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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE DETERMINATION OF ANTIBIOTIC DRUG IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Numerous infections are treated with Antibiotics. Moxifloxacin is utilized in numerous therapeutic procedures. A method involving an HPLC-diode array detector (DAD) was developed and proven for measuring moxifloxacin in pharmaceutical dose form. A simple, specific, and precise stability indicating reverse phase high performance liquid chromatography method was developed and validated as per the ICH guidelines for the simultaneous determination of Moxifloxacin and Prednisolone in bulk and combined dosage forms. The quantification was carried out by using hypersil BDS C_{18} (250mm*4.6mm, 5µ) column at ambient temperature with orthophosphoric Buffer pH 4 Acetonitrile: water (H_2O):Trifluro acetic acid in ratio of 45:55:0.2% V/V as mobile phase. The flow rate is 1 mL /min and the estimation was carried out by using PDA detector at 254 nm. The retention time of MFX and PDS were 3.20 and 4.73 minutes respectively. The linearity was observed from 25.10-275.3 µg /mL with correlation coefficient 0.9998 for Moxifloxacin and 30.06-90.18 µg/mL with correlation coefficient 0.9964 for Prednisolone. The LOD and LOQ of Moxifloxacin and Prednisolone were found to be 23.09 & 33.93µg/mL and 0.998 & 0.9964 µg/mL respectively and the Statistics data for the MFX and PDS were concluded that the method was found to be simple, reliable, selective, reproducible and accurate. The method was successfully used for quality control analysis of Moxifloxacin and Prednisolone.

KEYWORDS: Moxifloxacin (MFX), Prednisolone (PDS), RP-HPLC, Stability, and validation.

INTRODUCTION

Analytical Chemistry plays an important role in the resolution of a chemical composition of samples of matter. Analytical Chemistry plays an important role in the resolution of a chemical compound into its proximate or ultimate parts, determination of its elements or of the foreign substances it may contain. Its application extends to all parts of an industrial society.[1-6]

HPLC is a modern form of liquid chromatography that uses small-particle column through which the mobile phase is pumped at high pressure. This is chromatographic process, where a mixture of analytes is separated into two distinct bands as they migrate down the column filled with stationary phase. HPLC is used either in the liquid-solid adsorption chromatography mode or the liquid-liquid partition chromatography mode, either normal or reversed-phase. Both partition and adsorption chromatography operates on differences in solute polarity since polarity is important in determining both adsorption andsolubility.[7-8]

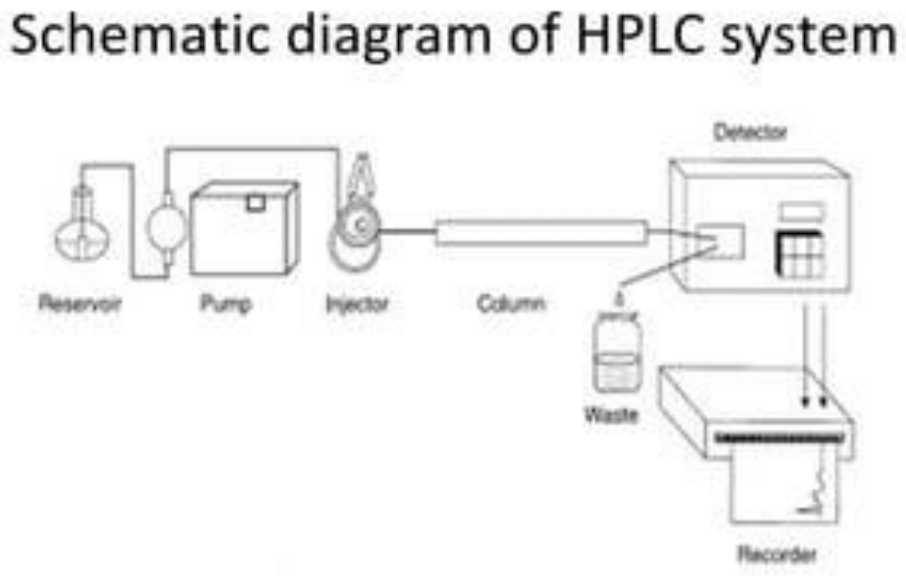

Fig. No. 1: Schematic diagram of HPLC system.

EXPERIMENTAL WORK

Prednisolone was procured from Yarrow Chem Laboratories Ltd, Mumbai India, Moxifloxacin Hydrochloride was procured from Yarrow chem laboratories Ltd, Mumbai India.

Details of Pure drug

Table no. 1: Details of API.

Drug	Supplied by	Quantity (g)	Purity(Assay) % w/w
Prednisolone	Yarrow Chem laboratories Ltd., Mumbai	5.0	99.67
Moxifloxacin Hydrochloride	Yarrow Chem laboratories Ltd., Mumbai	5.0	99.0

Details of marketed formulation: Brand name- GpMox-P

Contents- Moxifloxacin HCL-0.5%w/v

- Prednisolone Acetate-1%w/v

Mfg. By- Laborate pharmaceuticals India Ltd

Reagents and Chemicals

All reagents and chemicals used were of AR grade and HPLC grade

- Acetonitrile (HPLC grade)
- Trifluoroacetic acid (HPLC grade)
- Water (HPLC grade)
- Methanol (HPLC grade)
- Glacial acetic acid (HPLC grade)
- Sodium Acetate unhydrous (AR grade)
- Sodium hydroxide pellets (AR grade)
- Hydrochloric acid about 37% for analysis
- Hydrogen peroxide 30% for analysis

Instruments

Table No 2: Instruments Used.

Sr. No.	Instrument	Make	Model
1	UV-Visible Spectrophotometer	Thermo Electron	Double beamcarry-07 Bio
2	HPLC	1. Water Millipore 2. Water, 3. Water, 4. Water	600 E pump quaternary gradient, waters online degasser, 996 photo-diode array(PDA)detector, 515 Autoinjector.
3	Software	1. Microsoft (Intel)	1.EMPOWER
4	pH Meter	Hanna	-
5	Balance	Citizen	CY 104 (Micro Analytical Balance)

METHODOLOGY

Method Development

1. Selection of Analytical Technique

Chromatographic conditions:

HPLC was selected as an analytical technique for estimation of Moxifloxacin hydrochloride and Prednisolone.

- **Solubility Studies**

This study was carried out to find an ideal solvent in which drugs are completely soluble. Various solvents were tried for checking the solubility of Moxifloxacin and prednisolone. From solubility studies, it was concluded that Moxifloxacin and Prednisolone is freely soluble in Acetonitrile. Therefore Acetonitrile and water were selected as a suitable solvent for further studies of Moxifloxacin and Prednisolone.

- **Selection of chromatographic mode**

The reverse-phase HPLC (RP-HPLC) was selected because it was convenient and rugged than other forms of liquid chromatography and was more likely to give a well-resolved peak at a reasonable retention time.

- **Selection of chromatographic mode and Selection of stationary phase**

The analysis of the drug was carried out on HPLC model no 600 E pump quaternary gradient**,** Column Hypersil BDS C18 (250mm x 4.6 ID, Particle size: 5 micron), 600 E pump, UV/Vis Detector and running empower software.

- **Selection of flow rate**

Different mobile phase flow rates (1.00ml/min) were investigated. The optimum flow ratefor which the column plate number (N) was Maximum, with the best resolution obtained and with a short run time was selected.

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table No. 3: Chromatographic conditions (HPLC) details used during method.

Sr.NO.	Parameters	Specification
1	HPLC	600 E Pump quarternary gradient
2	Software	Empower

3	Column	Hypersil BDS C18(250mm×4.6,5µ)
4	Particle size packing	5µm
5	Stationary Phase	Hypersil BDS C18
6	Mobile Phase	ACN:Water:TFA(45:55:0.2)
7	Detection wavelength	254 nm
8	Flow rate	1.0 ml/min
9	Temperature	Ambient
10	Injection volume	20 µl
11	Run Time	12.0 min
12	Filter Paper	0.45 µm membrane filter paper

2. The analysis was done by the following parameter

Solubility

UV spectra and Determination of λmax HPLC chromatogram.

Preparation of Standard Stock Solution and selection of detection wavelength Standard Stock Solution

An accurately weighed quantity of about 25 mg of Moxifloxacin and 50.0 mg Prednisolone was transferred to the 50 ml volumetric flask, dissolved in acetonitrile and volume was made up to the mark with the same solvent.(conc. 500µg/ml of Moxi and 1000µg/ml of Pred).

From this standard stock solution, further 5 ml was transferred in 50 ml volumetric flask and acetonitrile was added up to mark to give a solution containing 50 ug/ml of Moxi and 100µg/ml of Pred. The solution was filtered to a 0.45µ membrane filter.

Determination of λmax by UV

The standard solution of Moxifloxacin and Prednisolone is scanned over the range of 200-400 nm. The wavelength of absorption (lambda max) was found to be 254 nm. So the wavelength selected for the determination of Moxifloxacin and Prednisolone is 254 nm.

3 Different method development trials took as follows

HPLC used for chromatographic condition applies to the Preparation of solution: Trial 1: Method development initiated by using moxi and prednisolone API Preparation of Mobile Phase

Methanol: Water (70:30)**Preparation of Diluent** Mobile phase.

Standard preparation

Moxifloxacin Standard Stock Solution: A weighed quantity of 10 mg of Moxifloxacin and 5mg of Prednisolone was dissolved in diluent (mobile phase) and the volume was filled with

50 ml mark to obtain a stock solution of 300 µg/ml. The resulting solution was filtered through a 0.45µ membrane filter and ultra-sonicated for 10 min with intermittent shaking.

Working standard solution

Pipette out 5 ml of a standard stock solution and further dilute it with 50 ml of diluent to obtain the working solution of 100µg/ml. This solution was filtered 0.45µ membrane filter paper and ultra-sonicated for 10 min with intermittent shaking. The resultant solution was used for further method development using RP-HPLC.

Chromatographic condition Constant parameter selected

Column	**Hypersil BDS C18 (250mm x 4.6 mm ID, Particle size: 5µ**
Flow rate	1.0 ml/minute.
Injection volume	20 µL.
Temperature	Ambient
Wavelength	254 nm
Run time	12 minutes
Elution	Isocratic

Trial – 02 change in the mobile phase

The trial was conducted by changing the mobile phase and keeping other parameters constant.

Preparation of Mobile Phase

Prepared a mixture of 0.1 m Sodium Acetate with Acetic acid and Methanol solution, pH 4.0 in the ratio 20:80 v/v, mix, and sonicated to degas. They were used as mobile phases.

Preparation of Diluent

Mobile phase.

Trial – 03 change in the mobile phase

The trial was conducted by changing the mobile phase and keeping other parameters constant.

Preparation of Mobile Phase

Prepared a mixture of Methanol and 0.1% Acetic acid solution in the ratio 80:20 v/v, mix, and sonicated to degas. They were used as mobile phase.

Preparation of Diluent

Mobile phase.

Trial – 04 change in the mobile phase

The trial was conducted by changing the mobile phase and keeping other parameters constant.

Preparation of Mobile Phase

ACN: Water: TFA (50:50:0.2v/v)

Preparation of Diluent

Mobile phase.

Trial – 05 change in the mobile phase

The trial was conducted by changing the mobile phase and keeping other parameters constant.

Preparation of Mobile Phase

ACN: Water: TFA (45:55:0.2v/v)

Preparation of Diluent

Mobile phase.

3. RP-HPLC method development trials and optimization

The standard solution of Moxifloxacin and Prednisolone was used for method development trials to optimize the method for the determination of Moxifloxacin and Prednisolone.

Selection of mobile phase

Each mobile phase was filtered through a 0.45 µ membrane filter. The mobile phase was allowed to equilibrate the phase until a steady baseline was obtained. The standard solutions containing Moxifloxacin and Prednisolone were run and different individual solvents, as well as combinations of solvents, were tried to get a stable peak. From the various mobile phases tried the mobile phase containing ACN: Water: TFA solution (45:55:0.2 v/v), was selected as it showed a sharp peak with symmetry and significant reproducible retention time for Moxifloxacin and prednisolone.

4. Optimization Chromatographic conditionss

The following chromatographic parameters were established on trial and error basis and were kept constant during experimentation.

