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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF PAROXETINE HYDROCHLORIDE AND CLONAZEPAM IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

The study describes development and subsequent validation of a stability indicating reverse-phase high-performance liquid chromatography method for the simultaneous estimation of Paroxetine hydrochloride and clonazepam in tablet dosage forms. A reversed-phase Kromasil ,C18, (150mm x 4.6 mm, particle size) column with mobile phase consisting of Acetonitrile and 0.1 % Orthophosphoric acid buffer 60:40 (v/v) was used. The flow rate was 1.2 mL min-1 and effluents were monitored at 260 nm. The retention times (tR) of Paroxetine and clonazepam were found to be 3.46 min and 4.55 min, respectively. The method was validated in terms of linearity, range, specificity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ). The linearity for both the drugs was found in the range of 125-750 µg/ml and 2.5-15 µg/ml for Paroxetine and clonazepam. The % recoveries of Paroxetine hydrochloride and clonazepam were found to be 99.4 -100.6 and 98.1-101.0 respectively. The utility of the procedure is verified by its application to marketed formulations that were subjected to accelerated degradation studies. The method distinctly separated the drug and degradation products even in actual samples. The products formed in marketed tablet dosage forms are similar to those formed during stress studies.

Key words: Paroxetine hydrochloride, Clonazepam, Stability indicating, RP-HPLC

INTRODUCTION

Paroxetine hydrochloride (PH) Chemically, (3S,4R)-3-[(2H-1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluoro phenyl)piperidine hydrochloride hemihydrate (Figure 1) belong to a class of antidepressant agents known as selective serotonin-reuptake inhibitor (SSRIs).It is used to treat major depressive disorder (MDD). Paroxetine likely inhibits the reuptake of serotonin at the neuronal membrane, enhances serotonergic neurotransmission by reducing turnover of the neurotransmitter, therefore it prolongs its activity at synaptic receptor sites and potentiates 5-HT in the CNS. It has the empirical formula of C19H20FNO3•HCl•1/2H_2O with a molecular weight 374.8. Paroxetine hydrochloride is an odorless, white to off-white crystalline powder. It is freely soluble in methanol, soluble in ethanol, sparingly soluble in dichloromethane and slightly soluble in water.

Clonazepam (CZ) chemically, 5-(2-chlorophenyl)-7-nitro-2,3-dihydro-1H-1,4-benzodia zepin-2-one (Figure2) belongs to the drug class benzodiazepines. It is prescribed for the treatment of anxiety and seizure disorders. Mechanism of action involves Allosteric interactions between central benzodiazepine receptors and gamma-minobutyric acid (GABA) receptors potentiate the effects of GABA. As GABA is an inhibitory neurotransmitter, this results in increased inhibition of the ascending reticular activating system. It's chemical formula and molecular weight is $C_{15}H_{10}ClN_3O_3$ and 315.7. Clonazepam is a light yellow crystalline powder

which is practically odorless. It is freely very soluble in methanol, ethanol, and acetone, and practically insoluble in water.

Literature survey states that Paroxetine hydrochloride and Clonazepam is official in IP [4], BP [5] and USP [6] with HPLC methods for the estimation individually. Combination of Paroxetine and clonazepam is not official in any monographs. Numerous UV[7],[24-28] Spectrophotometric [8][24-28], HPLC [9-21][29-32],HPTLC [22-23][33], LCMS [34-36] based methods have been reported for estimation of these drugs alone as well as in combination with other drugs in pharmaceutical dosage forms and biological fluids. A UV and Spectrophotometric methods was reported for the estimation of Paroxetine and Clonazepam in combined formulations [38-39]. But no stability-indicating assay method has been reported for the simultaneous determination of Paroxetine and Clonazepam in the presence of their degradants using the ICH [40- 41] approach of stress testing. Therefore, the present work was aimed to develop a simple, rapid, precise, and accurate isocratic reversed-phase stability indicating HPLC method for simultaneous determination of Paroxetine and Clonazepam in the tablet dosage forms. The developed method was validated as per ICH Guideline.

EXPERIMENTAL

Chemicals and reagents: Reference standards of Paroxetine and clonazepam were procured as gift samples from Dr.Reddys Laboratories, Hyderabad, India. HPLC grade Acetonitrile, water and Orthophosphoric acid were obtained from Merck India. Hydrochloric acid, NAOH, Hydrogen peroxide of Analytical grade were procured from Rankem, RFCL Limited, New Delhi, India.

