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Isocratic RP-HPLC Method Development, Validation and Stability Indicating Studies for Simultaneous Determination of Escitalopram and Clonazepam In the Combined Dosage Form

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ABSTRACT

A new rapid, sensitive and precise stability indicating HPLC method was developed on kromasil RP-C18 column (250mm X 4.6mm, 5µm), for the simultaneous analysis of Escitalopram and Clonazepam. Chromatographic separation was achieved at 229nm by using a mobile phase composed of methanol, acetonitrile and phosphate buffer (70: 28: 2, v/v). Retention time of escitalopram and clonazepam were found to be 2.67min and 3.46min respectively. Method validation was done as per the ICH guidelines. Good linearity was established in the concentration range of 50-300µg/ml and 2.5-15µg/ml by escitalopram and clonazepam respectively. Resolution of escitalopram and clonazepam was found to be 5.25. Percentage RSD of precision was found to be less than 2. The percentage assay of escitalopram and clonazepam in commercial formulation was found to be 99.18 and 99.22 respectively. Stability of the molecule was measured by inducing different stress conditions like acidic, basic, peroxide, thermal, light and UV light.

Keywords: Escitalopram, clonazepam, HPLC method and stability indicating studies.

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INTRODUCTION

Escitalopram [1-4] is chemically known as (1S)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile. It belongs to selective serotonin reuptake inhibitor category. It acts as antidepressant[5,6]. It is also used to treat major depressive disorders. Drowsiness, insomnia and diarrhoea are the common side effects. Clonazepam [7,8] belongs to the class of benzodiazepine tranquilizer. Chemically, it is known as 5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one. It is used to treat panic disorder, akathisia and seizures. Common side effects of clonazepam are sleepiness and agitation. Structures of escitalopram and clonazepam were shown in Figure 1 and Figure 2.

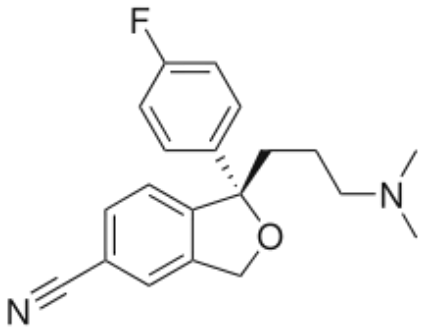


Figure 1: Structure of escitalopram

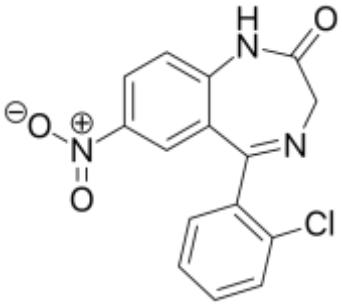


Figure 2: Structure of clonazepam

Numerous methods [9-18] were illustrated for the estimation of escitalopram and clonazepam in the pharmaceutical formulation. At the same time, several methods were reported for the determination of escitalopram and clonazepam in biological samples [19-21]. Few stability indicating studies[22,23] of escitalopram and clonazepam were described to measure the stability of the molecule under different stress conditions.

Instrumentation

PEAK chromatographic system equipped with LC-P7000 isocratic pump was used for the chromatographic separation. Wavelength of the maximum absorbance was determined with the help of Teccomp UV-2301 double beam UV-visible spectrophotometer. Sample solutions were

injected into HPLC system by Rheodyne injector fitted with 20µl fixed volume loop. Weighing of the standard and sample drugs was done on Denver electronic analytical balance (SI-234). Systronics digital pH meter was used to measure the pH of the solutions. Hitachi software was used to record the data.

Reagents and Material

Micro Labs Ltd and Unichem Laboratories Ltd gave the drug samples and escitalopram and clonazepam working standard as gift. Commercial formulation of escitalopram and clonazepam was procured from the local market. All the reagents used were of HPLC grade and buffer solutions (AR grade) were purchased from Merck Specialties Private Limited, Mumbai, India.

Preparation of standard stock solution

Initially, accurately weighed 10mg of escitalopram and 0.5mg of clonazepam were taken into 10ml volumetric flasks separately and then dissolved in 10ml of methanol. After sonication, the working standard solutions of escitalopram and clonazepam were obtained by the method of dilution of respective standard solutions with solvent.

