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Advances in Bioresearch

ORIGINAL ARTICLE

An RP-HPLC Method Development and Validation for the Quantification of Clonidine and Chlorthalidone Simultaneously

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ABSTRACT
The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. A simple and selective LC method is described for the determination of Clonidine and Chlorthalidone tablet dosage forms. Chromatographic separation was achieved on a Zorbax SB C18 (150mm×.4.6mm & 5µm) column using mobile phase consisting of Ammonium Acetate Buffer pH 4.0: Acetonitrile (70:30) %v/v were prepared with detection of 230 nm. Linearity was observed in the range 50- 150 µg/ml for Clonidine (r^2 =0.9999) and 50-150 µg /ml for Chlorthalidone (r^2 =0.9999) for drugs estimated by the proposed methods was in good agreement with the label claim.
Keywords: Clonidine and Chlorthalidone, Recovery, Precision, Linearity.

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INTRODUCTION

Chlorthalidone (CHR) is chemically is 2-chloro-5[(1RS)-1-hydroxy-3-oxo-2, 3-dihydro- 1Hisoindol-1yl) benzene-1-sulfonamide1. Chemical structure of chlorthalidone is shown in figure 1. Chlorthalidone is a diuretic drug used to treat hypertension and fluid retention caused by various conditions, including heart diseases. Chlorthalidone is very similar to hydrochlorothiazide and is used as an independent drug or in combination with other antihypertensive agents for lowering arterial blood pressure. Diuretics lower blood pressure by decreasing cardiac output and reducing plasma and extracellular fluid volume.

Chemically clonidine HCl (CLD) is chemically ((2-[2, 6-dichlorophenyl] amino)-2-imidazoline) preferentially stimulates central alpha (2)-adrenoceptors. Chemical structure of clonidine is shown in figure 2, which leads to inhibition of sympathetic tone, resulting in a lowering of arterial pressure and of heart rate. Clonidine HCl is a centrally acting alpha-agonist hypotensive agent used to treat hypertension (high blood pressure), attention deficit hyperactivity disorder, migraine etc. Clonidine HCl used to treat psychiatric disorders including stress, sleep disorders, other anxiety disorders. Mild sedative nature of Clonidine HCl implies its use as premedication before surgery or procedures (1-6).

Literature survey revealed UV-Visible spectrophotometric methods [7, 8] RP-HPLC [9, 10], HPTLC [11] and UPLC [12] Methods for the estimation of CHR and CLD alone or in combination With other drugs. The validation of methods was carried out as per ICH guidelines [13,14].

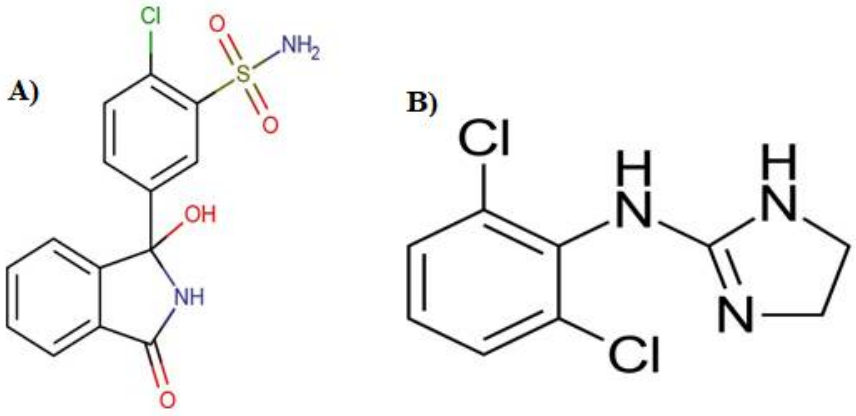

Fig. 1: Chemical structures of A) Chlorthalidone and B) Clonidine Hydrochloride.

MATERIAL AND METHODS

Materials and Chemicals

HPLC grade water, methanol, acetonitrile and analytical grade potassium dihydrogen, Triethyl amine and Orthophosphoric acid were obtained from Merck, India. Chlorthalidone and Clonidine Hydrochloride reference compounds acquired from Mylan laboratories, Hyderabad, India.

Instruments:

The list of instruments used in the research was given in Table 1.

Table 1: Instruments used

UV-Visible Spectrophotometer	Nicolet evolution 100
UV-Visible Spectrophotometer software	Vision Pro
HPLC software	Spin chrome (LC SOLUTIONS)
HPLC	Shimadzu(LC 20 AT VP)
Ultra sonicator	Citizen, Digital Ultrasonic Cleaner
pH meter	Global digital
Electronic balance	Shimadzu
Syringe	Hamilton
HPLC Column	Inertsil ODS 3V(250x4.6mm) 5µm

Buffer Preparation

Accurately weighed and transferred 0.77g of Ammonium acetate in to 1000mL of water, mixed well, adjusted pH 4.0 with diluted glacial acetic acid. Filtered through 0.45µ filter.

Mobile Phase Preparation

Mixed 700mL of Buffer and 300mL of Acetonitrile, degassed by sonication.

Preparation of standard solution

Weighed accurately 100 mg of Clonidine and 600mg of Chlorthalidone in 100 ml of volumetric flask and dissolve in 70ml of mobile phase and make up the volume with mobile phase from above stock solution 100µg/ml of Clonidine and 600µg/mL of Chlorthalidone were prepared by diluting 5mlto 50ml with mobile phase respectively.

