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Hemangi Parekh
Faculty of Pharmacy,
G. H. Patel Building, Donor`s Plaza, The M.S. University of Baroda, Vadodara, Gujarat, India

Rajashree Mashru
Faculty of Pharmacy,
G. H. Patel Building, Donor`s Plaza, The M.S. University of Baroda, Vadodara, Gujarat, India

Corresponding Author:
Hemangi Parekh
Faculty of Pharmacy,
G. H. Patel Building, Donor`s Plaza, The M.S. University of Baroda, Vadodara, India

Development and validation of analytical method for simultaneous estimation of paroxetine HCL and etizolam

Hemangi Parekh and Rajashree Mashru

Abstract
Paroxetine HCl and Etizolam are anxiolytics. Both drugs are used to treat anxiety, mental disorder etc. In combination of both the drug shows synergistic effect. UV Spectrometry and RP-HPLC method has been developed for paroxetine HCl and etizolam in marketed mixture. Absorbance Correction method, First derivative Zero Crossing Point (ZCP) and First derivative Q-Ratio method was developed base on UV Spectrometry. In RP-HPLC, mobile phase consist of ACN: phosphate buffer (50mm, 3.5pH) in ratio of 60:40. Drugs were detected at 249nm wavelength using flowrate 1mL/min.

Keywords: Validation, analytical method, simultaneous estimation, HCL and etizolam

Introduction
A new fixed dose combination containing Peroxetine HCl and Etizolam available in market in tablet dosage form (Etizola P). Paroxetine (figure 2) is an orally administered selective serotonin reuptake inhibitor drug commonly known as Paxil. Etizolam (figure 1) is a thienodiazepine which is chemically related to benzodiazepine (BDZ) drug class; it differs from BDZs in having a benzene ring replaced with a thiophene ring. It is an agonist at GABA-A receptors. Paroxetine helps to enhance the mood in anxiety patient by blocking the selective serotonin reuptake receptor. Etizolam helps to decrease excitation of the neuron by releasing GABA. They produce synergistic effect by using simultaneously. The combination of drugs is use to treat anxiety disorders, major depression, posttraumatic stress disorder, and symptoms of menopause.
The deep literature survey shows that there are several spectroscopic, chromatographic and calorimetric methods are reported for estimation of Paroxetine HCl and Etizolam alone and/or in combination with other drug. But there is no analytical method reported for simultaneous estimation of Paroxetine HCl and Etizolam.

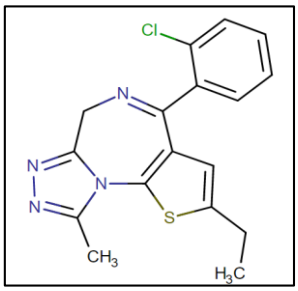

Fig 1: Etizolam [1]

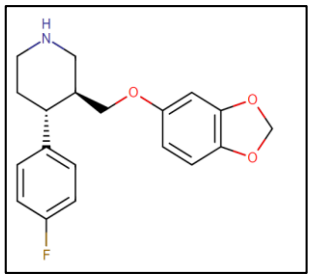

Fig 2: Paroxetine

1. UV Spectrometric Method

Shimadzu UV-1700 double beam spectrophotometer connected to a computer loaded with Shimadzu UV Probe 2.10 software was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-400 nm. The samples were weighed on electronic analytical balance (A×120, Shimadzu). Statistical Analysis of Data was accomplished using Microsoft Excel 2013.

Material and Reagent

Paroxetine HCl and Etizolam

Selection of solvent

Both drug were soluble in water in methanol. So, methanol was selected as solvent for estimation of both drug.

Preparation of Standard Stock Solution

Weighed accurately 5mg of Etizolam (ETZ) and transfer in to 10mL of volumetric flask, make up with methanol (500µg/mL). Similarly, weighed accurately 12.5mg of Paroxetine HCl (Paxil) and make up to 10mL with methanol in volumetric flask (1250µg/mL).

Preparation Test Stock Solutions

Take individual tablet of both the drugs from market, weighed 5mg and 12.5mg of etizolam and paroxetine, respectively. Mix both the drug and make it up to 10ml with methanol.

