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METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ALPRAZOLAM AND FLUOXETINE HCL IN A PHARMACEUTICAL FORMULATION BY RP-HPLC METHOD

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ABSTRACT

An isocratic Simultaneous estimation by RP-HPLC Method were developed and validated for the quantification of Alprazolam and Fluoxetine HCL in tablet dosage form. Quantification was achieved by using a reversed-phase C8 column (ZORBAX C_8 Column, 5µ, 150 mm × 4.6 mm) at ambient temperature with mobile phase consisting of Mixed 50mM Phosphate buffer: Acetonitrile (30:70 pH:4.0)). The flow rate was 1.0 ml/min. Measurements were made at a wavelength of 229nm. The average retention time for Alprazolam and Fluoxetine HCl were found to be 2.15 min and 3.14. The proposed method was validated for selectivity, precision, linearity and accuracy. The assay methods were found to be linear from 0.6-1.4µg/ml for Alprazolam and 48 to 112µg/ml for Fluoxetine HCl. All validation parameters were within the acceptable range. The developed method was successfully applied to estimate the amount of Alprazolam and Fluoxetine HCl in tablet dosage form.

KEYWORDS

Alprazolam, Fluoxetine HCL, RP-HPLC method, Zorbax C_8 Column, Methanol, Acetonitrile, KH_2PO4, Ortho phosphoric acid and Validation.

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INTRODUCTION

Alprazolam

Alprazolam (Figure No.1) is a short-acting anxiolytic of the benzodiazepine class of psychoactive drugs. Alprazolam is commonly used and FDA approved for the medical treatment of panic disorder, and anxiety disorders, such as generalized anxiety disorder (GAD) or social anxiety disorder (SAD).

Alprazolam is mostly used to treat anxiety disorders, panic disorders, and nausea due to chemotherapy. The FDA label advises that the physician should periodically reassess the usefulness of the drug. In the US alprazolam is FDA-approved for the management of anxiety disorders or the short-term relief of symptoms of anxiety. Anxiety associated with depression is responsive to alprazolam. Demonstrations of the effectiveness by systematic clinical study are limited to 4 months duration for anxiety disorder. In one study, some long term, high-dosage users of alprazolam developed reversible depression. In the UK, alprazolam is recommended for the short-term treatment (2-4 weeks) of severe acute anxiety. Alprazolam may be used in combination with other medications for chemotherapy-induced nausea and vomiting.

Side Effects

- Depressed mood, thoughts of suicide or hurting yourself, unusual risk-taking behavior, decreased inhibitions, no fear of danger;
- Confusion, hyperactivity, agitation, hostility, hallucinations;
- Feeling like you might pass out;
- Urinating less than usual or not at all;
- Chest pain, pounding heartbeats or fluttering in your chest;
- Uncontrolled muscle movements, tremor, seizure (convulsions); or
- Jaundice (yellowing of the skin or eyes).

Fluoxetine HCL

Fluoxetine hydrochloride (Figure No.2) is the first agent of the class of antidepressants known as selective serotonin-reuptake inhibitors (SSRIs). Fluoxetine is a racemic mixture of the R- and S-enantiomers and are of equivalent pharmacologic activity. Despite distinct structural differences between compounds in this class, SSRIs possess similar pharmacological activity. As with other antidepressant agents, several weeks of therapy may be required before a clinical effect is seen. SSRIs are potent inhibitors of neuronal serotonin reuptake. They have little to no effect on norepinephrine or dopamine reuptake and do not antagonize α- or β-adrenergic, dopamine D2 or histamine H1 receptors. During acute use, SSRIs block serotonin reuptake and increase serotonin stimulation of somatodendritic 5-HT1A and terminal autoreceptors. Chronic use leads to desensitization of somatodendritic 5-HT1A and terminal autoreceptors. The overall clinical effect of increased mood and decreased anxiety is thought to be due to adaptive changes in neuronal function that leads to enhanced serotonergic neurotransmission. Side effects include dry mouth, nausea, dizziness, drowsiness, sexual dysfunction and headache. Side effects generally occur within the first two weeks of therapy and are usually less severe and frequent than those observed with tricyclic antidepressants. Fluoxetine may be used to treat major depressive disorder (MDD), moderate to severe bulimia nervosa, obsessive-compulsive disorder (OCD), premenstrual dysphoric disorder (PMDD), panic disorder with or without agoraphobia, and in combination with olanzapine for treatment-resistant or bipolar I depression. Fluoxetine HCl is the most anorexic and stimulating SSRI.F.

