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Validation methods for a simultaneous determination of bisoprolol fumarate and hydrochlorothiazide multi-component products, examining system suitability, specificity, linearity and accuracy (Paper A)

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Abstract
At the present work a simultaneous Determination of Bisoprolol fumarate and Hydrochlorothiazide Multi-component Products, examining System Suitability, Specificity, Linearity and Accuracy via RP-HPLC method was carried out. The separation was achieved using Cyanide column (250 × 4.6mm, 5µm particle size), both components were determined by UV detector at fixed wavelength at 228nm, for simplicity of the method an isocratic elution was selected, the optimized mobile phase was composed of methanol and buffer solution (pH=5.0) at 82:18 ratio, with flow rate of 0.9ml/min, injection volume was 10µl, and the separation was performed at 30 ° C. Plot of average area versus prepared concentrations indicates a very good linearity correlation for, (R^2 =0.999) for both components. The limit of detection for bisoprolol fumarate and hydrochlorothiazide was found to be 1.8575µg/ml and 3.57811µg/ml, respectively; whereas the limit of quantitation was found to be 6.19184 µg/ml and 11.92µg/ml; respectively. The proposed method was found to be specific and accurate.

Keywords: validation, bisoprolol fumarate, hydrochlorothiazide, multi-component products, suitability, specificity, linearity and accuracy

1. Introduction

Hypertension is a major public health problem of worldwide distribution and is a major risk factor for cardiovascular disease morbidity and mortality (Kavita, *et al*., 2013).

In the next two decades, ALLHAT and other studies examined the comparability of outcomes with use of different classes and combinations of antihypertensive drugs. (Mohammad G. Saklayen and Neeraj V. Deshpande 2016) [13].

Antihypertensive drugs d is a class of drugs that has an important place in the range of medicinal products currently used to treat cardiovascular diseases. The most commonly used antihypertensive drugs are diuretics, angiotensin-converting enzyme (ACE) inhibitors, angiotensin antagonists and calcium channel blockers, and in some cases, is needed the combination of two or three of these. (Moisei *et al*., 2016) [9]. Based on the analysis of some literature and relative data from FDA, the advantages of fixed-dose combination are elucidated and formulations of common dual, triple combinations were summarized. Clinical practices proved that fixed-dose combinations had many benefits comparing with single drug and separate agents in terms of effects, convenience, compliance, and costs to a certain extent. From the patients' perspective, the fixed-dose combination therapy will be increasingly utilized in blood pressure control in the future. (Wald *et al*., 2009; Xinhuan Wan *et al*., 2014) [16, 17]. The molecular formula for Bisoprolol fumarate is: $(C_{18}H_{31}NO_4)_2$. $C_4H_4O_4$, Molecular Weight 766.98 and IUPAC name is (±)-1-[4-[[2-(1-methylethoxy) ethoxy] methyl] phenoxy]-3-[(1-methylethyl) amino]-2-propanol (E) -2-butenedioate (2:1) and the chemical structure is shown in Figure 1.

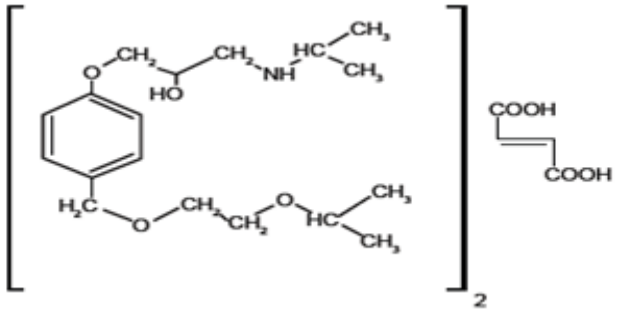


Fig 1: Is Bisoprolol Fumarate structure. (USP 2016)

Whereas the molecular formula for Hydrochlorothiazide (HCTZ) is $C_7H_8ClN_3O_4S_2$, Molecular Weight (297.74) and IUPAC name is 6-Chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfomanide 1, 1-dioxide and the chemical structure is shown in Figure 2.

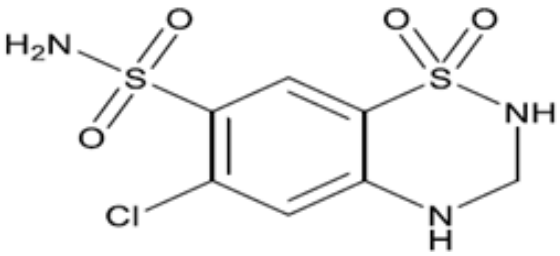


Fig 2: Is Hydrochlorothiazide structure. (USP 2016)

Bisoprolol fumarate is a cardio selective β 1 -adrenergic blocker. 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 beta-blocking activity. It is almost completely absorbed from the gastrointestinal tract and undergoes only minimal first pass metabolism resulting in an oral bioavailability of about 90%. It is bound to plasma proteins at about 30%.

