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A new validated simultaneous RP- HPLC method for estimation of escitalopram oxalate and etizolam in bulk and table dosage form

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ABSTRACT

A reversed phase HPLC method has been developed for the simultaneous determination of Escitalopram oxalate and Etizolam by using C18 (250 x 4.6 mm) column and mobile phase of 0.02M Potassium dihydrogen orthophosphate: Acetonitrile (40:60) at 254 nm. Retention times of Escitalopram oxalate and Etizolam were 2.8 min and 4.1 min respectively with resolution of 6.52. This method shows to be linear (r^2>0.99), precise (RSD<2%), accurate (recovery of 99.8% of Escitalopram oxalate and 99.46% of Etizolam), specific and robust. The Escitalopram oxalate contains in tablets was 99.8 % where Etizolam contains was 99.33 %

Key words: Escitalopram oxalate, Etizolam, reverse phase HPLC, validation.

INTRODUCTION

Escitalopram oxalate (ESC) is the pure s enantiomers of the racemic bicyclic phthalate derivative of citalopram. It is an antidepressant of the serotonin reuptake inhibitor class[1-2] is chemically S(+)-1-[3-(Dimethylamino)-propyl]-1-(4-fluorophenyl)-1,3-dihydro-5- Isobenzofurancarbonitrile oxalate [3] . ESC is not official in pharmacopoeia[4-5]. Few spectro photometric [6-7] and very few HPLC methods[8-10] are available for its determination. Etizolam(ETZ) chemically 4-(2-chlorophenyl)-2-ethyl-9-methyl-6H-thieno[3,2f] [1,2,4] triazolo[4,3- a] [1,4] diazepine[3] belong to a new class of diazepines. It has anxiolytic, anticonvulsant, hypnotic, sedative and skeletal muscle relaxant properties [4]. Literature review reveals that very few analytical method has been reported for the determination of ETZ in biological fluid which includes HPLC[11], HPTLC [12], LCMS [13-14] GCMS [15]. But recently very few methods has been developed [16-17] for the simultaneous estimation of Escitalopram oxalate and Etizolam by HPLC using methanol and phosphate buffer (70:30) and acetonitrile:0.005 M Hexane Sulfonic Acid pH 3.0 (adjusted with o-phosphoric acid) (40:60 v/v). The present study was aimed to developed recently a new simple economic and validated method and this validated method was applied for the analysis of tablet content of Escitalopram oxalate-Etizolam combination 10 + 0.5 mg.

MATERIALS AND METHODS

Materials:
Escitalopram oxalate and Etizolam bulk drugs were obtained from Chandra labs, Hyderabad, Andhra Pradesh, India as gift samples and marketed formulation Etizola plus (Escitalopram oxalate 10mg and Etizolam 5mg) manufactured by Macleods pharmaceutical Pvt. Ltd. was procured form local market of Hyderabad. Acetonitrile of HPLC grade and Potassium dihydrogen phosphate of AR grade, were purchased from obtained from local market.

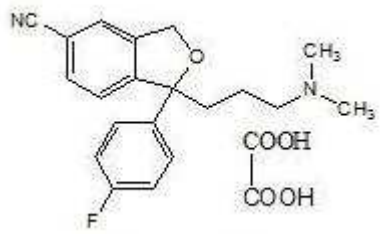

Fig: 1. Structure of Escitalopram Oxallate

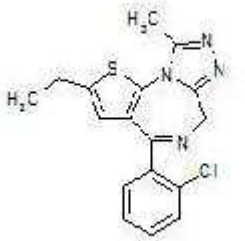

Fig: 2. Structure of Etizolam

Chromatographic condition:
A mobile phase consisted of Acetonitrile and Phosphate buffer in 60:40 ration was pumped at a flow rate of 1 ml/min. The elution was monitored at 254 nm and the injection volume was 20 µL. The validation of the method was done following the ICH guidelines.

