5.1. DRUG PROFILE

5.1.1. Escitalopram

Escitalopram(ESC) [1-4] is an antidepressant [5-6] and belongs to the class of a selective serotonin reuptake inhibitor. Ethanol uptake can be reduced effectively by the use of ESC. Occasionally ESC is used to treat dysmorphic and anxiety disorder [7-9]. Major depressive disorders (MDD) and generalized anxiety disorders (GAD) in adults and children over 12 years can be treated with ESC. Serotonin balance in brain can be restored by using ESC. ESC is more preferred than tricyclic antidepressants for the treatment of tardrive dyskinesia in depressed patients. Over dosage of ESC causes dizziness, vomiting, nausea, convulsions and sinus tachycardia. Common side effects of ESC generally include headache, drowsiness, diarrhoea, nausea, insomnia and ejaculatory disorder.

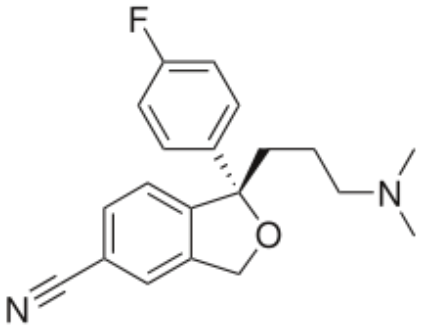


Figure.5.1. Structure of escitalopram

IUPAC name: (1S)-1-[3-(Dimethylamino) propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran- 5-carbonitrile

Molecular formula: $C_{20}H_{21}FN_2O$

Molecular weight: 324.4

Melting point: 147-148^{0}C (oxalate)

Brand name: Lexapro, Cipralex

5.1.2. Clonazepam

Clonazepam(CLZ) [10-11] is studied under the category of benzodiazepine tranquilizer. It can be used for the treatment of seizures, panic disorder and akathisia (movement disorder). The action of clonazepam starts within one hour and can be carried to six to twelve hours. Most regular side effects are agitation, poor coordination and sleepiness. This drug on continuous usage for long period of time may cause in dependence, tolerance and withdrawal symptoms. Confusion, drowsiness and dizziness like side effects may increase when CLZ and ESC are used together.

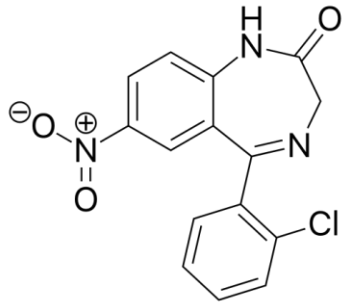


Figure.5.2. Structure of clonazepam

IUPAC name: 5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one

Molecular formula: $C_{15}H_{10}ClN_3O_3$

Molecular weight: 315.7

Melting point: 236.5-238.5^{0}C

Brand name: Klonopin, Rivotril, Clonotril

Table.5.1. List of brand names of combined formulations of escitalopram and clonazepam

S.No	Brand name	Available strength		Formulation	Manufacturer
1	Ection-LS	Escitalopram	5mg	Tablet	Mission Research Laboratories(I) Pvt,
		Clonazepam	0.25mg		
2	Depram	Escitalopram	10mg	Tablet	Intas Pharma Ltd
		Clonazepam	0.5mg		
3	Feliz Plus	Escitalopram	5mg	Tablet	Torrent Labs (P) Ltd
		Clonazepam	0.5mg		
4	Nexito Forte	Escitalopram	10mg	Tablet	Sun Pharma
		Clonazepam	0.5mg		
5	Stalopam	Escitalopram	10mg	Tablet	Lupin Laboratories
		Clonazepam	0.5mg		

5. 2. LITERATURE SURVEY

Several analytical methods were developed for the estimation of ESC and CLZ individually, in the combined dosage forms and in combination with other drugs. These methods generally consists of spectrophotometry[12-27], thin layer chromatography[28], high performance thin layer chromatography[29-32], liquid chromatography[33] and high performance liquid chromatography[34-43]. Stability studies of ESC and CLZ[44-49] in the tablet form were not available in plenty. Numerous methods were proposed for the estimation of ESC and CLZ in biological samples[50-68] also.

Kakde and Satone [13] developed a precise spectrophotometric method for the determination of ESC oxalate and CLZ in commercial formulation. This method utilized simultaneous equation method for the estimation. The wavelength chosen for ESC oxalate and CLZ was 238nm and 273nm respectively. Linearity was derived over the range of 5-100mµg/ml for ESC oxalate and 5-50mµg/ml for CLZ. Accuracy of the method was proved through recovery studies as mentioned in ICH guidelines.

Sakhreliya [17] et al were succeeded in developing three simple and cost effective UV spectroscopic methods for the determination of ESC oxalate and etizolam in pharmaceutical formulation. The concept of standard addition was employed in developing the proposed UV spectroscopic methods. ICH guidelines were followed for the validation of all the three methods and the results were shown in the **Table.5.2.**

Table.5.2. Results of spectrophotometric methods of escitalopram oxalate and etizolam

Parameter	Drug	Method I: Simultaneous equation method	Method II: Q ratio method	Method III: absorbance correction method
Wavelength	Escitalopram	238.2nm	238.2nm	238.2nm
	Etizolam	251.6nm	248.8nm	292.8nm
Linearity	Escitalopram	10-60µg/ml	10-60µg/ml	10-60µg/ml
	Etizolam	5-30µg/ml	5-30µg/ml	5-30µg/ml

Sri [24] et al illustrated a simple and economical method for the determination of ESC oxalate and CLZ in the pharmaceutical formulation. The method was developed basing on standard addition method and the results were mentioned in **Table.5.3**.

Table.5.3. Results of spectrophotometric methods of escitalopram oxalate and clonazepam

S.No	Parameter	Escitalopram oxalate	Clonazepam
1	Wavelength	238nm	222nm
2	Linearity	10-24µg/ml	2-14µg/ml
3	LOD	0.44	0.53
4	LOQ	1.33	1.61
5	% Label	97.5	95.8

Bhimanadhuni [37] et al proposed a simple and efficient RP-HPLC method for the estimation of ESC oxalate and CLZ simultaneously in the pharmaceutical dosage form. On hypersil ODS C18 column, chromatographic separation was achieved at a wavelength of 240nm. Mobile phase used for the detection was a composite of buffer and acetonitrile (50:50, v/v). At 1.0 ml/min flow rate, retention time exhibited by ESC oxalate was 2.840$\pm$0.007min and by CLZ was 4.007$\pm$0.006min. Good linearity was established when the concentration range was 20-120µg/ml for ESC oxalate and 1-6µg/ml for CLZ. Validity of the method was examined as per ICH guidelines. The proposed method was recognized to be specific, reproducible, rapid and robust.

Chakole [39] et al reported an efficient and sensitive RP-HPLC method for the determination of ESC and CLZ in commercial tablet forms and was done on C18 column at 248nm. Mobile phase of composition methanol and buffer in the ratio of 90:10 (v/v),

pH of 4.0 and a flow rate of 1.0ml/min were employed throughout the analysis. Retention times of ESC and CLZ were reported at 3.22min and 4.29min respectively. Linearity was established in the range of 2.5-80µg/ml for ESC and 0.125-4µg/ml for CLZ. The validation approach was carried out basing on the ICH guidelines. The run time was not more than 10min. The proposed method was successful in terms of precision, specificity, recovery and robustness.

Samanta [42] et al concluded a precise HPLC method for the evaluation of ESC in pharmaceutical formulation. The detection was carried out on Xterra RP C18 column (150mmx4.6mm, 5µm) at 238nm wavelength. Mobile phase utilized was a mixture of phosphate buffer (pH7.0), acetonitrile and methanol at a flow rate of 1.2ml/min. The analysis was done by injecting 10µl of solution and the run time was adjusted to 10min. The method was proved to be simple and accurate.

Reddy [44] et al derived a stability indicating HPLC method for the validation of paroxetine and CLZ in the combined pharmaceutical dosage form. The total process was carried out on an agilent zorbar sb-C18 column at 30^0C. Wavelength chosen for the estimation was 270nm at a flow rate of 0.8ml/min. A composite of orthophosphoric acid (0.2%) and methanol (60:40 v/v) was used as mobile phase for the chromatographic separation. Retention time of paroxetine was found to be 3.478min and that of CLZ was 3.964min. The method was validated as prescribed in ICH guidelines. The percentage RSD of recovery studies of both paroxetine and CLZ were found to be 97-103%. Different stress conditions like acidic, basic, peroxide, thermal and photolytic were applied on paroxetine and CLZ. Well resolved degradation products were identified from the degradation chromatograms. Hence the method can be applied for usual analysis due to high degree of precision, simplicity and rapidness.

