METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ANTI-HYPERTENSIVE DRUGS ATENOLOL AND CHLORTHALIDONE IN SOLID DOSAGE FORMS BY RP-HPLC

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ABSTRACT : ***A Novel Analytical simple, reproducible and efficient RP-HPLC method was developed for simultaneous estimation of Atenolol and Chlorthalidone in pure form and marketed combined pharmaceutical dosage forms. A column having Develosil ODS HG-5 RP C18, 15cmx4.6mm, i.d. Column in isocratic mode with mobile phase containing Methanol: Acetonitrile in the ratio of 85:15% v/v was used. The flow rate was 1.0 ml/min and effluent was monitored at 258nm. The retention times and linearity range for Atenolol and Chlorthalidone was found to be (2.217, 5861min) and (0-14, 0-28), respectively. The method has been validated for linearity, accuracy and precision, robustness and limit of detection and limit of quantitation. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.08µg/ml and 0.24µg/ml for Atenolol and 0.1µg/ml 0.3µg/ml for Chlorthalidone respectively. The proposed method was found to be accurate, precise and selective for simultaneous estimation of Atenolol and Chlorthalidone in pure form and marketed combined pharmaceutical dosage forms.***
Keywords: Atenolol and Chlorthalidone, RP-HPLC, Validation, Accuracy, Precision.

I.INTRODUCTION

Atenolol is a cardioselective beta-blocker that is widely used in the treatment of hypertension and angina pectoris. Atenolol[1] has been linked to rare cases of drug induced liver injury, some of which have been fatal. Atenolol is an ethanolamine compound having a (4-carbamoyl methyl phenoxy) methyl group at the 1-position and an N-isopropyl substituent. It has a role as a beta-adrenergic antagonist, an anti-arrhythmia drug, an antihypertensive agent, a sympatholytic agent, a xenobiotic and an environmental contaminant. It is a member of ethanolamines, a monocarboxylic acid amide and a propanolamine. Atenolol[2] is a cardioselective beta-blocker used in a variety of cardiovascular conditions. Sir James Black, a scottish pharmacologist, pioneered the use of beta-blockers for the management of angina pectoris in 1958 for which he received the Nobel Prize. Beta-blockers quickly became popular in clinical use and where subsequently investigated for use in myocardial infarction, arrhythmias, and hypertension during the 1960s. Later they continued to be investigated for use in heart failure throughout the 1970-1980s. Atenolol[3] itself was developed early on in this history by Alvogen Malta under the trade name Tenormin and received FDA approval in September, 1981. The IUPAC Name of Atenolol is 2-[4-[2-hydroxy-3-(propan-2-ylamino) propoxy] phenyl] acetamide. The Chemical Structure of Atenolol is following

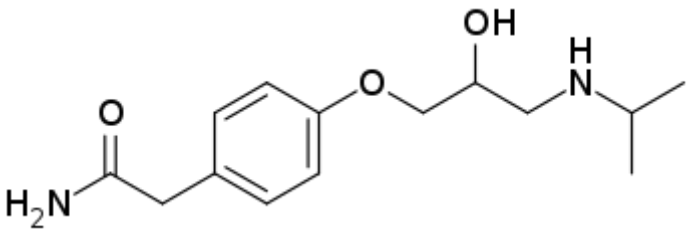

Fig.1. Chemical Structure of Atenolol

Chlorthalidone is a thiazide-like diuretic used for the treatment of hypertension and for management of edema caused by conditions such as heart failure or renal impairment. Chlorthalidone[4] improves blood pressure and swelling by preventing water absorption from the kidneys through inhibition of the Na+/Cl− symporter in the distal convoluted tubule cells in the kidney. The exact mechanism of chlorthalidone's anti-hypertensive effect is under debate, however, it is thought that increased diuresis results in decreased plasma and extracellular fluid volume, decreased cardiac output and therefore overall reduction in blood pressure. Chlorthalidone is considered first-line therapy for management of uncomplicated hypertension as there is strong evidence from meta-analyses

that thiazide diuretics such as Chlorthalidone[5] reduce the risk of stroke, myocardial infarction, heart failure, and cardiovascular all-cause mortality in patients with hypertension. In particular, the ALLHAT trial confirmed the role of thiazide diuretics as first-line therapy and demonstrated that Chlorthalidone had a statistically significant lower incidence of stroke and heart failure when compared to [DB00722], [DB00381], or [DB00590]. The IUPAC Name of Chlorthalidone[6] is 2-chloro-5-(1-hydroxy-3-oxo-2H-isoindol-1-yl) benzene sulfonamide. The Chemical Structure of Chlorthalidone is as follows