Instrumentation:
HPLC devise- shimadzu (LC10 ATVP), UV- Visible Spectrophotometer- Shimadzu SPD-10A, Ultrasonicator- Citizen, Digital Ultrasonic Cleaner, pH meter- Elico, Syringe- Hamilton, HPLC column ODS C18 column (250×4.6ID) were used.

Preparation of Solutions:
Preparation of 0.02M potassium dihydrogen ortho phosphate:

1.360gm of potassium dihydrogen phosphate was weighed and dissolved in 100ml of water and volume was made up to 500ml with water. Adjust the pH to 6 ±0.05 using triethylamine. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

Preparation of mobile phase.
A mixture of 40ml of Potassium di hydrogen orthophosphate Buffer, 60ml of Acetonitrile (HPLC grade). The mobile phase was sonicated for 10min to remove gases.

Preparation of standard stock solution of Escitalopram oxalate and Etizolam.
Standard stock solutions of Escitalopram oxalate and Etizolam (microgram/ml) were prepared by dissolving 100 mg of Escitalopram oxalate and 5mg of Etizolam in 10 ml of mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100 ml with mobile phase. Further dilution of 120 µg/ml of Escitalopram oxalate and 6 µg/ml was made by adding 1.2 ml of stock solution to 10 ml of mobile phase.

Preparation of sample solution of Escitalopram oxalate and Etizolam.
20 tablets (each tablet contain 10mg of Escitalopram oxalate and 0.5mg of Etizolam) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of Escitalopram oxalate and Etizolam (microgram/ml) were prepared by dissolving weight equivalent to 100mg of Escitalopram oxalate and 5mg of Etizolam in 10 ml of Methanol. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilution of 120 µg/ml of Escitalopram oxalate and 6 µg/ml of Etizolam was made by adding 1.2 ml of stock solution to 10 ml of mobile phase.

Wavelength Selection.
In simultaneous estimation of two drugs isobestic wavelength is used. Standard solution of Escilalopram oxalate and was prapared by weighing 100mg of Escitalopram oxalate was weighed and transferred in to 100ml volumetric flask and dissolved in 10ml of methanol and then make up to the mark with the mobile phase and prepare 100µg /ml of solution by diluting 1ml to 100ml with mobile phase and standard solution of Etizolam was prepared by weighing 5mg of Etizolam was weighed in to 100ml volumetric flask and dissolved in 10ml of Methanol and then dilute up to the mark with mobile phase and prepare 5µg /ml of solution by diluting 1ml to 10ml with mobile phase. The wavelength of maximum absorption (λ_{max}) of the drug of 10 µg/ml solution in mobile phase were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against mobile phase as blank. The λ_{max} was found to be 244 nm for Escitalopram oxalate, 284 nm for Etizolam and 254 nm for the combination shown in figure: 3