Bisoprolol is been used individually and in combination with other antihypertensive agents for the treatment of hypertension, heart attacks, and kidney problems. (Raju *et al*. 2016; Savita's Yadav and Janhavirrao. 2013; Bozal *et al*. 2013; Renuka *et al*. 2016) [11, 18, 4, 12]. Hydrochlorothiazide is a diuretic/antihypertensive agent. The diuretic drug hydrochlorothiazide (HCTZ) is used mainly for treatment of mild to moderate hypertension of edema in people with congestive heart failure, cirrhosis of the liver, or kidney disorders and is usually administered with other drugs. Hydrochlorothiazide binds to and inhibits the enzyme carbonic anhydrase.

It is frequently used alone or in combination with other medications for the treatment of hypertension, congestive heart failure, symptomatic edema, diabetes insipidus, renal tubular acidosis, hyperparathyroidism, and edema and prevention of kidney stones and used in the treatment of osteoporosis. (Savita's Yadav and Janhavirrao. 2013; Renuka *et al*. 2016; Nidhal S. Mohammed and Ahmed J. Mohammed. 2016) [18, 12, 8].

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

The method was validated for System Suitability specificity, linearity, precision, accuracy and Robustness.

There were many methods proposed for the analysis of bisoprolol fumarate and hydrochlorothiazide as combined drug or as separating drug or as a combined drug with other.

Different parameters affecting the method accuracy and reliability. etc. were also studied.

A high-performance liquid chromatography method for determination of bisoprolol Fumarate and Hydrochlorothiazide was investigated by many authors e.g. Patel. L. J *et al*. (2006) [10], Joshi *et al*. (2010) [6]; Ravi Varma Athota *et al*., (2016) [1]; and they used C_{18} column (250 × 4.6mm, 5µm) The limit of quantitation was 0.398 and 0.385µg/ml for bisoprolol and hydrochlorothiazide, respectively.

Bhoya *et al*. (2013) [2]; Savita's Yadav and Janhavirrao (2013) [18]; were used a high-performance thin-layer chromatographic for simultaneous determination of bisoprolol fumarate and hydrochlorothiazide using precoated silica gel HPTLC aluminum plate 60 F254, using chloroform, ethanol and glacial acetic acid in the ratio of 5**:**1.5**:**0.2 (v/v) as mobile phase.

The use of voltammetry, chromatographic, and spectrophotometric methods for determination of bisoprolol fumarate (BIS) and hydrochlorothiazide (HCZ) was reported by Bozal *et al*. (2013) [4]; Sevinc Kurbanoglu. *et al*, (2014) [7]; validated an Ultra-performance liquid chromatographic method for the simultaneous determination of bisoprolol fumarate and hydrochlorothiazide in their combined dosage forms and as well as in spiked human urine samples. Other authors were used HPLC-UV for determination of bisoprolol and hydrochlorothiazide such as Renuka, *et al*. (2016) [12] and Raju *et al*., (2016) [11] and they agreed in using C_{18} column (150 x 4.6mm, 5µm) and (250 x 4.6mm). Bobade, P. S. and Ganorkar, S. B. (2017) [3]; investigated spectrophotometric method for the determination of bisoprolol fumarate and Hydrochlorothiazide using 0.1N Sodium hydroxide as solvent. The objectives of the present work is a developing of assay methods for a simultaneous determination of bisoprolol fumarate and Hydrochlorothiazide multi-component products, examining System suitability, Specificity, Linearity, Accuracy and Robustness for each developed method and comparing the obtained results with the acceptance criteria of USP and ICH guidelines, finally application of the developed method for real sample assay.

2. Materials and Methods

2.1.1 Chemicals

Bisoprolol fumarate (purity: 99.30%), Hydrochlorothiazide (purity: 99.10%) and Tetra butyl ammonium hydroxide 40% were obtained from Aurobindo, India, Unichem India and Emplura India; respectively. Methanol was of HPLC grade and all other chemicals used were of analytical grade. Purified water from Milli-Q-system (Millipore, Bangalore, India) was used throughout the analysis.

