reporting and qualification thresholds are of prime importance in the development of impurity profile of a drug. The differentiation should also be made in the levels of stress-induced degradation products and the process-related impurities. The qualification of these levels of impurities is needed during new drug development, drug synthesis and post-marketing surveillance.[3,4]

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Though the drugs are subjected to the accelerated stability studies during the process drug development but forced degradation or stress testing studies are performed as severe conditions to examine whether a degradation product is formed or not.[5] As suggested by ICH and other regulatory bodies, the forced degradation studies are done by introducing the drug substances and drug products to stress conditions like oxidative, hydrolytic (which include acidic, basic and neutral hydrolysis), photolytic and thermal stress.[6-8] The degradation products formed are identified by using chromatographic techniques and the structure elucidation is done by employing spectroscopic techniques.[9]

In recent years, two stability studies for the Bisoprolol fumarate under the stress conditions have been reported in the literature with promising outcome. For example, I. Kasagić-Vujanović *et al.* studied the stability-indicating method using Hydrophilic Interaction Liquid Chromatography (HILIC)[10] and confirmed the formation of impurity A under acidic degradation. Also, kinetic studies were performed to check for their degradation behaviour. Another study was conducted by Ivana Mitrevska *et al.* for degradation of BF film-coated tablets in heat and its interaction with the diluents. The identified degradation product/impurity was concluded to be a product derived from the interaction of Bisoprolol fumarate with an excipient present that was calcium hydrogen phosphate.[11]

Some studies conducted on Bisoprolol fumarate corresponds to the analytical method development. To cite a few are, B. Jancic-Stojanovic *et al.* developed and validated a Hydrophilic Interaction Liquid chromatography (HILIC) method for determination of Bisoprolol fumarate[12] and Bueno Rolim *et al.* developed and validated a reverse phase HPLC method[13] for the dissolution study of the Bisoprolol fumarate which was found to be accurate and precise. V.K. Morothu *et al.* performed the isothermal stress testing and compatibility study of Bisoprolol and few excipients were performed and an HPLC method was developed and validated for the determination of the drug and stressed compounds.[14]

To the best of our knowledge, the degradation products formed in the present method are novel and they have not been reported till date. A stability-indicating HPLC method for Bisoprolol fumarate was developed and the characterization of degradation products was established by Q-TOF micromass (Electronspray Ionization-Mass Spectroscopy).

EXPERIMENTAL

Pure Bisoprolol fumarate was procured from Sigma Aldrich. HPLC grade water, methanol and acetonitrile were purchased from Merck, India. The other reagents used like orthophosphoric acid, hydrochloric acid, sodium hydroxide, hydrogen peroxide were of analytical grade purchased from Merck, India.

Instrumentation

The HPLC analysis was carried out by using Younglin Acme 9000, comprised of a quaternary solvent manager and sample manager in isocratic mode along with UV Visible detector. The output and processing of the data were done through Autocome 3000 software on Windows 7 for processing of the data. All pH measurements were carried out using pH meter (Acutek) and weighing was done with Shimadzu (1 mg sensitivity).

Waters, Micromass Q-TOF micro (Separation module: Waters Alliance 2795) was used for mass measurement. Ionization was used with Electrospray positive ES^+ and ES^-, MRM unit resolution, injection volume was 20 microlitres with a flow rate of 0.4 mL/min. For mass measurement, desolvation gases used were Nitrogen and Argon at 550 liters/hour, 300°C. Collision energy and capillary voltage was kept 4ev and 3000V respectively.

Forced Degradation Studies

The apprehension behind the forced degradation studies is to find out the ways through which the degradation products are formed, subjecting the solid-state active pharmaceutical ingredient to the stress conditions severe to that of the accelerated stability studies. It consisted of acidic hydrolysis where BF was refluxed with 3N HCl for 48 hr. Alkaline hydrolysis was achieved by refluxing the drug with 0.1N NaOH for 1 hr. For neutral hydrolysis drug in its solution form was refluxed for 48 hr. Oxidative degradation study was followed by subjecting the drug to 3%, 15% and 30% H_2O_2 for 7 days. Photolytic degradation was done for the drug in solution form as well as in solid-state exposing it to 1.2 million lux hours of UV light. To achieve thermal degradation, the drug in solid form was kept in an oven at 80°C for 7 days.