Mechanism of Action

Metabolized to norfluoxetine, fluoxetine is a selective serotonin-reuptake inhibitor (SSRI), it blocks the reuptake of serotonin at the serotonin reuptake pump of the neuronal membrane, enhancing the actions of serotonin on 5HT1A autoreceptors. SSRIs bind with significantly less affinity to histamine, acetylcholine, and norepinephrine receptors than tricyclic antidepressant drugs.

EXPERIMENTAL

Equipments

The chromatographic technique performed on a Shimadzu LC20-AT Liquid chromatography with SPD-20A prominence UV-visible detector and Spinchrom software, reversed phase C8 column (Zorbax 5µ, 150 mm × 4.6 mm) as stationary phase. Thermo Electron Corporation double beam UV-visible spectrophotometer (vision pro-software), Ultrasonic cleaner, Shimadzu analytical balance AY-220, Vaccum micro filtration unit with 0.45µ membrane filter was used in the study.

MATERIALS

Pharmaceutically pure sample of Alprazolam and Fluoxetine HCL were obtained as gift samples from Chandra Labs, Prashanthi nagar, Kukatpally, and Hyderabad, India. The purity of the drug was evaluated by obtaining its melting point and ultraviolet (UV) and infrared (IR) spectra. No impurities were found. The drug was used without further purification.

HPLC-grade Acetonitrile ware from standard reagents pvt ltd. KH_2PO_4 (AR grade) was from Merck.

A tablet formulation of Alprazolam and Fluoxetine HCL (0.25 mg and 20mg label claims) were procured from local market (Fludep AZ, Ultramark Healthcare Pvt Ltd, India).

Chromatographic conditions

The sample separation was achieved on a C8 (5 µ, 15 cm X 4.6 mm i.d.) Zorbax column, aided by mobile phase mixture of Phosphate Buffer buffer : Acetonitrile (30: 70, pH:4.0)), that was filtered and degassed prior to use, at a flow rate of 1ml/min. Injection volume is 20 µl and detected at 229 nm at ambient temperatures.

Preparation of mobile phase

Buffer Preparation

Weigh accurately about 6.8 gms of KH_2PO_4 and dissolve with 200ml of HPLC Grade water than make up to 1000 ml with HPLC grade water then adjust the pH:4.0 with ortho phosphoric acid.

Mobile phase

Then add 30 volumes of buffer, 70 volumes of Acetonitrile sonicated for 15 min and filtered through a 0.45 µ membrane filter.

Analysis of formulation

Preparation of standard solution

A 1mg of standard Alprazolam and 80 mg Fluoxetine HCl ware weighed and transferred to 100 ml of volumetric flask and dissolved in mobile phase. The flask was shaken and volume was made up to mark with mobile phase to give a primary stock solution containing 10µg/ml Alprazolam and 800µg/ml of Fluoxetine HCl. From the above solution 5ml of solution is pipette out into a 50 ml volumetric flask and volume was made up to mark with mobile phase to give a solution containing 1µg/ml Alprazolam and 80µg/ml of Fluoxetine HCl.

Preparation of sample solution (Tablet Formulation)

For the estimation of the drug in tablet formulation twenty tablets were weighed and their average weight was determined. The tablets were then finely powdered. Appropriate quantity equivalent to 1mg Alprazolam and 80 mg Fluoxetine HCl ware accurately weighed and The powder was transferred to 100 ml volumetric flask and shaken vigorously with mobile phase and sonicated for 15 min and volume made up to the mark with mobile phase. The solution was shaken vigorously and filtered by using whatmann filter no.41. from the above filtered clear solution 5ml of sample pipetted out into a 50 ml volumetric flask volume made up to the mark with mobile phase to give a solution containing 1µg/ml Alprazolam and 80µg/ml of Fluoxetine HCl.