2.1.2 Instruments

High Performance Liquid Chromatography HPLC
Type: HPLC prominence – i
Model: LC-2030C3D
Serial No: L21455300660AE
Company: Shimadzu Corporation
Origin: Japan

Analytical Balance
Type: AY220
Serial No: O4328143000
Capacity: 220 g
Readability: 0.1 mg
Company: Shimadzu Corporation
Origin JAPAN

Ultrasonic
Model: 621.05.003
Company: ISO Lab Laborgerate -GmbH
Origin: Germany

pH-meter
Model: PHS-550
Origin: Romania.

Magnetic Stirrer
Model: LMS, 1001
Serial No: 2016017862
Company: QAIHAN LAB TECH Co-LTD
Origin: Korea

2.2 Methods

2.2.1 Optimized chromatographic conditions

Cyanide column (250 × 4.6mm, 5µm), and simple isocratic elusion, were used (one pump required) with flow-rate of 0.9ml/min, both active ingredients were detected at 228nm, injection volume was 10µl (universal loop) and analysis temperature was 30°C.

2.2.2 Buffer Solution pH 5.0

1000 ml volumetric flask was Mixed 980 ml of deionized water, 10 ml of tetra butyl ammonium hydroxide 40% was added to the flask and adjusted to pH 5.0 with glacial acetic acid, and the volume was completed to the mark with deionized water.

2.2.3 Mobile Phase

Mixture of buffer and methanol were prepared in 82:18 v/v ratio, respectively. The mixture was shaken, filtered with vacuum filtration pump through 0.45µm nylon membrane filter, and then transferred to solvent reservoir and sonicated for 5 min.

2.2.4 Standard Stock Solution

0.2500g Hydrochlorothiazide and 0.1000g bisoprolol fumarate were weighed accurately, transferred quantitatively to the same 100ml volumetric flask, 50ml Methanol was added and sonicated to 5 min, cooled, and completed to with mobile phase.

2.2.5 System Suitability

Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 250µg/ml hydrochlorothiazide and 100µg/ml Bisoprolol Fumarate. System suitability solution was injected six times.

2.2.6 Specificity

a) **Standard:** Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 250µg/ml hydrochlorothiazide and 100µg/ml bisoprolol fumarate. System suitability solution was injected six times.
b) **Placebo:** A placebo equivalent to average weight of one tablet was transferred to 50-ml volumetric flask, the flask was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made in mobile phase with similar to those made for standard preparation.
c) **Sample:** Five tablets were taken in to clean and dry 100 ml volumetric flask and shacked with 10 ml methanol, sonicated for 10 min, cooled, then 50 ml mobile phase was added, sonicated to 20 min, leave to reach room temperature, and then completed to required volume with mobile phase. Then 5ml was diluted with mobile phase in to 25ml volumetric flask, passed through a suitable filter 0.45µm pore size.

2.2.7 Linearity

Subsequent dilutions were made from the stock solution with mobile phase to give concentrations of 100, 150, 200, 250, 300, 350 and 400µg/ml hydrochlorothiazide and 40.60,80,100,120,140 and 160µg/ml bisoprolol fumarate. Each solution was injected three times and results were collected, LOD and LOQ were calculated from the linear regression analysis.

2.2.8 Accuracy

a) **Standard:** Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 250µg/ml hydrochlorothiazide and 100µg/ml Bisoprolol Fumarate. System suitability solution was injected six times.
b) **Preparation of Test Solution:** Three 100-ml volumetric flasks were labeled; a placebo equivalent to tablets weight was transferred to each flask. A volume of standard stock solution required to produce 50%, 100%, and 150% tablets content of hydrochlorothiazide and bisoprolol fumarate was added each to different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase like those made for the standard preparation. Each solution was injected three times. The results were collected and subjected to statistical treatments.

3. Results and Discussion

3.1 Bisoprolol fumarate and Hydrochlorothiazide

3.1.1 System Suitability

System suitability results for bisoprolol fumarate and hydrochlorothiazide are shown in Table (1) and Table (2) respectively.

