ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 15, Issue 9, 2022

Online - 2455-3891
Print - 0974-2441

Research Article

SEPARATION AND IDENTIFICATION OF FORCED DEGRADATION PRODUCTS OF LOFEXIDINE BY USING LC-MS/MS

MASTANAMMA SK*, SATYA ANJALI T, TEJASWI J, CHIRANMAI M, HEMA LATHA K

Department of Pharmaceutical Analysis, University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India. Email: masthanamma.sk@gmail.com

Received: 05 May 2022, Revised and Accepted: 24 June 2022

ABSTRACT

Objectives: A rapid and reliable isocratic LC-MS/MS method was developed and validated for the separation and identification of stress degradation products (DPs) of lofexidine.

Methods: Lofexidine, a non-opioid centrally acting alpha2-adrenergic receptor agonist, was subjected to hydrolysis (acidic, alkaline, and neutral), oxidation, photolysis, and thermal stress as per International Council on Harmonization specified conditions. The drug showed extensive degradation under alkaline, acidic, oxidation, and photolytic stress condition.

Results: A total of 14 DPs were observed and the chromatographic separation of the drug and its DPs were achieved on waters symmetry C_{18} (150 × 4.6 mm, 3.5 µm) column using water and acetonitrile (75:25 v/v) as mobile phase. The DPs were separated and identified using LC-MS/MS. The LC-MS/MS method was validated with respect to specificity, linearity, accuracy, and precision.

Conclusion: The proposed method was used for impurity profiling and routine quality control tests of lofixidine.

Keywords: Lofexidine, Degradation products, Stress condition, LC-MS/MS.

 DOI: http://dx.doi.org/10.22159/ajpcr.2022v15i9.45117. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Lofiexidine, 2-[1-(2,6-dichlorophenoxy) ethyl]-4,5-dihydro-1H-imidazole (Fig. 1), Molecular Formulae-$_{C1}1_{H1}2C_i2_N2O$, Molecular Weight-259.132 g·mo^{l-}1, insoluble in water. Lofexidine is a non-opioid centrally acting alpha2-adrenergic receptor agonist. Lofexidine replaces the opioid driven inhibition of cAMP production by activating the alpha2-adrenergic receptor and moderating the symptoms of opioid withdrawal. It is of great importance to understand the stability of a drug molecule, that is, to know how the quality of the drug varies with time under the influence of a variety of environmental factors. The International Council on Harmonization (ICH) guideline entitled "stability testing of new drug substance and products" (Q1A) stated that stability-indicating method (SIAM) needs to develop to elucidate the inherent stability of the active substance by applying stress conditions. It suggests that stress degradation study should be carried out under a variety of conditions, including oxidation, hydrolysis wide pH range, photolysis, and thermal degradation. Through SIAM, the changes with time in the chemical, physical, or microbiological properties of the drug substances and drug product and that are selective can be detected so that the content of degradation products (DP) of the drug and explanation of side effects of active ingredient, DPs, and other components of interest can be accurately measured without interference. Moreover, characterization of degradants is useful to establish the mechanism of formation of drugs, recently hyphenated technique such as LC-MS/MS in combination with accurate mass measurement is widely adopted for the structural characterization of impurities/DPs of drug formed under various stress conditions.

For the present study, the drug lofexidine was selected because the extensive literature survey was carried out and revealed that there were no analytical and derivative methods reported for the estimation of drug. Hence, an attempt was made to develop a simple and effective analytical method for separation and identification of lofexidine DPs. Therefore, the aim of the work was to investigate degradation behavior of lofexidine to understand the stability of the drug molecule and to identify the DPs. This was accomplished by exposing the drug to ICH recommended stress conditions of hydrolysis, oxidation, thermal stress, and photolysis, and by analyzing the sample using optimized and validated stability indicating LC-MS/MS method. The structure of lofexidine is shown in Fig. 1.

METHODS

Chemicals and reagents

Pure lofexidine working standard (99.98%) was obtained as gift sample from Zydus Cadila, commercially available LUCEMYRA (0.2 mg). Lofexidine tablets were purchased from pharmacy.

Acetonitrile (Rankem, HPLC grade), methanol, orthophosphoric acid (Rankem), and water obtained from milli-Q water system (manufacturer inhouse production) (Millipore, Bedford, MA, USA) and it was used to prepare all solutions.

Instrumentation

High performance liquid chromatography instrument used was Waters e 2695, Empower software 2.0 version (Model-Aliance) with auto injector

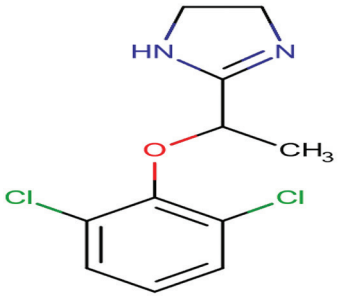

Fig. 1: Structure of lofexidine

and PDA detector, Pump-Isocratic model, Ultrasonicator-Unichrome, UCA 701(model), FTIR (bruker), pH meter (Eutech Instruments, Singapore), Weighing balance (Sartorius), and Vacuum filter pump.

The stress photodegradation was carried out in a photostability chamber cables of controlling the temperature range of ±25°C and ±5% RH, respectively. The chamber was equipped with illumination bank made of light source as described in the ICH guidelines. Chromatographic separation was carried out on a Water Symmetry C_{18} 150 × 4.6 mm, 3.5 µm with mobile phase composition consisting water and acetonitrile in the ratio of 75:25 v/v. The injection volume was 10 µL and the mobile phase flow rate was at 1.2 ml/min. All the stress samples were analyzed using PDA detector in a scan mode covering range of 200–400 nm and final chromatogram was extracted at 244 nm to detect all DPs.

Structural characterization was carried out using LC-MS/MS SCIEX QTRAP 5500 mass spectrometers equipped with electron spray ionization (ESI) interface to provide a compact and with class Empower-2 software. Post column splitting (10:1) was used to give optimal interface flow rate (1.0 mL/min) for MS detection. The mass spectrometer was obtained in: positive ESI mode, the drying gas temperature and flow rate: 120–250°C and 5L/min, respectively. Quantification was performed using multiple reaction monitoring of the transitions and calculating the molecular mass of analyte using version 2.0 (with Water) for Empower software.

Stress degradation study

Stress studies were performed starting with milder conditions followed by stronger conditions as to get sufficient degradation.

Hydrolytic degradation studies

Stress hydrolytic study was performed in acid, alkaline, and neutral condition with 0.1, 5N HCl, 0.1, 5N NaOH, and water, respectively. Acidic and alkaline hydrolytic studies were carried out at room temperature from 0.5 to 24 h to get optimum DP's. Neutral hydrolytic condition was performed in water after refluxing at 70°C for 12–24 h.

Oxidative degradation study

Oxidative stress studies were carried out at room temperature for 48 h in 3 and 30% H_2O_2.

Thermal degradation study

Thermal degradation study was also carried out in solid state by exposing pure lofixidine in a petri plate with a very thin layer to dry heat at 105°C for 24 h.

Photolytic degradation study

Photolytic stress studies were carried out to a total dose of 1.2 million lux h of fluorescent and 200 Wh/m^2 of UV-illumination. A parallel set of the drug solution was stored in dark at the same temperature to serve as control.

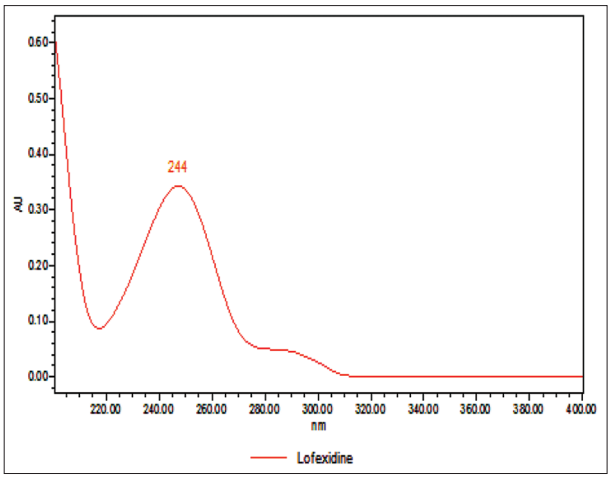

Fig. 2: PDA spectrum of lofexidine

Preparation of stock

Accurately weighed and transfer 7 mg of lofexidine working standard into a 10 ml clean dry volumetric flask, add diluents and sonicate to dissolve it completely (stock 1) and make volume up to the mark with the same solvent take 2.5 ml in 25 ml volumetric flask and make up with diluents (Stock 2).

