RESEARCH ARTICLE

Characterization of Degradation Products of Bisoprolol by LC-MS and *In-silico* Determination of Toxicity and Biological Activity Prediction of its Degradation Product: A Comprehensive Approach during Drug Development

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ABSTRACT

The stability of bisoprolol in tablet and bulk form was developed and validated using chromatographic methods. The bisoprolol was exposed to oxidizing agents like hydrogen peroxide, heat (dry and wet) and photolytic conditions, acid, alkali, and water hydrolysis. However, considerable degradation was observed in acid, alkali, oxidative, and wet heat thermal conditions. The drug's stability under photolytic and dry heat conditions was established. The drug and its degradation products were separated on ODS C-18 with the specification of (250 mm × 4.6 mm, 5 µm) and the mobile phase containing acetonitrile and phosphate buffer (20 mM, pH 8) (60:40, v/v). According to ICH Q2 (R1) all method validation parameters are verified. Determination of the active pharmaceutical ingredient was not interfered by excipients or degradation products. In the 6–14 µg/mL range, the response was shown to be linear. One degradation product's LC-MS m/z values and fragmentation patterns matched an impurity mentioned in the drug's European Pharmacopoeia monograph. The remaining three were unidentified degradation products. LC-MS fragmentation experiments were used to characterize the products. The findings may lead to the suggestion of a more thorough drug degradation mechanism. The Toxtree software (Version 3.1.1) was then used to forecast the toxicity of the degradation products. Additional toxicity determination was carried out by using *in-silico* method.

Keywords: Bisoprolol, RP-HPLC, Degradation products, Validation, *In-silico* toxicity, PASS.

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INTRODUCTION

Bisoprolol chemically contains secondary alcohol and a secondary amine. Using a cardioselective β1-adrenergic blocking medication, high blood pressure is treated.[1] FDA-approved beta-1-selective blockers used for treatments of heart-related disorders. Bisoprolol is cardioselective beta-blocker.[2]

Many reports are available in the literature on the degradation behavior of bisoprolol (BSP). The drug is established to be specifically sensitive to hydrolysis, oxidation and heat conditions. For this reason special handling precautions are employed to protect drug from hydrolysis, oxidation and heat conditions solid state. Additionally, Petrit *et al.* reported bisoprolol reactivity to hydrolytic degradation at different pH conditions, Ghauch *et al.* and Prachi *et al.* reported bisoprolol sensitivity to oxidative degradation conditions, Marothu *et al.* reported isothermal stress testing of bisoprolol fumarate.[3-6]

In literature, Pandey *et al.* reported thermal degradation of bisoprolol in stability indicating method.[7] Combination of bisoprolol with other drugs are used in hypertension treatment. Many stability indicating reports are available on the combination of bisoprolol with hydrochlorothiazide and amlodipine besylate. Various chromatographic stability-indicating methods was reported for bisoprolol and their combinations. Which include UPLC method, RP-HPLC, RP-HPLC and CE method and thin layer chromatography.[8-12] but there was lack of characterization and identification of degradation products with their toxicity prediction.

The current work's objective was to perform force degradation behavior of bisoprolol at hydrolytic, oxidative, thermal (wet and dry heat) and photolytic conditions and to characterize degradation products by using mass spectrometry. Additionally, toxicity properties of DPs (degradation products)

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were compared to drug. Further, those DPs are non-toxic in nature evaluated for biological activity by using PASS.

MATERIALS AND METHODS

Instruments and Equipment

The Shimadzu SCL-10Avp dual-pump HPLC system with a PDA detector and a sample injection port with a 10 mL loop was used to conduct the HPLC examination. Software used in HPLC analysis is LC solution (version 1.25), and separation and quantification ODS C18 column was used as stationary phase. A digital weighing scale (AUX 220, Shimadzu), a hot air oven (BTI-29), an ultrasonic bath (LMUC-3), are additional tools utilized in analysis.