Method Development

A. Absorption Correction Method

For analysis of both drugs, ETZ and Paxil have shown absorbance maxima at 242nm and 294nm, respectively. λmax of Etizolam (242nm), absorbance of Paroxetine HCl was there. But at the λmax of Paroxetine HCl, Etizolam showed negligible absorbance (figure 3). So for this combination Simultaneous equation method was developed. Absorptivity of ETZ and PAXIL were calculated at both the wavelengths.

The concentration of ETZ and PAXIL can be calculated from following equations:

$Cx = A_2ay_1 - A_1ay_2 / ax_2ay_1 - ax_1ay_2$

$Cy = A_2ax_1 - A_1ax_2 / ax_2ay_1 - ax_1ay_2$

A1 and A2 are the absorbances of the mixture at 242 nm and 294 nm respectively:

Whereas, ax_1=absorptivity of ETZ at 242nm,

ax_2=absorptivity of ETZ at 294nm.

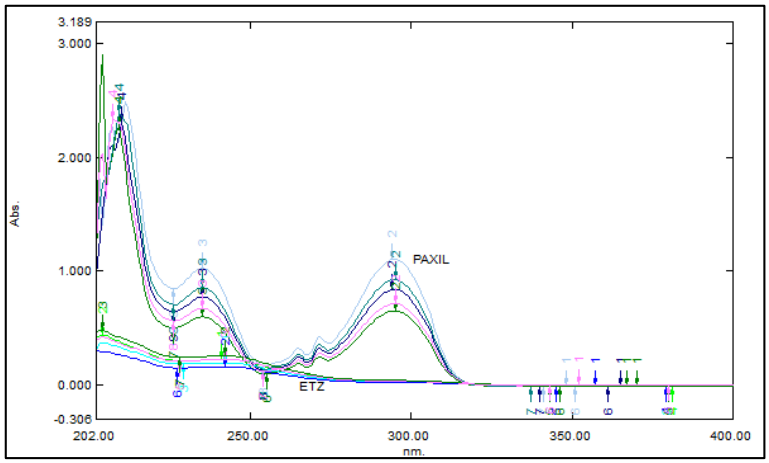

Fig 3: Zero order overlay spectra of ETZ and Paxil.

B. First Derivative Zero Crossing Point (ZCP) Method

The method measures the derivative spectra at particular wavelength, where derivative crosses the point at zero line. Interference of one component in determination of other component can be eliminated by zero crossing technique. Here, PAXIL shows ZCP at 255nm and ETZ shows zero crossing point at 307nm (figure 4). The absorption spectra were recorded in the range of 200 nm to 400 nm and were stored in the memory of the instrument and transformed to first derivative with Δλ = 10nm and scaling factor 5. Calibration curves were constructed with five different concentrations in the range between 2.5-3.5 µg/ml and 62.5-87.5 µg/ml for ETZ and PAXIL respectively.

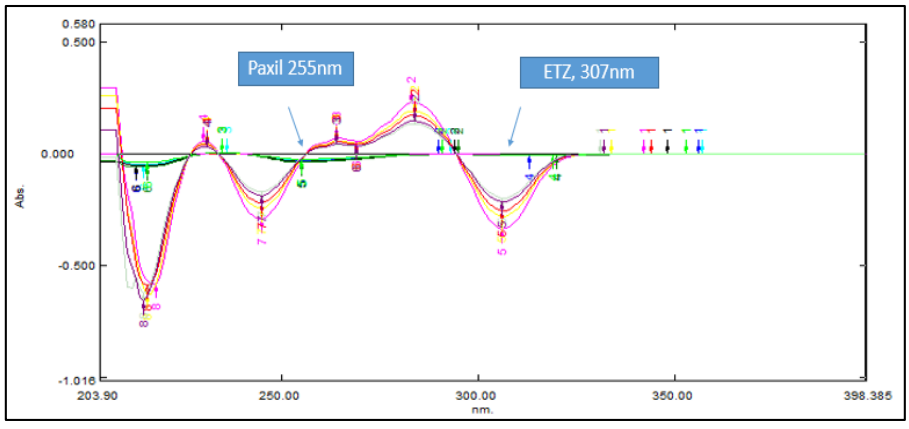

Fig 4: First Derivative overlay spectra of ETZ and Paxil

First Derivative Q-Ratio Method

A new UV Spectrophotometric first derivative Absorbance Ratio Method was developed for estimation of Etizolam and Paroxetine HCl. The method involved Q-absorption ratio analysis using two wavelengths, with one being the λmax of Paroxetine (307nm, λ2) and the other being the isoabsorptive point of both drugs (255nm, λ1) as shown in figure 4.