Preparation of sample solution

20tablets were weighed and taken into a mortar and crushed to fine powder and uniformlymixed. Weighed crushed powder equivalent to 100mgof Clonidine and 600mg of Chlorthalidone in to 100ml of mobile phase by 30min of sonication and make up the volumewith mobile phase. Centrifuged sample at 5000rpm for 10min.

From above stock solution 100µg/ml of Clonidine and 600µg/mL of Chlorthalidone were prepared by diluting 5ml to 50ml with mobile phase respectively.

RESULTS AND DISCUSSIONS

Determination of Working Wavelength (λmax)

Preparation of Standard solution

10mg of Clonidine was weighed and transferred in to100 ml volumetric flask and dissolvedin methanol and then make up to the mark with methanol and prepare 10 µg /ml of solutionby diluting 2ml to 20ml with methanol. 10mg of Chlorthalidone was weighed and transferred in to 100 ml volumetric flask and

dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 2ml to 20ml with methanol.

The wavelength of maximum absorption (λ max) of the solution of the drug in mobile phase were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400nm against mobile phase as blank. The absorption curve shows characteristic absorption maxima at 225 nm for Chlorthalidone and maxima at 244 nm for Clonidine, Isosbestic pointhas shown at 230nm so 230nm was selected as detector wavelength for the HPLC chromatographic method.

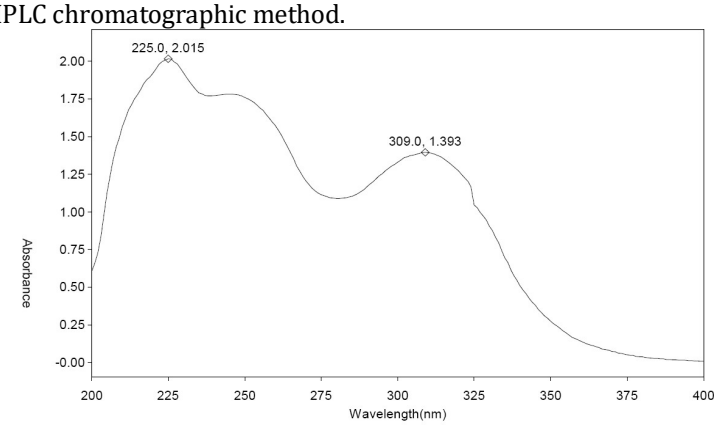

Fig. 2: UV-VIS spectrum of CHLORTHALIDONE (225 nm)

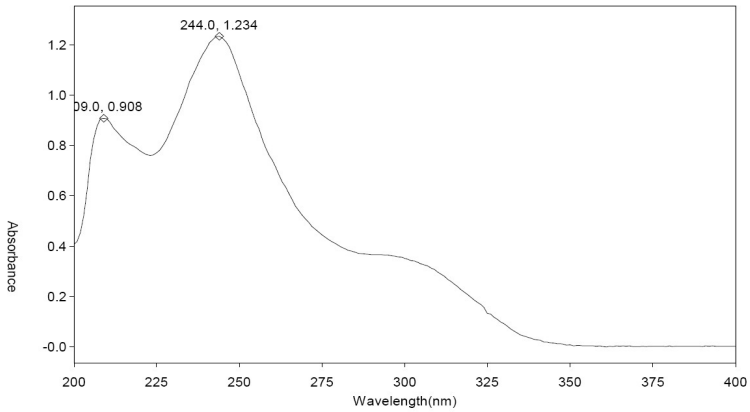

Fig. 3: UV-VIS spectrum of CLONIDINE (244 nm)

Optimized Chromatographic Conditions:

Column	:	Zorbax SB C18 (150mm×.4.6mm & 5µm)
Mobile phase	:	Ammonium Acetate Buffer pH 4.0: Acetonitrile
Ratio	:	70:30
Column Oven Temperature	:	30°C
Flow rate	:	1.0 mL/min
Detection wavelength	:	230nm
Injection volume	:	20µL
Run time	:	10 min

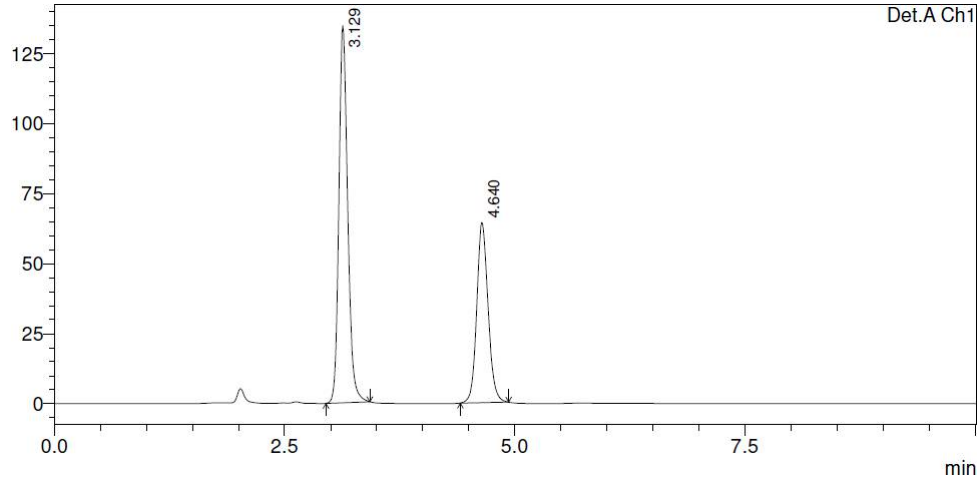

Fig. 4: Optimized chromatogram for both chlorthalidone and clonidine.

