Nat. Volatiles & Essent. Oils, 2021; 8(4): 16659-16689

NVEO
Natural Volatiles &
Essential Oils

Method Development And Validation For The Simultanious Estimation Of Chlordiazepoxide In Bulk And Tablet Dosage Form By Reverse Phase High Performance Liquid Chromatography

Linganaboina Sirisha [1] , Nagakanyaka Devi P[2*] and Rakesh Kumar Jat[1]

[1]Department of Pharmacy, Shri Jagdishprasad Jhabarmal Tibrewala University, Jhunjhunu, Rajasthan, India, 333001

[2]Department of Pharmaceutical Analysis, Max institute of Pharmaceutical Sciences, Khammam, Telangana, India, 507318

ABSTRACT

Chromatographic parameters were optimized to develop HPLC methods for individual drugs and for simultaneous estimation of combined dosage forms of Imipramine and Chlordiazepoxide in pharmaceutical formulations with short analysis time with acceptable resolution. In the present study a simple, rapid, accurate and robust HPLC method was developed for the estimation of Imipramine and Chlordiazepoxide in their pharmaceutical formulations. The optimum detection wavelength for Imipramine and Chlordiazepoxide combination was 255nm. The mobile phase optimized for Imipramine alone and Imipramine + Chlordiazepoxide combination consists of Methanol and disodium hydrogen phosphate buffer of pH 3.5 in the ratio of 45:55. The mobile phase optimized for Chlordiazepoxide alone was ammonium formate buffer of pH 3 and acetonitrile in the ratio of 67:33 v/v. Columns finalized for individual drugs are imipramine and chlordiazepoxide Thermosil RP C18 (4.6*150mm) 5µm were used as stationary phases. The solutions were chromatographed at a constant flow rate of for Imipramine alone and Imipramine + Chlordiazepoxide combination the flow rate was found to be ideal at 0.7ml/min and for Chlordiazepoxide alone the flow rate selected was 0.8ml/min. Linearity was set up over the focus scope of 10ppm to 50ppm for Imipramine, 15ppm to 75ppm for chlordiazepoxide and Correlation coefficient was seen as 0.999. The mean % Recovery for Imipramine bulk drug it was found to be 99.07%, 99.87% and 100.22% respectively and for Chlordiazepoxide it was found to be 99.41%, 100.02% and 99.67% respectively are within the limits. The mean percentage recovery for Imipramine and Chlordiazepoxide combination at 50%, 100% and 150% levels were found to be 99.64%, 100.04% and 99.89% respectively for Imipramine and 99.57%, 99.78% and 99.83% respectively for Chlordiazepoxide and are within the limits. The LOD value obtained for Imipramine and Chlordiazepoxide combination is 2.951 for Imipramine and 2.878 for Chlordiazepoxide and the LOQ result obtained is 9.902 for Imipramine and 9.731 for Chlordiazepoxide. The percentage purity of Imipramine and Chlordiazepoxide combination was found to be 98.62% for Imipramine and that of Chlordiazepoxide was found to be 99.60%

Keywords: Imipramine, Chlordiazepoxide Assay, RP-HPLC, Validation

INTRODUCTION:

Imipramine, sold under the brand name Tofranil, among others, is a tricyclic antidepressant (TCA) mainly used in the treatment of depression. It is also effective in treating anxiety and panic disorder. The drug is also used to treat bedwetting. Imipramine is taken by mouth.

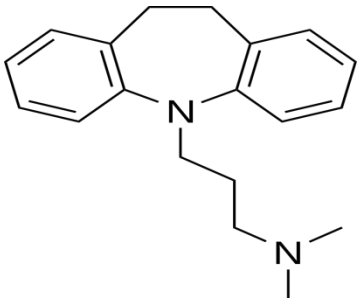


Figure 1: Chemical structure of Imipramine

Imipramine works by inhibiting the neuronal reuptake of the neurotransmitter's norepinephrine and serotonin. It binds the sodium-dependent serotonin transporter and sodium-dependent norepinephrine transporter reducing the reuptake of norepinephrine and serotonin by neurons.

Chlordiazepoxide is a sedative and hypnotic medication of the benzodiazepine class which is used to treat anxiety, insomnia and symptoms of withdrawal from alcohol and other drugs

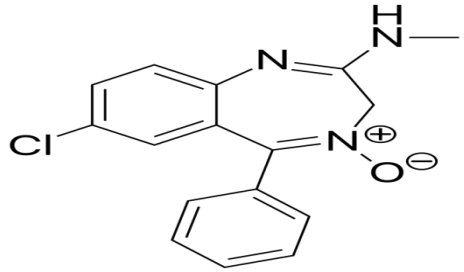


Figure 1: Chemical structure of Chlordiazepoxide

Chlordiazepoxide binds to stereospecific benzodiazepine (BZD) binding sites on GABA (A) receptor complexes at several sites within the central nervous system, including the limbic system and reticular formation. This results in an increased binding of the inhibitory neurotransmitter GABA to the GABA (A) receptor. BZDs, therefore, enhance GABA-mediated chloride influx through GABA receptor channels, causing membrane hyperpolarization.

MATERIALS AND METHODS:

Equipment: Chromatographic separation was conceded on WATERS HPLC system which is outfitted with the 515 dual head reciprocating pump & a 2489 UV Visible detector. The software used is Empower-2 software and Phenomenex kinetex C_{18} (250mm×4.6mm i.d, 5µm) column is used for the investigation.

Chemicals and reagents: Chlordiazepoxide and Imipramine (Assigned purity 99.98%) was obtained as a gift sample from RL Fine Chem Pvt Ltd, Bengaluru, Karnataka. India. Acetonitrile, methanol, HPLC grade water and mono sodium hydrogen orthophosphate and di sodium hydrogen ortho phosphate were procured from local manufacturers.

Preparation of buffer: 0.1gm of mono sodium hydrogen orthophosphate and 0.1gm of di sodium hydrogen ortho phosphate was precisely gauged and moved in to a 500ml volumetric jar, broken up by count HPLC water weakened stamp with water. Mix 51 ml of mono sodium hydrogen orthophosphate with 49 ml of di sodium hydrogen orthophosphate and adjust the pH to 6.8 with orthophosphoric acid.

Preparation of mobile phase: Methanol, Mono and disodium Hydrogen orthophosphate buffer of pH 6.8 and acetonitrile were blended in the proportion of 47:23:30 %V/V and the portable stage was then sifted through 0.45µm layer channel and sonicated for 5min in ultra sonicator shower and moved in to dissolvable repository staying away from air pockets.

Preparation of standard solutions: Accurately 25mg of Imipramine and 10mg of Chlordiazepoxide working standard was weighed and transferred into a 10ml clean dry volumetric flask and about 5ml of mobile phase was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent. From the above stock solution containing 2500µg/ml of Imipramine and 1000µg/ml of Chlordiazepoxide, 1ml was taken and transferred into 10ml volumetric flask and made up to volume with diluent to get a required dilution containing 250µg/ml of Imipramine and 100µg/ml of Chlordiazepoxide. For assay 1ml of standard stock solution was transferred to 25ml volumetric flask and made up to volume with diluents and injected into the HPLC system to calculate the percentage yield of drugs.

Preparation of the test solution: Accurately weighed 10 tablets were crushed in mortar and pestle and transferred amount equivalent to 10mg of Imipramine and 25mg of Chlordiazepoxide sample into a 10ml clean dry volumetric flask and about 5ml of diluent was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Selection of detection wavelength: The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. After optimization of all conditions for UV analysis it is scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the absorption maxima of selected drugs, so that the same wave length can be utilized in HPLC UV detector for estimating the drugs. The overlay spectrum was used for selection of wavelength for individual drugs and the isobestic point of combined formulations with maximum absorbance's is selected for the study.

METHOD DEVELOPMENT [4-6]

Optimized Chromatographic conditions:
Column: Phenomenex kinetex C_{18} (250mm×4.6mm i.d, 5µm) column
Mobile phase: Methanol: Mono and disodium Hydrogen orthophosphate buffer of pH 6.8: acetonitrile (47:23:30 %V/V)
Flow rate: 1ml/min
Injection volume: 20µl
Detection wavelength: 287nm
Mode of elution: Isocratic

Column temperature: Ambient

VALIDATION OF THE METHOD [7-10]

System suitability test: Solution for system suitability test was all set by moving 1ml of standard stock arrangement (1000µg/ml) into 10ml volumetric flagon, weakening to check with diluent and sonicated. This preparation was injected six times into the HPLC system for assessing parameters like number of hypothetical plates (N), peak area and tailing factor.

RESULTS AND DISCUSSION

Determination of Absorption Maxima by UV/Vis Spectrophotometry:

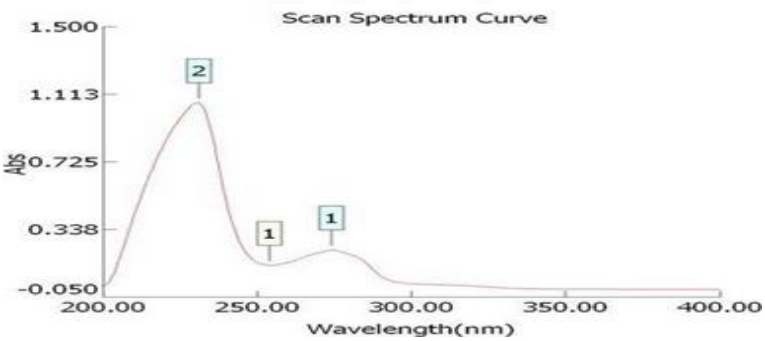


Figure 2: UV spectrum of Imipramine

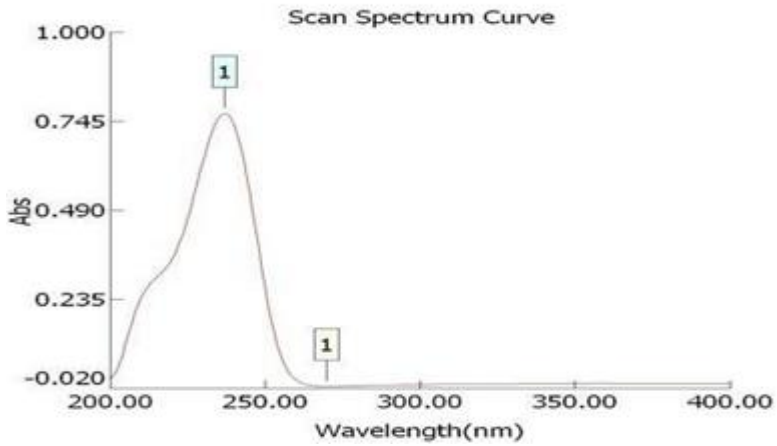


Figure 3: UV spectrum of Chlordiazepoxide

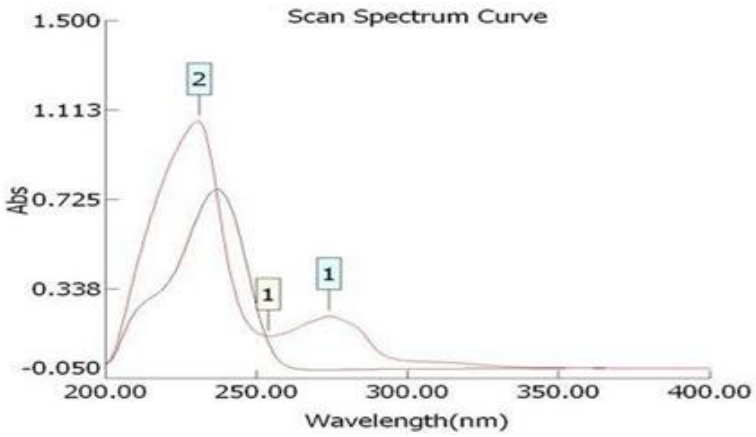


Figure 4: UV Overlay spectrum of Imipramine and Chlordiazepoxide

Observation: The absorbance maximum of both the drugs was found to be at 255nm. Hence 255nm wavelength was selected to carry out simultaneous estimation of drugs.

