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RP-HPLC method for simultaneous estimation of bisoprolol fumarate and hydrochlorothiazide in tablet formulation

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ABSTRACT

A simple, precise and stability-indicating HPLC method was developed and validated for the simultaneous determination of bisoprolol fumarate and hydrochlorothiazide in pharmaceutical dosage form. The method involves the use of easily available inexpensive laboratory reagents. The separation was achieved on an Inertsil ODS 3V (25 cm × 4.6 mm) 5 μm column with isocratic flow. The mobile phase at a flow rate of 1.0 mL min^{-1}, consisted of 0.1 M potassium dihydrogen phosphate buffer and acetonitrile (70:30, v/v). The UV detection was carried out at 228 nm. A linear response was observed over the concentration range 2.5–50 μg mL^{-1} of bisoprolol fumarate and the concentration range 6.25–125 μg mL^{-1} of hydrochlorothiazide. Limit of detection and limit of quantitation for bisoprolol fumarate were 0.01 and 0.03 μg mL^{-1}, respectively and for hydrochlorothiazide were 0.01 and 0.05 μg mL^{-1}, respectively. The method was successfully validated in accordance to ICH guidelines acceptance criteria for specificity, linearity, accuracy, precision, robustness, ruggedness and system suitability. Individual drugs (bisoprolol fumarate and hydrochlorothiazide), their combinations and the tablets were exposed to thermal, photolytic, hydrolytic and oxidative stress conditions. The resultant stressed samples were analyzed by the proposed method. The method gave high resolution among the degradation products and the analytes. The peak purity of analyte peaks in the stressed samples was confirmed by photodiode array detector. The method was used for accelerated stability study on marketed and in-house formulations. The analysis concluded that the method was selective for simultaneous estimation of bisoprolol fumarate and hydrochlorothiazide and was stability-indicating.

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1. Introduction

The parent guideline on drug stability testing Q1A (R2) issued by International Conference on Harmonization (ICH) [1] stipulates stress studies to be carried out on a drug in order to establish the drug's inherent stability characteristics. These stress studies can help in the identification of degradation products and support the suitability of the proposed analytical procedures. According to the guideline, analytical test procedures for stability samples should be stability-indicating and fully validated.

The aim of the present study, in accordance with the guideline, was to establish inherent stability of bisoprolol fumarate and hydrochlorothiazide through stress studies under a variety of ICH recommended test conditions [1,2] in order to develop a stability-indicating assay method [3]. For this study, beta-blocker bisoprolol fumarate (BF) and the diuretic hydrochlorothiazide (HZ) were used. The combination of these drugs, available as film coated tablets, is used in the therapy to treat high blood pressure.

Literature studies show various analytical methods reported for the estimation of HZ in biological fluids and for pharmaceutical formulations [4–7]. Several methods like HPLC with fluorescence detection, capillary liquid chromatography and liquid chromatography–tandem mass spectrometry (LC–MS/MS) are reported for the determination of BF in plasma [8–11]. Many analytical methods to quantify the combination of BF and HZ were reported by spectrophotometry [12,13], HPTLC [14] and HPLC [15]. None of these reports provide a stability-indicating method for BF and HZ.

The United States Pharmacopeia (USP) prescribes an HPLC method for the assay of BF and HZ tablets [16] using L11 packing and aqueous dibutyl ammonium phosphate with acetonitrile as an eluent using a gradient mode. For standard and sample preparation

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by this USP method, a mechanical stirring for 1 h and a sonication for 10 min is required which makes the method time consuming, expensive, cumbersome and tedious. An attempt was made in this study to develop a rapid, economical, precise and accurate stability-indicating assay method for simultaneous estimation of BF and HZ in tablet formulation in accordance with the ICH guidelines [17].

2. Experimental

2.1. Instrument and chromatographic conditions

Integrated HPLC system, Waters Alliance manufactured by Waters Corporation (Milford, USA) was used for method development, forced degradation and method validation. This system comprised of a ternary gradient pump and autosampler (2695 Separation module), column oven and a photodiode array detector (2998). PC installed Empower software, Version 2.6 was used to record and integrate the chromatograms.

Isocratic mobile phase consisted of 0.1 M potassium dihydrogen phosphate buffer and acetonitrile in the ratio of (70:30, v/v). A membrane filter of 0.45 μm porosity was used to filter and degass the mobile phase. Inertsil ODS 3V (250 mm × 4.6 mm, 5 μm) analytical column from GL Science, Tokyo, Japan was used as a stationary phase. The flow rate was 1.0 mL min^{-1} and the detector was set at 228 nm. The volume of the sample solution injected was 20 μL. The analysis was carried out at ambient temperature. Water bath of Thermo constant temperature (Mumbai, India) was used for solution degradation and dry oven was used for solid state thermal stress study. A walk-in stability chamber, from Newtronic (Mumbai, India), was used for stability studies. Photostability studies were performed in a photostability chamber, from Newtronic (Mumbai, India). The photostability chamber equipped with light bank comprising of two UV and four fluorescent lamps provided an overall illumination of not less than 1.2 million lx h and an integrated near ultraviolet energy of not less than 200 Wh m^{-2}, in compliance with Option 2 of ICH guideline Q1B [18]. Centrifuge (Eltec, Mumbai, India) and Whatman filter paper number 1 and glass-fiber filters (GF/C) were used to clear the sample solutions.