Geetharam and Praveen [49] demonstrated a stability indicating HPLC method for the estimation of paroxetine hydrochloride and CLZ in the pharmaceutical formulation simultaneously. Chromatographic separation was achieved on kromasil C18 column at 260nm by using acetonitrile and orthophosphoric acid buffer (0.1%) in 60:40 ratios as mobile phase. The validation parameters were calculated as per the ICH guidelines and were found to be within the acceptable range. The efficacy of the method was demonstrated through degradation studies. This method was succeeded in separation of the drug from its degradation products. The results were shown in **Table.5.4.**

Table.5.4. RP-HPLC results of paroxetine hydrochloride and clonazepam

S.No	Parameter	Paroxetine hydrochloride	Clonazepam
1	Retention time	3.49min	4.55min
2	Linearity	125 - 750µg/ml	2.5 – 15µg/ml
3	% Recovery	99.4 - 100.6	98.1 – 101.0

5.3. EXPERIMENTAL

5.3.1. Chemicals and solvents

The drug samples and the working standard of ESC and CLZ were gifted by Micro Labs Ltd and Unichem Laboratories Ltd respectively. The pharmaceutical formulations (Esilo-Forte brand: ECS10mg; CLZ 0.5mg) were procured from regional market. Methanol, acetonitrile and water were purchased from Merck Specialties Private Limited, Mumbai, India. Buffer chemicals (AR Grade) were also purchased from Merck Specialties Private Limited, Mumbai, India.

5.3.2. Preparation of standard stock solution

ESC and CLZ in the pure form were used for the preparation of standard stock solutions individually. Accurately weighed 10mg of ESC and 0.5mg of CLZ were transferred into 10ml volumetric flasks separately. Initially, 5ml of the methanol was added to both the volumetric flasks to dissolve the drugs. Sonication of these solutions was done for about 15min to ensure complete dissolution and were diluted with the required quantity of methanol to obtain the proposed volume. Solutions of different concentrations were prepared by diluting the above solutions to get the required concentration i.e. 50-300µg/ml for ESC and 2.5-15µg/ml for CLZ. Equal quantities of ESC and CLZ were mixed thoroughly and the resultant solution was used as standarad stock solution for the simultaneous analysis.

5.3.3. Preparation of sample solution

Sample solution was prepared from finely ground uniform size powder of twenty tablets. Into a 10ml volumetric flask accurately weighed 10mg of ESC was quantitatively transferred and then 5ml of the methanol was poured. The solution was kept under sonication for about 15min. The flask was filled up to the mark by making use of mobile phase. Filtered solution was properly diluted with the mobile phase to acquire a concentration of 200µg/ml of ESC. Simultaneously a concentration of 10µg/ml of CLZ was achieved as claimed on the label.

5.3.4. Preparation of phosphate buffer solution

Phosphate buffer solution was prepared by making use of potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4). Initially, 87.09g of potassium hydrogen phosphate was weighed accurately and was dissolved in 500ml of HPLC water. Similarly, 68.045g of potassium dihydrogen phosphate was weighed accurately and was dissolved in 500ml of HPLC water. The solution, which was obtained by mixing 19.2ml of potassium hydrogen phosphate solution with 80.8ml of potassium dihydrogen phosphate solution, exhibited the pH at 6.2.

5.4. METHOD DEVELOPMENT

A systematic study of effect of various factors was done by keeping all the parameters constant except one for the method development. Three primary factors influencing method development were wavelength, stationary and mobile phases. It was achieved through the following studies.

5.4.1. Detection of wavelength

Individually, the spectrum of diluted solutions of two drugs ESC and CLZ in methanol was recorded. On spectrophotometer the absorption spectrum was scanned in the UV region (200-400nm). The two active ingredients exhibited maximum overlapping at a wavelength of 229nm and hence the total analysis was carried out at this wavelength only.

5.4.2. Choice of stationary phase

Octadecyl columns with different types, configurations and from different manufactures were examined for the choice of stationary phase. Peak area response in each trial was compared and found that, kromasil RP-C18 was predicted as appropriate column for the determination of ESC and CLZ in combined dosage form.

5.4.3. Selection of mobile phase

Different mobile phases under isocratic conditions were examined in terms of sharp peak and base line separation. System suitability conditions in each case were studied. A mixture of methanol, acetonitrile and phosphate buffer in 70:28:02 (v/v) was recognized as a suitable mobile phase. This mobile phase exhibited high resolution good base line separation and column efficiency but theoretical plates were found to be less. Theoretical plate count was improved by adjusting the mobile phase to 6.2.

5.4.4. Flow rate

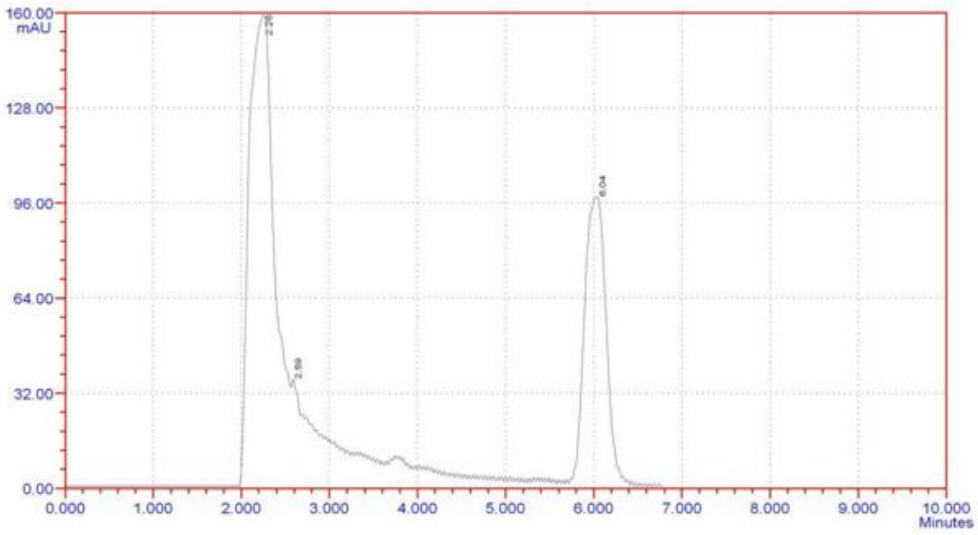
Flow rates of mobile phase were tested from 0.5-1.5ml/min to accomplish optimum separation. Minimum flow rate and minimum run time ensures best saving of the solvent. Successful elution of the analytes was reached when the flow rate was maintained at 1.0 ml/min.

5.4.5. Optimized chromatographic conditions

Several trials were conducted for the selection of optimum chromatographic conditions. Trial conditions were given in **Table.5.5** and the trial chromatograms were shown from **Figure.5.3.** to **Figure.5.10**. After several systematic trials, a sensitive, precise and rapid isocratic RP-HPLC method was established for the analysis of ESC and CLZ in commercial dosage forms. The optimized chromatographic conditions were furnished in **Table.5.6**. The chromatograms of blank, ESC single, CLZ single, standard and formulation were shown in **Figure.5.11, 5.12, 5.13, 5.14 and 5.15** respectively.

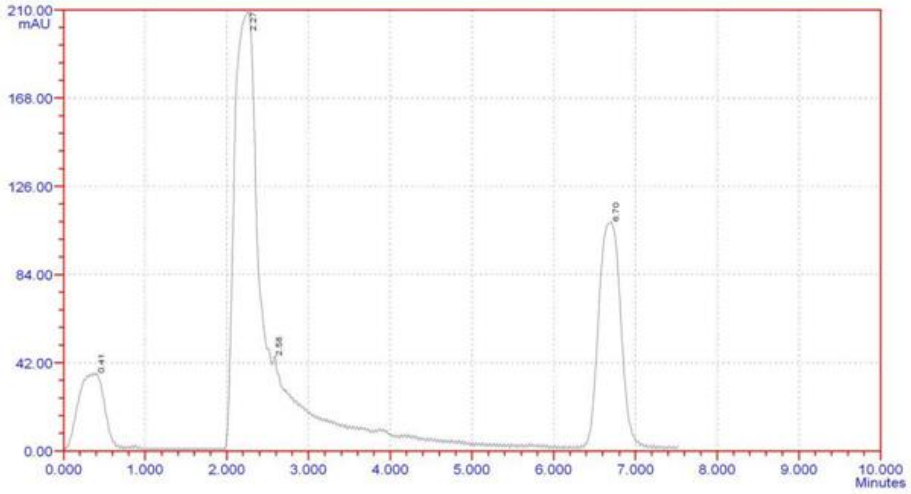
Table.5.5. Trial conditions of escitaloprm and clonazepam

Trial	Mobile phase (v/v)	Wavelength	pH of mobile	Column	Flow rate
I	ACN: MeOH 40:60	229nm	5.7	Kromasil RP- C18	1.0ml/min
II	MeOH: ACN: Water 50: 35: 15	229nm	5.7	Kromasil RP- C18	1.0ml/min
III	MeOH: ACN 80:20	229nm	5.8	Kromasil RP- C18	1.0ml/min
IV	MeOH: PB 80:10:10	229nm	5.4	Kromasil RP- C18	1.0ml/min
V	MeOH: Water: PB 80:10:10	229nm	5.8	Kromasil RP- C18	1.0ml/min
VI	ACN: Water: PB 80:10:10	229nm	6.8	Kromasil RP- C18	1.0ml/min
VII	MeOH: ACN: PB 70:20:10	229nm	6.4	Kromasil RP- C18	1.0ml/min
VIII	MeOH: ACN: PB 70:25:5	229nm	6.3	Kromasil RP- C18	1.0ml/min