Table 1: System suitability results for bisoprolol fumarate

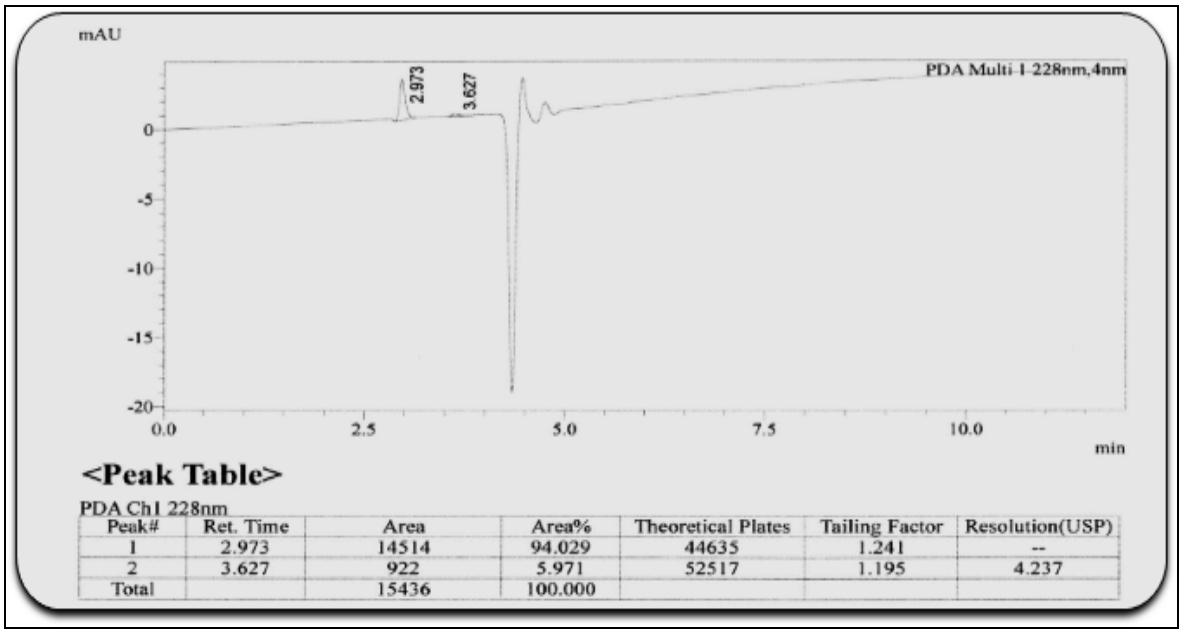
No	Area	Retention time	Tailing factor	Resolution	Theoretical plates
1	1476536	4.718	1.247	10.468	39604
2	1477324	4.71	1.241	10.438	39290
3	1475963	4.72	1.238	10.422	39390
4	1475068	4.722	1.236	10.421	39316
5	1470496	4.71	1.235	10.385	39211
6	1475461	4.719	1.234	10.358	39092
AVG	1475141.333	4.7165	1.2385	10.41533333	39317.16667
STDEV	2411.26321	0.005205766	0.00484768	0.038913579	173.3717586
RSD%	0.163459809	0.110373498	0.39141541	0.373618186	0.440956898

Table 2: System suitability results for hydrochlorothiazide

No	Area	Retention time	Tailing factor	Resolution	Theoretical plates
1	12358531	7.783	1.166	10.468	55637
2	12369606	7.775	1.161	10.438	55195
3	12365433	7.784	1.157	10.422	55143
4	12322841	7.785	1.154	10.421	55316
5	12323359	7.763	1.154	10.385	54845
6	12363664	7.779	1.151	10.358	54432
AVG	12350572.33	7.778166667	1.157166667	10.41533333	55094.66667
STDEV	21575.19108	0.008304617	0.005492419	0.038913579	413.9993559
RSD%	0.174689808	0.106768311	0.474643729	0.373618186	0.751432726

3.1.2 Specificity

Figure (3) Figure (4), Figure (5) and Figure (6) shows the specificity chromatograms for placebo, sample, standard for bisoprolol fumarate and hydrochlorothiazide and Fumaric acid; respectively.