Cx= {(QM-Qy)/(Qx-Qy)}* (A1/ax1)
Cy= {(QM-Qx)/(Qy-Qx)}* (A1/ay1)

Whereas, QM= A2/A1, Qx= ax2/ax2, Qy= ay2/ay1
ax_1= iso-absorptive point at 255nm
ax_2= iso-absorptive point at 307nm
ay_1=absorptivity of PAXIL at 255nm
ay_2= absorptivity of PAXIL at 307nm

Method Validation

Developed spectrophotometric methods for the estimation of drugs were validated according to ICH Q2 (R1) guidelines and data complying with the standards were obtained.

Linearity

The calibration curve constructed by plotting concentration of ETZ and Paxil versus their absorbance and the regression equation were calculated (figure 5(a)(b)). The linearity of the method was investigated by using five concentrations in range 2.5-3.5µg/mL for ETZ and 62.5-87.5µg/mL for Paxil (table 2). Limit of detection and limit of quantification were calculated from slop and standard deviation of calibration curve. Table shows the summary of various linearity parameter of ETZ and Paxil (table 3).

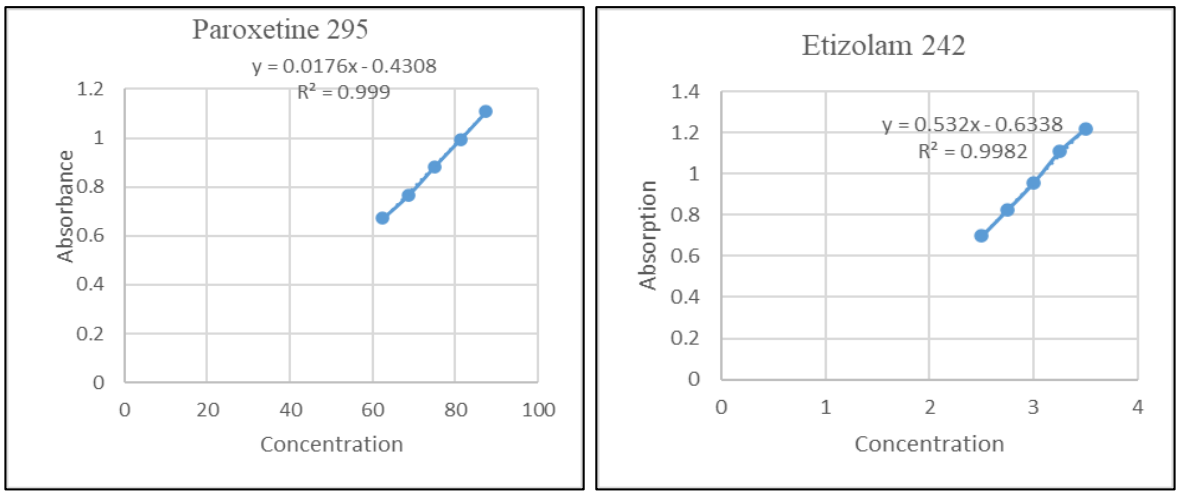

Fig 5: (a)(b) Calibration curve for Paroxetine HCl and Etizolam

Precision

Intraday and interday precision was determined in terms of %RSD. To perform intraday, experiment was repeated 3times a day and 3 different days for interday precision. The average %RSD was found to be less than 2 (table 4).

Accuracy

To check the accuracy for ETZ and Paxil, recovery studies were carried out from pre-analyzed sample at three different level of standard addition 80%, 100% and 120% (table 6). %Recovery of different method was found between 90% - 100%, which proves that all methods are accurate.

Ruggedness

Ability of an analytical method to remain unaffected by small variations in method parameters. The tests were carried out by injecting standard solution of sugammadex by different analyst (table 5).

Applicability of proposed method

These methods are applicable for simultaneous estimation of ETZ and Paxil in marketed formulation as mention in table 7. Paroxetine HCl and etizolam was taken individually from market and mix both the drug. Dilute the mixture with methanol to get 3µg/mL of etizolam and 75µg/mL of paroxetine HCl.