Instrumentation and Apparatus: Analysis was performed on Waters HPLC system connected with PDA detector and Empower 2 software for data acquisition, Kromasil ,C18, 150mm x 4.6 mm, 5µ.,xs 205 Dual range balance (Mettler Toledo),Bandelin sonex sonicator, volumetric flasks, Pipettes, Beaker,measuring cylinders of Borosil glass were used for the development .

Chromatographic conditions: The isocratic mobile phase was consisted of Acetonitrile and 0.1% Orthophosphoric acid buffer 40:60 (v/v) .The mobile phase was sonicated for 30 min and filtered through a 0.45 µmembrane filter paper. Flow rate of mobile phase was 1.2 mL min-1. Diluent Consists of Water and acetonitrile (50:50).The variable wavelength UV–visible detector was set at 260 nm. Column temperature was maintained at 30^{o} c, injection volume of 10 µl and runtime of 6 min were optimized for separation of Paroxetine and clonazepam**.** The stress-degraded samples and the solution stability Samples were analyzed using a photo diode array (PDA) detector covering the range of 200 - 400 nm.

Preparation of standard stock solution (500µg/ml Paroxetine & 10µg/ml Clonazepam)
50 mg of Paroxetine and 1 mg of Clonazepam were accurately weighed and transferred to 10 mL volumetric flasks add 7ml of diluent, sonicated for 30 minutes and make up to the final volume with diluents. From the above stock solutions, 1ml was pipeted out in to a 10ml Volumetric flask and then make up to the final volume with diluent.

Preparation of sample solution: Ten tablets (Xet CR Plus, Zydus Cadila Healthcare Ltd India) were weighed and calculate the average weight of each tablet then the weight equivalent to 10 tablets was transferred into a 100 ml volumetric flask and added 30 mL of Diluent. The solution was ultrasonicated for 30 min and filtered through 0.45 micron membrane filter. From the filtered solution 4ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent to obtain concentration of 500 µg/ml of Paroxetine and 10µg/ml of Clonazepam and were subjected to HPLC analysis as described earlier. From the peak area of Paroxetine and clonazepam, the amount of drugs in samples was computed.

Forced degradation studies: From the previously mentioned stock solutions of standard drug and sample, 5 mL of aliquots were diluted separately up to 10 ml with 3% H2O2 (v/v), distilled water, 0.1 M HCl, and 0.1 M NaOH to achieve a concentration of 500µg/ml and 10µg/ml each of Paroxetine and Clonazepam respectively. Solutions in water, 0.1M HCl, and 0.1M NaOH were heated at 80°C for 24 h. For oxidative degradation, drugs were stored at room temperature (RT) in 3% H2O2 (v/v) for 48 h. Degradation was also carried out in solid state by exposing pure drugs and drug product to dry heat at 80°C for 48 h. Photolytic studies were carried out by exposing a thin layer of solid Paroxetine and Clonazepam and their packaged (blister strip) and loose (removed from the blister pack) tablets placed in a Petri-dish as well as the solutions of drugs and samples in 0.1 M HCl,0.1 M NaOH, and water to light in the photo stability chamber for 30days. Suitable controls were maintained under dark conditions. Samples were withdrawn initially and subsequently at prefixed time intervals. Samples were neutralized by either acid or alkali and were diluted

with Diluent to yield starting concentrations of 500µg/ml and 10µg/ml each of Paroxetine and Clonazepam respectively. Appropriate blanks were injected before analysis of forced degraded samples.

Method validation

Specificity: Specificity was tested against standard compounds and against potential interferences in the presence of placebo. No interference was detected at the retention time of paroxetine and clonazepam in sample solution.

Linearity: Linearity is studied to determine the range over which analyte response is a linear function of concentration. This study was performed by preparing standard solutions at seven different concentrations and analyses were performed in triplicate. The responses were measured as peak area. The calibration curves were obtained by plotting peak area against concentration.

Precision: The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision was considered at two levels, i.e. repeatability and intermediate precision, in accordance with ICH recommendations. Repeatability, or intra-day precision, was determined by performing nine analyses at three concentrations on the same day. Intermediate precision was determined by analyzing the same sample in the same way on different days. Results from determination of repeatability and intermediate precision were expressed as Standard deviation and Relative standard deviation.