Trial & error methods:

Trial 1:

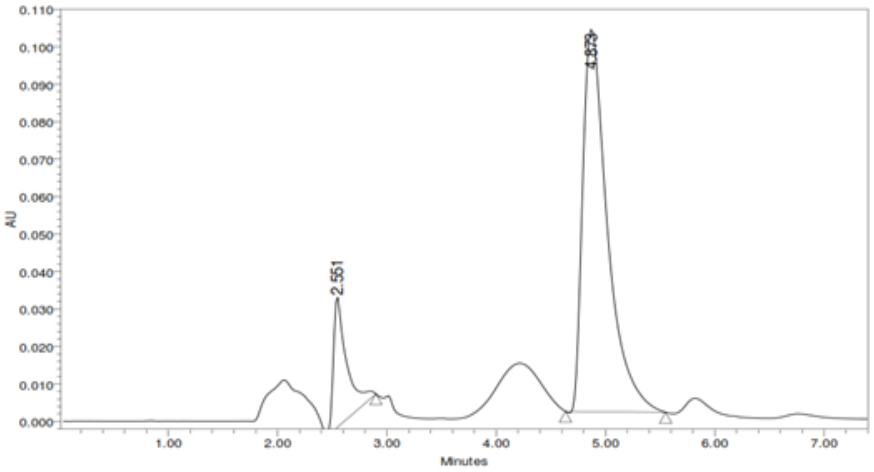
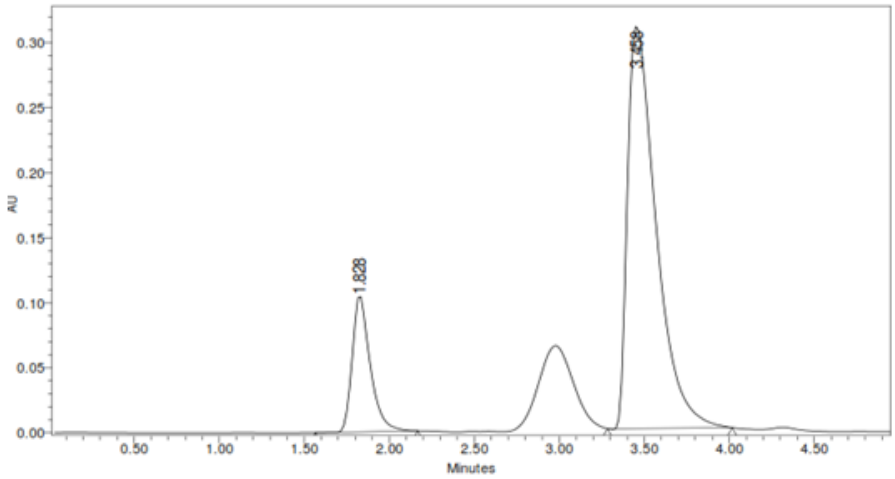


Figure 5: Trial-1 Chromatogram and result

Peak name	Rt	Area	Height	USP Plate count	USP tailing	USP resolution
Imipramine	2.551	8671924	460798	745	2.19	-
Chlordiazepoxide	4.879	2283694	179357	1911	2.79	1.45

Observation: Though Imipramine and Chlordiazepoxide were separated and two individual peaks are displayed but baseline was not proper. Hence, another trial was done.

Trial 2:

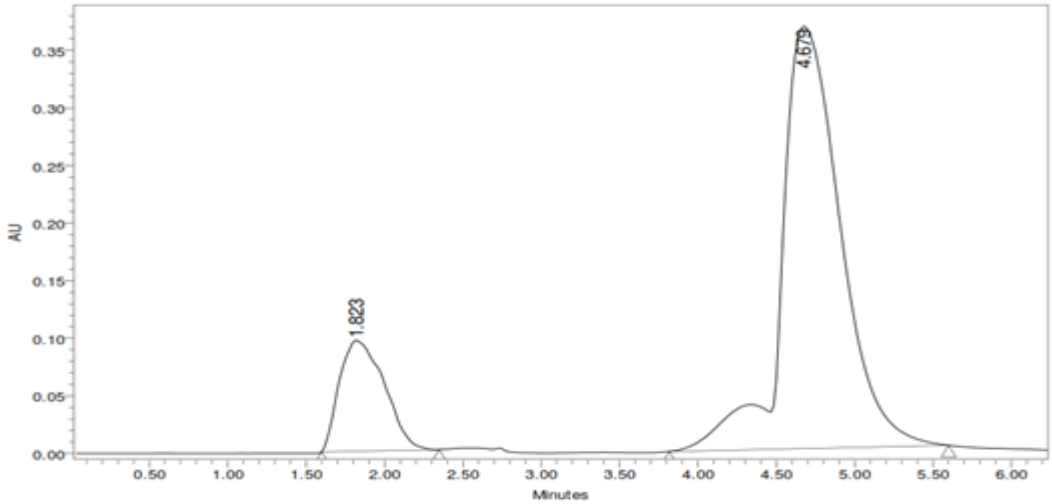


Peak name	Rt	Area	Height	USP Plate count	USP tailing	USP resolution
Imipramine	1.828	7913799	394185	722	2.21	-
Chlordiazepoxide	3.458	1853381	162758	2614	2.85	1.52

Figure 6: Trial-2 Chromatogram and result

Observation: Peaks symmetry is being improved when compared to the previous trial. Plate count was less and USP tailing was more. Further trials are conducted for better resolution.

Trial 3:



Peak name	Rt	Area	Height	USP Plate count	USP tailing	USP resolution
Imipramine	1.823	9849287	482363	198	1.97	-

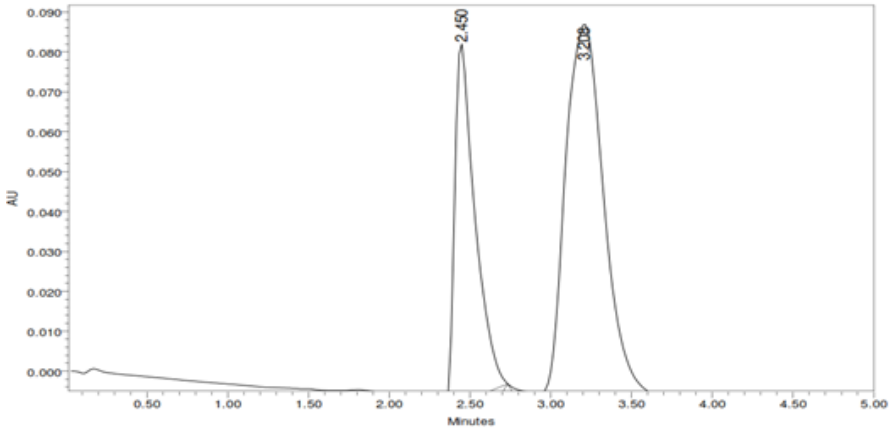
Chlordiazepoxide	4.679	3272312	356630	5036	1.15	4.23

Figure 7: Trial-3 Chromatogram and result

Observation:

There is noticeable improvement in resolution. But peak symmetry is not achieved. Hence another trial was made.

Trial 4:

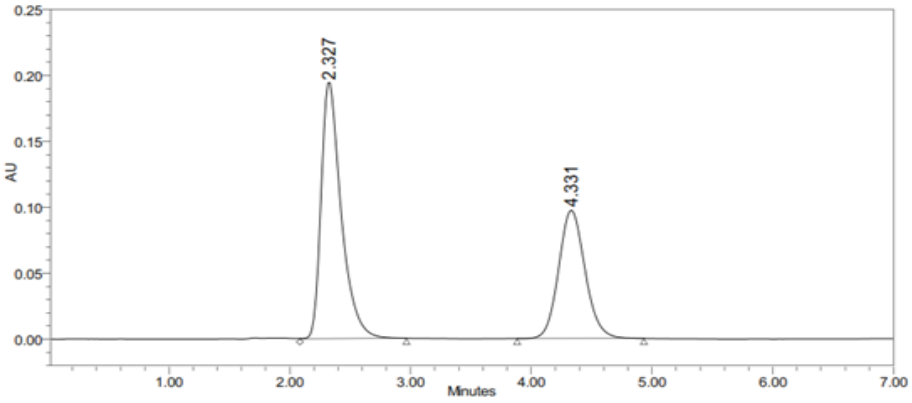


Peak name	Rt	Area	Height	Plate count	USP tailing	Resolution
Imipramine	2.450	11286305	813690	1587	1.46	-
Chlordiazepoxide	3.208	3443649	160557	616	1.80	1.46

Figure 8: Trial-4 Chromatogram and result

Observation: Resolution between the peaks was not good. Peak shapes were not proper. Hence another trial was done.

Trial 5:



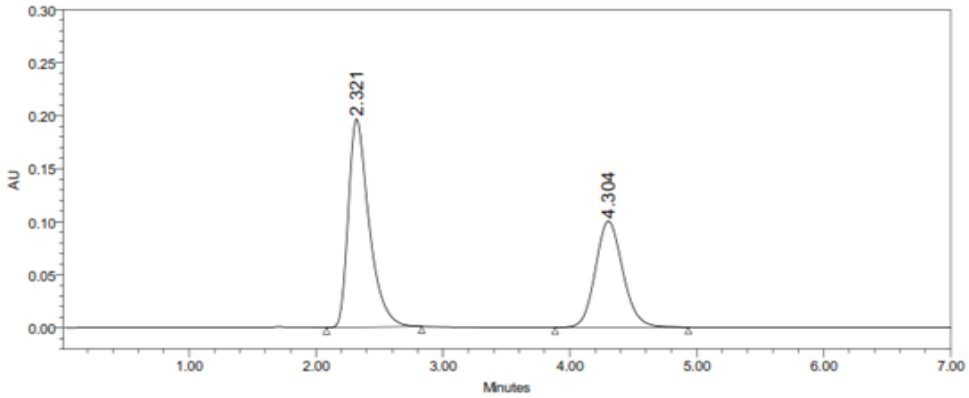
Peak name	Rt	Area	Height	Plate count	USP tailing	Resolution
Imipramine	2.327	1501841	155429	5105	1.3	-
Chlordiazepoxide	4.331	2239589	13239	3788	1.4	8.1

Figure 9: Trial-5 Chromatogram and result (Optimized trial)

Observation: The chromatogram is perfect with clear separation of components. The peak symmetry and system suitability parameters are within the limits. Hence this method is chosen as optimized one

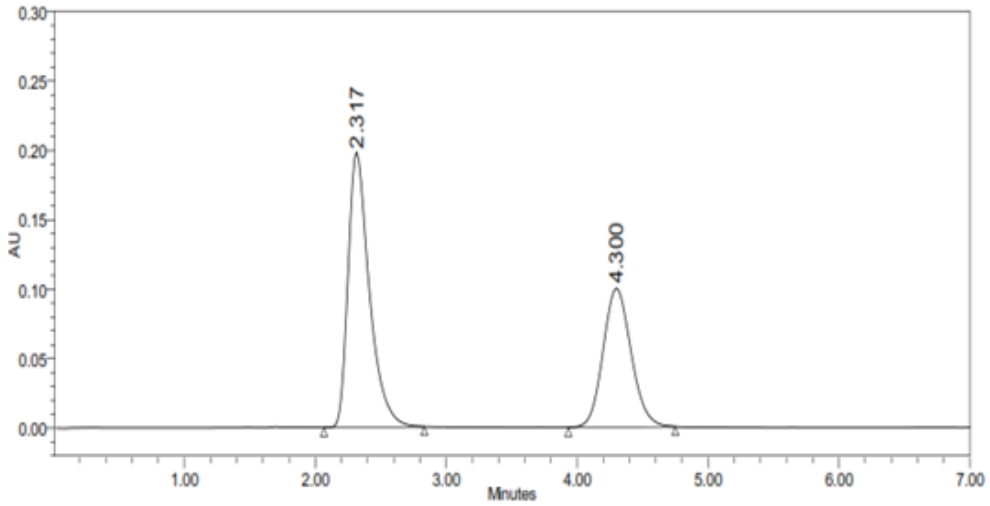
VALIDATION

SYSTEM SUITABILITY TEST (SST)



