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A MODIFIED LIQUID CHROMATOGRAPHIC METHOD DEVELPOMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF BISOPROLOL FUMARATE AND HYDROCHLOROTHIAZIDE IN BULK AND TABLET DOSAGE FORM

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Keywords:

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ABSTRACT: A simple, precise, accurate, reproducible and economical reverse phase liquid chromatography method was developed and validated for the quantitative simultaneous estimation of Bisoprolol Fumarate and Hydrochlorothiazide in bulk and marketed formulations. Estimation of drugs in this combination was done with a C18 column Kromasil 100-5C_{18} column [250mm x 4.6mm].using mobile phase of composition Acetonitrile and phosphate buffer (40:60 v/v, pH 3).The flow rate was 1 ml/min and the effluents were monitored at 228 nm. The retention time of Bisoprolol Fumarate and Hydrochlorothiazide were 3.3 min and 6.25 min respectively. The method was found to be linear over a concentration range of 20-100 µg/ml for both Bisoprolol Fumarate and Hydrochlorothiazide. The established method proved as reproducible one with a %RSD value of less than 2 and having the robustness and accuracy within the specified limits. Assay of marketed formulation was determined and find with 98.1% and 97.6% for Bisoprolol Fumarate and Hydrochlorothiazide respectively. The method was validated according to the guidelines of International Conference on Harmonization (ICH) and was successfully employed in the estimation of commercial formulations. This liquid chromatographic method can be applied for the qualitative and quantitative determination of selected drugs by the modern chemist.

INTRODUCTION: Bisoprolol fumarate is a cardioselective β_1 -adrenergic blocker. Chemically, Bisoprolol Fumarate is (±)-1-[4-[[2-(1-methylethoxy) ethoxy] methyl] phenoxy]-3-[(1-methylethyl)amino]-2-propanol *(E)* -2-butenedioate (2:1). Hydrochlorothiazide is thiazide diuretic and administered orally in the treatment of hypertension and oedema. Chemically, HCTZ is 6-chloro-3, 4-dihydro-2 *H* -1, 2, 4-benzothiadiazine-7-sulphonamide-1,1-dioxide. It is official in IP, BP and USP [1-4].

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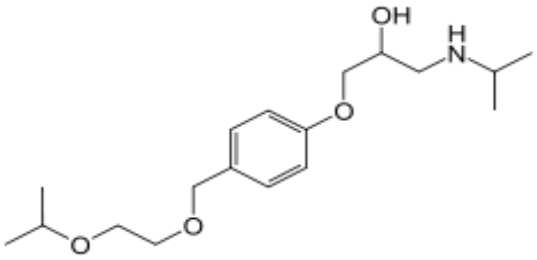
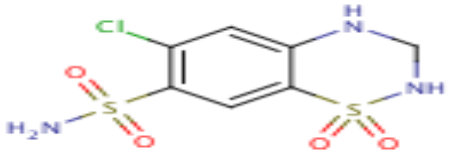


a) BISOPROLOL FUMARATE



b) HYDROCHLOROTHIAZIDE

FIG 1: CHEMICAL STRUCTURES OF a) BISOPROLOL FUMARATE b) HYDROCHLOROTHIAZIDE

Extensive literature survey proved that very few methods [5-10] were reported for the determination of Bisoprolol Fumarate and Hydrochlorothiazide by RP-HPLC. So we attempted to develop an accurate, rapid, precise, stable, sensitive and economically viable liquid chromatographic method for the simultaneous determination of selected drugs in the present research.

MATERIALS AND METHODS:

Equipment used:

The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20µl fixed loop. A reverse phase C18[Kromasil 250mm × 4.6 mm]was used. Lab India 3000$^+$ double beam UV visible spectrophotometer and Axis AGN204-PO electronic balance were used for spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals:

Pharmaceutical grade pure Bisoprolol Fumarate and Hydrochlorothiazide gift samples were procured from Mylan Laboratories, Hyderabad. Marketed tablet formulations (LODOZ 5) with of 5mg of Bisoprolol Fumarate and 6.25mg of Hydrochlorothiazide were procured from local market. (Mfd. by Merk Pharmaceuticals ltd). HPLC grade Acetonitrile and Water were commercially procured from Merck specialties private limited, Mumbai.