Materials

BSP was supplied as a gift sample by Unichem Laboratories Ltd., Goa. Its weight-based purity was verified to be 99.74%. The experiment called for HPLC-quality methanol and acetonitrile. Other chemicals included analytical laboratory-grade hydrogen peroxide, potassium dihydrogen orthophosphate, sodium hydroxide flakes, hydrochloric acid are procured from S D Fine-Chem Ltd. in Mumbai. Double distillation assembly used for preparation of double-distilled water. BSP film-coated tablets in the dosage of 5 mg (Concor 5 mg) were purchased from a pharmacy.

Methods

Chromatographic condition

The chromatographic conditions used during analysis contain stationary phase consisting of ODS C18 column (250 × 4.6 mm, 5 µm and mobile phase used as acetonitrile and potassium phosphate buffer 20 mM (pH 8.0) 60:40% v/v maintained at a flow rate of 1.2 mL/min. The eluents may be seen at 225 nm. Every analysis was performed at room temperature.

Forced degradation studies

Studies on forced degradation of BSP have been done. Every investigation was conducted with a 5 to 20% target degradation rate. For acid and alkali hydrolysis carried out at 70°C in acidic and alkaline conditions for 6 days. Additionally, an aqueous suspension of the medication was boiled under reflux for 48 hours at 70°C in order to perform the degradation under wet heat. At ambient temperature and in the dark for 48 hours, 30% v/v H_2O_2 was used to facilitate oxidative breakdown. Additionally, BSP was put through photolytic testing, which comprised exposing it to visible light for six hours and a dry heat study that involved placing the BSP in a hot air oven at 70°C for 48 hours. After being exposed, the drug samples were quenched. Lastly, the sample was to maintain a final concentration of 10 µg/mL drug, and analysis was performed on them. As a sign of degradation, it was thought that the BSP peak area had decreased and/or subsidiary peaks had begun to emerge. The errors were avoided each time by using the appropriate controls and blanks.

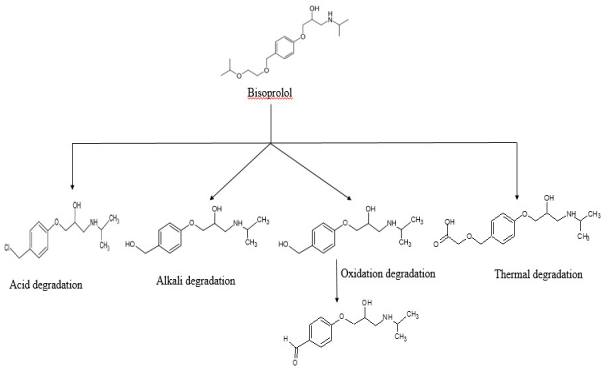

Figure 1: Possible degradation products of bisoprolol

Characterization of degradation products

Samples that had been stress under the various circumstances mentioned above were subjected to LC-MS analyses. The samples were examined using positive electrospray ionization (ESI) in an MS spectrometer. A mobile phase was compatible with MS (1 vol% formic acid: acetonitrile) and the HPLC technique to separate the samples. With the use of the LC-MS data, structure elucidation was carried out. In Figure 1, potential degradation products were depicted.

Validation of HPLC method

ICH Q2 (R1) guideline is commonly used for analytical method validation.[13] The standard BSP stock solution was added to the BSP tablet formulation stock solution to achieve 80, 100, and 120% QC range along the calibration curve to evaluate the precision and accuracy. Every study was done in triplicate over the course of three days. Low %RSD values were viewed as an indication of precision, and the percentage of recovery that reached the true added values was viewed as a sign of accuracy. The analytical data was evaluated for variance of analysis, and the intermediate precision was calculated by using F (theoretical) and F (observed). The following equation used for the determination of detection and quantitation limit:

DL = (3.3σ / S) and QL = (10σ / S), σ where is the standard deviation (SD) of response (y-axis) and is the calibration curve slope. The percentage organic concentration, flow rate, and wavelength of the system were slightly but purposefully changed under the ideal chromatographic circumstances mentioned previously, and the variations in the parameters were recorded. This was done to test the method's robustness. The suggested method's specificity was demonstrated by completely separating BSP and the degradation products it produced from the excipients. Specificity was considered when there were no peaks in the blank runs during the retention time of BSP and its degradation products.