Column : Hypersil BDS C18 (250mm x 4.6 ID, Particle size: 5µ) Detection wavelength: 254 nm

Injection volume : 20 µl

Flow rate : 1.0 ml/min

Temperature : Ambient

Mobile phases : ACN: Water: TFA (45:55:0.2 v/v)

5. Preparation of calibration curve

Preparation of mobile phase

Prepare a homogeneous mixture of 450 ml of HPLC grade Acetonitrile, 550 ml of HPLC grade water, and 2 ml of AR grade TFA in 1000 ml volumetric flask, mixed. Shake well and sonicate for 10 min filter this mobile phase through a 0.45 µm membrane filter, then degassed after filter again sonicate with digital ultrasound for 10 minutes.

Preparation of diluent

The mobile phase itself was selected as diluent for the preparation of standard and sample solutions.

Standard stock solution

An accurately weighed quantity of 25.1 mg of Moxifloxacin and 50.0 mg of Prednisolonewas dissolved in diluent and volume was made up to 50 ml with diluents. (1502 µg/ml)

From 1502µg/ml appropriate aliquots such as 5 ml, 7.5 ml, 10 ml, 12.50 ml, 15 ml taken in a 50 ml volumetric flask and made up the mark with diluents, whereby the resulting solution becomes 25.10, 37.65, 50.20, 62.75, 75.3 µg/ml.

Procedure

The mobile phase was allowed to equilibrate with the stationary phase until a steady baseline was obtained. Then each dilution of the drug was injected and the peak was recorded. The graph plotted as the concentration of the drug Vs peak area.

6. System suitability

System suitability is a pharmacopeial requirement and is used to verify, whether reproducibility of the chromatographic system are adequate for analysis to be done. The tests were performed by collecting data from 3 replicate injections of standard solution.

Preparation of standard drug solution Preparation of diluents

The mobile phase itself was selected as diluents for the preparation of standard and sample solutions.

Mix standard solution

Accurately weighed quantity of 25 mg Moxifloxacin and 50.0 mg of Prednisolone was dissolved in diluent and volume was made up to 50 ml mark by same to obtain 1502µg/ml stock solution. Pipette out 5 ml from standard stock solution and diluted it with 50 ml diluent to obtain 100 µg/ml of Moxifloxacin and Prednisolone.

Procedure

Filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20µL standard drug solution was injected which was made in three replicates and the system suitability parameters were recorded.

Acceptance criteria

1. Relative standard deviation of the area of analyte peaks in standard chromatograms should not be more than 2.0%.
2. Theoretical plates of analyte peak in Standard chromatograms should not be less than 2000.
3. Tailing Factor (Asymmetry) of analyte peaks in Standard Chromatograms should beless than 2.0

7. Application of the proposed method to marketed formulation

To estimate the content of drug (0.5%w/v Moxifloxacin and 1.0%w/v Prednisolone asper label claim) present in marketed ophthalmic suspension.

Preparation of diluent

The mobile phase itself was selected as diluents for preparation of standard and sample solutions.

Preparation of Standard drug Solution

Accurately weighed quantity of 25.0 mg Moxifloxacin and 50.0 mg of Prednisolone was dissolved in diluent and volume was made up to 50 ml mark by same to obtain 1502µg/ml stock solution. Pipette out 5 ml each from standard stock solution and diluted it with 50 ml diluent to obtain Moxifloxacin and Prednisolone 100µg/ml stock solution.

Preparation of test solution

Accurately weighed 5ml of Ophthalmic preparation equivalents to 0.5 %w/v of Moxifloxacin and 1%w/v of Prednisolone were taken and dissolved in diluent and a volume of up to 50 ml was prepared. It is subjected to sonication for 10 minutes with intermittent shaking and filtered through a syringe filter. Pipette 5 ml of the above solution into a 50 ml volumetric flask and dilute to the mark with diluent.

Procedure

Equal volumes(20µL) of standard and test solutions were injected separately after stationary phase equilibration. The chromatogram was recorded and the response which isthe peak area of the main peak was measured. The content of Moxifloxacin and Prednisolone was calculated by comparing a sampling peak with that of the standard.

Amount of drug in the ophthalmic suspension was calculated using the following formula-
Where,

%Label claim = (Ew × Wav/Lc × Ws) × 100

Ew = amount estimated in sample weight(mg)

Wav= weight of eye drop(mg)

Ws= Sample weight (mg)

Lc= Labeled claim(mg/ml)

Avg. wt = Average weight of the sample

Further, calculate the amount of moxi and pred present in % of assay using the following formula

$$\% \text{ assay} = \frac{{}^{wt}\text{moxi observed in formulation}}{\frac{wt}{v} - \text{label claimed}} \text{x100}$$

8. METHOD VALIDATION

Validation Approach

Validation of the analytical method shall be done to establish by laboratory studies, that the performance of the method meets the requirement for the intended analytical application.

a. Specificity

Preparation of diluent

The mobile phase itself was selected as diluents for preparation of standard and sample solutions.

Preparation of Standard drug Solution

Accurately weighed quantity of 25.0 mg Moxifloxacin was dissolved in diluent and volume was made up to 50 ml mark by same to obtain 500µg/ml stock solution. Pipette out 5 ml from standard stock solution and diluted it with 50 ml diluent to obtain 100µg/ml stock solution.

Preparation of test solution

Accurately weighed quantities of 25.9 mg of Moxifloxacin were taken and dissolved in diluent and a volume of up to 50 ml of the volumetric flask was prepared. It is subjectedto sonication for 10 minutes with intermittent shaking and filtered through a syringe filter. Pipette 5 ml of the above solution into a 50 ml volumetric flask and dilute to the mark with diluent.

Procedure

Equal volumes (20µL) of blank, standard, and test solutions were injected separately after stationary phase equilibration. The chromatogram was recorded and to check for interference from blank.

Acceptance Criteria

The blank should not show any interference from the peak at the retention time of Moxifloxacin and Prednisolone Standard and Sample Peaks.

b. Linearity and range

The linearity and range of the method were performed from the data obtained, the calibration graph was plotted using the peak area of the standard drug concerning the concentration for establishing the linearity and range of the method. Moxifloxacin and Prednislone was found

to be linear in the concentration range 25.10-75.3µg/ml and 30.06-90.18µg/ml. To asses the linearity of proposed method slope, interecept and correlation coefficient of standard curve were calculated.

c. LOD AND LOQ

Determination of LOD and LOQ is based on the comparison of the SD of the peak area and the slope of the calibration curve of Moxifloxacin and Prednisolone were found to be 23.09µg, 33.93µg, respectively, whereas, Moxifloxacin and Prednisolone were found to be 0.998µg/ml and 0.9964µg/ml.

d. Accuracy

In the present study the accuracy was checked by recovery studies, by addition of standard drug solution to preanalysed sample solution at three different concentration at 80%, 100% and 120% spiked level. It was ascertained based on recovery studies performed by spiked method (i.e. 80-120% of labeled claim).

Preparation of diluent

The mobile phase itself was selected as diluents for preparation of standard and sample solutions.

Preparation of Standard drug Solution

Stock standard solution

Accurately weighed quantity of 25 mg of Moxifloxacin was dissolved in diluent andvolume was made up to 50 ml mark by same to obtain 500µg/ml stock solution.

Working standard solution

Pipette out 5 ml from standard stock solution and diluted it with 50 ml diluent to obtain 100µg/ml solution.

Preparation of Test solution

An accurately weighed quantities of 25 mg of Moxifloxacin and 50.0 mg of Prednisolone was transferred in a 50 ml volumetric flask. Pipette 5 ml of the sample solution into three different 50 ml volumetric flasks and then a known amount of Moxifloxacin and Prednisolone was spiked over the range of 80%, 100%, 120%. The contents of the flask were shaken with mobile phase volume were adjusted up to the mark. The flask content issonicated for 10 min. The solution was filtered through a 0.45 µm- membrane filter.

Table No 4: Dilutions for Accuracy of Moxifloxacin.

Sample Name	Weight of Std Taken in mg	Amount of Sample takenin ml	Amount of accuracy stock solution added in µg/ml	Volume made up to(ml) with Diluent
Accuracy 80%	25	4	40	50
	25	4	40	50
	25	4	40	50
Accuracy 100%	25	5	50	50
	25	5	50	50
	25	5	50	50
Accuracy 120%	25	6	60	50
	25	6	60	50
	25	6	60	50

Table No 5: Dilutions for Accuracy of Prednisolone.

Sample Name	Weight of Std Taken in mg	Amount ofsample taken in ml	Amount of accuracy stock solution added in µg/ml	Volume made up to(ml) with Diluent
Accuracy 80%	50	4	80	50
	50	4	80	50
	50	4	80	50
Accuracy 100%	50	5	100	50
	50	5	100	50
	50	5	100	50
Accuracy 120%	50	6	120	50
	50	6	120	50
	50	6	120	50

Procedure

Equal volume (20µL) spiked solution of each level of 80%, 100%, 120% recovery were injected into three replicate each and then chromatogram was recorded. The % recovery of each level was calculated the standard deviation followed by %RSD of the recovery study was calculated.

Data Evaluation: For each level and each replicate, the following will becalculated:

(i) Amount added in mg (Amount actually added).

(ii) Amount recovered in mg (quantified against standard response with potencycorrection)

(iii) Percent Recovery = Amount recovered/Amount added x 100.

The Mean, Standard deviation and %RSD will be computed for the nine determinationsand reported along with the table.

Acceptance Criteria

The mean recovery for 80% - 120% should be in the range of 98%-102% and RSD shouldnot be more than 2.0%.

e. Precision

The study of Precision was carried out under two different condition

- Interday
- Intraday

Interday study

The samples of Moxifloxacin and Prednisolone were analyzed on different days by proposed method. The percent assay was calculated using same formula as in analysis of ophthalmic preparation

Intraday study

The samples of Moxifloxacin and Prednisolone were analyzed on different times on sameday by proposed method. The percent assay was calculated using same formula as in analysis of ophthalmic preparation.

Preparation of diluent

The mobile phase itself was selected as diluent for the preparation of standard and sample solutions.

Procedure: Injected standard preparation and sample preparations into the HPLC system record the chromatograms and measure peak responses for the Moxifloxacin and Prednisolone peak. Calculate the % assay for Moxifloxacin and Prednisolone for each of the test preparation. Calculate the mean of % assay of the preparation and % RSD for the two observation and record the observation. The precision study is expressed as S.D. or % R.S.D. of series of measurements.

Acceptance criteria

The mean assay percentage of Moxifloxacin and Prednisolone will be calculated &reported along with Standard deviation & Relative standard deviation of the two samples. The % RSD for the two determination shall be NMT 2.

f. Robustness

The robustness of the method was established by introducing small changes in various parameters like wavelength and flow rate. The changes made in wavelength ± 2nm, mobile phase composition and the flow rate was ± 0.1 ml/min, respectively. The robustness of the method was evaluated by calculating the % RSD values.

Experiment Preparation of diluent

The mobile phase itself was selected as diluents for preparation of standard and sample solutions. prepared standard and sample solutions the same as above. The samples along with standard injected in under different chromatographic conditions as shown below:

- Change in flow rate (±0.1ml/min).
- Change in wavelength (±2 nm)
- Change in mobile phase composition

Change in Flow Rate: (± 0.1ml/min)

The normal experimental condition for the flow rate is 1.0 ml/minute. Change in flow ratewas studied for actual ±0.1ml/minute.

Change in wavelength: (± 2nm)

Robustness studies at wavelengths 252 nm and 256 nm separately.

The chromatogram were recorded and the response i.e peak area, retention time of major peaks were measured.

Change in organic composition of mobile phase ± 10% (Acetonitrile & water): Prepared the solution ACN:Water:TFA(40:60:0.2v/v) and ACN: Water: TFA (50:50:0.2v/v) injected into the HPLC system at –10% and + 10% Acetonitrile & water compared with the test method buffer concentration.

Procedure

Injected standard solution into the HPLC system in normal conditions and followed by the robust conditions. Measure the peak response for the major peaks. Check the system suitability and record the results in the table.

Observation: The allowable variation in acetonitrile & water composition of method isfrom 90% to 110%.

Acceptance criteria

Overall RSD should not be more than 2.0%.

9. Stability Studies of moxi and pred

To establish whether the analytical method for the assay was stability-indicating, the pure active pharmaceutical ingredient (API) of Moxifloxacin and Prednisolone were subjected to various stress conditions to conduct forced degradation studies. Stress studies were carried out under the conditions of acid/base hydrolysis, oxidation, and dry heat, as mentioned in ICH Q1A (R2). Light degradation of drug substances and drug products wasperformed in the solid-state.

Forced degradation studies

Experimental Approach

Forced degradation or accelerated degradation is a process whereby the natural degradation rate of a product or material is increased by the application of additional stress. Forced degradation studies are used to identify reactions that may occur to degrade a processed product. Forced degradation is usually conducted before final formulation and it is done by applying external stress conditions and rapidly checked for material stabilities.