Sample Preparation

All the forced degradation studies were carried out by preparing 1mg/mL solution of BF in methanol, except for acid hydrolysis where 5 mg drug was dissolved in 5 mL of 3N HCl. For base hydrolysis, 5 mg drug was dissolved in 5 mL of 1 N NaOH. For oxidative degradation, 3 mL of H_2O_2 was added to the drug in its solution form. Each stock solution prepared was

further diluted to the concentration of 100 µg/mL with methanol. Before each HPLC analysis, the solution was filtered through Whatman filter paper grade 602 h and sonicated in a bath sonicator for 15 min.

Chromatographic Conditions

The method was found to be accurate and precise by using Cosmosil C-$_{18}$ PAQ column (4.6ID*250 mm) and mobile phase which consisted of methanol: phosphate buffer (pH 3.5 adjusted with orthophosphoric acid): acetonitrile in the ratio 45:35:20 in the isocratic mode. Flow rate of was 1.2 mL/min was maintaied throughout the analysis. The detection wavelength used was 220 nm and injection volume used was 1µl. The column temperature was maintained at 25°C.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

To develop an accurate, precise, reliable method of the proper separation of BF and its degradants several mobile phase compositions were experimented at different pH, flow rate, column and other dependent variables have experimented. Several mobile phase compositions were designed by altering the concentration of methanol, acetonitrile and phosphate buffer. The series of pH ranging from 2 to 5 was wandered during the course of study. Due to the polar nature of the drug the outstanding results were achieved by using a composition of methanol, buffer and acetonitrile in the ratio of 45:35:20.

Degradation Behavior of Bisoprolol Fumarate

A comprehensive study for the stress-induced degradation of BF was examined using HPLC-UV detector.

Hydrolytic Degradation

During the base and neutral hydrolysis, sufficient stability was found for the drug and no degradation products were formed (Figure S1). The acidic degradation study revealed that the degradants formed were already identified in the previously cited literratures.

Oxidative Degradation

The oxidative degradation study demonstrated that no degradants where formed when methanol was employed as diluents for the preparation of stock solution inspite of the nature of the drug which suggests its susceptibility towards oxidation. The same has been confirmed in various literatures. For this reason, authors conducted the studies in a varied manner to confirm the oxidative degradation. Oxidative degradations were performed in aqueous solution form of the drug by adding 15% hydrogen peroxide as stressor and 1mL of tertiary butyl hydrogen peroxide (TBHP). Observed degradation product's peak in the HPLC chromatogram did not reveal any degradation (Figure S2 and S3).

Photolytic Degradation

The drug in solution, as well as solid-state form, was exposed to 1.2 million lux hours of UV light and no degradation was observed (Figure S4).

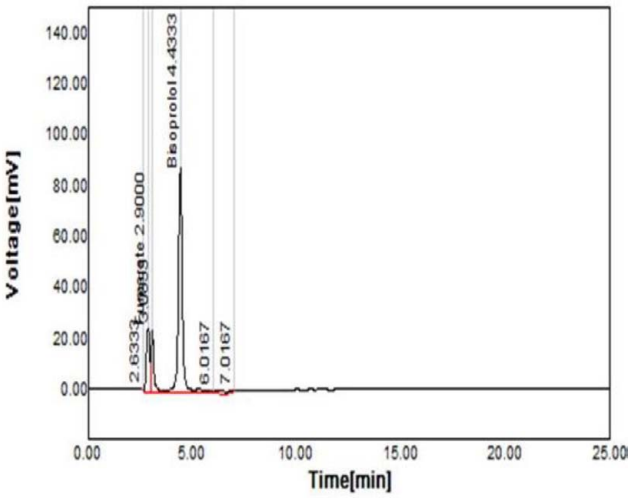

Figure S1: HPLC chromatogram of Basic Hydrolytic Degradation (3N NaOH) of Bisoprolol fumarate.