Preparation of sample solution

Sample solution was prepared from twenty tablets of escitalopram and clonazepam (Escilo-Forte brand), which were finely ground to uniform size powder. Initially, accurately weighed 10mg of escitalopram was transferred into 10ml volumetric flask and then dissolved in 5ml methanol. The resultant solution was sonicated for about 15min. Later, the solution in the flask was filled up to the mark with mobile phase. Finally, after filtration, the solution was diluted with mobile phase to a concentration of 200µg/ml. A concentration of 10µg/ml of CLZ was obtained simultaneously as claimed on the label. This combined solution was used for the analysis of escitalopram and clonazepam simultaneously in pharmaceutical formulation.

METHOD DEVELOPMENT

The purpose of the developed method was to resolve escitalopram and clonazepam under optimum chromatographic conditions. Appropriate wavelength was selected by scanning standard solutions of escitalopram and clonazepam on UV-visible spectrophotometer. The two active ingredients showed optimum separation near 229nm and hence the analysis was carried out at this wavelength only. Several trials were conducted for the selection of mobile phase and optimum separation was achieved by the utilization of mobile phase of methanol, acetonitrile and phosphate buffer in 70: 28: 2 (v/v). Temperature was found to show negligible effect on tailing factor and resolution and thus the entire analysis was done at room temperature. Optimum chromatographic conditions were shown in Table 1. Chromatogram of escitalopram and clonazepam standard was given in Figure 3.

Table 1: Optimized chromatographic conditions

S.no	Parameter	Results
1	MP	Methanol: Acetonitrile: pH 6.2 phosphate buffer in 70:28:02 (v/v)
2	Wavelength	229nm
3	Stationary Phase	Kromasil RP- C18
4	pH of MP	6.2
5	Flow Rate	1.0ml/min
6	Pump Mode	Isocratic
7	Pump Pressure	11.8±7MPa

METHOD VALIDATION

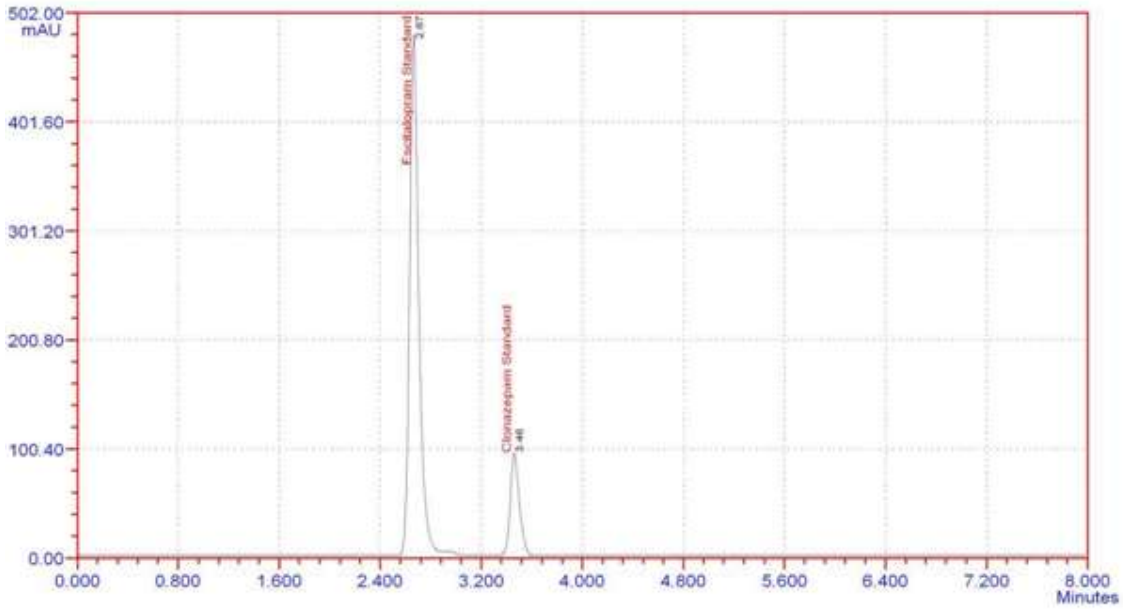
Validation parameters of the proposed method were determined by following ICH guidelines. Freshly prepared standard stock solution was used to evaluate the system suitability conditions. Results of system suitability conditions were listed in Table 2 Linearity of the method was determined by the preparation of various aliquots. Six different concentrations in the range of 50-300µg/ml for escitalopram and 2.5-15µg/ml for clonazepam were prepared from the standard stock solution by serial dilution. Linearity results were given in Table 3 Corresponding calibration curves were given in Figure 4 and Figure 5 Basing on the recovery studies, accuracy of the method was measured.

