International Journal of Pharmacy and Analytical Research (IJPAR)

ISSN: 2320-2831

IJPAR |Vol.12 | Issue 3 | July - Sept -2023
www.ijpar.com

Research article *Pharmaceutical Analysis*

Analytical method development and validation for simultaneous estimation of clonidine hcl and chlorthalidone in bulk and tablet dosage form by rp hplc method

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Published on: September 30, 2023

ABSTRACT

A simple, accurate and precise HPLC method for simultaneous determination of Clonidine HCl and Chlorthalidone in pure and tablet dosage form has been developed.HPLC of Waters (Model: Alliance 2695) with Phenomenex Luna C18 (4.6 mm I.D. × 250 mm, 5 µm) column was used for chromatographic separation. It contains waters injector and PDA Detector (Deuterium). Mobile phase consists of Methanol: Water (65:35% v/v) and flow rate adjusted was 1ml/min. Wavelength selected for detection was 220nm and injection volume was 10 µl. By using the developed method, retention time of Clonidine HCl and Chlorthalidone was found to be 3.2min and 5.4min respectively. The method has been validated for linearity, accuracy and precision. Linearity of Clonidine HCl and Chlorthalidone were in the range of 75–375µg/ml and 15–75µg/ml respectively. The percentage recoveries obtained for Clonidine HCl and Chlorthalidone were found to be in range of 99.3 – 99.6%. LOD and LOQ were found to be 12.5µg/ml and 38.1µg/ml for Clonidine HCl 3.7and 11.4µg/ml for Chlorthalidone. The developed HPLC method offers several advantages such as rapidity, usage of simple mobile phase and easy sample preparation steps. Further, improved sensitivity makes it specific and reliable for its intended use. Hence, this method can be applied for the analysis of pure drug and pharmaceutical dosage forms. From the present study it can be concluded that the proposed method is simple, sensitive, precise, specific, accurate andreproducible.Results ofvalidation parameters demonstrated that the analytical procedure is suitable for its intended purpose and meets the criteria defined in ICH Q2R1.

Keywords: Clonidine HCl, Chlorthalidone, Simultaneous Estimation, RP- HPLC

INTRODUCTION

Analytic method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest. This technical brief will focus on development and validation activities as applied to drug products[1-8]. The three critical components for a HPLC method are: sample preparation (% organic, pH, shaking/sonication, sample size, sample age) analysis conditions (%organic, pH, flow rate, temperature, wavelength, and column age), and standardization (integration, wavelength, standard concentration, and response factor correction). During the preliminary method development stage, all individual components should be investigated before the final method optimization[9-17]. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample

preparation, HPLC analysis and standardization) will first be investigated individually[18-24].

Drug Profile [25-28]

Table 1: Drug profile of Clonidine Hydrochloride

Drug Name	Clonidine hydrochloride
Synonym	Clonidine HCl
Drug category	Adrenergic Agents
IUPAC Name	N-(2,6-dichlorophenyl)-4,5-dihydro-1H-imidazol-2-amine hydrochloride.
Molecular Formula	$C_9H_9Cl_2N_3$
Molecular Weight	230.092 g/mol
Melting point	305°C
Solubility	Soluble in water

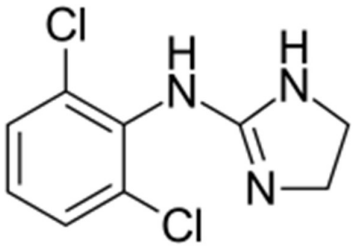

Fig 1: Clonidine Hydrochloride Structure

Table 2: Drug profile of Chlorthalidone

Drug	Chlorthalidone
Synonym	Chlortalidone and Chlortalidonum
Drug category	Antihypertensive Agent
IUPAC Name	2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1-yl)benzene-1-sulfonamide
Molecular Formula	$C_{14}H_{11}C_lN_2O_4S$
Molecular Weight	338.766 gm/mole.
Melting point	239 °C
Solubility	Water Solubility 0.0528 mg/mL