HPLC Instrumentation

Chromatography was performed on shimadzu. Chromatographic system equipped with Shimadzu LC – 20AT pump and shimadzu SPD-20AD absorbance detector. Sample were injected through a rheodyne 7725 injector valve with fix loop at 20µL. Data acquisition and integration was performed using Spincrome software. Analytical Technologies Limited C18 column (250mm x 4.6mm, 5µm) was used for separation.

Reagents and Chemicals

Etizolam and Paroxetine HCl
Acetonitrile (HPLC grade), Double Distilled water, Phosphate buffer (50mm), OPA.

Preparation of standard stock and working stock solution

Preparation of standard solution
Weighed accurately 5mg of Etizolam (ETZ) and transfer in to 10mL of volumetric flask, make up with methanol (500µg/mL). Further dilution made with ACN: Water (1:1). Similarly, weighed accurately 12.5mg of Paroxetine HCl (Paxil) and make up to 10mL with methanol in volumetric flask (1250µg/mL). Further dilution was made with ACN: Water (1:1).

Method Development

Chromatographic Condition

Chromatographic separation was carried out on C18 Colunm. Acetonitrile and 50mm phosphate buffer in ratio of 60:40 was use as mobile phase. pH of buffer adjusted to 3.5pH by using OPA (Ortho Phosphoric Acid) solution. Optimal retention

time was found to be 4.89min for paroxetine HCl and 9.8min for etizolam, at flow rate 1mL/min with UV detection at 249nm. Optimized runtime is 12min.

Method Validation

The developed method was validated in accordance to the current guidelines.

Specificity

The specificity of method for paroxetine and etizolam is demonstrated by injecting solution into the HPLC system.

- Standard solution (figure 6)
- Test Solution (figure 7)
- Blank Solution (figure 8)