The %degradation was evaluated by the following formula:

$$\% \text{ Degradation} = \frac{\text{Area of Unstressed} - \text{Area of stressed}}{\text{Area of Unstressed}} \times 100$$

Preparation of the solution

A. Preparation of the mobile phase

Prepare a homogeneous mixture of 450 ml of HPLC grade Acetonitrile, 550 ml of HPLC grade water, and 2 ml of AR grade TFA in 1000 ml volumetric flask, mixed. Shake well and sonicate for 5 min filter this mobile phase through a 0.45 µm membrane filter, then degassed after filter again sonicate with digital ultrasound for 10 minutes.

B. Preparation of diluent

The mobile phase itself was selected as diluents for the preparation of standard.

Forced degradation studies of standard drug solutions

C. Preparation of Stock Moxifloxacin and Prednisolone solution

Accurately weighed a quantity of 25 mg of Moxifloxacin and 50 mg of Prednisolone was

dissolved in diluent (mobile phase) and volume was made up to 50 ml mark with diluents, from this 5ml pipette out and diluted to 50 ml and filter it with a 0.45 µm membrane filter then sonicate it for 10 min. to obtain a 1502µg/ml stock solution.

Acid degradationProcedure

Subjected the test preparation to acid stress degradation by treating with, 5 ml of 0.1 N HCl solution and refluxed on a heating mantle at 60^{o}C for about 30 minutes. It was produced by acidic solution. After cooling then add drop by drop 0.1 N NaOH solution for frequently shaking to check to neutralize the above solution and finally volume made up to 50 ml to the mark with diluent and filtered it throgh 0.45 µm membrane filter beforeanalysis.

Base Degradation

Procedure

Subjected the test preparation to alkali stress degradation by treating sample with, 10 mlof 0.1 N NaOH solution and refluxed on a heating mantle at 60^{o}C for about 30 minutes. It was produced by a basic solution. After cooling then add drop by drop 0.1 N HCL solution for frequently shaking to check to neutralize the above solution and volumemade up to 50 ml to the mark with diluent and filtered it with 0.45 µm membrane filter before analysis.

Peroxide DegradationProcedure

Subjected the test preparation to peroxide stress degradation by treating the sample with, 10 ml of 0.3% H_2O_2 solution and refluxed on the heating mantle at 60^{o}C for about 12 hrs. After cooling, volume made up op to 50 ml to the mark with diluent and filtered it with a 0.45 µm membrane filter before analysis.

Thermal degradationProcedure

The pure drug was heated at the oven which is maintained at 105^{o}C for about 1 hr and cool at room temperature. Accurately weighed quantity of the above drug to 25 mg Moxifloxacin & 50 mg Prednisolone was dissolved in diluent (mobile phase) and volume was made up to 50 ml mark (stock solution).

Pipette out 5 ml from a standard stock solution of Moxifloxacin & Prednisolone were further diluted it with 50 ml diluent and filter it with a 0.45 µm membrane filter then sonicate it for 10 min. to obtain Moxifloxacin std and injected the solution to the chromatograph.

Photolytic degradation Procedure

Pure drug of Moxifloxacin and Prednisolone was placed in petri plate and exposed to UV light for 254nm in a UV chamber for 1 hr. accurately weighed quantity of 25 mg Moxifloxacin & 50 mg Prednisolone was dissolved in diluent (mobile phase) and volume was made up to 50 ml mark (stock solution).

Pipette out 5 ml from a standard stock solution of Moxifloxacin & Prednisolone were further diluted it with 50 ml diluent to obtain 100µg/ml stock solution.

Acceptance criteria to all condition

Result for % degradation and % area after degradation were shown in Table no 33 & 34.

RESULT AND DISCUSSION

Method development for the simultaneous estimation of Moxifloxacin and Prednisolone by using HPLC.

The HPLC method was developed for the simultaneous estimation of Moxifloxacin and Prednisolone. To obtain peak of good characters i.e. proper retention time number of trials performed has been shown in below. The methodwas developed using mobile phase Acetonitrile: Water:TFA in the ratio of (45:55:0.2 v/v), and detection was carried out at 254nm.

Table No. 6: Trials for method development of simultaneous estimation of Moxifloxacin and Prednisolone on HPLC.

Sr. No.	Chromatographic condition	PH	Result
1	Mobile Phase: 70% Methanol+ 30% Water+0.2% Trifluoroacetic acid, λmax: 254 nm, Flow rate:1mL/min	Not Modified	peaks with poorsymmetry
2	Mobile Phase: 0.1M Sod. Acetate: Methanol(20:80), λmax: 254 nm, Flow rate: 1 mL/min	Modified pHof buffer to 4.0 with Acetic Acid	Peaks with poor symmetry, Tailing factorfound out of acceptance criteria
3	Mobile Phase :Methanol:0.1%Aceticacid (80:20), λmax: 254nm, Flow rate: 1 mL/min	Not Modified	Good peak symmetry, but more resolution would be favourable
	Mobile Phase :ACN: Water:TFA(50:50:0.2), λmax: 254nm, Flow rate: 1 mL/min	Not Modified	Good shape peaks having
4			acceptable symmetry and resolution
5	Mobile Phase:ACN:Water:TFA(45:55:0.2), λ_{max}:254nm, Flow rate: 1mL/min	Not Modified	Good sharp resolved peaks having acceptable symmetry and resolution

Trial 1

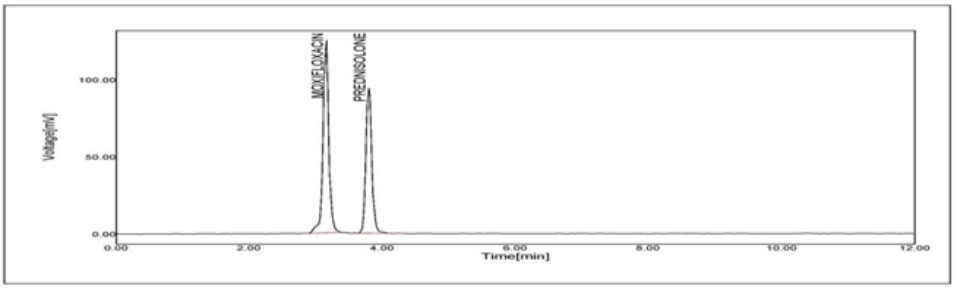

Fig No. 2: Chromatogram of Moxifloxacin and Prednisolone.

Observation: Peak splitting was observed at front of mofloxacin peak.

Conclusion: Change the proposition of mobile phase.

Trial 2

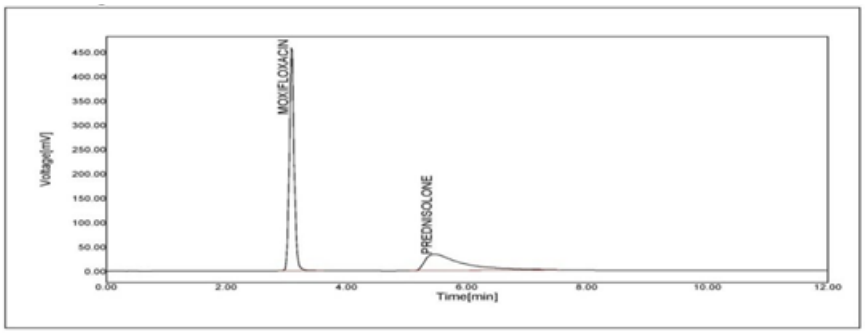

Fig No. 3: Chromatogram of Moxifloxacin and Prednisolone obtained by using 0.1M sod. Acetate: Methanol (20:80 v/v)

Observation: Tailing factor was not observed within acceptance criteria.

Conclusion: Change the proposition of mobile phase.

Trial 3

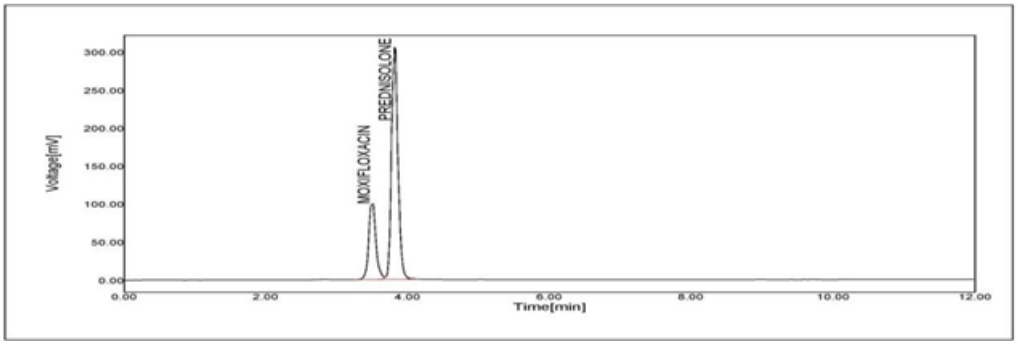

Fig. No. 4: Chromatogram of Moxifloxacin and Prednisolone obtained by using Methanol: 0.1% Acetic acid (80:20 v/v)

Observation: Good peak symmetry, but more resolution would be favourable

Conclusion: Change the proposition of mobile phase.

Trial 4

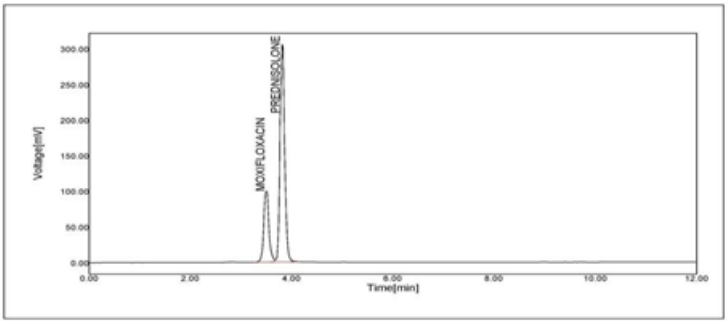

Fig. No. 5: Chromatogram of Moxifloxacin and Prednisolone obtained by using ACN: Water: TFA(50:50:0.2),v/v)

Observation: Good shape peaks having acceptable symmetry and resolution.

Conclusion: Method may need some optimization.

Trial 5 (Final and optimized trial)

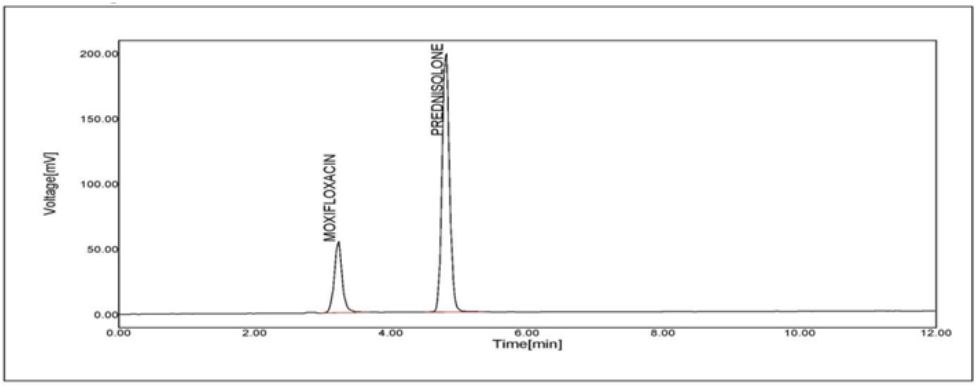

Fig. No. 6: Chromatogram of Moxifloxacin and Prednisolone obtained by using ACN: Water: TFA (45:55:0.2 v/v)

Observation: Peak shake is good, TP and TF observed well within accepatance criteria.

Conclusion: Hence the method seems suitable for estimation of Moxifloxacin and Prednisolone, so needs to do method validation to ensure that the developed method is suitable for intened use.

1. Preparation of Standard calibration curve

The various concentrations ranging from 25.10 to 75.3 µg/ml of Moxifloxacin & 30.06 to 90.18µg/ml of Prednisolone were injected and peaks were recorded. The graph was plotted as the concentration of drug verses peak area is depicted in fig.8 & 9.

Table No. 7: Observation of Standard Calibration curves for Moxi & Pred.