2.2. Materials and reagents

BF was purchased from Unichem Laboratories (Mumbai, India). BF related impurities B1, B3 and B4 were obtained from LGC Promochem (Bangalore, India). HZ and its related impurities H1, H2 were obtained from Ipca Laboratories Ltd. (Mumbai, India). Analytical reagent grade sodium hydroxide pellets, hydrochloric acid, potassium dihydrogen phosphate (KH_2PO_4), hydrogen peroxide (H_2O_2) and HPLC grade acetonitrile (ACN) were procured from Merck (Darmstadt, Germany). A Millipore Milli Q plus water purification system (Milford, USA), was used to prepare distilled water (>18 μΩ). Test samples, composed of BF 2.5 mg and HZ 6.25 mg per tablet, from in-house formulations and purchased from the local market (LODOZ 2.5, Merck, Mumbai, India), were used for the study.

2.3. Solution preparation

2.3.1. BF and HZ standard stock solution

BF (250 μg mL^{-1}) and HZ (625 μg mL^{-1}) standard stock solution was prepared by transferring approx 25 mg of BF and 62.5 mg of HZ reference standards to a 100 mL volumetric flask. A 50 mL diluent (water:acetonitrile, 70:30, v/v) was added. It was then sonicated for 2 min. The solution was diluted up to the volume with the diluent.

2.3.2. BF and HZ test stock solution

Ten whole tablets were weighed and disintegrated by shaking for 5 min with 10 mL water in a 100 mL volumetric flask. 30 mL acetonitrile was added. It was sonicated for 10 min and water was added to make up the volume in the flask.

Table 1
Results of forced degradation study.

Stress studies	Degradation condition	Imp B1	Imp H1	Assay HZ	Assay BF	Peak purity[a] of HZ peak	Peak purity[a] of BF peak
Acid hydrolysis	BF unexposed	ND	ND	NA	100.9	NA	Pure
	HZ unexposed	ND	ND	100.1	NA	Pure	NA
	BF + HZ unexposed	ND	ND	99.9	100.6	Pure	Pure
	BF 0 min	2.1	ND	NA	92.8	NA	Pure
	HZ 0 min	ND	3.2	97.5	NA	Pure	NA
	BF + HZ 0 min	2.2	1.9	99.1	97.6	Pure	Pure
	BF 30 min	41.2	ND	NA	49.9	NA	Pure
	HZ 30 min	ND	12.1	87.8	NA	Pure	NA
	BF + HZ 30 min	72.4	10.4	90.3	31.9	Pure	Pure
	BF 60 min	87.2	ND	NA	13.9	NA	Pure
	HZ 60 min	ND	25.5	74.9	NA	Pure	NA
	BF + HZ 60 min	79.4	24.8	70.3	13.1	Pure	Pure
	Tablet unexposed	ND	0.04	101.2	100.3	Pure	Pure
	Tablet 0 min	0.2	1.0	99.0	100.3	Pure	Pure
	Tablet 60 min	98.5	23.7	77.0	2.82	Pure	Pure
Alkaline hydrolysis	BF 0 min	0.6	ND	NA	96.8	NA	Pure
	HZ 0 min	ND	0.2	99.1	NA	Pure	NA
	BF + HZ 0 min	0.1	0.2	99.6	98.4	Pure	Pure
	BF 30 min	8.9	ND	ND	94.1	NA	Pure
	HZ 30 min	ND	0.4	98.2	NA	Pure	NA
	BF + HZ 30 min	4.8	1.1	98.1	96.6	Pure	Pure
	BF 60 min	84.9	ND	NA	16.0	NA	Pure
	HZ 60 min	ND	3.2	95.6	NA	Pure	NA
	BF + HZ 60 min	81.2	5.6	95.1	18.9	Pure	Pure
	Tablet 0 min	15.2	1.6	98.1	85.6	Pure	Pure
	Tablet 60 min	63.6	12.2	88.9	33.3	Pure	Pure
Oxidation	Tablet 4 h	30.4	17.2	82.6	69.9	Pure	Pure
Photostability	Tablet	ND	0.2	100.8	98.8	Pure	Pure
Thermal	Tablet	ND	0.4	99.2	98.2	Pure	Pure

ND: not detected; NA: not applicable; BF: bisoprolol fumarate; HZ: hydrochlorothiazide.
[a] Peak pure if peak angle is less than peak threshold.

Each stock solution was further diluted 10 times, with the diluent, to produce reference standard and test solutions containing BF (25 μg mL^{-1}) and HZ (62.5 μg mL^{-1}).

3. Method development

A variety of mobile phases were investigated in the development of a stability-indicating LC method for the analysis of BF and HZ in tablet dosage form. The suitability of mobile phase was decided on the basis of selectivity and sensitivity of the assay, stability studies and separation among impurities formed during forced degradation studies.

3.1. Forced degradation study

Forced degradation study was conducted on samples containing individual drugs, their combination and on tablets. Intentional degradation was carried out by exposing 10 mL of reference/test stock solution to 20 mL of 0.25N hydrochloric acid/sodium hydroxide for 60 min at 60 °C using a water bath. The solutions were withdrawn in a 10 mL volumetric flask, allowed to attain room temperature and then neutralized with acid or base (when necessary).

Oxidative degradation of sample solution was conducted on a water bath maintained at 60 °C for 4 h, by exposing equal volumes of standard/test stock solution and 10% hydrogen peroxide solution in a 10 mL volumetric flask. The solution was allowed to attain ambient temperature and diluted to mark with water.

