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A novel reversed-phase liquid chromatographic method for the simultaneous determination of potential impurities of bisoprolol fumarate and hydrochlorothiazide in a fixed dosage form

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ABSTRACT

In the present study 12 impurities of bisoprolol fumarate (BISO) and hydrochlorothiazide (HCTZ) were separated simultaneously in a single HPLC method. Out of these 12 impurities, five are potential degradants, which are validated as per The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. As the two active drug substances BISO and HCTZ have different solubilities and polarities, the most critical parameters in resolving the components from each other are pH, temperature, and solvents. The method is precise (RSD < 1.0%), accurate, linear ($r^2 > 0.999$), robust, and stability indicating in the range of limit of quantification (LOQ) to 150%. The HPLC method is then migrated to ultra-performance liquid chromatography (UPLC) to further reduce the run time and solvent consumption, and increase the sample throughput.

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KEYWORDS
Bisoprolol fumarate; HPLC; hydrochlorothiazide; potential impurities; simultaneous determination; UPLC

Introduction

Bisoprolol fumarate (BISO) and hydrochlorothiazide (HCTZ) tablet are indicated for the treatment of hypertension. The dosage form consists of two antihypertensive agents: one is a synthetic beta-selective (cardio-selective) adrenoceptor blocking agent (BISO) and the other is a benzothiadiazine diuretic (HCTZ). BISO is chemically described as (±)-1-[4-[[2-(1-methylethoxy) eth-oxy] methyl] phenoxy]-3-[(1-methylethyl) amino]-2-propanol (E)-2-butenedioate (2:1) (salt). Its empirical formula is $(C_{18}H_{31}NO_4)_2{\bullet}C_4H_4O_4$ having a molecular weight of 766.97. It possesses an asymmetric carbon atom in its structure and is provided as a racemic mixture. The S (-) enantiomer is responsible for most of the β-blocking activities. HCTZ is 6-chloro-3, 4-dihydro-2H-1,2,4-benzothiadia zine-7-sulfonamide 1,1-dioxide. HCTZ is a white, or practically white, practically odourless crystalline powder. It is slightly soluble in water, sparingly soluble in dilute sodium hydroxide solution, freely soluble in n-butylamine and dimethylformamide, sparingly soluble in methanol, and insoluble in ether, chloroform, and dilute mineral acids. Its empirical formula is $C_7H_8ClN_3O_4S_2$ and it has a molecular weight of 297.73.[1–3] The structures of BISO and HCTZ are shown in Fig. 1.

The BISO and HCTZ are official in the United States Pharmacopeia (USP) and European Pharmacopeia as a monotherapy and co-therapies.[4,5] The USP prescribes two chromatographic purity methods for the two actives and tablets dosage form. One is gradient separation using L11 packing and the other is an isocratic using L7 packing. These chromatographic purity procedures are inadequate to separate all 12 specified and unspecified impurities of BISO and HCTZ. Also the two methods monitor only two impurities separations. In the European Pharmacopoeia two separate HPLC methods are available for BISO impurities and HCTZ impurities. But these methods use more than one chromatographic procedure to determine all the impurities of BISO and HCTZ, which is very lengthy, time consuming, expensive, cumbersome, and tedious. A thorough literature survey revealed that one HPLC method was reported for the determination of bisoprolol and its conceivable impurities,[6] which employs a non-specific detection mode to determine the total assay, and its conceivable impurities. This method does not specify all the impurities of bisoprolol as listed in the European Pharmacopoeia. Another RP-HPLC method applicable for bisoprolol impurities was published by Yu Butao.[7] Several other publications like dissolution studies of bisoprolol in solid dosage form and determination of BISO in plasma samples are available for the determination of either bisoprolol or HCTZ alone or in combinations using various analytical techniques, like RP-HPLC, flourimetric, and TLC densitometry.[7–17] None of these reports provide the stability indicating method for the simultaneous determination