Degradation products toxicity is determined by using an in-silico approach.

TOXTREE (Toxic Hazard Estimation), version 3.1.1 was used to determine toxicity of BSP degradation products. The results of the toxicity were produced.[14]

Pharmacological activity prediction of alkali degradation product

More than 3500 different pharmacological activities are evaluated by PASS Online, including pharmacological effects, routes of action, hazardous and undesirable consequences, interactions, etc. The basis for predictions is the investigation of the structure-activity relationship for almost 250,000 physiologically active molecules, including medicines, drug candidates, leads, and hazardous chemicals. The robustness of the PASS algorithm was proved in special tests utilizing the primary compound from the MDDR database, which contained 18977 compounds and 124 activities. In 50 random divisions into two equal subsets of the compounds were made. For a total of 100 experiments, training and evaluation set is used. From the training set, 20, 40, 60, and 80% of the data (activity/ structure data) were at random excluded. The average accuracy of prediction (IAP) for each activity category was calculated. If Pa > 0.7, there is a high likelihood that the medication is an analogue of a well-known pharmacological agent as well as a high probability that it will show the activity in the experiment. The likelihood of the drug demonstrating the action in the experiment is lower if Pa is between 0.5 and 0.7 compared to other known pharmacological agents. The chemical is unlikely to show the experiment's action if Pa is less than 0.5. But if the experiment proves that this behavior exists, the compound might be a brand-new chemical substance.[15,16]

RESULTS AND DISCUSSION

Chromatographic Conditions for Analysis

Various mobile phases were used to properly retain BSP from the degradation products it produced. The retention of BSP was adequate at 4.599 minutes with acceptable system suitability when the stationary phase ODS C18 column used with a mobile phase of acetonitrile: potassium phosphate buffer (PH 8.0) (60:40, v/v) at flow rate of 1.2 mL/min. The excipients and degradation products that had formed could be easily separated from the peak of BSP. At the ideal wavelength of 225 nm, all eluents were detected. Figure 2 displays the bisoprolol chromatogram with optimized chromatographic conditions.

Forced Degradation Studies

BSP degradation was observed when exposed to conditions that contained acid, alkali, peroxide-mediated oxidative degradation, and heat. It was discovered that the peak area of BSP reduced in conjunction with the generation of DPs under

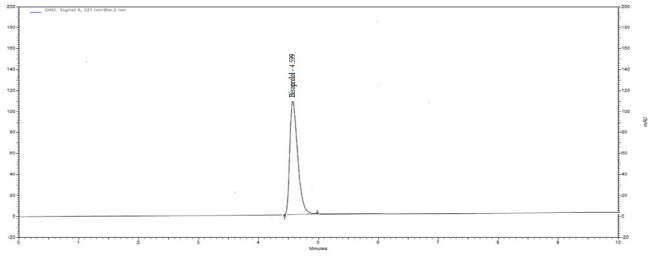

Figure 2: The chromatogram of bisoprolol with optimized chromatographic condition

Table 1: Summary of forced degradation behavior of BSP

Sr. no	*Forced degradation conditions*	*Retention time (Min)*	*% degradation*
1	Acid degradation - 0.1N HCl at 70°C for 6 days.	4.599, 6.511, 7.985	8.00
2	Alkali degradation at 70°C for 6 days in 0.1N NaOH.	4.599, 7.985	7.00
3	Oxidative degradation - at ambient temperature for 48 hr in 30 % H_2O_2.	4.594, 6.994,	6.50
4	Wet heat degradation to 70°C for 48 hours.	4.599, 7.512	5.50
5	Photolytic degradation 1.2 million lux hours of visible light and 400 W*hr/m^2 UV radiation	4.599	No degradation
6	Dry heat- 70°C for 48 hours	4.695	No degradation

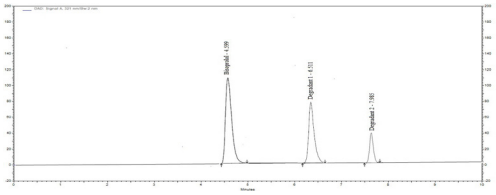

Figure 3: Chromatogram of acid degradation for 5 days of bisoprolol exposure to 0.1 N HCl at 70°C