For thermal stress study, the solid drug and tablets were kept in dry oven at 60 °C for 15 d.

Photolytic studies were carried out on solid drugs, their combination and their dosage form. The sample in a petri plate was spread as a thin layer (1 mm) and exposed to light in a photostability chamber.

Blank solutions were prepared by the aforementioned procedure wherein stock solutions were replaced with the diluent.

The method's analytical data were collected at a single wavelength of 228 nm. Additional PDA detector data were collected for the peak purity evaluation.

4. Method validation

The optimized chromatographic conditions were validated by evaluating specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability parameters in accordance with the ICH guideline Q2 (R1) [17].

4.1. Linearity

Standard stock solution of the drug was diluted to prepare linearity standard solutions in the concentration range of 2.5–50 μg mL^{-1} BF and 6.25–125 μg mL^{-1} HZ. For determination of the limits of detection and quantification based on the standard deviation of the response and slope as per ICH guidelines. The standard stock solution was diluted in the range of 0.025–2.5 μg mL^{-1}

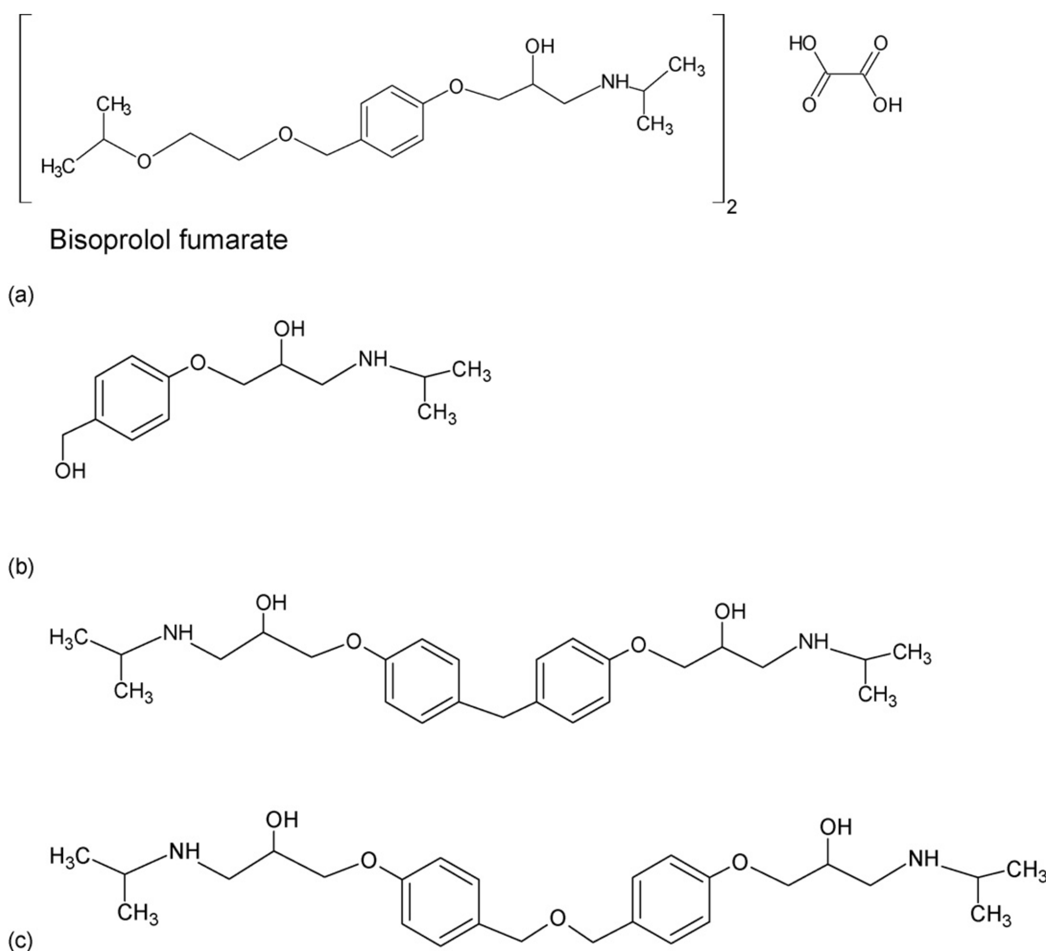

Fig. 1. Chemical structures of bisoprolol fumarate, its hydrolytic degradant: (a) impurity B1, and other impurities, (b) impurity B3, and (c) impurity B4.

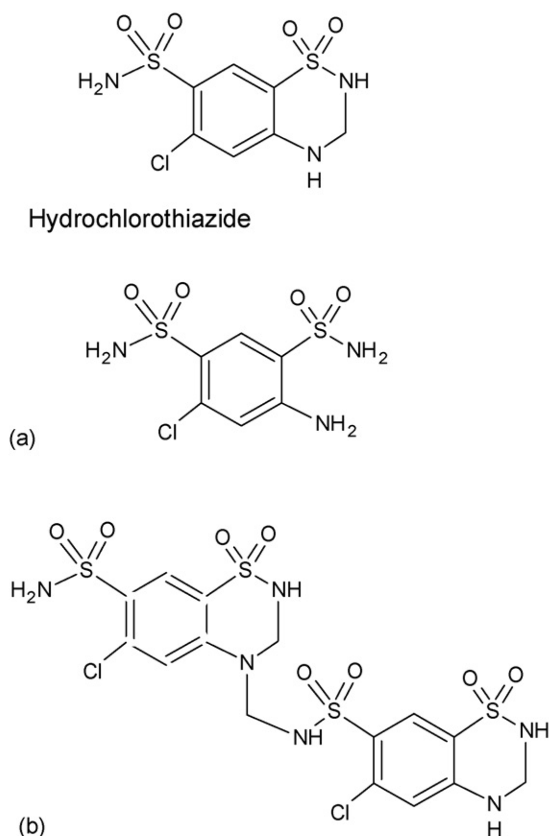

Fig. 2. Chemical structure of hydrochlorothiazide, its hydrolytic impurity: (a) impurity H1 and (b) impurity H2.