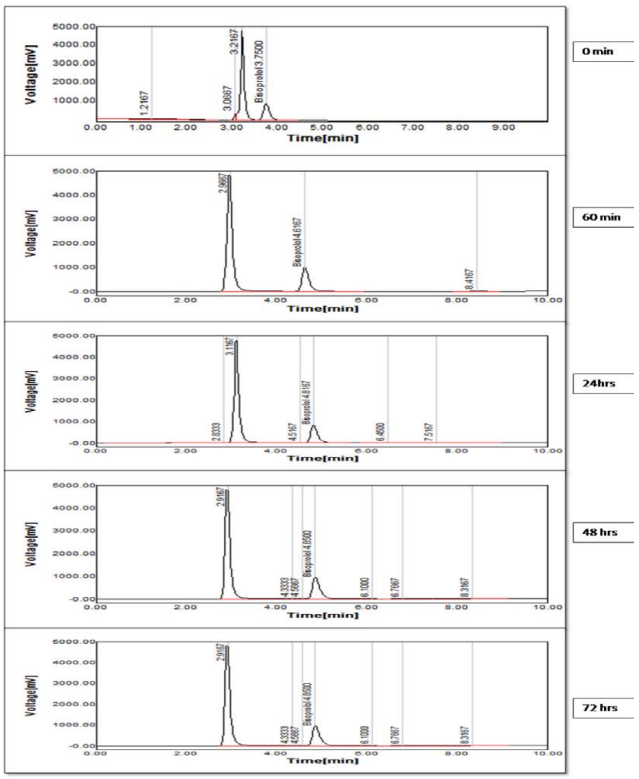

Figure S2: HPLC chromatogram of Oxidative Degradation (15% H_2O_2) of Bisoprolol Fumarate for 72 hr.

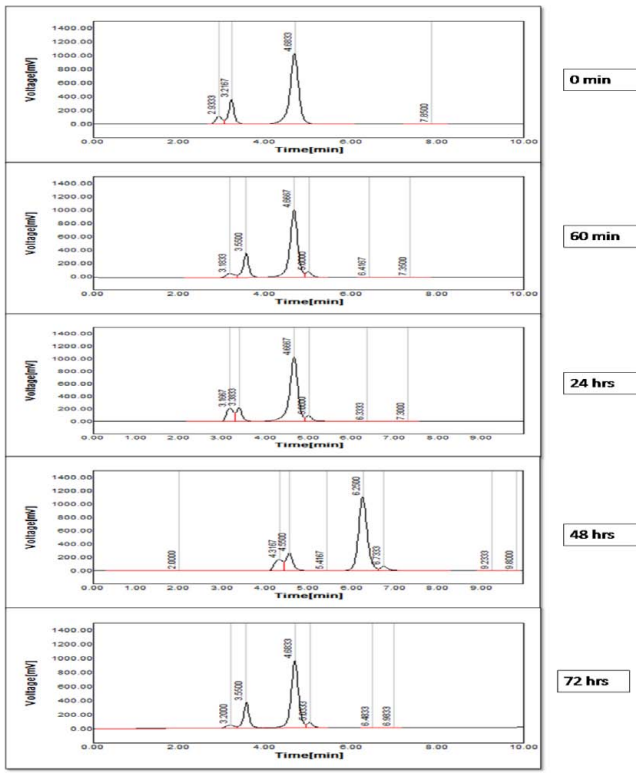

Figure S3: HPLC chromatogram of Oxidative Degradation (TbHP) of Bisoprolol Fumarate for 72 hr.

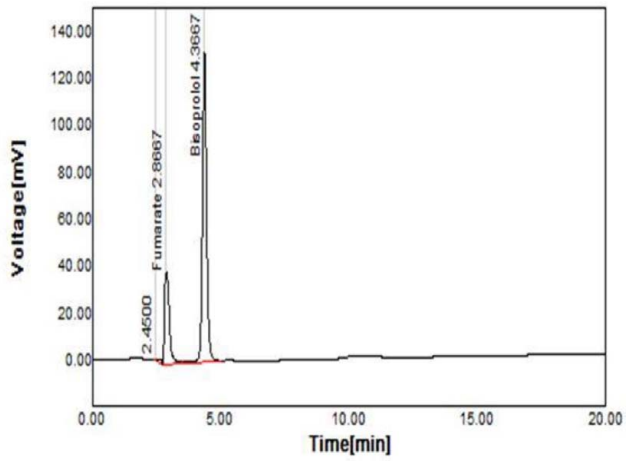

Figure S4: HPLC chromatogram of Photolytic Degradation (1.2 million lux hours) of Bisoprolol Fumarate for 7 days.