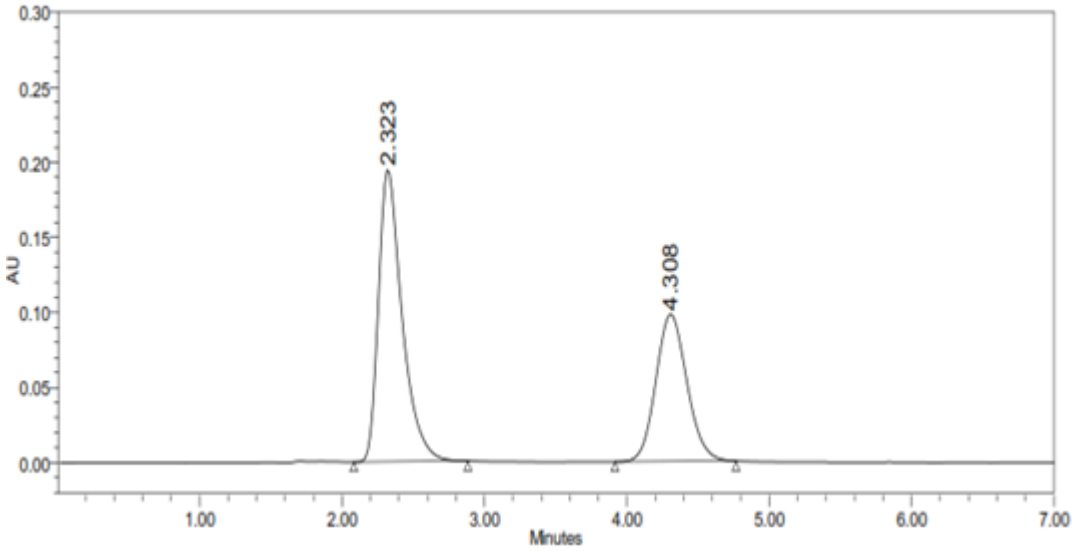
Name	Rt	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.321	1499952	156330	5216	1.319	-
Chlordiazepoxide	4.304	2241008	14028	3899	1.406	7.901

Figure 10: Chromatogram of System suitability (Injection 1)



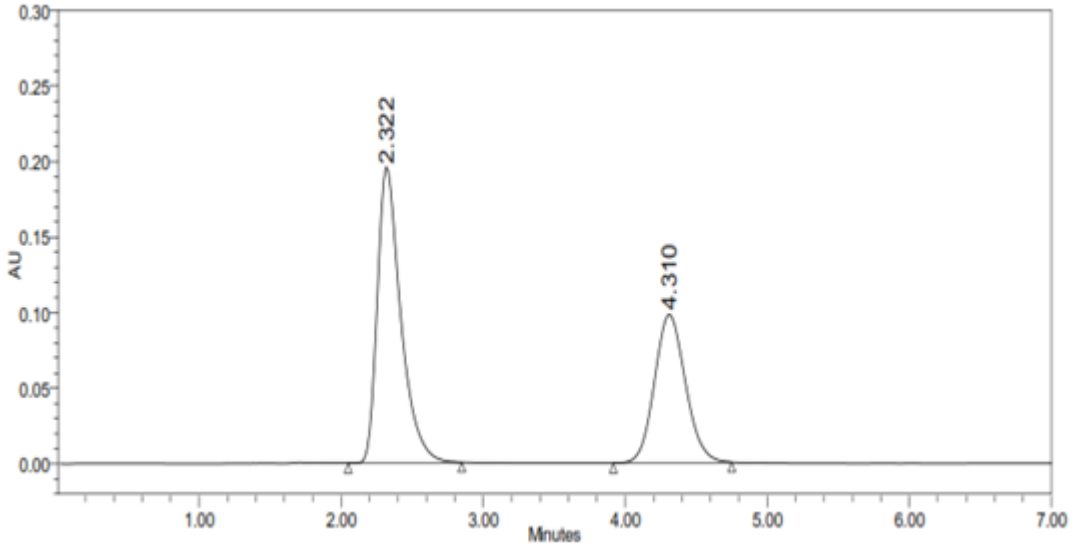
Name	Rt	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.317	1518863	159101	5199	1.326	-
Chlordiazepoxide	4.300	2239632	15001	3901	1.412	6.999

Figure 11: Chromatogram of System suitability (Injection 2)



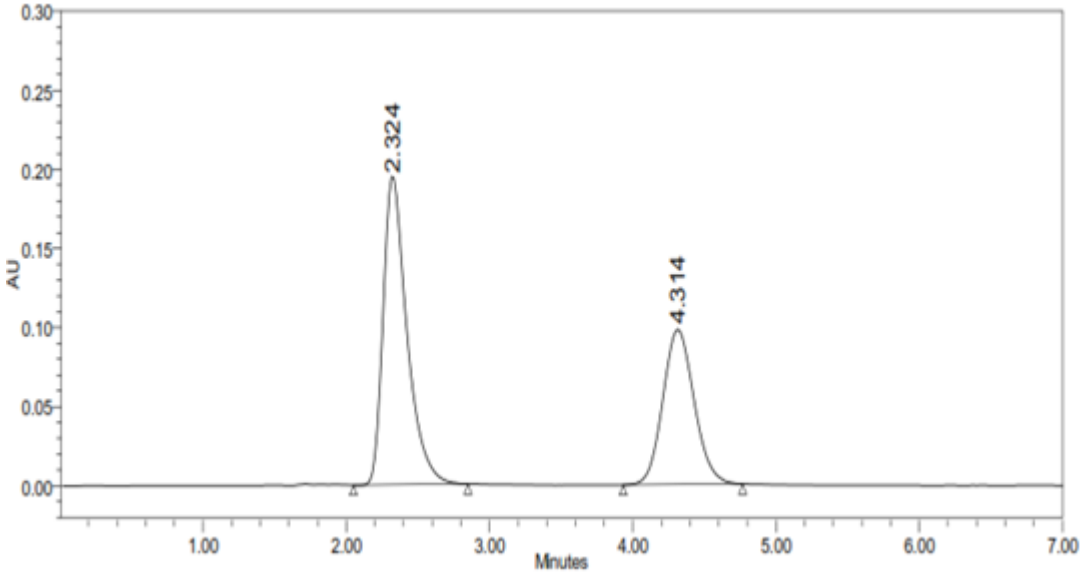
Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.323	1509976	160012	5286	1.342	-
Chlordiazepoxide	4.308	2240012	15992	4001	1.416	7.002

Figure 12: Chromatogram of System suitability (Injection 3)



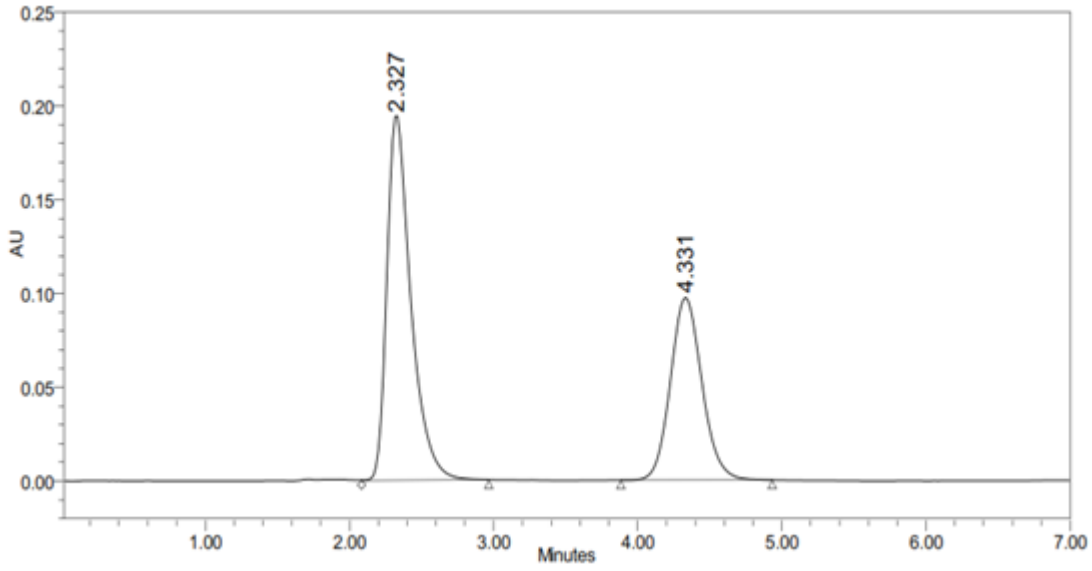
Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.322	1510154	161901	5319	1.315	-
Chlordiazepoxide	4.310	2239619	16345	4010	1.414	7.193

Figure 13: Chromatogram of System suitability (Injection 4)



Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.324	1498765	159918	5320	1.349	-
Chlordiazepoxide	4.314	2222999	15998	4019	1.409	7.806

Figure 14: Chromatogram of System suitability (Injection 5)



Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.327	1519503	159876	5355	1.335	-
Chlordiazepoxide	4.331	2231800	16001	4006	1.416	7.791

Figure 15: Chromatogram of System suitability (Injection 6)

Table 1: Peak results of system suitability for Imipramine

Injections	Retention time	Area	Theoretical plates	Tailing factor
1	2.321	1499952	5216	1.319
2	2.317	1518863	5199	1.326
3	2.323	1509976	5286	1.342
4	2.322	1510154	5319	1.315
5	2.324	1498765	5420	1.349
6	2.327	1519503	5455	1.335
Mean	2.322	1509535.5	5282.5	1.331
SD	0.0033	8885.68	62.292	0.013
%RSD	0.143	0.588	1.179	1.0001

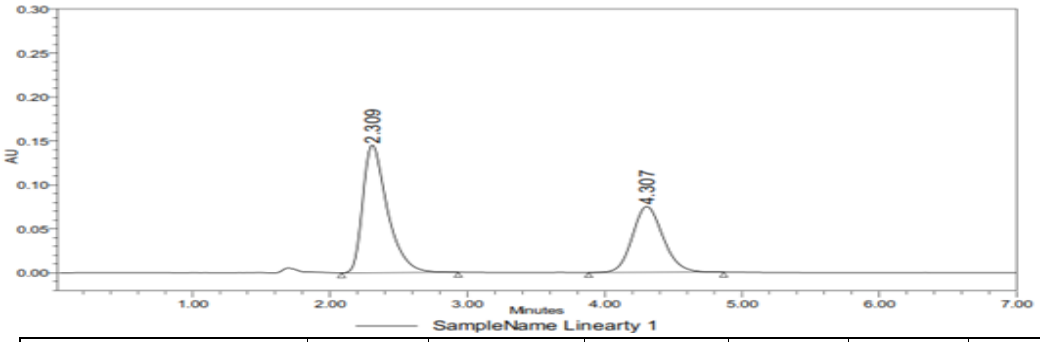
Table 2: Peak results of system suitability for Chlordiazepoxide

Injections	Retention time	Area	Theoretical plates	Tailing factor
1	4.304	2241008	3899	1.406
2	4.300	2239632	3901	1.412
3	4.308	2240012	4001	1.496
4	4.310	2239619	4110	1.434
5	4.314	2222999	4332	1.469
6	4.331	2231800	4620	1.476
Mean	4.311	2235845	3972.66	1.4121
SD	0.0108	7127.22	56.59	0.004
%RSD	0.251	0.318	1.424	0.284

Conclusion: From the results shown in the above table it is clear that the system suitability parameters meet the requirements of method validation**.** The chromatograms were shown in Figures 5.373 to 5.378.

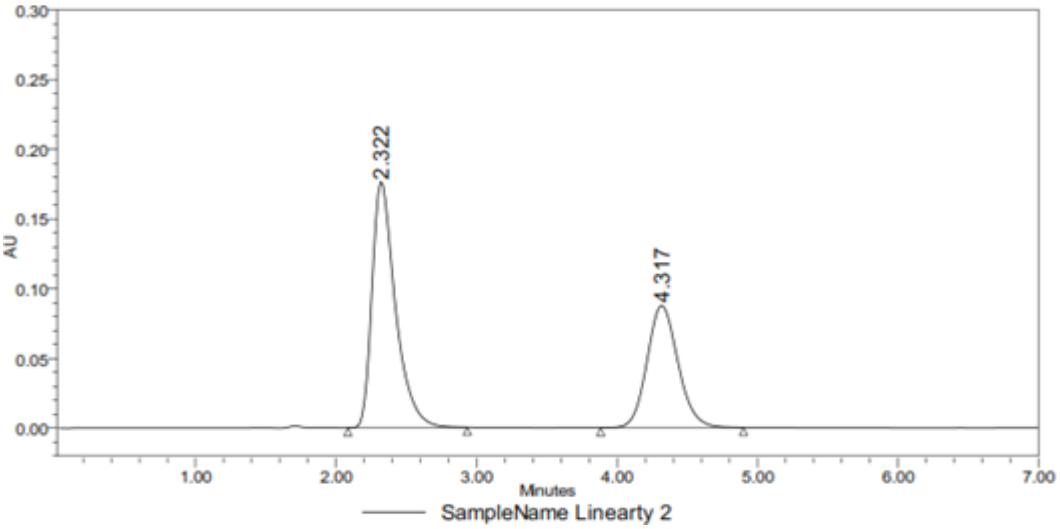
LINEARITY

It was demonstrated over the range of 10-50µg/ml for Imipramine and 15-75µg/ml for Chlordiazepoxide by plotting a graph taking area on Y-axis and attention on X-axis. The R^2, y-intercept, slope was suggested.