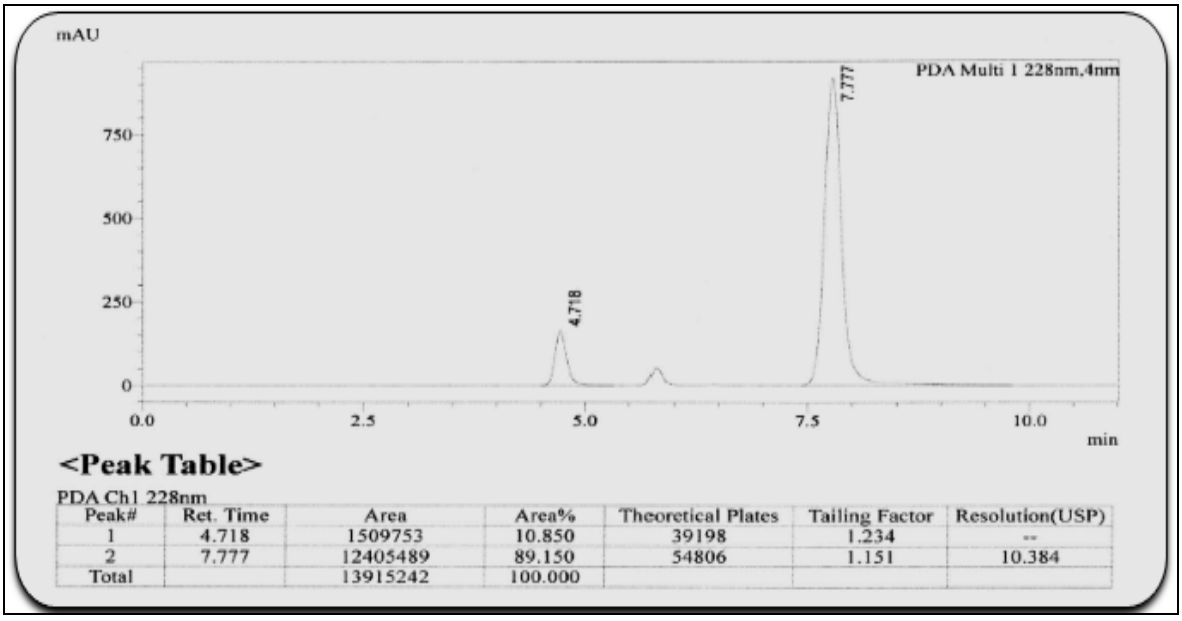


<Peak Table>

PDA Ch1 228nm

Peak#	Ret. Time	Area	Area%	Theoretical Plates	Tailing Factor	Resolution(USP)
1	2.973	14514	94.029	44635	1.241	--
2	3.627	922	5.971	52517	1.195	4.237
Total		15436	100.000			

Fig 3: Chromatogram for the Placebo of bisoprolol fumarate and hydrochlorothiazide

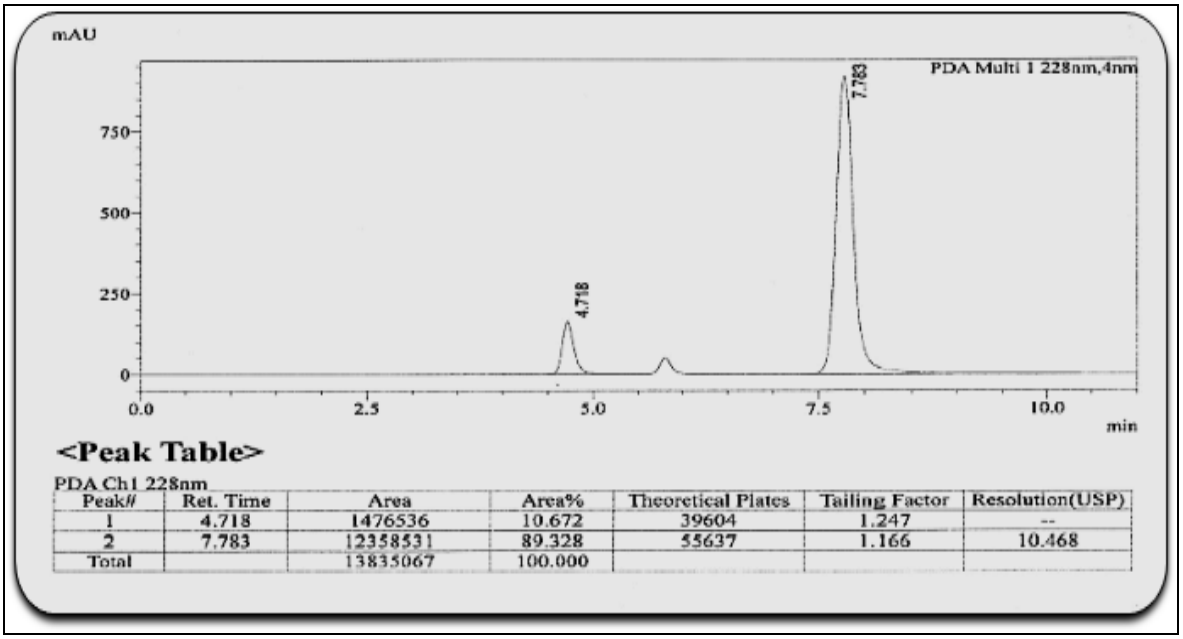


<Peak Table>

PDA Ch1 228nm

Peak#	Ret. Time	Area	Area%	Theoretical Plates	Tailing Factor	Resolution(USP)
1	4.718	1509753	10.850	39198	1.234	--
2	7.777	12405489	89.150	54806	1.151	10.384
Total		13915242	100.000			