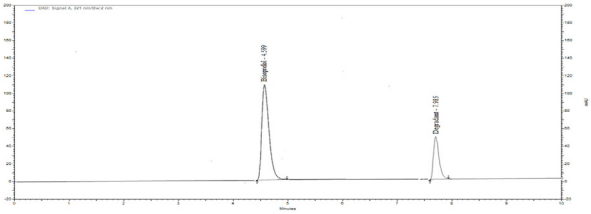

Figure 4: Chromatogram of alkali degradation for 6 days of bisoprolol exposure 0.1 N NaOH at 70°C

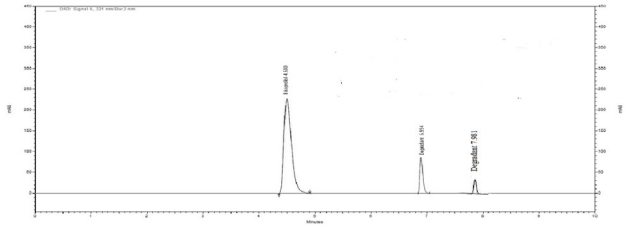

Figure 5: Chromatogram of oxidative degradation of bisoprolol exposed to 30% H_2O_2 at ambient temperature for 48 hours

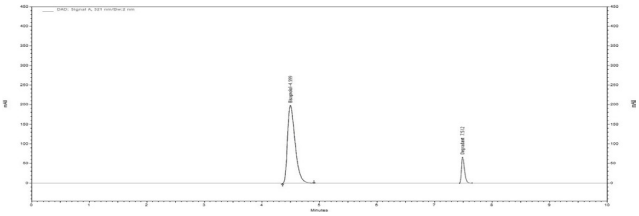

Figure 6: Chromatogram of Wet heat degradation of Bisoprolol exposed to 70°C for 48 hours

hydrolytic, oxidative, and thermal. However, under photolytic and humidity circumstances, the peak area of BSP decreased without producing any degradation products. Table 1 and Figures 3-6 show an overview of BSP's degradation behavior under various forced degradation studies.

Characterization of degradation Product by LC-MS

Acid degradation

Degradation products (DPs) produced during the acid degradation experiment showed how sensitive bisoprolol is to acid treatment. Using MS data, high concentrations of impurity A and other DPs were detected. The acid-degradant molecular ion peak was found at m/z 257 with specific fragment ions at m/z 239, 182, 133 and 180 in MS2. Mass spectra of the degradation product of acid was shown in Figure 7 and the fragmentation pathways in Figure 11.

Alkali degradation

High amounts of impurity A were identified using MS data. The protonated molecular ion of impurity A was detected at m/z 239 and specific fragments ions at m/z 222, 180, 164 and 134 in MS2. The alkali degradation product's mass spectra were shown in Figure 8; the fragmentation pathways are in Figure 12.

Oxidative degradation

High amounts of degradation identified using MS data. The molecular ion peak of oxidative degradation was found at m/z 237 with specific fragments ions at m/z 219, 162 and 133 in MS2. The oxidative degradation product's mass spectra were shown in Figure 9 and the fragmentation pathways in Figure 13.

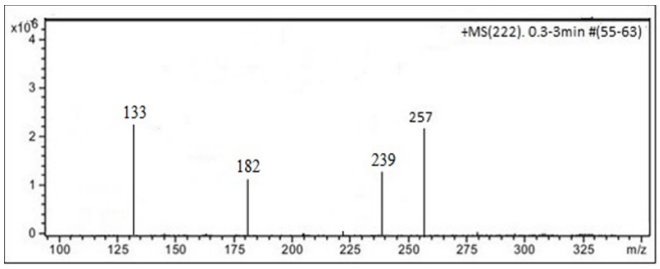

Figure 7: BSP acid degradation product mass spectrum.