Table 2: System suitability conditions

S.No	Parameter	Results
1	API Concentration	Escitalopram – 200µg/ml Clonazepam - 10µg/ml
2	RT	Escitalopram – 2.67min Clonazepam – 3.46min
3	Resolution	Escitalopram – ……. Clonazepam – 5.25
4	Area	Escitalopram – 242195 Clonazepam - 56040
5	Theoretical Plates	Escitalopram – 5540 Clonazepam - 7571
6	Tailing Factor	Escitalopram – 1.59 Clonazepam – 1.17

Table.3. Results of linearity

S.No	Escitalopram		Clonazepam	
	Concentration in µg/ml	Peak Area	Concentration in µg/ml	Peak Area
1	50	143700	2.5	25137
2	100	178120	5	36452
3	150	214072	7.5	46490
4	200	242195	10	56040
5	250	278571	12.5	67104
6	300	312949	15	78019



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Escitalopram Standard	2.667	47884	242195.1	82.748	1.59	5540	0.00
2	Clonazepam Standard	3.460	9983	56040.4	17.252	1.17	7571	5.25
	Sum:		57867	298235.5	100.0000			

Figure 3: Chromatogram of escitalopram and clonazepam standard

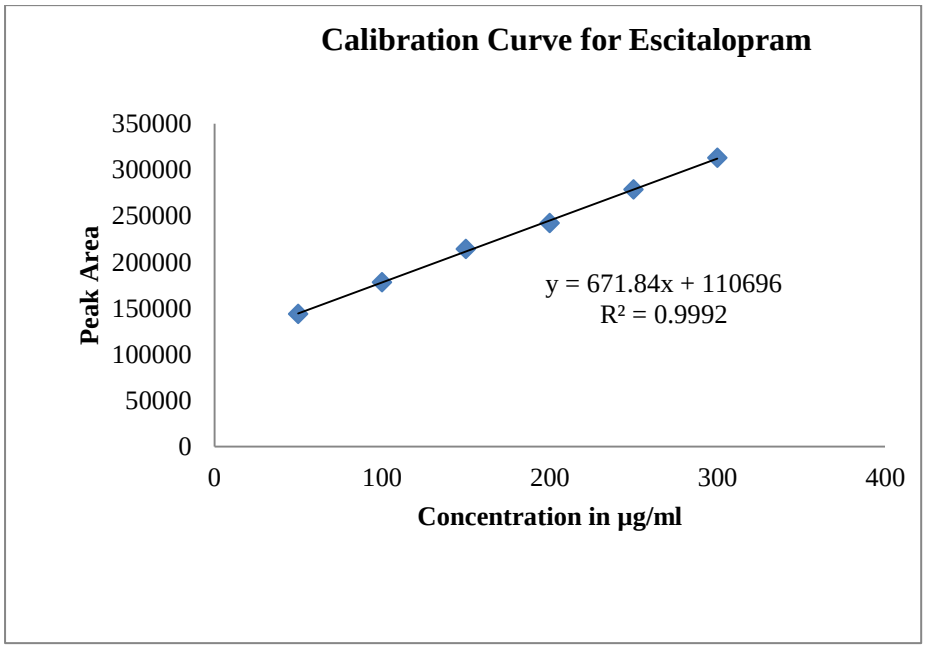


Figure 4: Calibration curve of escitalopram

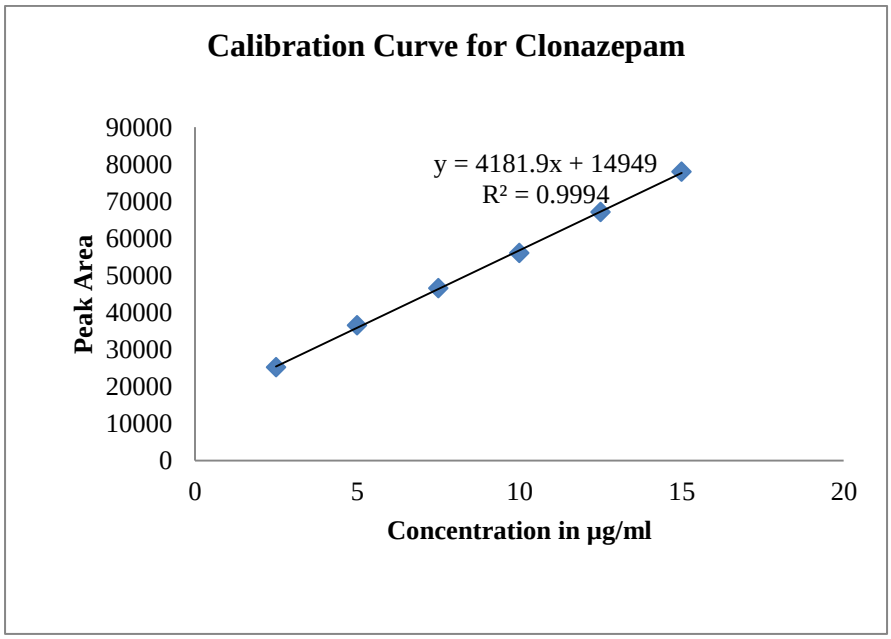


Figure 5: Calibration curve of clonazepam

Precision of the method was determined through the measurement of intra-day precision and inter-day precision. The percentage RSD in both the cases was found to be less than two. Small changes were introduced in