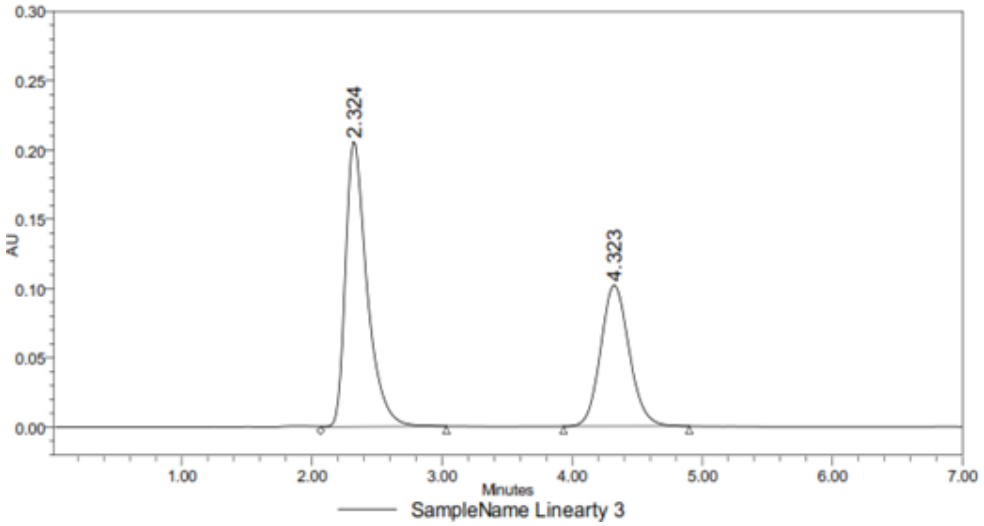
Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.309	514173	145957	5401	1.350	-
Chlordiazepoxide	4.307	860101	75128	4022	1.418	7.907

Figure 16: Chromatogram showing linearity of Imipramine (10µg/ml) & Chlordiazepoxide (15µg/ml)



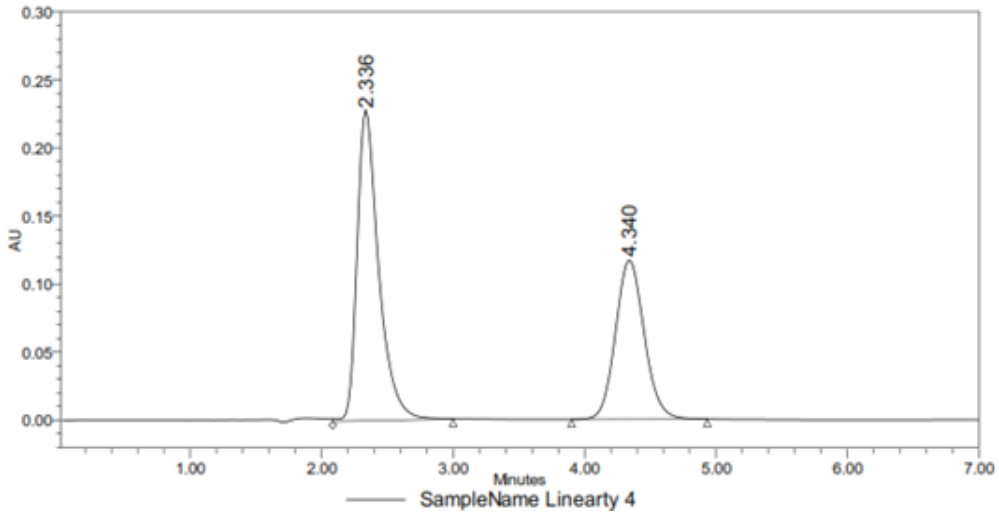
Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.322	1028535	176935	5387	1.356	-
Chlordiazepoxide	4.317	1620201	87703	4031	1.410	7.761

Figure 17: Chromatogram showing linearity of Imipramine (20µg/ml) & Chlordiazepoxide (30µg/ml)



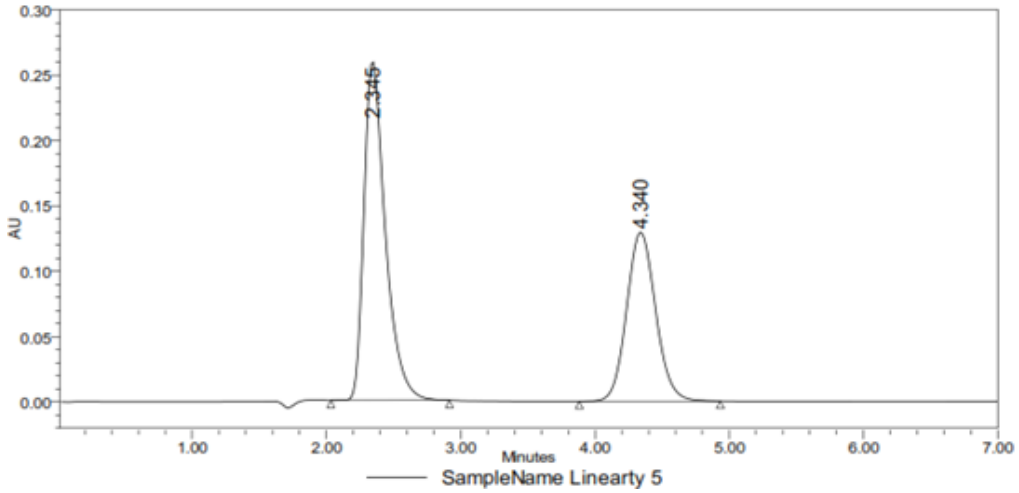
Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.324	1550090	206622	5376	1.378	-
Chlordiazepoxide	4.323	2367133	101999	4082	1.434	7.817

Figure 18: Chromatogram showing linearity of Imipramine (30µg/ml) & Chlordiazepoxide (45µg/ml)



Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.336	2017973	228576	5443	1.387	-
Chlordiazepoxide	4.340	3200179	117084	4067	1.462	7.900

Figure 19: Chromatogram showing linearity of Imipramine (40µg/ml) & Chlordiazepoxide (60µg/ml)



Name	Retention Time	Area	Height	USP Plate Count	USP Tailing	USP Resolution
Imipramine	2.345	2502319	259346	5499	1.355	-
Chlordiazepoxide	4.340	4069778	129409	4075	1.444	7.913

Figure 20: Chromatogram showing linearity of Imipramine (50µg/ml) & Chlordiazepoxide (75µg/ml)

Table 3: Table showing the results for the linearity of Imipramine

S.No	Concentration (µg/ml)	Rt	Peak area
1	10	2.309	514173
2	20	2.322	1028535
3	30	2.324	1550090
4	40	2.336	2017973
5	50	2.345	2502319
R2			0.999
Slope			50127
Intercept			15666

Table 4: Table showing the results for the linearity of Chlordiazepoxide

S.No	Concentration (µg/ml)	Rt	Peak area
1	15	4.307	860101
2	30	4.317	1620201
3	45	4.323	2367133
4	60	4.340	3200179

5	75	4.340	4069778
R2			0.999
Slope			53554
Intercept			11276

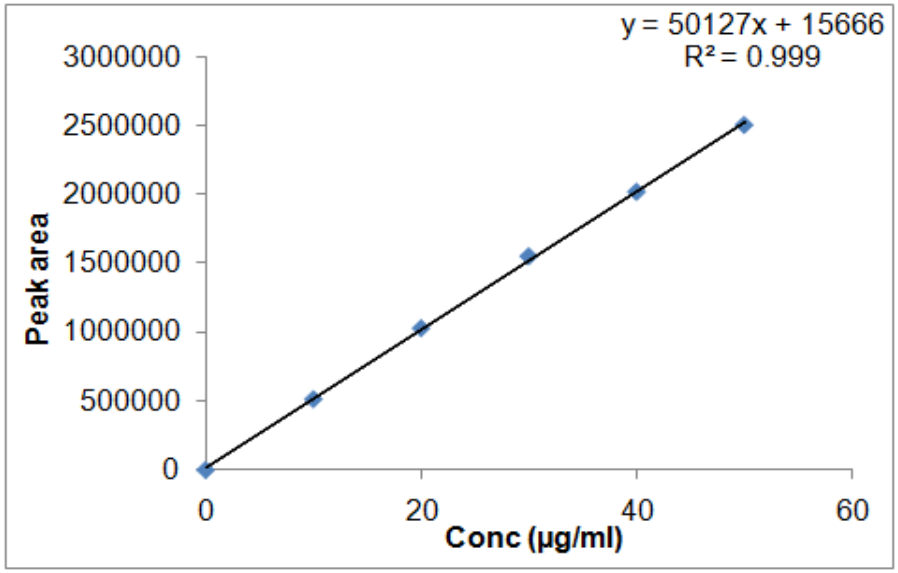


Figure 21: Linearity graph of Imipramine

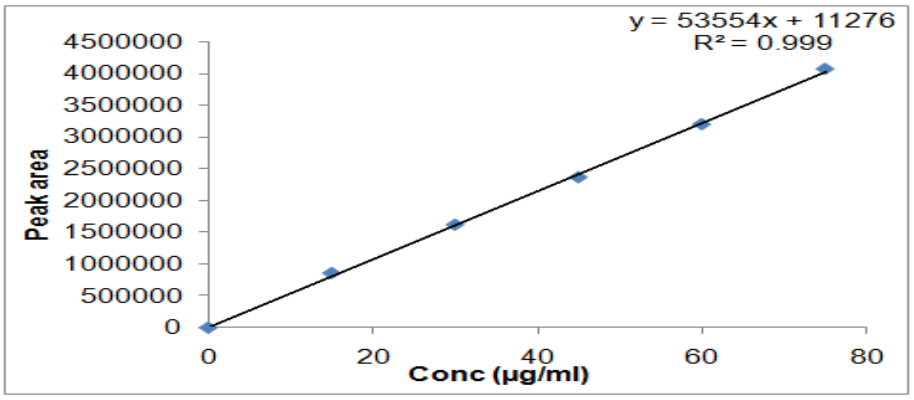


Figure 22: Linearity graph of Chlordiazepoxide

Report: The method was found to be linear in the range of 10 to 50ppm for Imipramine and 15 to 75ppm for Chlordiazepoxide and the R^2 values was found to be 0.999 for both Imipramine and Chlordiazepoxide.

PRECISION

SYSTEM PRECISION

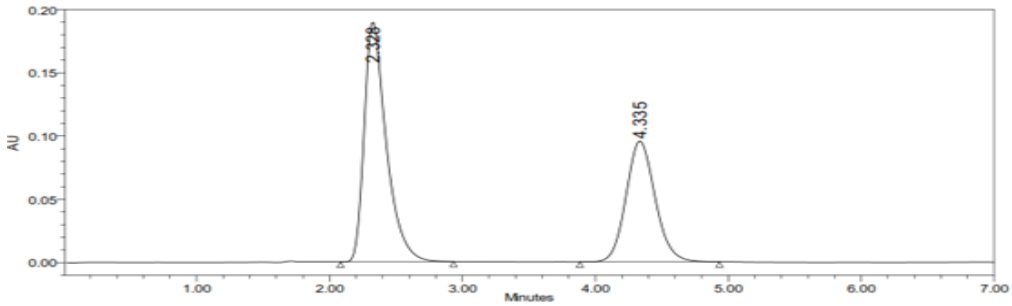


Figure 23: Representative chromatogram for system precision

Table 5: Peak results of system precision for Imipramine

Injections	Peak name	Rt	Area	Height	USP tailing	USP plate count
1	Imipramine	2.328	1500063	158976	1.320	5327
2	Imipramine	2.326	1529974	157998	1.335	5300
3	Imipramine	2.319	1510087	158889	1.353	5397
4	Imipramine	2.318	1521265	156997	1.326	5420
5	Imipramine	2.325	1509876	158109	1.350	5331
6	Imipramine	2.326	1520614	159003	1.346	5466
Mean		2.323	1515313.16	158328.66	1.338	5373.5
S.D		0.0041	10657.45	789.76	0.0134	64.251
%RSD		0.177	0.703	0.498	1.007	1.1957

Table 6: Peak results of system precision for Chlordiazepoxide

Injections	Peak name	Rt	Area	Height	USP tailing	USP plate count
1	Chlordiazepoxide	4.346	2252119	14100	1.427	3943
2	Chlordiazepoxide	4.311	2240743	14310	1.433	3876
3	Chlordiazepoxide	4.319	2251103	13989	1.447	4001
4	Chlordiazepoxide	4.321	2240720	14000	1.445	4019
5	Chlordiazepoxide	4.325	2234000	14001	1.430	4012

6	Chlordiazepoxide	4.342	2242911	14385	1.485	4015
Mean		4.327	2243599	14130.8	1.444	3977.7
Standard deviation		0.0137	6898.69	174.208	0.021	57.20
%RSD		0.3177	0.3074	1.232	1.483	1.438

Report: The Retention time and area for Imipramine and Chlordiazepoxide peaks obtained from six replicate injections are consistent as evidenced by the values of relative standard deviation. Hence it can be concluded that the system precision parameter meets the requirement of method validation.