Linearity and range: Six different concentrations (125,250,375, 500, 625, 750 and 2.5, 5, 7.5, 10, 12.5,15 µg/ml) of the mixture of two drugs were prepared for linearity studies. A typical HPLC chromatogram obtained during simultaneous determination of Paroxetine and clonazepam is given in Figure 3. The calibration curves obtained by plotting peak area against concentration showed linear relationship over a concentration range of 125 and 750 µg/ml for Paroxetine and 2.5-15 µg/ml for clonazepam. The linear regression equations for Paroxetine and clonazepam were found to be y = 3773x+124.25 and y = 65726x + 3870.3 respectively. The regression coefficient values (R2) were found to be 0.9999 and 0.9996 respectively indicating a high degree of linearity. Calibration curves of Paroxetine and Clonazepam are shown in Figure 4 & 5. Regression characteristics of the proposed HPLC method are given in Table 1.

Accuracy: The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as recovery (%), which is determined by the standard addition method.Samples were spiked with 50, 100, and 150% of the standard and analyzed. The experiment was performed in triplicate. Recovery (%) and RSD (%) were calculated for each concentration.

Limits of detection and limit of quantitation: The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines was used to calculate LOD and LOQ. Following formulae were used; LOD= 3.3×D/S and LOQ= 10×D/S, where, D is the standard deviation of the y-intercepts of regression line and S is the slope of the calibration curve.

RESULTS AND DISCUSSION

Method development: Several mobile phase compositions were tried to resolve the peaks of Paroxetine and Clonazepam. The optimum mobile phase containing acetonitrile and 0.1 % OPA buffer 40:60 was selected because it could resolve the peaks of Paroxetine (tR= 3.46 • }0.03 min) and Clonazepam (tR = 4.55 • }0.05 min) with a resolution factor of 4.92. UV overlaid spectra of Paroxetine and Clonazepam showed that both the drugs absorbed appreciably at 260 nm, so the same was selected as the detection wavelength during the studies.Optimun flow rate was tuned to 1.2 ml/min with 40:60 % of mobile phase composition gives good system suitability parameters.

Method validation

Linearity and range: Six different concentrations 125-750 µg/ml and 2.5-15µg/ml of the mixture of two drugs were prepared for linearity studies. A typical HPLC chromatogram obtained during simultaneous determination of Paroxetine and Clonazepam is given in Figure 3. The calibration curves obtained by plotting peak area against concentration showed linear relationship over a concentrations range of 125-750 µg/ml and 2.5-15µg/ml for both the drugs. The linear regression equations for Paroxetine and Clonazepam were found to be y = 3773x + 124.25 and y = 65726x + 3870.3 respectively. The regression coefficient values (R2) were found to be 0.9999 and 0.9996, respectively indicating a high degree of linearity. Calibration curves of Paroxetine and Clonazepam are shown in

Figure 3. Regression characteristics of the proposed HPLC method are given in Table 2.

Specificity: The specificity studies proved the absence of interference, since none of the peaks appeared at the retention time of Paroxetine and Clonazepam. The interaction study in standard solution was also carried out by comparing peak of each drug individually and in drug mixture.

Precision: From the standard stock solutions, mixed standards containing Paroxetine and Clonazepam were prepared. Standard solutions (n=3) were injected using a universal rheodyne injector with injection volume of 10 µL. The intra-day and inter-day precisions were assessed by analyzing standard solutions. The % RSD was found to be between 0.38 and 0.65 for both the drugs. The lower values of % RSD indicate that the method is precise. The results of Precission are shown in Table 3.

Accuracy: Recovery studies were carried out by applying the standard addition method. Known amounts of standard Paroxetine and Clonazepam corresponding to 80%, 100%, and 120% of the label claim were added to sample of tablet dosage form separately. The average % recoveries for Paroxetine and Clonazepam in marketed formulation were found to be 100.6 and 99.9 respectively. The results revealed that there was no interference of excipients. The results of accuracy are shown in Table 4.

Limit of detection (LOD) and limit of quantitation (LOQ): The limit of detection and limit of quantification were found to be 0.175 and 0.532 µg/ml for Paroxetine and 0.014 and 0.044 µg/ml for Clonazepam. The values indicate that the method is sensitive.