validation parameters (wavelength, pH and mobile phase) to measure the robustness of the method. Sensitivity of the method was determined through the determination of LOD and LOQ. Stability of the sample solution was tested by testing the solution at the regular time intervals. Freshly prepared sample solution was utilized to measure the percentage assay of the escitalopram and clonazepam formulation. Results of validation parameters were furnished in Table 4.

Table 4: Results of validation parameters

S.No	Parameter	Escitalopram	Clonazepam	Limit
1	% Recovery	98.1656 – 101.578	98.563 – 101.572	98 – 102%
2	Intra-day precision	0.802	0.672	Below 2
3	Inter-day precision	1.410	0.741	Below 2
4	Ruggedness	0.639	1.751	Below 2
5	Robustness	0.1366 – 1.6371	0.1106 – 1.4365	Below 2
6	LOD	1.5	5.0	---
7	LOQ	0.075	0.25	---
8	Solution stability	24hr	24hr	---
9	% Assay of formulation	99.184	99.22	98 – 102%

FORCED DEGRADATION STUDY

Stability of the escitalopram and clonazepam was measured through forced degradation studies. Different stress conditions like acid, aqueous, base, peroxide, UV light, thermal and light were

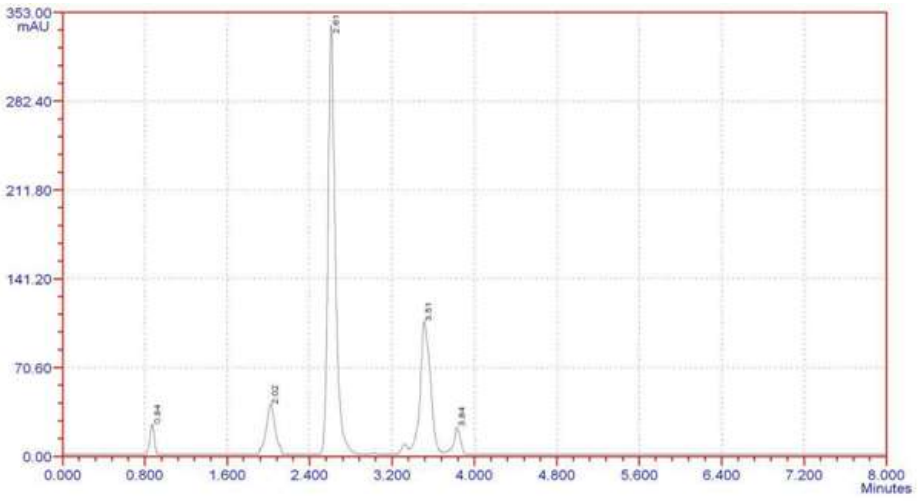
applied on the sample solution to determine the number of degradents in each and every case. Results of forced degradation study were tabulated in Table 5.