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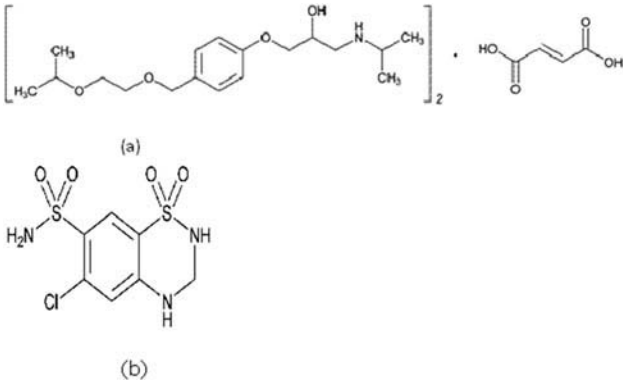

Figure 1. Chemical structures of (a) bisoprolol fumarate and (b) hydrochlorothiazide.

of impurities of bisoprolol and HCTZ in a combined dosage form due to the difficulty in separating the components of BISO and HCTZ, due to their different solubilities and polarities. To overcome these problems and to get a single separation, the present work is focused on the development of a single, selective, sensitive, and robust stability indicating HPLC method for the determination of five important specified and unspecified impurities of BISO and HCTZ simultaneously in a tablet formulation, using simple and inexpensive chemicals, solvents, and columns. The possible degradation impurities were also focused during the stress studies. Furthermore, the HPLC method was migrated to ultra-performance liquid chromatography (UPLC), to reduce the run time and increase the sample throughput.

The chemical structures of all the impurities of BISO and HCTZ are shown in Fig. 2 and their classification is shown in Table 1.

Experimental

Chemicals and reagents

Samples of bisoprolol and HCTZ tablets and placebo used were prepared at Mylan R&D Center (Hyderabad, India). Reference standards and impurity reference standards of BISO and HCTZ were procured from Unichem laboratories Ltd (Mumbai, India) and European pharmacopoeia. The analytical reagent-grade monobasic potassium phosphate used in the preparation of buffer solution was procured from Merck, India. The HPLC-grade acetonitrile and methanol were procured from Merck, India. Ortho-phosphoric acid used for adjusting the pH of the buffer solution was procured from Qualigens Fine Chemicals (Mumbai, India). HPLC water was obtained in-house from Milli-Q water Purification system, Millipore Corp. (Bedford, MA).

Instrument and chromatographic conditions

Integrated HPLC system consists of Waters Alliance 2695 (Waters Corporation, Milford, USA) equipped with a quaternary pump, an auto sampler, a column heater, and a Waters 2996 photodiode array detector (PDA). Integrated UPLC system consists of Waters acquity ultra performance LC, with a PDA detector, a column manager, a sample manager, and a binary solvent manager. Data collection and analysis were performed using Empower software 2 (Waters Corporation). The pH meter used to measure the pH of the buffer solution was manufactured by Inolab. The balance used for weighing the reference standards, impurities standards, and samples was from Metler Toledo (models: XP205 and UMX2).

HPLC separation was achieved on a Zodiacsil-C18 column from Zodiac life science with dimensions 250 mm × 4.6 mm (I.D) and a particle size of 5 µm. The UPLC method was developed on a performed ACQUITY BEH C18 column having dimensions 2.1 × 100 mm (1.7 µm particles). The elution is a linear gradient with mobile phase A consisting of a buffer solution (pH 3.60) containing 5 mM monobasic potassium phosphate in milliQ-water. Mobile phase B consists of a mixture of acetonitrile and methanol in the ratio 80:20 (v/v), respectively. The mobile phase B composition is ramped with respect to time as T/%B: 0/5, 12/10, 30/12, 37/28, 55/28, 60/35, 68/35, 70/5, and 80/5. The mobile phase flow rate was maintained at 0.8 ml/min throughout the run with a column temperature of 29°C. The injection volume was 10 µL, with detection at 226 nm.

Water bath of Thermo constant temperature (Mumbai, India) was used for solution degradation and a dry oven was used for solid-state thermal stress study. A walk-in chamber from thermo lab (Mumbai, India) was used for stability studies and a photostability chamber was equipped with UV and fluorescence lamps with an overall illumination of not less than 1.2 million lux hours and an integrated UV energy of not than 200 Whm^{-2} in compliance with ICH guidelines.