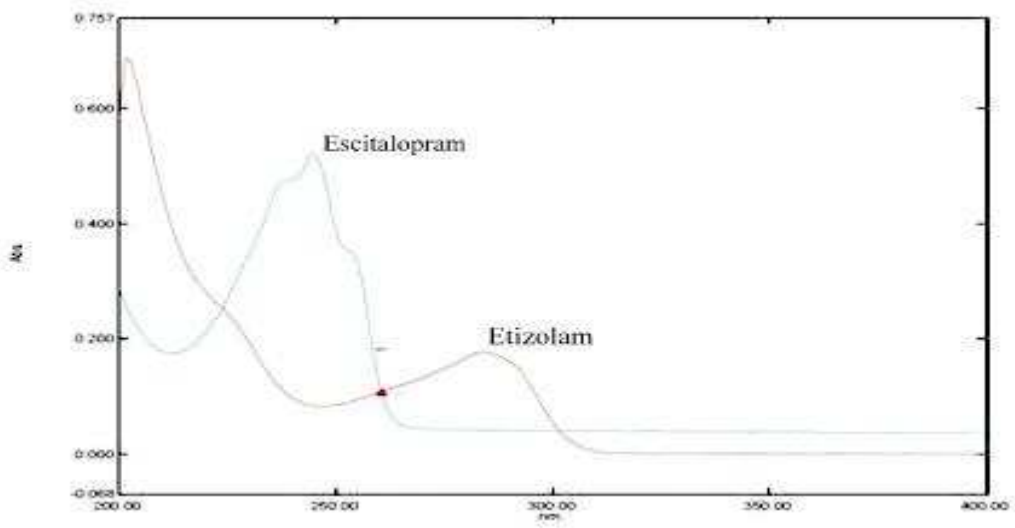

Fig: 3. Isobestic Point of both EST and ETZ

Selection of mobile phase for method Optimization and experimental condition:

Several trial has been taken for the proper optimization of RP HPLC method by changing different mobile phase with different ratio. And finally the mobile phase for optimised condition was selected and given follows. And the optimised parameters was for ESC and ETZ was given at table no. and in figure no.4.

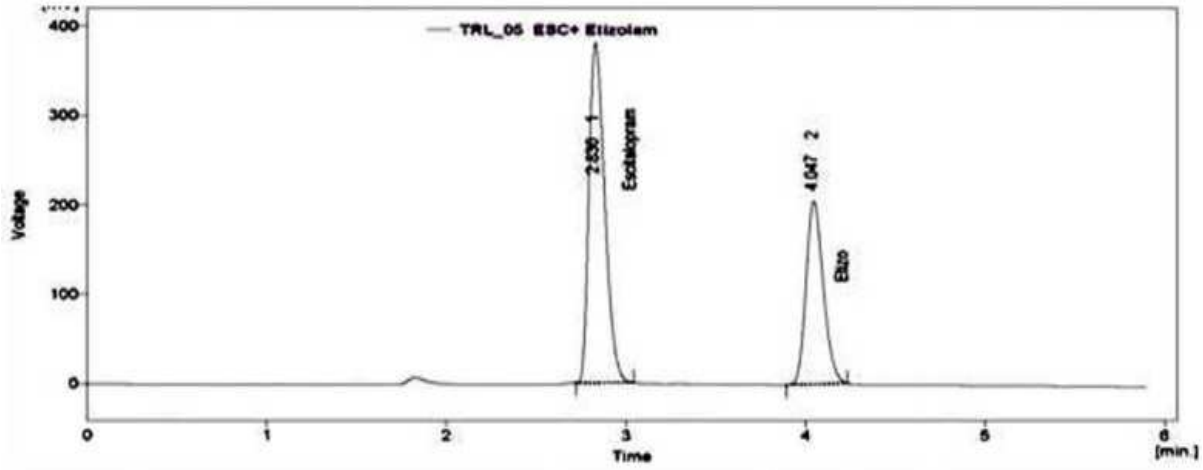

Fig: 4 A typical Optimised Chromatogram of ESC oxalate and ETZ

Chromatographic condition:

Mobile phase	: 0.02M Potassium dihydrogen orthophosphate : Acetonitrile (pH-6)
Ratio	: 40:60
Column	: ODS, C18(150×4.6× 5µ)
Wavelength	: 254nm.
Injection volume	: 6 min
Temperature	: 30^0C (± 2^0C)
Run time	: 6 min
Flow rate	: 1.0ml/min.

Assay:

Assay of marketed tablet formulation containing Etizolam 0.5 mg and Escitalopram oxalate equivalent to Escitalopram oxalate 10 mg was performed by preparing the sample solutions as described earlier in the preparation of the sample. Six injections of above prepared sample and standard solutions were injected. The assay of the commercial sample was calculated by comparing the areas of standard and sample peaks. The assay of marketed formulation ETIZOLA PLUS found within limit. And the chromatogram was given as figure 5.