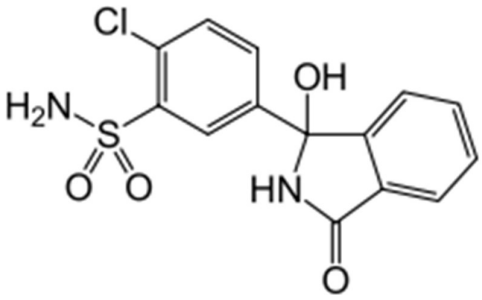

Fig 2: Chlorthalidone structure

A search through the literature revealed that the drug has been examined with a variety of analytical approaches, notably HPLC, RP-UPLC, UPLC-MS-MS etc.The development of a basic, precise, reliable, and repetitive HPLC method for the quantification of Vericiguat tablet dosage form is described in the current method withMethanol and Water as mobile phase and with Waters (Model: Alliance 2695) with Phenomenex Luna C18 (4.6 mm I.D. × 250 mm, 5 µm) column. [29-32]

Instruments used

Table 3: Instruments used

S. No	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Chemicals used

Table 4: Chemicals used

S. No	Chemical	Brand names
1	Clonidine (Pure)	Sura labs
2	Chlorthalidone (Pure)	Sura labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

MATERIALS AND METHODS

HPLC method development

Preparation of standard solution: Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 2.25ml of the above Clonidine HCl and Chlorthalidone and 0.45ml of the Clonidine stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure: Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization: Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and water in proportion 65:35 v/v respectively.

Optimization of Column: The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Luna C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Validation

Preparation of mobile phase

Preparation of mobile phase: Accurately measured 650ml (65%) of HPLC Methanol and 350ml of Water (35%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

Validation parameters

System suitability

Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Clonidine HCl and 0.45ml of the Chlorthalidone stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure: The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Specificity study of drug

Preparation of Standard Solution: Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Clonidine HCl and 0.45ml of the Chlorthalidone stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution: Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight ofClonidine HCl and Chlorthalidone sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 2.25ml of Clonidine HCl and Chlorthalidone above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Preparation of drugsolutions for linearity

Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (75ppm of Clonidine HCl and 15ppm of Chlorthalidone): Pipette out 0.75ml of the Clonidine HCl and 0.15ml of the Chlorthalidone from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Perform sonication for 10minutes.

Preparation of Level – II (150ppm of Clonidine HCl and 30ppm of Chlorthalidone): Pipette out 1.5ml of the Clonidine HCl and 0.3ml of the Chlorthalidone from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performs sonication for 10minutes.

Preparation of Level – III (225ppm of Clonidine HCl and 45ppm of Chlorthalidone): Pipette out 2.25ml of the Clonidine HCl and 0.45ml of the Chlorthalidone from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Perform sonication for 10minutes.

Preparation of Level – IV (300ppm of Clonidine HCl and 60ppm of Chlorthalidone): Pipette out 3.0ml of the Clonidine HCl and 0.6ml of the Chlorthalidone from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Perform sonication for 10minutes.

Preparation of Level – V (375ppm of Clonidine HCl and 75ppm of Chlorthalidone): Pipette out 3.75ml of the Clonidine HCl and 0.75ml of the Chlorthalidone from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Perform sonication for 10minutes.

Procedure: Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Precision

Preparation of Clonidine HCl and Chlorthalidone Product Solution for Precision:

Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Clonidine HCl and 0.45ml of the Chlorthalidone stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate precision

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

DAY 1:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

DAY 2:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

For preparation of 50% Standard stock solution: Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.12ml of the above Clonidine HCl and 0.225ml of the Chlorthalidone stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution: Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Clonidine HCl and 0.45ml of the Chlorthalidone stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution: Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3.37ml of the above Clonidine HCl and 0.675ml of the Chlorthalidone stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Clonidine HCl and Chlorthalidone and calculate the individual recovery and mean recovery values.