Table 5: Forced degradation studies

S. No	Condition	No of degradation Peaks Observed
1	Acidic	3
2	Aqueous	1
3	Base	2
4	Peroxide	2
5	Sun Light	1
6	UV light	2
7	Thermal	2

Acid degradation

In order to prepare acid hydrolyzed sample solution 300mg of sample was dissolved in 20ml of 0.1N hydrochloric acid. After 48hr, 5ml of this solution was initially neutralized with 5ml of 0.1N sodium hydroxide solution. Then, the solution was diluted to a volume of 25ml with diluents in a 25ml volumetric flask. Finally, the number of degradents were evaluated on comparison with the standard values. Acid degradation chromatogram was given in Figure 6.



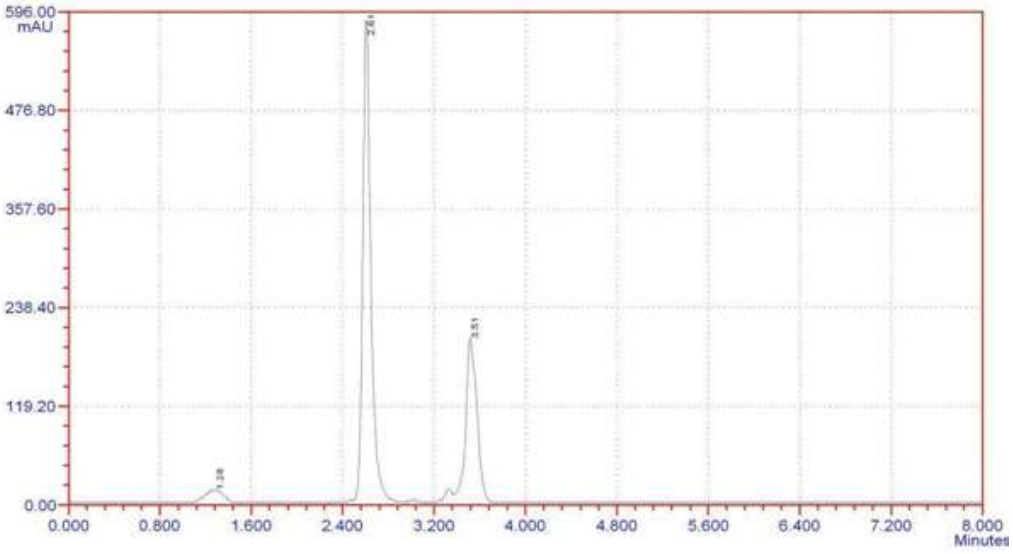
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		0.840	402	11931.5	3.739	2.86	1006	0.00
2		2.024	648	5838.3	1.829	1.07	3087	2.16
3		2.609	35894	237903.8	74.548	1.20	4087	2.64
4		3.510	9602	55948.4	17.532	1.18	7234	5.11
5		3.839	843	7506.9	2.352	0.80	3704	1.57
	Sum:		47389	319128.9	100.0000			

Figure 6: Chromatogram of acid degradation

Aqueous degradation

Aqueous sample solution was prepared by dissolving 100mg of sample in HPLC water and the solution was undisturbed for about two days. The resulting sample solution was diluted and then

20µl of the solution was injected into HPLC system to measure the degradation chromatogram (Figure 7).