METHOD VALIDATION

System Suitability and System Precision

Weighed accurately 100 mg of Clonidine and 600mg of Chlorthalidone in 100 ml of volumetric flask and dissolve in 70ml of mobile phase and make up the volume with mobile phase From above stock solution 100µg/ml of Clonidine and 600µg/mL of Chlorthalidone were prepared by diluting 5mlto 50ml with mobile phase respectively [15-18]. System suitability results were met with acceptance criteria, hence system is suitable

Table 2: System suitability results

Clonidine				
Name of the Standard	**Clonidine and Chlorthalidone**	**Tailing factor**	**Plate count**	**Retention time**
Standard-01	552352	1.26	30454	4.684
Standard-02	556622	-	-	4.672
Standard-03	555771	-	-	4.655
Standard-04	551536	-	-	4.640
Standard-05	545777	-	-	4.617
Average	552411	**-**	**-**	**-**
%RSD	0.8	**-**	**-**	**-**
Chlorthalidone				
Standard-01	905722	1.14	44763	3.138
Standard-02	904050	-	-	3.134
Standard-03	905074	-	-	3.132
Standard-04	900510	-	-	3.129
Standard-05	903263	-	-	3.120
Average	903723	**-**	**-**	**-**
%RSD	0.2	**-**	**-**	**-**

System Precision results were met with acceptance criteria, hence system is precise.

Table 3: System Precision results

Name of the Standard	Area of Chlorthalidone	Area of Clonidine
Standard-01	905722	552352
Standard-02	904050	556622
Standard-03	905074	555771
Standard-04	900510	551536
Standard-05	903263	545777
Standard-06	904064	554703
Average	903780	552793
%RSD	0.20	0.7

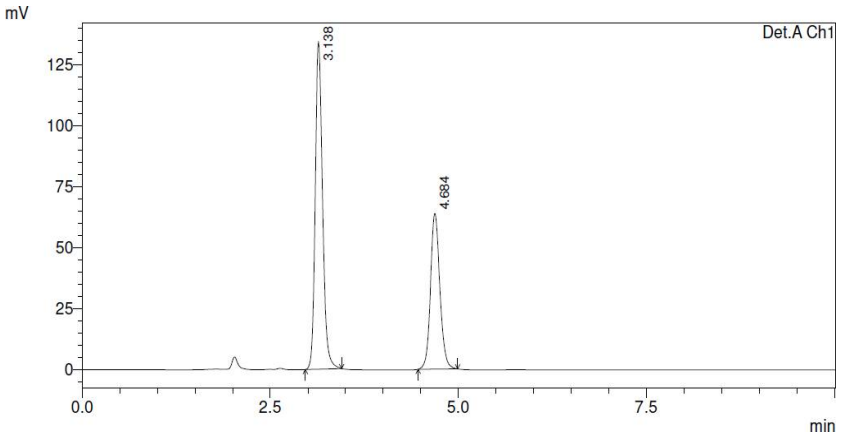

Fig. 5:Chromatograms of System suitability and System Precision

Specificity

Blank and Placebo Interference

Standard solution was prepared by weighing accurately 100 mg of Clonidine and 600mg of Chlorthalidone in 100 ml of volumetric flask and dissolve in 70ml of mobile phase and make up the volume with mobile phase[19-21]. From above stock solution 100µg/ml of Clonidine and 600µg/mL of Chlorthalidone were prepared by diluting 5mlto 100ml with mobile phase respectively.

Sample solution was prepared by taking weighed 20tablets and taken into a mortar and crushed to fine powder and uniformly mixed. Weighed crushed powder equivalent to 100mgof Clonidine and 600mg of Chlorthalidone in to100 ml of volumetric flask of mobile phase by 30min of sonication and make up the volume with mobile phase. Centrifuged sample at 5000rpm for 10min.

From above stock solution 100µg/ml of Clonidine and 600µg/mL of Chlorthalidone were prepared by diluting 5mlto 50ml with mobile phase respectively.

Preparation of Placebo solution

Weighed powder equivalent to 100mg of Clonidine and 600mg of Chlorthalidone in to50 mlof volumetric flask and dissolve in 35ml of mobile phase by 30min of sonication and make upthe volume with mobile phase[21-23]. Centrifuged sample at 5000rpm for 10min. From above stock solution diluting 5mlto50 with mobile phase respectively.

All the specificity solutions were injected into the chromatographic system and assessed for the interferences. There was no interference observed at the retention time of Clonidine and Chlorthalidone due to blank and Placebo Hence System is specific.