Robustness

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution: Accurately weigh and transfer 10 mg of Clonidine HCl and Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Clonidine HCl and 0.45ml of the Chlorthalidone stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions: The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded

Effect of Variation of mobile phase organic composition: The sample was analyzed by variation of mobile phase i.e. Methanol: Water was taken in the ratio and 60:40, 70:30 instead of 65:35, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Validation

System suitability

Table 5: Results of system suitability for Clonidine HCl

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Clonidine HCl	3.200	2391746	394171	8952	1.2
2	Clonidine HCl	3.248	2391647	381946	9561	1.2
3	Clonidine HCl	3.299	2381647	391746	6572	1.2
4	Clonidine HCl	3.297	2385631	386562	6452	1.2
5	Clonidine HCl	3.297	2385635	389164	7452	1.2
Mean			2387261			
Std.Dev.			4363.771			
%RSD			0.182794			

Table 6: Results of system suitability for Chlorthalidone

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Chlorthalidone	5.413	198362	7917	5272	1.1
2	Chlorthalidone	5.484	197486	7486	6291	1.1
3	Chlorthalidone	5.405	198354	7859	6184	1.1
4	Chlorthalidone	5.405	197352	7926	7145	1.1
5	Chlorthalidone	5.409	198453	7946	6946	1.1
Mean			198001.4			
Std.Dev.			535.1774			
%RSD			0.27029			

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitate Clonidine HCl and Chlorthalidone in drug product.

Clonidine HCl

Table 7: Specificity results of Clonidine HCL

S. No	Name	RT	Area	Height	USPTailing	USPPlateCount
1	Clonidine HCl	3.211	2397162	397161	1.2	9472
2	Clonidine HCl	3.222	2394721	389173	1.2	9745
3	Clonidine HCl	3.254	2389461	391723	1.2	8917

Chlorthalidone

Table 8: Specificity results of Chlorthalidone

S. No	Name	RT	Area	Height	USPTailing	USPPlateCount	Resolution
1	Chlorthalidone	5.414	198462	7811	1.1	8492	7.49
2	Chlorthalidone	5.453	198472	8193	1.1	8916	7.52
3	Chlorthalidone	5.424	198735	7972	1.1	9372	7.44

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Clonidine HCl and Chlorthalidone in pharmaceutical dosage form was found to be 99.2%.

Linearity

Clonidine HCl

Table 8: Linearity results of Clonidine HCL

Concentration Level (%)	Concentration µg/ml	Average Peak Area
60	75	909889
80	150	1583641
100	225	2395378
120	300	3185089
140	375	3943725