Chromatographic conditions:

Kromasil 100-5C_{18} column [250mm x 4.6mm] was used for the chromatographic separation at a detection wave length of 228nm. Mobile phase composition of Acetonitrile and Phosphate buffer pH 3in a ratio of 40:60 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 1ml/min and the injection volume was 20µl.

Preparation of Mobile phase:

Phosphate buffer pH 3 was prepared by dissolving 0.136 gm of Potassium dihydrogen phosphate and 2 ml of Triethyl amine in 80ml of HPLC grade water and adjusts the pH to 3.0 with orthophosphoric acid and volume was adjusted with water to produce100ml, which is then filtered through 0.45µ membrane filter and sonicated for 20 minutes.

Preparation of Standard solutions:

25mg each of Bisoprolol Fumarate and Hydrochlorothiazide were accurately weighed and transferred into two 25ml volumetric flasks respectively and dissolved in mobile phase as mentioned above and the volume was made up with the same solvent to obtain primary stock solutions A (Bisoprolol Fumarate) B (Hydrochlorothiazide) to achieve standard of concentrations of 1000µg/ml of each drug. From the primary stock solutions, 1ml of each solution was pipetted out and transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 100µg/ml of Bisoprolol Fumarate and Hydrochlorothiazide respectively and this solution is (working stock solution A).

Preparation of Sample Solution:

Twenty tablets of Bisoprolol Fumarate and Hydrochlorothiazide were weighed and crushed. Tablet powder equivalent to 5mg of Bisoprolol Fumarate and 6.25mg of Hydrochlorothiazide was weighed accurately and transferred to a 25ml volumetric flask. The content was dissolved with 10ml of mobile phase and then sonicated for 15min. The volume was made up with the mobile phase and filtered with 0.45µ membrane filter and sonicated for 20min. 1ml of this solution was pipetted out and transferred to a 10mlvolumetric flask and the volume was made up with the mobile phase to obtain a concentration of 150µg/ml of Bisoprolol Fumarate and 250µg/ml of Hydrochlorothiazide (working stock solution B).

Optimization of RP-HPLC method:

The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Bisoprolol Fumarate and Hydrochlorothiazide. For the method optimization, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Acetonitrile, Phosphate buffer pH 3 (40:60 v/v) using Kromasil 100-5C_{18} column [250mm x 4.6mm].

Validation of the RP-HPLC method:
Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability:
System suitability was carried out with five injections of solution of 100% concentration having 100µg/ml of Bisoprolol Fumarate and Hydrochlorothiazide of each in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factors (T) were reported in **Table 1**.

Linearity:
For the determination of linearity, appropriate aliquots were pipetted out from working stock solution A to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 20-100µg/ml of Bisoprolol Fumarate and Hydrochlorothiazide. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Bisoprolol Fumarate and Hydrochlorothiazide were shown in **Fig. 3** and **4** their corresponding linearity parameters were given in **Table 2**.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):
The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in **Table 2.**

Precision:
The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (100µg/ml of Bisoprolol Fumarate and Hydrochlorothiazide respectively) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in **Table 3**.

Accuracy:
Standard addition method was followed to ensure the reliability and accuracy of the method recovery studies. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in **Table 4**.

Specificity:
Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Bisoprolol Fumarate and Hydrochlorothiazide without any interference was shown in **Fig. 2**.

Robustness:
Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wave length detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the **Table 5.**

Assay of Marketed Formulations:
20µl of sample solution of concentration 150 µg/ml of Bisoprolol Fumarate and 250 µg/ml of Hydrochlorothiazide was injected into chromatographic system and the peak responses were measured. The solution was injected three times in to the column. The amount of drug present and percentage purity was calculated by comparing the peak areas of the standards with that of test samples. A typical chromatogram for assay of marketed formulation was shown in **Fig. 5** and the obtained values were reported in the **Table 6**.

