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Method Development and Validation for the Simultaneous Estimation of Propranolol Hydrochloride and Etizolam by Reverse Phase High Performance Liquid Chromatography

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ABSTRACT

RP-HPLC method has been developed for the quantitative estimation of Propranolol hydrochloride and Etizolam using C 18 column and a mobile phase consisting of Methanol and Water in the ratio 70:30. The mobile phase was pumped at a rate of 1ml/min and detection was carried out at 281.5 nm (iso-absorptive point). The linearity was found to be in range of 5-50 µg/ml and 0.5-5 µg/ml with regression coefficient ($r^2=0.999$, and $r^2=0.997$) for Propranolol hydrochloride and Etizolam respectively. The peaks obtained were sharp having baseline separation with a retention time of 2.35 and 6.57 min for Propranolol hydrochloride and Etizolam respectively. The LOD was found to be at the concentration of 0.1 µg/ml and 0.025 µg/ml for Propranolol hydrochloride and Etizolam respectively. The percentage recovery was found to be 98.5% to 100.58% for Propranolol hydrochloride and 95% to 106.6% for Etizolam. The method was validated statistically.

Keywords: Propranolol hydrochloride, Etizolam, RP-HPLC.

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INTRODUCTION

Propranolol (PROP) (Figure 1), chemically (RS)-1-(isopropylamino) 3-(1-naphthoxy) propan-2-ol¹ is a nonselective beta blocker and is mainly used in the treatment of hypertension by blocking the action of epinephrine and nor-epinephrine on both β_1 and β_2 adrenergic receptors and also used in the management of hypertension, angina pectoris, myocardial infarction, migraine, glaucoma etc². Etizolam (ETI) (Figure 2) belongs to an original chemical class of diazepines, namely thienotriazolodiazepines with antianxiety activity and chemically it is 4-(2-Chlorophenyl)- 2-ethyl-9-methyl-6H-thieno [3,2-f] [1,2,4] triazolo-[4,3-a][1,4] diazepine³. Etizolam has anxiolytic, anticonvulsant, hypnotic, sedative and skeletal muscle relaxant properties⁴. It is 6-10 times more potent than diazepam⁵. The combination of these drugs are used to treat anxiety. Literature review reveals that a few analytical methods that have been reported for the determination of etizolam by using solid phase extraction with GC-MS⁶, high performance liquid chromatography (HPLC)⁷ and capillary gas chromatography-mass spectrometry⁸. UV⁹, HPLC¹⁰, HPTLC¹¹ and GC¹² are reported for the estimation of propranolol in single and other combined dosage forms. PROP is official in IP¹³ and ETI is official in JP XV. However there is no analytical method reported for simultaneous estimation of both drugs in their combined tablet dosage form. Present work describes rapid, simple, sensitive, accurate and reproducible reverse phase HPLC method. The method was validated in compliance with ICH guidelines¹⁴⁻¹⁵

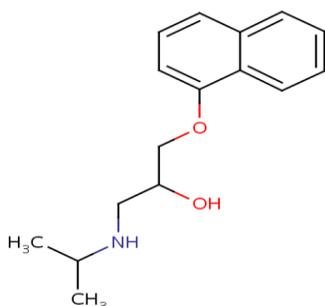


Figure 1: Structure of Propranolol

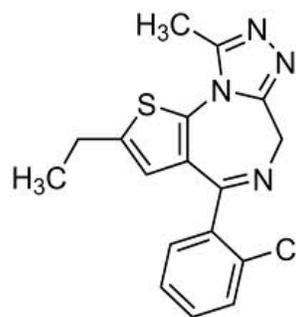


Figure 2: Structure of Etizolam

MATERIALS AND METHODS

Pure standards of Propranolol hydrochloride and Etizolam were obtained from Yarro chem Products and their marketed combination (ETIZOLA BETA) was purchased from the market. Methanol and water used were of HPLC grade and were purchased from Durga labs, Mangalore. The analysis was carried out by using LC JASCO UV detector, C18 column (250 X 4.6 I.D., 5 μ m particle size) with manual injector and the injection volume is 20 μ l.

Standard stock solution

Stock solutions of Propranolol hydrochloride (500 µg/ml) and Etizolam(50 µg/ml) were prepared separately using the mobile phase methanol: water(70:30) . From the standard stock solutions, mixed standard solutions of different concentrations ranging from 5 to 50 µg/ml of Propranolol hydrochloride and 0.5 to 5µg/ml of Etizolam were prepared by diluting with mobile phase.