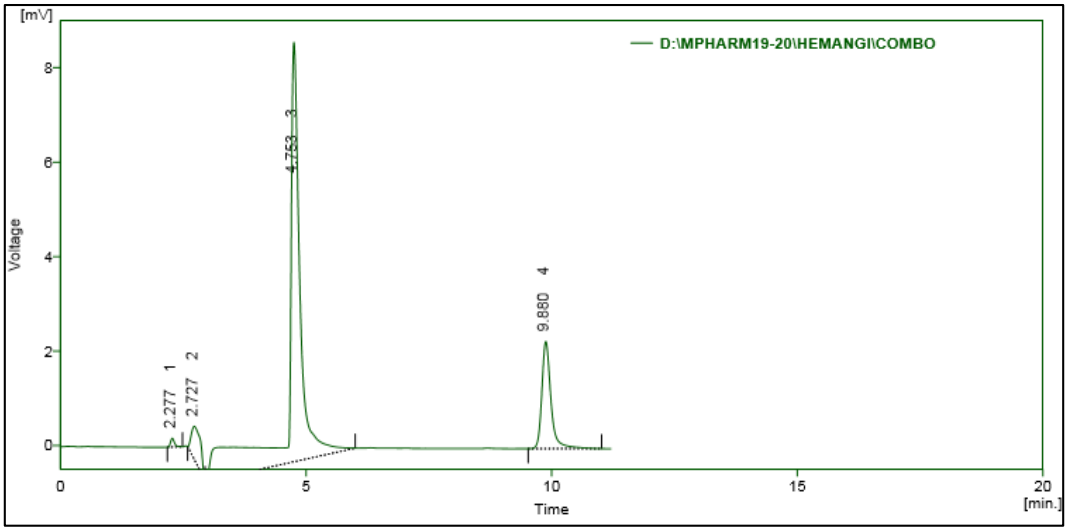

Fig 6: Chromatogram of standard drugs

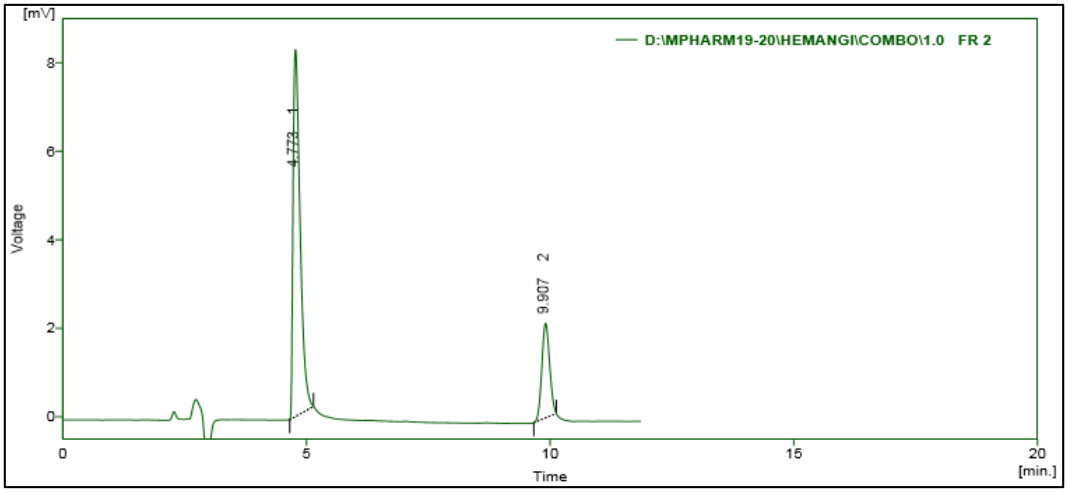

Fig 7: Chromatogram of Test Drugs

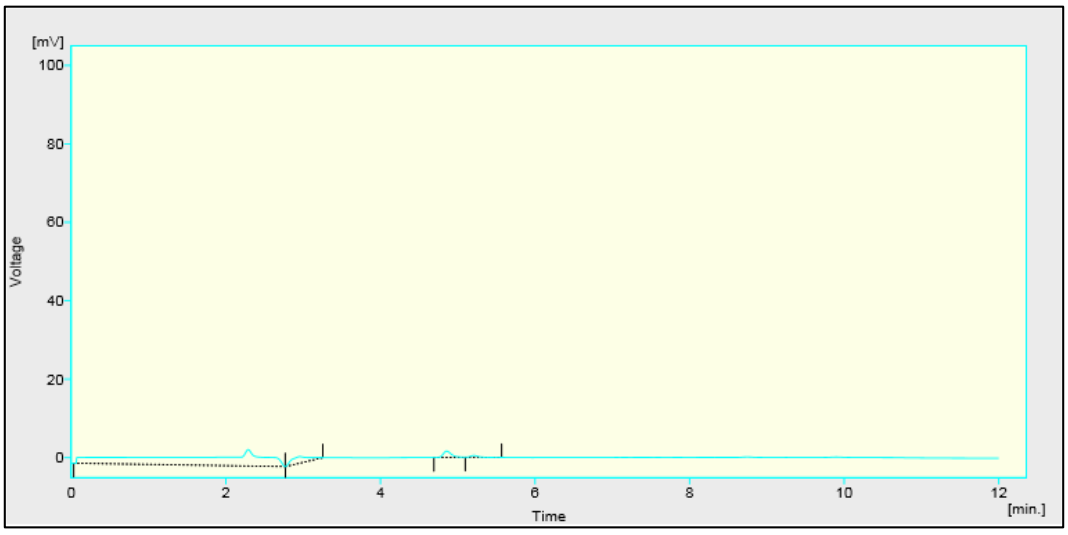

Fig 8: Blank solution of drugs

The method is considered to be specific as no peak was observed at the retention time of both the drugs in blank (table 1).

Table 1: Specificity of Paroxetine and Etizolam

Sr. no.	Sample Name	Analyte Name	Specificity
1	Blank	No peak	-
2	Standard	Paroxetine Etizolam	Specific
3	Test	Paroxetine Etizolam	Specific

System Suitability

Parameter were monitored by preparing mixture of 10µg/mL and 250µg/mL standard solution of etizolam and paroxetine, respectively. Further, the solution was injected into three replicates and measure parameter like retention time, theoretical plate, peak tailing. Then calculate %RSD and data shows that the system functioning correctly as %RSD observed within acceptable limit (table 2).

Linearity

The linearity of paroxetine HCl and etizolam was determined by analyzing at 5 independent level (figure 11). The range of 125-375µg/mL for Paroxetine HCl and 5-15µg/mL for Etizolam (table 9&10). The calibration curve of AUC of both drugs vs concentration was plotted and correlation coefficient and regression line equation was calculated (figure 9&10). The method considered to be linear as the correlation coefficient was found within acceptance criteria.