Table 4: Specificity results for Clonidine andChlorthalidone

S.No.	Solution details	Area of Chlorthalidone	Area of Clonidine
1	Standard	905722	558551
2	Blank	Not Detected	Not Detected
3	Placebo solution	Not Detected	Not Detected
4	Test solution	903649	546374

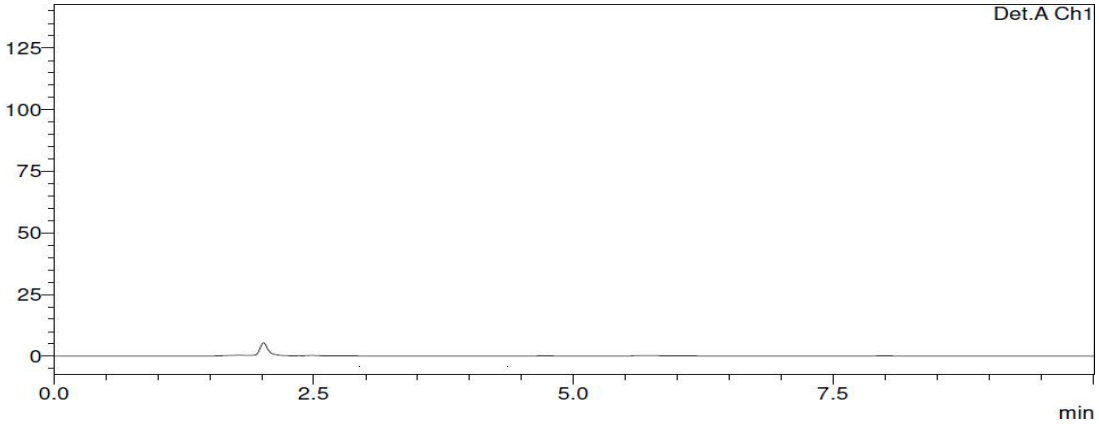

Fig. 6:Chromatogram of Blank

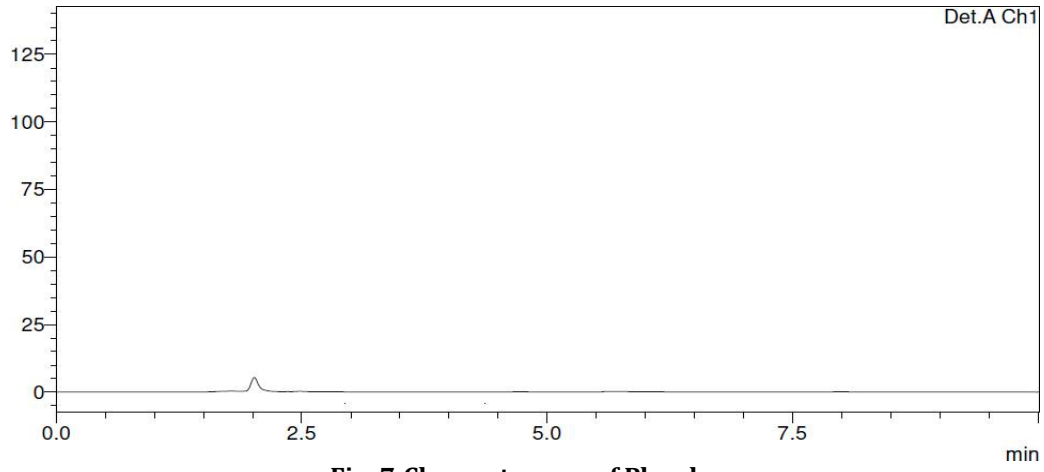

Fig. 7:Chromatogram of Placebo

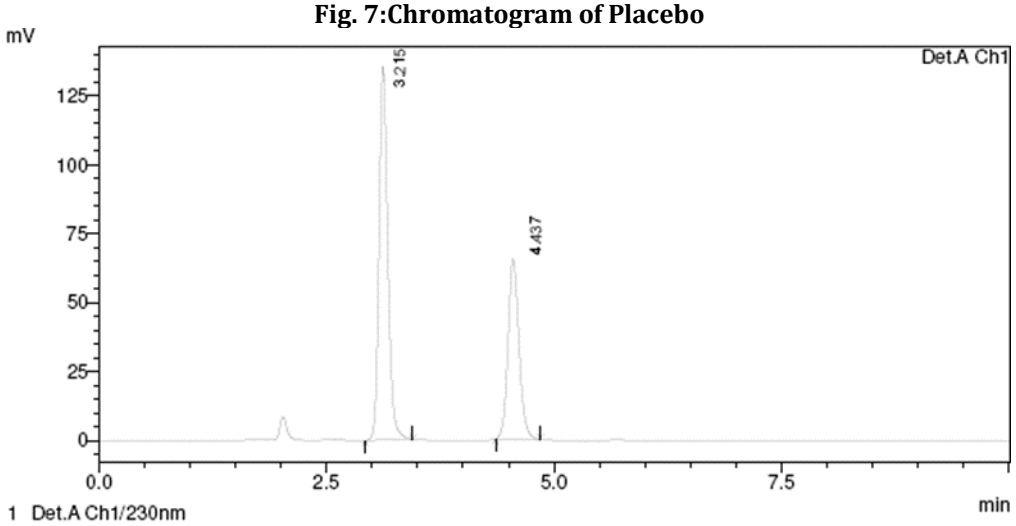

Fig. 8:Chromatogram of sample

Method Precision:

Standard and 6 sample solutions were prepared for the evaluation of method precision. The processed solutions were injected into the chromatographic system and evaluated for the percentage recovery by calculating the assay. Mean %Assay Obtained between 90.0 to 110.0%for Clonidine and Chlorthalidone[18]. The % RSD of % Assay results obtained from Test solution was obtained less than 2.0% for Clonidine and Chlorthalidone. Hence Method is Precise.