Preparation of Sample solution

Twenty tablets containing 20 mg of Propranolol hydrochloride and 0.5mg of Etizolam were weighed and finely powered. Accurately weighed 125.1mg of powder which is equivalent to 20mg of propranolol and 0.5mg of Etizolam was transferred into a clean, dry 100 ml volumetric flask. The powder was first dissolved in sufficient volume of mobile phase by sonication. The resulting suspension was then filtered through whatmann filter. The volume of filtrate was made up to 100 ml with mobile phase to get the concentration 200 µg/ml and 5 µg/ml of Propranolol and Etizolam respectively. Further diluted 1, 1.5, and 2ml of the above solution to 10ml with diluent and mixed to get 20, 30, and 40 µg/ml concentration of Propranolol hydrochloride and 0.5, 0.75 and 1 µg/ml of Etizolam.

METHOD VALIDATION

The method was validated as per ICH guidelines (ICH guidelines 1994 and 1996;).

Specificity:

The specificity was determined by injecting the placebo into the chromatograph. The placebo here represents the possible excipients of the formulation.

Accuracy:

The accuracy of the method was determined by recovery experiments. The recovery studies were performed by standard addition method at 80%, 100%, 120% level for both the drugs and the percentage recoveries were calculated.

Precision:

The precision of the method was demonstrated by inter day and intraday variation studies. In the intraday studies, six repeated injections of standard solutions were made and the response factor of drug peaks and percentage RSD were calculated. In the inter day variation studies, six repeated injections of standard solutions were made for three consecutive days and response factor of drug peaks and percentage RSD were calculated. Method Precision and system precision were determined by successive six injections of 20 µl of working standard solution (Mixture of 20 µg/ml Propranolol hydrochloride and 0.5 µg/ml of Etizolam) were injected and chromatograms recorded. The % relative standard deviation was calculated for the concentration of drug and for the peak area in replicates.

Linearity & Range:

The linearity of the method was determined at concentration levels ranging from 5 to 50 μ g/ml for Propranolol and 0.5 to 5 μ g/ml for Etizolam. The calibration curve was constructed by plotting response factor against concentration of drugs.

LOD:

The Limit of Detection (LOD) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. Visualization method was followed. In visualization method the detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

RESULTS AND DISCUSSION

UV overlain spectra of both Propranolol hydrochloride and Etizolam showed that both the drugs absorbs appreciably at 281.5 nm(Figure 3), so 281.5nm was selected as the detection wavelength in liquid chromatography. Different mobile phases were tried but satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase Methanol: Water in the ratio of 70:30 v/v. The retention time of propranolol hydrochloride was found to be 2.35min and that of Etizolam was found to be 6.57 min, respectively (Figure4). The amount of Propranolol hydrochloride and Etizolam in the tablet dosage form were found to 19.96mg and 0.48mg respectively (Table 1).

Table 1: Assay Report of Sample Propranolol hydrochloride and Etizolam.

Volume of stock Solution (ml)	Propranolol hydrochloride			Etizolam		
	Peak area	Concentration (μ g/ml)	Amount per tablet (mg)	Peak area	Concentration (μ g/ml)	Amount per tablet (mg)
1.0	78596	19.85	19.85	523	0.48	0.48
1.5	120998	29.98	19.98	813	0.71	0.473
2.0	160872	40.15	20.07	1022	0.97	0.485
Average			19.96	Average		0.48

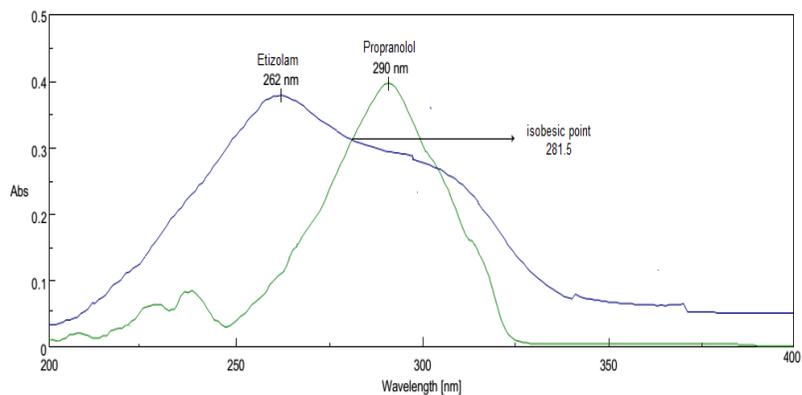


Figure 3: Overlaid spectra of Propranolol hydrochloride and Etizolam. Etizolam and Propranolol showed lambda max at 262nm and 290nm respectively. The isobestic point is 281.5nm