Preparation of mobile phase

Mobile phase was prepared by mixing water and ACN taken in the ratio 75:25. It was filtered through 0.45 µ membrane filter to remove the impurities which may interfere in the final chromatogram.

Sample solution preparation

Lofexidine laboratory synthetic mixture was prepared using suitable excipients which were mixed properly. From this accurately weighed and transferred equivalent to 7 mg of lofexidine into a 10 ml clean dry volumetric flask, add diluent and sonicate it up to 15 min to dissolve, and centrifuge for 15 min. To dissolve it completely and make volume up to the mark with the same solvent. Then, it is filtered through 0.45 µ injection filter. Further take 1 ml of the above solution into another 10 ml volumetric flask and made up to the mark with diluents (70 ppm of lofexidine).

METHODS DEVELOPMENT

Selection of solvent

Various solvents were studied for the solubility of lofexidine, and it was found to be 0.147 mg/ml in water and best soluble in acetonitrile; therefore, ACN and water were selected as a solvent of choice.

Development and optimization of chromatographic conditions

Chromatographic conditions

During the selection of chromatographic conditions, number of trails were carried out and the best trial was selected for optimized method. Respective chromatogram is shown in Fig. 3.

Validation of the proposed method

Specificity

Specificity is defined as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, and matrix components. For this purpose, blank chromatogram, excipient, standard chromatogram, and sample chromatogram were recorded. The chromatogram of blank shows no response at the retention times of drug which confirms the response of drug was specific. Fig. 4-6 shows the respective chromatograms for lofexidine standard and formulation.

Linearity and range

The linearity is its ability to elicit test results that are directly proportional to concentration of analyte in sample within given range. Linearity test was performed from the above Stock 1, series of aliquots were prepared by taking 0.1, 0.25, 0.5, 1, 1.25, 1.5, and 2 into different volumetric flasks (10 ml) and diluted up to the mark with diluents. Each concentration was injected 6 times and record the peak area and RT. Detector response for the proposed method determined to be linear over the range of 7 concentration levels prepared and injected, 7–105 ppm for lofexidine. A good linear relationship (r^2=0.9996) was observed between the concentration of lofexidine and the corresponding peak areas. The regression equation was found to be y=79349× + 20694. The slope, intercept, and the correlation coefficient of the drug. The results obtained were as shown in the Table 2 and Fig. 7.

Accuracy

Accuracy is expressed as the closeness of the results obtained from standard samples to that of the actual known amounts. To determine

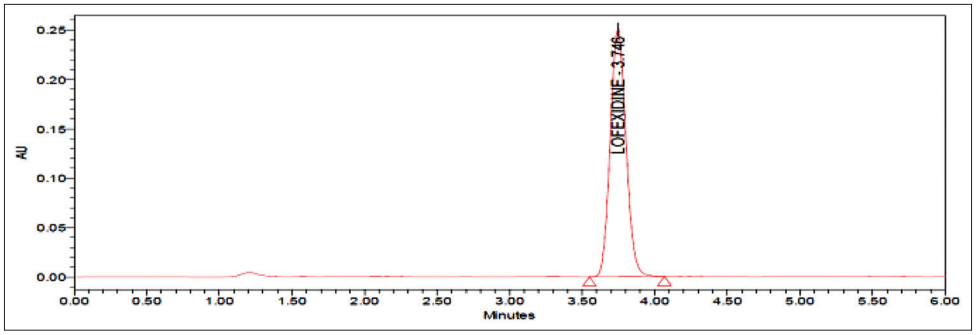

Fig. 3: Optimized chromatogram

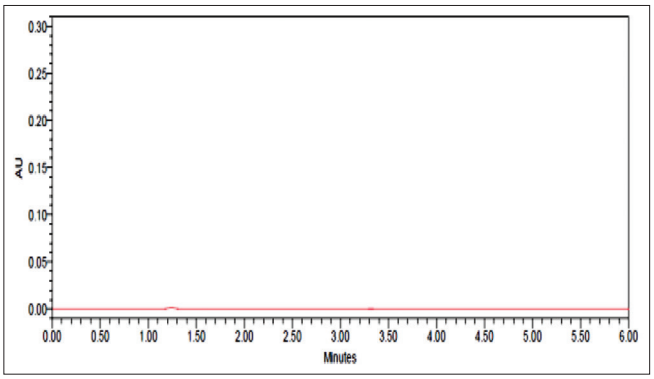

Fig. 4: Chromatogram of blank

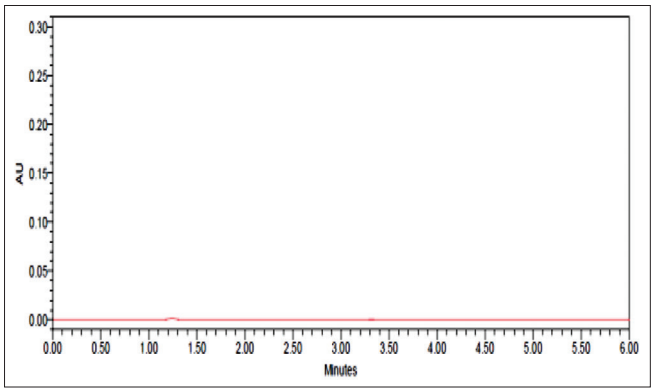

Fig. 5: Chromatogram of placebo

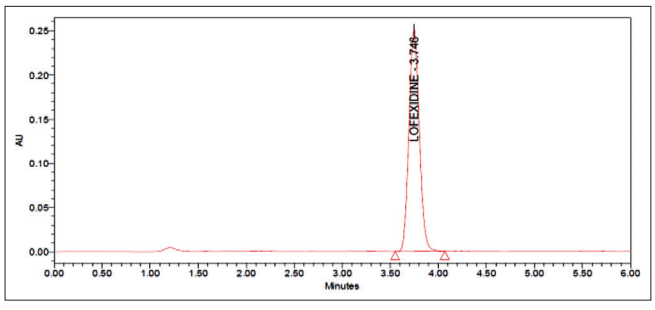

Fig. 6: Chromatogram standard

the accuracy of the proposed method, recovery studies were performed to the pre-analyzed formulation. The solutions were suitably diluted in the linearity range and then each of the dilution was injected 6 times. The percentage recovery of the drug was calculated. The results obtained are shown in Table 3.

Table 1: Optimized MASS parameters of lofexidine

Mass parameters	Lofexidine
Molecular ion (m/z)	259.6[M+H]$^+$
Product ion (m/z)	310.6[M+H]$^+$
Source dependent parameters (psi)	
Curtain gas (CUR)	22
Collisionally Activated Dissociation gas (CAD)	10
Nebulizer gas (NEU)	32
Compound dependent parameters (volts)	
Entrance potential	10
Focusing potential	25
De clustering potential	40
Collision cell entrance potential	45
Collision cell exit potential	15
Common mass parameters	
Ion spray voltage	5500 volts
Source temperature	500°C
Scan type	MRM
Dwell time	1 s
Mode	Positive ion
Type of ionization	Electron spray ionization

Table 2: Optical characteristics of lofexidine

S. No	Lofexidine	
	Conc(µg/ml)	Avg Peak area (n=6)
1	7	184880
2	17.5	459113
3	35	989432
4	52.5	1399934
5	70	1881430
6	87.5	2334297
7	105	2825863

Precision

Precision is the degree of repeatability of an analytical method under normal operative conditions.

System precision is checked using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of lofexidine, for six determinations were measured and % RSD should be calculated.

In method precision, a homogenous sample of single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this, analyze the sample 6 times and calculate the % RSD.

The precision of the proposed method, that is, the intra and inter day variations in the peak area of the drug solutions was calculated in terms of % RSD and the results were as shown in the Table 4 and 5.