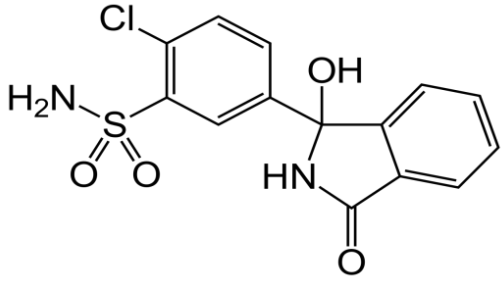

Fig.2. Chemical Structure of Chlorthalidone

II. MATERIALS AND METHODS

Table-1: List of Instrument used

S. No.	Instruments/Equipment/Apparatus
1.	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	T60-LAB INDIA UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator(Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5□m, 15mm x 4.6mm i.d.
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Table-2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Atenolol Standard Solution Preparation

Weigh accurately 10 mg of standard Atenolol and it transferred into a clean & dry 100 ml of volumetric flask. Add 10ml mobile phase and further do sonication[7] in order to dissolve. Finally make up to the volume up to mark with the mobile phase[8]. The final resulted solution contained about 100 µg/ml of Atenolol.

Chlorthalidone Standard Solution Preparation

Weigh accurately about 10 mg of standard Chlorthalidone and transferred into a clean and dry 100 ml volumetric flask. Add 10ml mobile phase and further do sonication in order to dissolve. Finally make up the volume with the same mobile phase i.e. same solvent system[9]. The volume was made up to the mark with same solvent. The final solution contained about 100µg/ml of Chlorthalidone.

Initialization of the HPLC instrument

First switched on the HPLC instrument. The selected column was washed with the HPLC grade water for 45 minutes. Then selected column[10] was saturated with the mobile phase for 45 minutes. Then keep the mobile phase for stabilization. The mobile phase was run to obtain the peaks. After completion of stabilization. After 20 minutes the standard drug solution was injected in HPLC[11].

Table- 3: Different Chromatographic used and their Optimizations

S.No.	Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
1	Symmetry C i8, 5pni, 25emx4.6mm i.d.	water: Methanol =30:70	1.0 ml/min	258nm	Peaks didn't Separate	Method rejected
2	Waters C18, 5pm, 25cmx4.6inini.d.	Water : ACN = 55:45	1.0 ml/min	258nm	Early elution of peak	Method rejected
3	Waters C18, 5pm, 25cmx4.6inini.d.	ACN: methanol= 60: 40	1.0 ml/min	258nm	Low resolution peak	Method rejected
4	Develosil ODS HG-5 RP C18, 5pin,15emx4.6mm i.d.	ACN: methanol 90:10	1.0 ml/ min	258nm	Resolution increases but Peak shapes not good	Method rejected
5	Develosil ODS HG-5 RP C18, 5pni, l5emx4.6mm i.d.	Methanol : Acetonitrile = 85:15	1.0 ml/min	258nm	Nice and Good Peaks	Method Accepted

Preparation of Mobile Phase: Mixed a mixture of methanol 850ml of HPLC grade Methanol and 150ml of HPLC grade[12] Acetonitrile and degassed in ultrasonic water bath for 15 minutes, filtered through 0.45µm membrane filter.

Diluent Preparation: Mobile phase is used as diluents.

III. RESULTS AND DISCUSSION

Method Development

Selection of Wavelength

Selectivity[13] of HPLC method that uses UV detector depends on proper selection of Wavelength. A wavelength which gives good response for the drug to be detected is to be selected. From the UV spectra 284 nm was selected as the wavelength for study. The λ max of this method can be determined as 284 nm.