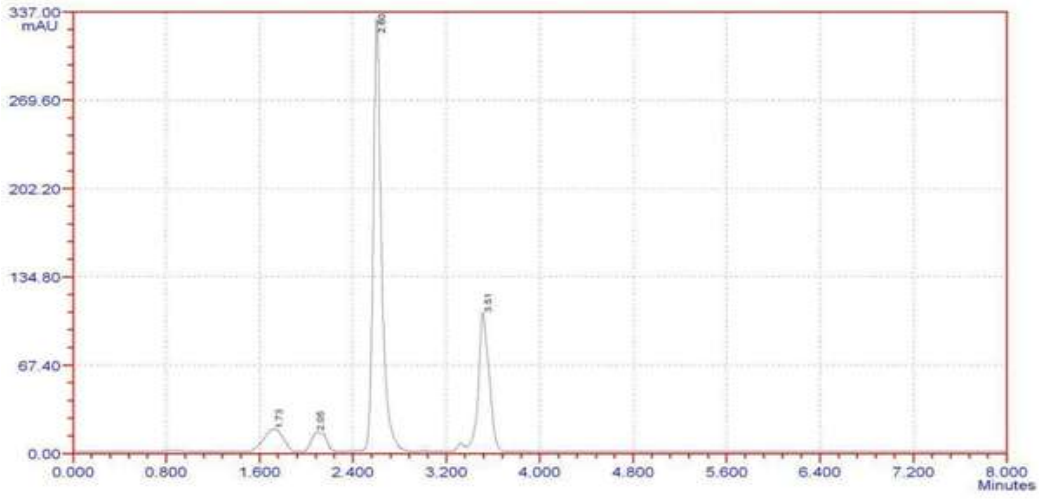


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.280	973	12087.3	3.904	0.91	1212	0.00
2		2.607	32615	241899.7	78.138	0.66	2462	4.72
3		3.515	9698	55592.1	17.957	1.05	7493	4.88
	Sum:		43286	309579.1	100.0000			

Figure 7: Chromatogram of aqueous degradation

Base degradation

Base degradation sample solution was prepared by dissolving 300mg of sample in 20ml of 0.1N sodium hydroxide solution. After 48hr, 5ml of 0.1N hydrochloric acid was used to neutralize 5ml of the above solution. Then, the resultant solution was made up to the mark with diluents in 25ml volumetric flask. Corresponding degradation chromatogram was shown in Figure 8.

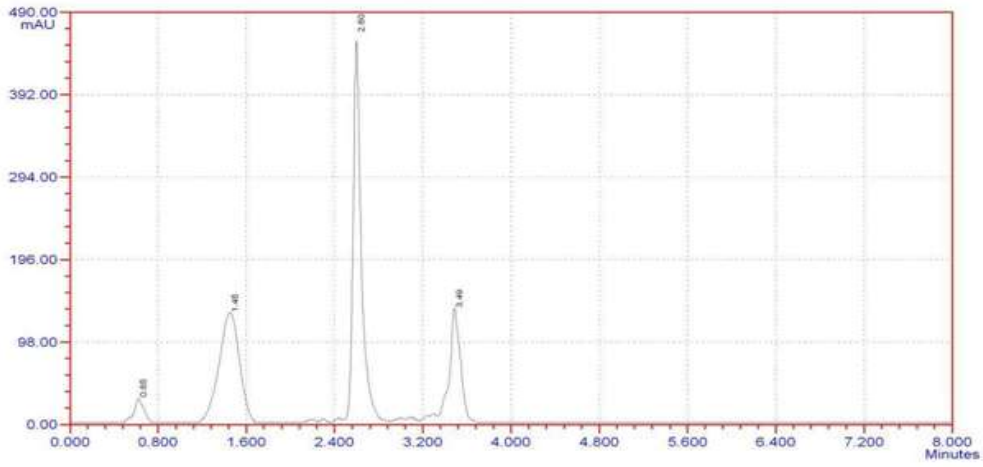


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.725	1871	23352.4	4.043	0.89	1381	0.00
2		2.603	34901	249693.4	43.230	0.66	2639	3.16
3		2.045	1884	6854.3	1.187	0.00	6298	3.65
4		2.603	34901	242617.0	42.005	0.68	2795	3.72
5		3.512	10157	55076.8	9.536	1.09	8359	5.18
	Sum:		83714	577594.0	100.0000			

Figure 8: Chromatogram of base degradation

Peroxide degradation

Initially, 300mg of sample was dissolved in 20ml of 3% hydrogen peroxide and allowed to stand for two days. In a 25ml volumetric flask, 5ml of this solution was diluted accurately up to the mark by using diluent. Degradation chromatogram was obtained (Figure 9) after injecting 20µl of the oxidized sample solution.