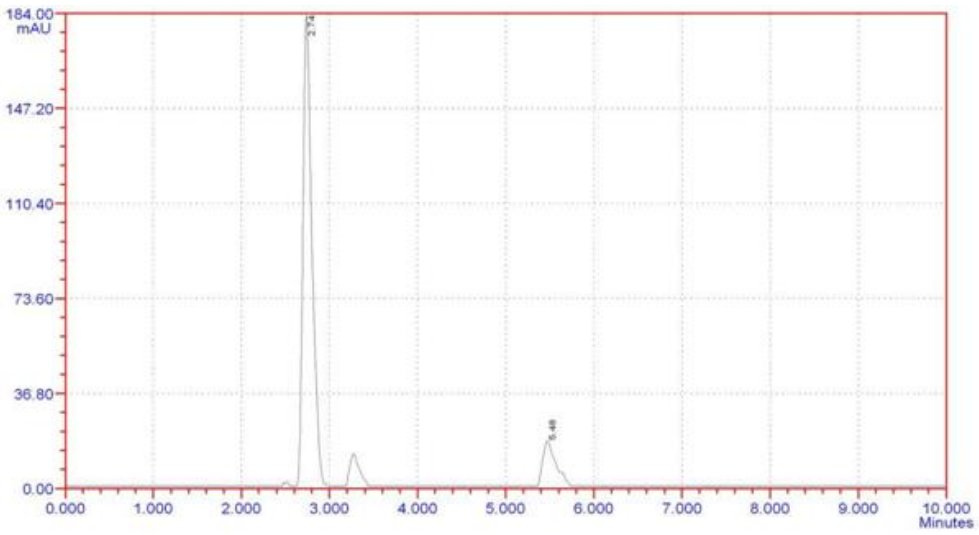
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.255	15895	318644.6	59.424	1.06	252
2		2.592	3303	53903.5	10.052	10.13	503
3		6.043	9685	163674.6	30.524	1.05	2549
	Sum:		28883	536222.7	100.0000		

Figure.5.3. Trial chromatogram I



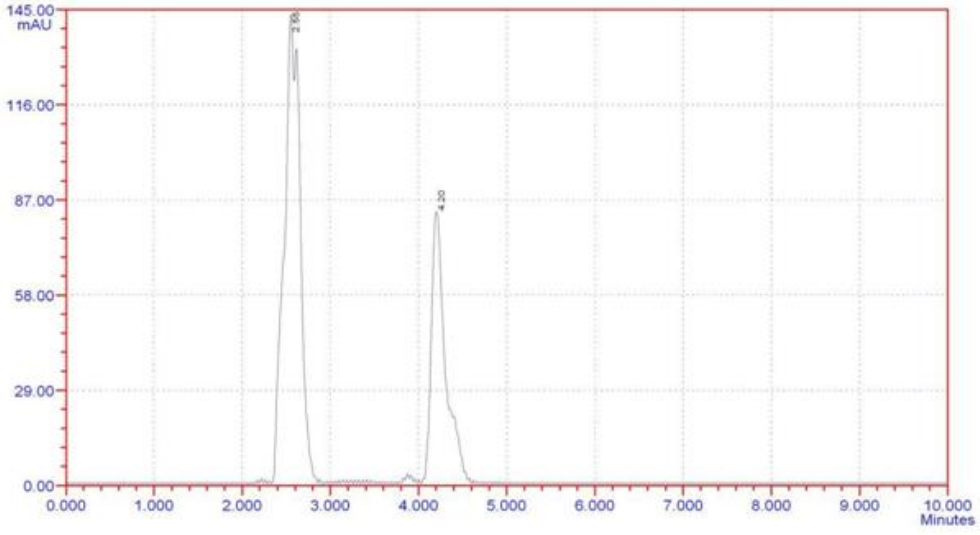
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		0.408	3737	82821.9	10.627	0.92	7
2		2.265	20963	410228.8	52.637	1.02	267
3		2.582	4299	82998.3	10.650	18.40	356
4		6.703	10817	203309.6	26.087	0.03	2535
	Sum:		39816	779358.6	100.0000		

Figure.5.4. Trial chromatogram II



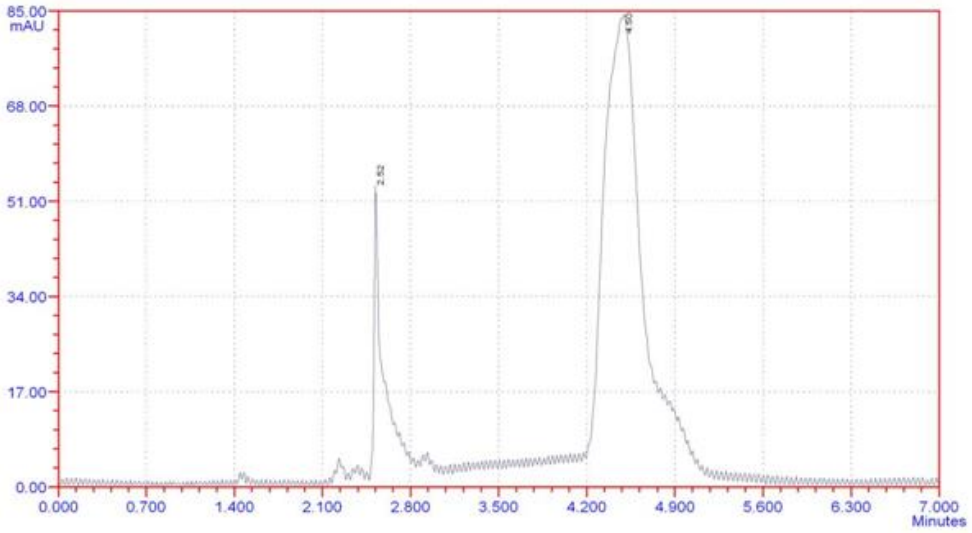
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.740	18496	134316.3	85.856	1.68	2838
2		5.475	1929	22127.1	14.144	1.63	4541
	Sum:		20425	156443.4	100.0000		

Figure.5.5. Trial chromatogram III



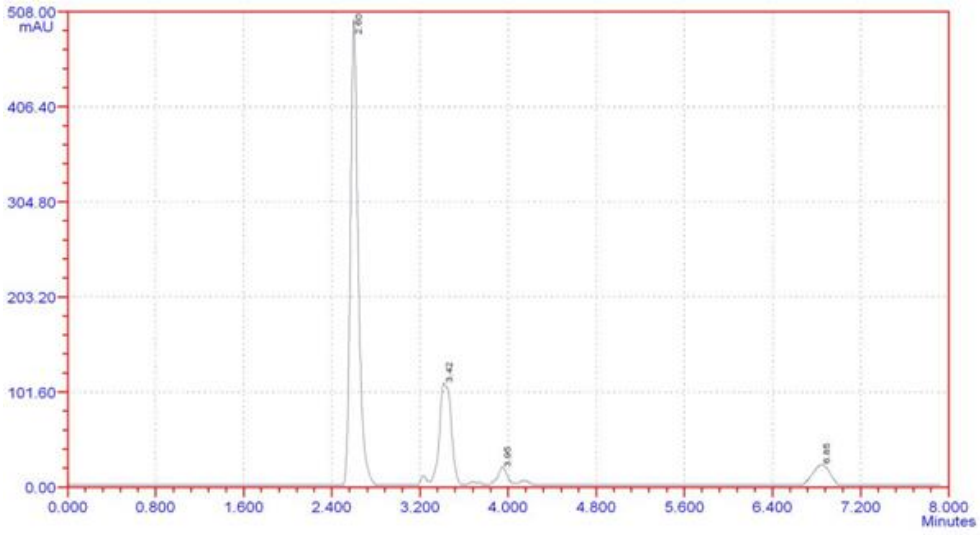
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.550	14301	181380.7	65.589	1.22	806
2		4.202	8351	95159.6	34.411	1.66	2710
	Sum:		22652	276540.2	100.0000		

