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Rp-Hplc Method Development and Validation for Estimation of Bisoprolol and Hydrochlorothiazide

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Abstract

The present study was to develop a rapid and sensitive method for the analysis of Bisoprolol and Hydrochlorothiazide by different analytical methods such as UV spectrophotometry and RP-HPLC in pharmaceutical dosage forms by using the most commonly employed C-18 column with UV-detection and validate it as per ICH guidelines**.** The retention time for Bisoprolol and Hydrochlorothiazide was found to be 2.1 min and 4.01 min respectively. A Resolution of greater than 2 was observed. The low % RSD values (≤ 2) indicated that the method was precise and accurate. The mean recoveries were found in the range of 99.1 – 99.9% w/w. Our approach was specific with good accuracy and precision when evaluating the commercial formulations without the interference of excipients and other additives. This method can be used to calculate the amounts of Bisoprolol and Hydrochlorothiazide in both single and mixed pharmaceutical formulations, as well as bulk samples.

Keywords: RP-HPLC, UV Spectrophotometry, Bisoprolol, Hydrochlorothiazide, Column.

1. Introduction

1.1 Bisoprolol Fumarate

Category : β_1 adrenergic receptor blocker, Antihypertive agent, Sympatholytics.
Physical properties : White, solid powder, soluble in water
Side effects : Bronchospasms, Bradycardia

Bisoprolol is a competitive, cardioselective β_1-adrenergic antagonist. Activation of β1-receptors (located mainly in the heart) by epinephrine increases heart rate and the blood pressure causing the heart to consume more oxygen. β1-adrenergic blocking agents such as bisopolol lower the heart rate and blood pressure and may be used to reduce workload on the heart and hence oxygen demands. They are routinely prescribed in patients with ischemic heart disease. In addition, β1-selective blockers prevent the release of renin, a hormone produced by the kidneys causes constriction of blood vessels. Bisoprolol is lipophilic and exhibits no intrinsic sympathomimetic activity (ISA) or membrane-stabilizing activity.[3],[16].

Bisoprolol is used with or without medication to treat high blood pressure (Hypertension) lowering high blood pressure helps prevent strokes heart attacks & kidney problems. This medication belongs to a class of drugs known as beta blockers. It works by blocking the action of certain natural chemicals in your body such as epinephrine on the heart & blood vessels. This effect lower the heart rate, blood pressure & strain on the heart.[22],[25].

1.2 Hydrochlorothiazide

Category : Diuretic

Physical properties : White, solid crystalline powder, very slightly soluble in water.

HCTZ-Poisoning : Over dosage with hydrochlorothiazide results in diuresis. Another effect is lethargy of various degrees that may progress to coma within a few hours, even without dehydration or electrolyte imbalance, or with minimal depression of respiration, and cardiovascular function.

The mechanism of central nervous system (CNS) depression is unknown. Gastrointestinal irritation and hypermotility may occur. Orthostatic hypotension, pancreatitis, potentiation of parathyroid hormone activity, skin rash, photosensitivity, and thrombocytopenia have been observed. Hypopotassaemia and hypomagnesaemia may be associated with ventricular ectopic activity. Hyponatraemia may be seen in older patients. Short-term potassium supplementation may induce a fall n blood pressure. .[3],[16].

- Hydrochlorothiazide, a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the sodium-chloride symporter (SLC12A3) in the distal convoluted tubule, which is responsible for 5% of total sodium reabsorption. Normally, the sodium-chloride symporter transports sodium and chloride from the lumen into the epithelial cell lining the distal convoluted tubule. The energy for this is provided by a sodium gradient established by sodium-potassium ATPases on the basolateral membrane. Once sodium has entered the cell, it is transported out into the basolateral interstitium via the sodium-potassium ATPase, causing an increase in the osmolarity of the interstitium, thereby establishing an osmotic gradient for water reabsorption. By blocking the sodium-chloride symporter, hydrochlorothiazide effectively reduces the osmotic gradient and water reabsorption throughout the nephron. [22],[25]. Thiazides such as hydrochlorothiazide promote water loss from the body (diuretics). They inhibit Na+/Cl- reabsorption from the distal convoluted tubules in the kidneys. Thiazides also cause loss of potassium and an increase in serum uric acid. Thiazides are often used to treat hypertension, but their hypotensive effects are not necessarily due to their diuretic activity. Thiazides have been shown to prevent hypertension-related morbidity and mortality. Thiazides cause vasodilation by activating calcium-activated potassium channels (large conductance) in vascular smooth muscles and inhibiting various carbonic anhydrases in vascular tissue. It is used for the treatment of high blood pressure and management of edema.[28]

1.3 UV spectrophotometry

Every chemical compound absorbs, transmits or reflects light (electromagnetic radiation) over a certain range of wavelength. Spectrophotometry is a measurement of how much a chemical substance absorbs or transmits. Spectrophotometry is widely used for quantitative analysis in various areas (e.g., chemistry, physics, biology, biochemistry, material and chemical engineering, clinical applications, industrial applications, etc). Any application that deals with chemical substances or materials can use this technique.[18] In biochemistry, for example, it is used to determine enzyme-catalyzed reactions. In clinical applications, it is used to examine blood or tissues for clinical diagnosis.

There are also several variations of the spectrophotometry such as atomic absorption spectrophotometry and atomic emission spectrophotometry. A spectrophotometer is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. Depending on the range of wavelength of light source, it can be classified into two different types. UV-visible spectrophotometer uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.[4],[6],[8]

1.4 High Performance Liquid Chromatography

HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch. The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process, if diffusion is minimized, a faster and effective separation can be achieved. The technique of high performance liquid chromatography is so called because of its improved performance when compared to classical column chromatography. Advances in column technology, high-pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages. Speed (many analysis can be accomplished in 20 min or less), Greater sensitivity (various detectors can be employed), Improved resolution (wide variety of stationary phases), Reusable columns (expensive columns but can be used for many analysis), Ideal for the substances of low viscosity, Easy sample recovery, handling and maintenance, Instrumentation leads itself to automation and quantification (less time and less labour), Precise and reproducible, Integrator itself does calculations.(Ashutosh Kar et.al.,2005).[10],[20],[24].

2. Materials: Bisoprolol Fumarate and hydrochlorothiazide bulk drug samples were obtained from Mylan Laboratories Ltd., Hyderabad.

2.1 Chemicals Used

Table 1 Chemicals used

Chemicals used	Suppliers
Acetonitrile	MERK chemicals limited.
Potassium dihydrogenorthophosphate	RANKEM chemicals limited.
Sodium hydroxide	S D fine chemicals limited.
Methanol	S.D Fine-Chem limited,Mumbai.
Methanol	MERCK Private limited, Mumbai.
Sulphuric acid	S.D fine chemicals limited.
Double distilled water	MERCK Private limited, Mumbai.

2.2 Instruments used

1. Axis digital balance.
2. 1.5LH Ultrasonic bath sonicator.
3. Agilent HPLC

3. Methodology

3.1 Method Development

3.1.1 Selection of Mobile Phase The standard solutions containing Bisoprolol and Hydrochlorothiazide were injected into the HPLC system and run in different solvent systems. By studying literature survey, different mobile phases in different proportions and different pH were tried in order to find the best conditions for the separation. It was found that Acetonitrile and Phosphate Buffer pH3 gives satisfactory results as compared to other mobile phases. This mobile phase system was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was determined to be Acetonitrile and Phosphate Buffer pH 3 (60:40).

3.1.2 Phosphate Buffer pH 3.0 : Dissolve 0.136 gm of potassium dihydrogen phosphate and 2 ml of triethylamine in 80 ml 0f water, adjust the pH to 3.0 with orthophosphoric acid and add sufficient water to produce 100ml.

3.1.3 Preparation of Mobile Phase Mobile phase was prepared by mixing Acetonitrile and Phosphate buffer pH3 in the ratio of 60:40 and was initially filtered through 0.45µm Millipore membrane filter and sonicated for 15 min before use.

3.1.4 Preparation of Standard Stock Solution The separate stock solutions of Bisoprolol and Hydrochlorothiazide were prepared by accurately weighing 25 mg each into a separate 25 ml volumetric flasks A and B and made up to the volume with mobile phase to get 1000µg/ml respectively (Working stock solution).