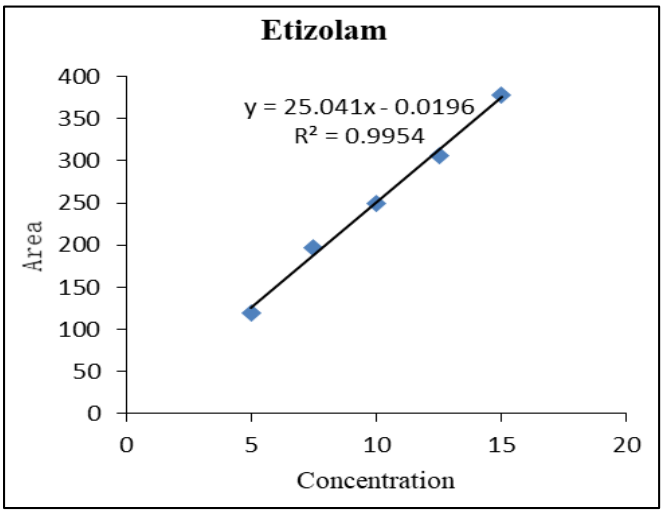

Fig 9: Calibration curve of Etizolam

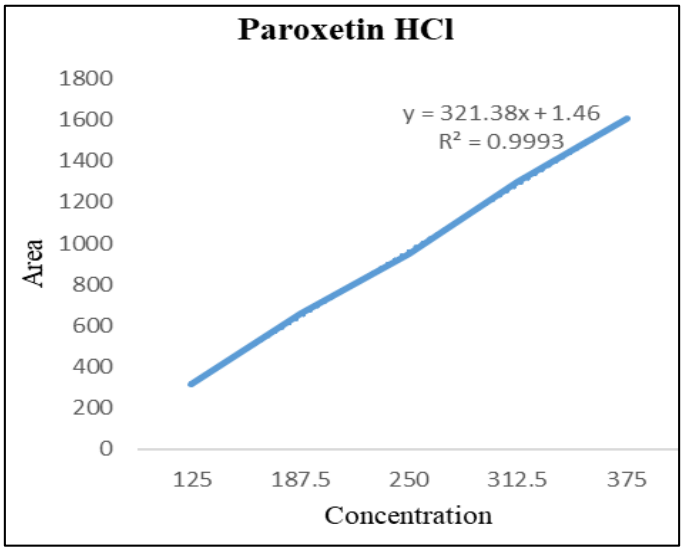

Fig 10: Calibration curve of Paroxetine HCl

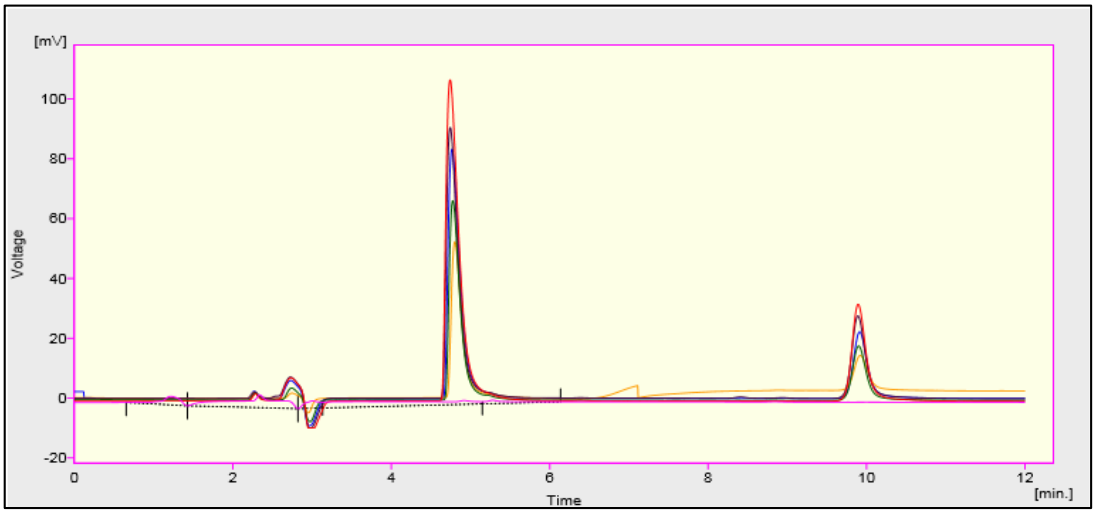

Fig 11: Linearity overlay Chromatogram of Paroxetine and Etizolam

Precision

Precision described at three level: repeatability, intermediate precision, reproducibility. Repeatability also term as intra-assay precision and performing the precision under same operating condition over short interval of time. Intermediate precision performed within - laboratories on different days. Reproducibility expresses the precision between laboratories. %RSD of repeatability, intraday and interday precision as shown in table 11.