Figure.5.6. Trial chromatogram IV



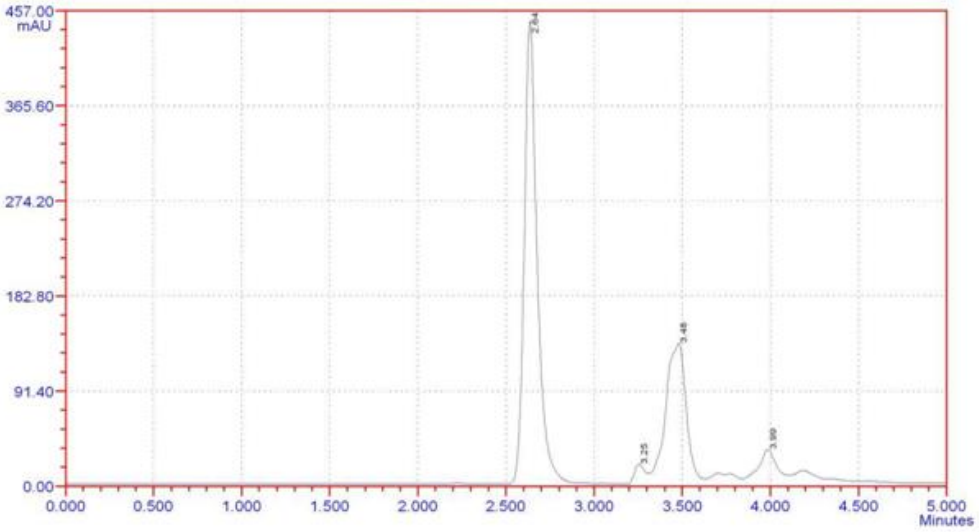
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.522	5433	32132.0	14.479	3.70	3623
2		4.495	8457	189793.0	85.521	1.49	800
	Sum:		13890	221925.0	100.0000		

Figure.5.7. Trial chromatogram V



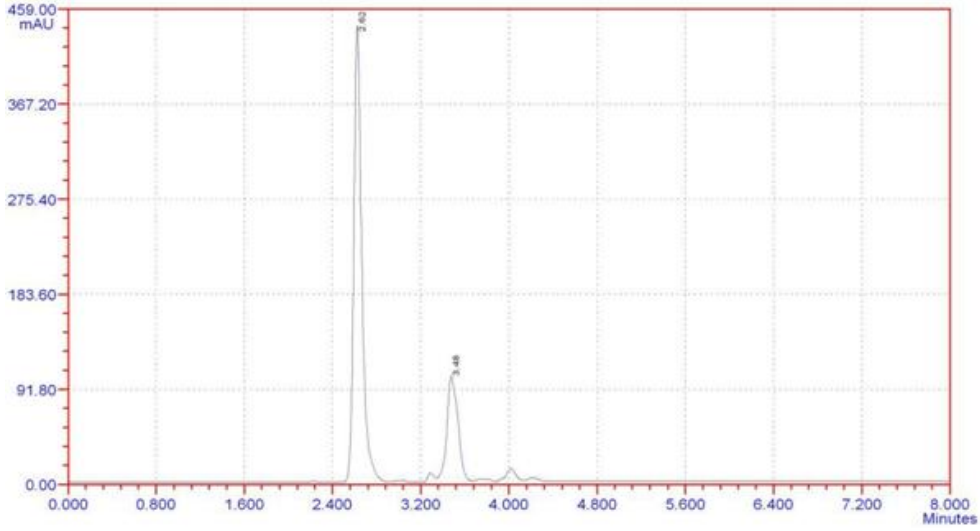
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.598	50193	252398.0	66.988	1.58	5321
2		3.418	10947	82310.3	21.846	1.16	4119
3		3.950	2030	13404.3	3.558	0.92	7132
4		6.848	2465	28666.3	7.608	0.92	6911
	Sum:		65635	376778.9	100.0000		

Figure.5.8. Trial chromatogram VI



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.637	45306	245467.8	74.721	1.65	4720
2		3.255	2124	9614.9	2.927	0.86	10305
3		3.480	13343	56334.9	17.148	2.89	13540
4		3.988	2763	17096.9	5.204	0.86	8280
	Sum:		63536	328514.5	100.0000		

Figure.5.9. Trial chromatogram VII

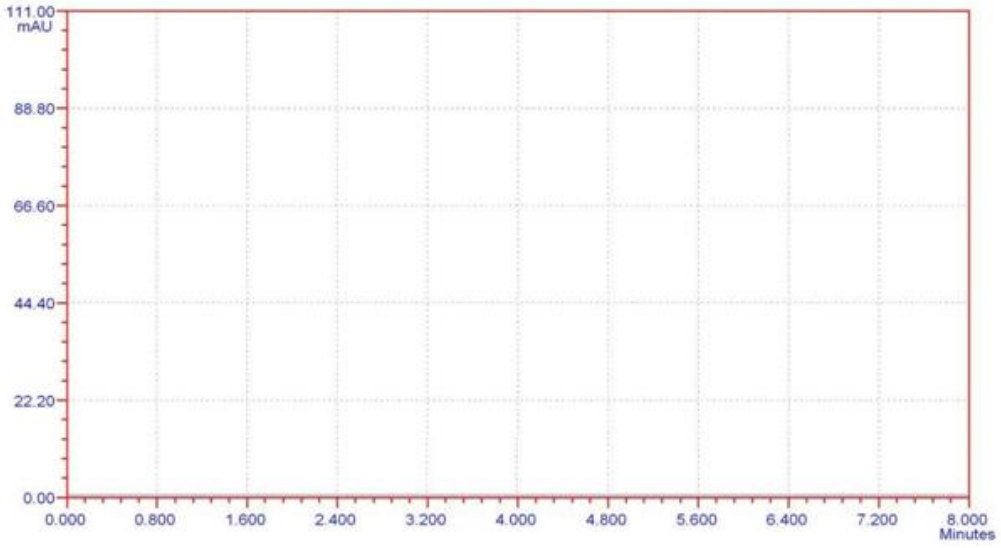


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.623	45159	245465.2	81.500	1.18	4642
2		3.477	10251	56682.0	18.500	4.27	7879
	Sum:		55410	302147.1	100.0000		

Figure.5.10. Trial chromatogram VIII

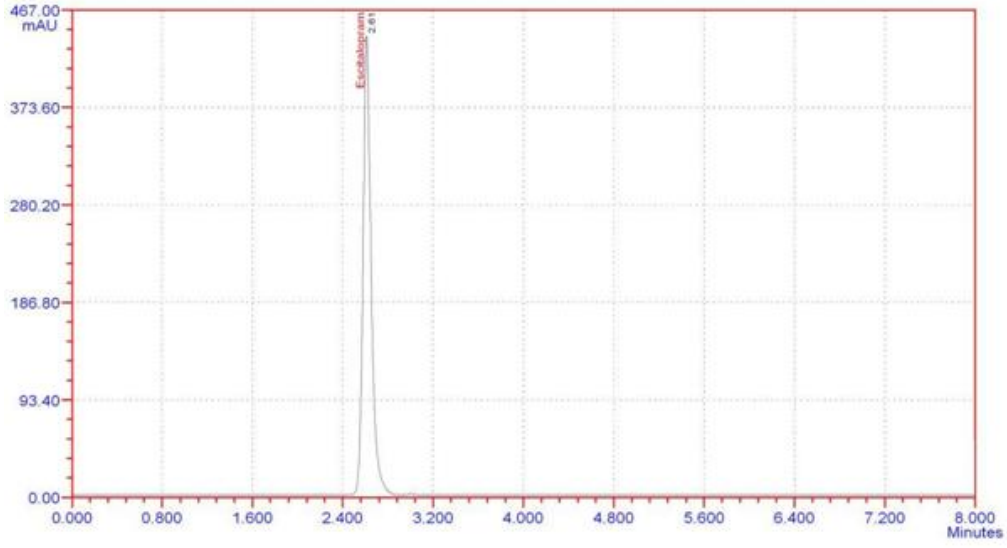
Table.5.6. Optimized chromatographic conditions of escitalopram and clonazepam

1	Pump mode	Isocratic	
2	Column	C 18 (250X4.6mm, 5µm)	
3	Injector	Rheodyne	
4	Injector Volume	20 µl	
5	Diluent	Methanol	
6	Mobile phase	Methanol: Acetonitrile: Phosphate buffer in 70:28:02 (v/v)	
7	Pump pressure	11.8±7MPa	
8	Mobile phase pH	6.2	
9	Wavelength	229nm	
10	Flow rate	1.0ml/min	
11	Run Time	8min	
12	Standard Concentration	Escitalopram	200µg/ml
		Clonazepam	10µg/ml
13	Retention Time	Escitalopram	2.67min
		Clonazepam	3.46min
14	Peak Area	Escitalopram	242195
		Clonazepam	56040
15	Theoretical Plates	Escitalopram	5540
		Clonazepam	7571
16	Tailing Factor	Escitalopram	1.59
		Clonazepam	1.17