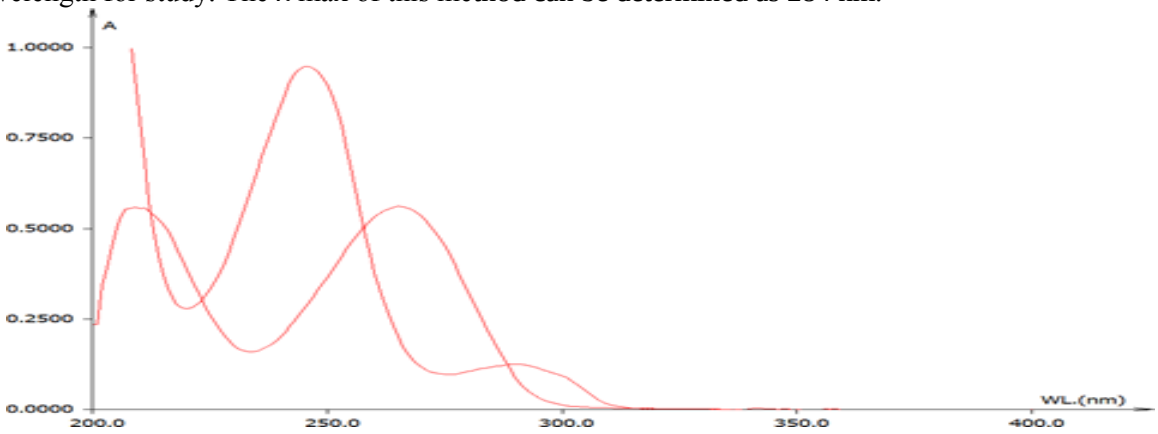

Fig.3. Isobestic point Atenolol and Chlorthalidone (258nm)

Optimized Chromatographic Conditions:

Table-4: Summary of Optimized Chromatographic Conditions

Mobile phase	Methanol: Acetonitrile 85:15% v/v
Column	Develosil ODS HG-5 RP C_{18}, 15cmx4.6mm, i.d.
Column Temperature	Ambient
Detection Wavelength	258 nm
Flow rate	1.0 ml/ min.
Run time	15 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic

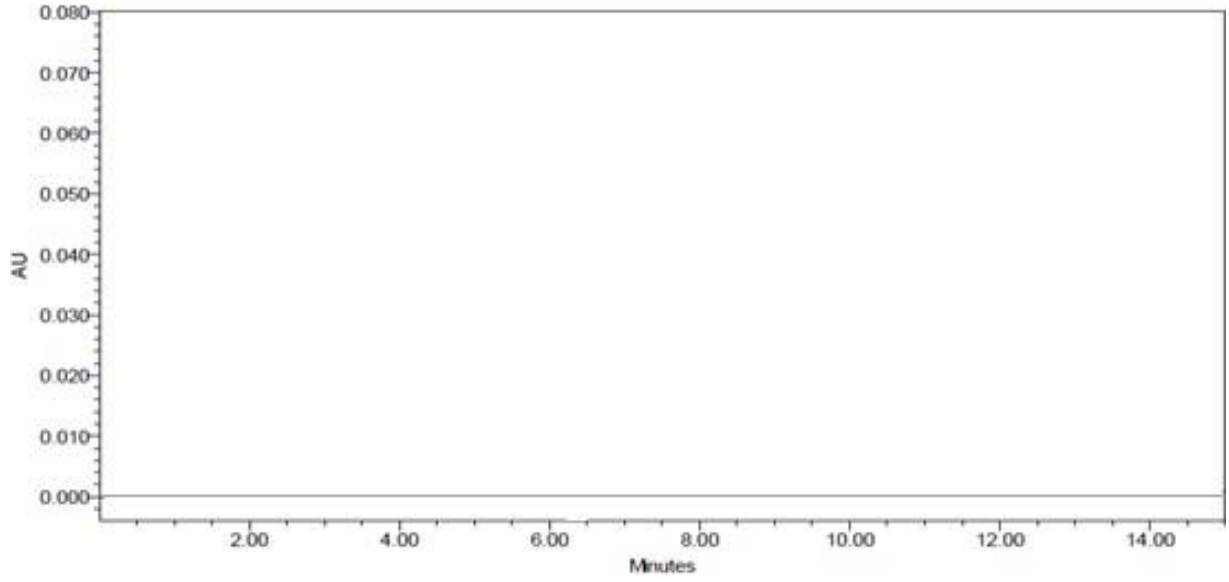

Fig.4. HPLC Spectrum of Atenolol and Chlorthalidone (Blank Solution)

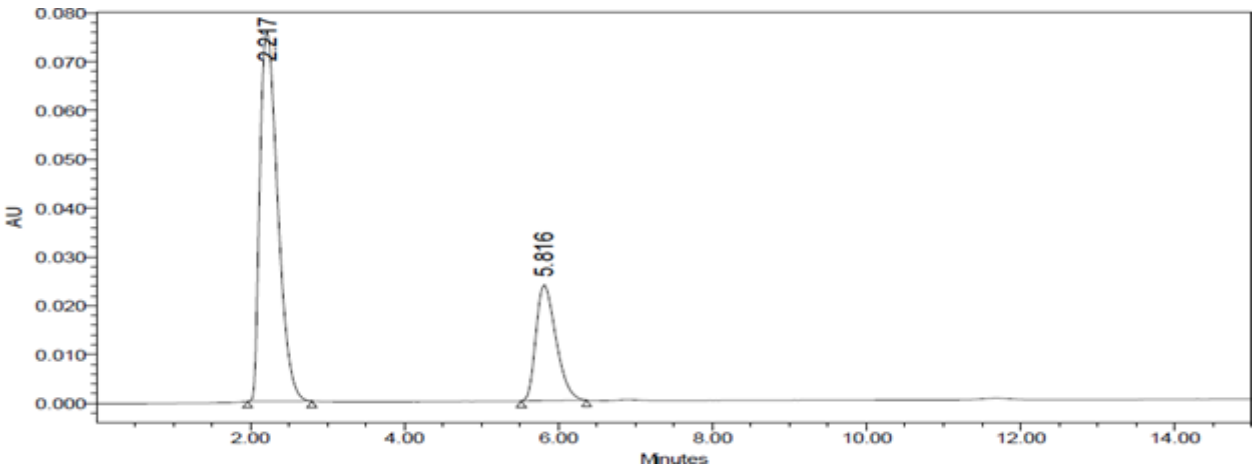

Fig.5. Chromatogram of Atenolol and Chlorthalidone in Optimized Condition

Method Validation

1. Linearity and Range:

Linearity[14] of developed HPLC method was studied by obtaining calibration curves of Atenolol and Chlorthalidone at different concentration levels ranging from 6-14µg/ml for Atenolol and 12-28µg/ml Chlorthalidone respectively. Table 5 and 6 shows the linearity data of Atenolol and Chlorthalidone. The equation for regression line[15] was y = 21516X+3613.2 (R2 = 0999) for Atenolol and y = 14059X+3514.9 (R2 = 0.9993) for Chlorthalidone respectively. The results show that an excellent correlation[16] exists between response factor and concentration of drugs within the concentration range indicated below