Table 3: recovery studies of lofexidine

Pre-analyzed formulation (mg)	% Concentration (at specification level)	Area	Amount added (mg)	Amount found (mg) (n=6)	% Recovery	Mean recovery (n=6)
7	50%	9863325	3.5	10.4	99.04	99.72
7	100%	1890631	7	14.1	100.71	
7	150%	2856287	10.5	17.4	99.42	

Table 4: Intermediate precision studies of lofexidine

Concentration of lofexidine (µg/mL)	Concentration of lofexidine precision found (µg/mL)±SD; %RSD (n = 6)			
	Day 1	Day 2	Different analyst	Different instrument
7	7.03 ± 0.12; 0.12	7.10 ± 0.40; 0.40	7 ± 0.11;0.24	6.9 ± 0.12;0.28
35	35.07 ± 0.09; 0.09	35.13 ± 0.17; 0.17	34.9 ± 0.13;0.18	34.8 ± 0.06;0.07
70	70.03 ± 0.02;0.02	70.07 ± 0.31; 0.07	70 ± 0.39;0.37	69.9 ± 0.22;0.21

Table 5: Repeatability studies of proposed method

Injection	Retention time (min)	Peak area	Theoretical plates	Tailing factor
1	3.744	1891923	5634	1.10
2	3.746	1895508	5623	1.10
3	3.746	1887820	5580	1.10
4	3.746	1891040	5600	1.10
5	3.752	1888217	5558	1.09
6	3.752	1893460	5567	1.09
Mean		1891328		
SD		2981.24		
%RSD		0.16		

The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 70 ppm of lofexidine.

Acceptance criteria

The % RSD for the peak area of six replicate injections should not be more than 2%.

Robustness

The robustness is a measure of its capacity to remain unaffected by small but deliberate changes in method parameters (such as flow rate, mobile phase composition, temperature variation was made to evaluate the impact on the method). The method conditions such as flow rate (±0.2 ml) and the organic phase (2%) were altered and the influence of these changes on the peak area, peak tailing, and number of theoretical plates was evaluated. The results obtained are shown in Table 6.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD is defines as a smallest level of analyte that gives a measurable response. Six replicates of analyte were measured. The LOQ is the concentration that can be quantitated reliably with a specified level of accuracy and precision. It is the lowest concentration at which the precision expressed by relative difference in the measured and true value is also <2%. Six replicates of analyte were measured and quantified. The LOD and LOQ of the drug were calculated using the following equation as per ICH guidelines. The results obtained are shown in Table 7.

LOD=3.3xσ/s LOQ=10xσ/s

LOD for lofexidine was found to be 0.07 µg/ml
LOQ for lofexidine was found to be 0.21 µg/ml

Forced degradation studies

Hydrolytic degradation under acidic condition

Pipette 1 ml of above stock solution into a 10 ml volumetric flask and 1 ml of 0.1N HCl was added and kept at room temperature. After 24 h neutralized with 1 ml of 0.1N and 1N NaOH and make up to 10 ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

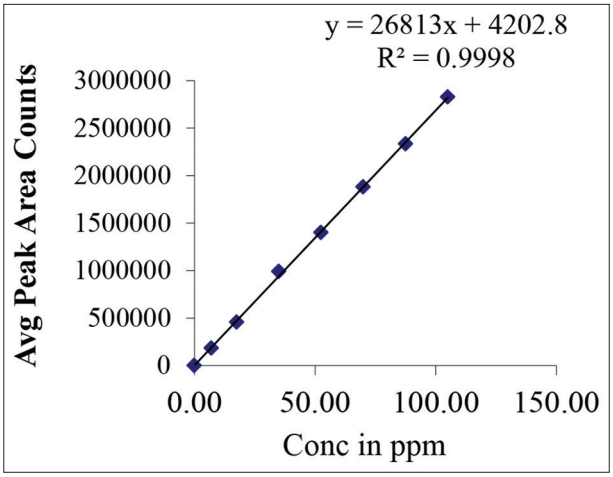

Fig. 7: Calibration curve of lofexidine

Pipette 1 ml of above stock solution into a 10 ml volumetric flask and 1 ml of 5N HCl was added. Then, the volumetric flask was kept at 60°C for 0, 6, 12, 18, 24 h and then neutralized with 1 ml of 5N NaOH and make up to 10 ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials and chromatograms were recorded.

Hydrolytic degradation under alkaline condition

Pipette 1 ml of above stock solution into a 10 ml volumetric flask and add 1 ml of 0.1N NaOH was added. After 24 h neutralized with 1 ml of 0.1N and 1N HCl and make up to 10 ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Pipette 5 ml of above stock solution into a 10 ml volumetric flask and add 1 ml of 5N NaOH. Then, the volumetric flask was kept at 60°C for 0, 6, 12, 18, and 24 h and then neutralized with 1 ml of 5 N HCl and make up to 10 ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials and chromatograms were recorded.

Peroxide degradation

Pipette 1 ml of above stock solution into a 10 ml volumetric flask, 1 ml of 3% and 30%w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 24 h. Filter the solution with 0.45 microns syringe filters and place in vials.

Table 6: Robustness studies of proposed method

Parameter	Lofexidine				
	Condition	Retention time (min)	Peak area	Tailing	Plate count
Flow rate change (ml/min)	Less flow (0.96 ml)	4.397	1812365	1.06	5361
	Actual (1.2 ml)	3.742	1886252	1.07	5645
	More flow (1.44 ml)	3.132	1834792	1.05	5169
Organic phase change	Less org (22.5:77.5)	4.251	1833129	1.1	5471
	Actual (25:75)	3.752	1850049	1.09	5512
	More Org (27.5:72.5)	3.173	1856921	1.06	5139

Table 7: Results of LOD and LOQ

Parameter	Result of lofexidine
LOD	0.07 µg/ml
LOQ	0.21 µg/ml

Pipette 5 ml above stock solution into a 10 ml volumetric flask, 1 ml of 30% w/v of hydrogen peroxide was added. Then, the volumetric flask was kept at 60°C for 0, 6, 12, 18, and 24 h and make up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials and chromatograms were recorded.

Hydrolytic degradation

From the working standard solution, 1 ml was taken in 10 ml volumetric flask, add 2 ml of diluent add 5 ml of water to disperse and dissolve and heated at 70°C for 0, 6, 12, 18, and 24 h on a water bath. Remove the flask from the water bath and allow the flask to cool at room temperature and diluted to volume with diluent and mixed. Then, the solution is injected in HPLC system to obtain chromatograms.

Reduction degradation

From the working standard solution, 1 ml was taken in 10 ml volumetric flask, add 3 ml of 10% sodium bisulfite contents are mixed well kept aside for 24 h. Then, make up to volume with mobile phase. The solution was injected in HPLC system to obtain chromatograms. From the working standard solution, 1 ml was taken in 10 ml volumetric flask, 1 ml of 10% sodium bisulfite was added; and heated at 70°C for 3 h on water bath and heated at 70°C for 0, 6, 12, 18, and 24 h on a water bath. Remove the flask from the water bath and allow the flask to cool at room temperature. Then, make up to volume with mobile phase. The solution was injected in HPLC system to obtain chromatograms.

Photolytic degradation study

The drug layer of 1 mm thickness was prepared in a Petri dish and exposed to ICH recommended photo stability conditions with the overall illumination of not <1.2 million lux h along with the integrated near ultraviolet energy of not <200 Wh/m^2. Another Petri dish containing the drug (1 mm layer thickness) was wrapped with aluminum foil and kept as control.

Sample solutions

The collected samples under various stress conditions were prepared by filtering the solution through 0.22 µm filter prior analysis. Samples were withdrawn at different time intervals and diluted with mobile phase before injection under optimized chromatographic conditions.

RESULTS AND DISCUSSIONS

A novel simple, accurate, precise, and economical LC-MS/MS method was developed for the estimation of lofexidine in the presence of its DPs which were obtained under various stress condition. The structure of lofexidine is shown in figure.

Determination of working wavelength (λ_{max})

The wavelength of maximum absorption of the solution of the drug in mixture of water and ACN (75:25) were scanned using PDA detector within the wavelength region of 200–400 nm against water and ACN (75:25) as blank. The absorption curve shows at 244 nm and was selected as detected wavelength for the HPLC chromatographic method.

Development and optimization of chromatographic conditions

Chromatographic conditions

During the selection of chromatographic and mass spectral conditions, number of trails were carried out and the best trial was selected for optimized method, respective optimized chromatogram was shown in Fig. 3.

Optimization of chromatographic and mass spectrometric conditions to achieve acceptable separation between the drug and its DPs, water, and acetonitrile (75:25) was used as mobile phase in isocratic elution mode and waters symmetry C18 (150 × 4.6 mm 3.5 µ) was used for successful separation of lofexidine and its DPs. Well resolved peaks with acceptable symmetry were achieved. The flow rate was 1.2 ml/min at detection wavelength 244 nm. The run time was 6 min. These optimized chromatographic conditions were used for separation of lofexidine and its DPs. The method was validated with respect to the parameters outlined in ICH guidelines Q1A R2. For LC-MS studies, same method was used as for HPLC. The Q-TOF ESI source conditions were also optimized to obtain a good signal and high sensitivity. The conditions such as drying gas flow, nebulizing gas flow, drying gas temperature, capillary voltage, spray voltage, and skimmer voltage were optimized to maximize the ionization in the source and sensitivity even at a very low concentration to identify and characterize the DPs.

