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ASSAY METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF PAROXETINE AND CLONAZEPAM BY RP- HPLC

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ABSTRACT

A simple, precise, rapid, specific and accurate reverse phase high performance liquid chromatography method was developed for simultaneous estimation of Paroxetine and Clonazepam in pharmaceutical dosage form. Chromatographic separation was performed on Agilent Eclipse XDB (C₈) (4.6mm x 150mm, 5m) column, with mobile phase comprising of mixture of buffer (pH7, adjusted with ammonium acetate), acetonitrile in the ratio of 82:18v/v, at the flow rate 0.8 ml/min. The detection was carried out at 265 nm. The retention times of paroxetine and clonazepam were found to be 2.36 and 3.14 mins respectively with a run time of 5 mins, theoretical levels for paroxetine and clonazepam were 6753 and 4693 respectively, with a resolution of 5.10. As per ICH guidelines the method was validated for linearity, accuracy, precision, limit of detection and limit of quantitation, robustness and ruggedness. Linearity of paroxetine was found in the range of 100-300µg/mL and that for clonazepam was found to be 2-6 µg/mL.

Keywords: Paroxetine, Clonazepam, RP-HPLC, Method development, Validation.

INTRODUCTION

Paroxetine is chemically a (3*S*,4*R*)-3-[(2*H*-1,3-benzodioxol -5 -yloxy) methyl]- 4- (4 fluorophenyl) piperidine. It is an antidepressant in a group of drugs called selective serotonin reuptake inhibitors (SSRIs) [1-5]. SSRIs are primarily classified as antidepressants. The mechanism action of Paroxetine affects neurotransmitters, the chemicals that nerves within the brain use to communicate with each other. Neurotransmitters are manufactured and released by nerves and then travel and attach to nearby nerves [6-10]. Paroxetine works by preventing the reuptake of one neurotransmitter, serotonin, by nerve cells after it has been released. Since reuptake is an important mechanism for removing released neurotransmitters and terminating their actions on adjacent nerves, the reduced uptake caused by paroxetine increases free serotonin that stimulates nerve cells in the brain [11-16].

Clonazepam is a benzodiazepine drug having

anxiolytic, anticonvulsant. Clonazepam is chemically 5-(2-chlorophenyl)- 1, 3- dihydro -7- nitro-2 #-1, 4- benzodiazepinone. Clonazepam is approved by the Food and Drug Administration for treatment of epilepsy and Panic Disorder. Clonazepam is a chlorinated derivative of nitrazepam and therefore a chloro-nitrobenzodiazepine. Clonazepam's primary mechanism of action is the modulation of GABA function in the brain, by the benzodiazepine receptor, located on GABA_A receptors, which, in turn, leads to enhanced GABAergic inhibition of neuronal firing. Clonazepam exerts its action by binding to the benzodiazepine site of the GABA receptors, which causes an enhancement of the electric effect of GABA binding on neurons, resulting in an increased influx of chloride ions into the neurons. This results in an inhibition of synaptic transmission across the central nervous system [16].

Literature survey revealed that few analytical

techniques are available for estimation of paroxetine alone as well as in combine dosage form such as UV, HPLC [4-8]. Similarly few analytical methods are available for estimation of clonazepam alone and its combination with drugs such as UV and HPLC, colorimetry [1-8] but there is few methods for their simultaneous estimation using RP-HPLC. The present study was designed to develop a simple, precise, and rapid analytical RP-HPLC procedure, which can be used for the analysis of assay method for simultaneous estimation of paroxetine and clonazepam as there was only individual methods reported for both drugs. The combination of these two drugs is not official in any pharmacopoeia; hence no official method is available for the simultaneous estimation of these two drugs in their combined dosage forms. Various validation aspects of the analysis accuracy, precision, recovery, the limits of detection and quantification etc have been measured as per ICH guidelines [15].

MATERIALS AND METHODS

Equipment

Chromatographic separation was performed on HPLC system-Water's, PDA Detector 2998 module, equipped with a solvent delivery pump, sample injector and column thermostats. EMPOWER2 Chromatographic system software was applied for data collecting and processing.

Chemicals and reagents

Methanol, Acetonitrile (HPLC grade) was used. Buffer used was pH-7 (pH adjusted with ammonium acetate). (Reference standards Paroxetine and Clonazepam were obtained from Rainbow Pharma Training Lab. paroxetine (25mg) and Clonazepam (0.5mg) manufactured by Zydus candela health care ltd were procured from local market.