METHOD PRECISION:

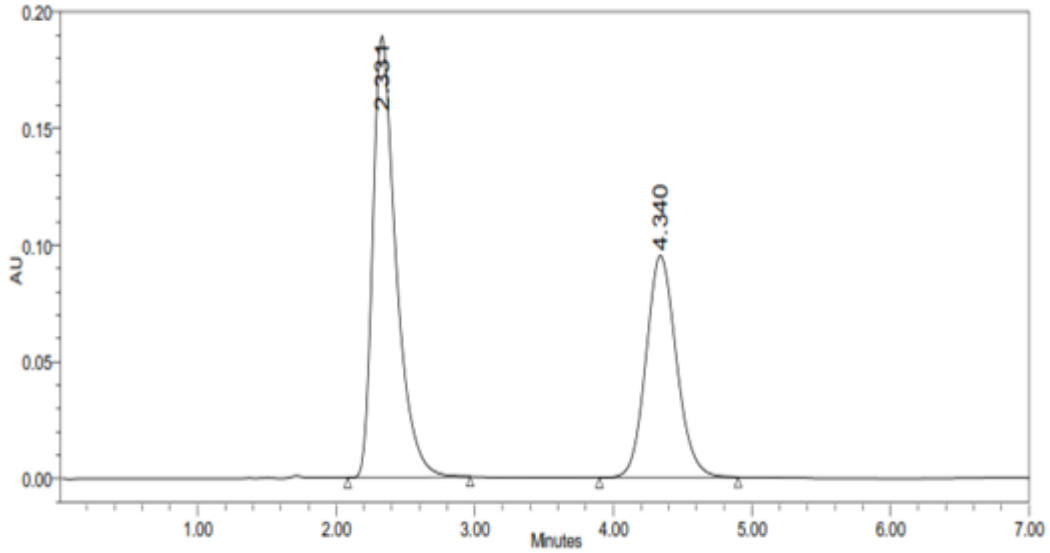


Figure 24: Representative chromatogram for method precision

Table 7: Peak results of method precision for Imipramine

Injections	Peak name	Rt	Area	Height	USP tailing	USP plate count
1	Imipramine	2.331	1511174	159887	1.314	5221
2	Imipramine	2.320	1510085	158009	1.337	5188
3	Imipramine	2.319	1518998	159990	1.349	5297
4	Imipramine	2.334	1500859	157888	1.326	5320
5	Imipramine	2.345	1510987	159210	1.350	5231
6	Imipramine	2.368	1509982	159114	1.346	5266
Mean		2.336	1510347.5	159016.3	1.337	5253.8
Standard deviation		0.0183	5764.67	898.99	0.014	49.64
%RSD		0.784	0.381	0.565	1.080	0.944

Table 8: Peak results of method precision for Chlordiazepoxide

Injections	Peak name	Rt	Area	Height	USP tailing	USP plate count
1	Chlordiazepoxide	4.340	2249108	14123	1.429	4069
2	Chlordiazepoxide	4.311	2241856	14567	1.428	4011
3	Chlordiazepoxide	4.321	2252194	14190	1.431	4052
4	Chlordiazepoxide	4.319	2256820	14246	1.440	4164
5	Chlordiazepoxide	4.317	2243532	14369	1.478	4032
6	Chlordiazepoxide	4.323	2250621	14345	1.454	4125
Mean		4.321	2249021	14306	1.443	4075.5
Standard deviation		0.0098	5566.43	157.5	0.019	58.188
%RSD		0.226	0.247	1.101	1.357	1.427

Report:

%RSD of peak areas for method precision of Imipramine was found to be 0.381 and for Chlordiazepoxide it was found to be 0.247. The values are found to be within the limits i.e., NMT 2%

INTERMEDIATE PRECISION (RUGGEDNESS): Ruggedness study was carried out by injecting 6 injections into HPLC system on different days and values are noted as shown in table

Table 9: Intermediate precision study of Imipramine

S. No	DAY 1	DAY 2
Injections	**Peak area**	**Peak area**
1	1512345	1554321
2	1517890	1545432
3	1524681	1517654
4	1536912	1529876
5	1548126	1530123
6	1517654	1539768
Mean	1526268	1536195.6
SD	13651.57	13016.97
% RSD	0.894	0.847

Table 10: Intermediate precision study of Chlordiazepoxide

S. No	DAY 1	DAY 2
Injections	Peak area	Peak area
1	2245678	2254567
2	2243456	2243210
3	2239876	2255432
4	2250123	2239865
5	2248765	2240012
6	2252345	2251468
Mean	2246707.1	2247425.6
SD	4603.95	7229.63
% RSD	0.204	0.321

Report: On day-1 %RSD of peak areas of Imipramine was found to be 0.894 and day-2 it was found to be 0.847. On day-1 %RSD of peak areas of Chlordiazepoxide was found to be 0.204 and day-2 it was found to be 0.321. The %RSD value obtained was found to be within the limits i.e., less than 2% and chosen method was found to be rugged.

5.11.3.5 ACCURACY

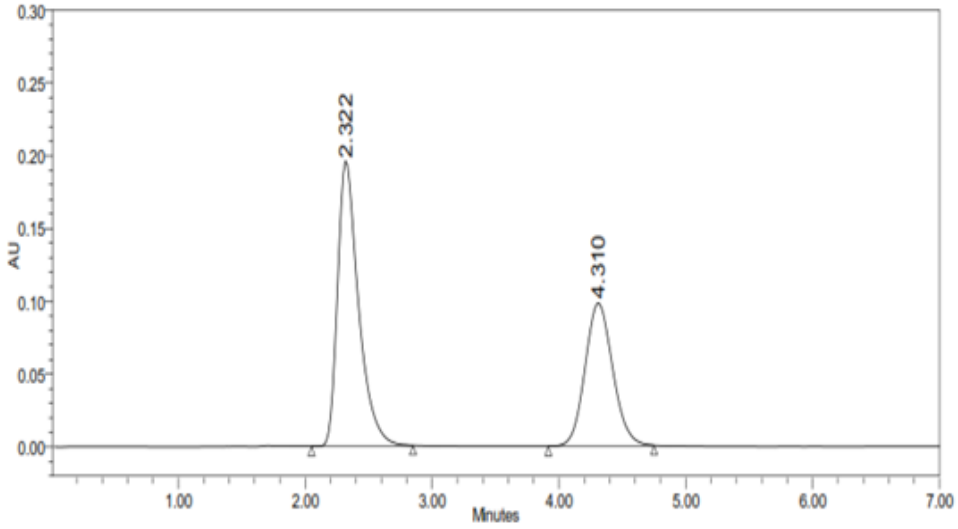


Figure 25: Chromatogram of 50% recovery-Injection 1

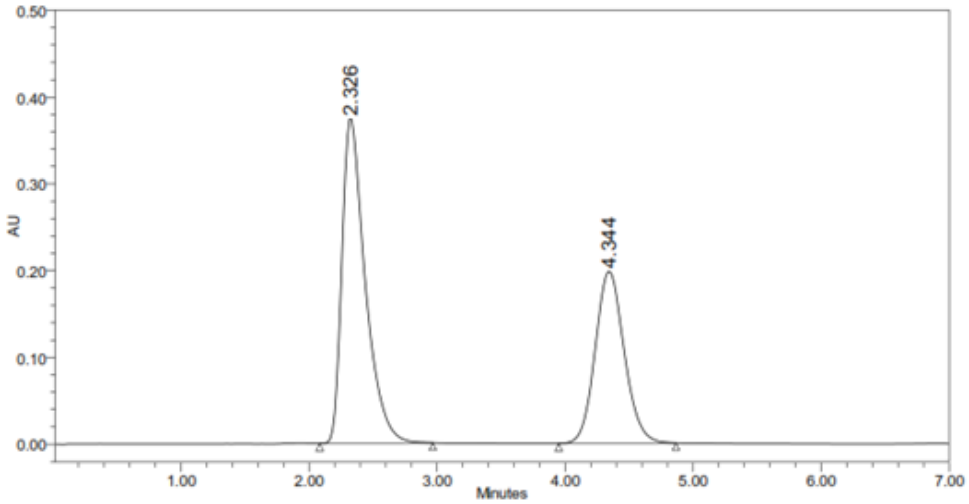


Figure 26: Chromatogram of 50% recovery-Injection 2

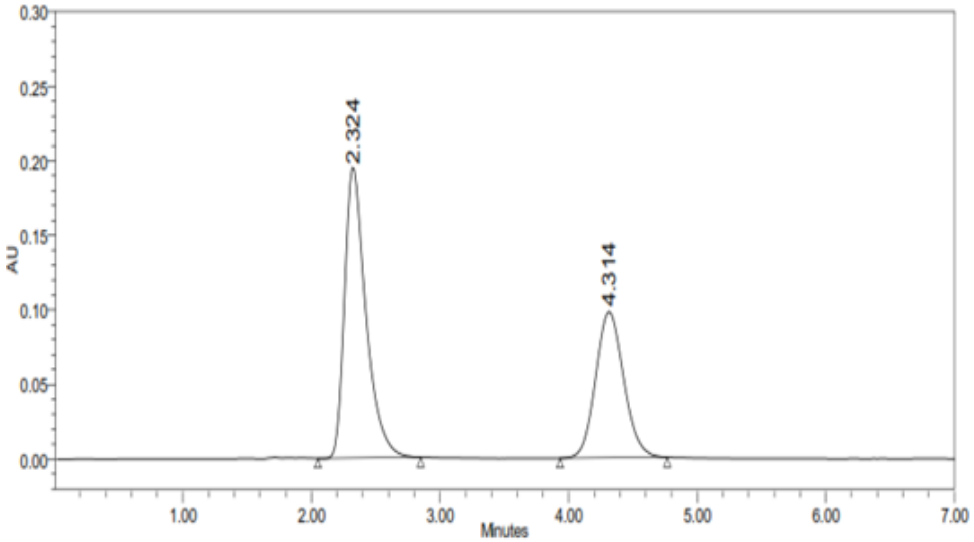


Figure 27: Chromatogram of 50% recovery-Injection 3

Table 11: Results of accuracy for sample concentration-50%

	Peak name	RT	Area	Height	USP tailing	USP plate count	USP resolution
1	Imipramine	2.322	855548	159832	1.390	5265	-
2	Chlordiazepoxide	4.310	1226871	14563	1.481	4197	7.981
3	Imipramine	2.326	855939	159209	1.382	5304	-
4	Chlordiazepoxide	4.344	1229311	14658	1.454	4179	7.910
5	Imipramine	2.324	855716	158982	1.390	5386	-
6	Chlordiazepoxide	4.314	1224105	14990	1.445	4281	7.987