RESULTS AND DISCUSSION:
After a number of trials with mobile phases of different composition, Acetonitrile, Phosphate buffer pH 3 in the ratio 40:60v/v was selected as mobile phase because of better resolution and symmetric peaks. Bisoprolol Fumarate and

Hydrochlorothiazide were found to show appreciable absorbance at 228nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Bisoprolol Fumarate and Hydrochlorothiazide at different R_Ts was shown in **Fig.2**.

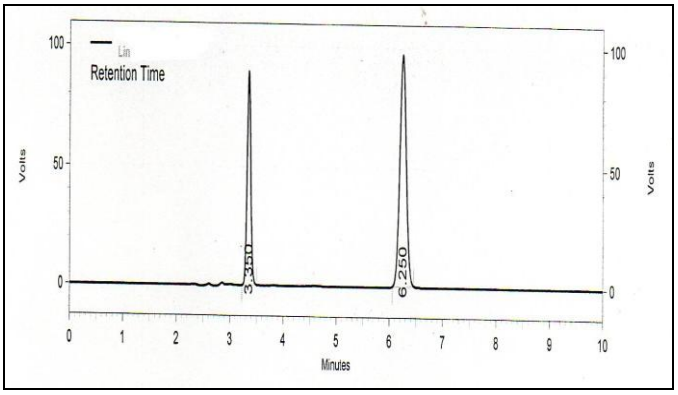


FIG 2: OPTIMIZED CHROMATOGRAM OF BISOPROLOL FUMARATE AND HYDROCHLOROTHIAZIDE

System suitability was carried out by injecting 5 replicate injections of 100% test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Bisoprolol Fumarate and Hydrochlorothiazide at 3.3min and 6.25min respectively without any interference. The parameters were given in **Table 1**.

TABLE 1: SYSTEM SUITABILITY PARAMETERS (n=5)

Parameters	Bisoprolol Fumarate	Hydrochlorothiazide
Retention time (min)	6.293	3.347
Theoretical plates (N)	6311	7240
Tailing factor (T)	1.15	1.16
Resolution (R_s)	2.946	

∗n= No. of determinants

Concentration range of 20-100µg/ml for Bisoprolol Fumarate and Hydrochlorothiazide were found to be linear with correlation coefficients 0.998 and 0.999 for Bisoprolol Fumarate and Hydrochlorothiazide respectively. The respective calibrations curve was shown in **Fig.3** and **4** respectively. The results were given in **Table 2.** The limits of detection for Bisoprolol Fumarate and Hydrochlorothiazide were found to be 0.544µg/ml and 0.658µg/ml respectively and the limits of quantitation were 1.64µg/ml and 1.99µg/ml respectively. Values were represented in **Table 2.**

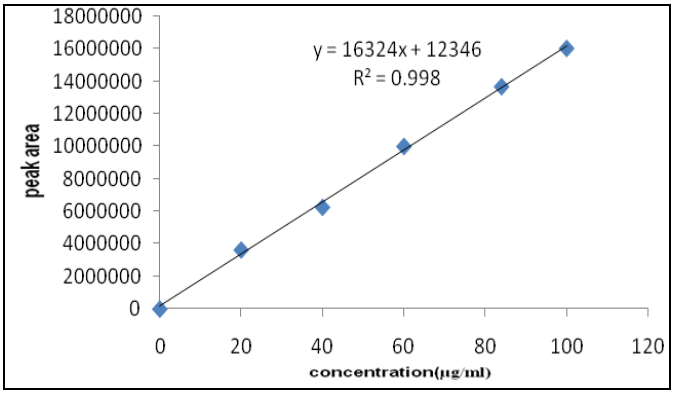


FIG 3: CALIBRATION PLOT OF BISOPROLOL FUMARATE

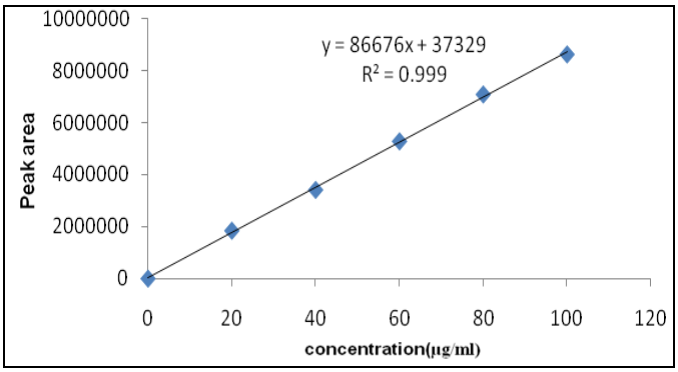


FIG 4: CALIBRATION PLOT OF HYDRO CHLORO THIAZIDE

TABLE 2: RESULTS FOR LINEARITY (n=3)