RESULTS AND DISCUSSION

Determination of Working Wavelength (λmax)

5 mg of the Alprazolam standard drug is taken in a 10 ml volumetric flask and dissolved in methanol and volume made up to the mark, from this solution 0.2ml is pipetted into 10 ml volumetric flask and made upto the mark with the methanol to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using methanol as blank. The λmax was found to be 248nm.

5 mg of the Fluoxetine HCl standard drug is taken in a 10 ml volumetric flask and dissolved in methanol and volume made up to the mark, from this solution 0.2ml is pipette into 10 ml volumetric flask and made upto the mark with the methanol to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using methanol as blank. The λmax was found to be 235nm.

The Isosbestic Point of Alprazolam and Fluoxetine HCl were found to be 229nm. The U.V Graph shown in Figure No.3.

After several initial trails with mixtures of methanol, water, ACN and buffer in various combinations and proportions, a trail with a mobile phase mixture of 50mM Phosphate Buffer buffer : Acetonitrile (30:70 pH:4.0) brought sharp and well resolved peaks. The chromatogram was shown in Figure No.4.

METHOD OF VALIDATION

Linearity

Linearity was studied by analyzing five standard solutions covering the range of 0.6-1.4µg/ml for Alprazolam and 48 to 112 µg/ml for Fluoxetine HCl of the drug. From the primary stock solution 0.6ml, 0.8ml, 1.0ml, 1.2ml, 1.4 ml of aliquots are pipette into 10 ml volumetric flasks and made up to the mark with the mobile phase to give a concentrations of 0.6µg/mL, 0.8µg/mL, 1.0µg/mL, 1.2µg/mL and 1.4 µg/mL of Alprazolam and 48µg/mL, 64µg/mL, 80 µg/mL, 96µg/mL, 112µg/mL of Fluoxetine HCL. Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method.

Method precision (repeatability)

The precision of the instrument was checked by repeated injections and measurement of peak areas and retention times of solutions (n = 6) for, 1 µg/ml of Alprazolam and 80 µg/ml of Fluoxetine HCl without changing the parameter of the proposed chromatographic method.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) (Table No.2) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (2) and (3), respectively.

$$LOD = 3.3\ \delta/S \quad \text{……. (3)}$$

$$LOQ = 10\ \delta/S \quad \text{…….. (4)}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of Alprazolam and Fluoxetine HCl by the standard addition method. Known amounts of standard solutions of Alprazolam and Fluoxetine HCl were added at 10% concentration to pre quantified sample solutions of Alprazolam (1.0, 1.2, 1.4µg/ml) and Fluoxetine HCl (80, 96, 112µg/ml). The amount of Alprazolam and Fluoxetine HCl recovered was estimated by using the following formulas.

$$\%\ Recovery = \frac{amount\ found}{Amount\ added} \times 100$$

$$Amount\ Found(mcg/ml) = \frac{Mean\ test\ area}{Mean\ standard\ area} \times Standard\ concentration$$

Specificity

In an assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay results are unaffected by the presence of these extraneous materials. There should be no interference of the diluents, placebo at retention time of drug substances (Table No.6).

Robustness

Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The results were shown in (Table No.4).

Ruggedness

The ruggedness of the method was studied by analyzing the sample and standard preparations by two analysts. The % RSD assay values between two analysts was calculated i.e., (limit <2%).

This indicates the method was rugged. The results were shown in Table No.5.