Accuracy

Recovery study obtained by using standard addition method of pure standards at three different levels in 80%, 100%, 120% to the sample in triplicate.[8] The spiked test solution was analyzed according to the proposed procedure. Then calculate the percent recovery as shown in table 12.

Robustness

Three replicate solution of paroxetine at 250µg/mL and etizolam at 10µg/mL were analyzed as per changes in level of factor as mention in table 13 i.e. changes in mobile phase and flow rate 1mL/min.

Assay of Drugs

Assays was performed by making stock solutions of mixture with using methanol. Further dilution made by ACN: water (1:1) to get 250µg/mL of paroxetine HCl and 10µg/mL of etizolam in ratio of 25:1 respectively. (table 14)

Result and Discussion

UV Spectrometry Data

Table 2: Linearity data

Paroxetine HCl			Etizolam		
Conc. (µg/mL)	**Mean Abs.**	**%RSD**	**Conc. (µg/mL)**	**Mean Abs.**	**%RSD**
62.5	0.673	1.23	2.5	0.697	0.52
68.75	0.768	0.64	2.75	0.826	0.39
75	0.884	0.79	3	0.958	0.65
81.25	0.995	0.68	3.25	1.11	0.54
87.5	1.108	0.36	3.5	1.22	0.49

Table 3: Linearity parameter

Parameter	Absorption Correction Method		First Derivative Zero Crossing Point method	
	Paroxetine	**Etizolam**	**Paroxetine**	**Etizolam**
Linearity Range(µg/mL)	62.5-87.5	2.5-3.5	62.5-87.5	2.5-3.5
Analytical Wavelength	295	242	307	255
Regression equation	y = 0.017x +0.43	y = 0.532x +0.633	y = 0.0064x + 0.229	y = 0.033x + 0.053
Correlation co-efficient	0.999	0.998	0.999	0.998
Slope	0.0176	0.532	0.0064	0.033
Standard error on slop	0.0006	0.003	0.0002	0.0005
Intercept	0.4308	0.6338	0.2298	0.053
Standard error in intercept	0.0061	0.013	0.004	0.0013
LOD	1.12	0.09	2.14	0.14
LOQ	3.38	0.26	6.49	0.42

Table 4: Precision data

Parameter	Absorption Correction Method		First Derivative Zero Crossing Point method	
	Paroxetine	**Etizolam**	**Paroxetine**	**Etizolam**
Intraday Precision (%RSD)	1.14	0.92	1.41	1.33
Interday Precision (%RSD)	0.92	0.89	0.84	1.3
Repeatability	1.2	1.24	1.45	1.81

Table 5: Ruggedness data

Parameter	Absorption Correction Method		First Derivative Zero Crossing Point method	
	Paroxetine	**Etizolam**	**Paroxetine**	**Etizolam**
Analyst I	0.92	0.86	1.41	1.83
Analyst II	1.5	1.86	1.39	1.32

Table 6: Accuracy data

Spike level % (n = 3)	Amount present in mixture (µg/mL)		Amount Added (µg/mL)		Amount Recovered		% Recovery	
Absorption Correction Method								
	Paxil	ETZ	Paxil	ETZ	Paxil	ETZ	Paxil	ETZ
80	75	3	60	2.4	125.7	4.9	93.11	90.71
100	75	3	75	3.0	143.07	5.48	95.38	91.32
120	75	3	90	3.6	156.48	6.36	94.83	96.38
First Derivative Zero Crossing Point method								
80	75	3	60	2.4	132.04	5.29	97.8	97.91
100	75	3	75	3.0	145.72	5.82	97.14	96.98
120	75	3	90	3.6	165.27	6.61	100.2	100.61