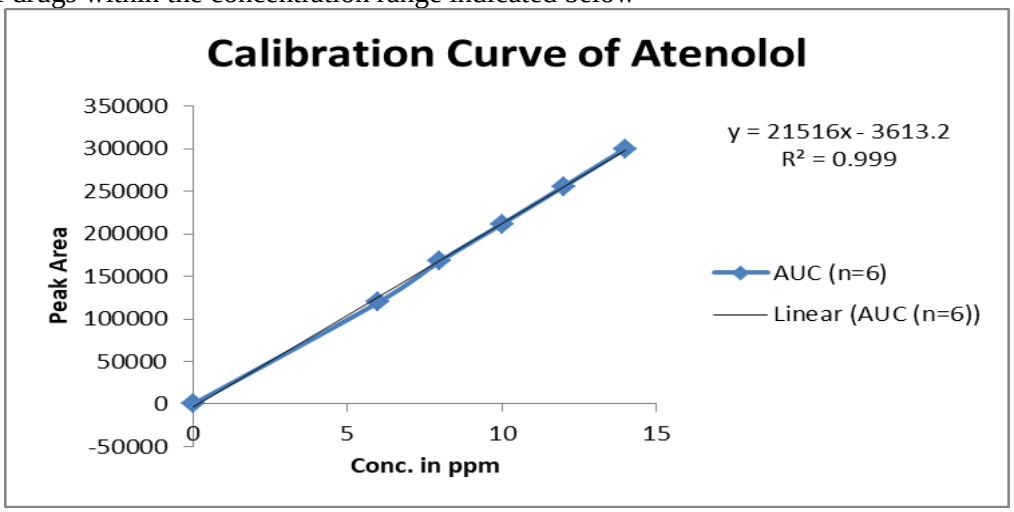

Fig.6. Standard curve for Atenolol

Table-5: Linearity Results for Atenolol

CONC. (µg/ml)	AUC (n=6)
0	0
6	119571
8	167873
10	211264

12	255428
14	299987

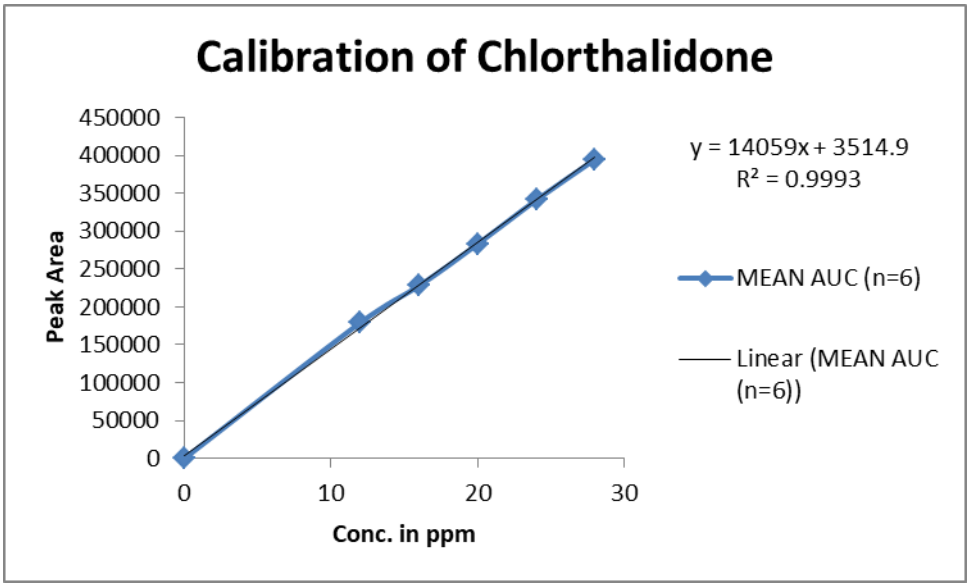

Fig.7. Standard curve for Chlorthalidone

Table-6: Linearity Results for Chlorthalidone

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	179371
16	227893
20	283264
24	341428
28	394987

2. Accuracy: Atenolol

The accuracy of the method was determined by recovery experiments. The recovery studies[17,18] of Atenolol were carried out at three levels of 80, 100 and 120% in triplicate and the percentage of recovery was calculated shown in Table 7. The mean recovery of the drug was found to be in the range of 98- 102% and % of RSD is less than 2, indicating a high degree of accuracy[19] for the developed method.