Validation of proposed method

Specificity

The chromatogram of blank shows no response at the retention times of drug which confirms the response of drug was specific. Fig. 4-6 show the respective chromatograms for Lofexidine blank, placebo, and standard.

Linearity

The method was found to be linear over a concentration range of 7–105 µg/mL of lofexidine. The proposed method was found to be linear with correlation coefficient of 0.9998 and linear regression equation of y = 26813×+4202.8. The obtained calibration curve of lofexidine is shown in Fig. 7. The results are shown in Table 2.

Accuracy

The accuracy was established by the addition of known quantities of standard to the synthetic mixture of API and excipients which are taken in the formulation of the drug product. Each solution was injected in 6 times (n=6) and the percentage recovery was calculated. The percentage mean recovery was found to be 99.72 %. Results are given in Table 3.

Table 8: Results of lofexidine laboratory synthetic marketed formulation

Drug	Sample Area	Avg sample area	Labeled amount	Estimated amount (n = 6)	% Assay
Lofexidine	1891923 1895508	1891328	70 mg	69.99 mg	99.99

Table 9: Results of forced degradative studies for lofexidine

Results: % Degradation results	Lofexidine					
	Area		% Label claim after degradation	% Degradation	Purity Angle	Purity Threshold
Control	-	1892596	100.1	-0.1	1.257	5.604
Acid	0.1N	1846327	97.6	2.5	0.059	0.321
	1N	1573674	83.2	16.9	0.066	0.281
	5N-Ini	1759241	93	7	1.252	5.527
	6 h	1642575	86.9	13.2	1.211	5.526
	12 h	1425694	75.4	24.6	1.114	5.534
	18 h	1285614	68	32	1.135	5.569
	24 h	1256324	66.4	33.7	1.259	5.627
Base	0.1N	1825964	97.6	2.4	0.049	0.265
	1N	1557425	82.4	17.6	0.056	0.328
	5N-Ini	1815345	96	4	1.235	5.673
	6 h	1754896	92.8	7.2	1.247	5.629
	12h	1623578	85.9	14.1	1.239	5.534
	18 h	1425896	75.4	24.6	1.225	5.542
	24 h	1246257	65.9	34.1	1.264	5.675
Reduction	0.1N	1812603	95.9	4.2	0.058	0.261
	1N	1589865	83.4	16.6	0.062	0.365
	5N-Ini	1802754	95.3	4.8	1.225	5.623
	6 h	1753624	92.7	7.3	1.263	5.452
	12 h	1635954	86.5	13.5	1.238	5.517
	18 h	1524617	80.6	19.4	1.247	5.539
	24 h	1259306	66.6	33.4	1.241	5.635
Peroxide	0.1N	1843256	97.5	2.6	0.052	0.362
	1N	1520364	80.4	19.6	0.057	0.267
	5N-Ini	1812542	95.9	4.1	1.259	5.623
	6 h	1756245	92.9	7.1	1.254	5.624
	12 h	1625784	86	14	1.236	5.532
	6 h	1756245	92.9	7.1	1.254	5.642
	12 h	1625784	86	14	1.236	5.532
	1 h	1465287	77.5	22.5	1.255	5.618
	24 h	1268952	67.1	32.9	1.273	5.684
Photo	0.1N	1825321	96.5	3.5	0.047	0.355
	1N	1558967	82.4	17.6	0.051	0.274
	5N-Ini	1759628	93.1	6.9	1.235	5.421
	6 h	1698574	89.8	10.3	1.257	5.636
	12 h	1569823	83.1	16.9	1.243	5.754
	18 h	1456287	77	23	1.241	5.626
	24 h	1257630	66.5	33.5	1.227	5.618
Thermal	0.1N	1812684	95.9	4.1	0.049	0.285
	1N	1592697	84.2	15.9	0.058	0.266
	5N-Ini	1796325	95	5	1.257	5.628
	6 h	1712630	90.6	9.4	1.269	5.613
	12 h	1630246	86.2	13.8	1.254	5.627
	18 h	1445022	76.4	23.6	1.239	5.611
	24 h	1269451	67.1	32.9	1.242	5.639
Hydrolysis	0.1N	1836933	97.1	2.9	0.058	0.313
	1N	1548759	81.9	18.1	0.066	0.281
	5N-Ini	1823641	96.4	3.6	1.258	5.627
	6 h	1702365	90.1	9.9	1.234	5.634
	12 h	1623014	85.8	14.3	1.287	5.661
	18 h	1469285	77.7	22.3	1.365	5.525
	24 h	1236952	65.4	34.7	1.205	5.677

Precision

The repeatability of the method was established by analyzing six injections of the standard drug at 100 % level and the % RSD was found to be 2. The intermediate precision of the method was investigated by analyzing the drug at three different concentrations different days (interday precision), different column (with different lot number), different analyst, and different instrument within the same laboratory. % RSD was determined for the concentration of lofexidine found at

each level as shown in Table 4 and 5. The developed method has good precision as low % RSD values were obtained.

Robustness

Flow rate was (1.2±0.24 mL/min) and change in organic phase was (2.5%). The peak area of the injections (n=6) was taken as a measure for calculation for determining the robustness of the method. There was no significant variation in the assay of the components indicating the method to be robust as shown in Table 6.

The LOD and LOQ

LOD for lofexidine was found to be 0.07 µg/ml and LOQ for lofexidine was found to be 0.21 µg/ml. The results reveal that the proposed method was sensitive and it was used in cleaning validation as shown in Table 7.

Forced degradation study behavior

Alkali-induced degradation study

Six DPs (DP-4 to DP-9) were formed under the alkaline hydrolysis. stress conditions prone to the formation of oxidation products of lofexidine which are highly polar in nature when compared to the lofexidine drug. The chromatogram of lofexidine under alkaline stress conditions is shown in Fig. 8. The DPs are formed at different retention times and the HPLC leads to achieve the better separation of chromatographic peaks and further transferred to LC-MS/MS instrument which had the capability to resolute the precursor ions with a resolution of around 50,000. The precursor ions were subjected to fragmentation studies (MS/MS) in parallel by applying argon as collision-induced dissociation gas. The precursor ions HRMS formula, error (ppm), and fragment ions for corresponding parent ions of each degradation product are depicted in Table 10.