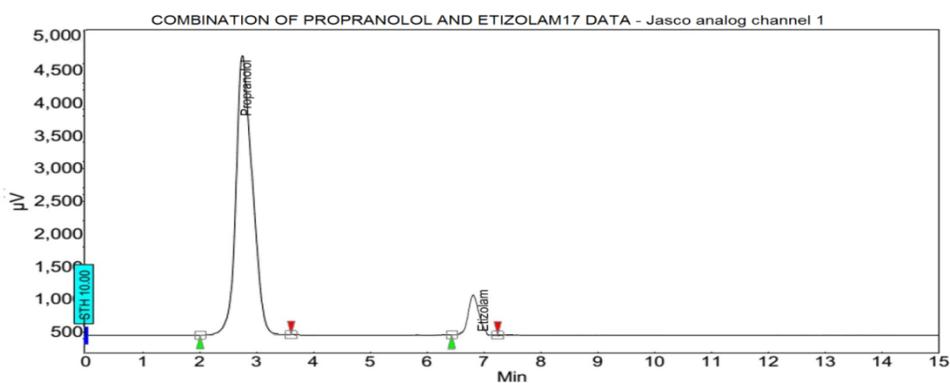


Figure 4: Chromatogram of mixture of Propranolol hydrochloride and Etizolam. Propranolol and Etizolam showed the retention time at 2.35 and 6.57mins.