3.1.5 Selection of Analytical Wavelength By appropriate dilution of each standard stock solution with mobile phase, various concentrations of Bisoprolol and Hydrochlorothiazide were prepared separately. Each solution was scanned using double beam UV visible spectrophotometer between the range of 200 nm to 400 nm and their spectra was overlaid.

3.1.6 Optimized Chromatographic Conditions Mobile phase consisting of Acetonitrile and Posphate Buffer pH3 (60:40 v/v) was used in isocratic mode. The mobile phase was initially filtered through 0.45µm Millipore membrane filter and sonicated for 15 min before use. The flow rate was maintained at 1.5 ml/min and the injection volume was 20µl. UV detection was performed at 220 nm and the separation was achieved at ambient temperature.

3.1.7 Selection of Analytical Concentration Range and Construction of Calibration Curve for Bisoprolol and Hydrochlorothiazide Appropriate aliquots ranging from 0.2 ml to 1 ml were pipetted out from the working stock solution (1000 µg/ml of Bisoprolol and Hydrochlorothiazide) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 20-100 µg/ml of Bisoprolol and Hydrochlorothiazide respectively. Chromatograms representing linearity was shown in figure 26 to 30. Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20 µl of each concentration of the drug were injected into the HPLC system three times separately and their chromatograms were recorded under the same chromatographic conditions as described above.

Peak areas were recorded for all the peaks and a standard calibration curve of area against concentration was plotted as concentration of the drug Vs peak area (figure 31 and 32). The results were shown in table 36. Both the drugs follow the Beer's Lambert's law in the concentration range of 20-100 µg/ml of Bisoprolol and Hydrochlorothiazide.

The linearity of calibration curves and adherence of the system to Beer's Lambert's law was validated by high value of correlation coefficient and less than 2% percent relative standard deviation (%RSD) for the intercept value.

3.2 Analysis of Tablet Formulation 20 tablets were initially powdered and an amount equivalent to 25 mg of Hydrochlorothiazide and 100 mg of Bisoprolol was accurately weighed into a 25 ml volumetric flask, mixed with 25 ml of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 5 minutes. The solution was then filtered through 0.45µm Millipore membrane filter. The solution contains 1000 µg/ml of Hydrochlorothiazide and 4000 µg/ml of Bisoprolol (Stock solution-'A'). From the above stock solution-'A' 1ml aliquot was transferred in to a 10 ml volumetric flask, volume was made up to the mark with mobile phase to obtain a final concentration of 100 µg/ml Hydrochlorothiazide and 400 µg/ml Bisoprolol. This solution was used as the sample stock solution-'B'.

2ml of the sample stock solution -'B' was transferred in to a 10 ml volumetric flask, volume was made up to the mark with mobile phase to obtain a final concentration of 20 µg/ml Hydrochlorothiazide and 80 µg/ml Bisoprolol. 20µl of sample solution was injected into chromatographic system and the peak responses were measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of the standard.

3.3 Method Validation The method was validated according to ICH Q2 B guidelines for validation of analytical procedures in order to determine system suitability, linearity, sensitivity, precision, accuracy and robustness for the analytes (ICH Q2B, 1996).

3.3.1. System Suitability: The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from six replicate injections for Bisoprolol and Hydrochlorothiazide retention times and peak areas. System suitability was carried out by injecting 100% concentration (sample having 100µg/ml of Bisoprolol and 100µg/ml of Hydrochlorothiazide into the HPLC system. This was repeated for six times under similar condition.

3.3.2 Accuracy: To confirm the accuracy of the proposed method, recovery experiments were performed by standard addition technique. In this method a known quantity of pure drug was added at three different levels i.e. 50 %, 100% and 150% to pre-analyzed sample solutions and calculated the recovery of Bisoprolol and Hydrochlorothiazide for each concentration.

3.3.3 Linearity and Range: The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. Linearity of the method was determined by means of calibration curve using different concentration of the drugs. Linearity was evaluated by visual inspection of a calibration curve shown in figure 31 and 32. The linearity of the method was determined in concentration range of 20-100µg/ml for Bisoprolol and Hydrochlorothiazide. Each solution was injected in triplicate.

3.3.4 Precision The precision of an analytical method was studied by performing intraday and inter day precision.

Intraday Precision Variation of results within the same day was analyzed. Intraday precision was determined by analyzing a set of six combined standard solutions of Bisoprolol and Hydrochlorothiazide (100 µg/ml) in linearity range as 100% concentration at 220 nm in different time intervals on same day.

3.3.5 Specificity and Selectivity The specificity of the RP-HPLC method was determined by complete separation of Bisoprolol and Hydrochlorothiazide with parameters like retention time (Rt), resolution (Rs) and tailing factor (T_f). Here tailing factor for peaks of Bisoprolol and Hydrochlorothiazide was less than 2% and resolution was also more than 2%. The average retention time and standard deviation for Bisoprolol and Hydrochlorothiazide were found to be satisfactory for six determinations of sample solution containing 100µg/ml of Bisoprolol and Hydrochlorothiazide respectively. The peaks obtained for Bisoprolol and Hydrochlorothiazide were sharp and have clear baseline separation as none of the excipients interfered with the analytes of interest.

3.3.6 Robustness The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different column temperature, different analytical wavelength, different flow rate. The solution containing 100 µg/ml of Bisoprolol and Hydrochlorothiazide Hydrochlorothiazide was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate (±0.1ml/min) and detection wavelength (± 2 nm).

3.3.7 Ruggedness The evaluation of ruggedness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different instruments, analysts, laboratories, reagents, days etc. The solution containing 100 µg/ml of Bisoprolol and Hydrochlorothiazide was injected into HPLC three times under different parameters like different analysts.

3.3.8 LOD and LOQ The LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of the responses and S is mean of the slopes of the calibration curves).

4. Results

4.1 Simultaneous Estimation of Bisoprolol and Hydrochlorothiazide by RP-HPLC Method

4.1.1 Chromatographic parameters of Trial-1

Mobile phase : Phosphate buffer pH 6.8: Acetonitrile (50:50%v/v)
Flow rate : 1 ml/min
Column : C_{18}(Agilent ODS UG 5 Column 250mmX4.5 mm)
Detector wavelength : 220 nm
Injection volume : 20 µL
Run time : 10 min

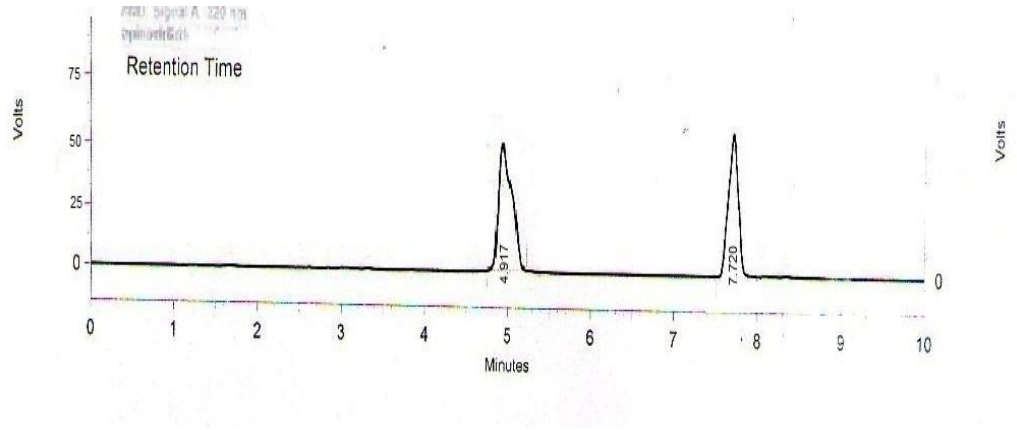

Figure 1 Chromatographic Trial-1

Observation: Tailing is observed, Peak broading is observed

4.1.2 Chromatographic parameters of Trial -2:

Mobile phase : Phosphate buffer pH 3: Acetonitrile (50:50 v/v)
Flow rate : 1 ml/min
Column : C 18(Agilent ODS UG 5 Column 250mmX4.5 mm)
Detector wavelength : 220 nm
Injection volume : 20 µL
Run time : 20 min