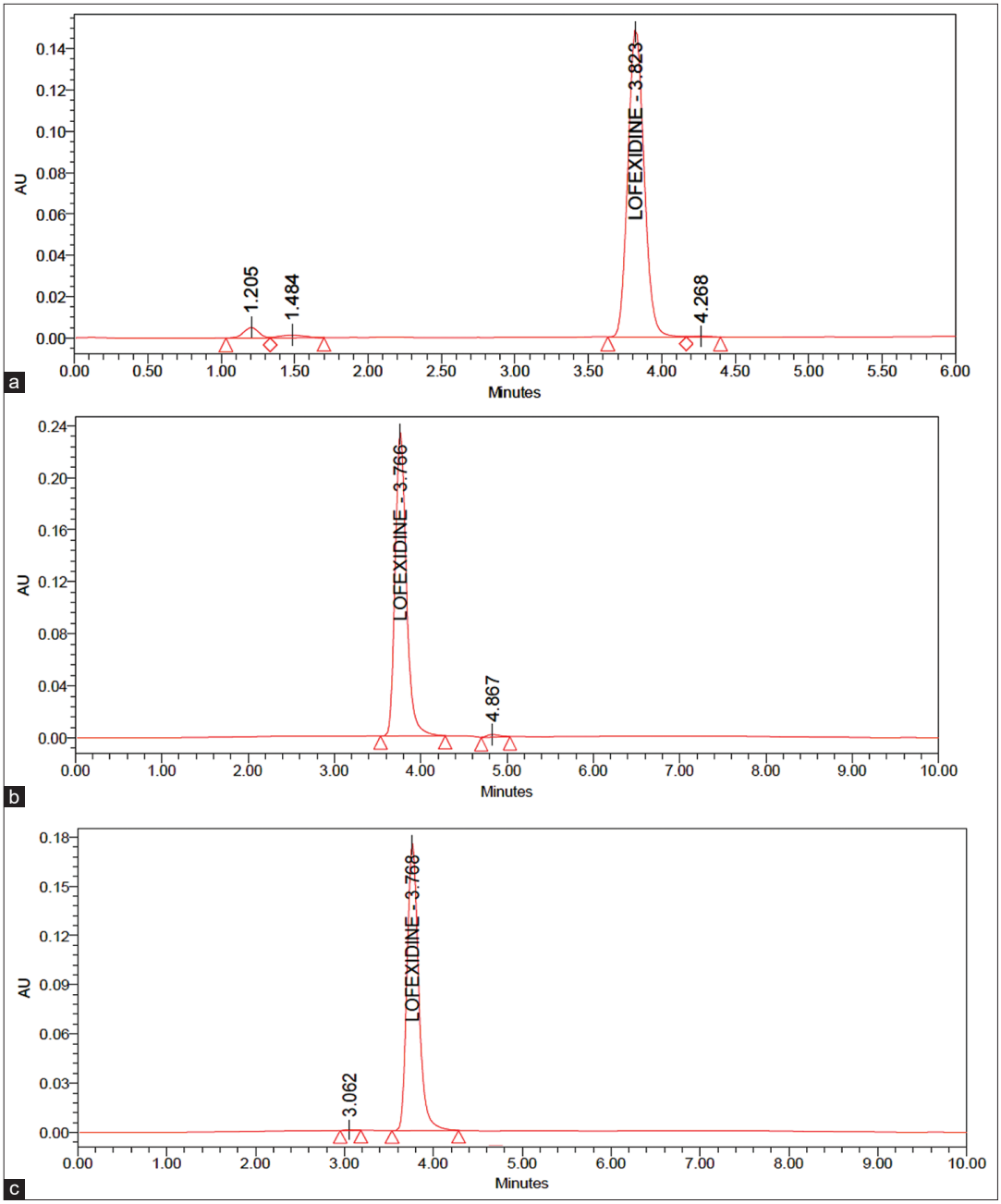

Fig. 8: (a) Chromatogram of Alkali degradation (1N), (b) Chromatogram of Alkali degradation (5N-6 h), (c) Chromatogram of Alkali degradation (5N-12 h)

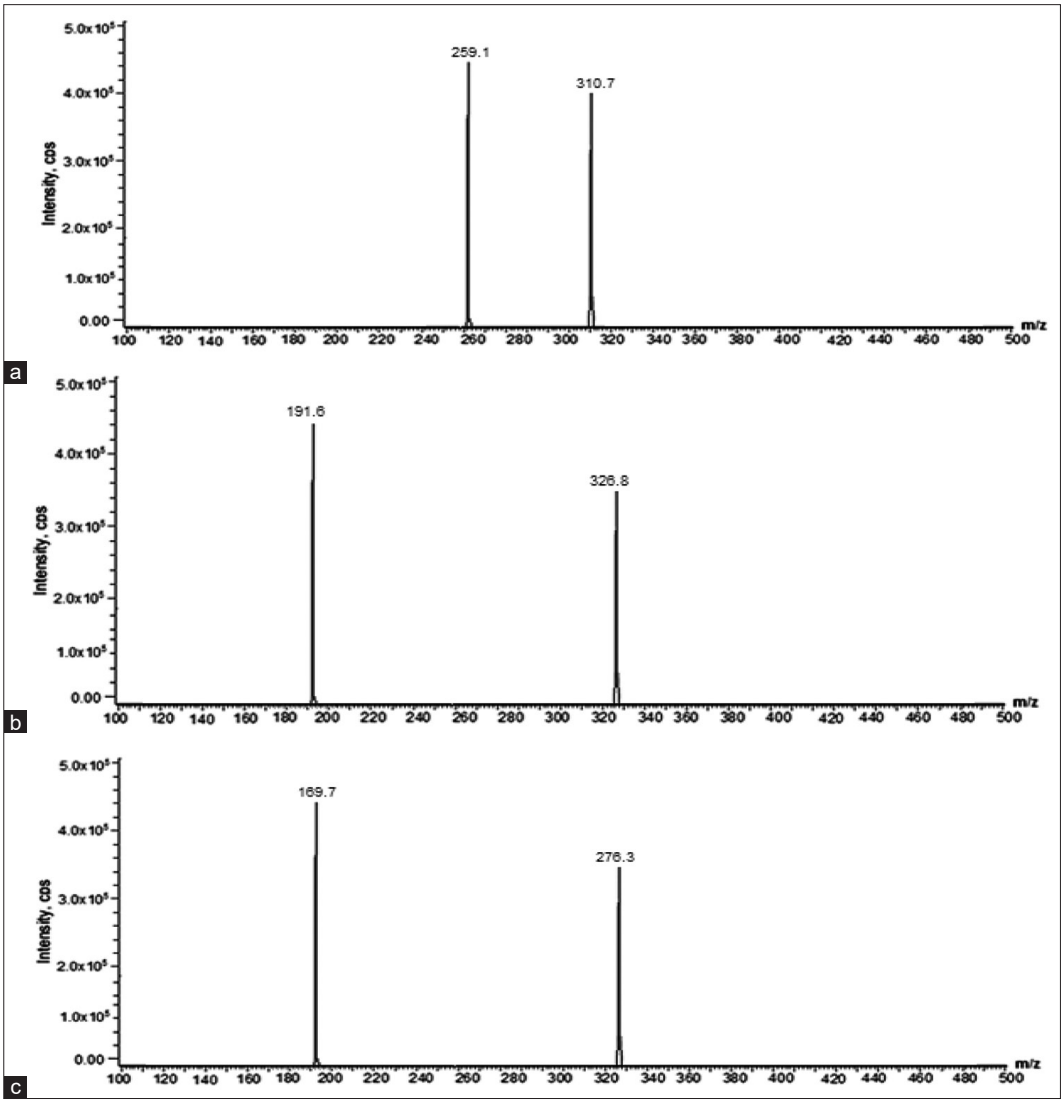

Fig. 9: (a) MS/MS Spectra of RT 3.764, (b) MS/MS Spectra of RT 4.829, (c) MS/MS Spectra of RT 6.954

Table 10: LC/MS-MS data of lofexidine and its degradation products

S. No	Degradation product	RT Time (min)	Calculated HRMS (M/Z)	Observed HRMS (M/Z)	Error (PPM)	Fragment IONS (M/Z)	Conditions
1.	DP1	1.189	288.7	288.5	0.2	245.8	1N ACID, 24 h
2.	DP2	3.45	211.5	211.3	0.2	182.7	5N ACID, 6 h
3.	DP3	4.650	308.4	308.2	0.2	216.2	5N ACID, 24 h
4.	DP 4	1.205	326.8	326.5	0.3	196.6	1N Alkali, 24 h
5.	DP5	1.484	326.8	326.9	-0.1	196.6	1N Alkali, 24 h
6.	DP6	4.268	326.8	326.5	0.3	196.6	1N Alkali, 24 h
7.	DP7	4.867	326.8	326.5	0.3	196.6	5N Alkali, 6 h
8.	DP8	3.062	310.7	310.5	0.2	259.1	5N Alkali, 12 h
9.	DP9	6.954	278.3	278.2	0.1	189.7	5N Alkali, 24 h
10.	DP10	8.838	352.4	352.2	0.2	246.9	3% H2O2 18 h
11.	DP11	6.166	348.2	348.3	-0.1	222.6	Hydro 6 h
12.	DP12	9.672	257.6	257.5	0.1	194.2	Hydro 12 h
13.	DP13	4.959	254.8	254.7	0.1	195.6	Photo 6 h
14.	DP14	8.381	328.8	328.9	-0.1	234.7	Reduction 6 h

ALKALI DEGRADATION

Acid-induced degradation study

When lofexidine was subjected to acidic degradation, the study leads to the formation of three DPs (DP-1, DP-2, and DP-3) in which one degradation product (DP-1) was observed in both alkaline (0.1N NaOH) and acidic (0.5 M HCl) stress conditions as oxidation product. The HPLC chromatographic separation was depicted in Fig. 10 and 11 and the MS/MS studies for the above three DPs and molecular ion formula, error (ppm), and fragment ions are shown in Table 10.