Standard and sample preparations

A mixture of diluted orthophsophoric acid (2 in 100 mL) and acetonitrile mixed in the ratio of 80:20 v/v, respectively, was used as a diluent for preparing all the solutions. Standard solution was prepared by dissolving appropriate amounts of BISO and HCTZ reference standards in diluent and suitably diluted, to obtain a concentration of 2.5 and 12.5 μgmL^{-1}, respectively. The sample solution was prepared by dissolving the tablet powder equivalent to 10 mg of BISO in 20 mL of the

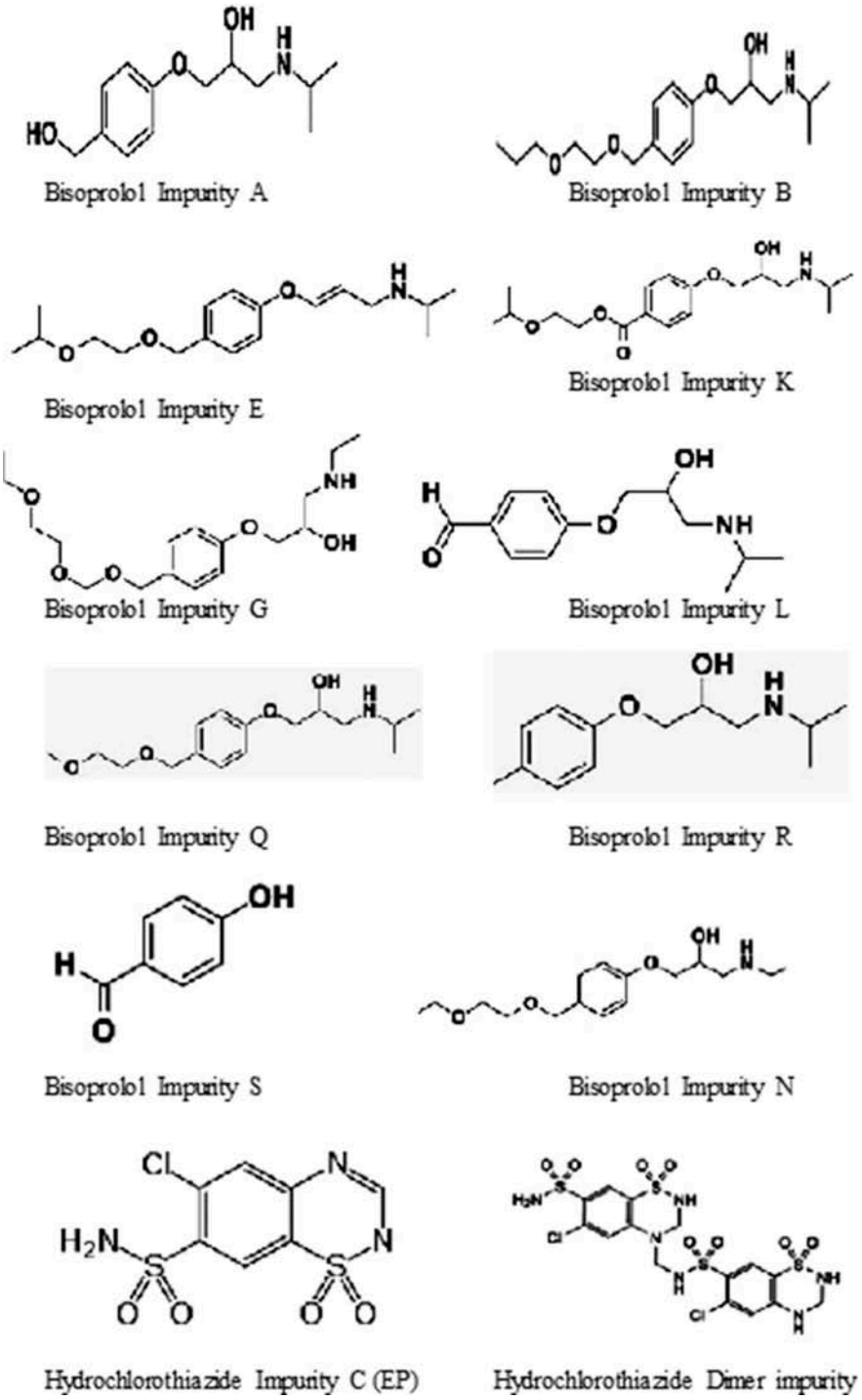

Figure 2. Chemical names and structures of impurities of bisoprolol and hydrochlorothiazide.