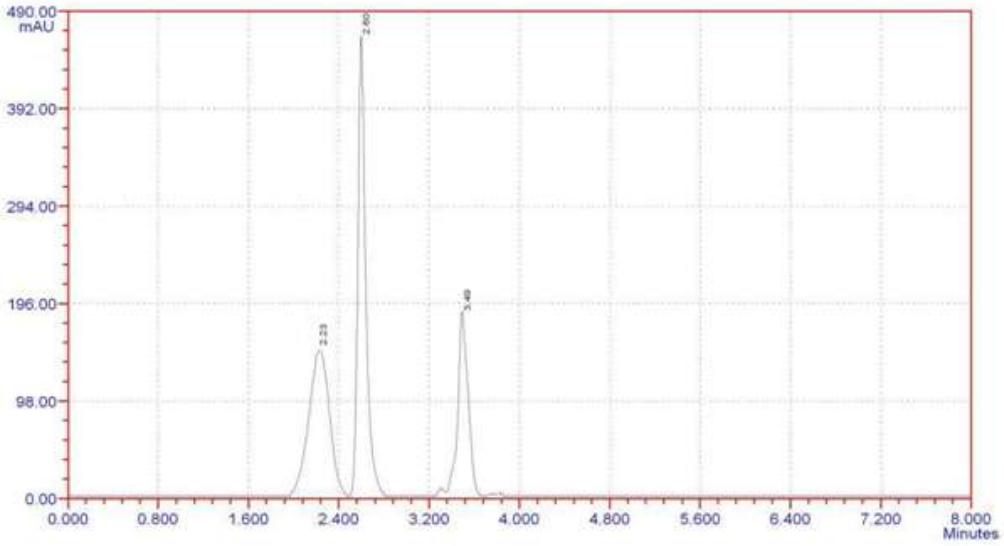


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		0.650	125	1473.5	0.484	0.90	1761	0.00
2		1.452	4780	58276.9	19.162	0.82	2283	2.36
3		2.598	11942	190678.6	62.698	0.57	5528	2.87
4		3.487	3987	53693.5	17.655	0.92	9336	2.13
	Sum:		20834	304122.5	100.0000			

Figure 9: Chromatogram of peroxide degradation

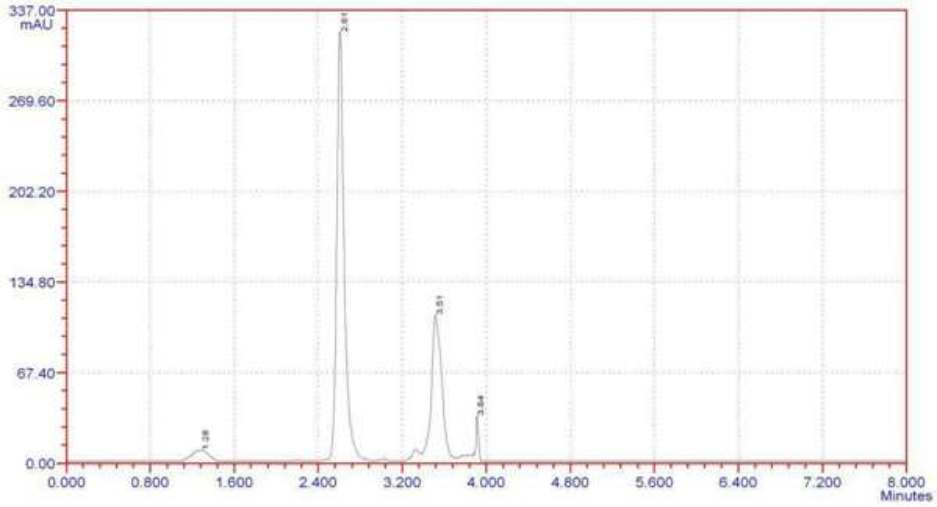
Light and UV light degradation

Open petri dish was used to expose the sample under normal light and UV light for about two days. Two sample solutions were injected into HPLC system separately and the corresponding degradation chromatograms were shown in Figure 10 and Figure 11 respectively.