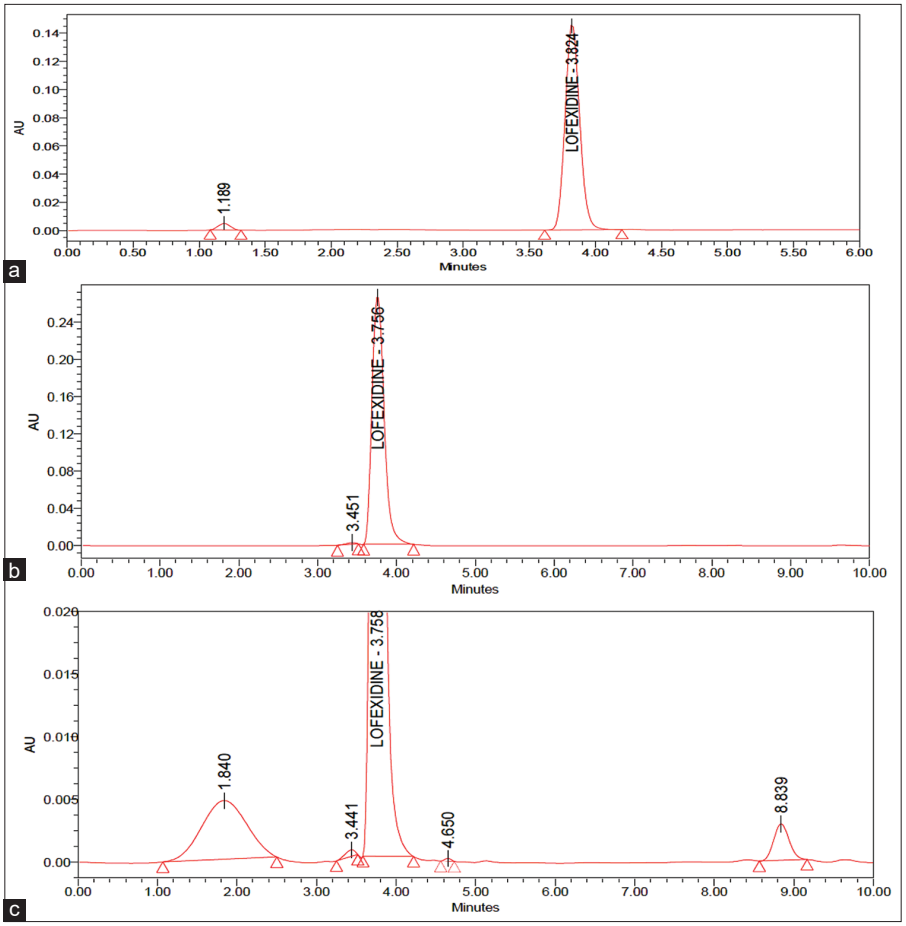

Fig. 10: (a) Chromatogram of acid degradation (1N), (b) Chromatogram of Acid degradation (5N-6 h), (c) Chromatogram of acid degradation (5N-24 H Zoomed)

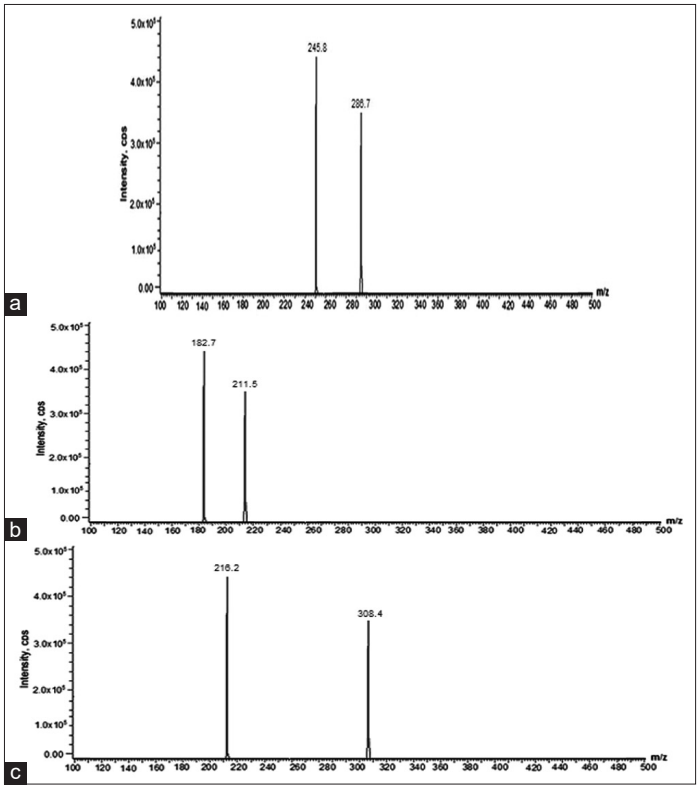

Fig. 11: (a) MS/MS Spectra of RT 1.840, (b) MS/MS Spectra of RT 3.441, (c) MS/MS SPECTRA OF RT 4.650

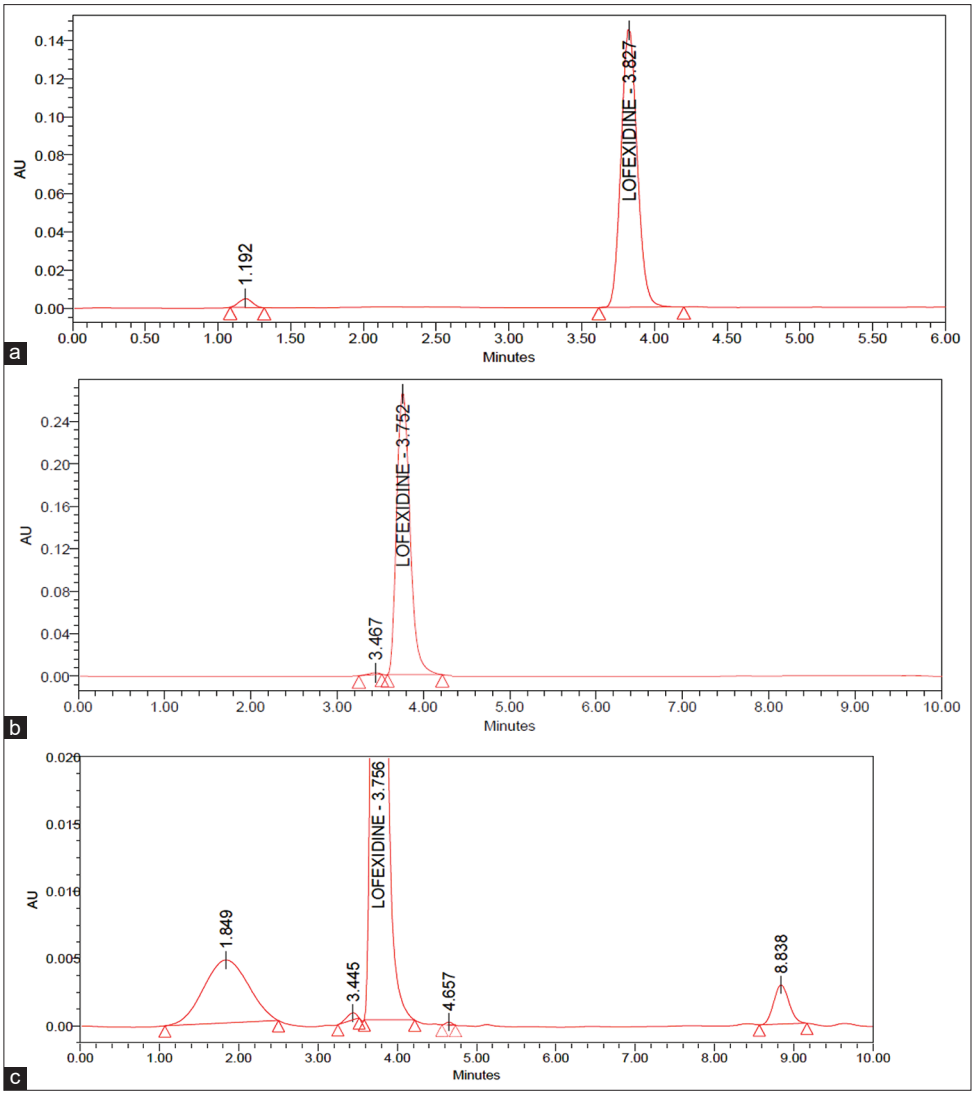

Fig. 12: (a) Chromatogram of peroxide degradation (3%), (b) chromatogram of peroxide degradation (30%-6 h), and (c) chromatogram of peroxide degradation (30%-24 h Zoomed)