diluent (conc. 500 ppm), with sonication for 20 min and intermittent shaking in an ice-cold bath. The solution is then filtered through a 0.45 µm polyvinylidene fluoride (PVDF) filter. The sample solution is kept on a bench top to attain room temperature and injected into the chromatographic system. A sample solution spiked with all impurities was prepared by dissolving appropriate amounts of all impurities and tablet powder equivalent to 10 mg of BISO in 20 mL diluent with sonication for 20 min and intermittent shaking in an ice-cold bath. Placebo solutions were prepared by dissolving placebo powder equivalent to 10 mg of BISO in 20 mL of the diluent with sonication for 20 min and intermittent shaking in an ice-cold bath.

Table 1. List of impurities with their classification.

Active	Name of the impurity	Nature of the impurity	Process related or degradant
Bisoprolol Fumarate	A	Specified	Degradant
	B	Unspecified	Process related
	E	Specified	Degradant
	G	Specified	Degradant
	K	Unspecified	Process related
	L	Unspecified	Degradant
	N	Unspecified	Process related
	Q	Unspecified	Process related
	R	Unspecified	Process related
	S	Unspecified	Process related
Hydrochlorothiazide	Benzothiadiazine	Specified	Degradant
	Chlorothiazide	Specified	Degradant

Forced degradation study

Forced degradation studies were conducted on samples and on the plain placebo with individual drugs. Intentional degradation was carried out by exposing 10 mL of test solution to 0.1 N hydrochloric acid and 0.01 N sodium hydroxide at 60°C for 1 h and 30 min, respectively, using a water bath. The solutions were withdrawn, allowed to attain room temperature, and then neutralized with base/acid and injected into the chromatograph. Oxidative degradation of the sample solution and placebo was conducted using 3% hydrogen peroxide in a 20 mL volumetric flask. The solution was allowed to attain ambient temperature, further diluted up to mark with the diluent, and injected into the chromatograph. The tablets and placebo were exposed to humidity at 25°C and 90% RH for 48 h to study the effect of humidity.

For thermal stress study, the tablets and placebo were kept in a dry oven at 60°C for 72 h. Photolytic studies were carried out on solid dosage form. The samples in a Petri dish were spread as a thin layer (1 mm) and exposed to light (1.2 million lux hours) in a photo-stability chamber. The method's analytical data were collected at a single wavelength of 226 nm. Additional PDA detector data was collected for the peak purity evaluation. The solution stability of standard and sample was carried out by leaving both the test solutions of sample and standard at room temperature for 24 h. The mobile phase stability study was also carried out by keeping the mobile phase at room temperature for 3 days and by evaluating the system's suitability.

Filter validation

Filters used (0.45 µ PVDF) in the sample preparation were evaluated for their suitability. A spiked sample was prepared as described above in the specified diluent. A portion of the sample was filtered through PVDF, and the other portion was centrifuged. These samples were then chromatographed. The results obtained from the filtered samples were compared with those obtained with the centrifuged sample.

Results and discussion

Method development and optimization

BISO has three specified impurities and seven unspecified impurities. HCTZ has two specified impurities. Since no single HPLC method was available, the main aim was the development of a single, accurate, reproducible, stability indicating RP-HPLC method for routine quality control analysis.