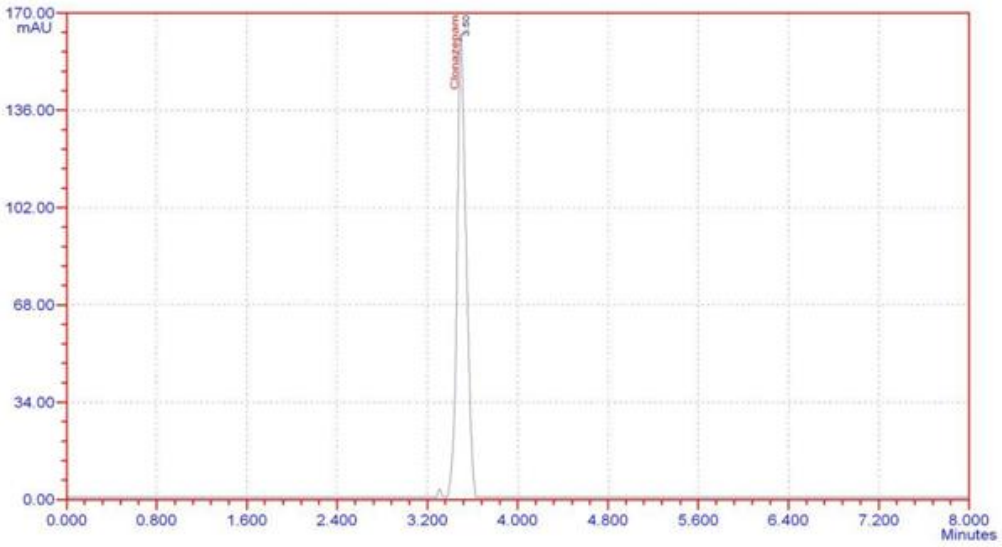
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
	Sum:		0	0.0	0.0000			

Figure.5.11. Chromatogram of blank of escitalopram and clonazepam



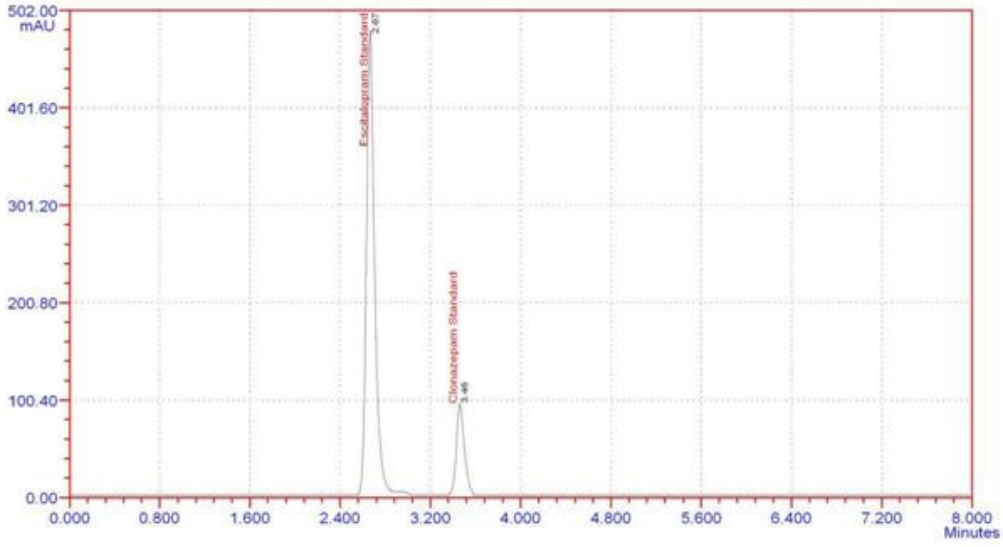
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Escitalopram	2.610	45166	245544.6	100.000	0.95	4594	0.00
	Sum:		45166	245544.6	100.0000			

Figure.5.12. Chromatogram of escitalopram single



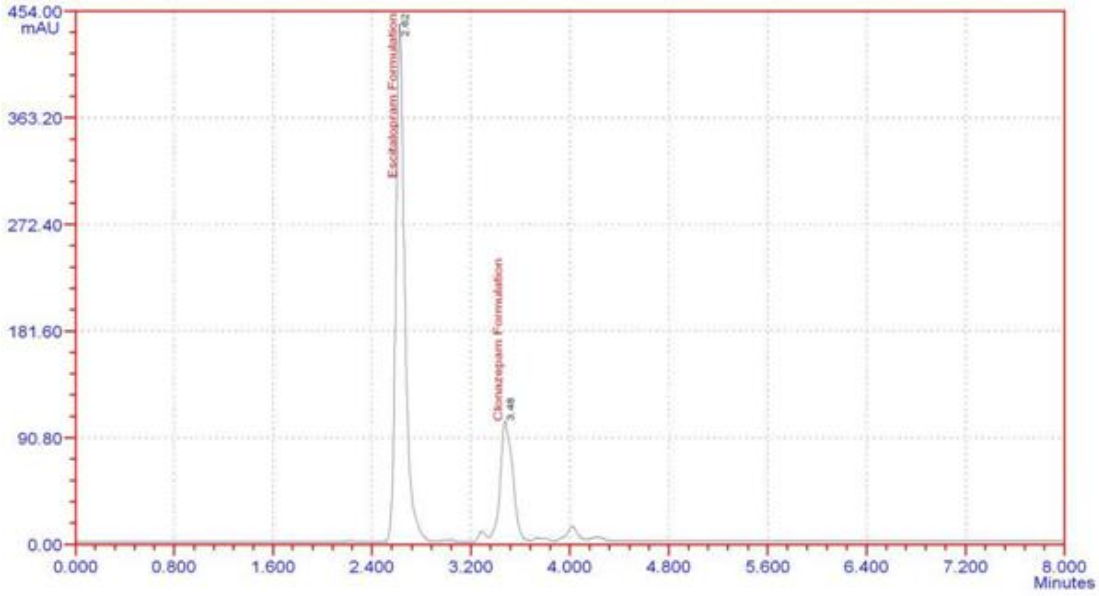
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Clonazepam	3.495	10244	56470.9	100.000	1.95	8013	0.00
	Sum:		10244	56470.9	100.0000			

Figure.5.13.Chromatogram of clonazepam single



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.5.14. Chromatogram of escitalopram and clonazepam standard



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Escitalopram Formulation	2.623	45159	240218.1	81.204	1.01	4847	0.00
2	Clonazepam Formulation	3.477	10251	55603.2	18.796	2.90	8188	5.61
	Sum:		55410	295821.3	100.0000			

Figure.5.15. Chromatogram of escitalopram and clonazepam formulation

5.5. METHOD VALIDATION

The proposed method was validated as per ICH guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification and solution stability.

5.5.1. Specificity

Specificity of the method was tested by comparing the chromatograms of blank, standard and sample. The detailed study of chromatograms of standard and sample confirmed the fact that there was no interference of diluents and placebo in the assay. Thus the method was proved to be specific. The retention times of ESC and CLZ were 2.67min and 3.46min respectively.

5.5.2. System suitability

System suitability conditions were evaluated by utilizing the freshly prepared standard stock solution of ESC and CLZ. In a volumetric flask equal volumes of standard concentration of ESC and CLZ were mixed properly and 20µl of this solution was injected into HPLC system. The results obtained were tabulated in **Table.5.7** to convey the system suitability of proposed method.

Table.5.7. Results of system suitability of escitalopram and clonazepam

S.No	Parameter	Escitalopram	Clonazepam
1	API concentration	200µg/ml	10µg/ml
2	Retention Time	2.67min	3.46min
3	Resolution	……	5.25
4	Peak Area	242195	56040
5	Theoretical Plates	5540	7571
6	Tailing Factor	1.59	1.17

5.5.3. Linearity

Linearity of the proposed method was determined by injecting a series of six different concentration levels into the HPLC system. Linearity was evaluated by the construction of calibration curves (concentration against peak area) for ESC and CLZ. Least square method was utilized for the calculation of regression. In both the cases the correlation coefficient obtained was 0.999. The linearity results were tabulated in **Table.5.8** and the calibration plots were shown in **Figure.5.9 & 5.10**.