Table 5: Method Precision Results

S.No.	Solution details	%Assay of Chlorthalidone	%Assay of Clonidine
1	Test solution preparation-1	100.8	99.9
2	Test solution preparation-2	99.5	100.7
3	Test solution preparation-3	100.1	99.0
4	Test solution preparation-4	99.9	98.6
5	Test solution preparation-5	99.4	98.3
6	Test solution preparation-6	98.2	100.5
Average		103.2	99.5
Std Dev		0.81	0.97
%RSD		0.80	0.9

Intermediate Precision

Table 6: Intermediate Precision Results

S.No.	Solution details	% Assay of Chlorthalidone
1	Test solution preparation-1	98.8
2	Test solution preparation-2	99.2
3	Test solution preparation-3	99.1
4	Test solution preparation-4	99.8
5	Test solution preparation-5	100.2
6	Test solution preparation-6	100.1
Average		99.5
Std Dev		0.63
%RSD		0.6
S.No.	**Solution details**	**%Assay of Clonidine**
1	Test solution preparation-1	99.0
2	Test solution preparation-2	99.0
3	Test solution preparation-3	98.9
4	Test solution preparation-4	99.6
5	Test solution preparation-5	100.9
6	Test solution preparation-6	99.9
Average		99.6
Std Dev		0.84
%RSD		0.8

Anlyst-01 vs. Analyst-02

Table 7: Intermediate Precision results

S.No.	Solution details	%Assay Chlorthalidone
Analyst-01	Test solution preparation-1	100.8
	Test solution preparation-2	99.5
	Test solution preparation-3	100.1
	Test solution preparation-4	99.9
	Test solution preparation-5	99.4
	Test solution preparation-6	98.2
Analyst-02	Test solution preparation-1	98.8
	Test solution preparation-2	99.2
	Test solution preparation-3	99.1
	Test solution preparation-4	99.8
	Test solution preparation-5	100.2
	Test solution preparation-6	100.1
Average		**99.8**
Std Dev		**0.47**
%RSD		**0.45**

Observation:

- Mean %Assay obtained between 90.0 to 110.0% for Clonidine and Chlorthalidone
- The % RSD of % Assay results was obtained from Test solution less than 2.0% for Clonidine and Chlorthalidone (Performed by Analyst-II)

- Cumulative % RSD of % Assay results was obtained less than 2.0 for both analysts-I & II of Clonidine and Chlorthalidone
- Cumulative Mean of %Assay for both analysts-I & II obtained 90.0 to 110.0% for Clonidine and Chlorthalidone
- Hence Method is Rugged

Linearity and Range

Preparation of the Standard Stock

Weighed accurately 100mgof Clonidine and 600mg of Chlorthalidonein 100 ml of volumetric flask and dissolve in 70ml of mobile phase and make up the volume with mobile phase[16, 20].

Table 8: Linearity results

S.No	Name of the Solution	Area of Chlorthalidone	Area of Clonidine
1	Linearity solution, Level-1	454530	264727
2	Linearity solution, Level-2	733496	443366
3	Linearity solution, Level-3	923374	554516
4	Linearity solution, Level-4	1100148	670888
5	Linearity solution, Level-5	1388503	842245
Slope		9315	11526
Intercept		11571	21168
Correlation coefficient		0.9999	0.9999

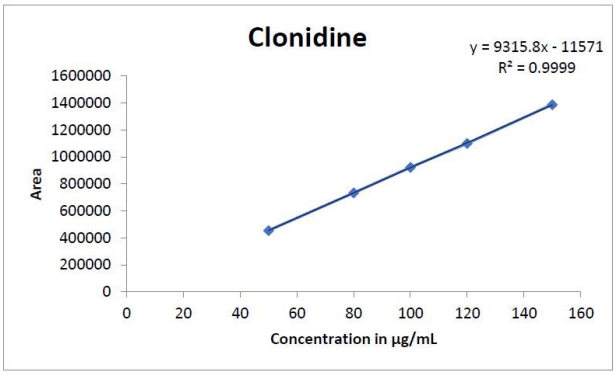

Fig. 9: Linearity Graph for clonidine

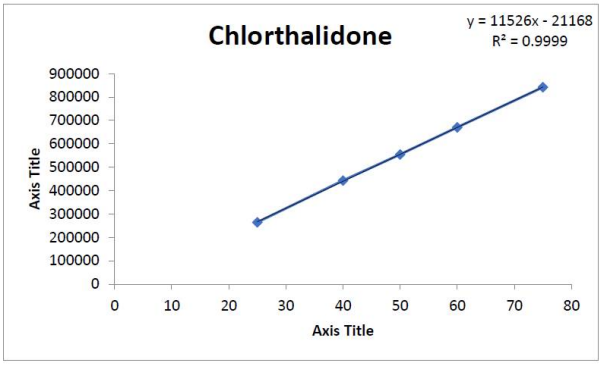

Fig. 10: Linearity Graph for chlorthalidone.