BF and 0.0625–6.25 μg mL^{-1} HZ. Three sets of such solutions were prepared. Each set was analyzed to plot a calibration curve. Standard deviation (SD), slope, intercept and coefficient of determination (r^2) of the calibration curves were calculated to ascertain linearity of the method.

4.2. Recovery

Recovery of the method was determined by spiking the marketed sample with 80%, 100% and 120% standard solutions. These mixtures were analyzed by the proposed method. The experiment was performed in triplicate and recovery (%), RSD (%), bias (%) and standard error of mean (SEM) of spiked drugs were calculated.

4.3. Precision

The precision of the proposed method was evaluated by carrying out six independent assays of test sample. RSD (%) of six assay values obtained was calculated. Intermediate precision was carried out by analyzing the samples by a different analyst on another instrument.

4.4. Limit of detection and limit of quantification

The detection and quantification limits were evaluated from calibration curves plotted in concentration ranges of 0.025–2.5 μg mL^{-1} BF and 0.0625–6.25 μg mL^{-1} HZ. The acceptance criterion for these replicate injections was RSD not more than 30% for LOD concentration and not more than 10% for LOQ concentration.

The formulae used were LOD = 3.3σ/S and LOQ = 10σ/S (where σ = standard deviation of response and S = slope of calibration curve). The standard drug solutions, for each value of LOD and LOQ concentration were injected 6 times. % RSD values for the area of replicate injections were calculated.

4.5. Robustness and system suitability

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by ±0.2 mL min^{-1}), mobile phase composition (acetonitrile ± 7%), buffer pH (altered by ±0.2) and use of LC columns from different batches. These chromatographic variations were evaluated for resolution between impurity H1 and HZ in a system suitability solution with respect to retention time R_T and % assay of drugs. The filter compatibility was studied by comparing % assay of test solution filtered through various filters such as Whatman 1 and GFC vis-á-vis test solution clarified by centrifugation.

4.6. Solution stability

To assess the solution stability, standard and test solutions were kept at 25 °C (laboratory temperature) for 24 h. These solutions were compared with freshly prepared standard and test solutions.

4.7. System suitability

The system suitability parameters with respect to theoretical plates, tailing factor, repeatability and resolution between impurity H1 peak and HZ peak were defined.

5. Results and discussion

5.1. HPLC method development

The maximum absorption wavelength of the reference drug solution and of the forcefully degraded drug solution was found to be 228 nm. This was observed from the UV absorption spectra (Fig. 4) and was selected as detection wavelength for LC analysis. The main objective of this chromatographic method was separation of degraded impurities from both the drugs. Forced degradation study revealed a critical separation of closely eluting impurity H1, formed from the HZ peak. This impurity co-eluted with HZ, in void volume, when stationary phases C 18, C 8, phenyl and cyano were used with some mobile phases (different ratios of acetonitrile with ammonium phosphate, 0.1% orthophosphoric acid solution). Inertsil ODS 3V column helped in retaining HZ peak as the column had

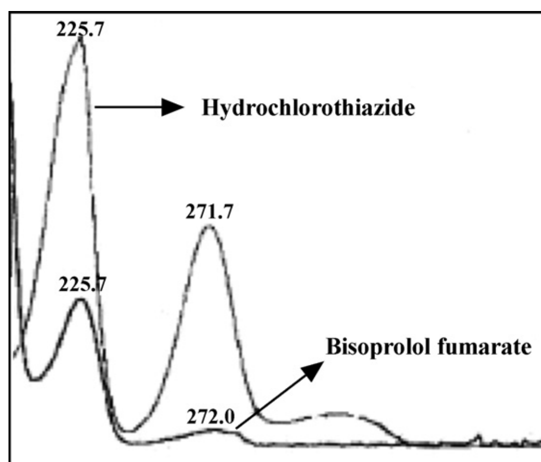

Fig. 3. Overlay spectra of the two drugs.