Table.5.8. Results of linearity of escitalopram and clonazepam

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

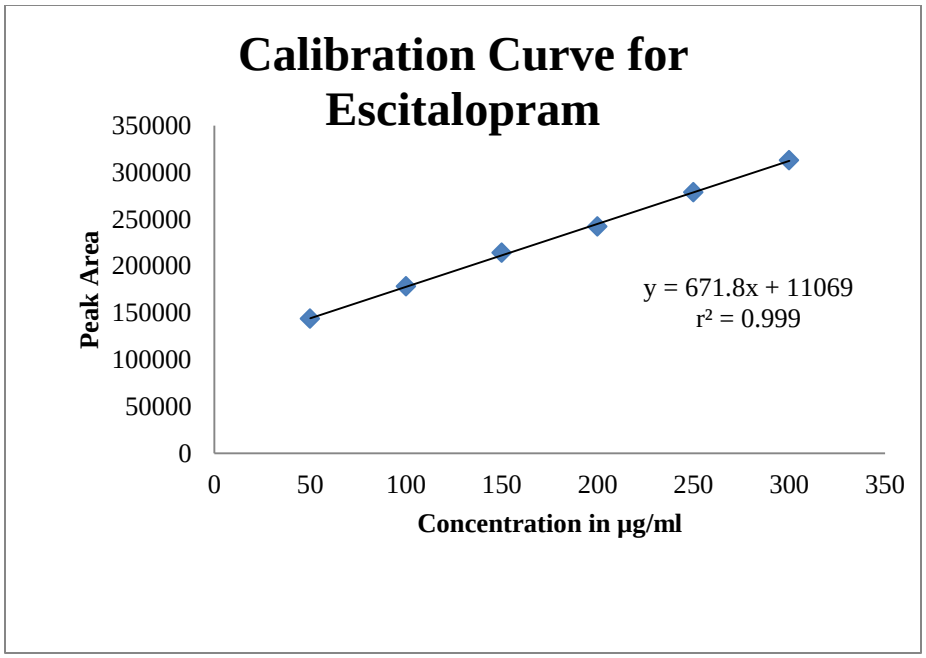


Figure.5.16. Calibration curve of escitalopram

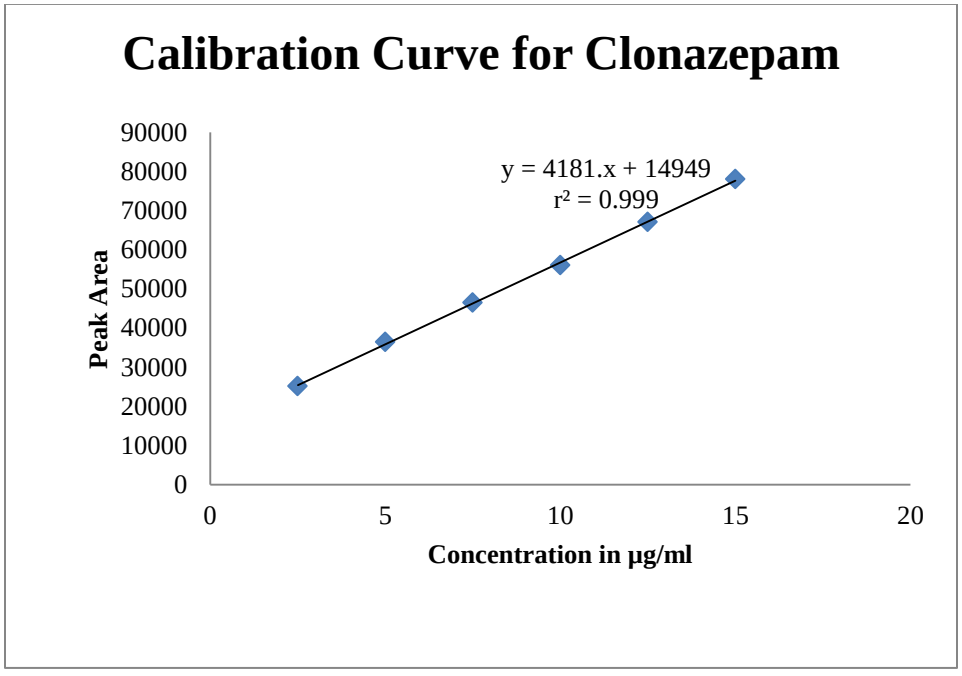


Figure.5.17. Calibration curve of clonazepam

5.5.4. Accuracy

Standard addition method was employed to measure the accuracy of recommended method. Three concentration levels (50%, 100% and 150%) were spiked with the reference solution and at each level the measurements were made in triplicate. Percentage RSD was used to calculate the recovery of the two drugs ESC and CLZ and the results were presented in **Table.5.9& 5.10** respectively. Comfortable recoveries ranging from 98.165 to 101.578 for ESC and from 98.563 to 101.572 for CLZ were obtained.

Table.5.9. Recovery results of **e**scitalopram

S. No	Spiked Level	Concentration in µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	100	50	150	149.13	99.4198
2		100	50	150	147.248	98.1656
3		100	50	150	148.473	98.9821
4	100%	100	100	200	203.079	101.539
5		100	100	200	202.704	101.352
6		100	100	200	199.874	99.9368
7	150%	100	150	250	245.785	98.3139
8		100	150	250	251.055	100.422
9		100	150	250	253.945	101.578

Table.5.10. Recovery results of clonazepam

S. No	Spiked Level	Concentration in µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	5	2.5	7.5	7.52291	100.305
2		5	2.5	7.5	7.41966	98.9288
3		5	2.5	7.5	7.50129	100.017
4	100%	5	5	10	9.85635	98.5635
5		5	5	10	9.96913	99.6913
6		5	5	10	9.93808	99.3808
7	150%	5	7.5	12.5	12.6965	101.572
8		5	7.5	12.5	12.6799	101.44
9		5	7.5	12.5	12.4436	99.5485

5.5.5. Precision

The degree of repeatability of an analytical method under ordinary conditions is termed as precision. Measurement of precision depends on both intra-day and inter-day precisions. In order to measure intra-day precision six replicate standard solutions of ESC and CLZ were injected and for the measurement of inter-day precision six replicate standard solutions of ESC and CLZ were injected on three consecutive days. The percentage RSD calculated in both the cases and was found to be in good agreement. The results of intra-day precision and inter-day precision were furnished in **Table.5.11 and 5.12** respectively. Repeatability of the method was established from the results of both intra-day and inter-day precisions.

Table.5.11. Results of intra-day precision of escitalopram and clonazepam

S.No	Escitalopram at 200µg/ml	Clonazepam at 10µg/ml
1	242194	56040
2	246878	55657
3	245467	56334
4	247946	56523
5	245465	56682
6	246466	56516
%RSD	**0.802**	**0.676**

Table.5.12. Results of inter-day precision of escitalopram and clonazepam

S.No	Escitalopram at 200µg/ml	Clonazepam at 10µg/ml
1	249471	55865
2	246846	55477
3	246858	56376
4	248955	56368
5	242753	56103
6	240767	56632
%RSD	**1.410**	**0.741**

5.5.6. Ruggedness

Ruggedness is the measure of reproducibility of the proposed method. Ruggedness was estimated by different analyst using different columns on different days. Six dilutions were tested and the results were shown in **Table.5.13**. The percentage RSD obtained for ESC was 0.639 and for that of CLZ was 1.751. Thus the proposed method was confirmed to be reproducible.

Table.5.13. Results of ruggedness of escitalopram and clonazepam

S.No	Escitalopram at 200µg/ml	Clonazepam at 10µg/ml
1	243358	56663
2	244428	57643
3	246765	57038
4	246825	55345
5	247236	55179
6	244994	55844
%RSD	**0.639**	**1.751**

5.5.7. Robustness

Small deliberate changes were introduced with respect to mobile phase, pH and wavelength to measure the robustness of the method. The effect of these changes on chromatographic parameters was studied. In particular retention time, tailing factor and number of theoretical plates were compared with the standard solution. Percentage change in the results was calculated and was found to be within the acceptable limit. Hence the method is valid within the given limits. The results of robustness were tabulated in **Table.5.14**.

Table.5.14. Results of robustness of escitalopram and clonazepam

S.No	Condition	Change	Escitalopram		Clonazepam	
			Area	% Change	Area	% Change
1	Standard	NO Change	242195	……..	56040	…….
2	MP 1	MeOH: ACN: PB 65:33:02 (v/v)	242525	0.13667	56102	0.11064
3	MP 2	MeOH: ACN: PB 75:23:02 (v/v)	245683	1.44058	54996	1.863
4	pH 1	6.3	246159	1.63712	56129	0.15882
5	pH 2	6.1	239414	1.1478	55820	0.3926
6	WL 1	224nm	242525	0.13667	55235	1.4365
7	WL 2	234nm	247238	2.08263	55482	0.9957

5.5.8. Limit of detection and limit of quantification

Limit of detection enables to measure detectable response at the lowest possible concentration. On the other hand limit of quantification requires the measurement of quantified response with enough accuracy and precision at a minimum concentration level. Solutions of different concentrations were prepared and all the solutions were investigated repeatedly to evaluate LOD and LOQ values. LOD values of ESC and CLZ were 0.075µg/ml and 0.25µg/ml. LOQ values of ESC and CLZ were 1.5µg/ml and 5.0µg/ml. The resultant values were shown in **Table.5.15.**

Table.5.15. Results of LOD and LOQ of escitalopram and clonazepam

Drug	LOD	LOQ
Escitalopram	1.5µg/ml	0.075µg/ml
Clonazepam	5.0µg/ml	0.25µg/ml

5.5.9. Solution stability

In order to measure the stability of ESC and CLZ, standard solution was prepared and was kept aside for about two days. At regular intervals of time the solution was injected and the corresponding chromatographic parameters were compared with the freshly prepared standard solution. The results were presented in **Table.5.16**. The solution exhibited stability up to 24hr.

