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Development and validation of a new simple, sensitive and validated RP-HPLC method for the estimation of moxonidine in bulk form and marketed formulation

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ABSTRACT

A novel, simple, accurate, precise, sensitive and specific analytical RP-HPLC method was developed and validated for the quantitative estimation of Moxonidine in bulk drugs and pharmaceutical dosage form. Chromatographic separation was achieved on an Symmetry ODS C18 (4.6×250mm, 5µm) analytical column using mobile phase composition of methanol and Phosphate Buffer in ratio of (35: 65 v/v) that was set at a flow rate of 1.0µl/min with detection of 235 nm. The retention time of Moxonidine was found to be 3.006min. The drug was analyzed by following the guidelines of International conference on Harmonization (ICH). This drug showing linearity in the concentration range of 6-14µg/ml and the correlation coefficient showing R2 = 0.9996. The % Recoveries showing within the limits. The presentation of the method was validated according to the present ICH guidelines for accuracy, precision and robustness, Linearity, limit of quantification, limit of detection linearity.

Keywords: Moxonidine, RP-HPLC, Method Development, Accuracy, Precision.

INTRODUCTION

Moxonidine is an organohalogen compound and a member of pyrimidines. Moxonidine[1] is a new-generation centrally acting antihypertensive drug approved for the treatment of mild to moderate essential hypertension. It is suggested to be effective in cases where other agents such as thiazides, beta-blockers, ACE inhibitors, and calcium channel blockers are not appropriate or irresponsive. As well, Moxonidine[2] has been shown to present blood pressure-independent beneficial effects on insulin resistance syndrome. Antihypertensive agent whose site of action is the Central Nervous System (CNS), specifically involving interactions with I1- imidazoline and alpha-2-adrenergic receptors within the rostral Ventrolateral medulla (RSV). Stimulation of central alpha 2-adrenergic receptors is associated with sympathoadrenal suppression and subsequent reduction of blood pressure. As this class was further explored it was discovered that sympathoadrenal activity can also be suppressed by a second pathway with a newly discovered drug target specific to imidazolines 5. Specifically, Moxonidine[3] binds the imidazoline receptor subtype 1 (I1) and to a lesser extent αlpha-2-adrenoreceptors in the RSV causing a reduction of sympathetic activity, reducing systemic vascular resistance and thus arterial blood pressure. Moxonidine is an imidazoline/α-2 receptor agonist used to treat hypertension, especially in cases where ACE inhibitors, β-blockers, calcium channel blockers, and thiazides are not appropriate or provide inadequate blood pressure control. The IUPAC Name of

Moxonidine is 4-chloro-N-(4, 5-dihydro-1H-imidazol-2-yl)-6-methoxy-2-methyl pyrimidin-5-amine.

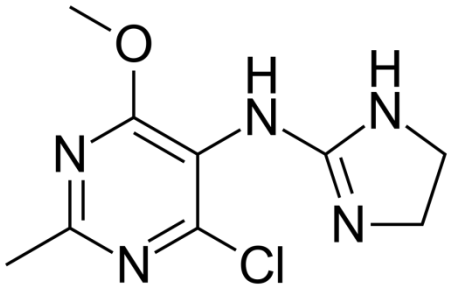

Fig 1: Chemical Structure of Moxonidine

Experimental

Table 1: Instruments Used

S.No.	Instruments and Glass wares	Model
1	HPLC	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
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

Table 2: Chemicals Used

S.No.	Chemical	Brand Names
1	Moxonidine (Pure)	Local Market
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Moxonidine 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 0.1ml of the above Moxonidine 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[29-30].

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 35:65% v/v.

Optimization of Column

The method was performed with various C18 columns like, X-bridge column, Xterra, and C18 column[4]. Symmetry ODS C18 (4.6 x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Preparation of Buffer and Mobile Phase

Preparation of Potassium dihydrogen Phosphate (KH2PO4) Buffer (pH-3.6)

Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration[5] and ultra-sonication.

Preparation of Mobile Phase

Accurately measured 350 ml (35%) of Methanol, 650 ml of Phosphate buffer (65%) were mixed and degassed in digital

ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration[6].