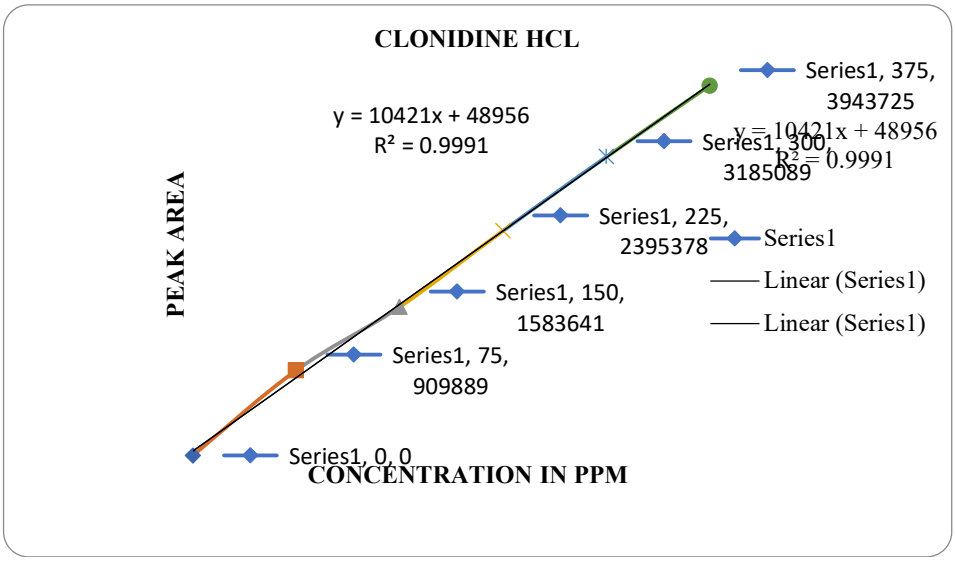

Fig 3: Calibration Curve of Clonidine HCl

Chlorthalidone

Table 9: Linearity results of Chlorthalidone

Concentration Level (%)	Concentration µg/ml	Average Peak Area
60	15	61953
80	30	130213
100	45	198697
120	60	267002

140	75	321658

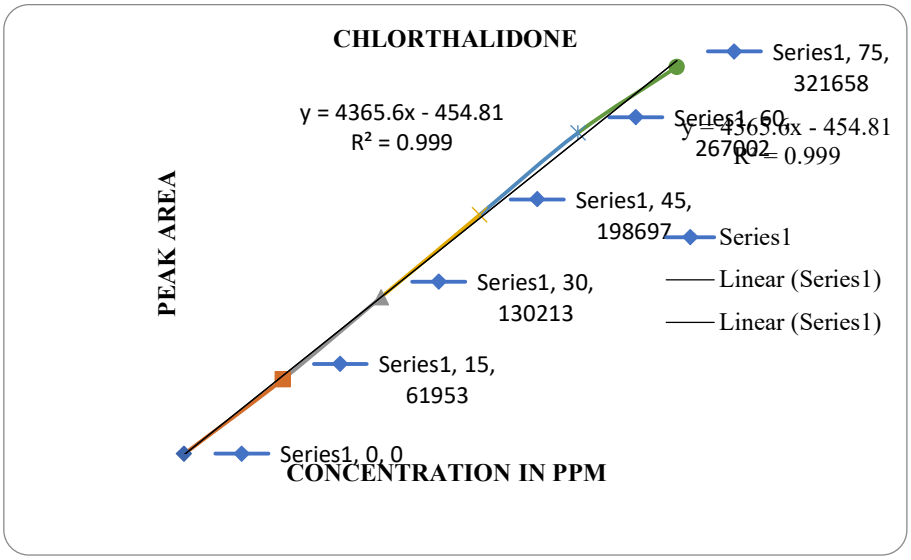

Fig 4: Calibration Curve of Chlorthalidone

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Table 10: Results of repeatability for Clonidine HCl

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Clonidine HCl	3.213	2397164	381741	8155	1.2
2	Clonidine HCl	3.253	2391741	371742	9174	1.2
3	Clonidine HCl	3.297	2371846	391746	7154	1.2
4	Clonidine HCl	3.215	2361748	391847	9917	1.2
5	Clonidine HCl	3.254	2371649	384622	9247	1.2
Mean			2378830			
Std.dev			14958			
%RSD			0.628797			

Table 11: Results of repeatability for Chlorthalidone

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Chlorthalidone	5.441	198464	7291	6274	1.1
2	Chlorthalidone	5.442	193643	7219	6592	1.1
3	Chlorthalidone	5.409	196462	7194	6028	1.1
4	Chlorthalidone	5.520	194644	8174	6927	1.1
5	Chlorthalidone	5.424	198464	8653	5920	1.1
Mean			196335.4			
Std.dev			2190.191			
%RSD			1.115536			

Intermediate precision

Table 12: Results of Intermediate precision for Clonidine HCl

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Clonidine HCl	3.211	2389572	395275	9375	1.2
2	Clonidine HCl	3.211	2391847	392175	9275	1.2
3	Clonidine HCl	3.210	2319472	312947	8265	1.2
4	Clonidine HCl	3.212	2306842	310585	6254	1.2
5	Clonidine HCl	3.211	2375972	310694	9028	1.2
6	Clonidine HCl	3.297	2396746	358373	8928	1.2
Mean			2363409			
Std.Dev.			39730.83			
%RSD			1.681082			