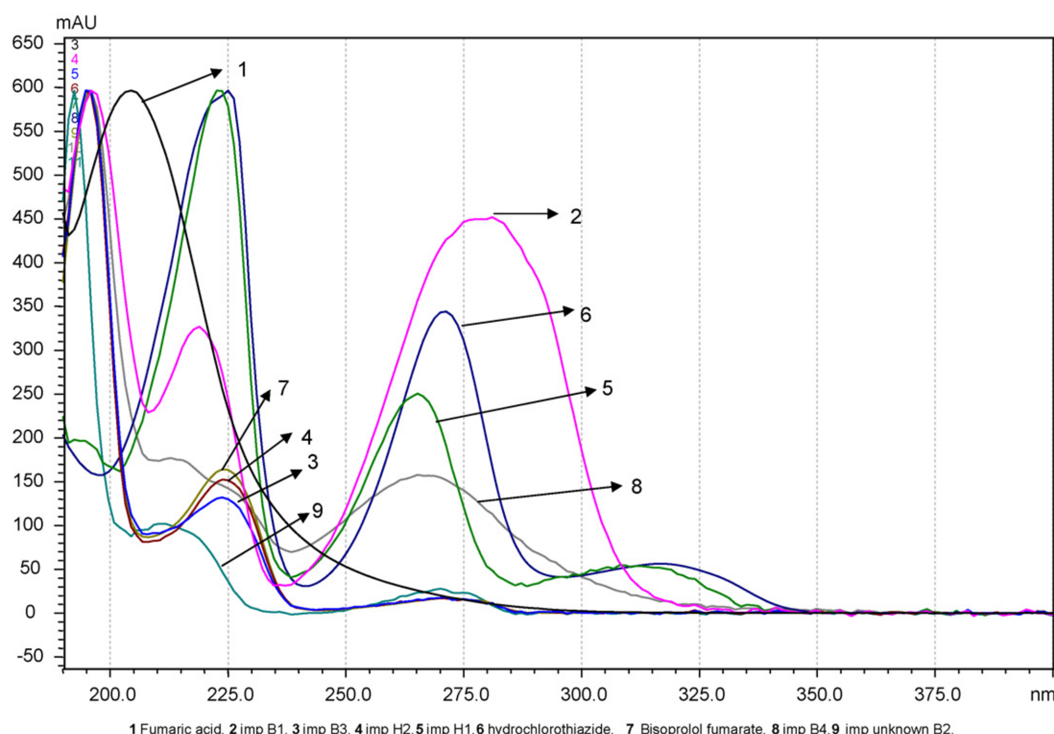

Fig. 4. Spectra of impurities and drug.

higher carbon loading approx 15% against conventional ODS. This effect was observed by using the mobile phase 0.1% orthophosphoric acid (pH 2.2) and acetonitrile in the ratio of 80:20 (% v/v). However, the HZ peak revealed from the PDA analysis, was not pure which suggested co-elution of some impurity peak(s).

Increasing the pH of mobile phase to 4.5 using 0.1 M potassium dihydrogen phosphate helped to sharpen the HZ peak, probably due to increase in hydrophobic interactions between stationary phase and less unionized analyte. BF showed no significant change in retention with change in composition. After several trials, using 25 cm × 4.6 mm, 5 μm Inertsil ODS 3V column, the mobile phase, consisting of buffer 0.1 M potassium dihydrogen phosphate (pH 4.5) and acetonitrile (70:30, % v/v), at flow rate 1.0 mL min^{-1} gave sharp and well resolved peaks of both the drugs. The satisfactory separation of impurities H1, H2 and impurities B1, B3, B4 from BF and HZ was observed with the help of the aforementioned chromatographic conditions. The set up gave good resolution of 2.67 of HZ from its impurity H1, symmetry of about 1.25 for bisoprolol peak and 1.14 for HZ and low R_T (5.601 min for HZ and 6.616 min for bisoprolol) reducing the overall run time to 15 min (Fig. 5a and b). Since BF is highly soluble in water and HZ is practically insoluble, mixture of water:acetonitrile 70:30 (% v/v) was confirmed for use as this helped in easy extraction of both the analytes from the whole tablets.

5.2. Results of forced degradation studies

Subsequently, different forced degradation samples were analyzed. Both the drug peaks in acid, alkaline, oxidation, thermal and photo-degraded solutions passed the purity test (Table 1). Results of forced degradation study showed that impurity B1 was formed as a result of hydrolysis of BF during acidic and alkaline stress studies (Fig. 1a). Unknown impurity B2 was formed during acidic hydrolysis (Fig. 1b) and impurities B3, B4 were not formed during the forced degradation (Fig. 1c–e). Similarly forced degradations results revealed that impurity H1 of HZ was hydrolytic degradation product (Fig. 2a) and impurity H2 was formed as a result of prolonged exposure to hydrolysis for more than 6–8 h under reflux conditions at 60 °C (Fig. 2b) (Figs. 3 and 4). Drug solutions, in combinations, showed similar pattern of degradation, except in acid hydrolysis. An unknown impurity, whose spectra resembled that of BF, was formed at rrt 1.87 (Fig. 5a–g). Mass balance (% assay + % degradants + % impurities) was calculated for each stress sample, average mass balance was found to be 100.6% for bisoprolol and 99.8% for HZ.

5.3. Method validation

The calibration plot for the method was linear over the concentration range of 2.5–50 μg mL^{-1} for BF and 6.25–125 μg mL^{-1} for HZ. The determination coefficients (r^2) were 0.9996 and 0.9998 for BF and HZ, respectively. Values of recovery (%), RSD and standard error of mean (SEM), indicating the method accuracy, are listed in Table 2. For precision study, % RSD of BF was about 0.34 and the value for HZ was about 0.54. RSD (%) in intermediate precision study was about 0.28 for BF and 0.85 for HZ. The % RSD results of precision and intermediate precision for both the drugs were within 2.0%, confirming good precision of the developed analytical method. The LOD and LOQ of BF using calibration curve in the range of 0.025–2.5 μg mL^{-1} were 0.01 and 0.03 μg mL^{-1}, respectively, while those of HZ using calibration curve in the range of 0.0625–6.25 μg mL^{-1} were 0.01 and 0.05 μg mL^{-1}, respectively. RSD (%) of six replicate injections of BF at LOD (0.01 μg mL^{-1}) and LOQ (0.03 μg mL^{-1}) were 15.28 and 3.79, respectively. Similarly % RSD of six replicate injections of HZ at LOD (0.01 μg mL^{-1}) and LOQ (0.05 μg mL^{-1}) were 17.93 and 4.53, respectively. These values indicated that the method was very sensitive to quantify both the drugs (Table 2).