Table-7: Accuracy Readings for Atenolol

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	7.997368	115949	99.9671	Mean= 100.7003%
S_2 : 80 %	8	8.106622	117485	101.3328	S.D. = 0.6884036
S_3 : 80 %	8	8.064087	116887	100.8011	% R.S.D.= 0.683616%
S_4 : 100 %	10	9.904901	142767	99.04901	Mean= 100.36157%
S_5 : 100 %	10	10.02966	144521	100.2966	S.D. = 1.346221
S_6 : 100 %	10	10.17391	146549	101.7391	R.S.D.= 1.3413706%
S_7 : 120 %	12	12.01807	172476	100.1506	Mean= 100.183756%
S_8 : 120 %	12	11.88079	170546	99.00657	S.D. = 1.19411
S_9 : 120 %	12	12.16729	174574	101.3941	% R.S.D. = 1.19191%

Recovery study: Chlorthalidone

The accuracy[20] of the method was determined by recovery experiments. The recovery studies[21] of Chlorthalidone were carried out at three levels of 80, 100 and 120% in triplicate and the percentage of recovery was calculated shown in Table 8. The mean recovery of the drug was found to be in the range of 98- 102% and % of RSD is less than 2, indicating a high degree of accuracy for the developed method.

Table-8: Accuracy Results for Chlorthalidone

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	16	16.08685	229679	100.5428	Mean= 100.54488%
S_2 : 80 %	16	15.93079	227485	99.56745	S.D. = 0.97847%
S_3 : 80 %	16	16.2439	231887	101.5244	R.S.D.= 0.9731%

S_4 : 100 %	20	20.07632	285767	100.3816	Mean= 99.97095% S.D. = 0.395406 % R.S.D.= 0.39552%
S_5 : 100 %	20	19.98769	284521	99.93847	
S_6 : 100 %	20	19.91856	283549	99.59279	
S_7 : 120 %	24	23.75432	337476	98.97634	Mean= 100.27718%
S_8 : 120 %	24	24.11494	342546	100.4789	S.D. = 1.21262 % R.S.D. = 1.20927%
S_9 : 120 %	24	24.33032	345574	101.3763	

Precision:

Repeatability: The precision[22,23] at 100 % concentration of the assay method was evaluated by six replicate injections and measurement of peak areas by determining the % RSD of Atenolol and Chlorthalidone. The calculated values of % RSD for Atenolol and Chlorthalidone are mentioned in Table 9. The results indicated a high degree of repeatability[24].

Table-9: **Data showing repeatability analysis for Atenolol & Chlorthalidone**

HPLC Injection Replicates	**AUC for Atenolol**	**AUC for Chlorthalidone**
Replicate – 1	113568	241022
Replicate – 2	113241	240137
Replicate – 3	115408	242911
Replicate – 4	117412	245245
Replicate – 5	112541	241941
Replicate – 6	112546	240444
Average	**114119.3333**	**241356.6667**
Standard Deviation	**1925.83838**	**1416.95812**
% RSD	**1.68756**	**0.58708**

Intermediate Precision:

Intra-assay & inter-assay: The intra & inter day[25,26] variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Atenolol and Chlorthalidone revealed that the proposed method is precise.

Table-10: Results of intra-assay & inter-assay

Conc. of Atenolol (API) (µg/ml)	Observed Conc. of Atenolol (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	8.09	0.97	8.03	0.96
10	10.05	0.45	10.04	0.47
12	11.98	0.37	11.90	0.12

Table-11: Data for Chlorthalidone intra-assay & inter-assay analysis

Conc. of Chlorthalidone (API) (µg/ml)	Observed Conc. of Chlorthalidone (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	7.97	0.27	8.09	0.59
10	10.14	1.29	9.95	0.64
12	12.08	0.61	11.94	0.26

Result and discussion:

The Intraday and interday related studies shows that the % RSD was found to be within limit i.e. (≤2%). So it is indicated that the developed is within the limits. Hence finally we concluded that the developed method was found to be precise[27].

Limit of detection (LOD) & Limit of quantification (LOQ):

The detection limit[28] (LOD) and quantitation limit[29] (LOQ) may be expressed as:

$$L.O.D. = 3.3\ (SD/S).$$

$$L.O.Q. = 10\ (SD/S)$$

Where, SD = Standard deviation[30] of the response

S = Slope of the calibration curve[31]

Result & Discussion:

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24 µg/ml respectively for Atenolol.

The LOD was found to be 0.1 µg/ml and LOQ was found to be 0.3 µg/ml for Chlorthalidone which represents that sensitivity[32] of the method is high.