Table.5.16. Results of solution stability of escitalopram and clonazepam

S No	Time in Hours	Escitalopram		Clonazepam	
		Area	% Stability	Area	% Stability
1	1	245259	101.2651	55988	99.90721
2	2	246855	101.9241	55504	99.04354
3	4	240625	99.35176	55325	98.72413
4	8	239504	98.88891	55082	98.29051
5	12	240446	99.27785	54933	98.02463
6	24	238315	98.39799	55034	98.20485

5.5.10. Formulation

20µl of freshly prepared sample of ESC and CLZ was injected and the corresponding peak response was measured. The percentage assay was found to be 99.184 and 99.22 for EST and CLZ respectively. Results were introduced in **Table.5.17**.

Table.5.17. Results of escitalopram and clonazepam formulation

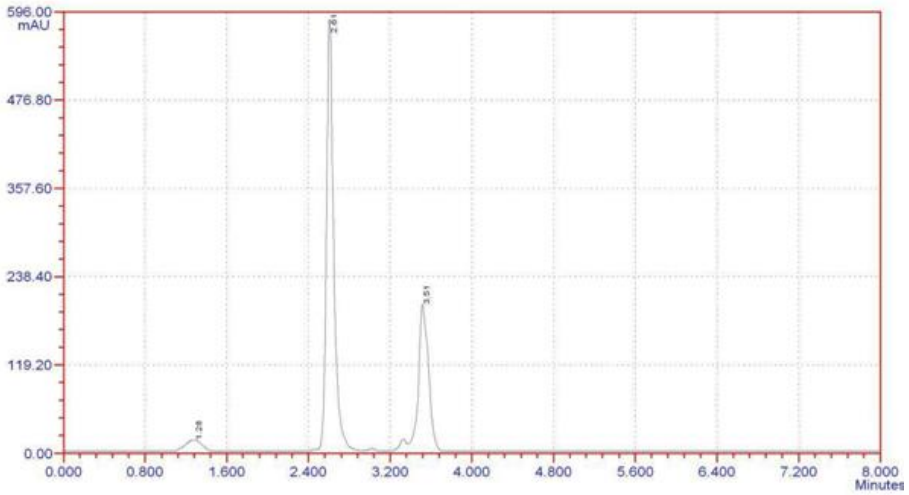
S.No	Drug	Brand	Dosage	Amount Prepared	Amount Found	%Assay
1	Escitalopram	Esilo-Forte	10mg	200µg/ml	198.367µg/ml	99.184
2	Clonazepam		0.5mg	10µg/ml	9.922µg/ml	99.22

5.6. FORCED DEGRADATION STUDIES

The stability of ESC and CLZ formulation under different stress conditions were studied and the results were presented in **Table.5.17**. Degradation chromatograms of aqueous, acidic, basic, peroxide, thermal, light and UV light were shown from **Figure.5.18** to **Figure.5.24**.

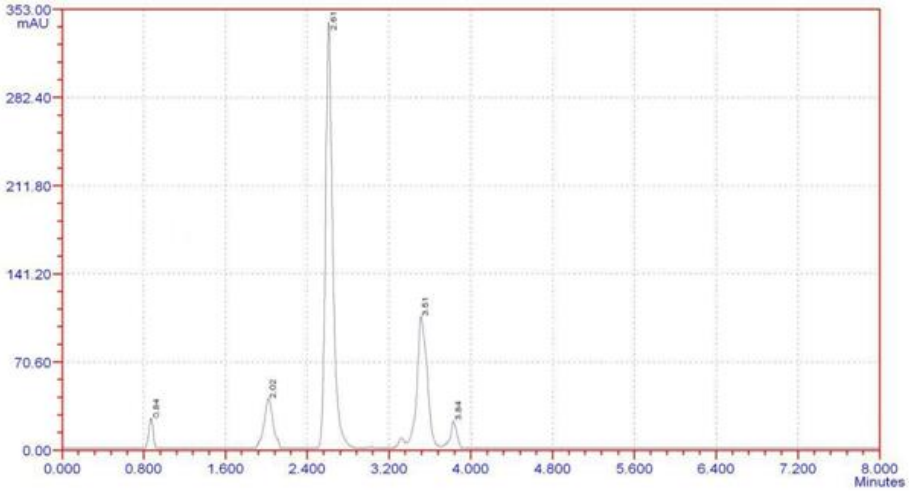
Table.5.18. Forced degradation studies of escitalopram and clonazepam

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



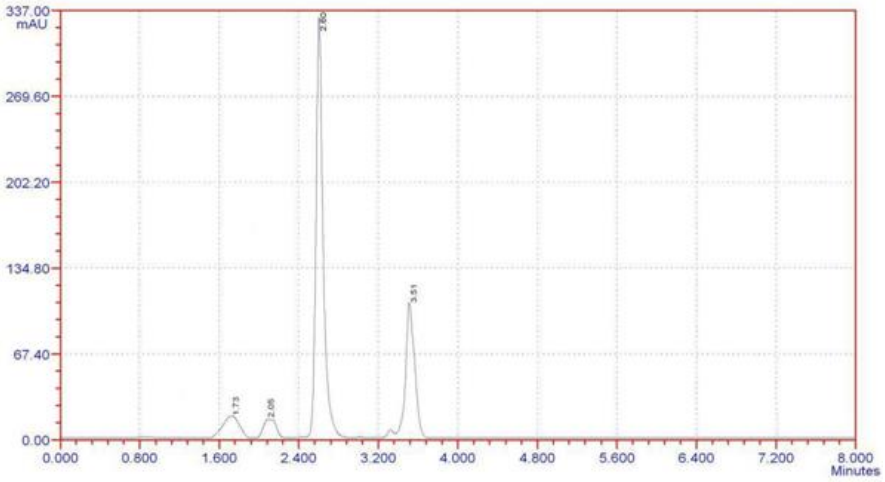
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.280	973	12087.3	3.904	0.91	1212	0.00
2		2.607	32615	241899.7	78.138	0.66	2462	4.72
3		3.515	9698	55592.1	17.957	1.05	7493	4.88
	Sum:		43286	309579.1	100.0000			

Figure.5.18. Chromatogram of aqueous degradation



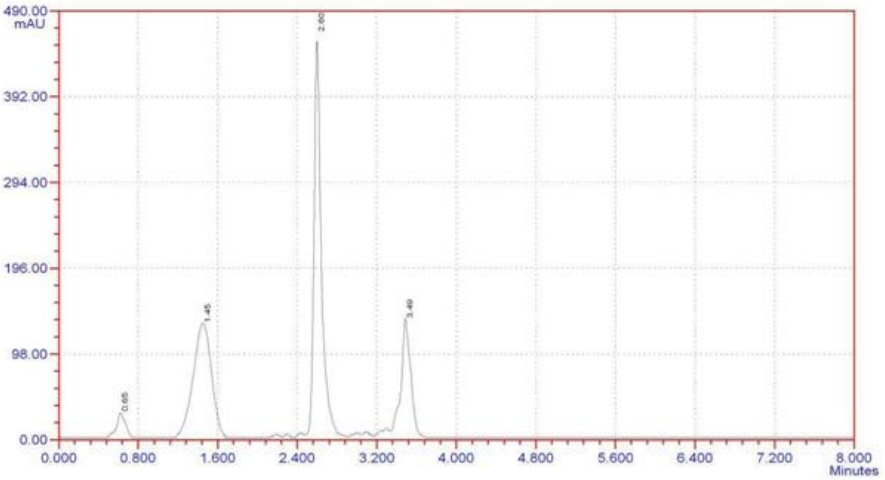
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		0.840	402	11931.5	3.739	2.86	1006	0.00
2		2.024	648	5838.3	1.829	1.07	3087	2.16
3		2.609	35894	237903.8	74.548	1.20	4087	2.64
4		3.510	9602	55948.4	17.532	1.18	7234	5.11
5		3.839	843	7506.9	2.352	0.80	3704	1.57
	Sum:		47389	319128.9	100.0000			

Figure.5.19. Chromatogram of acid degradation



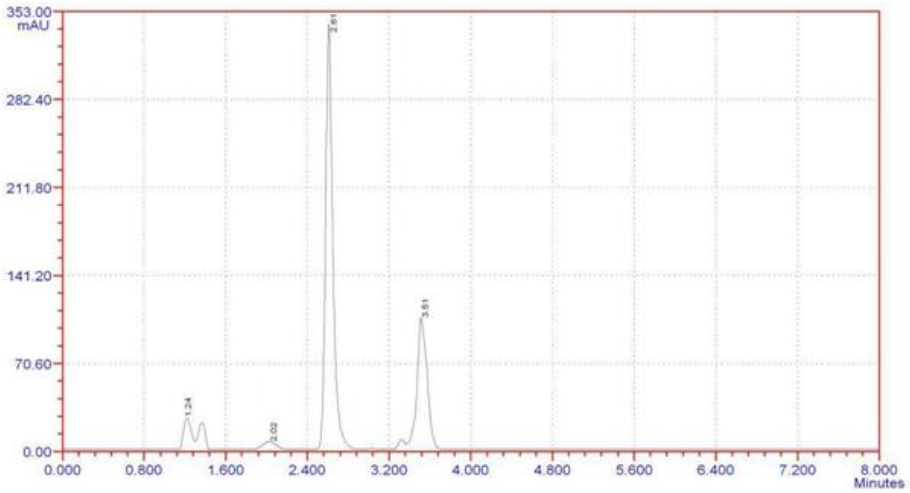
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.725	1871	23352.4	4.043	0.89	1381	0.00
2		2.603	34901	249693.4	43.230	0.66	2639	3.16
3		2.045	1884	6854.3	1.187	0.00	6298	3.65
4		2.603	34901	242617.0	42.005	0.68	2795	3.72
5		3.512	10157	55076.8	9.536	1.09	8359	5.18
	Sum:		83714	577594.0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