Fig 4: Chromatogram for the sample of bisoprolol fumarate and hydrochlorothiazide

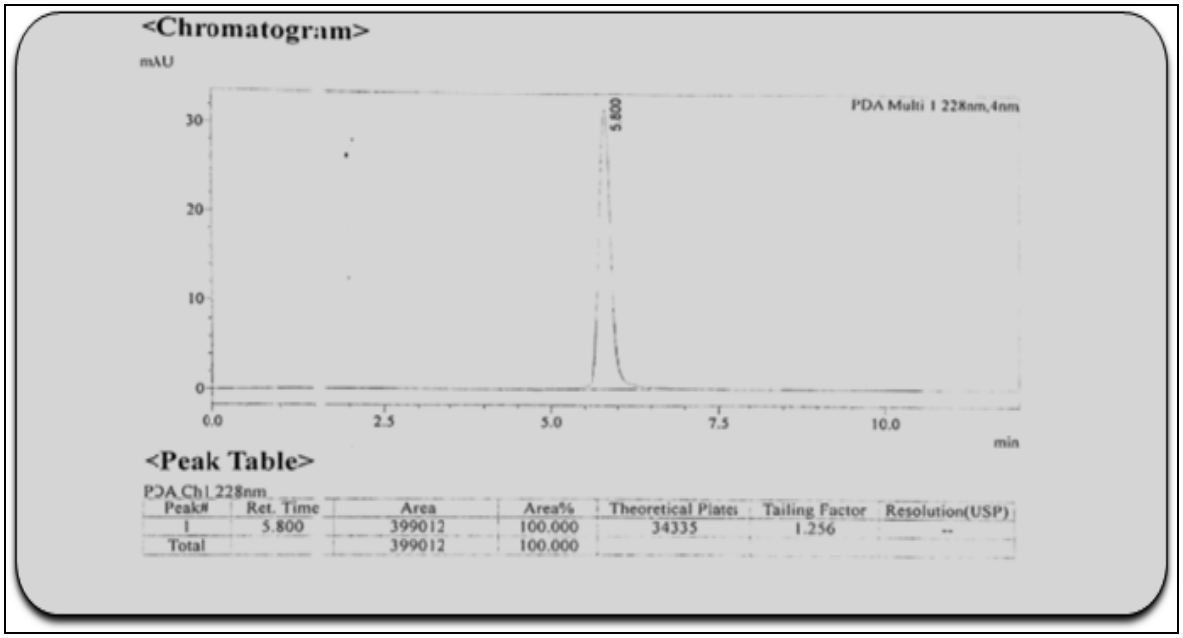


<Peak Table>

PDA Ch1 228nm

Peak#	Ret. Time	Area	Area%	Theoretical Plates	Tailing Factor	Resolution(USP)
1	4.718	1476536	10.672	39604	1.247	--
2	7.783	12358531	89.328	55637	1.166	10.468
Total		13835067	100.000			

Fig 5: Chromatogram for mixed standard of bisoprolol fumarate and hydrochlorothiazide



<Peak Table>

PDA Ch1 228nm

Peak#	Ret. Time	Area	Area%	Theoretical Plates	Tailing Factor	Resolution(USP)
1	5.800	399012	100.000	34335	1.256	--
Total		399012	100.000			

Fig 6: Chromatogram for fumaric Acid

3.1.3 Linearity, LOD and LOQ

i) Bisoprolol fumarate

Linearity results for bisoprolol fumarate, was shown in Table (3)

Table 3: linearity results for bisoprolol fumarate

%	40%	60%	80%	100%	120%	140%	160%
C. µg/ml	40	60	80	100	120	140	160
Area – 1	567911	856268	1123553	1395731	1691940	1973292	2229507
Area – 2	567682	855959	1123277	1395368	1691052	1968819	2229121
Area – 3	568128	855616	1121591	1394959	1690548	1977723	2248307
AVG	567907	855947.666	1122807	1395352.66	1691180	1973278	2235645
STDEV	223.0269	326.1477	1062.0904	386.2283	704.7723	4452.016	10967.312
RSD%	0.0393	0.0381	0.0946	0.0277	0.0417	0.2256	0.4906