Conc.(µg/ml) Moxi	Conc(µg/ml) Pred	Area Moxi	Area Pred	RT(Min) Moxi	Retention Time(Min) Pred
25.10	30.06	621.5026	1151.55	3.22	4.68
37.65	45.09	990.6368	1826.52	4.68	4.68
50.20	60.12	1211.8732	2182.35	3.20	4.68
62.75	75.15	1532.1552	2773.93	3.20	4.70
75.3	90.18	1800.0417	3227.76	4.70	4.70
Mean		1231.242	2232.42	3.8	4.688
±SD		459.3827	809.239	0.812527	0.010954

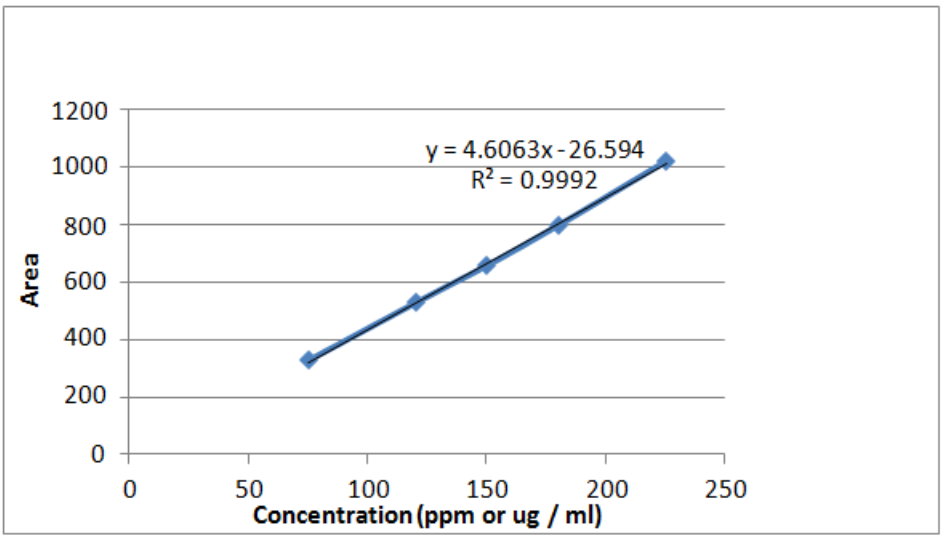

Fig. No. 7: Standard calibration curve for Moxifloxacin Hydrochloride.

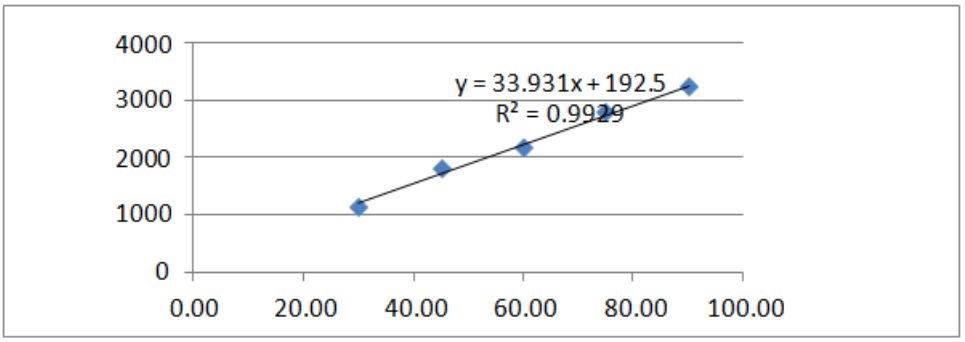

Fig. No. 8: Standard calibration curve for Prednisolone.

Acceptance Criteria

The correlation coefficient should not be less than 0.999.

Observation

The correlation coefficient for Moxifloxacin and Prednisolone is 0.999. Therefore, the HPLC Method for the Assay of Moxifloxacin and Prednisolone is linear.

2. System Suitability

The system suitability parameter established for the present development method includes the number of theoretical plates, tailing factor.

Table No 8: Result for system suitability.

Sr.No	Peak Area		Retention Time		Tailing Factor		Theorotical Plate	
	Moxi	Pred	Moxi	Pred	Moxi	Pred	Moxi	Pred
1	1172.61	2103.0293	3.20	4.77	1.14	0.98	6001	10888
2	1159.87	2135.6909	3.20	4.75	1.04	1.04	4856	12546
3	1150.71	2082.6541	3.20	4.67	1.13	1.08	4335	10975
Mean	1161.06	2107.1248	3.20	4.73	1.1033	1.03333	5064	11469.67
±SD	10.99866	26.7545	0.000	0.0529	0.0550	0.05033	852.25	933.14
% RSD	0.95	1.27	0.00	1.12	4.9917	4.87086	16.829	8.1357

Acceptance criteria

1. The relative standard deviation of the area of analyte peaks in standard chromatograms should not be more than 2.0%.
2. Theoretical plates of the analyte peak in Standard chromatograms should not beless than 2000.
3. Tailing Factor (Asymmetry) of analyte peaks in Standard Chromatograms shouldbe less than 2.0.

Observation

The data demonstrates that the system suitability is within the acceptance criteria, thus the system is suitable.

3. Analysis of marketed formulation

Thus the results obtained for such method are given as follow

Table No. 9: Summary of the Moxifloxacin marketed formulation.

Weight of standard(mg)	Peak areaof standard	Weightof test(ml)	Peak area of Sample	Amount pred observed in % w/v	Amount claim in %w/v	% Assay
25.0	1172.6195	5.0	1138.5800	0.4903	0.5	98.06
	1159.8712	5.0	1159.9272	0.4995	0.5	99.90
	1150.7175	**Mean**				98.48
Mean	1161.0694					
SD	11.0001					
% RSD	0.95					

Table No. 10: Summary of Prednisolone marketed formulation.

Weight of standard(mg)	Peak areaof standard	Weightof test(ml)	Peak area of Sample	Amount pred observed in % w/v	Amount claim in %w/v	% Assay
50.0	2103.0293	5.0	2112.1863	1.0024	1.0	100.24
	2135.6909	5.0	2131.9578	1.0118	1.0	101.18
	2082.6541	**Mean**				100.71
Mean	2107.1248					
SD	26.7545					
% RSD	1.27					

Acceptance criteria

The % assay as between 98-102%.

Observation

Experimental results of the amount of Moxifloxacin and Prednisolone in ophthalmic formulation expressed as a percentage of assay were in good agreement with the label claims. The mean % assay was 98.48 for Moxifloxacin and 100.71 for Prednisolone with % RSD values was NMT 2.0% indicates the developed method was successfully applied for the analysis of marketed formulation. All the result found was is good agreement withthe label claimed of the marketed formulation.

4. Validation

Specificity

Blank interference: A study to establish the interference of blank was conducted.

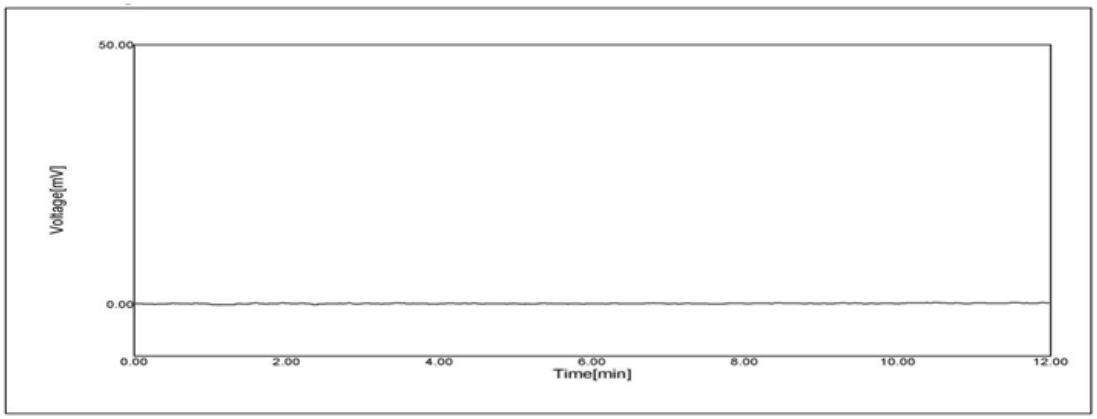

Fig No8: Blank Chromatogram: Diluent (Mobile phase)

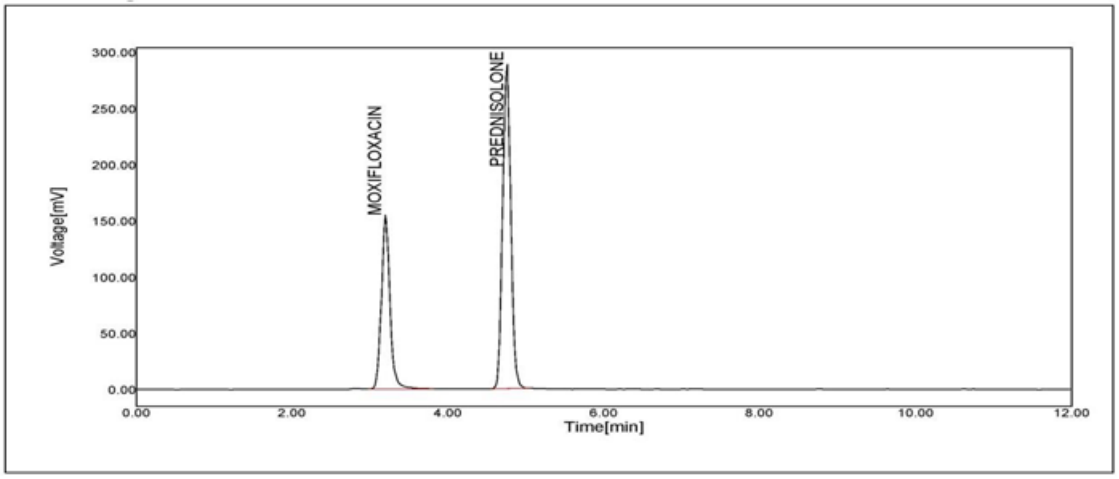

Fig No 9: Specificity Chromatogram (Moxifloxacin and Prednisolone Standard)

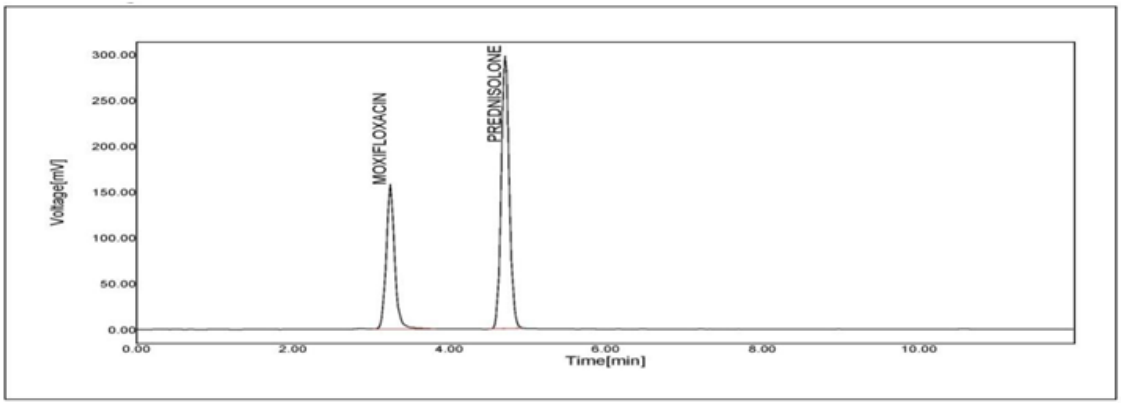

Fig No 10: Specificity Chromatogram (Moxifloxacin and Prednisolone Test)

Table No. 11: Result of specificity.

Sr. No.	Area	RT (Min)	TF	TP
Blank Injection	-	-	-	-
Standard Injection	1172.6195	3.20	1.14	6001
Test Injection	1171.8270	3.25	1.07	4804

Mean	1172.223	3.225	1.105	5402.5
±SD	0.560382	0.035355	0.049497	846.4068

Acceptance criteria

The blank solution should not show any interference at the retention time of Moxifloxacin and prednisolone Standard and Sample Peaks.

Observation

Chromatogram of blank solution showed no peaks at the retention time of Moxifloxacin and Prednisolone standard and test peaks. This indicating that the diluent used in sample preparation do not interfere in the estimation of Moxifloxacin and Prednisolone in formualation. It means that my method was specific to the HPLC system.

Linearity and Range

Linearity regression data showed a good linear relationship between concentrations of peak area over a concentration range of 25.10-75.3 µg/ml for Moxifloxacin and 30.06 to 90.18 µg/ml Prednisolone. To assess the linearity of the proposed method slope, intercept and correlation coefficient of the standard curve were calculated and were given in fig 8 & 9.