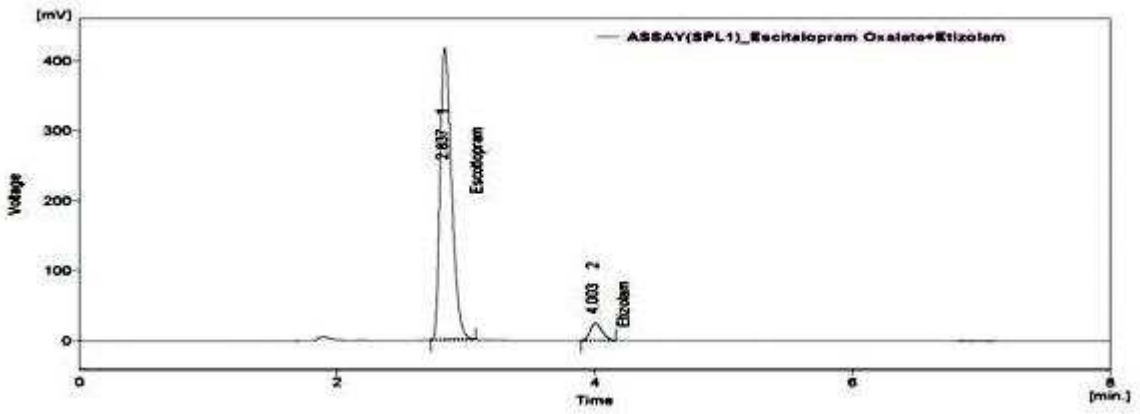

Fig: 5. Chromatogram for the Assay of marketed Formulation

This optimized method was validated in terms of linearity, accuracy, precision, specificity, limit of detection, limit of quantification and solution stability as per ICH guidelines.

Linearity:
Calibration curve was constructed by plotting concentrations of Escitalopram oxalate and Etizolam vs. peak areas, and the regression equations were calculated. The linearity of this method was investigated by using the concentrations 20,40,60,80, 100 and 120 µg/ml for Escitalopram oxalate and 1,2,3,4,5 and 6 µg/ml for Etizolam. These concentrations were prepared by diluting appropriate volume of working standard with mobile phase.

System suitability:
Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated.

Accuracy:
Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 80%, 100%, 120%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 80%, 100%, 120%.

Precision:
Intra-day precision was calculated from results obtained from six-fold replicate analysis of samples at three different concentrations of on the same day. Inter-day precision was calculated from results from the same samples analyzed on six consecutive days. Standard solution was prepared as per the test method and injected six times as per the test procedure. And Relative standard deviation was calculated.

Specificity:
A solution containing a mixture of tablet was prepared using sample preparation procedure and injected into the system, to evaluate possible interfering peaks.

Robustness:
Robustness of the method was demonstrated by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 ml/min to 0.8 ml and 1.2 ml/min. The wavelength was changed from 254nm to 256nm and 252nm, temperature of column oven ($\pm 2^0$C) unit. The mobile phase composition also little changed.

LOD and LOQ:
Combine standard solution were prepared by sequential dilutions and injected on to the chromatograph, at a decreasing concentration in the range of 0.050 to 15µm/ml for EST and 0.045 µm/ml to 10 µm/ml for ETZ The limit of detection was define as the concentration for which a signal-to-noise ratio of 3 was obtained and for Quantitation limit, a signal-to-noise ratio of 10 was considered.

RESULTS AND DISCUSSION

Method Development:

The isobestic point of ETP and ETZ was determined spectrophotometricaly to select the detection wavelength. It was found to be 254 nm was shown in figure no 3.

The chromatographic parameters was optimised using ODS, C18(150×4.6× 5µ) column, and 0.02M Potassium dihydrogen orthophosphate: Acetonitrile (pH-6) as mobile phase with flow rate of 1ml/ min. Under these condition the retention tine for ETP and ETZ was found to be 2.5 and 4.1 min respectively, with a resolutions of 6.928. Other parameters related to optimized value was given in table: 3 and in optimized chromatogram of Escitalopram oxalate and Etizolam was given in figure 4. The assay of marketed formulation ETIZOLA PLUS was performed and found within limit. The result was given in table 1.