Initially the molecules were studied for the spectrophotometer absorbance in the UV-VIS region to establish the λmax. The UV spectra shown in Fig. 3 show λmax for both the actives . A common wavelength at 226 nm was chosen based on two reasons. The first reason is that the majority of impurities and drug substances have significant absorbance at 226 nm and secondly a good signal to noise ratio with no interference from the placebo and diluent was found at the retention time (RT) of analyte peaks. The next primary aim was to choose a column and separate all 12 impurities from each other and from the active peaks without compromising the resolution. An initial attempt was made using the USP chromatographic purity procedure. The gradient programme given in the monograph was extended to check separation. The attempt was unsuccessful, as only few impurities along with bisoprolol and HCTZ were seen. A fresh development was initiated with a long C18 column (250 mm × 4.6 mm, 5 µ) with phosphate buffer (KH_2PO_4, 0.05 M). Initially an isocratic mode with 20% methanol was used and injected a spiked sample containing all impurities. Little separation was observed between all the impurities except for impurities N, R, and G, which were observed to be close eluting. As the two actives differ in polarities, an attempt was made by reducing the pH to 3.5 and introdu-

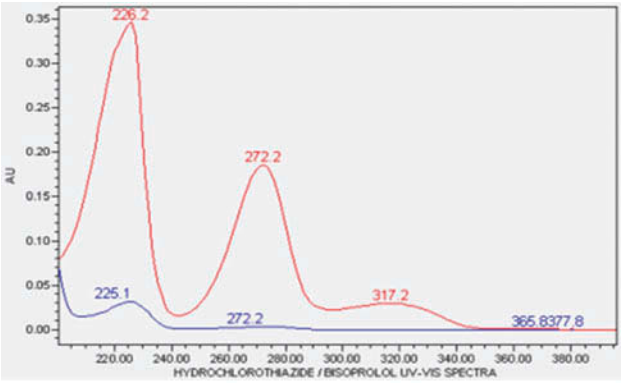

Figure 3. Overlaid UV-VIS spectra of bisoprolol and hydrochlorothiazide.

cing a gradient programme. All impurities were well separated except for impurity G. By slight modification of the pH to 3.60 and introducing acetonitrile in methanol in the ratio 80:20 as a mobile phase B composition, the desired separation was achieved between these impurities. Figure 4 shows the chromatogram of sample spiked with all impurities.

Method validation

The optimized chromatographic conditions were validated for 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) [18] .

System suitability

System suitability parameters were measured so as to verify the system performance. System precision was determined on six replicate injections of standard preparations. Chromatographic performance parameters like retention factor and peak resolution were measured.

Specificity

The specificity of the method was determined by analysing the blank solution, placebo solution, and the spiked sample. The chromatograms of blank, placebo, and spiked sample were compared and no interference was found at the retention times of individual impurities and active peaks. Samples of bisoprolol and HCTZ tablets, placebo were subjected to stress conditions as discussed in the section "Forced Degradation Study". The results obtained from the forced degradation studies are tabulated in Table 2.

LOD and LOQ

The LOD and LOQ of the individual known impurities were established by using the signal to noise ratio approach. Diluted solutions of impurities were injected into the chromatograph and the signal to noise ratio was determined and is reported in Table 3.

Linearity

Linearity of the detector response was established by injecting the potential impurities at concentrations ranging from LOQ to about 150% (LOQ, 20%, 50%, 80%, 100%, 120%, and 150%) of the target concentration A linearity graph was plotted between the detector response and the concentration. The correlation coefficient obtained was 0.999. The linearity data is shown in Table 3.

Accuracy

Accuracy of the method was evaluated by performing recovery study for the known impurities of BISO and HCTZ, respectively, in triplicate by spiking the sample preparation with known impurities at 50%, 75%, 100%, 125%, and 150%. The average recoveries and %RSD were calculated. The recovery of six impurities from the excipients ranged from 94.9% to 109.3% and the % RSD of the individual preparations was between 0.1% and 6.5%. The accuracy data is shown in Table 3.