Chromatogram

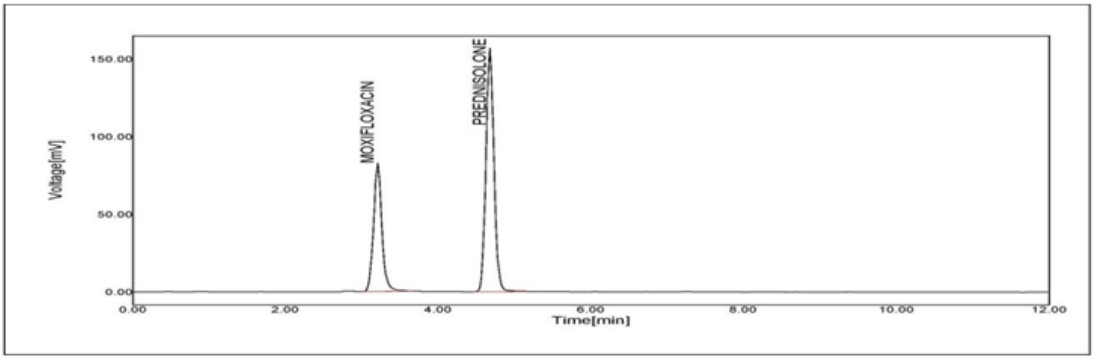

Fig No. 9: Linearity chromatogram of Moxifloxacin and Prednisolone in 25.10 µg/ml.

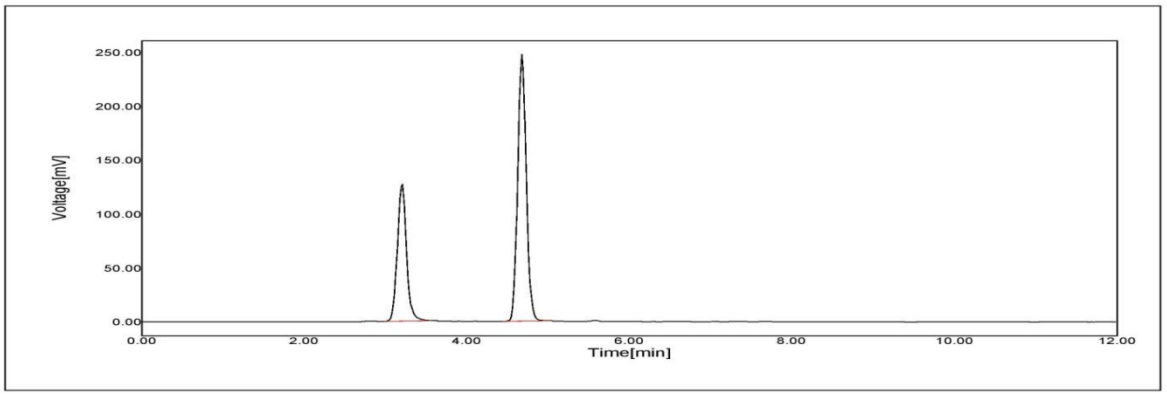

Fig No. 10: Linearity chromatogram of Moxifloxacin and Prednisolone in 37.65 µg/ml.

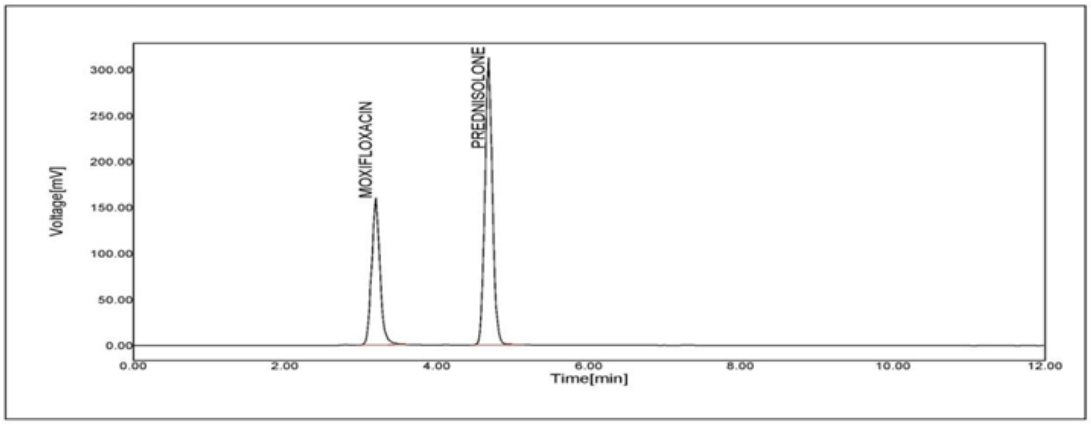

Fig No. 11: Linearity chromatogram of Moxifloxacin and Prednisolone in 50.20 µg/ml.

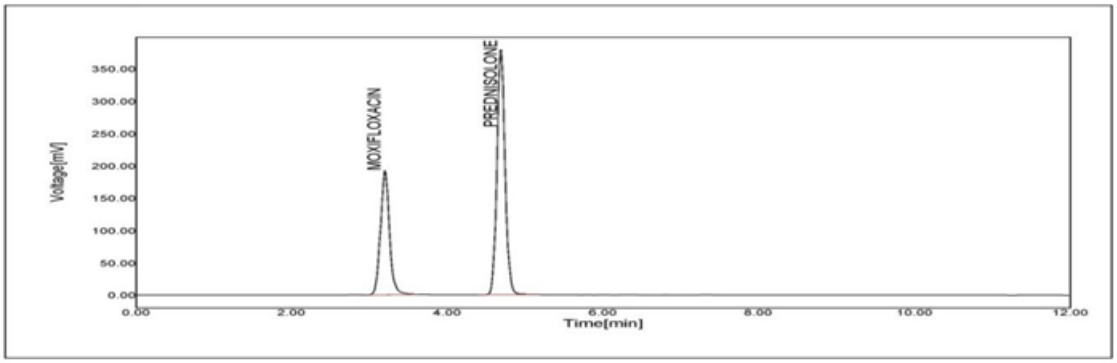

Fig No. 12: Linearity chromatogram of Moxifloxacin and Prednisolone in 62.75 µg/ml.

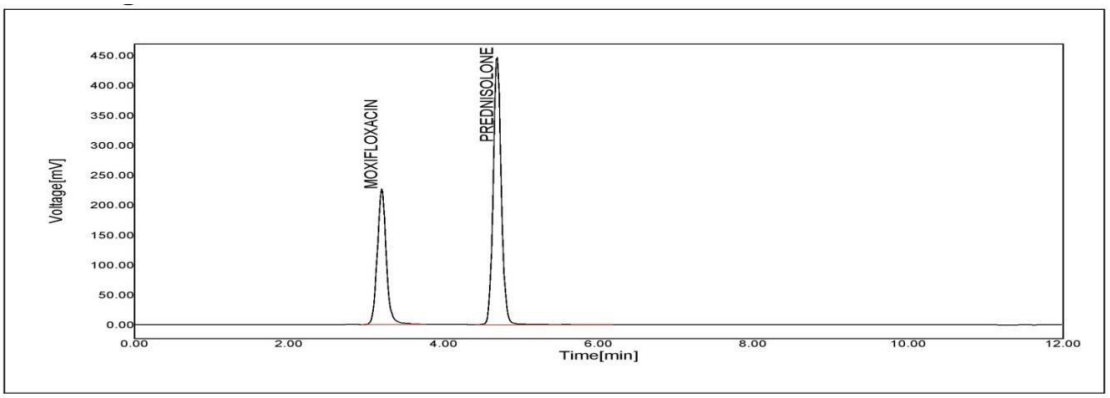

Fig No. 13: Linearity chromatogram of Moxifloxacin and Prednisolone in 75.3 µg/ml.

Table No. 12: Linearity for Moxifloxacin.

Concentration (µg/ml)	Area	RT(min)	Statistical analysis	
25.10	621.5026	3.22	egression Equation	4.606x - 26.59
37.65	990.6368	3.22		
50.20	1211.8732	3.20	Slope	4.066
62.75	1532.1552	3.20	Intercept	26.59
75.3	1800.0417	3.20	Correlation Coefficient	0.999
Mean	1231.242	3.208		
SD	459.3827	0.010954		

Table No. 13: Linearity for Prednisolone.

Concentration (µg/ml)	Area	RT(min)	Statistical analysis	
30.06	1151.5575	4.68	Regression Equation	33.93x+192.5
45.09	1826.5272	4.68		
60.12	2182.3501	4.68	Slope	33.93
75.15	2773.9309	4.70	Intercept	192.5
90.18	3227.7622	4.70	Correlation Coefficient	0.992
Mean	2232.426	4.688		
SD	809.2373	0.010954		

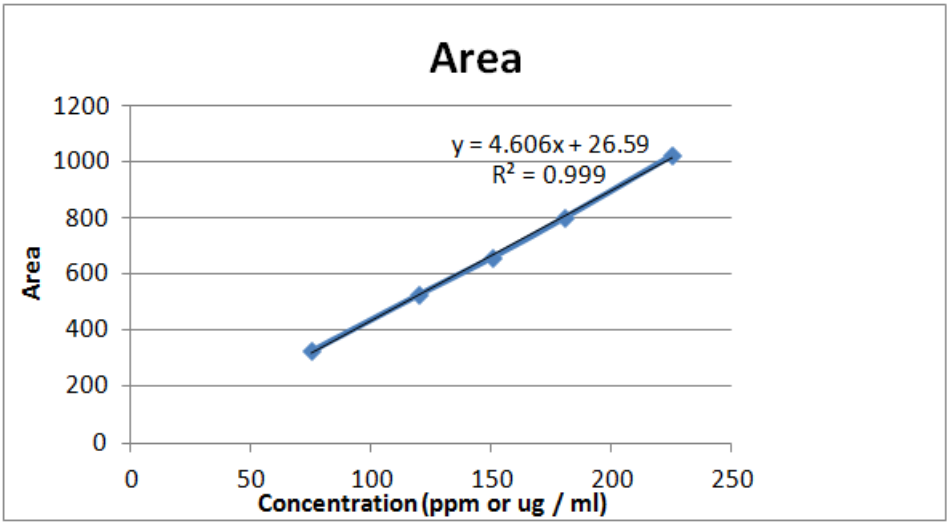

Fig No.14: Calibration graph of moxifloxacin Hydrochloride.

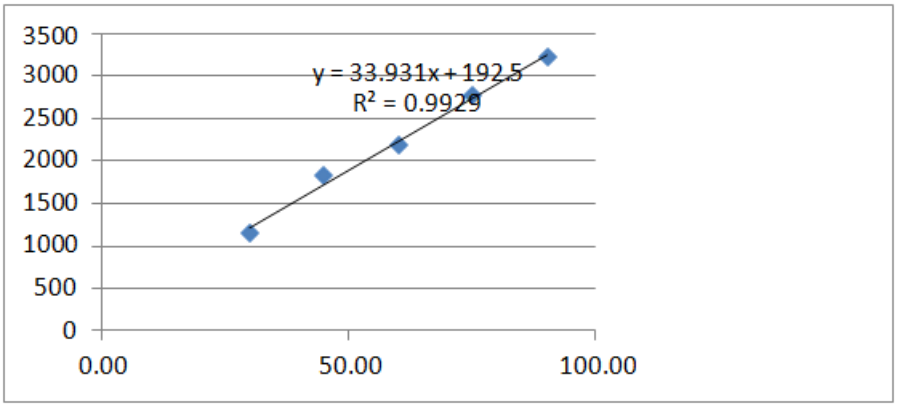

Fig.No.15: Calibration graph of PrednisoloneAcceptance.

Criteria

The correlation coefficient should not be less than 0.999.

Observation

The detector response was linear with a correlation coefficient of 0.999. A typical calibration curves has the regression equation of y = 4.606x +26.59 & y = 33.93x + 192.5. The result shows that an excellent correlation exists between concentration and mean peak areas within the concentration range. R-value (≥ 0.999) confirmed the good linearity of the method.

Table No. 14: LOD and LOQ of developed method for determination of moxi and pred.

Sr.no	Drugs	LOD(µg/ml)	LOQ(µg/ml)
1	Moxifloxacin HCL	0.13	0.38
2	Prednisolone	0.27	0.82

Accuracy

The proposed method when used for estimation of moxifloxacin and prednisolone from pharmaceutical dosage form after spiking with the additional drug, afforded recovery of 80-120%.