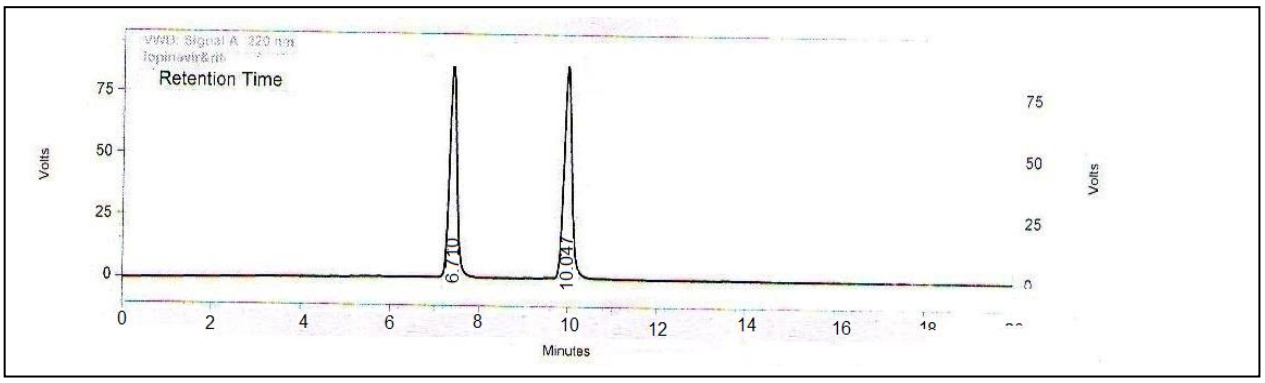

Figure 2 Chromatographic Trial-2

Observation: Sharp peaks are observed but long elution time

4.1.3 Chromatographic parameters of Trial -3:

Mobile phase : Phosphate buffer pH 3: Acetonitrile (50:50v/v)
Flow rate : 1.5 ml/min
Column : C 18(Agilent ODS UG 5 Column 250mmX4.5 mm)
Detector wavelength : 220 nm
Injection volume : 20 µL
Run time : 20 min

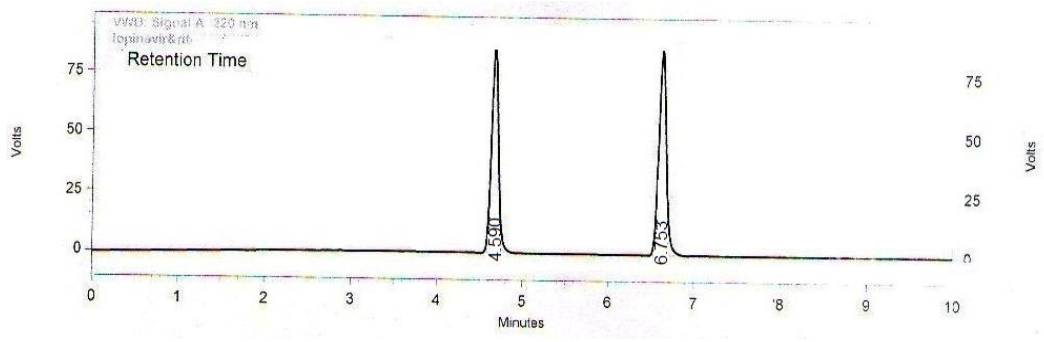

Figure 3 Chromatographic Trial-3

Observation: Sharp peaks are observed but long elution time.

Table 2: Optimized Chromatographic Conditions

Parameters	Method
Stationary phase (column)	C_{18} column (Agilent ODS UG column, 250mm x 4.5mm)
Mobile phase	Acetonitrile: Phosphate Buffer pH3 (60:40v/v)
Flow rate	1.5 ml/min
Column temperature	Ambient
Volume of injection	20 µl
Detection wavelength	220 nm
Retention time	Bisoprolol- 2.1min , Hydrochlorothiazide- 4.0min

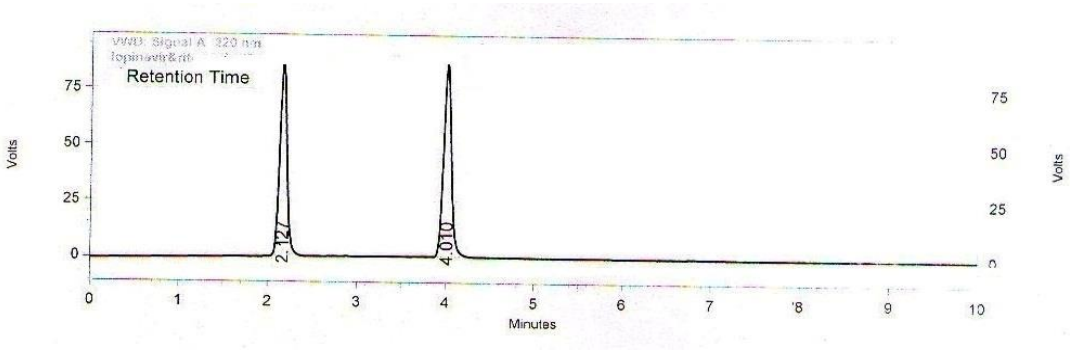

Figure 4 Optimized Chromatogram

4.2 Method Validation

4.2.1 System suitabiliy

Table 3: System Suitability Data of Bisoprolol and Hydrochlorothiazide

S. No	Bisoprolol			Hydrochlorothiazide		
	Conc. (µg/mL)	R_t (min)	Peak Area	Conc. (µg/mL)	R_t (min)	Peak Area
1	100	2.42	20481698	100	4.01	22575114
2	100	2.42	20073583	100	4.07	22347591
3	100	2.43	20415432	100	4.01	22753473
4	100	2.41	19740006	100	4.01	21896076
5	100	2.42	20515025	100	4.03	22391680
6	100	2.42	20272436	100	4.07	22481473
Mean		2.42	20249696	**Mean**	4.03	22407567.83
SD		0.00632	297660	**SD**	0.037	209363
% RSD		0.261	0.147	**% RSD**	0.726	0.934

Table 4: Summary of System Suitability Parameters

Parameters	Bisoprolol	Hydrochlorothiazide
Retention Time (min)	2.1	4.01
Resolution (R_s)	2	
Tailing Factor (T)	1.2	1.4
Theoretical Plates (N)	11456	10366