Method Robustness: Influence of small changes in chromatographic conditions[33] such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-12, % RSD < 2%) the developed RP-HPLC method for the analysis of Atenolol (API).

Table-12: Result of Method Robustness Test

Change in parameter	**% RSD**
Flow (1.1 ml/min)	1.05
Flow (0.9 ml/min)	0.67

Temperature (27^0C)	0.58
Temperature (23^0C)	0.61
Wavelength of Detection (280 nm)	0.38
Wavelength of detection (270 nm)	0.17

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^0C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-13, % RSD < 2%) the developed RP-HPLC method for the analysis of Chlorthalidone (API).

Table-13: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.09
Flow (0.9 ml/min)	0.07
Temperature (27^0C)	0.28
Temperature (23^0C)	0.74
Wavelength of Detection (235 nm)	0.86
Wavelength of detection (240 nm)	0.67

System Suitability Parameter: It is an integral part of so many analytical procedures. The parameters are based on the idea that the equipment, electronics, analytical operations and the samples to be analyzed constitute as an integral system which can be examined. Finally system suitability test parameters[34] are established. The obtained data is shown in the following table-14.

Table-14: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs < 2	3.65
2	Asymmetry	T ± 2	Atenolol = 0.35 Chlorthalidone = 0.23
3	Theoretical plate	N < 2000	Atenolol = 3771 Chlorthalidone = 2437

Estimation of Atenolol and Chlorthalidone in Pharmaceutical Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase

and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicated to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded. A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in Table-15.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area
AS = Standard preparation Peak Area
WS = Working standard weight taken in mg
WT = Sample weight taken in mg
DS = Standard solution dilution
DT = Sample solution dilution
P = Working standard percentage purity

The assay[35] was performed as explained in the previous chapter (Above). The results which are obtained are following:

Table-15: Recovery Data for estimation Atenolol and Chlorthalidone in Tenoric 50

Brand name of Atenolol and Chlorthalidone	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Tenoric 50 Tablets (Pfizer Pharmaceutical Co., Ltd.)	50 & 12.5mg	49.687 (±0.598)/11.998 (±0.857)/	99.875 (±0.598)/99.698 (± 0.467)

Result & Discussion: The %purity of Atenolol & Chlorthalidone for Tablets was found to be 99.875% and 99.698% respectively.

Stability Studies

Results of Degradation Studies: The results of the stress studies indicated the specificity of the method that has been developed. Atenolol and Chlorthalidone were stable only in photolytic stress conditions and little bit in thermal stress conditions. The results of forced degradation studies are given in the following Table-16.

Table-16: Results of Forced Degradation Studies of Atenolol and Chlorthalidone API

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.62	4.38	100.00

Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.13	2.87	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.24	3.76	100.00
UV (254nm)	24Hrs.	95.43	4.57	100.00
3% Hydrogen peroxide	24Hrs.	96.16	3.84	100.00

IV. SUMMARY

Isocratic elution is easy, needs only one pump & flat standard splitting up for easy and also reproducible results. So, it was preferred for the present research over gradient elution.

In case of RP-HPLC various columns are offered, however below Develosil ODS HG-5 RP C18, 5µm, l5cmx4.6mm i.d. column was preferred since using this column top shape, resolution as well as absorbance were great.

Mobile stage & diluent for preparation of different examples were completed after researching the solubility of API in various solvents of our disposal (methanol, Acetonitrile, water, 0.1 N NaOH, 0.1 N HCl).

Discovery wavelength was picked after checking the basic remedy of drug over 200 to 400nm. From the U.V spectrum of Atenolol and also Chlorthalidone it is evident that the majority of the HPLC work can be achieved in the wavelength variety of 200-300 nm easily. Even more, a circulation rate of 1ml/min & an injection volume of 10µl were found to be the best evaluation.

CONCLUSION

A delicate & selective stability fingering RP-HPLC approach has actually been developed & validated for the simultaneous evaluation of Atenolol and Chlorthalidone in bulk form as well as marketed pharmaceutical dosage form.

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