Table No. 15: Result of Accuracy study (Assay) of Moxifloxacin.

Weight of Standard (mg)	Peak areaof Standard	Weight of Test(mg)	Peak areaof Test	Amount observed in mg	% Assay
25	1172.6195	25.2	1171.8270	24.65	100.13
	1159.8712	25.1	1166.7961	24.65	100.09
	1150.7175	-	**Mean**	24.65	100.11
Mean	1161.0694				
SD	11.0001				
%RSD	0.95				

Table No. 16: Result of Accuracy study (Assay) of Prednisolone.

Weight of Standard (mg)	Peak areaof Standard	Weight of Test(mg)	Peak areaof Test	Amount observed in mg	% Assay
50.0	2103.0293	50.9	2138.0020	49.31	99.67
	2135.6909	50.0	2085.1021	49.31	98.95
	2082.6541	-	**Mean**	49.31	99.31
Mean	2107.1248				
SD	26.7545				
%RSD	1.27				

Table No. 17: Result of Recovery Study of Moxifloxacin.

Sample No.	Peak areaof the test	Amount of drug added in µg/ml w.r.t test	Amount found in µg/ml w.r.t. test	Amount recovered in µg/ml	% Recovery
Accuracy80%	2170.1267	40.0000	90.2805	40.2805	100.70
	2157.7153	40.0000	89.7641	39.7641	99.41
	2155.9728	40.0000	89.6916	39.6916	99.23
Accuracy100%	2383.5549	50.0000	99.1594	49.1594	98.32
	2396.5584	50.0000	99.7003	49.7003	99.40
	2409.6217	50.0000	100.2438	50.2438	100.49

Accuracy120%	2651.074	60.0000	110.2886	60.2886	100.48
	2628.6524	60.0000	109.3558	59.3558	98.93
	2625.5417	60.0000	109.2264	59.2264	98.71
				Mean	1201.8807
				SD	18.0263404
				%RSD	1.50

Table No 18: Result of recovery study of PrednisoloneStatistical validation data for Accuracy.

Sample No.	Peak area of the test	Amount of drug added in µg/ml w.r.t Test	Amount found in µg/ml w.r.t. test	Amount recoveredin µg/ml	% Recovery
Accuracy80%	3951.7256	80.0000	179.8381	79.8381	99.80
	3968.5595	80.0000	180.6042	80.6042	100.76
	3942.0593	80.0000	179.3982	79.3982	99.25
Accuracy100%	4391.2451	100.0000	199.8401	99.8401	99.84
	4362.5148	100.0000	198.5326	98.5326	98.53
	4424.8261	100.0000	201.3683	101.3683	101.37
Accuracy120%	4823.1641	120.0000	219.4961	119.4961	99.58
	4852.7956	120.0000	220.8446	120.8446	100.70
	4817.0084	120.0000	219.2160	119.2160	99.35
				Mean	2197.3799
				SD	12.8559
				%RSD	0.59

Acceptance criteria

Mean recovery should be in the range of 98.0% to 102.0%. RSD should not be more than 2.0%.

Observation

The mean % recovery was found in the range of 99.90 ± 1.20 and that of the assay was 98.06%. The result of accuracy revealed that the method was accurate.

Precision

The precision study was performed using an interday and intraday precision method. The proposed method was determined by analyzing the Moxifloxacin and Prednisolone solution at different time intervals and on different days.

Table No. 19: Result of Interday study for Moxifloxacin HCL.

Weight of Standard (mg)	Standard peak area	Day	Weightof Test(mg)	Sample peak area	Moxifloxacin observed in mg	% Assay
25	1177.5211	1	24.9	1145.2997	24.65	99.67
	1161.7196	2	25.1	1169.8681	24.65	98.63
	1158.4543	-	-	-	-	-
Mean	1165.8983	**Overall mean**				99.30
SD	10.1972	**Overall SD**				0.61
%RSD	0.87	**Overall %RSD**				0.62

Table No. 20: Result of interday precision study for Prednisolones.

Weight of Standard (mg)	Standard peak area	Day	Weightof Test (mg)	Sample peak area	Prednisolone observed in mg	% Assay
51.0	2155.8596	1	50.0	2064.8809	50.30	98.87
	2115.7839	2	50.1	2086.4158	50.30	99.70
	2119.4186	-	-	-	-	-
Mean	2130.3540	**Overall mean**				99.30
SD	22.1631	**Overall SD**				0.4494
%RSD	1.04	**Overall %RSD**				0.45

Table No. 21: Result of Intraday precision study for Moxifloxacin.

Weight of Standard (mg)	Standard Peak area	Analysis	Weight of Test (mg)	Sample peak area	Moxifloxacin observed in mg	% Assay
25.0	1162.3787	1	25.3	1188.1864	24.65	98.05
	1181.4428	2	25.1	1166.7961	24.65	99.67
	1169.0097	3	25.2	1152.6123	24.65	100.27
Mean	1170.9437	**Over all mean**				99.53
SD	9.6781	**Over all SD**				0.57
%RSD	0.83	**Over all % RSD**				0.57

Table No. 22: Result of intraday precision study for Prednisolone.

Weight of Standard (mg)	Standard Peak area	Analysis	Weight of Test (mg)	Sample peak area	Prednisolone Observed in mg	% Assay
49.9	2104.5923	1	51.1	2178.1748	49.21	99.96
	2149.5215	2	49.8	2101.9708	49.21	98.98
	2129.5214	-	-	-	-	-
Mean	2127.8784	**Over all mean**				99.39
SD	22.5096	**Over all SD**				0.5047
%RSD	1.06	**Over all % RSD**				0.51

Acceptance criteria

The % RSD should not be more than 2.0%.

Observation

Standard peak area found with % RSD NMT than 2% which was in agreement with system suitability. The precision was expressed in terms of standard deviation and % RSD. The RSD of assay results obtained in interday and intraday precision studies was within 0.53% and 0.54% respectively this confirms that the method was precise.

Robustness studies

The robustness of the method was established by introducing small changes in various parameters like flow rate and wavelength. The robustness of the method was evaluated by calculating the % RSD values.

Table No 23: Results of robustness studies change in flow rate 0.9ml/min (Moxifloxacin).

Change in Flow (0.9 ml/min)					
Weight of Standard (mg)	**Standard peak area**	**Weight of Test (mg)**	**Test peak area**	**Moxifloxacin observed in mg**	**% Assay**
25.3	1373.6577	24.8	1304.5201	24.95	98.63
	1327.6258	25.1	1338.0957	24.95	99.96
	1346.7859	**Over all mean Over all SD Over all %RSD**			99.30
Mean	1349.3565				0.62
SD	23.1234				0.62
%RSD	1.71				

Table No. 24: Result of robustness studies change in flow rate 0.9ml/min(Pred).

Change in Flow (0.9 ml/min)					
Weight of Standard (mg)	**Standard peak area**	**Weight of Test (mg)**	**Test peak area**	**Prednisolone observed in mg**	**% Assay**
50.4	2422.9119	50.5	2404.8315	49.70	100.23
	2378.4428	50.2	2373.4128	49.70	99.51
	2382.3122	**Over all mean Over all SD Over all %RSD**			99.59
Mean	2394.5556				0.5266
SD	24.6333				0.53
%RSD	1.03				

Table No. 25: Results of robustness studies change in flow for moxifloxacin (1.1ml/min).

Change in flow (1.1 ml/min)					
Weight of Standard (mg)	**Standard peak area**	**Weight of Test (mg)**	**Test peak area**	**Moxifloxacin observed in mg**	**% Assay**
25.1	1090.1599	24.9	1066.3313	24.75	98.39
	1112.5148	25.1	1091.4128	24.75	99.90
	1074.8909	**Over all mean Over all SD Over all %RSD**			99.23
Mean	1092.5219				0.69
SD	18.9228				0.70
%RSD	1.73				

Table No. 26: Result of robustness studies change in flow for Prednisolone (1.1ml/min).

Change in flow (1.1 ml/min)					
Weight of Standard (mg)	**Standard peak area**	**Weight of Test (mg)**	**Test peak area**	**Prednisolone observed in mg**	**% Assay**
50.1	1996.4718	49.9	1971.6971	49.41	99.26
	1976.5417	50.1	2003.0958	49.41	100.43
	2010.3099	**Over all mean Over all SD Over all %RSD**			99.58
Mean	1994.4411				0.6427
SD	16.9754				0.65
%RSD	0.85				

Table No. 27: Results of robustness studies change in wavelength at 252 nm(Moxi).

Change wavelength (252 nm)					
Weight of Standard (mg)	**Standard Peak area**	**Weight of Test (mg)**	**Test peak area**	**Moxifloxacin observed in mg**	**% Assay**
25.1	1162.1759	25.0	1142.5553	24.75	98.59
	1182.2784	25.2	1165.4559	24.75	99.77
	1146.1018	**Over all mean Over all SD Over all %RSD**			99.25
Mean	1163.5187				0.57
SD	18.1256				0.57
%RSD	1.56				

Table No. 28: Results of robustness studies change in wavelength at 252 nm(Pred)

Change wavelength (252 nm)					
Weight of Standard (mg)	**Standard Peak area**	**Weight of Test (mg)**	**Test peak area**	**Prednisolone observed in mg**	**% Assay**
50.0	2137.4053	50.3	2154.4253	49.31	99.32
	2163.0049	50.0	2128.8802	49.31	98.73
	2168.6128	**Over all mean Over all SD Over all %RSD**			99.17

Mean	2156.3410		0.4148
SD	16.6368		0.42
%RSD	0.77		

Table No. 29: Results of robustness studies change in wavelength at 256 nm (Moxifloxacin)

Change wavelength (256 nm)					
Weight of Standard (mg)	**StandardPeak area**	**Weight of Test (mg)**	**Test peakarea**	**Moxifloxacin observed in mg**	**% Assay**
25.0	1185.6793	25.0	1174.4045	24.65	99.67
	1199.8126	25.1	1188.9037	24.65	98.95
	1159.9975	**Over all mean Over all SD Over all %RSD**			99.55
Mean	1181.8298				0.52
SD	20.1848				0.52
%RSD	1.71				

Table No. 30: Results of robustness studies change in wavelength at 256 nm (Prednisolone)

Change wavelength (256 nm)					
Weight of Standard (mg)	**StandardPeak area**	**Weight of Test (mg)**	**Test peakarea**	**Prednisolone observed in mg**	**% Assay**
50.1	2042.3147	50.1	2039.212	49.41	99.77
	2022.8561	50.3	2057.1947	49.41	100.25
	2066.4219	**Over all mean Over all SD Over all %RSD**			99.66
Mean	2043.8642				0.5377
SD	21.8242				0.54
%RSD	1.07				

Table No. 31: Result of robustness studies change in organic composition of mobilephase.

Sr. No.	**System Suitability Parameter**		**Observations**			**Limits**
			As Such	**- 10%**	**+10%**	
1	The % RSD ofpeak area response for three replicate injections	Moxifloxacin	0.95	0.56	0.72	NMT2.0
		Prednisolone	1.27	0.75	0.76	
2	Theoroticalplates	Moxifloxacin	6001	6785	6735	NLT2000
		Prednisolone	10888	10569	13080	
3	Tailing factor	Moxifloxacin	1.14	1.07	1.11	NMT2.0
		Prednisolone	0.98	1.07	1.02	
4	RetentionTime	Moxifloxacin	3.20	3.65	2.82	
		Prednisolone	4.77	5.28	4.10	

Acceptance criteria

System suitability should meet as per the test method at each variable condition. Overall RSD should not be more than 2.0.

Observation

The result of assay of two test preparation was not affected by varying the condition. The fully agree with the result obtained under the original condition. The % RSD for (Retention time, peak area, and % Assay) was not more than 2% for (Moxifloxacin and Prednisolone) which was in agreement with system suitability. The above results indicate that the test method is Robust for all variable conditions outlined in the above tables.

The observation and result obtained for each of parameter like system suitability, specificity, linearity and range, accuracy, robustness etc. lies well within the acceptance criteria. So the proposed method was simple, specific, linear, accurate precise, robust and used for estimation of drug.

5. Forced degradation studies

5.1 Acid degradation

The moxifloxacin and prednisolone was subjected to forced degradation by acid hydrolysis using 10ml of 0.1N HCl maintained at 60^{o}C for 30min. The sample after stress was neutralized with 10 ml of 0.1N NaOH and diluted with diluent and filter through a 0.45-µm membrane filter. From this, 20µl was injected into the system. From the peak area found in the chromatograph, the % degradation was calculated.