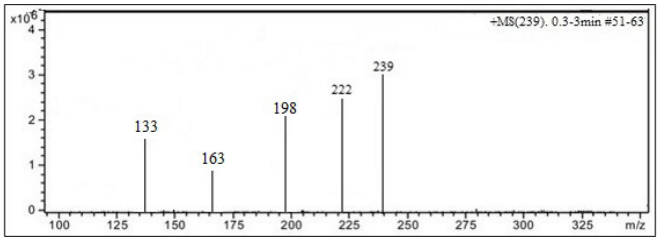

Figure 8: BSP alkali degradation product mass spectrum

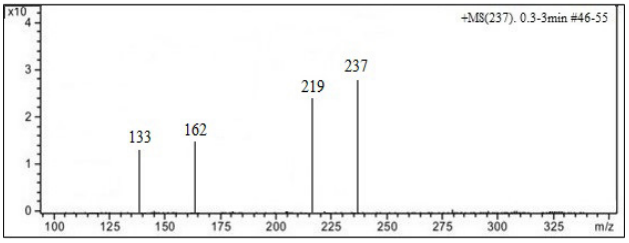

Figure 9: BSP Oxidative degradation products Mass Spectrum

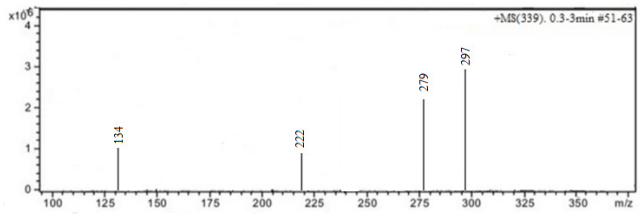

Figure 10: BSP thermal (Wet) degradation products Mass Spectrum

Thermal degradation

High amounts of degradation were identified using MS data. The protonated molecular ion peak of thermal (wet heat) degradation was detected at m/z 297 with specific fragments ions at m/z 279, 222 and 134 in MS2. Mass spectra of the thermal degradation product was shown in Figure 10 and the fragmentation pathway are in Figure 14.

Method validation

Table 2 provides a summary of the findings from the precision and accuracy experiments. The accuracy of the suggested procedure is demonstrated by the fact that the mean amount found was determined to be relatively near the sum that was

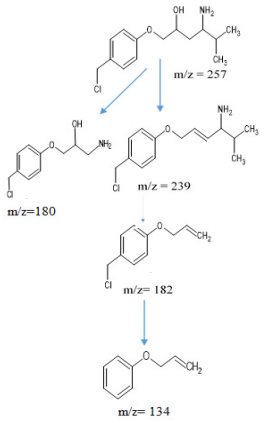

Figure 11: Fragmentation pattern of acid degradation product

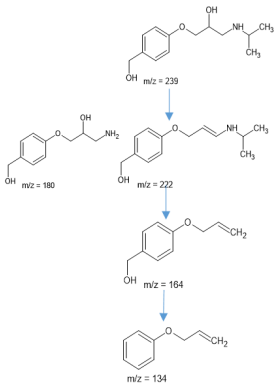

Figure 12: Fragmentation pattern of alkali degradation product

Table 2: Data of Accuracy and precision

Quantity Added	*Quantity Found (µg/mL)*			*Within mean square*	*Between mean square*	*F value*
	Day 1	*Day 2*	*Day 3*			
80% (10.8 µg/mL)	10.614	10.614	10.636	0.000216	0.000198	0.9182
	10.614	10.615	10.650			
	10.615	10.615	10.614			
Mean	10.614	10.614	10.633			
Recovery (%)	96.9	96.9	97.16			
SD	0.0005	0.0005	0.0181			
%RSD	0.0054	0.0054	0.1706			
100% (12.0 µg/mL)	11.803	11.824	11.840	0.0000809	0.000352	4.347
	11.807	11.831	11.847			
	11.820	11.820	11.833			
Mean	11.81	11.825	11.84			
Recovery (%)	96.83	97.08	97.33			
SD	0.0088	0.0055	0.007			
%RSD	0.0751	0.0470	0.0591			
120% (13.2 µg/mL)	13.237	13.236	13.201	0.0000761	0.000483	6.3435
	13.201	13.222	13.215			
	13.187	13.216	13.230			
Mean	13.208	13.224	13.215			
Recovery (%)	100.13	100.4	100.5			
SD	0.0257	0.0102	0.0145			
%RSD	0.195	0.0775	0.1097			