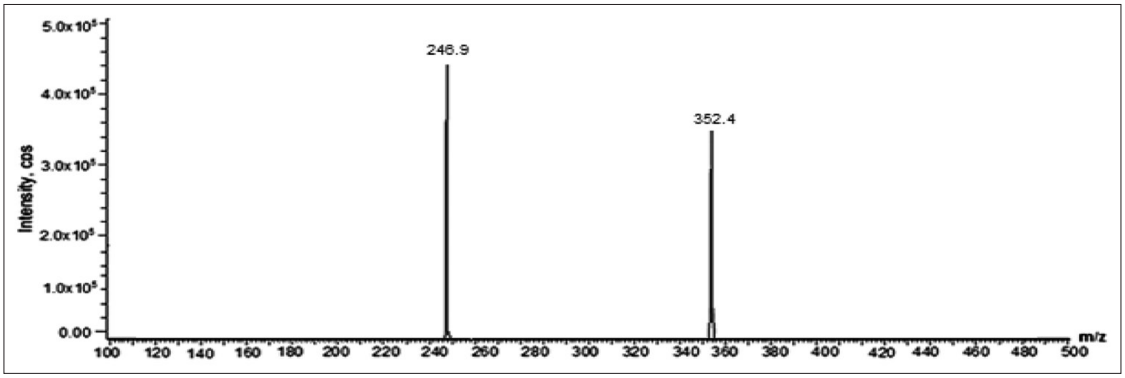

Fig. 13: MS/MS spectra of RT 8.838

ACIDDEGRADATION

Oxidative-induced degradation study

Under oxidative stress conditions with 3 % v/v H2O2, reflux conditions, 60°C one degradation product was formed at 24 h intervals. The HPLC chromatographic separation was depicted and the MS/MS studies for the formed DPs were mentioned and HRMS formula, error (ppm), and fragment ions are indicated in Table 10.

Hydrolytic degradation

Under hydrolytic stress conditions with water reflux for 24 h, two DPs were formed at 24 h intervals. The HPLC chromatographic separation

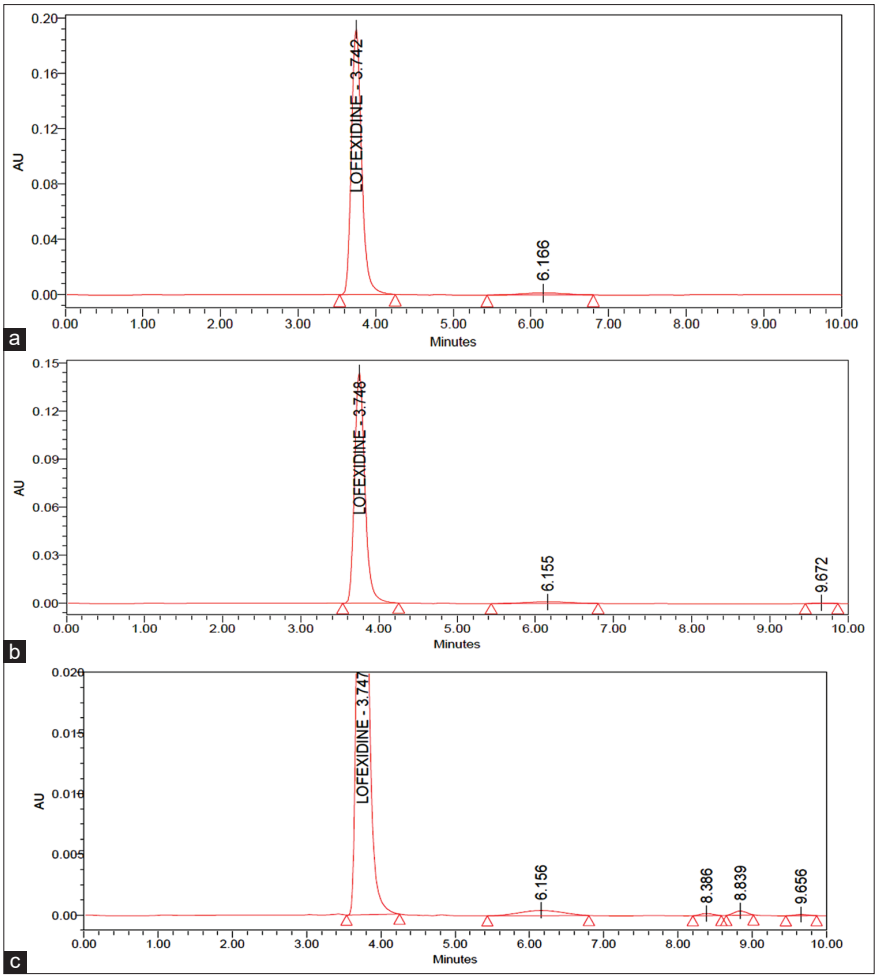

Fig. 14: (a) Chromatogram of hydrolytic degradation (6 h), (b) chromatogram of hydrolytic degradation (12 h), and (c) chromatogram of hydrolytic degradation (24 h Zoomed)

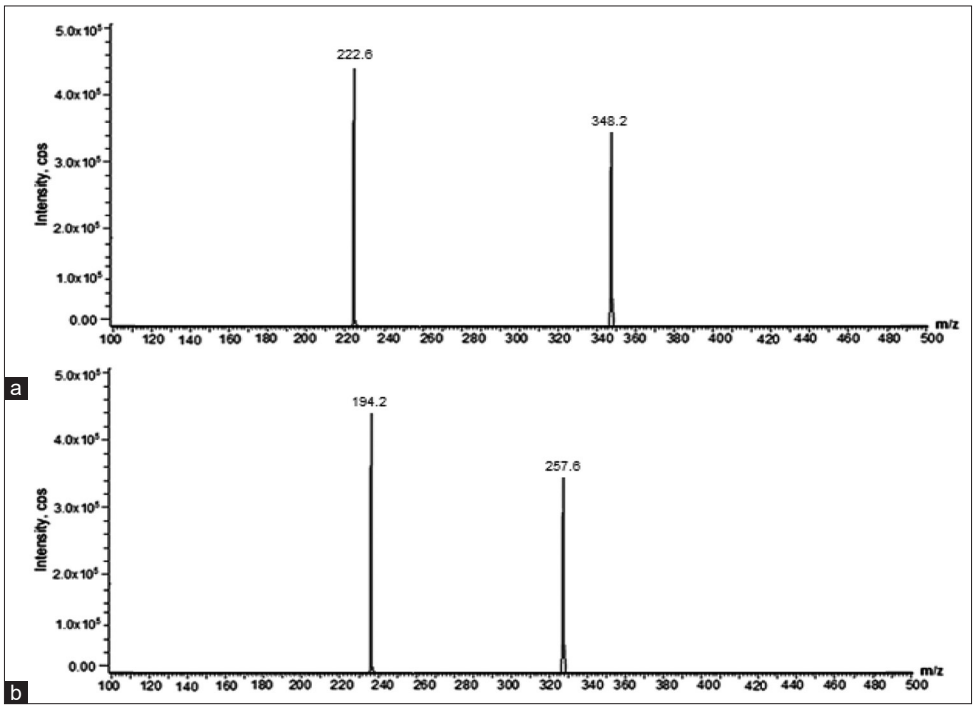

Fig. 15: (a) MS/MS spectra of RT 6.156, (b) MS/MS Spectra of RT 9.656

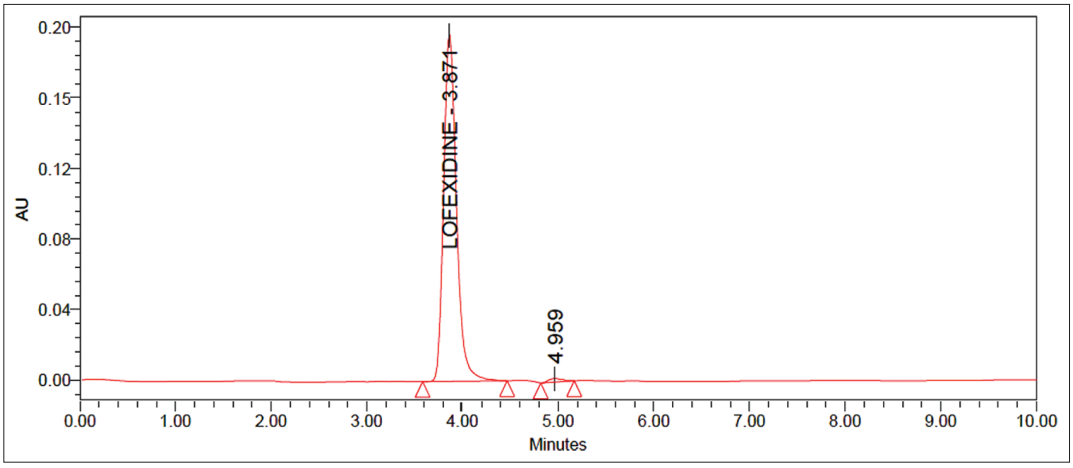

Fig. 16: Chromatogram of photolytic degradation (6 h)