System suitability parameters: For system suitability parameters, seven replicate injections of mixed standard solution were injected and parameters such as the resolution, capacity factor, tailing factor, theoretical plate, retention volume and asymmetry factor of the peaks were calculated. The results are shown in Table 5.

Degradation studies

Acidic conditions: Both the drugs were found to be labile to acid hydrolysis in 0.1M HCl at 80°C. It was observed that Paroxetine and Clonazepam gradually degraded on heating at 80°C in 0.1M HCl for 24 h, forming degradation products showing retention time 5.65 and 7.47 min. Clonazepam showed higher degradation as compared to Paroxetine. At the end of 12 h, around 25% fall in Clonazepam peak area was observed. After refluxing for 24 h, drug was degraded by 60% with corresponding increase in concentration of the degradation products. Refer Figure 6 for chromatogram.

Degradation in alkali: Paroxetine was found to be highly labile to alkaline hydrolysis. Around 60% degradation of the drug was observed in 0.1M NaOH at 80°C within 2 h. The degradation peaks appeared at tR 2.29, 2.549 and 7.571 min whereas mild degradation was seen in Clonazepam in alkaline condition. It was observed that around 10–12% of the drug degraded on heating it in 0.1M NaOH for 24 h at 80°C. Refer Figure 7 for chromatogram.

Neutral (water) conditions: In neutral condition, Paroxetine and clonazepam was found to be relatively stable. Upon heating the drug solution in water at 80°C for 24 h, on further heating up to 48 h, there was no rise degraded peaks. Refer Figure 8 for chromatogram.

Oxidative conditions: Paroxetine was found to be relatively stable following exposure to oxidative condition (3% H2O2 at RT for 48 h) resulting in 4–5% degradation while Clonazepam was found to degrade more than 25%. Mild degradation was seen in clonazepam with appearance of single peak at 8.384 min whereas the degradation products of paroxetine appeared at tR 2.29 and 3.99 min. Refer Figure 9 for chromatogram.

Thermal stress: Thermo stable property of clonazepam was clearly observed when it was exposed to dry heat at 80°C for 48 h. profound degradation (15–20%) in paroxetine was seen with a single degradation peak at 2.54min. On the other hand, Clonazepam was found to be relatively stable in the study. Refer Figure 10 for chromatogram.

Photolytic conditions: Mild decomposition was seen on exposure of Paroxetine and Clonazepam solid drug powder and their tablets to light in the photo stability chamber. The photolytic exposure (30 days) of SS in 0.1M HCl and 0.1M NaOH resulted in 45% and 12% degradation, respectively.On the other hand standard Paroxetine and its tablet were found to be more stable under acidic photolytic stress conditions, resulting in 25% decomposition. Paroxetine and Clonazepam API and the pharmaceutical tablets were found to be sufficiently stable under neutral photolytic degradation conditions. Refer Figure 11 for chromatogram.

Analysis of marketed formulation: The developed method was successfully applied to analyze

Paroxetine and Clonazepam in marketed tablet formulations. A clear separation of the drugs and degradation products was achieved in tablet with no interference from excipients Figure 5. Analysis of marketed tablets (Xet CR Plus, Zydus Cadila Healthcare Ltd India) was carried out using optimized mobile phase and HPLC conditions. The average % drug content of tablets obtained by the proposed method for Paroxetine and Clonazepam were found to be 100.3 and 100.6 respectively, which showed that the estimation of dosage forms was accurate within the acceptance level of 95% to 105%. The results are given in the Table 6.

CONCLUSIONS

The present study envisages the stability behavior of Paroxetine and Clonazepam individually and in combination as per the ICH guidelines. Paroxetine was found to be more susceptible under stress conditions in comparison to Clonazepam and the results were given in Table No: 7. The method was found to be accurate and precise with good and consistent recoveries at all levels studied. The good % recovery in tablet forms suggests that the excipients present in the dosage forms have no interference in the determination. The %RSD was also less than 2% showing high degree of precision of the proposed method. In addition, simple isocratic elution and easy extraction procedure offered rapid and cost-effective analysis of Paroxetine and Clonazepam. The proposed method can be used for routine analysis of Paroxetine and Clonazepam in combined dosage form. It can be also used in the quality control in bulk manufacturing.