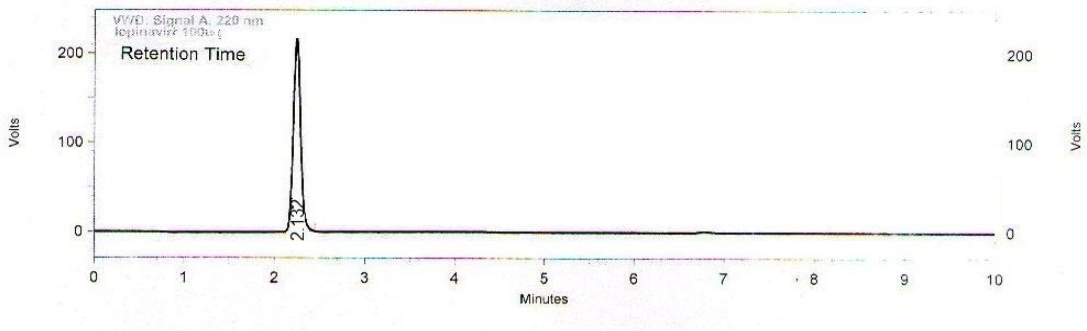

Figure 5: RP-HPLC Chromatogram of Bisoprolol

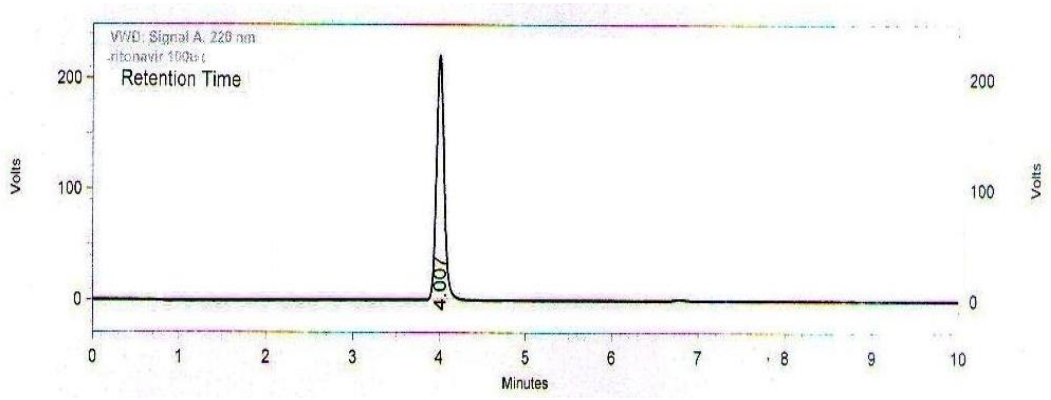

Figure 6: RP-HPLC Chromatogram of Hydrochlorothiazide

4.2.2 Linearity:

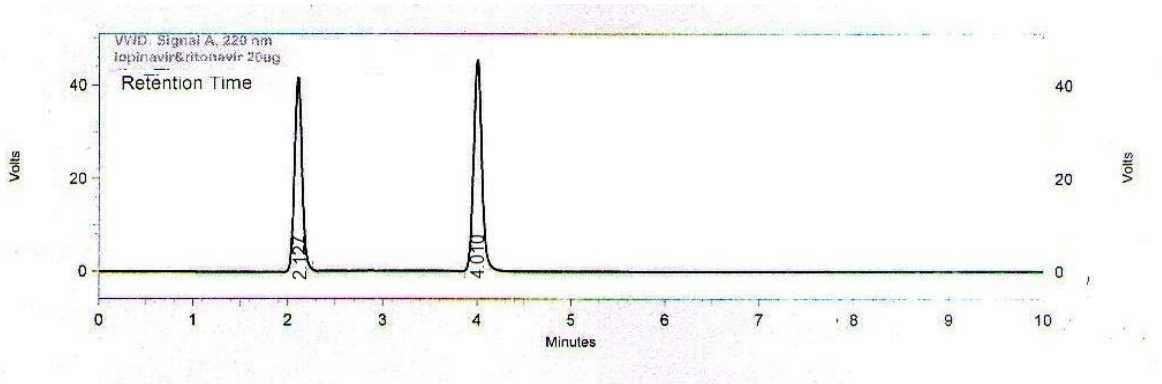

Figure 7: RP-HPLC Chromatogram of Linearity-1 (20 µg/ml of Bisoprolol and Hydrochlorothiazide)

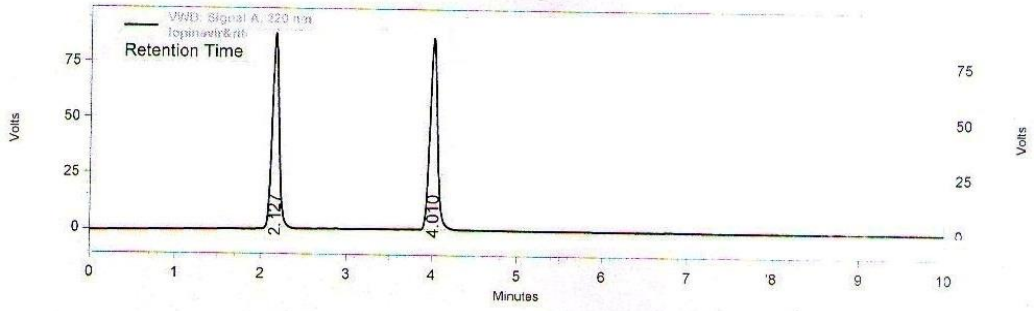

Figure 8: RP-HPLC Chromatogram of Linearity-2 (40 µg/ml of Bisoprolol and Hydrochlorothiazide)

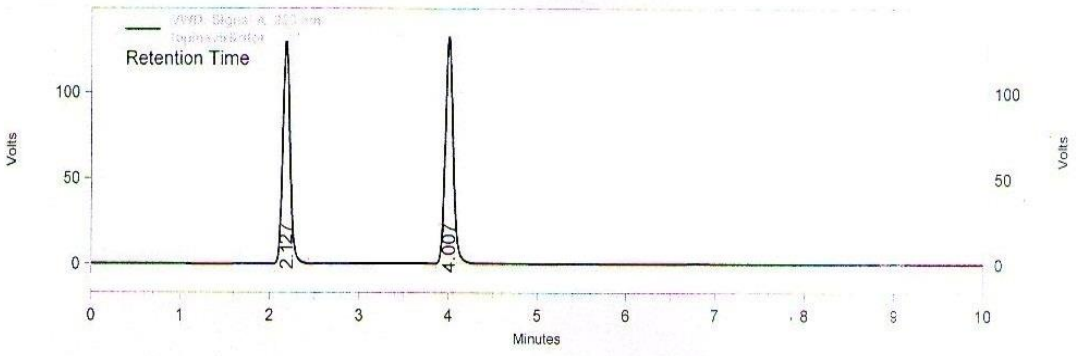

Figure 9: RP-HPLC Chromatogram of Linearity-3 (60 µg/ml of Bisoprolol and Hydrochlorothiazide

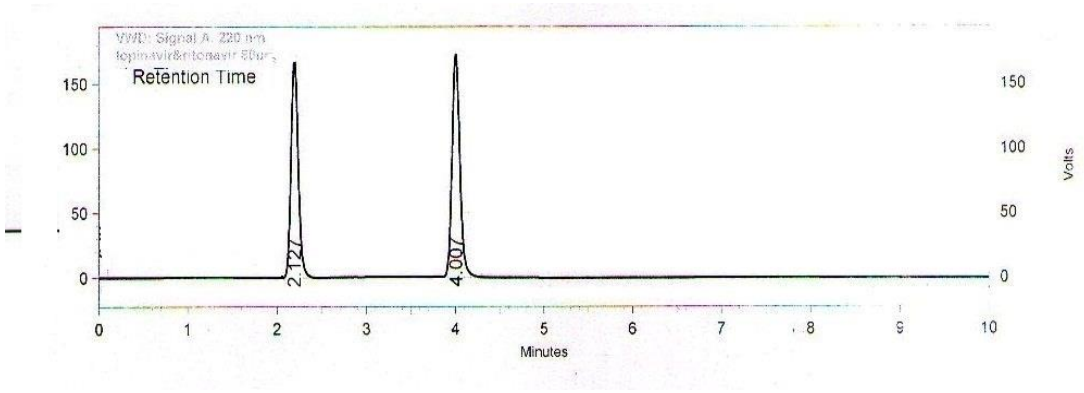

Figure 10: RP-HPLC Chromatogram of Linearity-4 (80 µg/ml of Bisoprolol and Hydrochlorothiazide)

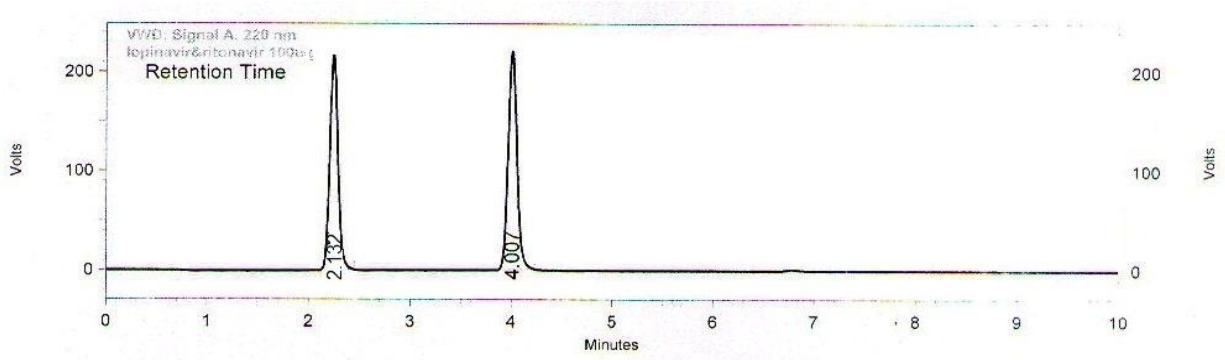

Figure 11: RP-HPLC Chromatogram of Linearity-5 (100 µg/ml of Bisoprolol and Hydrochlorothiazide)