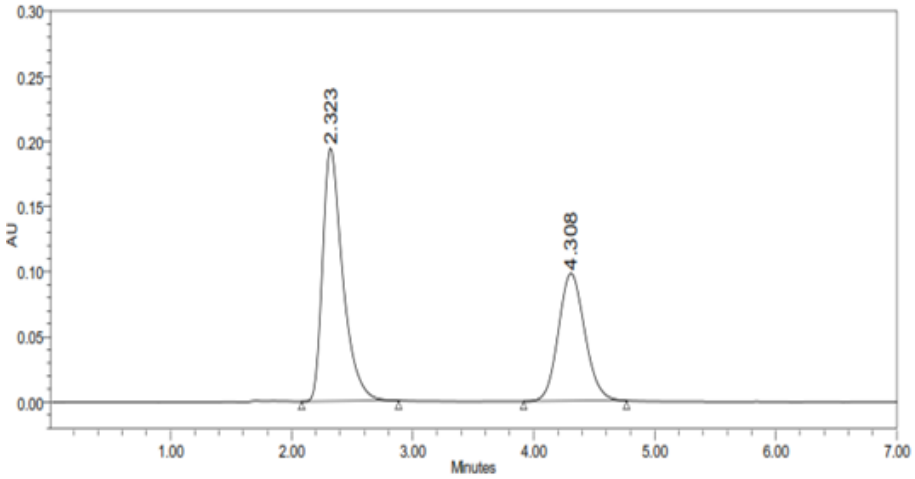


Figure 28: Chromatogram of 100% recovery-Injection 1

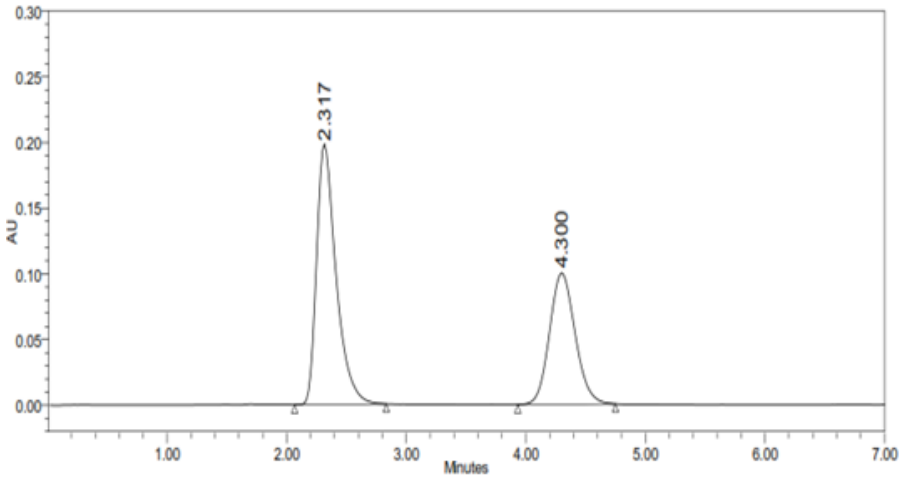


Figure 29: Chromatogram of 100% recovery-Injection 2

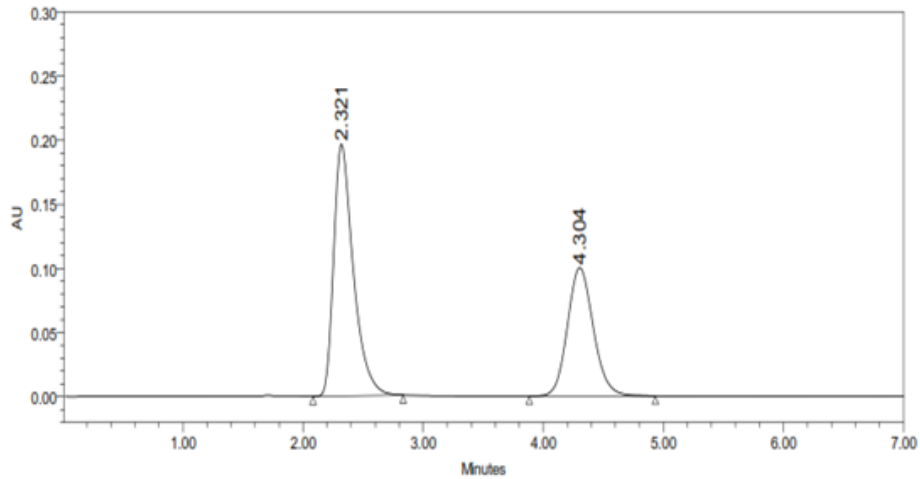


Figure 30: Chromatogram of 100% recovery-Injection 3

Table 12: Results of accuracy for sample concentration-100%

	Peak name	RT	Area	Height	USP tailing	USP plate count	USP resolution
1	Imipramine	2.323	1511096	158911	1.371	5286	-
2	Chlordiazepoxide	4.308	2253742	14654	1.448	4152	7.898
3	Imipramine	2.317	1511879	159102	1.367	5240	-
4	Chlordiazepoxide	4.300	2258621	14468	1.439	4187	7.907
5	Imipramine	2.321	1511432	159887	1.389	5290	-
6	Chlordiazepoxide	4.304	2248210	14824	1.431	4166	7.951

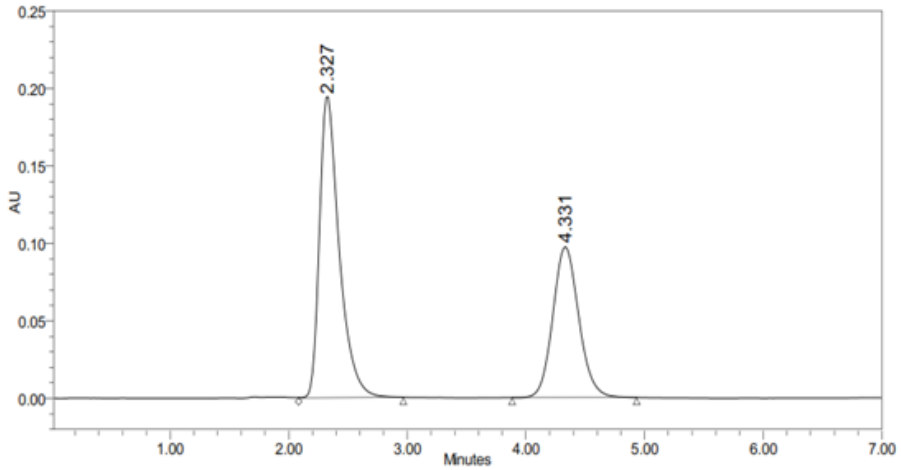


Figure 31: Chromatogram of 150% recovery-Injection 1

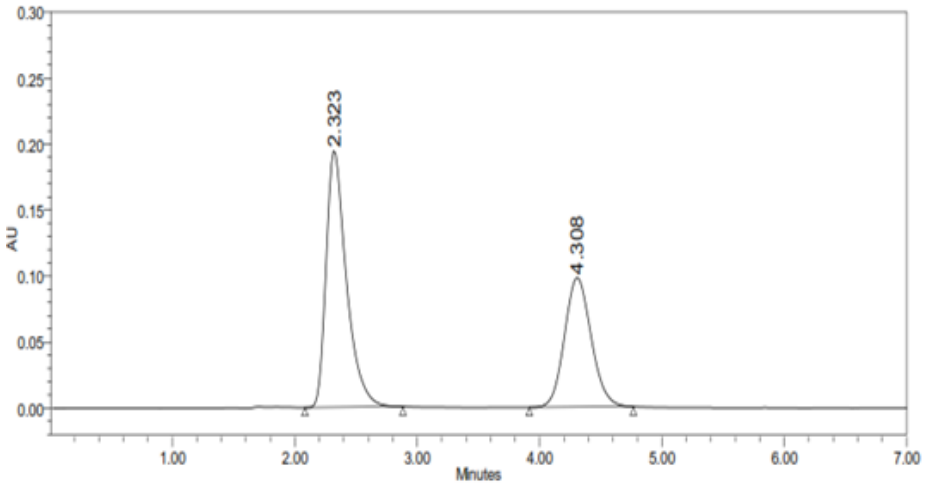


Figure 32: Chromatogram of 150% recovery-Injection 2

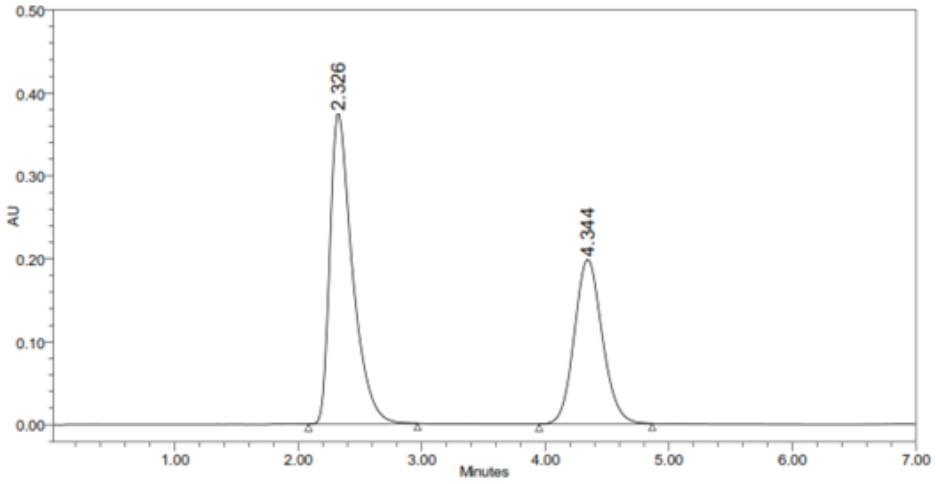


Figure 33: Chromatogram of 150% recovery-Injection 3

Table 13: Results of accuracy for sample concentration-150%

	Peak name	RT	Area	Height	USP tailing	USP plate count	USP resolution
1	Imipramine	2.327	2366644	159900	1.390	5301	-
2	Chlordiazepoxide	4.331	3380613	14846	1.482	4263	7.963
3	Imipramine	2.323	2367819	159820	1.394	5361	-
4	Chlordiazepoxide	4.308	3387932	14981	1.496	4290	7.974
5	Imipramine	2.326	2267148	159779	1.395	5311	-
6	Chlordiazepoxide	4.344	3372315	14943	1.474	4235	7.967

Table 14: Accuracy-%Recovery of Imipramine

S.no	Level	Amount present (mg)	Amount of standard drug added(mg)	Final amount in mg	Total amount recovered in mg	% Recovery	Mean % recovery
1	50%	15	7.5	22.5	22.34	99.28	99.64
2	50%	15	7.5	22.5	22.41	99.6	
3	50%	15	7.5	22.5	22.51	100.04	
1	100%	15	15	30	30.06	100.2	100.04
2	100%	15	15	30	30	100	
3	100%	15	15	30	29.98	99.93	
1	150%	15	22.5	37.5	37.41	99.76	99.89
2	150%	15	22.5	37.5	37.47	99.92	

3	150%	15	22.5	37.5	37.5	100	

Table 15: Accuracy-%Recovery of Chlordiazepoxide

S.no	Level	Amount present (mg)	Amount of standard drug added(mg)	Final amount in mg	Total amount recovered in mg	% Recovery	Mean % recovery
1	50%	15	2.5	17.5	17.47	99.82	99.57
2	50%	15	2.5	17.5	17.39	99.37	
3	50%	15	2.5	17.5	17.42	99.54	
1	100%	15	5	20	19.87	99.35	99.78
2	100%	15	5	20	19.98	99.9	
3	100%	15	5	20	20.02	100.1	
1	150%	15	7.5	22.5	22.49	99.95	99.83
2	150%	15	7.5	22.5	22.52	100.08	
3	150%	15	7.5	22.5	22.38	99.46	

Data interpretation: The mean percentage recovery for Imipramine at 50%, 100% and 150% levels were found to be 99.64%, 100.04% and 99.89% respectively and the mean percentage recovery for Chlordiazepoxide at 50%, 100% and 150% levels was found to be 99.57%, 99.78% and 99.83% respectively and are within the limits. The excellent mean recoveries suggested the good accuracy of the proposed method.

SPECIFICITY: In this study the blank, standard and sample solutions are injected to detect the interference of any impurity at the Rt of the sample or standard peak. The chromatograms are shown in Figures 5.397 to 5.399.