TABLE 1: ANALYSIS OF THE MARKETED FORMULATION

Formulation	Labelled claimed		Amount found		% of Assay*	
	ESC	ETZ	ESC	ETZ	ESC	ETZ
Etizola Plus	10 mg	0.5 mg	9.88 mg	0.49mg	99.8	99.33

***ESC** stands for Escitalopram oxalate and **ETZ** stands for Etizolam. *Assay has been done by using the average six determinations.*

Validation:

System suitability tests were carried out on freshly prepared standard solution and all the parameters are within limit. Summary of all parameters was given in table no 2.

TABLE 2: SYSTEM SUITABILITY STUDY OF ESCITALOPRAM OXALATE AND ETIZOLAM

Parameters	Escitalopram Oxalate (± %RSD)	Etizolam (± %RSD)
Retention Time	2.84 ± 0.26	4.01 ± 0.48
Theoretical plate	4162 ± 0.18	7736 ± 0.49
Tailing Factor	1.74 ± 0.18	1.36 ± 0.87
Resolution	-----	6.51 ± 0.83

±%RSD = Percentage Relative Standard Deviation.

The method was linear in the concentration range of 20-120 µg/ml for ESC and 1-6 µg/ml for ETI with a correlation coefficient of 0.998 and 0.998 for respective drugs. The regression equations for ESC and ETI were y = 20.59x + 123.6 and y = 26.04x + 6.997 for ESC and ETI respectively. The results are shown in table 3. And figure 6. and 7 for ESC and ETZ respectively.

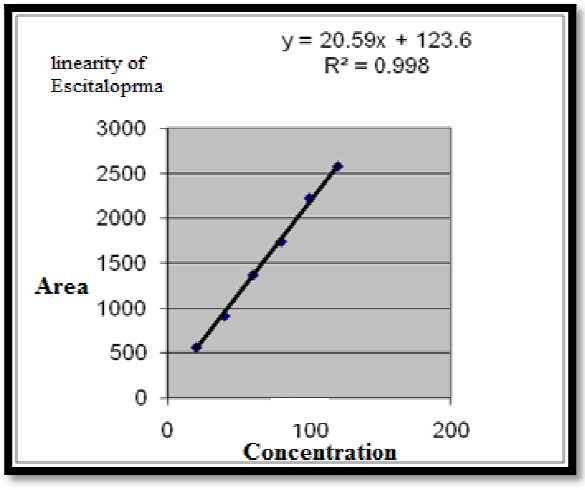

Fig: 6 Linearity of ESC

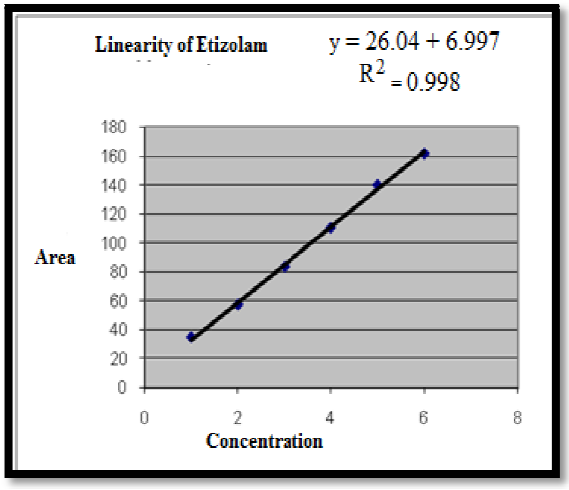

Fig: 7. Linearity of ETZ

The study of accuracy of the developed method has been done. The recovery was found in the range of 99.40-101.2 % for ESC and 99.5-100 % for ETI shown in Table.4 , indicating the accuracy of method. And the RSD of recovery of Escitalopram oxalate and Etizolam is 0.056 and 0.34 respectively.