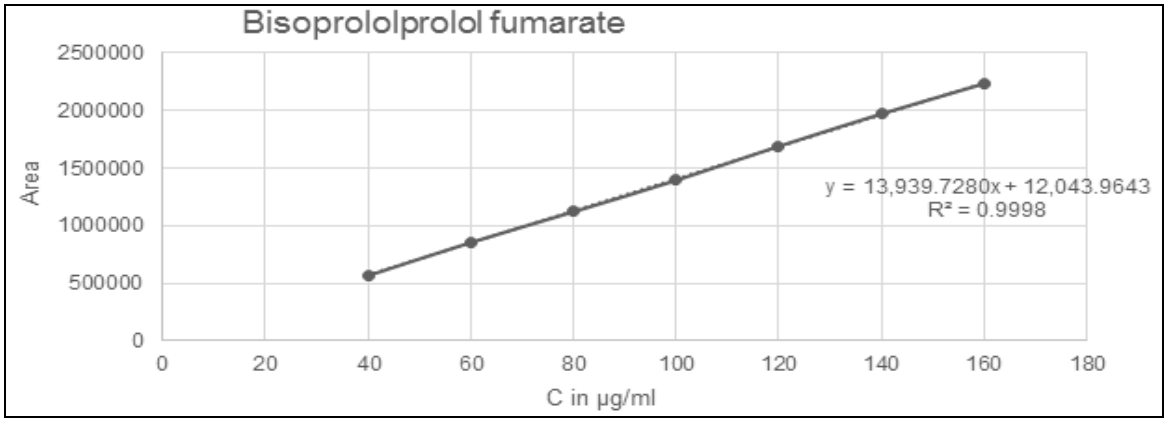


Fig 7: Plot of average area versus concentration for bisoprolol fumarate

Table (4) Shows the Summarize data of regression parameters for Bisoprolol fumarate by excel program.

Table 4: For the Summarize data of regression parameters for Bisoprolol fumarateh5

Parameter	Value
Regression Coefficient R^2	0.999829
Root Mean Squire Error (RME)	8631.25704
Slope (S)	13939.728
Intercept	12043.9643

Limit of detection and limit of quantitation

LOD =3*RMSE/S

= 3* 8631.257043/13939.728= 1.857552111µg/ml

LOQ=10*RMSE/S

=10*8631.257043/13939.728=6.19184µg/m

ii) Hydrochlorothiazide

Linearity results for bisoprolol fumarate, was shown in Table (5)

Table 5: linearity result for Hydrochlorothiazide

%	40%	60%	80%	100%	120%	140%	160%
C. µg/ml	100	150	200	250	300	350	400
Area – 1	4671998	6863543	9069697	11397175	13575926	15737025	17890700
Area – 2	4646667	6862508	9086374	11395711	13581165	15679591	17893591
Area – 3	4670935	6863214	9045076	11367125	13551764	15716988	17865704
AVG Area	4663200	6863088	9067049	11386670	13569618	15711201	17883331
STDEV	14327.859	528.81975	20775.951	16942.57552	15682.62396	29150.9900	15334.2898
RSD%	0.3072538	0.0077052	0.2291369	0.148793063	0.115571592	0.18554271	0.08574627

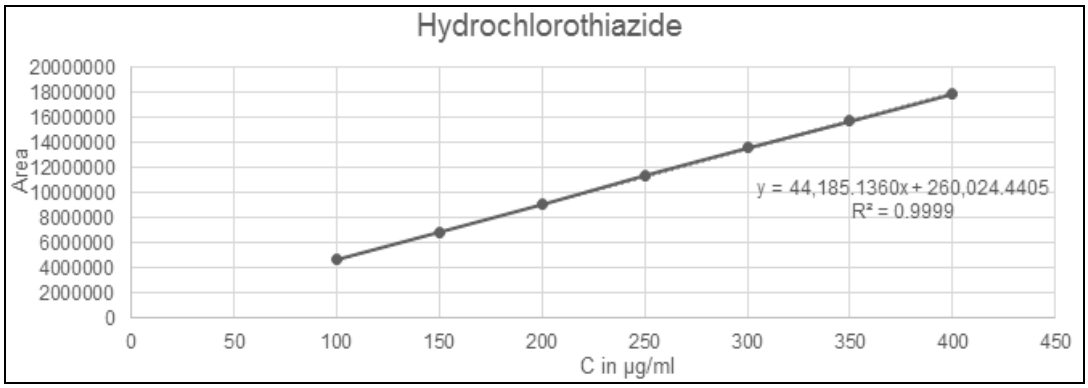


Fig 8: Plot of average area versus concentration for hydrochlorothiazide

Table (6) shows the Summarize data of regression parameters for Hydrochlorothiazide by excel program.