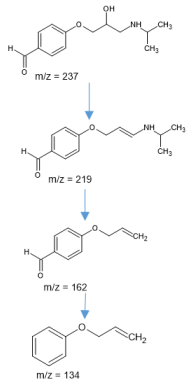

Figure 13: Fragmentation pattern of Oxidative degradation product

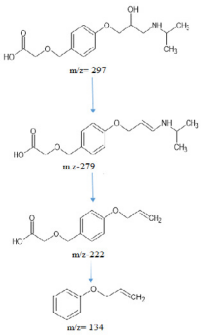

Figure 14: Fragmentation pattern of Thermal degradtion products

contributed. Low readings of %RSD demonstrate that the technique is accurate at each QC level on each day. The F values at each levels observed will less than the F (theoretical) [F (2,3) = 9.55 at = 0.05] when the data obtained from precision were used to calculate the analysis of variance and value prove that intermediate precision are within given calibrated range. The DL and QL were found to be, respectively, 1.16 and 3.5 µg/mL when they were calculated using the response of SD and calibration curve slope. Because the chromatographic parameters were optimized for the %of organic content, flow rate, and detection wavelength, the BSP peak exhibited appropriate system suitability, indicating the technique's versatility. When the BSP were compared before and after being subjected to the specified forced degradation circumstances, there was no interference peak to be seen at the retention time of BSP. Furthermore, the specificity of the method depends on the separation of BSP degradation products.

Degradation products toxicity is determined by using in-silico approach

Acid degradant toxicity data reveal the Cramer Rule in high toxic (Class III), class III (unspecific reactivity), low probability of lifetime cancer, and class III (unspecific reactivity). Structural modification for *S. typhimurium* mutagenicity and class I (easily biodegradable substance), non for nongenotoxic carcinogenicity, and structural modification for genotoxic carcinogenicity. Degradant of alkali or impurities a toxicity test indicates class I low toxicity, class III general reactivity, and genotoxic carcinogenicity is absent, non-

Table 3: Pharmacological activity of alkali degradant

Pa	*Pi*	*Activity*
0.831	0.005	Regulation of lipid metabolism
0.705	0.004	Anesthetic
0.679	0.003	Local anesthetic
0.670	0.035	Fibrinilytic
0.602	0.006	Ophthalmic drug

genotoxic carcinogenicity negative, There is no structural alert for *S. typhimurium*, class I (easily biodegradable product) mutagenicity. Results of oxidative degradant toxicity indicate Class I low toxicity, class III general reactivity, and negligible danger, minimal lifetime cancer risk, negative for nongenotoxic carcinogenicity, structure with genotoxic carcinogenic warning *S. typhimurium* structural alert, class I (easily biodegradable substance).

Pharmacological activity prediction of alkali degradation product

PASS predicted some novel activities for these alkali degradation compounds in addition to the recognized antihypertensive activities. Predicted a possible activity of Alkali degradant for the treatment of regulation of lipid metabolism, anesthetic, local anesthetic, fibrinolytic and ophthalmic drug. Table 3 shows Pa and Pi score of alkali degradation products.

CONCLUSION

The estimate of BSP in bulk formulation with its produced degradation products has been developed and validated. The drug and its degradation products were separated on ODS-C18 column and acetonitrile and phosphate buffer (pH 8) (60:40 v/v) at a 1.2 mL/min flow rate. Each eluent was distinguished at a wavelength of 225 nm. However, it was found that the BSP remained stable in the presence of dry heat and photolysis while degrading in the presence of hydrolysis, wet heat, and peroxide-treated oxidative. Under the mentioned chromatographic conditions, the drug was determined to be liner at 06 to 14 µg/mL. The degradation product formed in FDS was characterized by using mass spectrometry. The detected acid degradation product was found to be very harmful, according to the *in-silico* toxicity prediction, while alkali and oxidative degradation showed minimal toxicity. Further biological activity prediction using PASS shows that alkali degradants show various pharmacological activity apart from antihypertensive.

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