Thermal Degradation

By keeping the drug at 80°C for 7 days, it was observed that the drug did not withstand its stability and HPLC chromatogram revealed the formation of a degradation product was observed at retention time 6.00 min (Figure 1). For analyzing the degradation product, the test compound was subjected to Q-TOF Micromass ESI-MS/MS (Figure 2). The proposed fragmentation pattern of the test compound in order to determine the

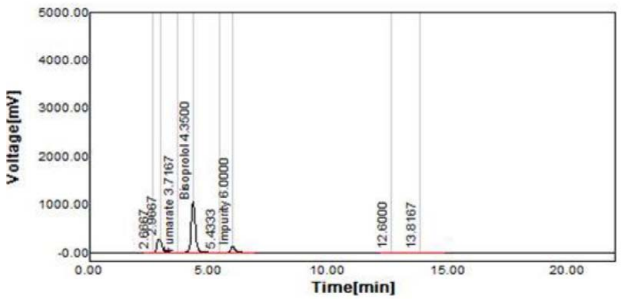

Figure 1: HPLC chromatogram of Thermal Degradation (80°C) of Bisoprolol Fumarate for 7 days.

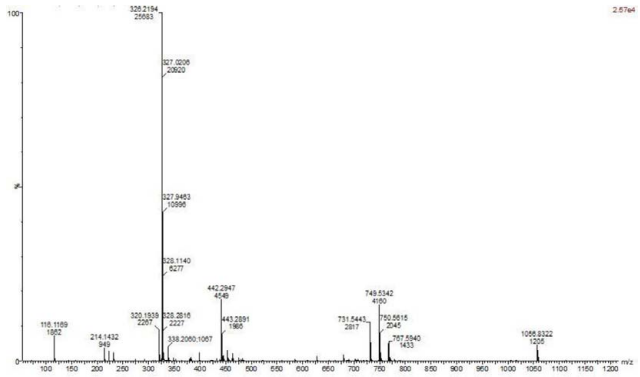

Figure 2: ESI/MS/MS Spectrum of Bisoprolol (m/z 326), fumaric acid (m/z 116), impurity IA (m/z749), fragmentation ion IF (m/z 731).

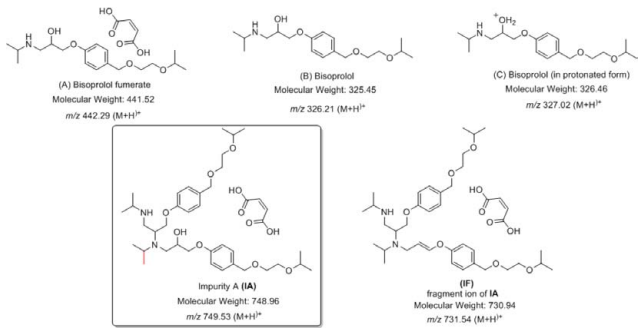

Figure 3: Proposed structures of protonated degradation products of Bisoprolol Fumarate.

formation of expected degradants in the presence of heat has been depicted with the elemental composition and molecular mass in Figure 3.

Q-TOF Micromass ESI-MS/MS study of Bisoprolol Fumarate and its degradants and Projected mechanism for the formation of the degradation product

From the HPLC chromatogram as shown in Figure 1, three different peaks correspond to fumaric acid, bisoprolol, unknown impurities were observed at RT 2.966 min, 4.35 min and 6.00 min, respectively. To

investigate about the possible structure of unknown impurity, the mass spectrum of degradant was studied based on the *m/z* values of their $[M+H]^+$ ions. As shown in Figure 2, the *m/z* at 326 and 116 displayed the presence of bisoprolol and fumaric acid, whereas the appearance of *m/z* at 442 validated the existence of bisoprolol fumarate. Furthermore, an increase in the mass of molecular ion by 01 AMU (*m/z* 327) may be attributed to the protonated form of bisoprolol under stress conditions.