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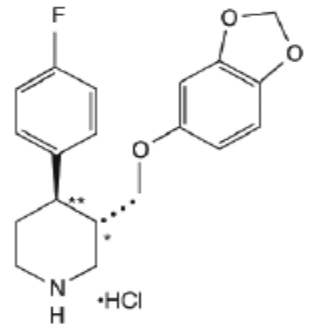

Fig 1: Structure of Paroxetine

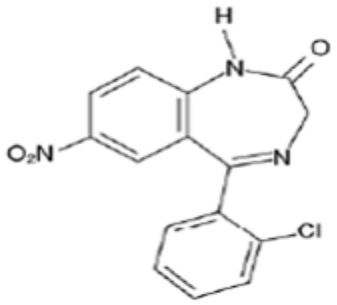

Fig 2: Structure of Clonazepam

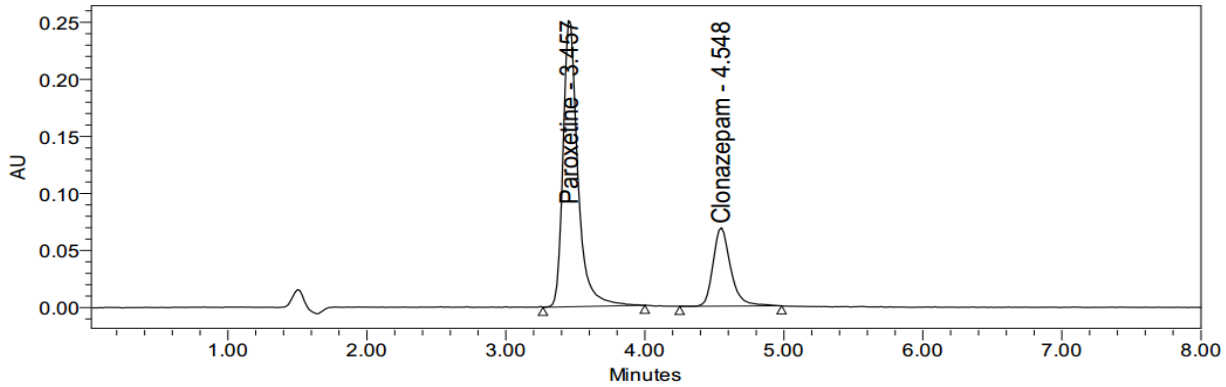

Figure 3 : Chromatogram of paroxetine and Clonazepam

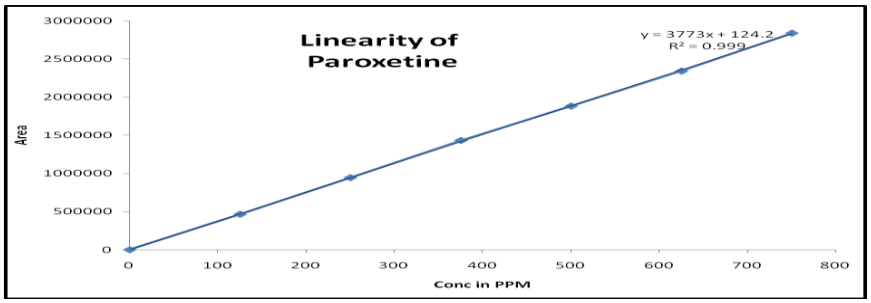

Figure 4: Calibration curves of Paroxetine

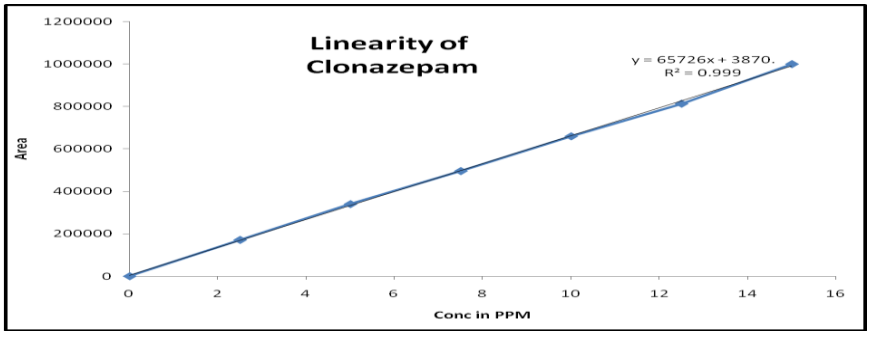