TABLE: 3 SUMMARY OF SYSTEM SUITABILITY AND VALIDATIONPARAMETERS

Sl. No	Parameter	ESC (± % RSD)	ETZ (± % RSD)
1.	Retention time	2.84 ± 0.26	4.016 ± 0.48
2.	Theoretical plate	4162 ± 0.19	7736 ± 0.49
3.	Tailing factor.	1.74 ± 0.18	1.36 ± 0.87
4.	Resolution	----	6.517 ± 0.83
5.	Linearity	20-120 µg/ml	1-6µg/ml
6.	Regression line Equation	y = 20.59X + 123.6	Y = 26.04X + 6.997
7.	Co relation coefficient (R^2)	0.998	0.998
8.	Intra Day Precision	2.84 ± 0.26	4.01 ± 0.49
	Inter Day Precision	2.84 ± 0.11	4.03 ± 0.10
9.	Accuracy (% Recovery)	99.99	99.79
10.	Limit Of Detection	0.085 µg/ml	0.05 µg/ml
11.	Limit Of Quantification	0.283 µg/ml	0.168 µg/ml

***ESC** stands for Escitalopram oxalate and **ETZ** stands for Etizolam. ±%RSD = Percentage Relative Standard Deviation.*

TABLE: 4 ACCURACY DATA OF THE ANALYSIS OF ESCITALOPRAM OXALATE AND ETIZOLAM

Concentration of Spiked level %	Amount Std added µg/ml		Total amount found µg/ml		% Recovery µg/ml		Mean	
	ESC	ETZ	ESC	ETP	ESC	ETZ	ESC	ETZ
80	80	4	79.47	3.98	99.4	99.55		
100	100	5	99.05	4.93	99.10	99.83	99.99	99.79
120	120	6	121.6	6	101.2	100		

***ESC** stands for Escitalopram oxalate and **ETZ** stands for Etizolam.*

The results for intraday and interday precision studies and repeatability are listed in Table 3, indicating that the method was precise and reproducible. Specificity studies indicated that there is no interference from excipients, impurities and degradation products and assured that the peak response was due to Escitalopram oxalate and Etizolam only.

The limit of detection was found to be 0.085µg/ml for Escitalopram oxalate and 0.05µg/ml for Etizolam. The limit of quantification was found to be 0.283µg/ml for Escitalopram oxalate and 0.168 µg/ml for Etizolam. These values indicate that the method is sensitive. Robustness study indicates at various conditions the system suitability parameters are unaffected with small, deliberate changes results of robustness expressed in table.5.

TABLE 5. ROBUSTNESS STUDY OF THE DEVELOPED METHOD

Parameters	Escitalopram oxalate		Etizolam	
	SD of t_R*	%RSD	SD of t_R*	%RSD
Flow Rate (± 0.2ml/min)	0.055	1.89	0.058	1.45
Wavelength (± 2nm)	0.028	1.03	0.011	0.29
Temperature (± 2^0C)	0.041	1.55	0.035	0.81
Mobile phase (± 2)	0.045	1.75	0.074	1.67

***t_R** stands for Retention time. **SD** indicates Standard Deviation. **RSD** indicates Relative Standard Deviation.*

A simple and selective LC method is described for the determination of Etizolam and Escitalopram oxalate in tablet dosage forms. Chromatographic separation was achieved on a c_{18} column using mobile phase of a mixture of buffer and Acetonitrile (40:60) with detection of 254 nm. Less retention time of both the drugs makes this method rapid with a good resolution of 6.52. Linearity was observed in the range 20-120 µg /ml for Escitalopram oxalate and 1-6µg /ml for Etizolam (r^2 =0.998) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim.

The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing % RSD less than 2. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

CONCLUSION

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation of Escitalopram oxalate and Etizolam was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively

applied for routine analysis in research institutions, quality control department in the industries, approved testing laboratories, bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

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