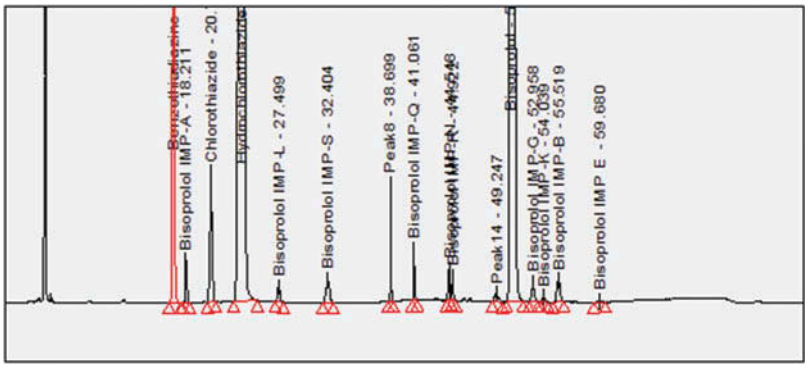

Figure 4. Spiked sample chromatogram.

Table 2. Results obtained from forced degradation studies.

		% Impurity levels						
Degradation parameters	Conditions	I	II	III	IV	V	VI	Total impurities
Acid	0.1 NHCl at 60°C for 1 h	1.99	–	–	–	0.42	0.01	2.61
Base	0.01 N NaOH at 60°C for 30 min	0.40	–	–	–	0.23	0.01	0.78
Peroxide	3% of H_2O_2	0.27	0.08	–	–	0.18	0.02	2.13
Thermal	60°C for 72 h	0.08	–	0.12	0.14	0.54	0.05	4.53
Photolytic	1.2MILLION LUX HOURS	–	0.04	–	–	0.05	–	0.26

I=Bisoprolol imp A; II= Bisoprolol imp L; III= Bisoprolol imp G; IV= Bisoprolol imp K; V=Benzothiadiazine; VI=Chlorothiazide.

Table 3. Summary of analytical method validation results.

Parameter	Impurity A	Impurity G	Impurity L	Chlorothaizide	Benzothiazidine
Accuracy					
LOQ	99.5	95.8	102.6	103.8	98.4
50%	109.3	103.2	100.9	96.4	101
100%	106.5	102.8	102.6	95.2	98.1
150%	105	101.8	100.8	95.4	97.8
LOD(%w/w)	0.0196	0.0191	0.0016	0.0019	0.0010
LOQ(%w/w)	0.0593	0.0577	0.0048	0.0058	0.0030
S/N Ratio (LOD)	4	3	3	4	3
S/N Ratio (LOQ)	10	10	10	12	12
Regression equation (y)					
Slope	40314.2	25878.1497	49373.8586	87043.9882	136461.9018
Intercept	367.8611	358.6578	3043.5713	4617.2669	10452.1079
Correlation coefficient	0.999	0.999	0.999	0.999	0.999
Precision(%RSD)[a]	0.60	0.70	0.70	0.00	0.00
Intermediate Precision(%RSD)[a]	0.90	0.90	1.00	0.50	0.40
Precision at LOQ(%RSD)	1.9	1.2	4.0	1.1	2.0

Linearity range is LOQ-150%.

Precision

The system precision was established by injecting the BISO and HCTZ standard preparations at a concentration of 0.0025 and 0.125 mg/mL, respectively. RSD (%) was calculated for the areas of BISO and HCTZ peaks. The precision of the proposed method was evaluated by carrying out six independent sample preparations of bisoprolol and HCTZ tablets of 1.25 mg strength. RSD (%) was calculated from the obtained six sample results. Intermediate precision was studied by analysing the samples by a different analyst on another instrument and on a different day to evaluate the ruggedness of the method. The precision data is shown in Table 3.

Filter validation

PVDF filters were used to filter the sample matrix containing insoluble placebo powder. To assess the impact of filters on the absorption of drug during the sample preparation, filter validation study was carried out. For this samples were filtered through PVDF and compared with that of the centrifuged sample. Table 4 shows the filter validation data.

The above results show that there was no interference of impurities due to the filter at the retention times, suggesting PVDF filters are suitable for filtering the tablets powder in the sample preparation.