Preparation of Standard Solution

Accurately weighed and transferred 50 mg of Paroxetine and 1.0 mg of Clonazepam working standard into a 50mL clean dry volumetric flask and added about 50mL of diluent. It was sonicated to dissolve completely and made volume up to the mark with the same diluent. (Stock solution) (1000µg/mL). From this, 5 ml of the solution was pipetted into another 25ml volumetric flask and diluted up to the mark with diluent (200µg/mL).

Preparation of Sample Solution

Accurately weigh 10 tablets and transferred tablet powder equivalent to 50 mg of Paroxetine and 1.0 mg of Clonazepam into a 50mL clean dry volumetric flask and added about 50mL of diluent. It was sonicated to dissolve completely and made volume up to the mark with the same diluent. (Stock solution). From this, 5 mL of the solution was pipetted into another 25ml volumetric flask and diluted up to the mark with diluents

Preparation of Buffer

Weigh 7.708gms of ammonium acetate in to a 1000mL beaker, dissolve and diluted to 1000ml with HPLC grade water. The pH was adjusted to 7 with ammonium acetate buffer.

Preparation of Mobile Phase

A mixture of above prepared buffer 820 mL (82%), 180mL of HPLC grade Acetonitrile (18%) were mixed and degassed in ultrasonic water bath for 5 minutes. The mobile phase was filtered through 0.45 µ filter under vacuum.

Diluent Preparation

A mixture of HPLC grade acetonitrile and water mixed in 50:50 ratio was prepared degassed in ultrasonic water bath for 5 minutes and filtered through 0.45 µ filter under vacuum.

Optimized chromatographic conditions are as follows

- Column : Agilent Eclipse XDB C8 (4.6mm×150mm×5µ)
- Mobile Phase : 82:18(ammonium acetate: Acetonitrile)
- Flow Rate : 0.8ml/min
- Volume : 5ul
- Column Temperature : 30^oc
- Sample temperature : 25^oc
- Detector wavelength : 265nm (PDA)
- Run Mode : Isocratic
- Instrument : Waters HPLC

METHOD VALIDATION

Linearity

Solutions were prepared containing 100µg/ml, 150µg/ml, 200µg/ml, 250µg/ml, 300µg/ml, concentrations of Paroxetine and 2 µg/ml, 3 µg/ml, 4 µg/ml, 5µg/ml, 6 µg/ml, concentrations of Clonazepam which corresponding to 50,75,100,125 and 150% respectively of the test solution concentration. Each solution was injected, linearity was evaluated by linear-regression analysis.

Accuracy

Accuracy was determined by the recovery studies at three different concentrations (corresponding to 50, 100 and 150 % of the test solution concentration) by addition of known amounts of standard to pre-analysed sample preparation. For 50%,150% concentration five sets and for 100% three sets were prepared and injected.

Precision

Intraday and interday variations were determined by using six replicate injections of one concentration and analyzed on the same day and different days. Precision of An analytical method is usually expressed as the standard deviation correlative standard deviation (coefficient of variation) of series of measurements.

Robustness

The robustness was evaluated by assaying test solutions after slight but deliberate changes in the analytical conditions. The factors chosen for this study were the flow rate (± 0.1 ml/min), temperature.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ was calculated from linear curve using formulae

$$\text{LOD} = 3.3 * \sigma / \text{slope}, \text{LOQ} = 10 * \sigma / \text{slope}$$

(Where σ = the standard deviation of the response and S = Slope of calibration curve).

Specificity

Specificity was checked for the interference of impurities in the analysis of blank solution and injecting sample solution under optimized chromatographic conditions to demonstrate separation of both paroxetine and clonazepam from impurities.

RESULT AND DISCUSSION

Several mobile phase compositions were tried to

resolve the peak of paroxetine and clonazepam. The mobile phase containing buffer: Ammonium acetate : Acetonitrile in proportion of 82:18v/v was found ideal to resolve the peak of paroxetine and clonazepam. Retention time of paroxetine and clonazepam were 2.1 and 3.3 min respectively (Figure 3). Result of assay is shown in Table-2. The proposed method was found to be linear in concentration range 100-300 $\mu\text{g/ml}$ for paroxetine and 2-6 $\mu\text{g/ml}$ for clonazepam. The data was shown in Table-3(a&b) and Figure-10&11. System suitability parameters were evaluated and results shown in (Table-4), which were within acceptance criteria. The mean percentage recovery for paroxetine and clonazepam was found to be 100% and 100% respectively, which are well within the limit and hence the method was found to be accurate (Table-5). LOD and LOQ values were 2.752 $\mu\text{g/ml}$ and 9.174 $\mu\text{g/ml}$ for paroxetine and 2.40 $\mu\text{g/ml}$ and 8.0 $\mu\text{g/ml}$ for clonazepam (Table-6). Results of intraday and interday precision were shown in the (Table-7a&7b). The robustness of the method was investigated by varying experimental conditions such as changes in flow rate and temperature. The result obtained implies method is robust for routine qualitative analysis (Table-8).