Diluent Preparation

The Mobile phase was used as the diluent[7].

METHOD VALIDATION PARAMETERS

System Suitability

Accurately weigh and transfer 10 mg of Moxonidine 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 0.1ml of the above Moxonidine stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

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

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Moxonidine 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 0.1ml of the above Moxonidine stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution

Weight 10 mg equivalent weight of Moxonidine 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 0.1ml of Moxonidine 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[8] by using formula:

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

Linearity

Accurately weigh and transfer 10 mg of Moxonidine 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 (6ppm of Moxonidine): Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – II (8ppm of Moxonidine): Take 0.8ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – III (10ppm of Moxonidine): Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – IV (12ppm of Moxonidine): Take 0.12ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – V (14ppm of Moxonidine): Take 0.14ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Procedure: Inject each level into the chromatographic system[9] 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[10].

Precision

Repeatability

Preparation of Moxonidine Product Solution for Precision

Accurately weigh and transfer 10 mg of Moxonidine 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 0.1ml of the above Moxonidine stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

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[11].

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:

Analyst 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.

Analyst 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 Moxonidine 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 0.05ml of the above Moxonidine stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 100% Standard Stock Solution

Accurately weigh and transfer 10 mg of Moxonidine 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 0.1ml of the above Moxonidine stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 150% Standard Stock Solution

Accurately weigh and transfer 10 mg of Moxonidine 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 0.15ml of the above Moxonidine stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

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 Moxonidine 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 Moxonidine 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 0.1ml of the above Moxonidine stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

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: Phosphate Buffer was taken in the ratio and 40:60, 30:70 instead (35:65), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Method Development

Optimized Chromatographic Conditions

Mobile phase ratio: Methanol: Phosphate Buffer (35:65) V/V
Column : Symmetry ODS C18 (4.6×250mm, 5µm)
Column temperature: Ambient
Wavelength : 235nm
Flow rate : 1ml/min
Injection volume : 10µl
Run time : 8min

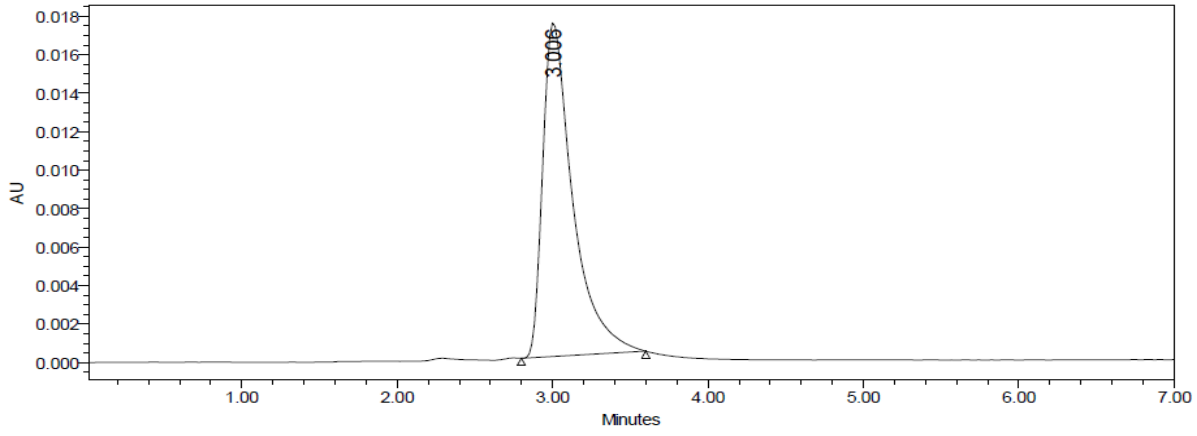

Fig 2: Optimized Chromatographic Condition of Moxonidine

In this trial it shows proper separation of peak and more plate count in the chromatogram and the tailing factor is within the limit. So it is an optimized chromatogram.

Method Validation

The proposed method[12] was validated for specificity, accuracy, and precision, the limit of detection, the limit of quantitation as well as the robustness of the method as per ICH guidelines[13]. Replicate injections of the standard and sample were used to carry out all the studies.