Table 6: For the Summarize data of regression parameters for Hydrochlorothiazide

Parameter	Value
Regression Coefficient R^2	0.9998984
Root Mean Squire Error (RME)	52699.89639
Slope (S)	44185.13595
Intercept	260024.4405

Limit of detection and limit of quantitation

LOD =3*RMSE/S

= 3* 52699.89639/ 44185.13595= **3.578µg/ml**

LOQ=10*RMSE/S

=10*52699.89639/44185.13595=**11.927µg/ml**

3.1.5 Accuracy

Table (7) shows the results of mixed standard of bisoprolol fumarate and hydrochlorothiazide while the accuracy results for bisoprolol fumarate and hydrochlorothiazide samples were shown in Table (8) and Table (9), respectively; summary of accuracy results for both components is shown in Table (10).

Table 7: Results of hydrochlorothiazide and bisoprolol fumarate standard for accuracy test

No	Bisoprolol fumarate	Hydrochlorothiazide
1	1412198	12000697
2	1413126	12006732
3	1413927	12011592
4	1413575	12006457
5	1416091	12027056
6	1414754	12013025
AVG	1413945.17	12010926.5
STDEV	1350.53995	9024.636209
RSD%	0.096	0.075

Table 8: Accuracy results for bisoprolol fumarate

Content	50%	100%	150%
1	726795	1415612	2169404
2	726784	1416273	2141343
3	725474	1415666	2169404
AVG	726351	1415850	2160050
STDEV	759.524	367.035	16201.026
RSD%	0.10457	0.02592	0.75003
Recovery	51.371	100.135	152.768
Recovery%	102.741	100.135	101.845

Table 9: Accuracy results for hydrochlorothiazide

Content	50%	100%	150%
1	6044602	12026875	18223119
2	6024101	12033751	18290031
3	6045482	12028800	18316606
AVG	6038062	12029809	18276585
STDEV	12098.296	3547.238	48172.028
RSD%	0.20037	0.02949	0.26357
Recovery	50.271	100.157	152.166
Recovery%	100.543	100.157	101.444

Table 10: Summary of accuracy results for bisoprolol fumarate and hydrochlorothiazide

Content%	Recovery % of Bisoprolol fumarate	Recovery% of Hydrochlorothiazide
50	102.74	100.54
100	100.14	100.16
150	101.85	101.44
AVG	101.57	100.71
STDEV	1.32402	0.66045
RSD%	1.30351	0.65576

3.2 Discussion

A simple and sensitive RP-HPLC method was developed for the determination of hydrochlorothiazide and bisoprolol fumarate in their combined pharmaceutical formulations. The separation was achieved using Cyanide column (250 × 4.6mm, 5µm particle size), both components were determined by UV detector at fixed wavelength at 228nm, for simplicity of the method an isocratic elution was selected, the optimized mobile phase was composed of methanol and buffer solution at 82:18 ratio, with flow rate of 0.9ml/min, injection volume was 10µl, and the separation was performed at 30°C. Linearity of this method was checked using seven solutions centered with the target concentration, the concentrations range was (40–160) µg/ml for bisoprolol fumarate and (100–400) µg/ml for hydrochlorothiazide. Each solution was injected in triplicate. Plot of average area versus prepared concentrations indicates a very good linearity correlation for, (R^2 =0.999) for both components. The limit of detection for bisoprolol fumarate and hydrochlorothiazide was found to be 1.8575µg/ml and 3.57811µg/ml, respectively; whereas the limit of quantitation was found to be 6.19184 µg/ml and 11.92µg/ml; respectively.

In specificity tests, none of placebo peaks had same retention time of active ingredients peaks. The second peak in Sample and standard was confirmed as fumarate by injection of fumaric acid alone and given the same retention time as the combination. This indicates that the excipients used in the formulation did not interfere in the estimation when we used this method for assay in tablets. Accuracy was evaluated for bisoprolol fumarate and hydrochlorothiazide using three concentrations in content of 50%, 100%, and 150 of target concentration. The recovery percentage for bisoprolol fumarate at the above concentrations was found to be 102.741, 100.135 and 101.845, respectively; while for hydrochlorothiazide, it was 100.543, 100.157 and 101.444 respectively. The average of recovery percentage for bisoprolol fumarate and hydrochlorothiazide was 101.5736% and 100.7146%, respectively.

3.3 Conclusions

The proposed method is simple and sensitive for simultaneous determination of bisoprolol fumarate and hydrochlorothiazide in tablet as well as in pharmaceutical preparations. Statistical analysis of the results has been carried out revealing high accuracy, good system suitability, specificity and linearity with limit ICH guidelines and USP.

4. References

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