Figure 5: Calibration curves of clonazepam

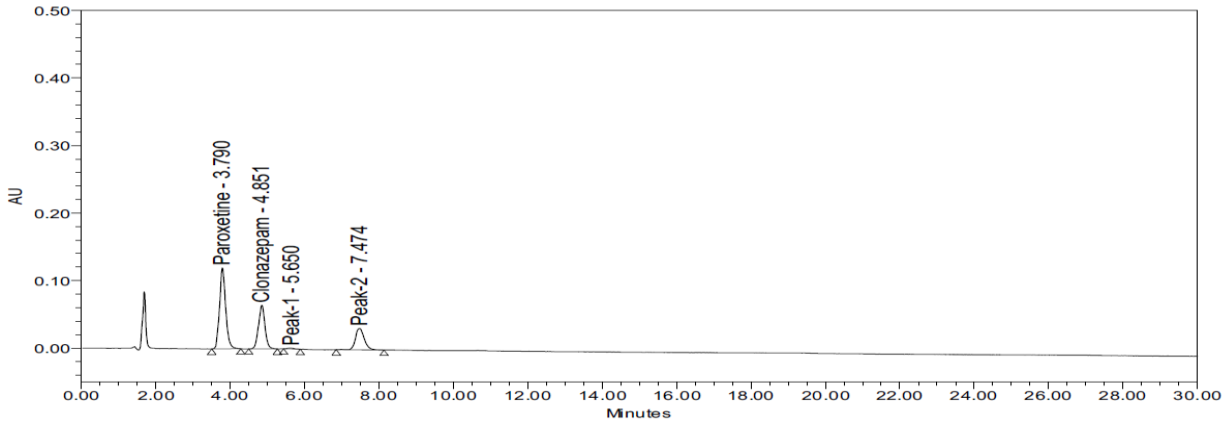

Figure 6: Acid degradation chromatogram of Paroxetine and Clonazepam

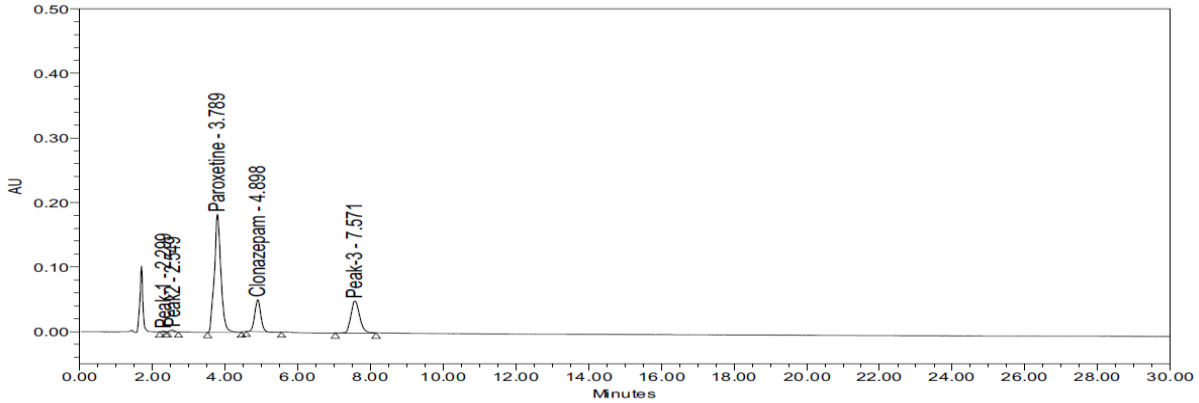

Figure 7: Alkali degradation chromatogram of Paroxetine and Clonazepam

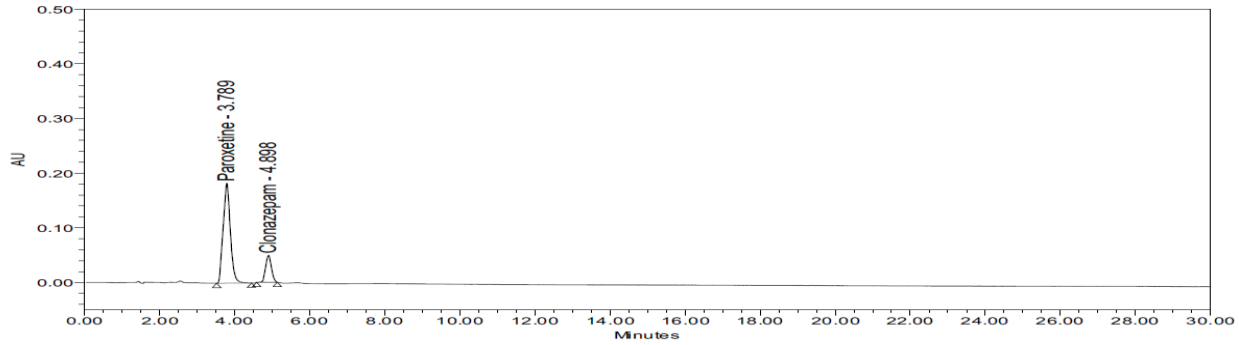

Figure 8: Neutral degradation degradation chromatogram of Paroxetine and Clonazepam