System Suitability

Standard solution of Moxonidine was injected 5 times for testing system suitability parameters. The results for system suitability[14] are shown in Table 3. From Table 3, it is concluded that system suitability parameters were within the acceptance criteria.

Table 3: Results of System Suitability for Moxonidine

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Moxonidine	3.008	1652847	185647	6589	1.24
2	Moxonidine	3.005	1653658	186254	6587	1.26
3	Moxonidine	3.001	1654521	185475	6584	1.28
4	Moxonidine	3.000	1653564	186594	6582	1.29
5	Moxonidine	3.001	1658745	185684	6895	1.24
Mean			1654667			
Std. Dev.			2355.764			
% RSD			0.142371			

Specificity

The ICH documents define specificity[15] 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 quantitates Moxonidine in drug product

.

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

The % purity[16] of Moxonidine in pharmaceutical dosage form was found to be 99.86%.

Linearity

Linearity is the ability to obtain experimental results which are proportional to the conc. of the analyte present in the sample. The calibration curve was obtained by using five different concentrations in 6, 8, 10, 12, and 14µg/ml and the linearity was established by applying linearity expression y = mx+c and slope was calculated. The calibration curve[17] for Moxonidine was shown in Figure 3. The Results of linearity was shown in Table 4. From Table 3, the response of Moxonidine was linear[18] at a range of 6 to 14µg/ml concentration. The correlation was not less than 0.95, so linearity parameter was passed.

Table 4: Data for Linearity of Moxonidine

Concentration µg/ml	Average Peak Area
6	1078475
8	1461129
10	1808358
12	2211573
14	2593778

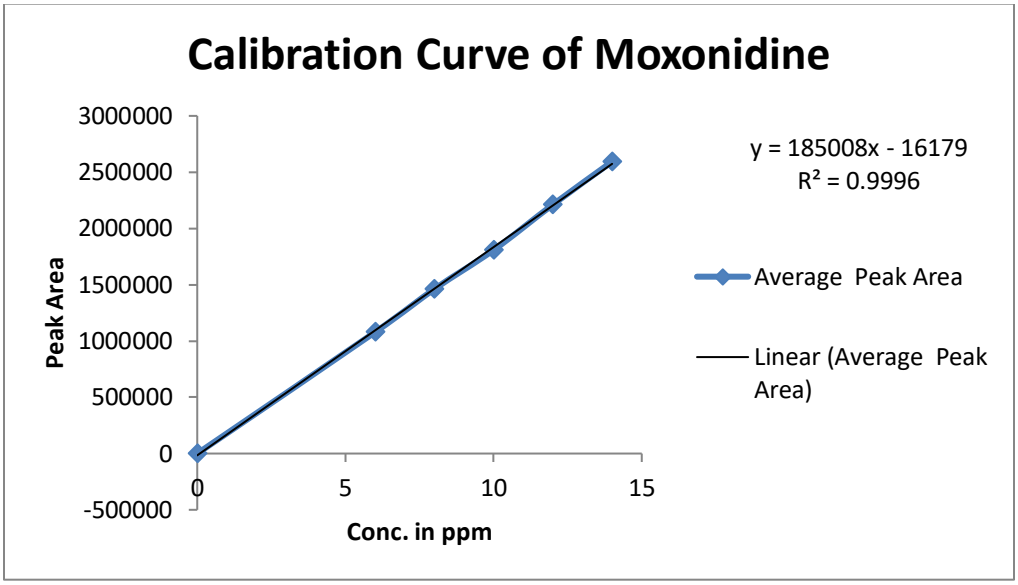

Fig 3: Linearity Curve of Moxonidine

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Moxonidine is a straight line.

Y = mx + c
Slope (m) = 185008
Intercept (c) = 16179
Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
Correlation Coefficient[19] (r) is 0.99, and the intercept is 0.16179. These values meet the validation criteria.

Precision

The precision[20] 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[21].