Observation:

- The correlation coefficient value obtained 0.9999 for Clonidine and 0.9999 forChlorthalidone

Accuracy and Recovery

Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzedsample), the reference standards of the drugs were added at the level of 50%, 100% & 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table [22]. To check the accuracy of the method,recovery studies were carried out by addition of standard drug solution to pre-analyzed samplesolution at three different levels 50%, 100% & 150%.

Results of Accuracy and Recovery:

Table 9: Results for Recovery of chlorthalidone

Recovery level	Accuracy chlorthalidone			Average % Recovery
	Area	Average area	%Recovery	
50%	451018	453782.0	100.4	100.4
	456169			
	454159			
100%	907152	905442.0	100.2	
	909748			
	899426			
150%	1398530	1362485.3	100.5	
	1348013			
	1340913			

Table 10: Results for Recovery of clonidine

Recovery level	Accuracy clonidine			Average % Recovery
	Area	Average area	%Recovery	
50%	277733	275714.7	99.8	100.5 %
	271508			
	277903			
100%	552544	553863.0	100.2	
	554904			
	554141			
150%	840433	840703.0	101.4	
	840251			
	841425			

Observation

The % Recovery obtained between 98.0 to 102.0% for Clonidine and Chlorthalidone. Mean % Recovery obtained between 98.0 to 102.0% for Clonidine and Chlorthalidone. The %RSD obtained for All %recoveries less than 2.0%. Hence method is Accurate.

Limit of Detection

Chlorthalidone

The LOD for this method was found to be 7.18µg/ml

LOD=3.3*(2028.74) /9315.8

=7.18µg/ml

Clonidine

The LOD for this method was found to be 1.22µg/ml

LOD =3.3*(4295.38) / 11526

=1.22µg/ml

Limit of Quantification

Chlorthalidone:

LOQ =10*(2028.74)/9315.8

=2.17µg/ml.

Clonidine

The LOQ for this method was found to be 3.72µg/ml

LOQ=10*(4295.38)/11526

=3.72µg/ml.

Robustness

The % RSD of the area response of Clonidine and Chlorthalidone peak obtainedfrom the five injections of standard solution should be not more than 2.0[17]. The Theoretical plates for 1st injection should be NLT 1500 for Clonidine andChlorthalidone peak. The Tailing factor for 1st injection should be NMT 2.0 for Clonidine and Chlorthalidone peak. System suitability met the acceptance criteria in robustness parameters hence method is Robust.

Table 11: Robustness results.

Chlorthalidone			
Name of the Parameter	**%RSD**	**TheoreticalPlates**	**Tailing factor**
Low Flow(0.8mL/min)	0.47	30458	1.2
High Flow(1.2mL/min)	0.40	30487	1.1
Lower Wavelength(228nm)	0.27	33021	1.1
Higher Wavelength(232nm)	0.30	29874	1.0
Clonidine			
Low Flow(0.8mL/min)	0.36	42877	1.3
High Flow(1.2mL/min)	0.37	40587	1.1
Lower Wavelength(228nm)	0.41	40693	1.2
Higher Wavelength(232nm)	0.44	40754	1.1

Forced degradation

Forced degradation is a technique where different stress conditions are applied over drug Product and which in turn different degradation products are produced. These studies are alsocalled as stress testing or stress degradation studies. These methods are mainly used for the determination of stability of molecule under accelerated conditions[13, 14].

It is known that regulatory documentation process, selection of proper storage and package conditions, and selection of formulation are dependent on the stability of molecules. In forced degradation process, general conditions such as light, heat, humidity, and oxidation are accelerated individually or in combination with automated stress to accelerate the degradationof the molecule by physical or chemical means. Studies on forced degradation of drug molecules are very important in the following aspects.

1. To develop methods to determine stability.
2. To determine the degradation pathways.
3. For determination of intrinsic stability of drug in dosage forms.
4. To study the chemical properties of molecules.
5. For production of stable formulations.
6. To determine the structure of decomposition products.
7. To solve problems related to stability.
8. To generate a degradation profile under ICH conditions.

Thermal Degradation:

Stress testing is likely to be carried out on single batch of the drug substance (API). Thermolyticdegradation may lead to hydrolysis / dehydration / isomerization / epimerization / decarboxylation / rearrangements and some kinds of polymerization reactions. ICH guidelines suggest that thermolytic degradation study should be carried out at temperatures (in 10 increments e.g. 50°C, 60°C, etc.) above that for accelerated testing and withdraw the sample at different time intervals during reaction condition. If reasonable degradation (i.e. 5-20%) has seen, testing can be stopped at this point.