Figure.5.20. Chromatogram of base degradation



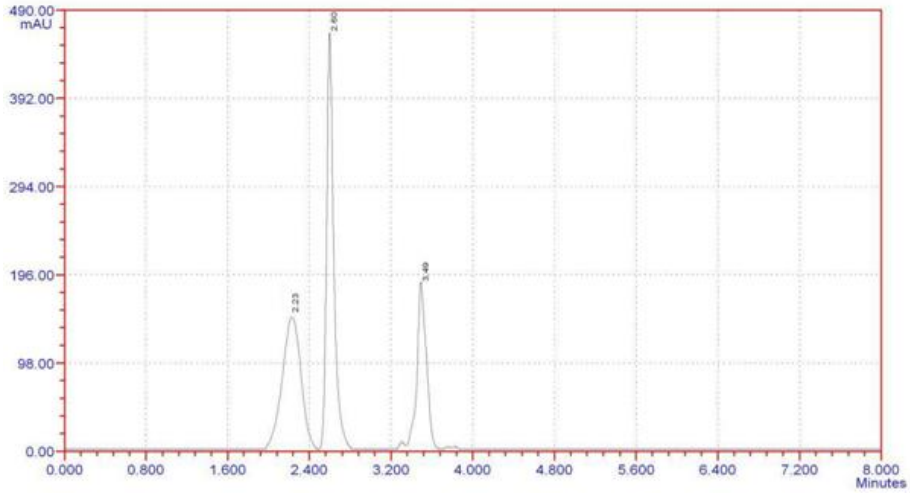
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		0.650	125	1473.5	0.484	0.90	1761	0.00
2		1.452	4780	58276.9	19.162	0.82	2283	2.36
3		2.598	11942	190678.6	62.698	0.57	5528	2.87
4		3.487	3987	53693.5	17.655	0.92	9336	2.13
	Sum:		20834	304122.5	100.0000			

Figure.5.21. Chromatogram of peroxide degradation



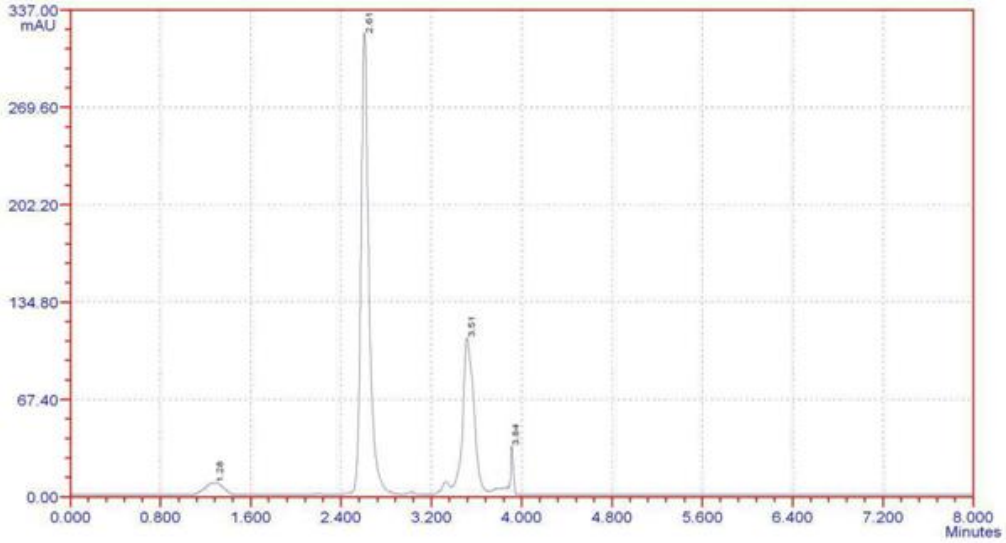
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		1.235	1407	26020.8	7.422	0.90	2602	0.00
2		2.024	1914	35602.7	10.156	0.78	3440	1.50
3		2.609	35894	234405.5	66.864	1.14	5894	1.64
4		3.510	9602	54539.0	15.557	1.37	7613	5.21
	Sum:		48817	350568.0	100.0000			

Figure.5.22. Chromatogram of thermal degradation



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.5.23. 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.5.24. Chromatogram of UV light degradation

5.7. RESULTS AND DISCUSSION

The proposed method was aimed to develop a stability indicating HPLC method for the simultaneous estimation of ESC and CLZ in commercial formulation**.** UV spectrophotometer was used to monitor the wavelength selection. Maximum absorption was exhibited by the both drugs at 229nm and thus the entire work was carried out at this wavelength only. Numerous trials were conducted for the choice of column, stationary phase and mobile phase. Finally, successful results were obtained when the process utilized kromasil RP-C18 column with mobile phase of methanol, acetonitrile and phosphate buffer in 50: 28: 02 (v/v). The entire analysis was carried out more comfortably at ambient temperature. At a pH of 6.2, optimized chromatographic conditions were achieved and the results were tabulated in **Table.5.6**.

Method validation was initiated with earlier measurement of specificity. The chromatograms of blank (**Figure.5.11**), ESC single (**Figure.5.12),** CLZ single **(Figure.5.13)**, ESC and CLZ standard (**Figure.5.14**) and formulation of ESC and CLZ (**Figure.5.15**) were carefully studied and it was concluded that excipients have no effect in the analysis. Various properties of chromatogram were studied especially tailing factor (<2.0), resolution (>2.0) and theoretical plates (> 2000) and were given in **Table.5.7.** The simple analytical conditions gave a very less time (<4 min) for both the drugs and facilitated the analysis of more number of samples on a less time interval. Linearity of ESC was observed in the concentration range of 50-300µg/ml and corresponding regression equation was y = 671.8x + 11069 (r^2 = 0.999). Linearity of CLZ was found in the range of 2.5-1µg/ml with regression equation of y = 4181.x + 14949 (r^2 = 0.999). Corresponding linearity results were presented in **Table.5.8**. Accuracy of the method was predicted from the recovery studies. The recovery studies were conducted at three spiked concentrations and the results **(Table.5.9 & 5.10)** were within the requisite range.

Sufficient number of aliquots of a homogeneous sample was injected to determine the precision of the method. Intra-day precision and inter-day precision measurements were used to ensure the precision of the method. The calculated percentage RSD for ESC and CLZ were within the agreeable range (<2). The results were furnished in **Table.5.11 and 5.12**. Ruggedness was performed by different analyst on three different days using six replicate injections. The results shown in **Table.5.13** were in comfortable range. Small changes were introduced in chromatographic parameters to analyze robustness. The results of robustness were presented in **Table.5.14** and were found to be in the suitable range. The LOD and LOQ values were shown in **Table.5.15**. Stability study (**Table.5.16**) was conducted over a period of 48hr and it was found that the solution was stable up to 24hr.

The validated method was applied for the assay of commercial formulation of ESC and CLZ (Esilo-Forte brand: ECS10mg; CLZ 0.5mg). The results were furnished in **Table.5.17**. Thus, the developed stability indicating isocratic RP-HPLC method was found to be precise, sensitive and rapid. Method development and validation were the major steps of the drug analysis. The stability of the molecule was measured through degradation studies. Various stress conditions such as acidic, basic, peroxide, thermal and photolytic were applied for inducing stress in the molecule. The results were tabulated in **Table.5.18**.

5.8. CONCLUSIONS

In the present thesis the author has planned to create an interest on the work by attaching degradation studies. The proposed method utilized very short period of time i.e. less than 4min for the analysis of ESC and CLZ which was favourable in terms of time and cost. The reported method exhibited linear response in the proposed range and was found to be precise, accurate and rapid. Different stress conditions were applied to the standard solution of ESC and CLZ to measure the stability of the molecule. Thus the proposed method was proved to be superior over the most of the developed methods for the regular determination of ESC and CLZ simultaneously in the tablet dosage forms.

Forced degradation studies provide valuable information that is highly useful during the manufacturing, formulation development and packing of ESC and CLZ in pharmaceutical formulation. Degradation studies are highly useful in investigating degradation path ways, mechanism of action and degradation products. This provides a valuable route for the extension of the proposed work in future.

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