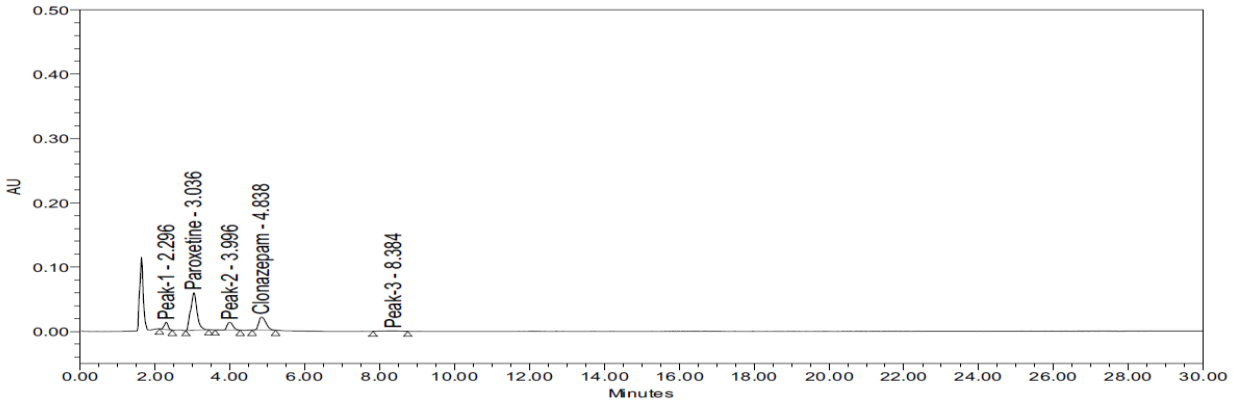

Figure 9:oxidative degradation chromatogram of Paroxetine and Clonazepam

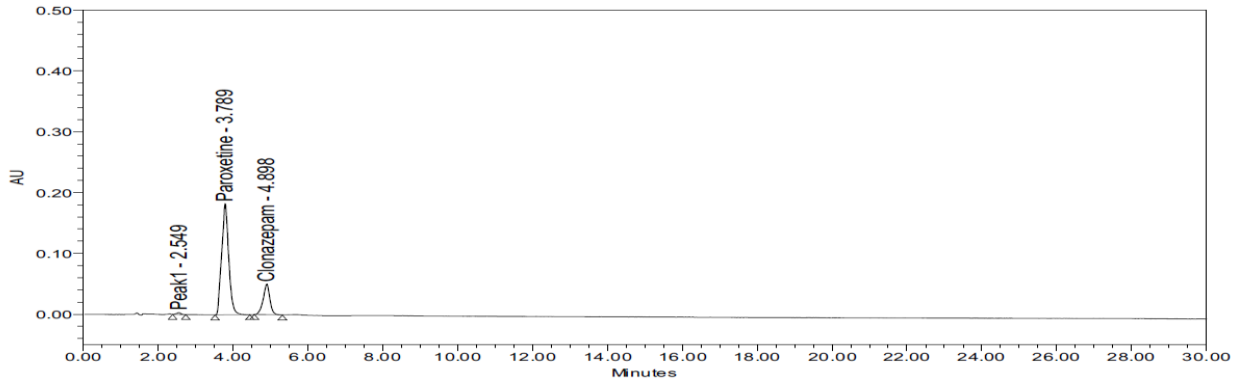

Figure 10: Thermal degradation chromatogram of Paroxetine and Clonazepam

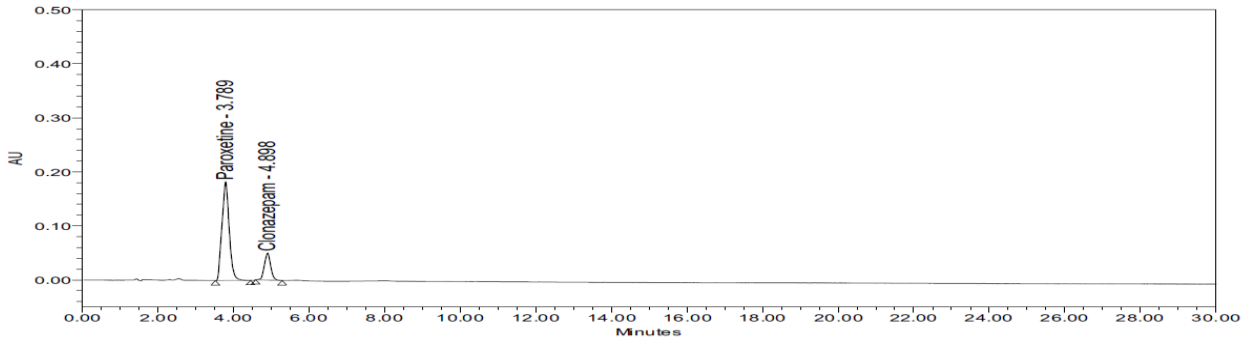

Figure 11: Photolytic degradation degradation chromatogram of Paroxetine and Clonazepam

Table-1: Optimized chromatographic conditions

Parameters	Condition
Mobile phase	0.1 % OPA Buffer and Acetonitrile taken in the ratio 60:40.
Diluent	Water and Acetonitrile (50:50)
Column	Kromasil 150mm x 4.6 mm, 5µ.
Flow rate	**1.2**ml/min
Wavelength	260nm
Injection volume	10µL

Table-2: Results of Linearity

Paroxetine Hcl		Clonazepam	
Concentration (µg/mL)	**Area**	**Concentration (µg/mL)**	**Area**
125	465158.7	2.5	171202
250	944795	5	339724.3
375	1432596	7.5	495364.3
500	1882528	**10**	659232
625	2341670	12.5	812853.7
750	2838249	15	999341

Table-3: Results of Precission

S.no.	Area of Paroxetine Hcl	S.no :	Area of Clonazepam
1	99.76374	1	100.28
2	100.577	2	100.4944
3	99.96671	3	98.6962
4	100.3912	4	99.60619
5	100.8058	**5**	99.4687
6	100.1551	6	99.39862