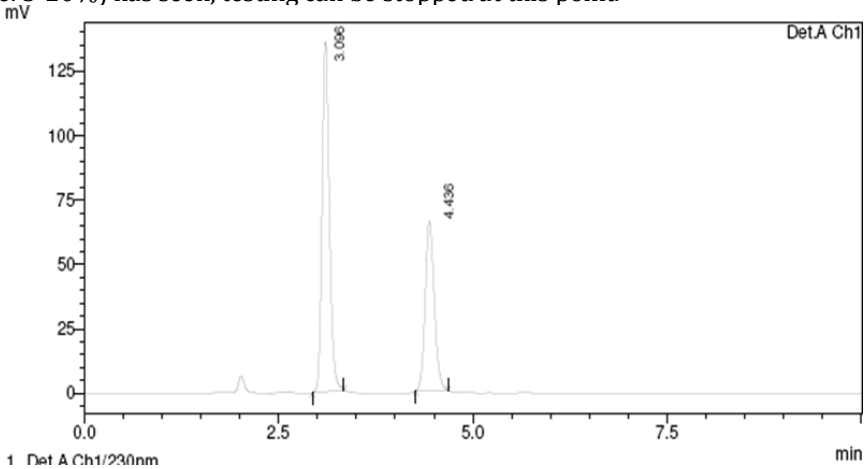

Fig. 11:Chromatogram of Thermal Sample (120°C/48Hrs)

Photolytic Degradation: Photo degradation is a surface-mediated phenomenon. The photolytic studies should be carried out by exposure to light, using either a combination of cool white and ultraviolet fluorescent lamps, or one among the xenon and metal halide lamps. Exposure energy should be minimum of 1.2 million Lux-h fluorescent light and 200Wh/m²UVand if decomposition is not seen, the drug can be

declared photostable. Total exposure of drugsubstance to light measured with the help of Lux meter/Watt meter. The light sources describedbelow may be used for photo stability testing.

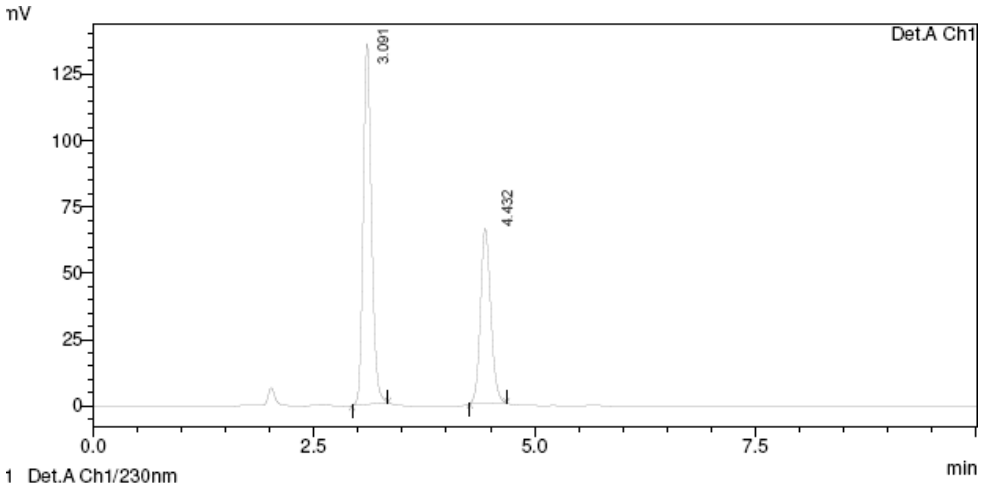

Fig. 12:Chromatogram of Photolytic Sample (1.2mil LUX hrs)

Acidic Degradation:

Sample solution (100µg/ml) prepared and transferred into a 50ml volumetric flask and dissolve in mobile phase up to 75% then sonicate it for 10 minutes then add 1 ml of 0.1N HCl then keptin oven at 60^0c for 1 hour then cool and add 1 ml of 0.1N NaOH it then make up the volume up to 50ml with mobile phase, then place the sample in the vial and measure the chromatogram.

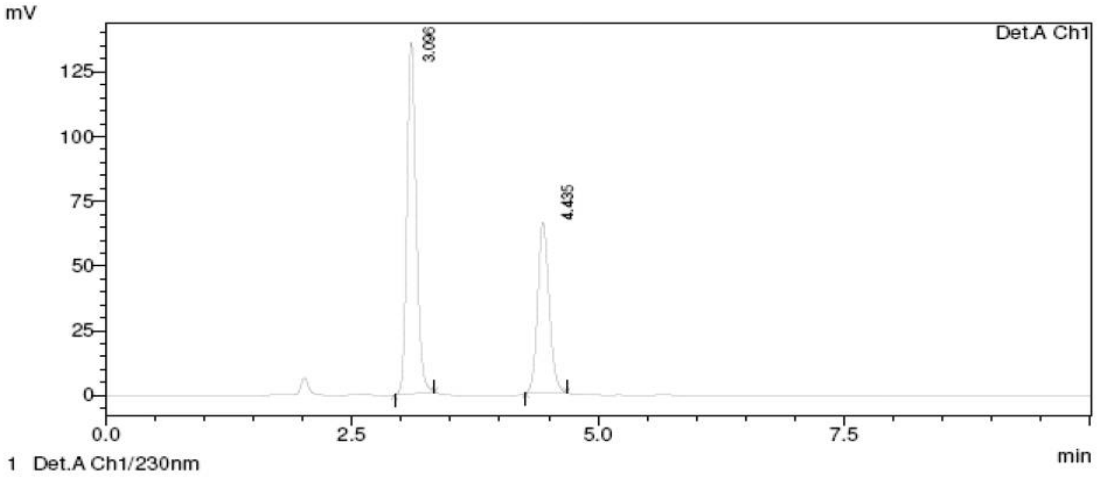

Fig. 13:Chromatogram of Acid Sample preparation (3N HCl /12Hrs/80°C)