Table 1. Observations of sample Chromatogram

Sl.No	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Paroxetine	2.360	2360112		1.30	6753
2	Clonazepam	3.140	6610849	5.10	1.46	4693

Table 2. Analysis data of formulation

Injection	Label claim (mg)	Assay (%)
Paroxetine	25	99
Clonazepam	0.5	100

Table 3(a). Linearity Observation of Paroxetine

Conc %	Area	ug/ml
50	1141690	100
75	1722681	150
100	2298287	200
125	2870955	250
150	3440881	300

Table 3(b). Linearity Observation of Clonazepam

Conc%	Area	ug/ml
50	3231484	2
75	4857955	3
100	6469948	4
125	8083511	5
150	9708063	6.00

Table 4. System suitability parameters

Parameters	Paroxetine	Clonazepam	Acceptance criteria
Theoretical plates	6753	4693	Not less than 2000
Tailing factor	1.30	1.46	Not more than 2
Resolution	-	5.10	Not less than 2

Table 5.Recovery studies for Paroxetine and Clonazepam

Drug	Spiked level%	Amount taken (µg/ml)	Amount found (µg/ml)	Percent recovery	Mean recovery
Paroxetine	50	99.10	98.85	100	100
	100	198.2	197.98	100	
	150	297.3	297.38	100	
Clonazepam	50	1.99	1.99	100	100
	100	3.98	3.99	100	
	150	5.98	5.98	100	

Table 6. LOD and LOQ for Paroxetine and Clonazepam

Drug	LOD (µg/ml)	LOQ (µg/ml)
Paroxetine	2.752	9.174
Clonazepam	2.40	8.0

Table 7(a).Results of Precision

S.No	Sample Weight	Sample Area-1	Sample Area-2	% Assay
1	434.00	2292161	6461645	99
2	434.00	2294813	6465231	99
3	434.00	2290710	6460277	99
4	434.00	2299117	6460179	99
5	434.00	2293760	6468029	99
6	434.00	2293668	6469412	99
Average Assay:				99
STD				0.12
%RSD				0.13

n- Number of replicate injections

Table 7(b). Results of precision

Drug	Conc. (µg/ml)	Peak area (n=6)	% RSD
Paroxetine	200	2294038.2	0.13
Clonazepam	4	5493712.2	0.06

Table 8. Results of Robustness study

S.no	Parameter	Condition	Theoretical levels		Tailing factor		Retention time	
			PARA	CNZ	paroxetine	clonazepam	PARA	CNZ
1.	Flow rate1	0.6 ml/min	7569	4868	1.32	1.49	2.343	3.076
	Flow rate2	1.0ml/min	6958	4689	1.30	1.50	1.957	2.58
2.	Temp 1	20 ^o c	7281	5234	1.32	1.39	2.347	3.09
	Temp 2	30 ^o c	7113	4857	1.31	1.48	2.346	3.08

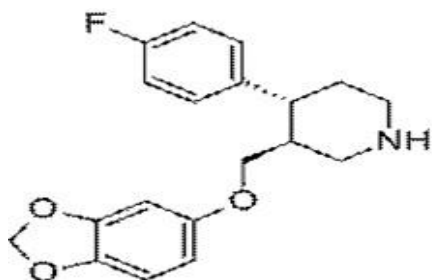
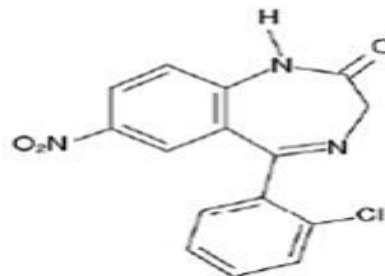
Figure 1. Molecular Structure of paroxetine**Figure 2. Molecular structure of clonazepam**