Table 5: Linearity Data of Bisoprolol and Hydrochlorothiazide at 220 nm by RP−HPLC Method

S. No	Bisoprolol			Hydrochlorothiazide		
	Conc. (µg/ml)	R_t (min)	Peak Area	Conc. (µg/mL)	R_t (min)	Peak Area
1	20	2.5	3870150	20	4.1	4528516
2	40	2.5	8122762	40	4.1	8747174
3	60	2.5	12421486	60	4.1	13568359
4	80	2.5	16411684	80	4.1	17809809
5	100	2.5	20481898	100	4.1	22575114

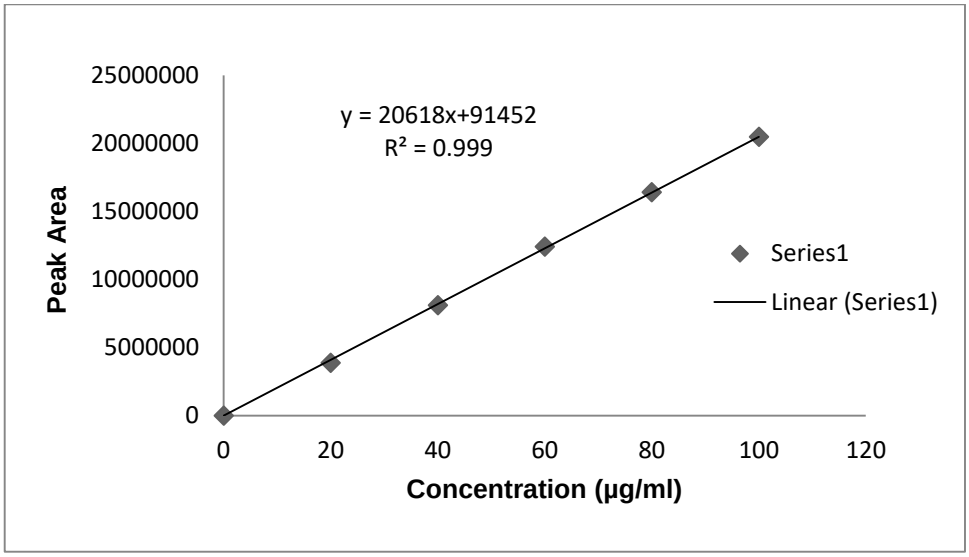

Figure 12: Calibration Curve of Bisoprolol at 220 nm by RP−HPLC Method

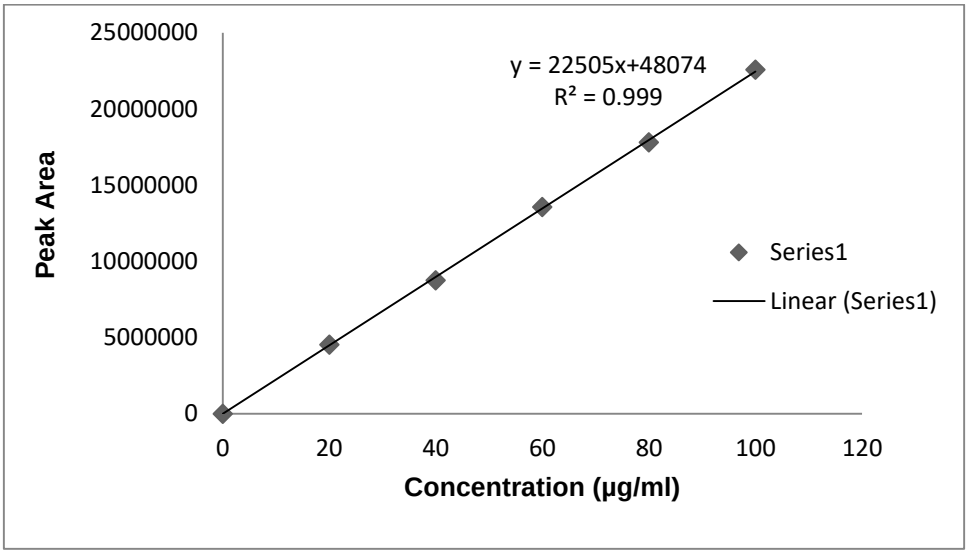

Figure 13: Calibration Curve of Hydrochlorothiazide at 220 nm by RP−HPLC Method

Table 6: Statistical Data of Bisoprolol and Hydrochlorothiazide at 220 nm by RP−HPLC Method

Parameter	**Bisoprolol**	**Hydrochlorothiazide**
Linearity Range (µg/ml)	20-100	20-100
Regression Equation	Y=20618x+91452	Y=22505x+48074
Slope (m)	20618	22505
Intercept (c)	91452	48074
Regression Coefficient (r^2)	0.999	0.999

Limit of Detection (µg/ml)	0.316	0.433
Limit of Quantitation (µg/ml)	0.949	1.28

4.2.3 Assay

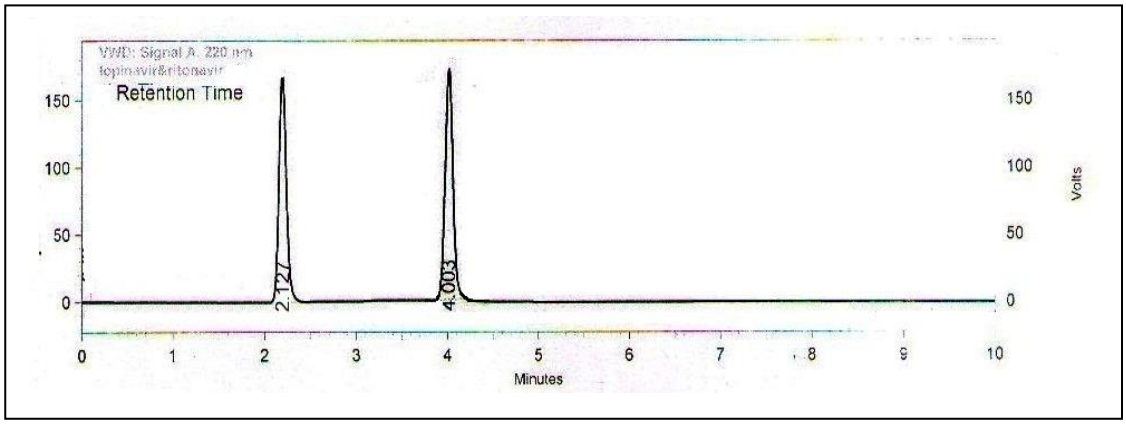

Table 7: Assay of Bisoprolol and Hydrochlorothiazide Tablet Formulation

S. No	Amount Present in (mg/tab)		Amount Obtained in (mg/tab)		% Purity(w/w)	
	BIS	HCTZ	BIS	HCTZ	BIS	HCTZ
1	200	50	198.96	49.35	99.48	98.7

4.2.4Accuracy:

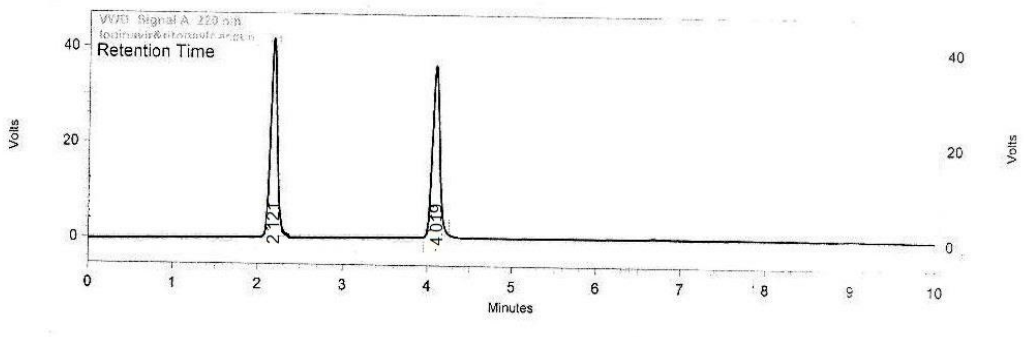

Figure 15: RP-HPLC Chromatogram of 50% Recovery Level

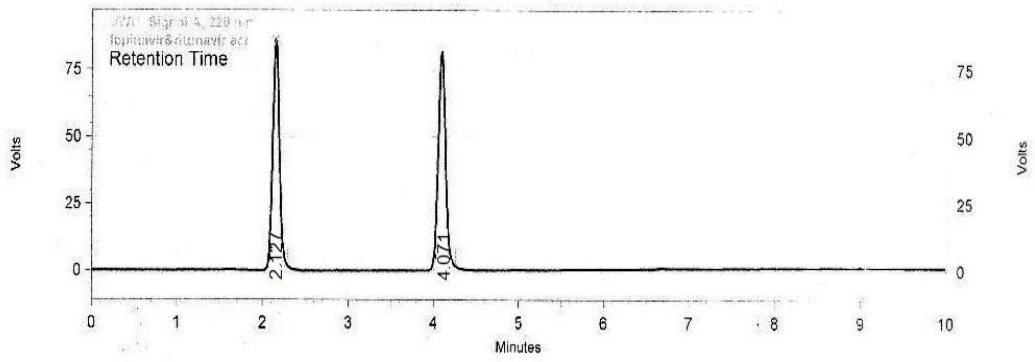

Figure 16: RP-HPLC Chromatogram of 100% Recovery Level

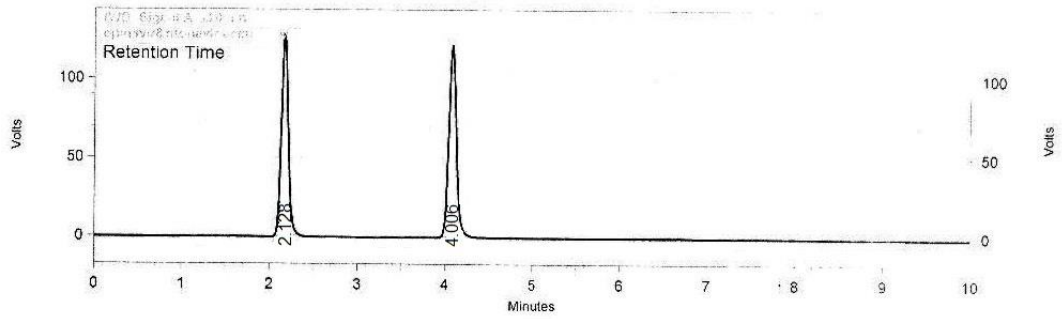

Figure 17: RP-HPLC Chromatogram of 150% Recovery Level

Table 8: Determination of Accuracy for Bisoprolol by RP-HPLC Method

Recovery level	Amount added (µg/ml)		Conc (µg/ml)	Amount found (µg/ml)	% Recovery
	std	test			
50%	4	16	20	19.8	99.1
100%	24	16	40	39.9	99.7
150%	44	16	60	61.02	101.6
Mean re-covery	**99.1-101.6%**				

Table 9: Determination of Accuracy for Hydrochlorothiazide by RP-HPLC Method

Recovery level	Amount added (µg/ml)		Conc (µg/ml)	Amount found (µg/ml)	% Recovery
	std	test			
50%	16	4	20	19.6	98.01
100%	36	4	40	39.5	98.75
150%	56	4	60	60.02	100.3
Mean re-covery	**98.01-100.3%**				

4.2.5Precision:

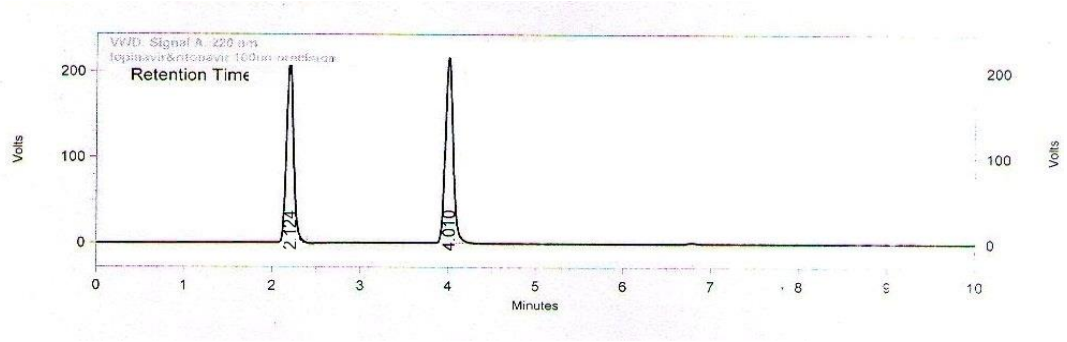

Figure 18: RP-HPLC Chromatogram to Show Intraday Precision of Bisoprolol and Hydrochlorothiazide-

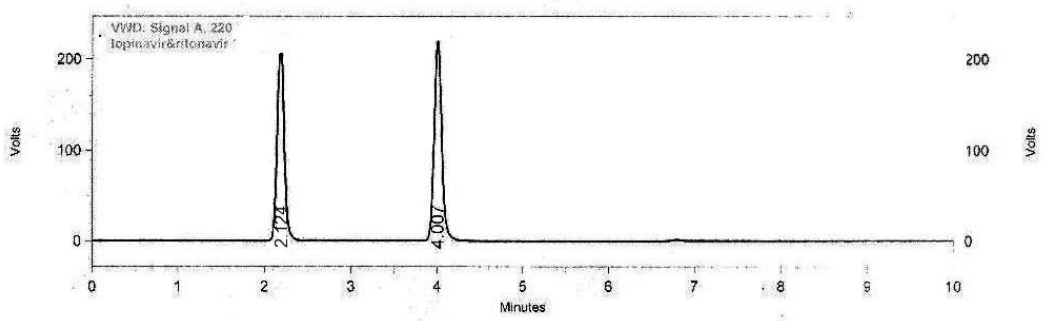

Figure 19: RP-HPLC Chromatogram to Show Intraday Precision of Bisoprolol and Hydrochlorothiazide-2

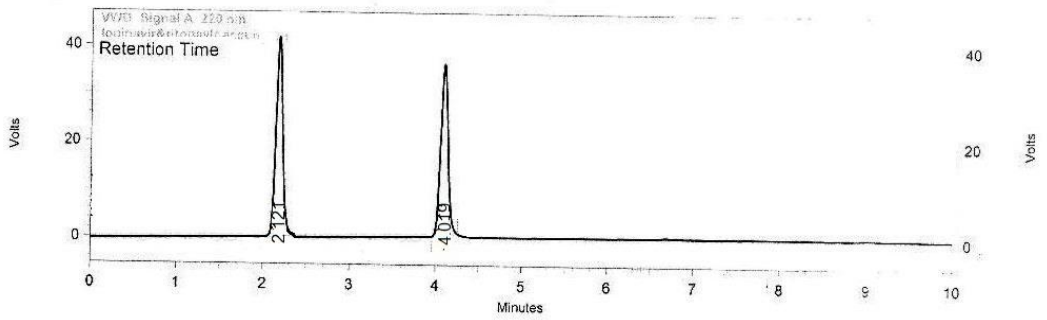

Figure 20: RP-HPLC Chromatogram to Show Intraday Precision of Bisoprolol and Hydrochlorothiazide-3

Table 10: Determination of Intraday Precision for Bisoprolol and Hydrochlorothiazide by RP-HPLC

S. No	Peak area	
	Bisoprolol(100µg/ml)	**Hydrochlorothiazide(100µg/ml)**
1	20481698	22575114
2	20073583	22347591
3	20415432	22753473

4	19740006	21896076
5	20515025	22391680
6	20272436	22481473
Mean	20249696	22407567
SD	297660	209363
% RSD	0.147	0.934

4.2.6Specificity:

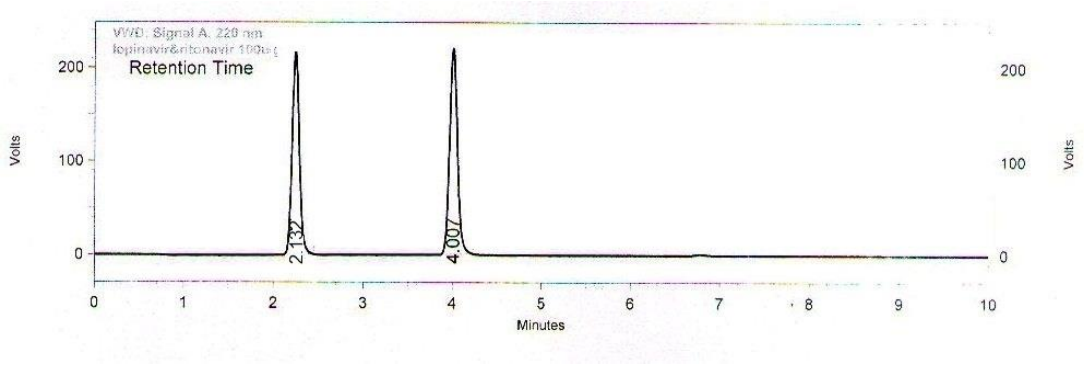

Figure 21: RP-HPLC Chromatogram to Show Specificity of Sample Solution

Table 11: Specificity Parameters of Bisoprolol and Hydrochlorothiazide by RP-HPLC

Parameters	Bisoprolol	Hydrochlorothiazide
Retention Time (min)	2.1	4.0
Resolution (R_s)	2	
Tailing Factor (T)	1.17	1.35
Theoretical Plates (N)	11750	10587

4.2.7Robustness:

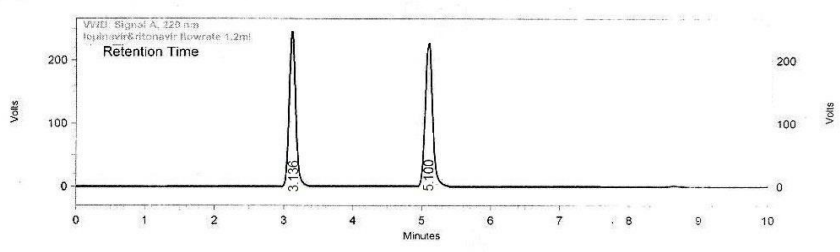

Figure 22: RP-HPLC Chromatogram of Robustness Study for Bisoprolol and Hydrochlorothiazide at Flow rate 1.2 ml/min

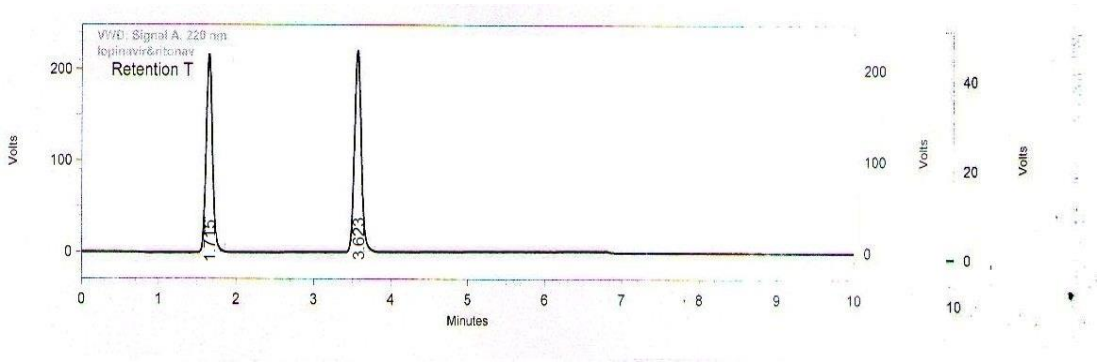

Figure 23: RP-HPLC Chromatogram of Robustness Study for Bisoprolol and Hydrochlorothiazide at Flow rate 1.7 ml/min

Table 12: Robustness Data with Change in Flow Rate

Chromatographic Parameters	Bisoprolol		Hydrochlorothiazide	
	Retention time	Peak Area	Retention time	Peak Area
Flow Rate (ml/min)				
1.2	3.13	20457846	5.100	22385435
	3.11	20852365	5.117	22563224
	3.09	20456685	5.113	22632541
MEAN	3.10	20653624	5.112	22425325
SD	0.0025	0.0026	0.0031	0.0032
%RSD	0.47	0.36	0.52	0.66
1.7	1.715	20369525	3.623	22654255
	1.718	20865265	3.622	22635582
	1.714	20852333	3.625	22533321
Mean	1.716	20645622	3.622	22568844
SD	0.0032	0.0021	0.0026	0.0028
%RSD	0.36	0.31	0.25	0.38

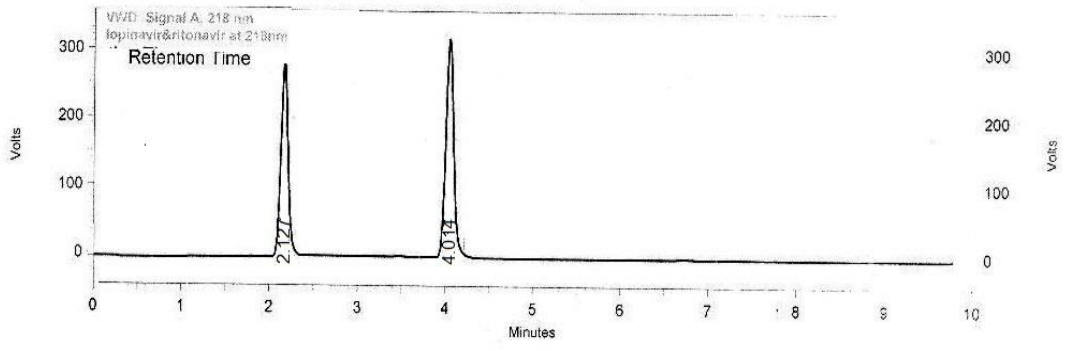

Figure 24: RP-HPLC Chromatogram of Robustness Study for Bisoprolol and Hydrochlorothiazide at Detection Wavelength 218nm

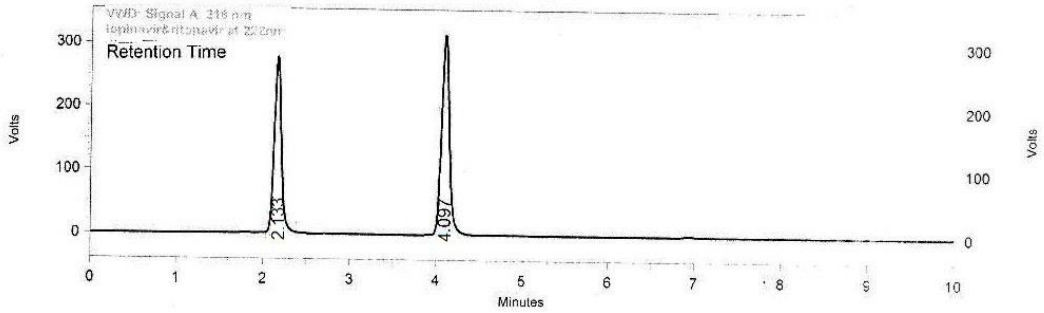

Figure 25: RP-HPLC Chromatogram of Robustness Study for Bisoprolol and Hydrochlorothiazide at Detection Wavelength 222nm

Table 13: Robustness Data with Change in Detection Wavelength

Chromatographic Parameters	Bisoprolol		Hydrochlorothiazide	
	Retention time	Peak Area	Retention time	Peak Area
Wave length nm				
218	2.12	20185198	4.01	22037198
	2.16	20352365	4.03	22263224
	2.14	20456685	4.05	22132541
MEAN	2.13	20253624	4.04	22125325
SD	0.0025	0.0026	0.0031	0.0032
%RSD	0.27	0.36	0.32	0.66
222	2.12	20433342	4.01	22875307
	2.13	20365265	4.03	22635582
	2.16	20252333	4.08	22533321
Mean	2.14	20245622	4.05	22568844
SD	0.0029	0.0041	0.0035	0.0028
%RSD	0.34	0.61	0.56	0.38