Robustness study, conducted by deliberate changes in pH of buffer, mobile phase composition, flow rate and different batches of column, revealed that there was no significant variation in % assay, retention time R_T, tailing factor and resolution (Table 3). The stud-

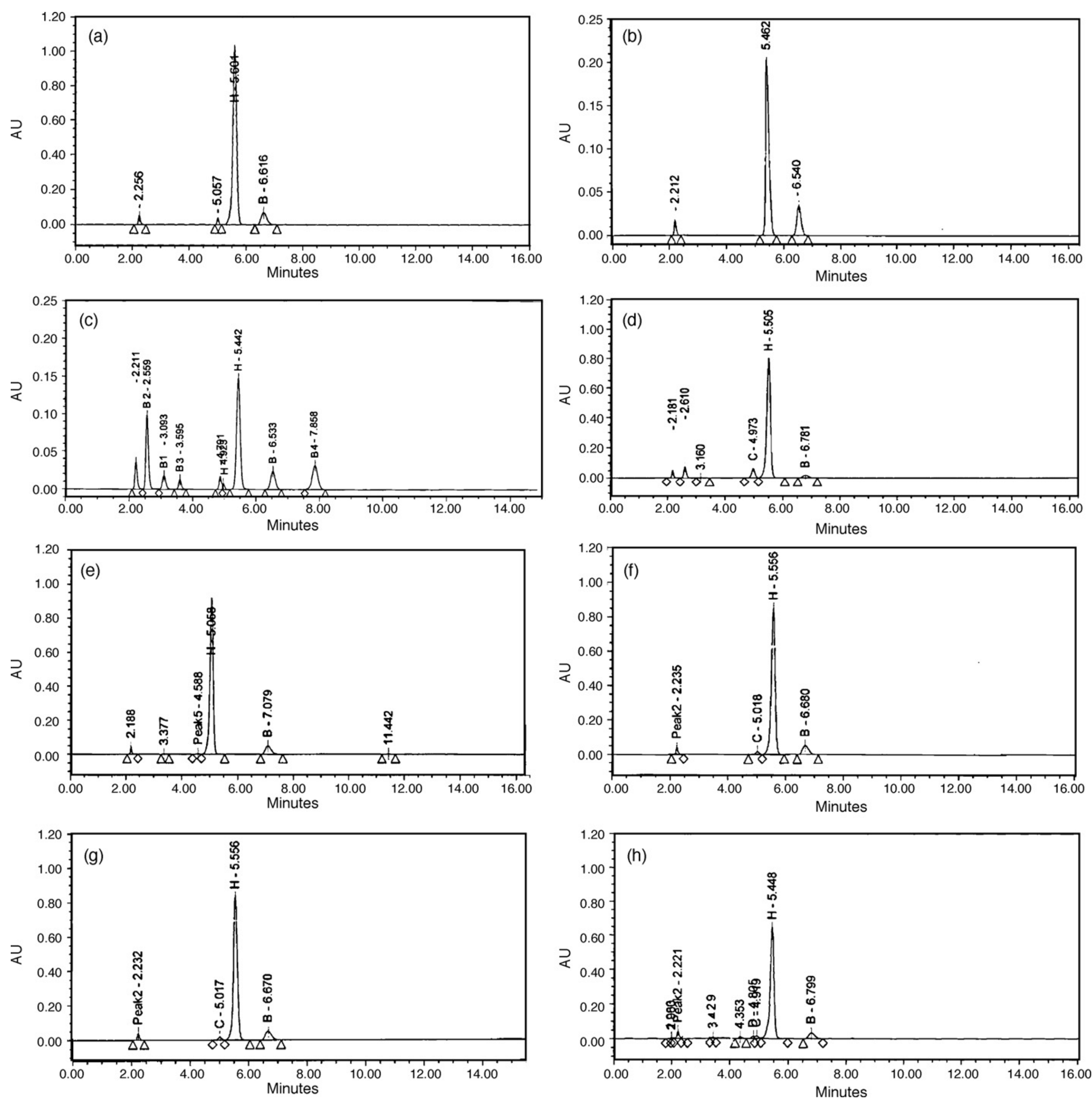

Fig. 5. Chromatograms of (a) system suitability (fumaric acid R_T 2.256, impurity H1 R_T 5.057, hydrochlorothiazide R_T 5.601, bisoprolol R_T 6.616), (b) standard, (c) separation among impurities, (d) alkaline hydrolysis, (e) acidic hydrolysis, (f) thermal, (g) photostability, and (h) oxidation.

ied filters were found suitable for assay of drug product as there was no significant change in % assay values. Solution stability of reference and test solutions revealed the solutions were stable up to 10 h, after which 0.1% of the impurity H1 was formed. The method complied with limits stipulated by USP for system suitability (theoretical plate number, tailing factor, resolution and repeatability) for the analyte peaks (Table 4).