Robustness

No significant effect was observed on the system suitability parameters such as capacity factor, resolution, and theoretical plates of the respective components when small but deliberate changes were made to chromatographic conditions. To study the effect of flow rate on the resolution, three different flow rates were studied (0.6, 1.0, and 1.2 mL/min) and the impact of column temperature on the resolution was studied at 25°C, 27°C, 30°C, 32°C, 35°C, and 40°C. The effect of variation of organic composition in the mobile phase was also studied. The impact of the mobile phase pH on the retention times was studied between ±0.2 units.

HPLC method migration to UPLC

A need was envisioned to develop a faster method to reduce the run time, because the above-proposed method though very precise and robust was nevertheless a time-consuming exercise. An attempt was made to improve analyst productivity by taking advantage of smaller particles column, which would improve resolution speed and sensitivity without compromising accuracy.

Acquity UPLC™ System (Waters Corporation, Milford, MA, USA) was used to transfer the method from conventional HPLC to UPLC™. The separation

Table 4. Filter validation data.

	Filter Validation data			Solution stability data		
Name of the peak	Unfiltered result centrifuge result (%w/w)	PVDF (%w/w)	Absolute difference	Initial impurity level (%w/w)	Solution stability after 24 h (%w/w)	Absolute difference
Bisoprolol A	0.56	0.57	0.01	0.56	0.57	0.01
Bisoprolol G	0.61	0.61	0	0.63	0.64	0.01
Bisoprolol L	0.56	0.56	0	0.55	0.56	0.01
Chlorothiazide	0.34	0.34	0	0.34	0.34	0
Benzothiadiazine	1.05	1.05	0	1.05	1.06	0.01
Bisoprolol E	0.1	0.1	0	0.1	0.1	0
Total Impurities	7.52	7.51	0.01	7.56	7.66	0.1

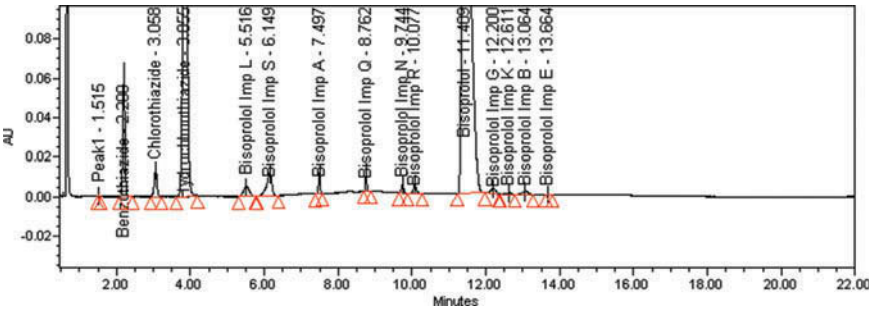

Figure 5. UPLC migrated chromatogram of the spiked sample.

Table 5. UPLC gradient programme.

Time	Flow (mL/min)	Buffer (%A)	Organic Composition (%B)	Curve type
0	0.2	95	5	6
5	0.2	95	5	6
8	0.2	75	25	6
15	0.2	75	25	6
16	0.2	80	20	6
18	0.2	95	5	6
22	0.2	95	5	6

was scaled from a C_{18}, 250 × 4.6 mm (5 µm particles) HPLC column to a 2.1 × 100 mm ACQUITY UPLC™ BEH C18 column (1.7 µm particles). The separation was achieved using sodium perchlorate buffer (pH 3.0) and a mixture of methanol and acetonitrile (20:80) as organic solvent. The gradient profile was tweaked to suit the UPLC™ analysis. The mobile phase flow rate was kept at 0.25 mL/min, and the run time was reduced to 22 min as compared to 80 min in HPLC. Complete resolution was observed for all the relevant analytes as seen in the chromatogram (Fig. 5). An approximately four-fold reduction was achieved in the run time (Table 5).

Conclusions

The gradient RP-LC method developed for the simultaneous determination of potential degradant and process impurities of bisoprolol and HCTZ in a single chromatographic run is precise, accurate, linear, robust, and selective. The method was validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for assessing the stability of bisoprolol or HCTZ or in combination of both in fixed dosage forms. Finally, we succeeded in transferring the method from RP-HPLC to UPLC to further reduce the run time, solvent consumption, and increase the sample throughput.

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