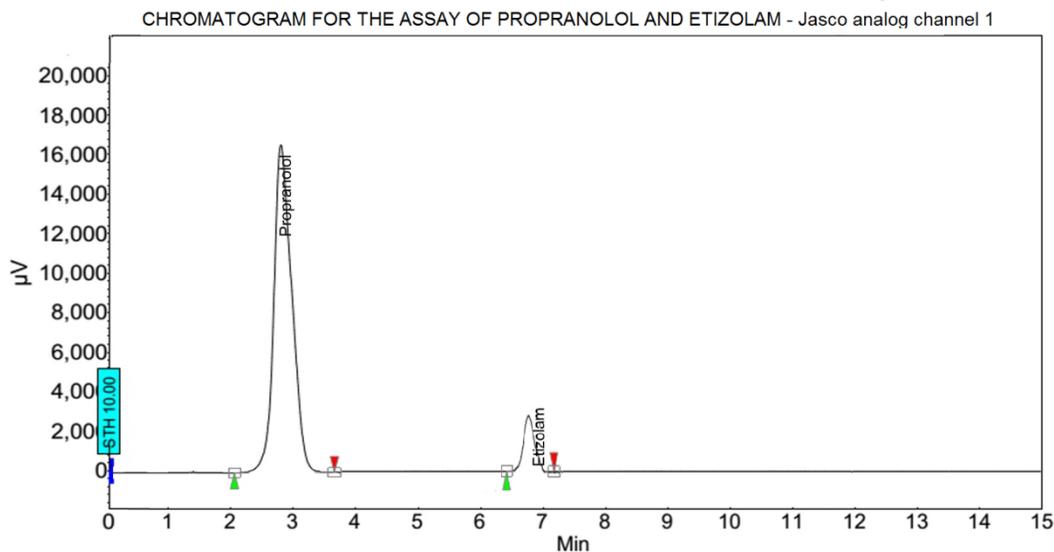


Figure 5: Chromatogram for the assay of marketed formulation

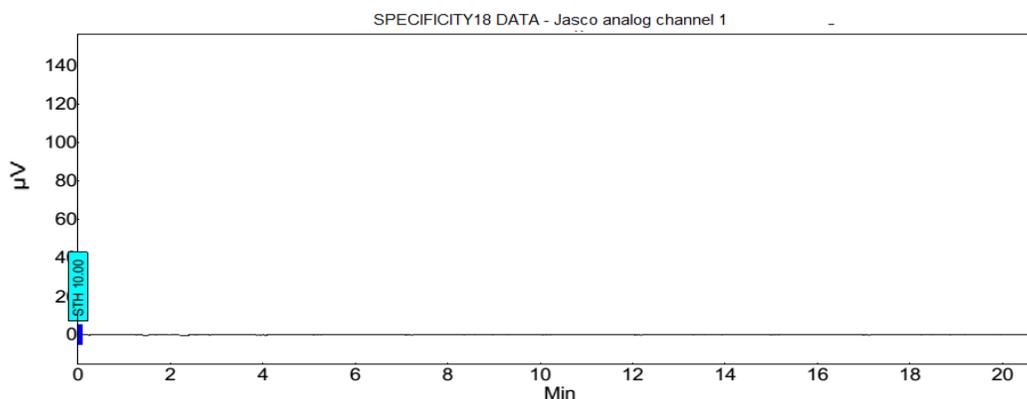


Figure 6: Chromatogram for specificity

Specificity:

No peaks were found at retention time of 2.35 and 6.57 min, the proposed method was specific for detection of Propranolol hydrochloride and Etizolam.

Linearity and Range:

Was performed by plotting a calibration graph by taking different concentrations in the range of 5–50 $\mu\text{g/mL}$ for Propranolol hydrochloride and 0.5 – 5 $\mu\text{g/mL}$ for Etizolam. The slope of the linearity graph was found to be 3786 and 1220 for Propranolol hydrochloride and Etizolam. The intercept was found to be 4911 and - 150.7 for propranolol hydrochloride and Etizolam respectively. The linearity graph for both the drugs was satisfactory as observed from the correlation coefficient values which were 99.9% and 99.7% for Propranolol hydrochloride and Etizolam respectively.

Table 2: Linearity data of Propranolol and Etizolam

Propranolol hydrochloride		Etizolam	
Concentration ($\mu\text{g/ml}$)	Peak area	Concentration ($\mu\text{g/ml}$)	Peak area
5	24563	0.5	542
10	42253	1.0	1102
15	60235	1.5	1695
20	79652	2.0	2256
25	99862	2.5	2854
30	121253	3.0	3354
35	139652	3.5	4123
40	155421	4.0	4635
45	172364	4.5	5502
50	195234	5.0	5998

Table 3: Linearity Report of Propranolol and Etizolam

Parameters	Propranolol hydrochloride	Etizolam	Acceptance Criteria
Linearity Range	5-50 $\mu\text{g/ml}$	0.5-5 $\mu\text{g/ml}$	-

Regression Equation	$y = 3786.x + 4911$	$y = 1220.x - 150.7$	-
Correlation Coefficient	0.999	0.997	0.99
Percentage curve	99.9%	99.7%	99%
Fitting			
Intercept	4911	- 150.7	-
Slope	3786	1220	-