6. Discussion

In recent years, LC methods have been published for simultaneous analysis of BF and HZ in tablet dosage form [15] and a method is also described in USP [16]. The reported method [15] involved the use of cyano column and also stability-indicating nature was not explored. Sample preparation for composition of 5 mg BF and 12.5 mg HZ in a tablet involved a tedious procedure and many solvents, such as methanol, 0.1 M phosphate buffer, acetonitrile and tetrahydrofuran. Real time application was not studied for this method. USP method in terms of assay can be considered time consuming, expensive, cumbersome and tedious because the method involves gradient elution, elaborate sample preparation, use of corrosive reagent such as aqueous dibutyl ammonium phosphate and high flow rate of 3 mL min^{-1}.

Table 2
Summary of validation parameters.

Components	System suitability test		Precision				Linearity and range ($n=9$)	LOD	LOQ
			Repeatability ($n=6$)		Intermediate ($n=12$)				
	RSD of standard injections ($n=6$)	Resolution between impurities H1 and HZ	Mean% assay	RSD	Mean% assay	RSD	Coefficient of determination	$\mu g\ mL^{-1}$	$\mu g\ mL^{-1}$
Bisoprolol fumarate	0.14	–	100.2	0.34	100.4	0.28	0.9996 (2.5–50 $\mu g\ mL^{-1}$)	0.01	0.03
Hydrochlorothiazide	0.18	2.67	100.6	0.54	99.9	0.85	0.9998 (6.25–125 $\mu g\ mL^{-1}$)	0.01	0.05

Components	At 80% level ($n=3$)			At 100% level ($n=3$)			At 120% level ($n=3$)		
	%Recovery	% RSD	SEM	%Recovery	% RSD	SEM	%Recovery	% RSD	SEM
Bisoprolol fumarate	100.5	0.2	0.2	100.6	0.1	0.1	100.5	0.1	0.3
Hydrochlorothiazide	100.1	0.3	0.5	99.8	0.2	0.3	99.1	0.01	0.02

Table 3
Robustness study.

Studied parameters	Change in column		Change in pH			Change in ACN composition			Change in flow rate			Filter paper		
	Ideal 25 cm × 4.6 mm Inertsil ODS 3V	25 cm × 4.6 mm Zorbax ODS	Buffer in MP pH 4.3	Buffer in MP pH 4.5	Buffer in MP pH 4.7	−7% Buffer:ACN (72:28)	Ideal composition Buffer:ACN (70:30)	+7% Buffer:ACN (68:32)	0.8 ml/min	Ideal 1.0 ml/min	1.2 ml/min	Whatman 1	GFC	Centrifuge
% assay of bisoprolol fumarate	100.2	100.4	100.4	100.2	100.9	100.4	100.2	100.3	100.9	100.2	100.2	100.7	100.5	100.1
% assay of hydrochlorothiazide	100.6	99.2	101.1	100.6	99.4	100.3	100.6	101.0	100.0	100.6	100.8	100.6	99.8	100.3
Resolution between impurity H1 and hydrochlorothiazide	2.67	2.51	2.49	2.67	3.0	2.98	2.67	2.55	3.01	2.67	2.57	–	–	–

Table 4
Table for system suitability.

Components	Retention time in min (R_T)	Tailing factor	Theoretical plates/meter	% RSD	Resolution	Area
Fumaric acid	2.287	1.32	220,287	1.82	–	200,380
Impurity H1	5.046	1.27	30,705	0.78	16.35	393,786
Hydrochlorothiazide	5.628	1.14	38,085	1.1	2.67	7,959,095
Bisoprolol	6.916	1.25	43,585	0.67	4.84	788,784

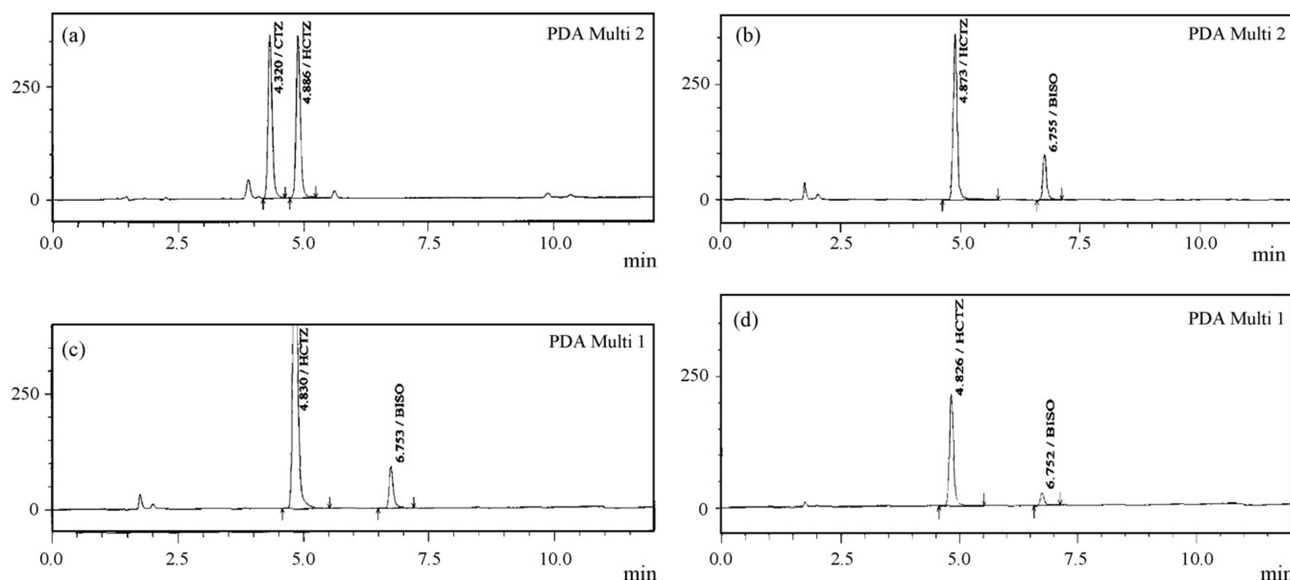

Fig. 6. Chromatogram of analysis of market sample as per US pharmacopeial method: (a) system suitability solution, (b) standard preparation, (c) bisoprolol fumarate assay preparation, and (d) hydrochlorothiazide assay preparation.