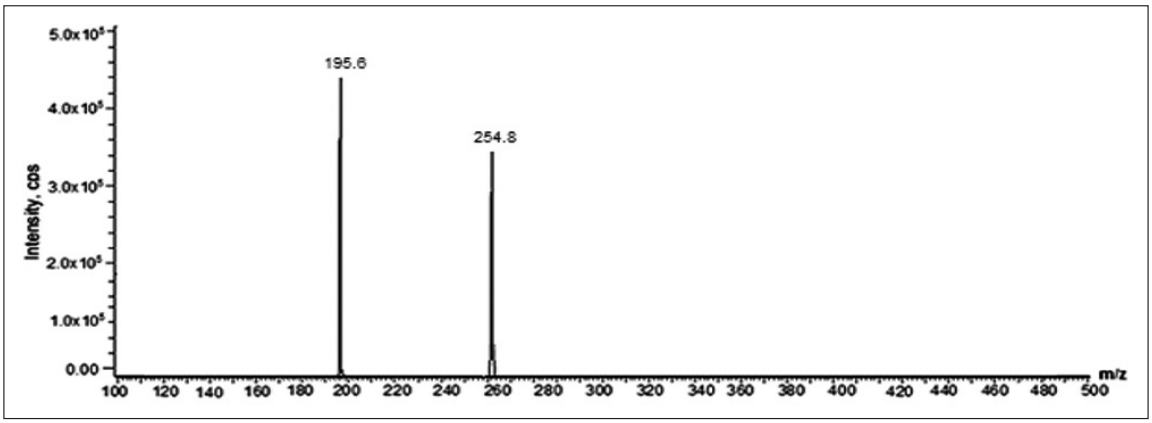

Fig. 17: MS/MS Spectra of RT 4.965

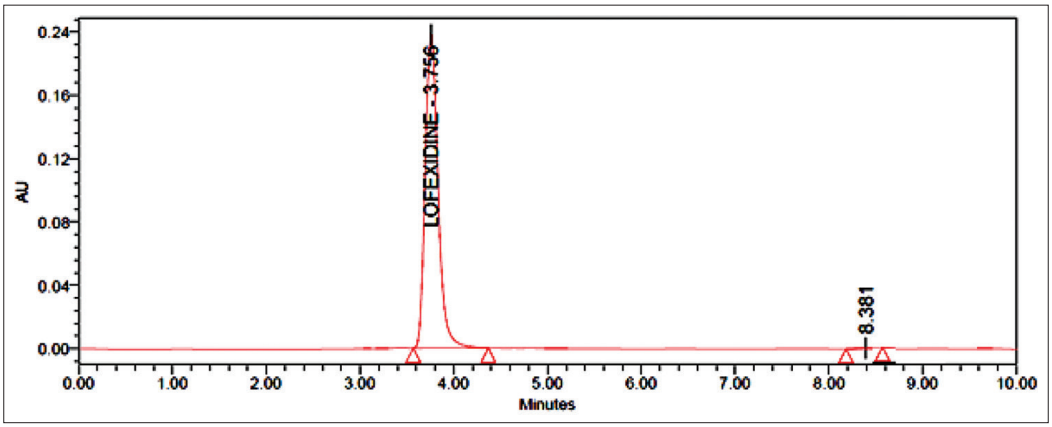

Fig. 18: Chromatogram of reduction degradation (6 h)

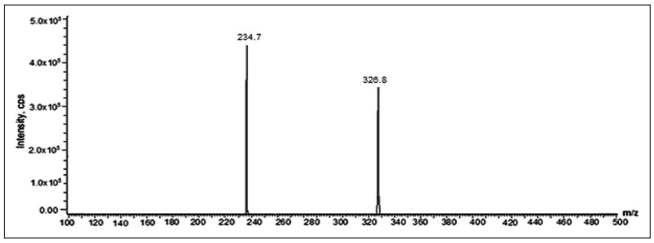

Fig. 19: MS/MS Spectra of RT 8.397

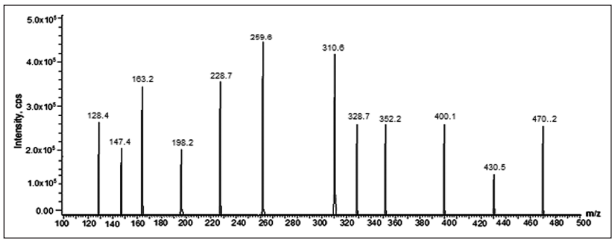

Fig. 20: Purity control MS/MS spectra of lofexidine

was depicted and the MS/MS studies for the formed DPs were mentioned in the Fig. 14 and 15 and HRMS formula, error (ppm), and fragment ions are indicated in Table 10.

HYDROLYSIS DEGRADATION

Photolytic degradation

Lofexidine drug was subjected to photolytic degradation for 24 h and one degradation product was formed at 24 h intervals. The HPLC chromatographic separation was depicted and the MS/MS studies for the formed DPs and HRMS formula, error (ppm), and fragment ions are indicated in Table 10.

Reduction degradation

Lofexidine drug was subjected to reduction degradation for 24 h and one degradation product was formed at 24 h intervals. The HPLC chromatographic separation was depicted and the MS/MS studies for the formed DPs and HRMS formula, error (ppm), and fragment ions are indicated in Table 10.

CONCLUSION

The developed LC-MS/MS method for the estimation of selected drug was simple, rapid, accurate, precise, robust, and economical. The study explored the stress degradation behavior of lofexidine under acidic, alkaline, oxidative, photolytic, hydrolytic, and thermal conditions were carried out according to ICH guidelines. The drug showed extensive degradation in acidic, alkaline, oxidation, and photolytic condition. DPs were separated and identified using LC-MS/MS. The study indicates that under various conditions as per ICH, they showed maximum degradation. For further proceed for the purification through preparative HPLC and toxicity studies for the identification of genotoxic impurities.

ACKNOWLEDGMENTS

1. The authors are thankful to shree icon laboratories, Vijayawada for providing necessary instruments to carry out the research work.
2. The authors express their gratitude to university college of pharmaceutical sciences for providing library and laboratory facilities to carry out the research work.

REFERENCES

1. Drug Random House. Vol. 1. Unbridge. Available from: https://dictionary.com [Last accessed on 2007 Sep 20].
2. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. QlA. Stab Test New Drug Substitute Products. Geneva, Switzerland: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 2006. p. R2.
3. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (R1): Validation of Analytical Procedures: Text and Methodology. In: International Conference on Harmonization. Geneva: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 2005.
4. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Impurities in New Drug Substances Q3A. In: International Conference on Harmonisation. Vol. R2. Geneva, Switzerland: International Federation of Pharmaceutical Manufacturers Associations; 2006.
5. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Impurities in New Drug Products Q3B. In: International Conference on Harmonisation 2006. Vol. R2. Geneva, Switzerland: International Federation of Pharmaceutical Manufacturers Associations; 2006.
6. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Impurities: Guideline for Residual Solvents Q3C. In: International Conference on Harmonisation. Vol. R6. Geneva, Switzerland: International Federation of Pharmaceutical Manufacturers Associations; 2016.
7. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Impurities: Guideline for Elemental Impurities. International Conference on Harmonisation 2014. Vol. Q3D. Geneva, Switzerland: International Federation of Pharmaceutical Manufacturers Associations; 2004.
8. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Q6A, Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances. Geneva, Switzerland: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 2006.
9. Heyden YV, Nijhuis A, Smeyers-Verbeke J, Vandeginste BG, Massart DL Smith; *et al*. Guidance for robustness/ruggedness tests in method validation. J Pharm Biomed Anal 2001;24:723-53. doi: 10.1016/s0731-7085(00)00529-x, PMID 11248467.
10. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. Harmonised tripartite Guideline q2(R_1), Current. 4th Version. Parent Guideline. Geneva: Step Publishing; 1994.
11. Al9Ghananeem AM. Pharmacokinetics of lofexidine hydrochloride in healthy volunteers. J Pharm Sci 2009;98:319-26. doi: 10.1002/jps.21401, PMID 18393298.
12. Yu E, Miotto K, Akerele E, O'Brien CP, Ling W, Kleber H, *et al*. Clinical pharmacokinetics of lofexidine, the α 2-adrenergic receptor agonist, in opiate addicts plasma using a highly sensitive liquid chromatography tandem mass spectrometric analysis. Am J Drug Alcohol Abuse 2009;34:611-6.