Figure 3. Chromatogram of sample

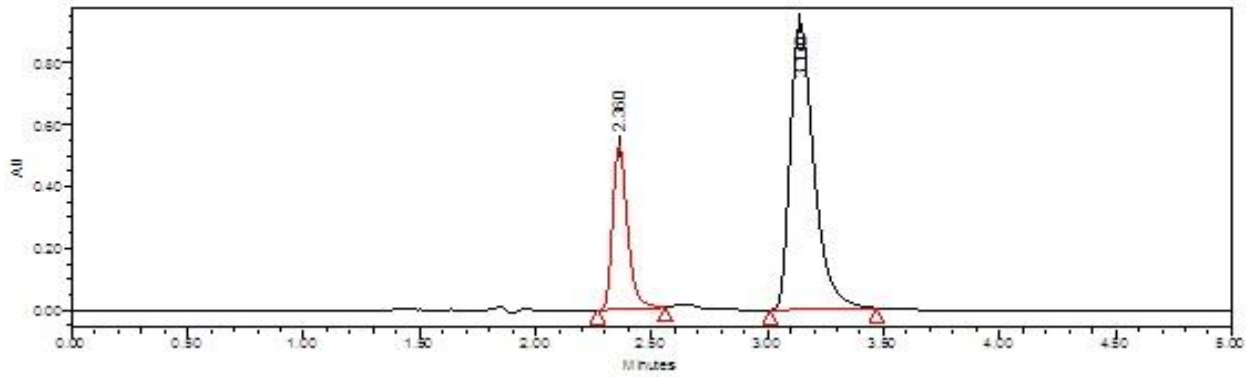


Figure 4. Standard chromatogram - 1

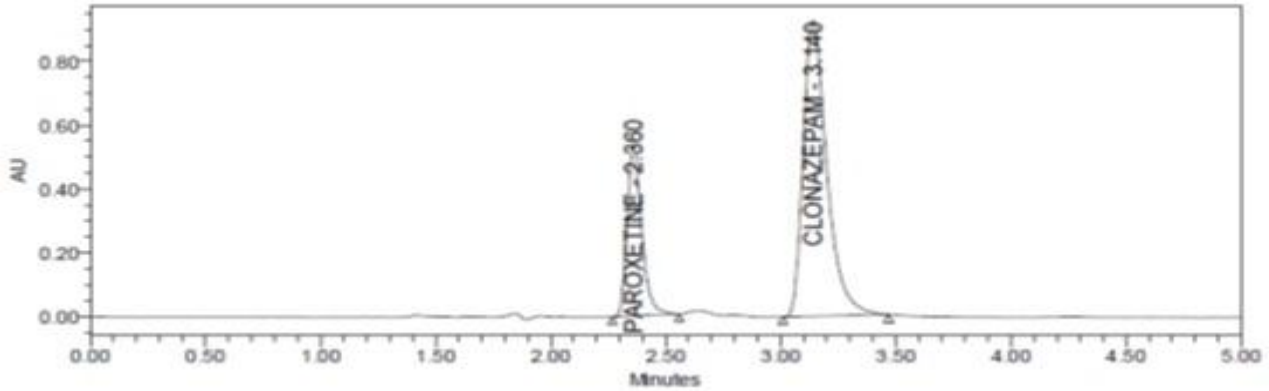


Figure 5. Standard Chromatogram - 2

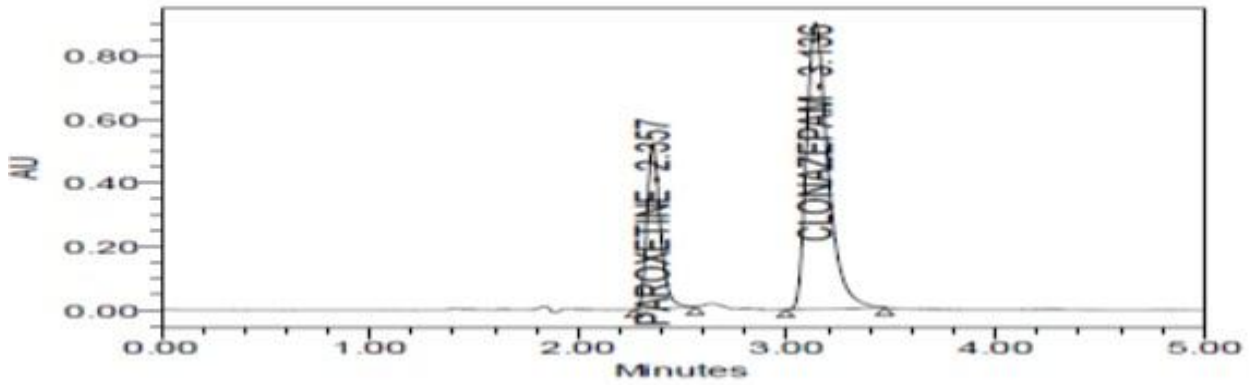


Figure 6. Standard Chromatogram - 3

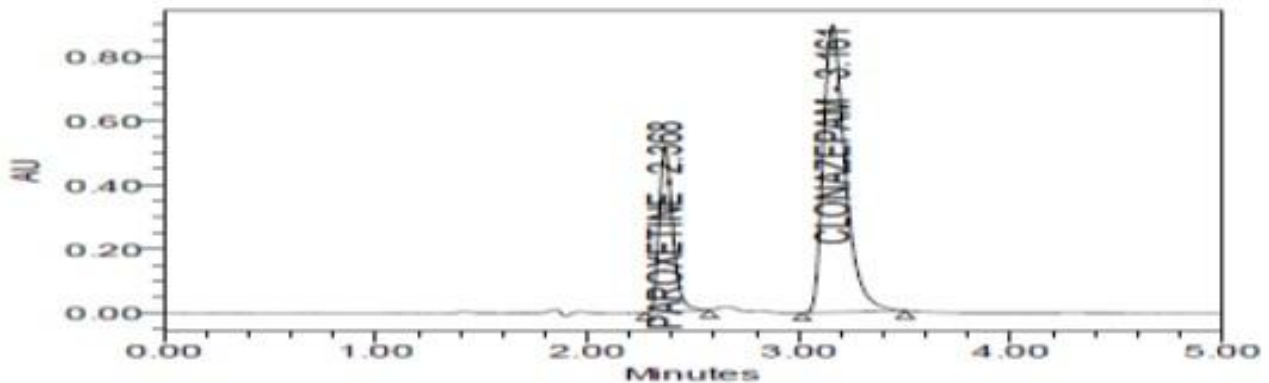


Figure 7. Standard Chromatogram -4

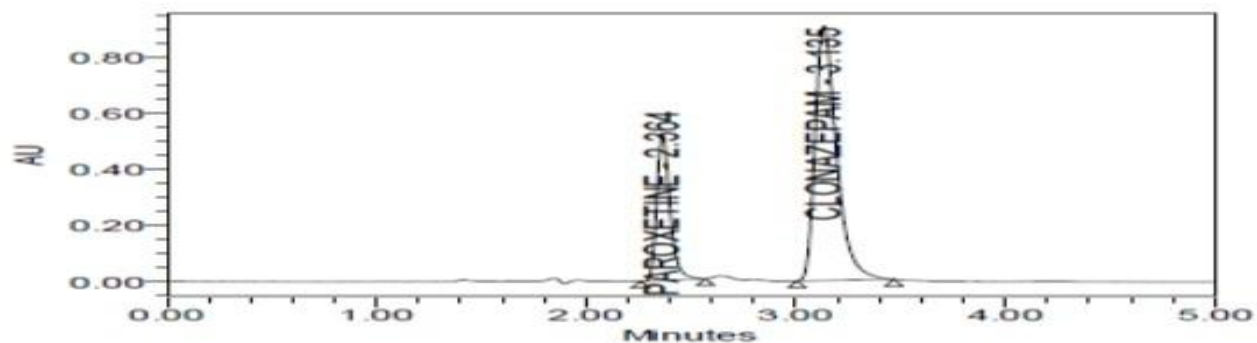