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		2.230	995	17461.6	5.723	1.02	2489	0.00
2		2.597	10762	231264.6	75.799	0.57	5929	3.35
3		3.493	4315	56376.3	18.478	1.45	11942	5.83
	Sum:		16072	305102.5	100.0000			

Figure 10: Chromatogram of light degradation

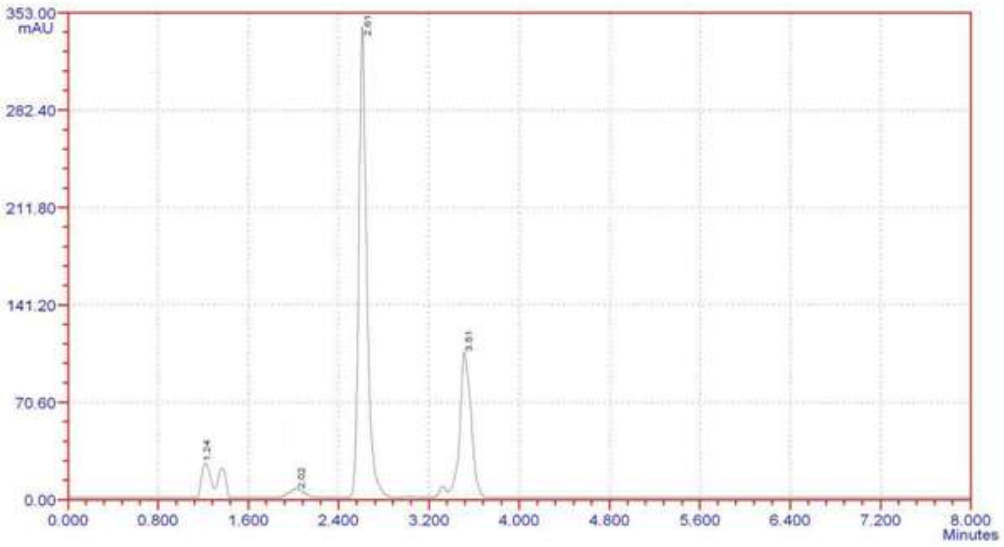


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.280	1017	13233.5	4.256	0.99	1193	0.00
2		2.607	32613	231782.1	74.547	0.72	2681	4.65
3		3.515	9698	53988.9	17.364	1.29	7945	5.06
4		3.845	966	11917.7	3.833	1.31	1936	1.30
	Sum:		44294	310922.2	100.0000			

Figure 11: Chromatogram of UV light degradation

Thermal degradation

Sample was taken in a petri dish and was placed in oven maintained at 40^0C to 80^0C for about 48hr. 20µl of this sample solution was injected into HPLC system and the resultant chromatogram (Figure 12) was recorded.



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.235	1407	26020.8	7.422	0.90	2602	0.00
2		2.024	1914	35602.7	10.156	0.78	3440	1.50
3		2.609	35894	234405.5	66.864	1.14	5894	1.64
4		3.510	9602	54539.0	15.557	1.37	7613	5.21
	Sum:		48817	350568.0	100.0000			

Figure 12: Chromatogram of thermal degradation

RESULTS AND DISCUSSION

In the present investigation an attempt was made to develop a precise, sensitive and rapid HPLC method for the determination of escitalopram and clonazepam in the commercial formulation. Optimum chromatographic separation was achieved at a wavelength of 229nm by the use of a mixture of methanol, acetonitrile and phosphate buffer (70: 28: 2, v/v). At a flow rate of 1.0ml/min, the elution was very rapid and the elution was completed in less than 4min. The results obtained were found to be within the expected limits.

Linearity range reported for escitalopram and clonazepam was found to be good. Recovery results revealed the accuracy of the method. Precision of the two drugs was examined by injecting six replicates. The results supported the preciseness of the method.

Reliability of the method was tested with respect to deliberate variations in method parameters. The percentage change calculated in each and every changed condition was found to be less than 2%. LOD and LOQ values of escitalopram and clonazepam emphasized the sensitivity of the

proposed method. The sample solution was found to be stable up to 24hr. The percentage assay of escitalopram and clonazepam were found to be 99.184 and 99.22 respectively. In addition to method validation, the current investigation also provides stability studies of escitalopram and clonazepam.

CONCLUSION

The present approach i.e. the stability indicating method development and validation studies of escitalopram and clonazepam, provides priceless information to quality control and pharmaceutical industries. Thus, the proposed method is suitable for the investigation escitalopram and clonazepm in pharmaceutical formulation.

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