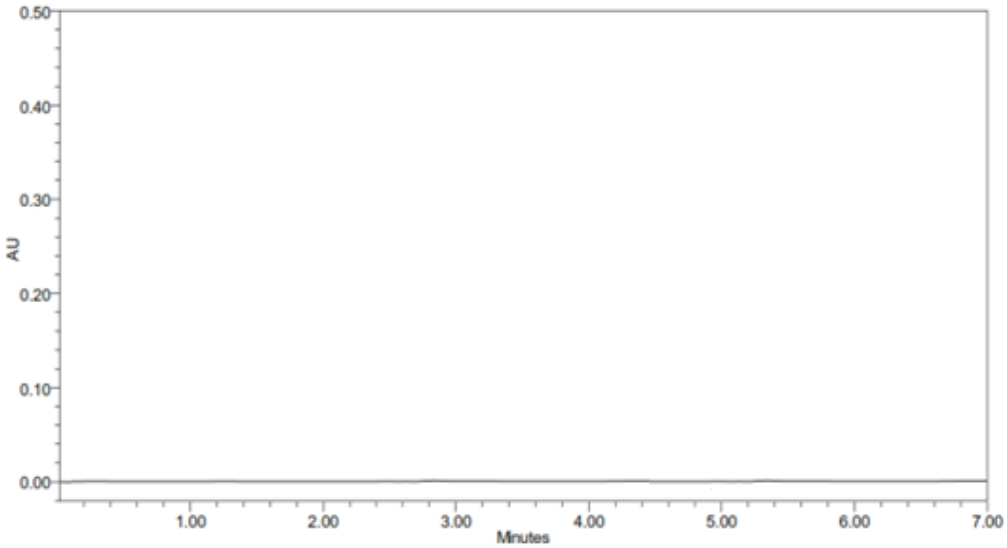
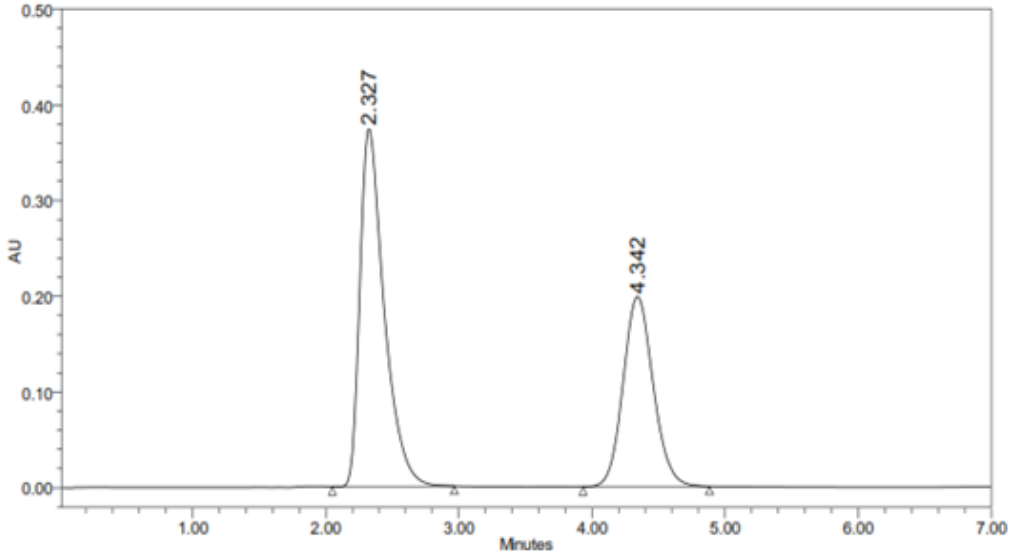
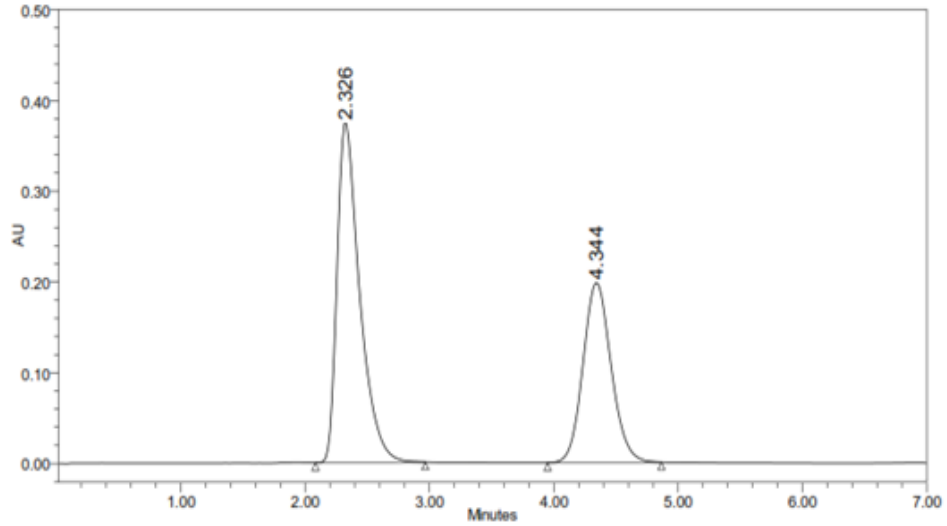


Figure 34: Chromatogram showing blank (mobile phase preparation)



Peak name	RT	Area	Height	USP plate count	USP tailing	USP resolution
Imipramine	2.327	1518764	159876	5764	1.378	-
Chlordiazepoxide	4.342	2245310	16143	4213	1.421	7.854

Figure 35: Chromatogram showing standard injection



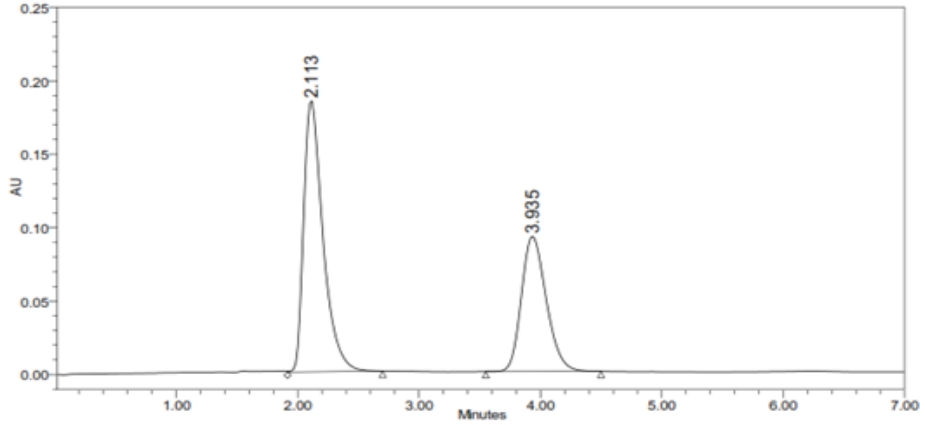
Peak name	RT	Area	Height	USP plate count	USP tailing	USP resolution
Imipramine	2.326	1521398	159934	5543	1.363	-
Chlordiazepoxide	4.344	2243457	16273	4542	1.432	7.952

Figure 36: Chromatogram showing sample injection

Conclusion: There was no interference of blank at the retention time of standard and sample solutions of both the drugs. Hence the method was found to be specific.

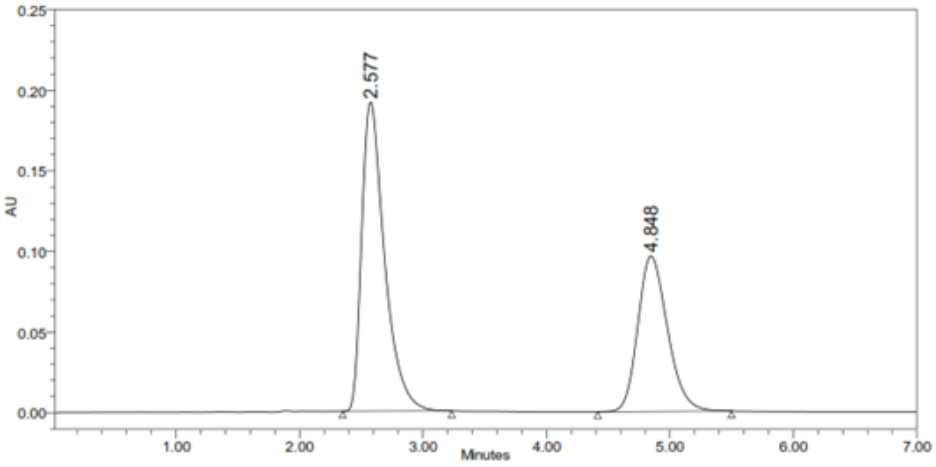
5.11.3.7 ROBUSTNESS: The robustness was performed for the flow rate variations from 0.5ml/min to 0.9ml/min, wavelength variation from 253 to 257nm and mobile phase ratio variation from more organic phase to less organic phase ratio for Imipramine and Chlordiazepoxide.

Variation in flow



Peak name	RT	Area	Height	Plate count	USP tailing	Resolution
Imipramine	2.113	1653210	160123	5318	1.374	-
Chlordiazepoxide	3.935	2414568	15391	4011	1.419	7.160

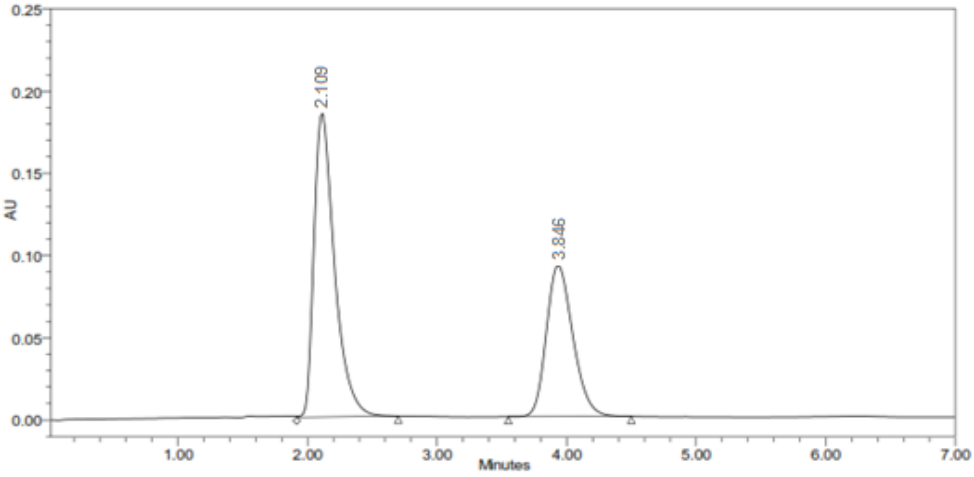
Figure 37: Chromatogram showing more flow of 0.9ml/min



Peak name	RT	Area	Height	Plate count	USP tailing	Resolution
Imipramine	2.577	1401105	159998	5206	1.401	-
Chlordiazepoxide	4.848	2099876	16082	4001	1.423	7.589

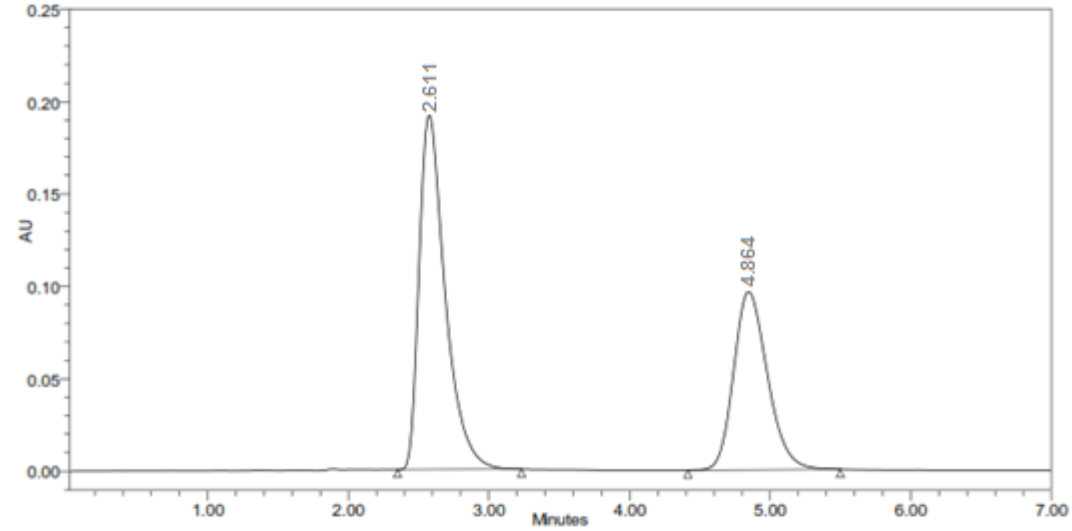
Figure 38: Chromatogram showing less flow of 0.5ml/min