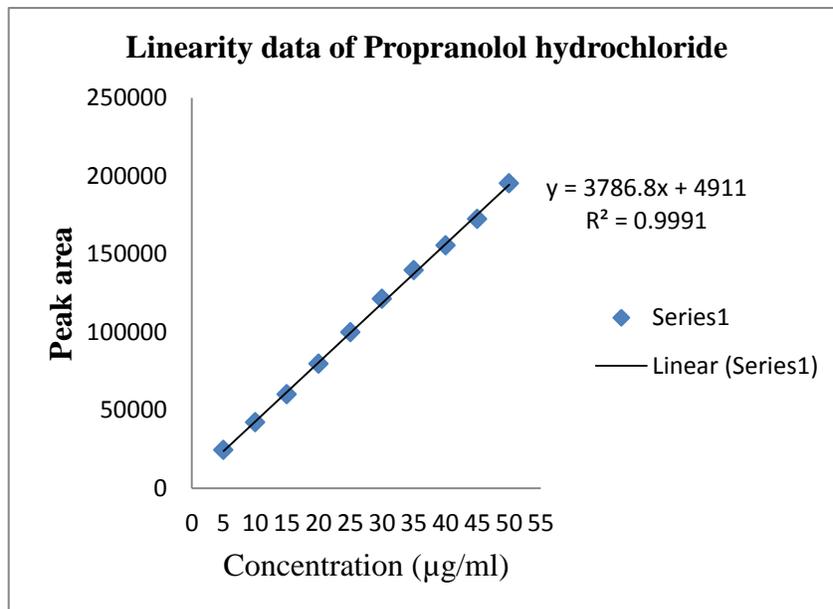


Figure 7: Linearity curve of propranolol hydrochloride

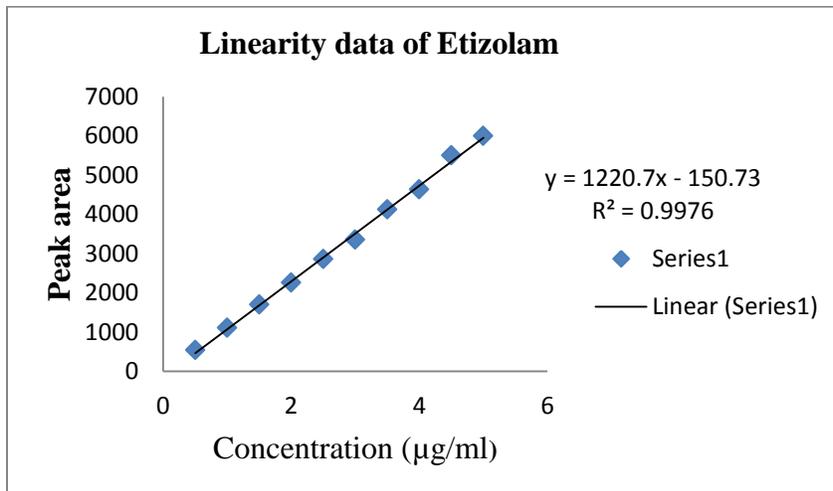


Figure 8: Linearity curve of Etizolam

Precision:

Six replicated injections were performed into the HPLC system from the stock solution within the same day and different days and for method precision and system precision. From the peak areas the %RSD was calculated for repeatability and inter day precision. The %RSD was found to be less than 2.

Table 4: Precision data of Propranolol hydrochloride and Etizolam

Precision Parameters	Propranolol hydrochloride % RSD	Etizolam % RSD
Method Precision	0.1665	1.182
System Precision	0.7145	0.3445
Intra- day Precision	0.3046	0.3393
Inter day Precision	0.348	0.5032

Accuracy:

The accuracy was determined through recovery study of the drug by spiking the standard drug of Propranolol hydrochloride and Etizolam at three different levels of 80 %, 100 % and 120 % with previously analyzed samples of known fixed concentration. The percentage recovery was found to be 98.5% to 100.58% for Propranolol hydrochloride and 95% to 106.6% for Etizolam. The percentage recovery was in total agreement with acceptance criteria of 90 % – 110 %.

Table 5: Accuracy data of Propranolol hydrochloride and Etizolam

Level %	Propranolol hydrochloride			Etizolam		
	Conc. (µg/ml)	Amount Recovered (µg/ml)	%Recovery	Conc. (µg/ml)	Amount Recovered (µg/ml)	%Recovery
80%	8	7.88	98.5	0.4	0.38	95
100%	10	9.99	99.9	0.5	0.5	100
120%	12	12.07	100.583	0.6	0.64	106.6

Limit of detection:

The LOD was determined by visualization method and found to be at the concentration of 0.1 µg/ml and 0.025 µg/ml for Propranolol hydrochloride and Etizolam respectively.

Table 6: LOD data of Propranolol hydrochloride and Etizolam

Drug	LOD (µg/ml) Visualization
Propranolol hydrochloride	0.1
Etizolam	0.025

Table 7: Summary of Validation parameters for proposed HPLC method

Parameters	Propranolol hydrochloride	Etizolam	Acceptance
Retention time(min)	2.35min	6.57min	-
LOD visualization(µg/ml)	0.1 µg/ml	0.05 µg/ml	-
Linearity(µg/ml)	5-50 µg/ml	0.5-5 µg/ml	-
Accuracy(% Recovery)	98.5 -100.56%	95 -106.6 %	90-110%
Precision %RSD	Method	0.17	1.18
	System	0.71	0.34
	Intra day	0.30	0.34
	Inter day	0.35	0.50
			<2%
			<2%
			<2%
			<2%

Specificity	No peaks detected upto retention time of 15 min	Peaks should not be detected
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CONCLUSION

A precise RP – HPLC method was developed for the simultaneous estimation of Propranolol hydrochloride and Etizolam. The method was validated as per the ICH guidelines and the method was found to be simple, precise, linear, accurate. Hence the proposed method was found to be satisfactory and could be used for the routine analysis of Propranolol hydrochloride and Etizolam in their marketed tablet dosage formulations.

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