Table 13: Results of Intermediate precision for Chlorthalidone

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Chlorthalidone	5.411	197284	7194	8264	1.2
2	Chlorthalidone	5.410	197849	7294	9174	1.2
3	Chlorthalidone	5.420	196572	7147	9164	1.2
4	Chlorthalidone	5.423	195028	7927	9733	1.2
5	Chlorthalidone	5.419	199474	8238	9194	1.2
6	Chlorthalidone	5.409	197482	7638	8973	1.2
Mean			197281.5			
Std.Dev.			1466.354			
%RSD			0.74328			

Table 14: Results of Intermediate precision Day 2 for Clonidine HCl

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP PlateCount	USP Tailing
1	Clonidine HCl	3.211	2389562	391741	9264	1.2
2	Clonidine HCl	3.233	2381654	391047	9746	1.2
3	Clonidine HCl	3.244	2381946	391748	9816	1.2
4	Clonidine HCl	3.297	2391741	391746	9917	1.2
5	Clonidine HCl	3.297	2386452	381641	9742	1.2
6	Clonidine HCl	3.202	2374763	381645	9017	1.2
Mean			2384353			
Std.Dev.			6183.339			
%RSD			0.25933			

Table 15: Results of Intermediate precision Day 2 for Chlorthalidone

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP PlateCount	USP Tailing
1	Chlorthalidone	5.411	197486	7582	6272	1.1
2	Chlorthalidone	5.410	197486	7184	6174	1.1
3	Chlorthalidone	5.420	196746	7456	5184	1.1
4	Chlorthalidone	5.405	195862	7814	6194	1.1
5	Chlorthalidone	5.409	196582	7194	6292	1.1
6	Chlorthalidone	5.463	198463	7745	6191	1.1
Mean			197104.2			

Std.Dev.	903.542
%RSD	0.458408

Accuracy

Accuracy at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

Table 16: Accuracy results for Clonidine HCl

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1217218	112.5	112.4	99.6	99.3
100%	2397141	225	225	100	
150%	3514547	337.5	332.5	98.5	

Table 17: Accuracy results for Chlorthalidone

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	98598.67	22.5	22.4	99.9	99.6
100%	198359.7	45	45	100	
150%	291512.3	67.5	66.8	99	

Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD= 3.3 × σ / s

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Clonidine HCl:

Result:

=3.3×39762/10421

=12.5µg/ml

Chlorthalidone:

Result:

=3.3×5008/4365

=3.7µg/ml

Quantitation limit:

The quantitation limit of an individual analytical procedure is the lowest amount ofanalyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Clonidine HCl:

Result:

=10×39762/10421

=38.1µg/ml

Chlorthalidone:

Result:

=10×5008/4365

=11.4µg/ml

ROBUSTNESS:

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Clonidine HCl and Chlorthalidone. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard sample of Clonidine HCl and Chlorthalidone were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor and plate count.

CONCLUSION

The developed HPLC method offers several advantages such as rapidity, usage of simple mobile phase and easy sample preparation steps. Further, improved sensitivity makes it specific and reliable for its intended use. Hence, this method can be applied for the analysis of pure drug and pharmaceutical dosage forms.

From the present study it can be concluded that the proposed method is simple, sensitive, precise, specific, accurate and reproducible. Results of validation parameters demonstrated that the analytical procedure is suitable for its intended purpose and meets the criteria defined in ICH Q2A/R1.

REFERCENES

1. Dr. Kealey, Haines PJ. Analytical chemistry. 1st ed. Bios Publisher; 2002. P. 1-7.
2. BraithWait A, Smith FJ. Chromatographic methods. 5th ed. Kluwer Academic Publishers; 1996. P. 1-2.
3. Weston A, Phyllisr. Brown, HPLC principle and practice. 1st ed. Academic press; 1997. P. 24-37.