Based on the molecular ion peak observed at *m/z* 749 in MS/MS of degradant, mass higher than 307 Da of bisoprolol fumarate is expected to be found impurity after thermal degradation, as shown in HPLC chromatogram at RT 6.00 min. The anticipated structure of impurity (IA) based on MS/MS with *m/z* 749 has been shown in Figure 3. We therefore, believe that the formation of impurity (IA) is possible during the stress condition via intramolecular nucleophilic substitution reaction of bisoprolol fumarate as depicted in Figure 3. We also observed the *m/z* at 731 amu, which is expected to be the fragmentation ion (IF) of target impurity (IA). The difference in the mass of 18 Da between IA and IB clearly suggested the formation of IF *via* elimination reaction/dehydration of target impurity IA (Figure 4).

Method Validation

Stability indicating HPLC method was developed and validated as per the ICH Q2 (R1) guidelines. The method was found to be accurate and precise.

Specificity

The stressed samples of BF were analyzed for the specificity of the proposed stability-indicating method to ensure capability of the method to separate the degradation products from the pure bulk drug BF. The specificity of the method was established by determining the resolution factor and peak purity of stressed samples of BF. Assay of the Pure BF was determined against the stress BF samples. The determined peak purity shows the undoubted specificity of the method.

Linearity and Range

For the determination of linearity, BF samples in the range of 20-100 µg/mL were analyzed and the correlation coefficient was found to be 0.999 (Figure 5). The range analyzed was used for 6 concentrations and consecutively three days in the same range. All the data obtained satisfies the criteria required to have a linear relationship between the dependent and independent variable (Table 1). Residual plots for individual drug show a random distribution of data points around the regression line indicates a good linear relationship (Figure 5).

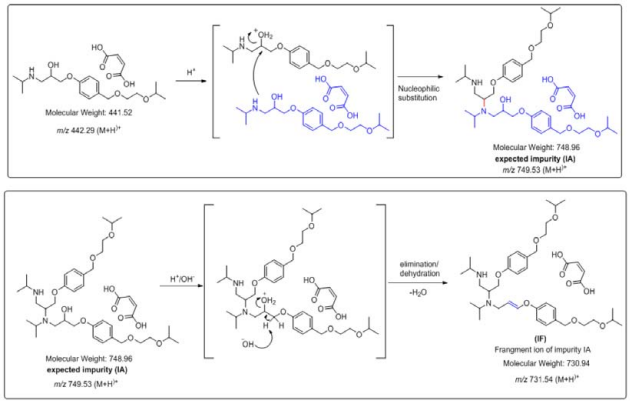

Figure 4: Proposed fragmentation pathway of protonated Bisoprolol Fumarate.

Table 1: Parameters for linear regression equation.

Parameters		Unknown	Criterion	Comment
Regression statistics	Multiple R	0.999	Smaller value indicates good fit	
	R Square	0.999		
	Adjusted R Square	0.999		
	Standard error	96.83		
	Total observations	5		
ANOVA	*F* value (Conc. Variable)	4086.353	*F* value > F Critical	F Critical = 10.12
	F Significance (Conc. Variable)	8.43E-06	F significance <α	Alpha = 0.05
	T Stat (Conc. Variable)	63.924	Should not lie between ± T Critical (-2.77 to +2.77)	T Critical = 2.77
	P-value (Conc. Variable)	8.43E-06	*P* value < α	
	Lower 95% - Upper 95% (Conc. Variable)	93.005- 102.75	Zero should not lie between upper and lower 95% interval	
	The standard error of Y-intercept	101.565	Used for Standard deviation calculation.	