4.2.8Ruggedness:

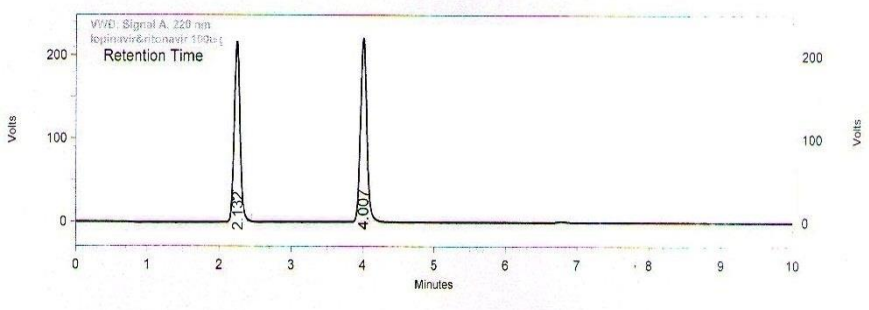

Figure 26: RP-HPLC Chromatogram of Ruggedness Study for Bisoprolol and Hydrochlorothiazide by Analyst-I

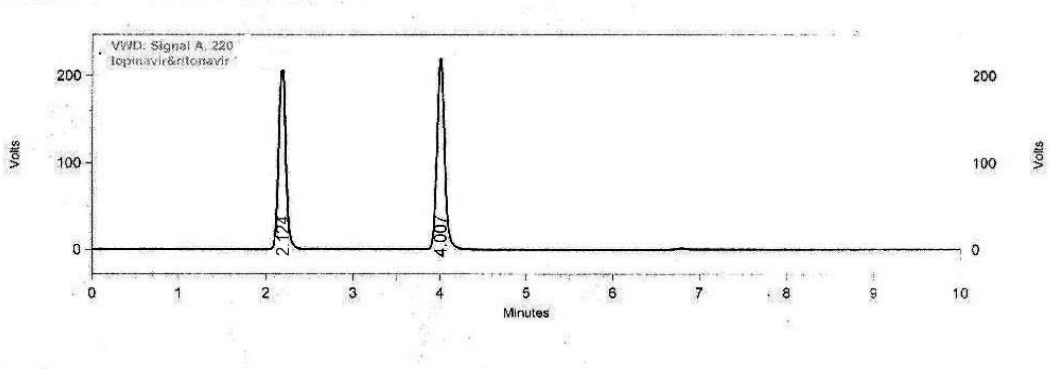

Figure 27: RP-HPLC Chromatogram of Ruggedness Study for Bisoprolol and Hydrochlorothiazide by Analyst-II

Table 14: Ruggedness Data of Bisoprolol and Hydrochlorothiazide

S. No.	Condition	Bisoprolol		Hydrochlorothiazide	
		Rt	Peak Area	Rt	Peak Area
1	Analyst-1	2.13	20481698	4.07	22575114
2		2.12	20415432	4.10	22753473
3		2.12	20373583	4.04	22347591
Mean		2.12	20323571	4.07	22558726
SD		0.005	2409016	0.03	203436
%RSD		0.208	0.81	0.73	0.90
1	Analyst-2	2.12	20515025	4.01	22391680
2		2.11	20740006	4.03	22481473
3		2.12	20472436	4.07	22196076
Mean		2.12	20575822	4.03	22356409
SD		0.01	143772	0.035	145931
%RSD		0.41	0.69	0.86	0.65

Table 15: Summarized Results of RP-HPLC Method

Parameter	Results	
	Bisoprolol	Hydrochlorothiazide
Detection Wavelength	220	
R_t (min)	2.1	4.01
Beer's Law Range (µg/ml)	20-100	20-100
Regression Equation	Y= 20618x+91452	Y= 22505x+48074
Correlation Coefficient (r^2)	0.9993	0.9997
Accuracy (w/w)	99.1-101.6%(w/w)	98.01-100.3%(w/w)
LOD (µg/ml)	0.16	0.33
LOQ (µg/ml)	0.49	1.01
Assay (% purity) (w/w)	99.93% (w/w)	99.56% (w/w)

Precision (%RSD)		
Intraday Precision	0.14	0.93
Robustness		
Flow Rate 1.2 ml/min	0.36	0.66
Flow Rate 1.7 ml/min	0.31	0.38
Detection Wavelength 218 nm	0.38	0.66
Detection Wavelength 222 nm	0.56	0.38
Ruggedness		
Analyst 1	0.81	0.90
Analyst 2	0.42	0.65

5. Discussion

Simultaneous Estimation of Bisoprolol and Hydrochlorothiazide by RP-HPLC Method

In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to separate title ingredients

The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of Bisoprolol and Hydrochlorothiazide in bulk drug and pharmaceutical dosage form by using the most commonly employed C-18 column with UV-detection.

Initially, various mobile phase compositions were tried to elute the drug. Mobile phase ratio and flow rate were selected based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor), run time and resolution. The system with Acetonitrile and Posphate Buffer pH3 (60: 40 v/v) and 1.5 ml / min flow rate was selected.

The optimum wavelength selected was 220 nm from the overlain spectra obtained, at which better detector response for the drug was obtained. The retention time for Bisoprolol and Hydrochlorothiazide was found to be 2.1 min and 4.01 min respectively. The linearity was observed in concentration range of 20-100 µg/ ml for Bisoprolol and Hydrochlorothiazide respectively. Calibration curves of the respective drugs were shown in figure 12 and 13. Summary of validation parameters were given in table 15.

System suitability was assessed by injecting 5 replicate injections of 100% test concentration. Number of theoretical plates was more than 2000 for both the drugs and tailing factor was less than 1.5 for both Bisoprolol and Hydrochlorothiazide was reported. A Resolution of greater than 2 was observed. The relative retention times of six replicate injections and system suitability parameters were given in table 3 and 4.

The low % RSD values (≤ 2) indicated that the method was precise and accurate. The mean recoveries were found in the range of 99.1 – 99.9% w/w.

Specificity of the chromatographic method was tested by injecting sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Bisoprolol and Hydrochlorothiazide at 2.1 min and 4.01min respectively without any interference. Thus the developed method was specific to Bisoprolol and Hydrochlorothiazide and the parameters were given in table 11.

The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters such as change in flow rate to 1.5 ± 0.2 ml and changing detection

wavelength 220nm ± 2nm. The obtained values were given in table 12 and 13. These values with low % RSD (<2) indicated that the method was quite robust.

Ruggedness of the proposed method was determined by analysis of aliquots from homogeneous slot by different analysts, using similar operational and environmental conditions; the % RSD reported was found to be less than 2 and these values were listed in table 14.

The proposed method was validated in accordance with ICH parameters and was applied for analysis of the same in marketed formulations. The content of each component in the formulation was estimated by comparing the peak area of the test sample with that of the peak area of the standard and the results were given in table 7 which were found to be 99.93% w/w for Bisoprolol and 99.56% w/w for Hydrochlorothiazide respectively. High % recovery and low % RSD suggested that the method can be applicable for the routine analysis of commercial formulations.

Hence, the developed HPLC method can be adopted for the routine analysis of Bisoprolol and Hydrochlorothiazide in pharmaceutical formulations.

6. Conclusion

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the methods developed were complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of Bisoprolol and Hydrochlorothiazide in single and combined pharmaceutical dosage forms. The developed and RP-HPLC methods were validated according to ICH guidelines and were found to be applicable for the routine analysis of Bisoprolol and Hydrochlorothiazide in their single and combined dosage forms.

The proposed RP-HPLC method were simple, sensitive and reliable with good precision and accuracy. This method was specific while estimating the commercial formulations without interference of excipients and other additives. Hence, this method can be used for the estimation of Bisoprolol and Hydrochlorothiazide in bulk samples and their pharmaceutical formulations individually and in combination by simultaneous estimation method.

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