Repeatability: Obtained Five (5) replicates of 100% accuracy[22] solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table 5: Results of Repeatability for Moxonidine

S. No.	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Moxonidine	3.008	1658954	186958	1.26	6785
2	Moxonidine	3.000	1658745	187548	1.27	6854
3	Moxonidine	3.013	1659865	189854	1.26	6852
4	Moxonidine	3.006	1653254	186985	1.25	6784
5	Moxonidine	3.001	1654781	189542	1.24	6895
Mean			1657120			
Std. Dev			2913.592			
%RSD			0.175823			

Intermediate Precision

The Intermediate Precision[23-24] consists of two methods:-

Intra Day: In Intra Day process, the 50%, 100% and 150% concentration are injected at different intervals of time in same day.
Inter Day: In Inter Day process, the 50%, 100% and 150% concentration are injected at same intervals of time in different days.

Table 6: Results of intra-assay & inter-assay

Conc. of Moxonidine (API) (µg/ml)	Observed Conc. of Moxonidine (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
50	49.38	0.56	49.45	0.56
100	100.17	0.71	99.70	0.77
150	150.89	0.89	149.91	0.85

The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Moxonidine revealed that the proposed method is precise. Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery[25] was calculated.

Table 7: The Accuracy Results for Moxonidine

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109068.3	5	5.021	100.420%	100.72%
100%	202187	10	10.054	100.540%	
150%	297032.3	15	15.181	101.206%	

Limit of Detection

The detection limit[26] 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.

$$LOD = 3.3 \times \sigma / s$$

Where
σ = Standard deviation of the response
S = Slope of the calibration curve
Result: = 1.2µg/ml

Quantitation Limit

The quantitation limit[27] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$LOQ = 10 \times \sigma / S$$

Where
σ = Standard deviation of the response
S = Slope of the calibration curve
Result: = 3.6µg/ml

Robustness

The robustness[28] 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 Moxonidine. The method is robust only in less flow condition. The standard of Moxonidine was injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table 8: Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.68
Flow (0.9 ml/min)	0.39
Temperature (27^0C)	0.54
Temperature (23^0C)	0.63
Wavelength of Detection (280 nm)	0.91
Wavelength of detection (270 nm)	0.93

Estimation of Moxonidine in TABLET Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 10 mg of drug were transferred to 10 ml volumetric flask, and 8 ml of mobile phase was added and solution was sonicated for 15 minutes, there after volume was made up to 10 ml with same solvent. Then 1ml of the above solution was diluted to 10 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1.0 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded. A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded.

ASSAY

% Assay=AT/AS×WS/DS×DT/WT×P/100×AW/LC×100

Where:
AT = Peak Area of Moxonidine obtained with test preparation
AS = Peak Area of Moxonidine obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg
DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard
Results obtained are tabulated below:

Table 9: Assay of Moxonidine Tablets

Brand Name of Capsules	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=5)	Assay + % RSD
Moxilong-0.2 Tab (Micro Labs Limited)	0.2mg	0.168 (± 0.568)	99.574 % (± 0.694)

The %Purity of Moxilong-0.2 Tablets containing Moxonidine was found to be 99.574 % (± 0.694).

Forced Degradation Studies

Following protocol was strictly adhered to for forced degradation of Moxonidine Active Pharmaceutical Ingredient (API). The API (Moxonidine) was subjected to keep in some stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. It is one type of accelerated stability studies of the drugs that is used to help us to determining the total fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing. The different types of forced degradation pathways/studies are studied here are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

Results of Degradation Studies

The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Moxonidine were stable only in oxidation, photolytic and acidic stress conditions. The results of stability studies are given in the following Table-10.

Table 10: Results of Forced Degradation Studies of Moxonidine API

Stress Condition	Time (hours)	Assay of Active Substance	Assay of Degraded Products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	96.854	3.146	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	81.632	18.368	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	86.475	13.525	100.00
UV (254nm)	24Hrs.	97.866	2.134	100.00
3% Hydrogen Peroxide	24Hrs.	98.654	1.346	100.00

SUMMARY AND CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Moxonidine, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the

current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS C18 (4.6×250mm, 5µm) column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, acetonitrile, water, 0.1N NaOH, 0.1NHCl). Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Moxonidine it is evident that most of the HPLC work can be accomplished in the wavelength range of 235 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 10µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay which can help in the analysis of Moxonidine in different formulations.

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