Robustness

For estimating how robust the method is, some small and purposeful variations were done in the flow rate, column temperature, and mobile phase. Flow rate was changed by 0.2 mL/min (i.e. 0.8mL/min to 1.2mL/min) that show %RSD of 0.78%. The column temperature was varied by 5°C (i.e. 25 to 30°C) and the % RSD found was 1.23%. Similarly when the mobile phase was altered by changing the pH from 3.5-4.5 the %RSD found was 1.45%. All the above-stated robustness parameters qualified the guidelines demonstrating that the method is robust enough.

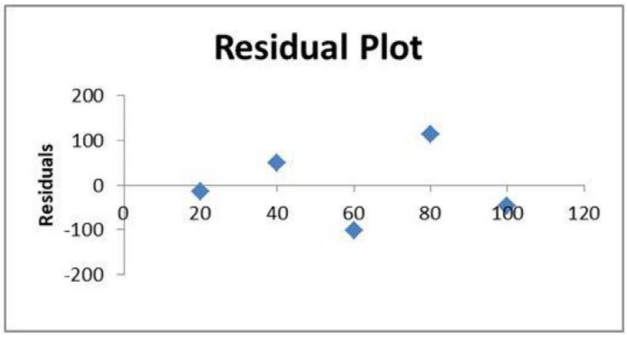

Figure 5: Residual plot in the observed range (20-100µg/ml).

Accuracy

According to the British Pharmacopoeia the accuracy study was carried out in the levels of 50%, 100% and 150 %. The concentrations chosen (20, 40 and 60 µg/mL), were evaluated in triplicate. The % recovery was calculated and was found in the range of 99.81-102.22 % (Table 2).

Precision

Inter and intraday precisions were performed by analyzing the samples on the same day in triplicate and in successive for 3 days. Determining the peak area in the concentrations chosen 20, 40, and 60µg/mL depicted that the method is precise. The value of p found greater than the significance level and F Value were smaller than F-Critical value. It indicates excellent reproducibility of the method. The results are denoted in Table 3 and 4.

Table 2: Inter day precision (Data analysis through Anova Single factor).

Summary						
Groups	**Count**	**Sum**	**Average**	**Variance**		
A	3	119.9	39.96667	411.3265		
B	3	120.5	40.16667	413.5385		
C	3	120.55	40.18333	394.1858		
D	3	120.41	40.13667	404.333		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	0.0894	3	0.0298	7.34E-05	0.999999	4.066181
Within Groups	3246.768	8	405.846			
Total	3246.857	11				

Table 3: Inter day precision (Data analysis through Anova Single factor).

Summary						
Groups	**Count**	**Sum**	**Average**	**Variance**		
	3	120.58	40.19333	419.2266		
	3	120.93	40.31	396.6433		
	3	120.65	40.21667	384.2433		
	3	120.72	40.24	399.8721		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	0.022867	3	0.007622	1.91E-05	1	4.066181
Within Groups	3199.971	8	399.9963			
Total	3199.994	11				

Table 4: Recovery Data.		
Amount Added	**Accuracy/Calculated Spiked concentration**	**Recovery**
(µg/ml)	**(µg/ml) ± SD; RSD**	**(%)**
20	20.16 ± 0.34; 1.68	100.8
40	40.89 ± 0.40; 0.98	102.25
60	59.89 ± 0.33; 0.55	99.81

CONCLUSION

The degradation studies induced on Bisoprolol fumarate were as per the ICH guideline (hydrolytic, photolytic, thermal and oxidative stress). These studies revealed the formation of one degradation product and one fragmented product of the degradant during the thermal stress due the interaction of the drug product with heat which induced a nucleophilic substitution reaction. The degradation behaviour was justified through the plausible mechanism by which the degradation products were formed. The degrdation products formed were identified for their mass by ESI-QTOF-Mass Spectroscopy.