Base Degradation:

Sample solution (100µg/ml) prepared and transferred into a 50ml volumetric flask and dissolve in mobile phase up to 75% then sonicate it for 10 minutes then add 1 ml of 0.1N NaOH then kept in oven at 60°C for 1 hour then cool it and add 1 ml of 0.1N HCl then makeup the volume up to 50ml with mobile. Phase, then place the sample in the vial and measure the chromatogram

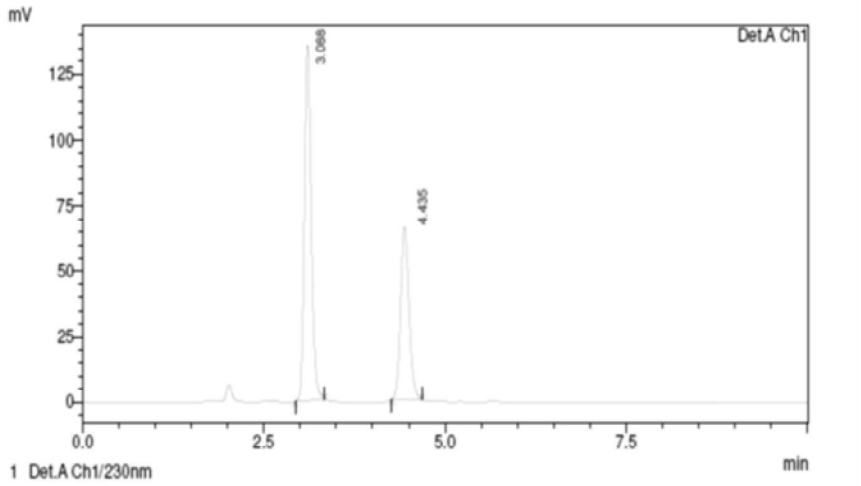

Fig. 14:Chromatogram of Base Sample preparation (3N NaOH /12Hrs/80°C)

Peroxide Degradation:

Sample solution (100µg/ml) and 1 ml of 20% hydrogen peroxide (H2O2) was mixed. For HPLC study, 100µg/ml was injected into the system and the chromatogram was recorded to assess the stability of sample.

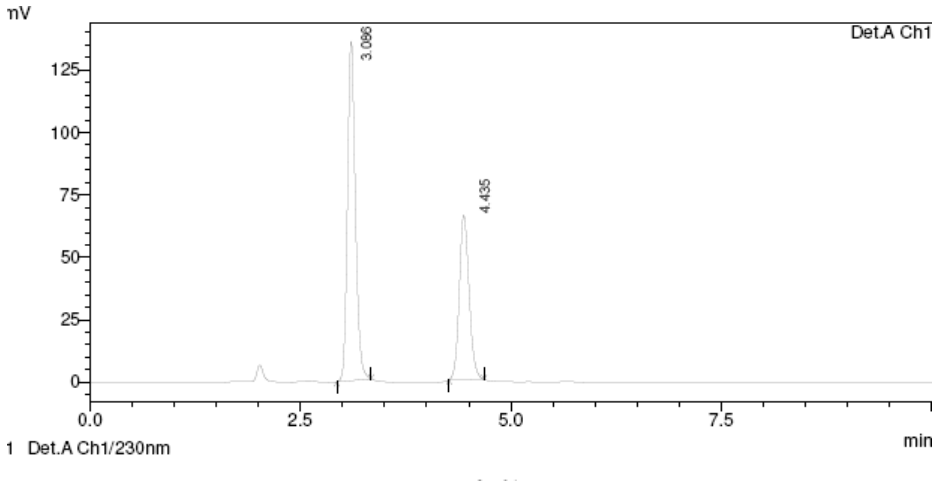

Fig. 15:Chromatogram of Peroxide Sample preparation (30%H2O2/Bench top/4hrs)

Table 12: Forced Degradation results For Chlorthalidone and Clonidine

Injection	Chlorthalidone			
	Condition	**Area**	**% Assay**	**% Degraded**
1	Thermal	876533	98.95	0.75
2	Photolytic	874563	98.73	0.97
3	Acid Hydrolysis	875426	98.83	0.87
4	Base Hydrolysis	875963	98.89	0.81
5	Peroxide Hydrolysis	875421	98.83	0.87
Injection	**Clonidine**			
	Condition	**Area**	**% Assay**	**% Degraded**
1	Thermal	598745	98.96	0.74
2	Photolytic	597458	98.75	0.95
3	Acid Hydrolysis	598632	98.94	0.76
4	Base Hydrolysis	598453	98.91	0.79
5	Peroxide Hydrolysis	598542	98.93	0.77

CONCLUSION

A simple and selective LC method is described for the determination of Clonidine and Chlorthalidone tablet dosage forms. Chromatographic separation was achieved on a Zorbax SB C18 (150mm×.4.6mm &

5µm) column using mobile phase consisting of Ammonium Acetate Buffer pH 4.0: Acetonitrile (70:30) %v/v were prepared with detection of 230nm. Linearity was observed in the range 50-150 µg/ml for Acebrophylline (r2 =0.9999) and 50-150µg /ml for Clonidine (r2 =0.9999) for drugs estimated by the proposed methods was in good agreement with the label claim. The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

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