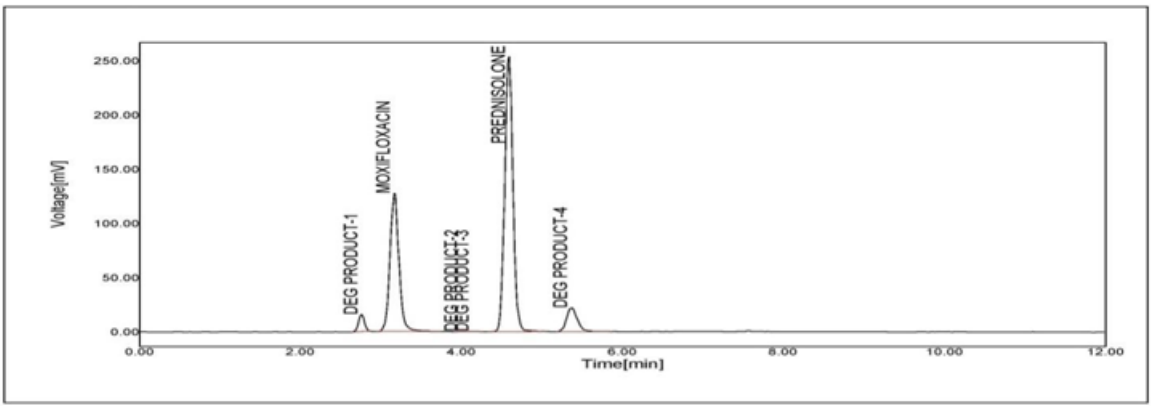

Fig No 16: Chromatogram for Acidic Degradation Study.

Observation

In this study drug show, six peak two was of the standard drug, and another four was of the degraded product, from the peak area of standard drug and degraded product the drug was degraded up to 90.52% and actual 9.48% degradation was, from this condition it was found that the drug was degraded in acidic condition.

5.2 Basic degradation

The moxi and pred was subjected to forced degradation by base hydrolysis using 0.1NaOH maintained at 60°C for 30min. The sample after the stress was neutralized with 0.1 N HCL and diluted with diluent and filter through a 0.45-µm membrane filter. From this, 20lµl was injected into the system. From the peak area found in the chromatograph, the % degradation was calculated.

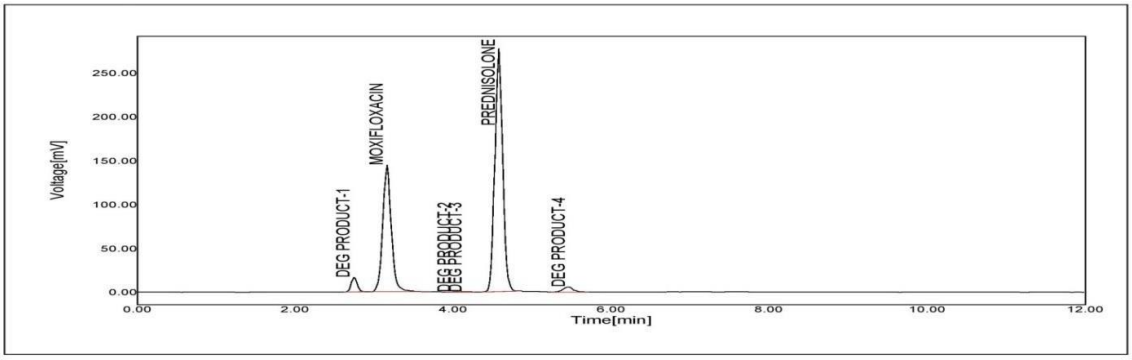

Fig No. 17: Chromatogram for Basic Degradation Study.

Observation

In this study drug show, six peak two was of the standard drug, and another four was of the degraded sample, from the peak area of standard drug and degraded product the drug was degraded up to 95.49% and actual 4.51% degradation was, from this condition it was found that the drug was degraded in basic condition.

Peroxide degradation

To study the effect of oxidizing conditions, 10ml of the stock solution was added to 10 ml of 0.3 %H_2O_2 and refluxed at 60°C for about 12 hrs. From this, 20µl was injected into the system. From the peak area found in the chromatograph, the % degradation was calculated.

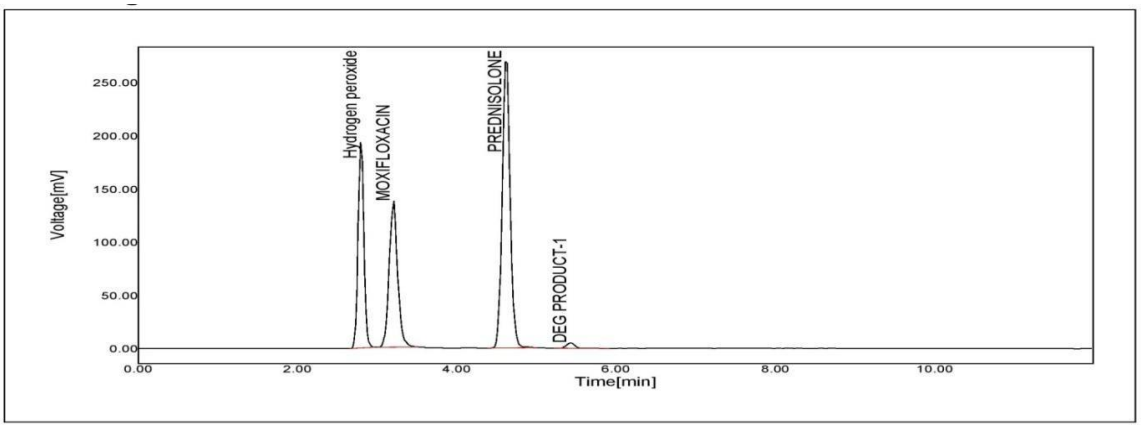

Fig No. 18: Chromatogram of Peroxide degradation study.

Observation

In this study drug show, four peak two was of the standard drugs, and another one was of the degraded product, and one of unreacted hydrogen peroxide, from the peak area of standard drug and degraded product the drug was degraded up to 99.01% and actual 0.99% degradation was, from this condition it was cleared that the drug was not degraded in peroxide condition.

Thermal degradation

To study the effect of thermal degradation, the standard drug in solid form was placed in the oven at 105^{o}C. A sample was withdrawn after 1hr, weighed, and dissolved in the mobile phase to get a solution of of moxi and pred and then injected. From the peak area found in the chromatogram, the % degradation was calculated.

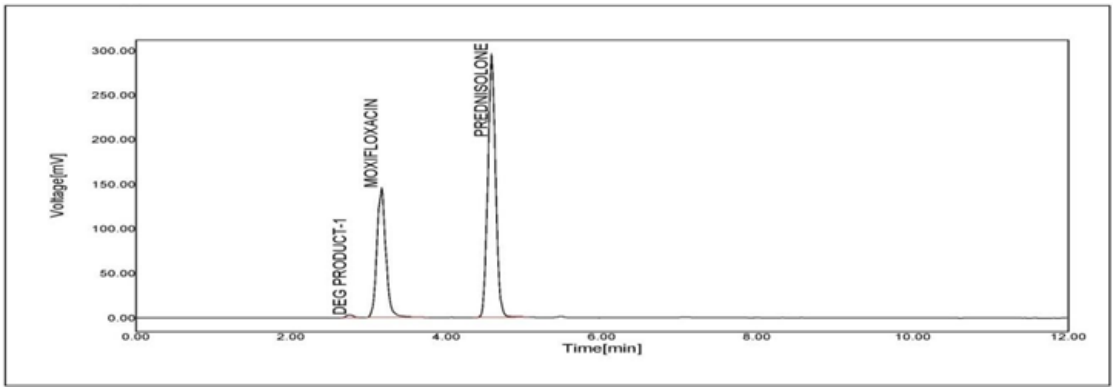

Fig No. 19: Chromatogram for Thermal Degradation Study.

Observation

In this study drug show, three peak two was the standard drug, and another one was of the degraded product, from the peak area of standard drug and degraded product the drug was

degraded up to 99.36% and actual 0.65% degradation was, from this condition it was cleared that the drug was not degraded in peroxide condition.

Photolytic degradation

To study the effect of photolytic degradation, Pure drug of moxi and pred was taken in Petri plate and spread as a thin layer and this is exposed to UV light at 254 nm in a UV chamber for 1 hr. Sample was weighed, dissolved in the mobile phase to get solution of moxi and pred and then injected. From the peak area found in the chromatograph, the % degradation was calculated.

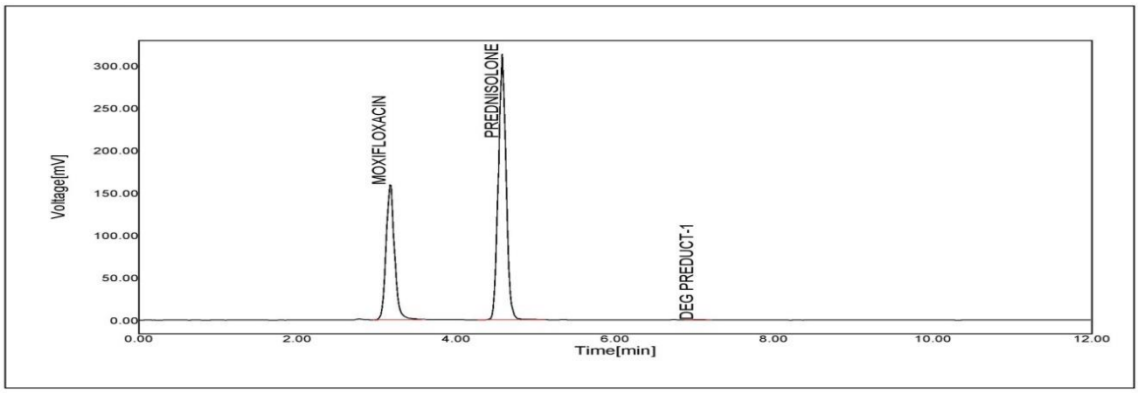

Fig No. 20: Chromatogram for Photolytic Degradation Study.

Observation

In this study drug show, three peak two was the standard drug, and another one was of the degraded product, from the peak area of standard drug and degraded product the drug was degraded up to 99.88% and actual 0.12% degradation was, from this condition it was cleared that the drug was not degraded in peroxide condition.

Table No. 32: Result of forced degradation study for Moxifloxacin.

Stress Condition	Time	%Area Moxi Observed after Degradation	% of Degradation
Acidic	30 Min	32.05	9.48
Basic	30 Min	34.66	4.51
Peroxide	12 Hr	26.18	0.99
Thermal	1 Hr	35.63	0.65
Photolytic	1 Hr	35.43	0.12

Table No. 33: Result of forced degradation study for Prednisolone.

Stress Condition	Time	%Area Pred Observed after degradation	% of Degradation
Acidic	30 Min	58.47	9.48
Basic	30 Min	60.83	4.51
Peroxide	12 Hr	47.97	0.99
Thermal	1 Hr	63.73	0.65
Photolytic	1 Hr	64.45	0.12

DISCUSSION

Moxifloxacin in combination with prednisolone is used for treatment of bacterial infection and inflammation of the eye. As, no reports available for the stability of the drug and their possible degraded product to date. Since the literature has not mentioned any method for the determination of this drug from the bulk drug, as well as its formulation. It has been proposed to a new RP-HPLC method was to develop and validated for moxi and pred as per ICH guideline and used as a stability-indicating method.

Selection of analytical wavelength

The standard disposition of moxi and pred is examined in the range of 200-400 nmfor the Acetonitrile as blank. From the spectrum, the detecting wavelength selected for the estimation of the drug was 254nm.

Method development and optimization

This project aimed to develop simple, easy, economical, and reproducible of a validated analytical method for estimation of moxi and pred from pure and form the marketed formulation. A number of the trial were made by changing the mobile phase by varying its composition as well as by changing the solvents. All these trials have resulted either in low asymmetric peaks or peaks with more tailing factor. However, finally, the Hypersil BDS C18 column (250 mm x 4.6 mm, 5 µm) column with flow rate 1.00ml/min of mobile phase as ACN: water: TFA (45: 55: 0.2) had resulted in a good peak shape was observed with low retention and run time. The detection was carried out at 254 nm. The retention time obtained for Moxi and pred was 3.20 and 4.77 min respectively with C18 stationary phase.

System suitability

The system suitability parameter established for the present developed RP-HPLC method includes the number of theoretical plates, tailing factor. The result is all within acceptable limits.