Variation of mobile phase organic composition



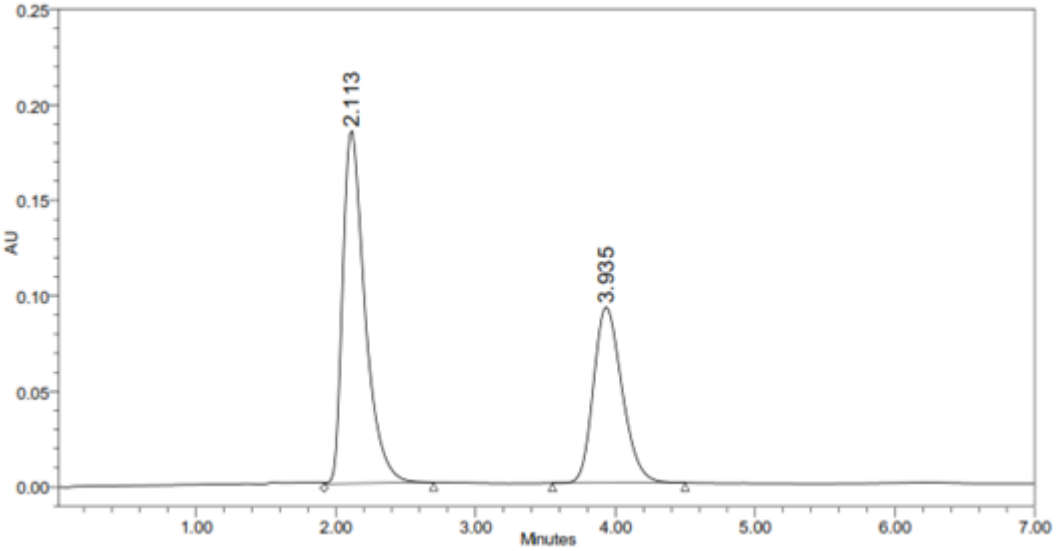
Peak name	RT	Area	Height	Plate count	USP tailing	Resolution
Imipramine	2.109	1622418	161004	5232	1.349	-
Chlordiazepoxide	3.846	2436998	15990	4102	1.422	7.614

Figure 39: Chromatogram showing more organic composition



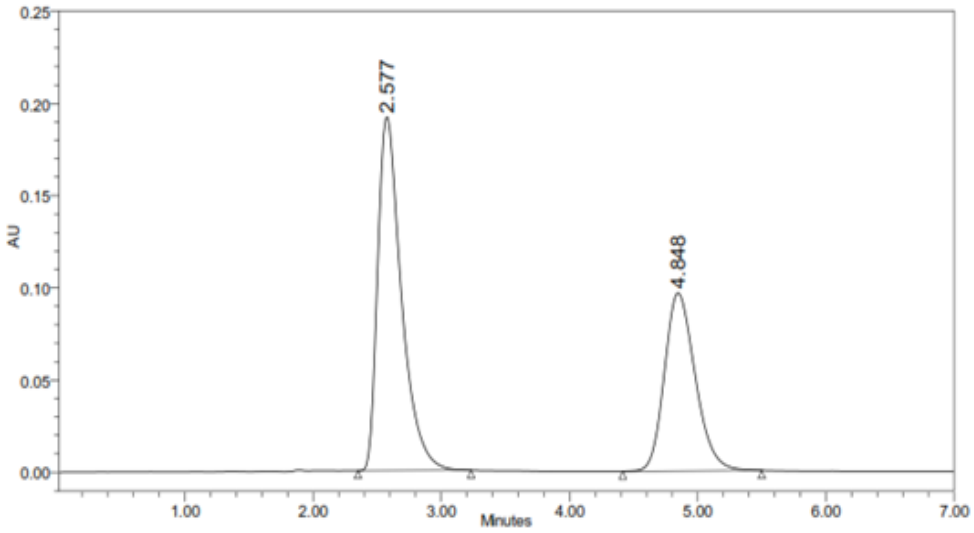
Peak name	RT	Area	Height	USP plate count	USP tailing	USP resolution
Imipramine	2.611	1412351	158865	5301	1.386	-
Chlordiazepoxide	4.864	2076512	14981	4005	1.411	7.601

Figure 40: Chromatogram showing less organic composition Change in wavelength



Peak name	RT	Area	Height	USP plate count	USP tailing	USP resolution
Imipramine	2.113	1436543	159876	5276	1.354	-
Chlordiazepoxide	3.935	2098761	14321	4009	1.413	7.803

Figure 41: Chromatogram showing increased wavelength (257nm)



Peak name	RT	Area	Height	Plate count	USP tailing	Resolution
Imipramine	2.577	1612368	157654	5119	1.397	-
Chlordiazepoxide	4.848	2399864	14987	4015	1.401	7.762

Figure 42: Chromatogram showing decreased wavelength (253nm)

Table 5.171: Robustness of Imipramine

S.no	Parameter	Retention time	Average Area	Tailing factor	USP plate count
1	Initial Imipramine	2.322	1509535.5	1.331	5282.5
2	Flow (-0.2ml/min)	2.577	1401105	1.401	5206
3	Flow (+0.2ml/min)	2.113	1653210	1.374	5318
4	Organic (-)	2.611	1412351	1.386	5301
5	Organic (+)	2.109	1622418	1.349	5232
6	Wavelength (+)	2.113	1436543	1.354	5276
7	Wavelength (-)	2.577	1612368	1.397	5119

Table 16: Robustness of Chlordiazepoxide

S.no	Parameter	Retention time	Area	Tailing factor	USP plate count
1	Initial Chlordiazepoxide	4.311	2235845	1.4121	4212.6
2	Flow (-0.2ml/min)	4.848	2099876	1.423	4011
3	Flow(+0.2ml/min)	3.935	2414568	1.419	4001
4	Organic (-)	4.864	2076512	1.411	4005
5	Organic (+)	3.846	2436998	1.422	4102
6	Wavelength (+)	3.935	2098761	1.413	4009
7	Wavelength (-)	4.848	2399864	1.401	4015

Acceptance criteria: There was no significant change in the parameters like resolution, tailing factor and plate count. The %RSD obtained for change of flow rate, change in wavelength and variation in mobile phase ratio was found to be within the acceptance criteria. Hence the method is robust.

SUMMARY AND CONCLUSION:

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of individual and combined drugs was done by RP-HPLC

The optimum detection wavelength for Imipramine and Chlordiazepoxide combination was 255nm. The mobile phase optimized for Imipramine alone and Imipramine + Chlordiazepoxide combination

consists of Methanol and disodium hydrogen phosphate buffer of pH 3.5 in the ratio of 45:55. The mobile phase optimized for Chlordiazepoxide alone was ammonium formate buffer of pH 3 and acetonitrile in the ratio of 67:33 v/v. The mean percentage recovery for Imipramine and Chlordiazepoxide combination at 50%, 100% and 150% levels was found to be 99.64%, 100.04% and 99.89% respectively for Imipramine and 99.57%, 99.78% and 99.83% respectively for Chlordiazepoxide and are within the limits.

The excellent mean recoveries suggested the good accuracy of the proposed methods.

There was no interference of blank at the retention time of standard and sample solutions of both the individual drugs and the combined dosage forms. Hence the selected methods were found to be specific.

Robustness study was carried out with change in flow rate, mobile phase combination and wavelength. The % RSD for individual drugs and for selected formulations were found to be not more than 2.0 % for variation in each parameter. The LOD value obtained for Imipramine and Chlordiazepoxide combination is 2.951 for Imipramine and 2.878 for Chlordiazepoxide and the LOQ result obtained is 9.902 for Imipramine and 9.731 for Chlordiazepoxide. The percentage purity of Imipramine and Chlordiazepoxide combination was found to be 98.62% for Imipramine and that of Chlordiazepoxide was found to be 99.60%

The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

ACKNOWLEDGEMENTS: The authors acknowledge to RL Fine Chem Pvt Ltd, Bengaluru, Karnataka. India for providing gift sample of Chlordiazepoxide and Imipramine

CONFLICT OF INTEREST: The authors declare that they have no conflict of interests.

REFERENCES

[1] Radhakrishna T, Sreenivas Rao D, Om Reddy G. Determination of pioglitazone hydrochloride in bulk and pharmaceutical formulations by HPLC and MEKC methods. J Pharm Biomed Anal. July 2002; 29(4): 593-607.

[2] Radhakrishna T, Satyanarayana J, Satyanarayana A. LC determination of rosiglitazone in bulk and pharmaceutical formulation. J Pharm Biomed Anal. July 2002; 29(5): 873- 80.

[3] Rajan V. Rele. (2016), Development of Analytical Method by RP-HPLC Technique for Determination of Alprazolam in Pharmaceutical Dosage Form. International Journal of Pharm Tech Research IJPRIF; 9(9): 408-414.

[4] Rajan VS Thiruvengada, Saleem TS Mohamed, S Ramkanth, M Alagusundaram, K Ganaprakash, and Chetty C Madhusudhana. (2010), A Simple RP-HPLC Method for Quantitation of Itopride HCl in tablet dosage form. J Young Pharm.; 2(4): 410–413.

[5] Ramadevi, S. Srikanth, and A. A. Kumar. (2015), RP-HPLC method development and validation for simultaneous quantitative estimation of mebeverine hydrochloride and chlordiazepoxide in capsules. International Journal of Pharmacy and Pharmaceutical Sciences; 7(2): 314-318.

[6] Ramulu K, Kumar TT, Krishna SR, Vasedev R, Kaviraj M. Identification, isolation and characterization of potential degradation products in pioglitazone hydrochloride drug substance. Pharmazie. 2010 Mar; 65(3): 162-8.

[7] Rane VP, Shinde DB. A Validated Chiral LC Method for the Enantiomeric Separation of Repaglinide on amylose based stationary phase. Chrom. Oct 2007; 66 (7-8): 583
[8] Rani P, Sekaran BC, Arachna N. Determination of Repaglinide in pharmaceutical formulations by RP-HPLC method. J Applied Sciences Research. 2009; 5(10): 1500- 04.
[9]Rania N. El-Shaheny, Fathalla F. Belal. (2015), Simultaneous HPLC Determination of Chlordiazepoxide and Mebeverine HCl in the Presence of Their Degradation Products and Impurities. Journal of Chemistry; 3(8): 8-16.
[10] Rao NVSM, Benjamin B,Ramesh M,Srinvas R. Simple method for the determination of rosiglitazone in human plasma using a commercially available internal standard. Biomedical Chrom. 2003; 17: 417–20.
[11] Rathinavel G, Nath UU, Valarmathy J, Samueljoshua L, Tanuja CS, Ganesh M,Sivakumar T,Priyadarsini R. RP-HPLC Method for the Simultaneous estimation of Rosiglitazone and Gliclazide in Tablets. E J Chem. 2009; 6(4): 1188- 92.
[12] Raveendra, V., Vemula, B., & Sharma, P.K. (2013), Analytical method development and validation for simultaneous estimation of imipramine and diazepam in tablet dosage form by RP-HPLC. International Journal of Pharmacy and Pharmaceutical Sciences; 5(3): 249-253.
[13] Ravindra N, Singhvi I. Spectrophotometric Estimation of Glimepiride from Pharmaceutical Dosage Forms. Asian J Chem. (2008); 20(6): 4379-4382.
[14] Reddy B.V, M. M. Eswarudu, M. Anitha, N. Gayathri and T. Chaitanya. (2007), A validated RP-HPLC method for the simultaneous estimation of Fluxetine hydrochloride and Olanzapine in pharmaceutical dosage form. International Research Journal of Pharmacy; 3(4): 310-313.
[15] Reddy, G. S., S. L. N. P. Reddy, and L. S. K. Reddy. (2014), Development and validation of a stability indicating liquid chromatographic method for the simultaneous estimation of paroxetine and clonazepam in bulk and its pharmaceutical formulations. International Journal of Pharmacy and Pharmaceutical Sciences; 6(10): 397-02.
[16] Reddy Krishna KVSR, Babu JM, Mathad VT, Eswaraiah S, Reddy MS et all Impurity profile study of repaglinide. J Pharm Biomed Anal. July 2003; 32(3): 461-467.
[17] Reddy, Y. Krishna, Battu.Prasanna Reddy and Ramachandran, Dittakavi. (2018). Determination of Pantoprazole Sodium in Individual Tablet Dosage Forms by RP-HPLC Using Single Mobile Phase. E-Journal of Chemistry; 6: 489-494.
[18] Reena Paghadal, Mayank Bapna, Jigisha Patel, Hiren Jani, Bhavik Jani. (2014), Development and validation of analytical method for estimation of fluoxetine hydrochloride in oral solution. Journal of Chemical and Pharmaceutical Research; 6(6):1166-1172.
[19] Rimawi FA. Development and validation of an analytical method for metformin hydrochloride and its related compound (1-cyanoguanidine) in tablet formulations by HPLC-UV. Talanta. Oct 2009; 79(5): 1368-71.
[20] Ruzilawati AB, Mohd SAW, Ahmad I, Zabiddah I, Siew HG. Method development and validation of repaglinide in human plasma by HPLC and its application in pharmacokinetic studies. J Pharm Biomed Anal. 2007; 43 1831– 35.