4. Kazakevich Y, Lobrutto R. HPLC for pharmaceutical scientists. 1st ed. Wiley Interscience A JohnWiley & Sons, Inc Publishing House; 2007. P. 15-23.
5. Chromatography [online]. Wikipedia. Available from: http://en.wikipedia.org/wiki/Chromatography.
6. Meyer VR. Practical high-performance liquid chromatography. 4th ed. England: John Wiley & Sons Ltd; 2004. P. 7-8.
7. Sahajwalla CG a new drug development. Vol. 141. New York: Marcel Dekker, Inc; 2004. P. 421-6.
8. Introduction to column [online]. Available from: http://amitpatel745.topcities.com/index_files/study/columncare.pdf.
9. Detectors used in HPLC (online). Available from: http://wiki.answers.com/Q/What_detectors_are_used_in_HPLC.
10. Detectors [online]. Available from: http://hplc.chem.shu.edu/NEW/HPLC_Book/Detectors/det_uvda.html.
11. Detectors [online]. Available from: http://www.dionex.com/enus/webdocs/64842-31644-02_PDA-100.pdf.
12. Detectors [online]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8867705.
13. Detectors [online]. Available from: http://www.chem.agilent.com/Library/applications/59643559.pdf.
14. Detectors [online]. Available from: http://hplc.chem.shu.edu/new/hplcbook/detector.
15. Draft ICH. Guidelines on Validation of Analytical Procedures Definitions and terminology. Fed Regist. 1995;60:1126.
16. Code. Q2B, validation of analytical procedures; methodology. ICH harmonized tripartite guidelines. 1996:1-8.
17. Introduction to analytical method validation [online], available from. Available from: http://www.standardbase.hu/tech/HPLC%20validation%20PE.pdf.
18. Data elements required for assay validation [online] available from. Available from: http://www.labcompliance.com/tutorial/methods/default.aspx.
19. Snyder LR practical HPLC method development. 2nd ed. New York: John Wiley & sons; 1997. P. 180-2.
20. Skoog DA, West DM, Holler FJ. Introduction of analytical chemistry. Sounder college of publishing, Harcourt Brace college publishers; 1994. P. 1-5.
21. Sharma BK. Instrumental method of chemical analysis Meerut; 1999. P. 175-203.
22. Breaux J, Jones K. Understanding and implementing efficient analytical method development and validation. Pharm Technol. 2003;5:110-4.
23. Willard H, Merritt LL, Dean JA, Settle FA. Instrumental methods of analysis. 7th ed CBS publisher and distributors. New Delhi; 1991. P. 436-9.
24. ICH. 'validation of analytical methods, definitions and terminology', ICH Harmonized tripartite guideline; 1999. p. Q2A.
25. Available from: https://www.drugbank.ca/drugs/DB00575.
26. Available from: https://en.wikipedia.org/wiki/Clonidine. Wikipedia.
27. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/clonidine#section=General-MS.
28. Available from: https://www.drugbank.ca/salts/DBSALT000538.
29. AlRabiah H, Attia SM, Al-Shakliah NS, Mostafa GAE. Development and validation of an HPLC-UV detection assay for the determination of clonidine in mouse plasma and its application to a pharmacokinetic study. Molecules. 2020;25(18):4109. doi: 10.3390/molecules25184109, PMID 32911847.
30. Khan HN, Asha C, Zameeruddin M, Bharkad VB. Development anValidation of RP-HPLC method for simultaneous estimation of clonidine HCl and chlorthalidone in bulk form. Ijppr. Human. 2017;10(4):202-9.
31. Panchal RS, Thakkar D, Patel MB. Development and validation of analytical methods for simultaneous estimation of clonidine HCl and chlorthalidone in their combined dosage forms. J PharmSci Bioscientific Res. 2015;5(5):463-9.
2. Saravanan G, Padmaja M, Gowthami AL, Sri Krish Anjaneyulu I. Stability indicating RP-HPLC method for estimation of hydrochlorothiazide and clonidine hydrochloride in bulk and formulation; July-September 2014.