DISCUSSION

In RP HPLC method, the primary requirement for developing a method for analysis is that the using different solvents and buffers and columns to get better retention time and theoretical plates, and better cost effective and time saving method than the previously developed methods. The Iso bestic Point of Alprazolam and Fluoxetine HCl were found to be 229nm by scanning in UV region. The chromatographic method was optimized with mobile phase consisting of 50mM KH_2PO_4 Buffer: Acetonitrile (30: 70) and C18 Zorbax column. All the validation parameters were studied at a wavelength 229nm. Accuracy was determined by calculating the recovery (Table No.3) and the results were in acceptable range (limit 98-102%). The method was successfully used to determine the amount of Alprazolam and Fluoxetine HCl present in the Tablet. The results obtained were in good agreement with the corresponding labeled amount (Table No.3). The method was linear in the concentration range of 0.6 to 1.4 µg/ml for Alprazolam and 48 to 112µg/ml for Fluoxetine HCl (Figure No.5 and 5.1), Table No.1 and 2). Precision was calculated as repeatability (% RSD) for the drug (Table No.7). Robustness and ruggedness results were in acceptable range (Table No.4 and Table No.5). Summary of all validation parameters for method is given in Table No.8. By observing the validation parameters, the method was found to be simple, sensitive, accurate and precise. Hence the method can be employed for the routine analysis Alprazolam and Fluoxetine HCl in tablet dosage form.

Table No.1: Linear in the concentration range of Alprazolam

S.No	Concentration (µg/ml)	Peak Area
1	0.6	79.106
2	0.8	95.497
3	1	111.227
4	1.2	128.697
5	1.4	146.368

Table No.1.1: Linear in the concentration range of Fluoxetine HCl

S.No	Concentration (µg/ml)	Peak Area
1	48	1200.622
2	64	1483.069
3	80	1750.565
4	96	2015.579
5	112	2311.699

Table No.2: LOD and LOQ values Calculated from calibration curve

S.No	Alprazolam			Fluoxetine HCl	
	Parameters	Mcg	Area	Mcg	Area
1	LOD	0.03	3.12	1.79	39.25
2	LOQ	0.09	9.96	5.43	118.43

Table No.3: Recovery data

S.No	Level	Amount of Sample taken (%)	Amount of Standard Spiked (%)	% Recovery of Alprazolam	% Recovery of Fluoxetine HCl
1	I	80	10%	101.03%	98.40%
		80	10%		
		80	10%		
2	II	100	10%	100.99%	100.63%
		100	10%		
		100	10%		
3	III	120	10%	99.47%	99.05%
		120	10%		
		120	10%		

Table No.4: Results of Robustness study

S.No	Parameter	Rt of Alprazolam	Tailing factor	Theoretical Plates
1	Temparature-25°C	2.137	1.038	2934
2	20°C	2.150	1.500	2928
3	30°C	2.137	1.500	2930
4	Wave Length 229nm	2.137	1.038	2934
5	231nm	2.147	1.483	2936
6	227nm	2.147	1.467	2927

S.No	Parameter	Rt of Fluoxetine Hcl	Tailing factor	Theoretical Plates
1	Temperature(25°C)	3.140	1.008	4013
2	20°C	3.150	1.005	4015
3	30°C	3.140	1.004	4021
4	Wave Length 229nm	3.140	1.008	4013
5	231nm	3.143	1.006	4077
6	227nm	3.14	1.008	4011

Table No.5: Results of Ruggedness

S.No	Analyst	Drug	Std Area	Spl Area	% Assay	% RSD
1	Analyst-1	Alprazolam	124.069	124.524	100.21	0.06%
2	Analyst-2		124.584	124.587	100.04	
3	Analyst-1	Fluoxetine HCl	1889.865	1888.254	100.08	0.45%
3	Analyst-2		1887.562	1886.024	99.03	

Table No.6: Assay Results

S.No	Alprazolam			Fluoxetine HCL		
1	Standard Area	1	122.4		1841.2	---
		2	123.8		1848.7	
		3	119.2		1847.5	
		4	123.8		1848.7	
		5	118.5		1788.9	
		Average	121.5	Average	1835.0	
2	Sample area	1	116.8		1842.7	
		2	113.5		1843.2	
		3	122.4		1841.2	
		4	118.5		1795.4	
		5	123.2		1865.5	
		Average	118.9	Average	1837.6	
3	Tablet average weight		150.2	Mg	150.2	mg
4	Standard weight		1.0	Mg	80.0	mg
5	Sample weight		600.7	Mg	600.7	mg
6	Label amount		0.25	Mg	20	mg
7	std.purity		99.2	%	99.3	%
8	Cal.:		0.25	Mg	19.89	mg
		% Assay	99.0	%	99.5	%