Table 7: Assay Results

Methods	**% Assay** (Synthetic Mixture) ± %RSD	
	Paroxetine HCl	**Etizolam**
Absorption Correction Method	99.2% ± 0.95	97.8% ± 0.72
First Derivative ZCP method	98.5% ± 1.68	104.1% ± 1.21

RP-HPLC Data

Table 8: System Suitability data of paroxetine HCl and etizolam

Parameters	**Paroxetine HCl**		**Etizolam**	
	Mean (n=3)	**%RSD**	**Mean (n=3)**	**%RSD**
RT (min)	4.77	0.04	9.9	0.15
Theoretical plates	4958	1.68		
Tailing Factor	1.3	0.93		

Table 9: Regression data analysis RP-HPLC method of Paroxetine HCl and Etizolam

Parameters	**Paroxetine HCl**	**Etizolam**
Wavelength	249nm	249nm
Range	125 - 375 µg/mL	5 – 15 µg/mL
Regression Equation	y = 321.38x - 1.46	y = 25.041x - 0.0196
Slop (m)	321.38	25.041
Intercept (c)	1.46	0.0196
Correlation coefficient	0.999	0.995
LOD	0.09	0.04
LOQ	0.27	0.13

Table 10: Linearity data of Etizolam and Paroxetine HCl

Etizolam			**Paroxetine HCl**		
Conc. (µg/ml)	**Mean Area**	**%RSD**	**Conc. (µg/ml)**	**Mean Area**	**%RSD**
5	119.9	1.95	125	470.8	0.96
7.5	197.3	1.42	187.5	665.9	0.41
10	250.13	1.33	250	906.5	0.28
12.5	305.8	1.26	312.5	1298.3	0.42
15	378.7	1.06	375	1603.5	0.34

Table 11: Precision data of paroxetine HCl and etizolam

Precision	**Paroxetine HCl (250µg/mL)**		**Etizolam (10µg/mL)**	
	Mean	%RSD	Mean	%RSD
Repeatability	903	0.6	248	1.55
Intraday	906	0.66	244.86	0.72
Interday	908	0.92	246.9	1.95

Table 12: Accuracy data of Paroxetine HCl and Etizolam

Spike level % (n = 3)	**Amount present in mixture (µg/mL)**		**Amount Added (µg/mL)**		**Amount Recovered**		**% Recovery**	
	Paxil	ETZ	Paxil	ETZ	Paxil	ETZ	Paxil	ETZ
80	250	10	200	8	444.97	17.76	98.88	98.67
100	250	10	250	10	498.18	20.02	99.63	100.11
120	250	10	300	12	545.16	21.94	99.12	99.73

Table 13: Robustness data of Paroxetine HCl and Etizolam

Factor	**Level Change**	**Etizolam**		**Paroxetine HCl**	
		Mean	%RSD	Mean	%RSD
Mobile Phase	KH_2PO_4: CAN= 42:58	10.04	0.26	5.03	0.04
	KH_2PO_4: ACN= 40:60	9.91	0.10	4.77	0.10
	KH_2PO_4: ACN= 38:62	9.43	0.27	4.67	0.68
Flow Rate (mL/min)	0.999	10.14	0.24	4.79	0.084
	1.00	9.90	0.15	4.77	0.048
	1.001	9.84	0.21	4.74	0.04
Wavelength (nm)	248	229.22	0.62	792	1.53
	249	244.70	0.86	899.56	0.32
	250	235.55	0.74	837.15	0.73

Table 14: Assay data of paroxetine HCl and etizolam

	Paroxetine			Etizolam		
Sr. No	**% Drug Recovered (n = 6)**	**Mean %Drug Recovered**	**%RSD of Drug Recovered**	**% Drug Recovered (n = 6)**	**Mean %Drug Recovered**	**%RSD of Drug Recovered**
1	98.8	99.14	0.60	96.9	99.09	0.155
2	99.2			98.2		
3	99.6			101.1		
4	100.0			98.6		
5	98.7			100.6		
6	98.4			99.0		

Conclusion

Simultaneous estimation of paroxetine HCl and etizolam offers advantage in terms of less time consuming for analysis. The developed RP-HPLC method validated according to ICH guideline. From the review of literature, it was found that no analytical method developed for paroxetine HCl and etizolam. Developed method found to be simple, precise and accurate. It produces satisfactory results of combination paroxetine HCl and etizolam.

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