Average	100.2766	Average	99.65736
Standard Deviation	0.389172	Standard Deviation	0.650756
% RSD	0.388099	% RSD	0.652993

Table-4: Results of Accuracy

Level	Concentration added (µg/mL)		Concentration found (µg/mL)		% Recovery		Mean recovery	
	Paroxetine Hcl	Clonazepam	Paroxetine Hcl	Clonazepam	Paroxetine Hcl	Clonazepam	Paroxetine Hcl	Clonazepam
50 %	250	5	251.44	4.95	100.6	98.9		
100%	500	10	502.65	10.00	100.5	100.0	100.6	99.9
150%	750	15	756.0	15.11	100.8	100.7		

Table-5: Results of System suitability

Parameters		Results
Theoretical plates	Paroxetine	5874
	Clonazepam	6474
Tailing factor	Paroxetine	1.36
	Clonazepam	1.33
Resolution		4.962
Retention time	Paroxetine	3.39
	Clonazepam	4.41
Linarity Range µg/ml	Paroxetine	125-750µg/ml
	Clonazepam	2.5-15 µg/ml
% RSD	Paroxetine	1.4
	Clonazepam	1.0

Table-6: Assay Results of Market formulation

Market formulation	Label claim (mg)		Quantity of API found (mg)		Assay %	
Xet CR Plus, ZydusCadila Healthcare Ltd	Paroxetine Hcl	Clonazepam	Paroxetine Hcl	Clonazepam	Paroxetine Hcl	Clonazepam
	12.5	0.25	12.48	0.25	99.9	100.1

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