In the present paper, we have applied newer strategies for validation of the developed method that concurrently satisfies the concept of Quality by Design (QbD). General parameter of validation of method depends on linearity, standard deviation and standard error. the coefficient of determination is a common method fro the verification of linearity and its taken that is R^2 value approaches closely to 1 then there is a linear relationship, but the same not suggested in the literatures.[15] To rationalize the same we have opted for regression analysis and residual analysis for validating the linear relationship between the dependent and independent variable for clear understanding and better interpretation of the results obtained. One way ANOVA is used for the precision study which is more apparent and acceptable approach as compared to Relative standard deviation. Further, a HPLC method was developed and validated that can be used in the determination of stability studies and routine qualitative analysis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ICH: International Council for Harmonization; **API:** Active pharmaceutical ingredient; **BF:** bisoprolol fumarate; **RSD:** Relative standard deviation; **SIM:** Stability indicating method; **RT:** Retention Time; **AMU:** Atomic Mass unit; **ESI-QTOF:** Electronspray Ionization- Quadrapole Time of Flight; **Nm:** namometer; **mg/mL:** milligram per milliliter; **H:** hours.

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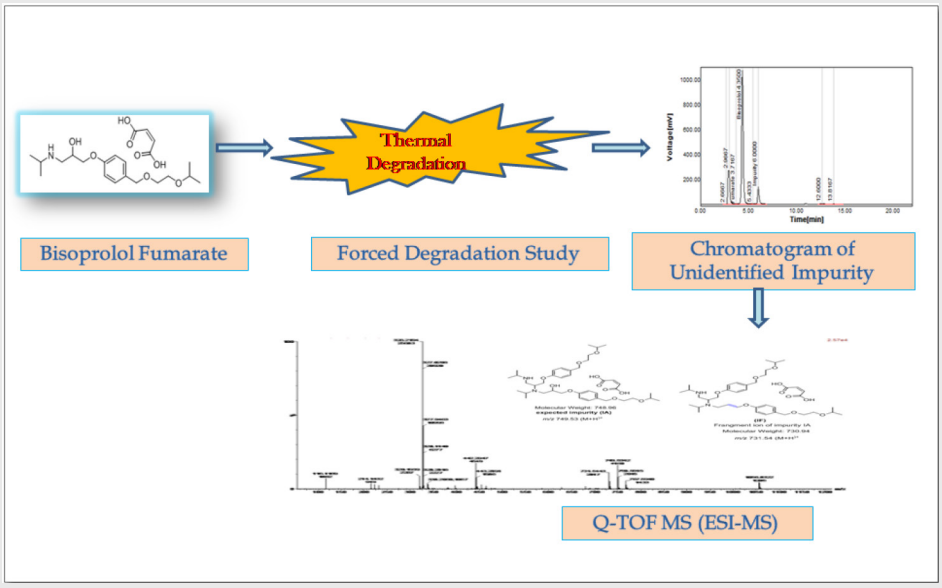
PICTORIAL ABSTRACT



SUMMARY

The degradation studies induced on Bisoprolol fumarate were as per the ICH guideline (hydrolytic, photolytic, thermal and oxidative stress). These studies revealed the formation of one degradation product and one fragmented product of the degradant during the thermal stress due the interaction of the drug product with heat which induced a nucleophilic substitution reaction. The degradation behaviour was justified through the plausible mechanism by which the degradation products were formed. The degrdation products formed were identified for their mass by ESI-QTOF-Mass Spectroscopy. In the present paper, we have applied newer strategies for validation of the developed method that concurrently satisfies the concept of Quality by Design (QbD). General parameter of validation of method depends on linearity, standard deviation and standard error. The coefficient of determination is a common method for the verification of linearity and it's taken that is R^2 value approaches closely to 1 then there is a linear relationship, but the same not suggested in the literatures. The major findings in the presented work are the degradation products formed in the course of thermal degradation. The complete thermal degradation products were identified by HPLC, Q-TOF micromass (ESI-MS). The developed HPLC method was validated as per the ICH guidelines. The use of superior statistical tools for validation ensures the efficiency, quality and reproducibility of the presented method. Results obtained were agreeable and ensured the quality and reproducibility of the method. To rationalize the same we have opted for regression analysis and residual analysis for validating the linear relationship between the dependent and independent variable for clear understanding and better interpretation of the results obtained. One way ANOVA is used for the precision study which is more apparent and acceptable approach as compared to Relative standard deviation. Further, a HPLC method was developed and validated that can be used in the determination of stability studies and routine qualitative analysis.

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