Table No.7: Method Precision (Repeatability)

S.No	Alprazolam		Fluoxetine HCL	
	Rt	Area	Rt	Area
1	2.177	126.269	3.200	1888.705
2	2.167	127.364	3.187	1869.940
3	2.160	128.698	3.150	1891.833
4	2.143	127.421	3.137	1884.028
5	2.137	130.354	3.137	1882.877
6	2.153	126.087	3.157	1878.600
avg	2.1562	127.699	3.161	1882.664
stdev	0.0149	1.605	0.026	7.757
% RSD	0.69	1.26	0.84	0.41

Table No.8: Validation parameters of evaluated method

S.No	Parameter	Limit	Value Obtained
1	Accuracy (% Recovery)	98-102%	99.47 to 101.03% (Alprazolam) 98.40 to100.63% (Fluoxetine HCl)
2	Linearity concentrations Range (µ g/mL) Regression coefficient (R2 value)	NLT 0.99%	0.6 to 1.4µg/ml (Alprazolam)R^2=0.999 and 48 to 114µg/ml(Fluoxetine HCl)R^2=0.999
3	Precision (% RSD) Method precision (Repeatability) (% RSD, n = 6)	NMT 1% (For Rt) NMT 2% (For Area)	% RSD of Rt=0.69% and % RSD of Area 1.26% (Alprazolam) % RSD of Rt=0.84% and % RSD of Area 0.41% (Fluoxetine HCl)
4	Robustness (Intermediate Precision)	It should meet System suitability criteria	Complies
5	Ruggedness (% RSD analyst to analyst variation)	NMT 2%	0.06% For Alprazolam and 0.45% for Fluoxetine HCl

SD = Standard deviation, LOD = Limit of detection, LOQ = Limit of quantification, RSD = Relative standard deviation.