Comparative study of USP method and developed method was done by performing repeatability study on the market sample (Fig. 6a–d). Statistical evaluation of the obtained result showed no significant difference in terms of % assay, which was confirmed using student *t* test and *F* test (Table 5). The proposed method was found superior in terms of volume of mobile phase required for analysis, speed, easily available laboratory columns, reagents, flow rate, total time consumed for analysis, number of sample preparation steps and separation of degradation products (Table 6) (Fig. 6a–d).

6.1. Study of the stability of commercial tablets and in-house tablets

The assay contents of BF and HZ, commercially available and in-house formulated tablets were analyzed by the proposed method after exposure to accelerated storage conditions (i.e. 40 °C/75% RH, 30 °C/65% RH). The results were in the range of 101.4–100.4% for BF and 100.5–97.3% for HZ in marketed tablets at 40 °C/75% RH and 99.8–98.9% for BF and 100.4–97.4% for HZ in in-house formulated tablets at 40 °C/75% RH (Table 7). These results con-

Table 5
Statistical evaluation of results obtained from analysis of marketed formulation by proposed and US pharmacopeial methods.

	Assay value of HZ		Assay value of BF	
	Developed method	USP method	Developed method	USP method
	100.7	98.0	100.3	99.9
	100.1	100.8	100.4	100.1
	100.9	99.3	100.4	100.8
	100.8	99.4	100.2	100.0
	100.2	99.4	100.1	101.9
	100.7	100.5	99.9	101.4
Mean	100.6	99.6	100.2	100.7
SD	0.33	1.00	0.19	0.83
t test t_{tab} = 2.45 (at 95% probability level when n = 6)	t_{cal} = 2.33; $t_{cal} < t_{tab}$ shows values differ numerically but are statistically significant		t_{cal} = 1.34; $t_{cal} < t_{tab}$ shows values differ numerically but are statistically significant	
F test F_{tab} = 0.20 (at $n-1$)	F_{cal} = 0.11; $F_{cal} < F_{tab}$ shows that the difference is not significant		F_{cal} = 0.055; $F_{cal} < F_{tab}$ shows that the difference is not significant	

Table 6
Comparative parameters and results of assay of market sample (LODOZ 2.5) by USP and developed methods.

Parameter	Specifications as per USP	Developed isocratic method	USP pharmacopeial method
Resolution	Between Imp H1 and HZ NLT 1.5	2.67	3.1
Tailing factor	For the HZ peak is not more than 1.3	HZ = 1.06 BF = 1.08	HZ = 1.1 BF = 1.2
% RSD	For replicate injections is not more than 2.0%.	HZ = 0.14 BF = 0.18	HZ = 0.7 BF = 0.8
Assay	NLT 90–110% of labeled amounts of bisoprolol fumarate and hydrochlorothiazide	HZ = 100.6 BF = 104.2	HZ = 99.6 BF = 104.7
Column	L11 packing	Inertsil ODS 3V 25 cm × 4.6 mm, particle size 5 μm	Phenyl 10 cm × 8 mm, particle size 10 μm
Mobile phase	Flow rate	1 ml/min	3 ml/min
	Elution	Isocratic	Gradient
	Volume required to analyze six samples	About 600 mL	About 3000 mL
	Reagent	Easily available AR grade reagent	Costly, corrosive PIC D4 reagent (dibutylammonium phosphate)
Sample preparation time	–	About 7 min	About 1 h, 10 min
Total time consumed for analysis	–	Approx 4.5 h sample preparation (two injections each)	Approx 10–12 h sample preparation (four injections each)
Number of samples to be prepared including standard.	–	7 samples	13 samples

Table 7
Study of the stability of commercial and in-house tablets.

Duration	Market formulation LODOZ 2.5				In-house formulation			
	40 °C/75% RH		30 °C/65% RH		40 °C/75% RH		30 °C/65% RH	
	BF	HZ	BF	HZ	BF	HZ	BF	HZ
Initial control	101.4	100.5	101.4	100.5	99.8	100.4	99.8	100.4
1 month	101.2	99.4	100.8	999	98.7	98.9	99.4	99.2
3 months	100.6	98.2	100.7	98.8	98.9	98.1	99.2	98.4
6 months	100.4	97.3	100.1	98.2	98.9	97.4	98.7	98.1
9 months	Not applicable				Not applicable		98.6	97.9
12 months	Not applicable				Not applicable		98.4	96.1

firmed the use of the proposed method as a stability-indicating method.

7. Conclusion

The developed and validated LC method is stability-indicating and enables specific, accurate, robust and precise simultaneous analysis of bisoprolol and hydrochlorothiazide in tablet formulations. The method is sensitive enough for quantitative detection of the analytes in pharmaceutical preparations. The proposed method can thus be used for routine analysis, quality control and for studies of the stability of pharmaceutical tablets containing these drugs.

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