Parameter	Bisoprolol Fumarate	Hydrochlorothiazide
Linearity Range (µg/ml)	20-100	20-100
Regression Equation	y = 16324x+12346	y = 86676x + 37329
Slope (m)	16324	86676
Intercept (c)	12346	37329
Regression Coefficient (r^2)	0.998	0.999
Limit of Detection (µg/ml)	0.544	0.658
Limit of Quantitation (µg/ml)	1.64	1.99

∗n= No. of determinants

The proposed method was found to be precise and reproducible with %RSD of 0.68 and 0.510 for Bisoprolol Fumarate and Hydrochlorothiazide respectively. %RSD was reported in **Table 3**.

TABLE 3: RESULTS OF PRECISION (n=6)

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Bisoprolol Fumarate	0.68	0.71
Hydrochlorothiazide	0.510	0.63

∗n= No. of determinants

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 98.7% to 99.0% for Bisoprolol Fumarate and 99.1 to 99.9% for Hydrochlorothiazide. This indicates that the method was accurate. Values obtained were given in **Table 4**.

TABLE 4: RESULTS FOR ACCURACY (n=3)

Recovery level	Bisoprolol Fumarate				Hydrochlorothiazide			
	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery
	std	test			std	Test		
80%	20	60	79.1	98.8	20	60	79.9	99.9
100%	40	60	98.7	98.7	40	60	98.9	99.1
120%	60	60	118.5	99.0	60	60	118.9	99.3
Mean recovery	98.7-99.0				99.1-99.9			

∗n= No. of determinant

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in **Table 5**.

TABLE 5: RESULTS FOR ROBUSTNESS (n=3)

Parameters (n=3)	%RSD	
	Bisoprolol Fumarate	Hydrochlorothiazide
Detection wavelength at 226nm	0.17	0.18
Detection wavelength at 230nm	0.75	0.19
Flow rate 0.8ml/min	0.25	0.235
Flow rate 1.2ml/min	0.443	0.390

∗n= No. of determinant

The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 98.1% for Bisoprolol Fumarate and 97.6% for Hydrochlorothiazide. The typical chromatogram for assay of marketed formulations was shown in **Fig. 5** and Values obtained were given in **Table 6.**

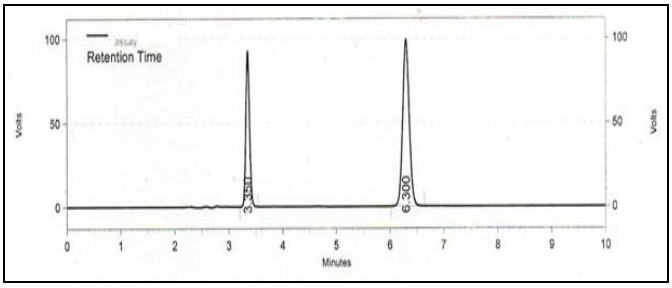


FIG.5: A TYPICAL CHROMATOGRAM FOR ASSAY OF MARKETED FORMULATION CONTAINING 150µg/ml OF BISOPROLOL FUMARATE AND 250µg/ml OF HYDROCHLOROTHIAZIDE

TABLE 6: RESULTS FOR ASSAY (N=3) OF MARKETED FORMULATION (LODOZ 5)

Drug	Label claim (mg/tab)	Amount recovered	% Amount found in drug
Bisoprolol Fumarate	5	4.90	98.1
Hydrochlorothiazide	6.25	6.05	97.6

∗n= No. of determinants

CONCLUSION: The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Bisoprolol Fumarate and Hydrochlorothiazide from their formulations. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, rugged and robust. So the established method can be employed in the routine analysis of the marketed formulations.

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