Analysis of marketed formulation

Analysis of marketed ophthalmic suspension was carried out using the above said optimized mobile phase and HPLC condition. The % drug content of ophthalmic suspension obtained by the proposed method for moxi and pred was found to be 98.06 and 100.24 respectively reviling that the estimation of dosage form was accurate within the acceptance level of 95% to 100%.

Method validation

The result of the analysis in all the method was validated in terms of specificity, linearity and range, accuracy, precision, and ruggedness.

Specificity

Blank interference: The result for specificity as compared to a study to establish the interference of blank. Chromatogram of blank solution showed no interference at the retention time of moxi and pred peak indicating that the diluent solution usedin standard and test preparation do not interfere in the estimation of moifloxacin formulation.

Linearity and range

The result showed that there was an excellent correlation between peak and analyte concentration. The detector response was linear with a correlation coefficient of 0.999. The slope and intercept of the calibration plot of moxifloxacin and pred were y = 4.066x-26.59.

Accuracy

The accuracy of the method was determined by calculating the recovery of moxi and pred using the spiked method. The average % recovery values are within the limit (98.71% and 100.70%). The mean % recovery was found in the range of 100.70 ± 0.49 and that of the assay was 99.34.

Precision

The precision study was performed using an intraday and intraday precision method. The proposed method was determined by analyzing the moxi and pred solution at different time intervals and on different days. The precision was expressed in terms of standard deviation and % RSD. The result was given in table and. The result was calculated in terms of % RSD for both interday and intraday precision study which was found to be < 2.0% this confirms that the method was precise.

Robustness

The robustness method was determined by varying the method parameter, such as a change in flow and a change in wavelength. The robustness was calculated as the % assay and RSD. The result of the assay of two test preparation was not affected by varying the condition. The fully agree with the result obtained under the original condition.

Degradation study

The moxi and pred pure drug was used for the study and stress under Acid, Base, Peroxide, Thermal, and Photolytic condition. Moxi and pred was found to be stableunder peroxide, thermal, and photolytic condition. Chromatogram of pure drug and stress samples are shown in Fig 20-24. The HPLC analysis of the stressed sample has shown that degradation was observed in acid and base stressed samples, but not in peroxide, thermal, and photolytic stressed samples. The result of all degradation studies is given in Table 34,35.

SUMMARY AND CONCLUSION

Combined dose ophthalmic formulation containing moxifloxacin and Prednisolone is available in market(GpMox-P) for the treatment of bacterial infection in eye.

Multicomponent formulations are gaining precedence over single component formulations owing to the following reasons

- ✓ Synergism of effects.
- ✓ Reduction of cost of treatment.
- ✓ Increased patient compliance.

Due to this rise in the multicomponent formulations, the challenges faced by the analytical chemist are on the rise. Estimation of drugs from a multicomponent formulation requires a method capable of discriminating the two or more components. Approaches to multicomponent analysis can be broadly categorized into those which rely on physical separation of components prior to analysis (e.g. chromatographic methods) and those that do not actually separate the components (e.g. simultaneous equations method in spectroscopy). The present work involved the development of accurate, precise, simple and suitable RP-HPLC method for estimation of the drugs in multicomponent ophthalmic formulations. A thorough literature survey revealed few spectrophotometric methods were reported like simultaneous equation method and colorimetric method for simultaneous estimation of these drugs in pharmaceutical formulations. Simple, sensitive and reliable spectroscopic methods for estimation of moxifloxacin and prednisolone in combined dosage form have been attempted. In RP-HPLC method, the analyte were resolved using ACN:Water: TFA (45:55:0.2), at a flow rate of 1 ml/min, on HPLC autosamler system containing UV- visible detector with Empower Software and Hypersil BDS C18 column (4.6 x 250 mm,5µm). The detection was carried out at 254nm. The method gave the good resolution and suitable retention time. The results of analysis in all the method were validated in terms of accuracy, precision, ruggedness, linearity and range. From the studies it can be concluded that RP-HPLC technique can be successfully used for the estimation of moxifloxacin and prednisolone in their combined dosage ophthalmic formulations. The method shows good reproducibility Compared to UV-spectrophotometric methods. The RP-HPLC method is accurate, precise, specific, reproducible and sensitive. No interference of additives, is encountered in these methods. Further studies on other pharmaceutical formulations would throw more light on these studies. The methods were found to be sensitive, reliable, reproducible, rapid and economic also.

CONCLUSION

The observations and results obtained for each parameter including sensitivity and selectivity, recovery, linearity and range, accuracy and precision, lies well within the acceptance criteria. A method for the determination of Moxifloxacin Hydrochloride and Prednisolone in pharmaceutical dosage form was developed and the method was shown to be selective, accurate and precise and the limit of quantitation was sufficient for the purpose of a study. The method was validated as per US-FDA Guidelines[40] with compliance to acceptance criteria. Since the results within the acceptance criteria for all validation parameters, the

method was considered as validated and suitable for intended use.

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REFERENCES

1. Sethi PD, Quantitative analysis of pharmaceutical formulations, CBS publishers and distributors, New Delhi, 2001(1): 3-5.
2. Srivastava VK, and Srivastava KK. Introduction to chromatography theory and practice, 1991; (14): 66-67.
3. Ayoub BM, Mowaka S.LC-MS/MS. Determination of ketoprofen journal of chromatographic science, 2017; 55(7): 742-747.
4. Eldindahshan H.Helal, M.A., Mostafa, S.M., & SalehElgawish, M. Development and validation of an HPLC-UV method for simultaneous deterrmination of Sildenafil and Tramadol in biologicl fluids. Application to Drug-Drug Interaction Study, Journal of Pharmaceutical And Biomedical Analysis, 2019; 101: 253-284.
5. Chilbule A, Singh R, Mann B, Arora, S, Sharma R, Singh Rao, P. Development and validation of an analytical method for determination of bronopol and kathon preservative in milk, journal of food science and technology, 2019; 56(6): 3170-3176.
6. Snyder LR, Kirkland J, Joseph Glajch L.Practical HPLC method development, john wiley and sons, INC, Canada, 1997; (2): 2-11.
7. Quantitative Bioanalytical Method Validation and Implementation:best practice for chromatographic and ligand binding assay-3rd AAPS/FDA Bioanalytical workshop Gedaway A, Al-Salami, H, & Dass CR. Development and validation of a new analytical HPLC method for simultaneous determination of NSAIDS. Journal of food and drug analysis 2018.
8. Department of Quality Control and Quality Assurance, Smilax Laboratories limited, Hyderabad 500055, A.P, India: 1111-1117.
9. Zhang Y-Long H, et al. Simultaneous determination of cortisol and prednisolone in body fluids by using HPLC–DAD coupled with second-order calibration based on

alternating trilinear decomposition, Journal of Chromatography B, 2006; (840): 116–123.

10. Razzaq NS, et.al. Stability indicating HPLC method for the simultaneous determination of moxifloxacin and prednisolone in pharmaceutical formulations, chemistrycentral journal, 2012; 6-94: http://journal.chemistrycentral.com/content/6/1/94.
11. Raut GS, et.al. Simultaneous Determination Of Prednisolone Acetate and Moxifloxacin Hydrochloride in Bulk and in eye drop using Rp–Hptlc (11 Dec 2013), Journal of Liquid Chromatography & Related Technologies, 2014; (37): 528–537. doi: 10.1080/10826076.2012.749495.
12. Adrian M, et.al. Development and validation of a new stability indicating reversed phase liquid chromatographic method for the determination of prednisolone acetate and impurities in an ophthalmic suspension, Journal of Pharmaceutical and Biomedical Analysis, 2014; 1-22. http://dx.doi.org/doi:10.1016/j.jpba.2014.09.023.
13. Ileana A, Ionita. Development of a sensitive and selective method for the quantitative analysis of cortisol, cortisone, prednisolone and prednisone in human plasma. Journal of Chromatography B, 2009; (877): 765–772. doi:10.1016/j.jchromb.2009.02.019.
14. Qadir MA, et.al. Validated Stability Indicating RP-LC Assay for Determination of Gatifloxacin and Prednisolone Acetate in Ophthalmic Preparations and Biological Samples. Latin American Journal of Pharmacy, 2016; 35(5): 912-20.
15. Maria NG. Determination of Prednisolone and Prednisone in Plasma, Whole Blood, Urine, and Bound-to-Plasma Proteins by High-Performance Liquid Chromatography, Journal Of Chromatographic Science, April 2005; 43.
16. Nariman A, El-Ragehy, Maha A, Hegazy G, AbdElHamid, and Samia A. Tawfik. Validated Chromatographic Methods for the Simultaneous Determination of Sulfacetamide Sodium and Prednisolone Acetate in their Ophthalmic Suspension. Journal of Chromatographic Science, 2017; 55(10): 1000–1005. doi:10.1093/chromsci/bmx064.
17. Aburuz S, et.al. Simple liquid chromatography method for the rapid simultaneous determination of prednisolone and cortisol in plasma and urine using hydrophilic lipophilic balance solid phase extraction cartridges, Journal of Chromatography B, 2003; (798): 193–201.doi:10.1016/j.jchromb.2003.09.0.
18. Naga Raju Potnuri, et.al. A Novel Stability indicating RP-HPLC method for the simultaneous estimation of moxifloxacin and prednisolone in bulk and their combined dosage form., IJPSR, 2015; 6(5): 1965-1973.

19. Doppenschmitt SA, Scheidel B, Harrison F, Surmann JP. Simultaneous determination of prednisolone, prednisolone acetate and hydrocortisone in human serum by high performance liquid chromatography, Journal of Chromatography B, 1995; (674): 237-246.
20. Barot HN, Dave JB and Patel CN .Development and validation of spectrophotometric method for simultaneous determination of prednisolone acetate and ofloxacin in eye drop, IJPSR, 2012; 3(6): 1817-1821.
21. Devika GS, Sudhakar M and Venkateshwara Rao, Simultaneous Estimation of Cefixime and Moxifloxacin in Bulk and its Pharmaceutical Dosage form by RP- HPLC, Oriental Journal Of Chemistry, 2012; 28(4): 1743-1750.
22. Patel D, Patel M, Patel K. Simultaneous RP-HPLC Estimation of Moxifloxacin Hydrochloride and Ketorolac Tromethamine in Ophthalmic Dosage Forms Asian
23. Boubakar B, Renaud Etienne BA, Dominique Ducint, Claudine Quentin, a Marie-Claude Saux Determination of moxifloxacin in growth media by high- Performance liquid chromatography, Journal of Chromatography B, 2001; (754): 107–112.
24. Julie De Smet, Koen Boussery, et.al (2009), Pharmacokinetics of fluoroquinolones in critical care patients: A bio-analytical HPLC method for the simultaneous quantification of ofloxacin, ciprofloxacin and moxifloxacin in human plasma, Journal of Chromatography B, 2009; 877: 961–967.
25. Panchumarthy Ravisankar, Saranu Anusha, Sai Sneha Latha K, Shaik Anjum Kausar. Development and Validation of A Stability-Indicating Reversed Phase HPLC Method for Determination of Moxifloxacin in Bulk and Pharmaceutical Dosage Form, Int. J. Pharm. Sci. Rev. Res., 2019; 54(1): Article No. 06, Pages: 33 -40.
26. Predrag Djurdjevic, Andrija Ciric, Aleksandra Djurdjevic, Milena Stankov, Optimization of separation and determination of moxifloxacin and its related substances by RP- HPLC, Journal of Pharmaceutical and Biomedical Analysis, 2009; 50: 117–130.
27. Pradipbhai D. Kalariya, Deepak Namdev, Srinivas R, Gananadhamu S, Application of experimental design and response surface technique for selecting the optimum RP- HPLC conditions for the determination of moxifloxacin HCl and ketorolac tromethamine in eye drops, Journal of Saudi Chemical Society, 2014; 1-10.
28. Lemoine T, Breilh D, Ducint D, Dubrez J, Jougon J, Velly JF, Saux MC. Determination of moxifloxacin (BAY 12-8039) in plasma and lung tissue by high- performance liquid chromatography with ultraviolet detection using a fully automated extraction method with a new polymeric cartridge, Journal of Chromatography B, 2009; (742): 247–254.

29. Dewani AP, Barik BB, Kanungo SK, Wattyani BR and Chandewar AV. Development and Validation of RP-HPLC Method for the Determination ofMoxifloxacin in Presence of Its Degradation Products, American-Eurasian Journal of Scientific Research, 2011; 6(4): 192-200.