Figure 8. Standard Chromatogram -5

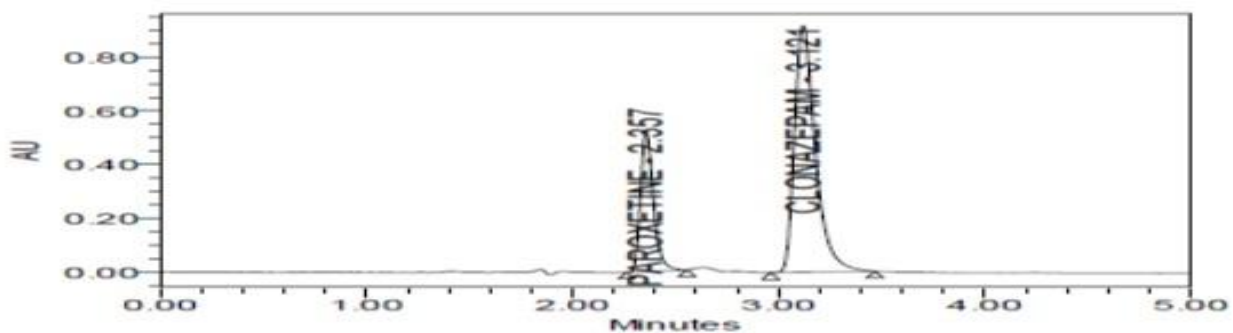


Figure 9. Standard Chromatogram -6

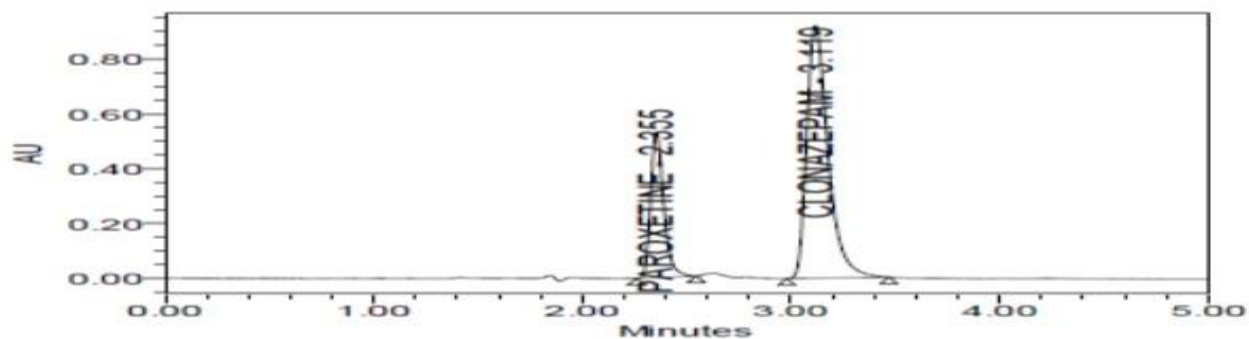


Figure 10. Calibration curve of Paroxetine

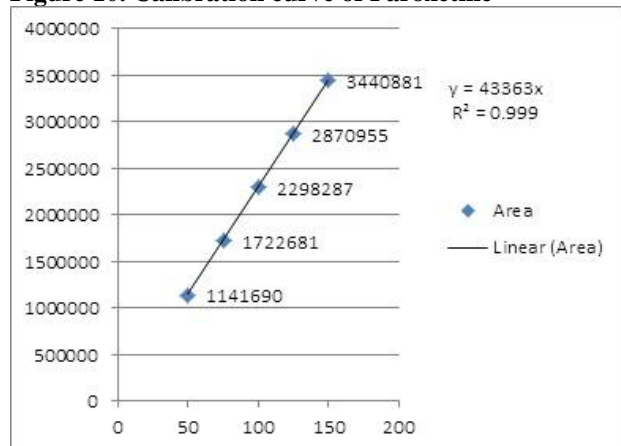
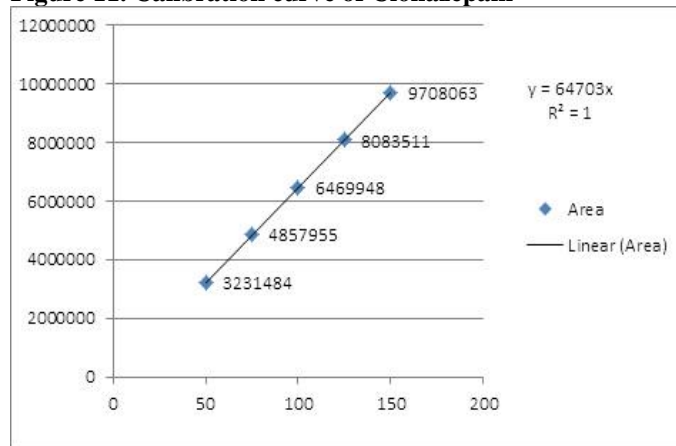


Figure 11. Calibration curve of Clonazepam



CONCLUSION

The proposed RP-HPLC method was used for the simultaneous estimation of paroxetine and clonazepam was found to be sensitive, accurate, precise, simple, and rapid. Hence the present RP-HPLC method may be used for routine analysis of the raw materials, in vitro dissolution

study of combinational dosage formulations containing paroxetine and clonazepam.

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