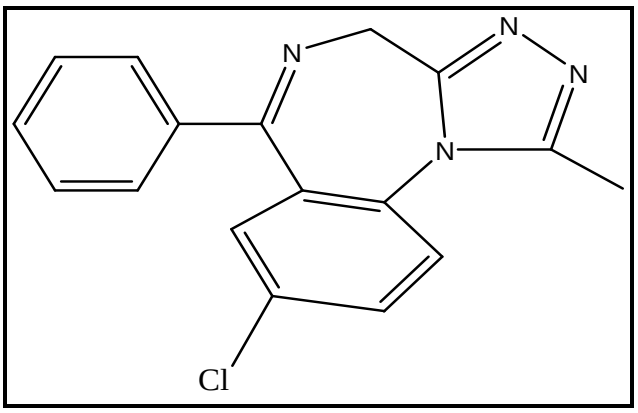


Figure No.1: Structure of Alprazolam

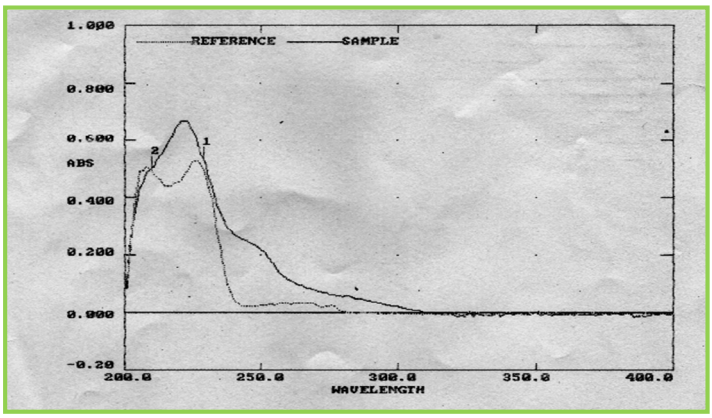


Figure No.3: U.V Graph of Alprazolam and Fluoxetine HCL

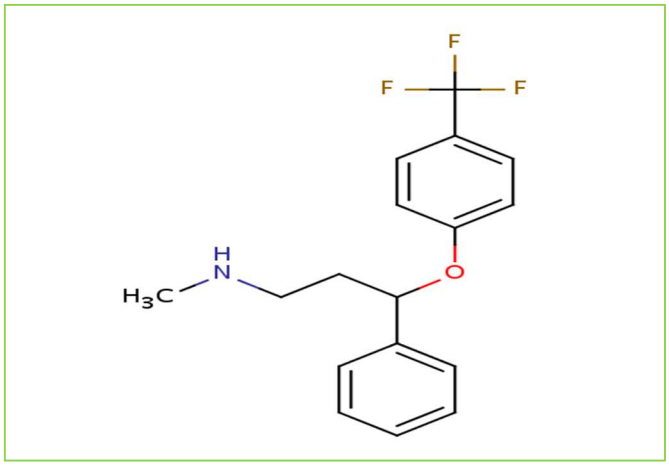


Figure No.2: Structure of Fluoxetine HCL

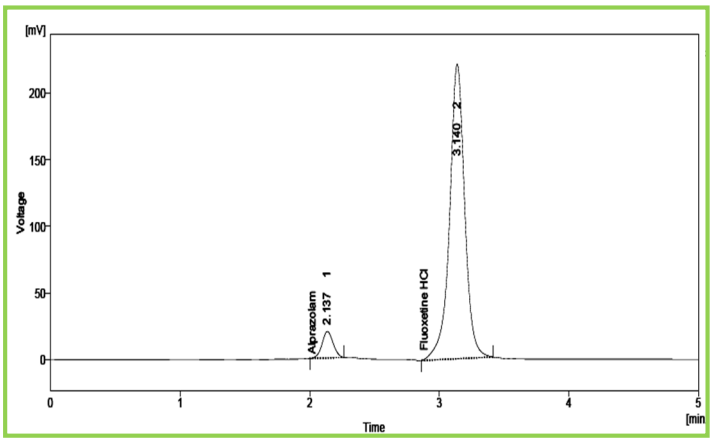
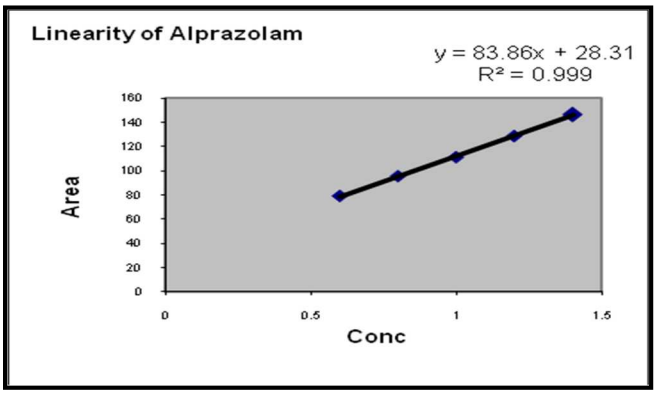


Figure No.4: Chromatogram of Alprazolam and Fluoxetine HCL



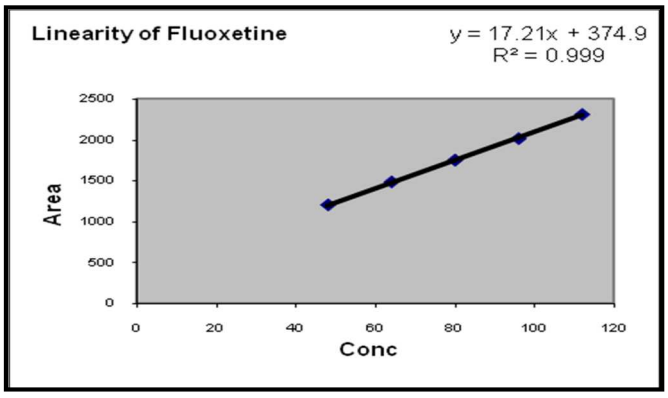


Figure No.5 and 5.1: Linearity (calibration) curve of Alprazolam and Fluoxetine HCl

CONCLUSION

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation of Alprazolam and Fluoxetine HCL 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 meant in industries, approved testing laboratories.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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