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RP-HPLC Method Development and Its Validation for Simultaneous Estimation of Alprazolam and Fluoxetine Hydrochloride in Pharmaceutical Dosage Form

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Abstract

A simple, specific, accurate and precise reverse phase high pressure liquid chromatographic method has been developed for the simultaneous determination of Alprazolam(Alp) and Fluoxetine Hydrochloride(Flx) in tablet dosage form by reverse phase C18 column, Phenomenex (250mm x 4.60mm), Particle Size 5 µ. The samples were analyzed by using Acetonitrile: Water in the ratio of 75:25 (pH adjusted to 2.75 with 0.1% orthophosphoric acid) as a mobile phase at the flow rate of 1.1 ml/min in isocratic mode and detection wavelength 224 nm. Both the drugs were eluted within 5 minutes and give sharp peak with high theoretical plate count and low tailing factor. The retention time for Fluoxetine Hydrochloride and Alprazolam was found to be 2.06 and 3.14 min respectively. The validation was carried according to ICH guidelines. In linearity curve correlation coefficients for Alprazolam and Fluoxetine Hydrochloride were found to be 0.9989 and 0.9994 respectively. The percent recovery was 99.56 for Alprazolam and 99.96 for Fluoxetine Hydrochloride indicating accuracy and reliability of method. So the method can be used for estimation of combination of these drugs in tablet dosage form.

Keywords:

Alprazolam; Fluoxetine hydrochloride; RP-HPLC

1. Introduction

Alprazolam is a anxiolytic drug belonging to the class of benzodiazepine, chemically it is 8-Chloro-1-methyl-6-phenyl-4H-(1,2,4)triazolo(4,3-α)(1,4)-benzodiazepine. It is used in the treatment of anxiety disorders, agoraphobia, panic disorders and depression [1, 2]. Some spectrophotometric and HPLC methods are reported in literature for estimation of Alprazolam in combination with other drugs and its metabolite in biological fluid and urine [3-9]. Spectrofluorimetric assay is also available for the photo degradation products of Alprazolam [10].

Fluoxetine Hydrochloride (Flx) is an antidepressant drug; chemically it is *N*-Methyl-*γ*-[4-trifluoromethyl) phenoxy] benzenepropanamine. It is used to treat major depression; anxiety states especially obsessive compulsive disorders, premenstrual tension and bulimia nervosa, also panic disorders [11]. Various analytical methods are reported for estimation of

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Fluoxetine and norfluoxetine in human plasma as well as combination of Fluoxetine with other antidepressant drugs [12-16].

However there is no RP-HPLC method reported for the simultaneous estimation of these drugs in combined dosage forms. The objective of the present work is to develop and validate new analytical method for simultaneous determination of Alprazolam (Alp) and Fluoxetine Hydrochloride (Flx) in tablet dosage form.

2. Material and Method

2.1. Chemical and Reagents

Pure samples of Alprazolam (Alp) and Fluoxetine Hydrochloride (Flx) were obtained from Alembic limited, vadodara. HPLC grade Acetonitrile, glacial acetic acid, orthophosphoric acid procured from Merck and methanol from Rankem. Highly pure water was prepared by using Millipore system. The pharmaceutical preparations of combination of Alprazolam (Alp) and Fluoxetine Hydrochloride (Flx) are FIDEN-AZ (Condor Biotech Ltd, Solan) and FLUWEL (Bestochem Formulation Ltd, Delhi). The commercial formulation of Alp and Flx is available in the ratio of 1:80 (0.25/20 mg).

2.2. Instrumentation

A High performance liquid chromatography system LC- $10AT_{VP}$ Shimadzu with photo diode array detector (SPD-M10 AVP-Shimadzu) was used for analysis. The data was recorded by using the software CLASS-LC10/M10A. The column used for separation was octadecyl silane (C_{18}) with length 250 mm and internal diameter 4.6 mm (Phenomenex).

Table 1. The optimized chromatographic conditions

Seperation Variable	Optimized condition
Chromatography	LC- 10ATVP Shimadzu
Column	$C_{18.}$ 5 µ, (250 mm x 4.6 mm)
Mobile phase	Water and Acetonitrile (25:75) Ph 2.75 adjusted by 0.1% OPA.
Diluent	Acetonitrile
Flow rate	1.1 mL min^{-1}
Temperature	Ambient
Detection wavelength	224 nm
Retention time – Fluoxetine hydrochloride	2.06 min
Retention time – Alprazolam	3.14 min

2.3. Solubility determination

Solubility of Alprazolam and Fluoxetine Hydrochloride was determined in different solvents. Both the drugs were found to be soluble in acetonitrile and Methanol.

2.4. Wavelength selection

The λmax for Alprazolam is 221 nm and for Fluoxetine Hydrochloride it is 226 nm. The isobestic point for both the drugs was found to be 224 nm so it is selected as detection wavelength.

2.5. Preparation of stock solution

Standard stock solution of Alprazolam and Fluoxetine Hydrochloride were prepared by dissolving 25 mg of drug in 20 mL of diluent (Acetonitrile) separately and it was sonicated for 10 min, then volume was made up to 25 mL (1000 µg mL^{-1}).

2.6. Calibration Curve

To establish the linearity of analytical method, a series of dilutions ranging from 1.5-7.5 µg mL^{-1} for Alprazolam and 120-600 µg mL^{-1} for Fluoxetine Hydrochloride was prepared. All the solution were filtered through 0.22 µm membrane filter and injected, the chromatograms were recorded and it was repeated for six times. A calibration graph was plotted between the mean peak area vs. respective concentration and the regression equation was derived. The correlation coefficient for Alprazolam was 0.9989 and for Fluoxetine hydrochloride 0.9994.

2.7. Preparation of mix standard

From the stock solutions, dilutions of different concentration were prepared in the ratio of 1:80 for Alp and Flx respectively and chromatogram was recorded after injecting the mix standard (Fig 1).

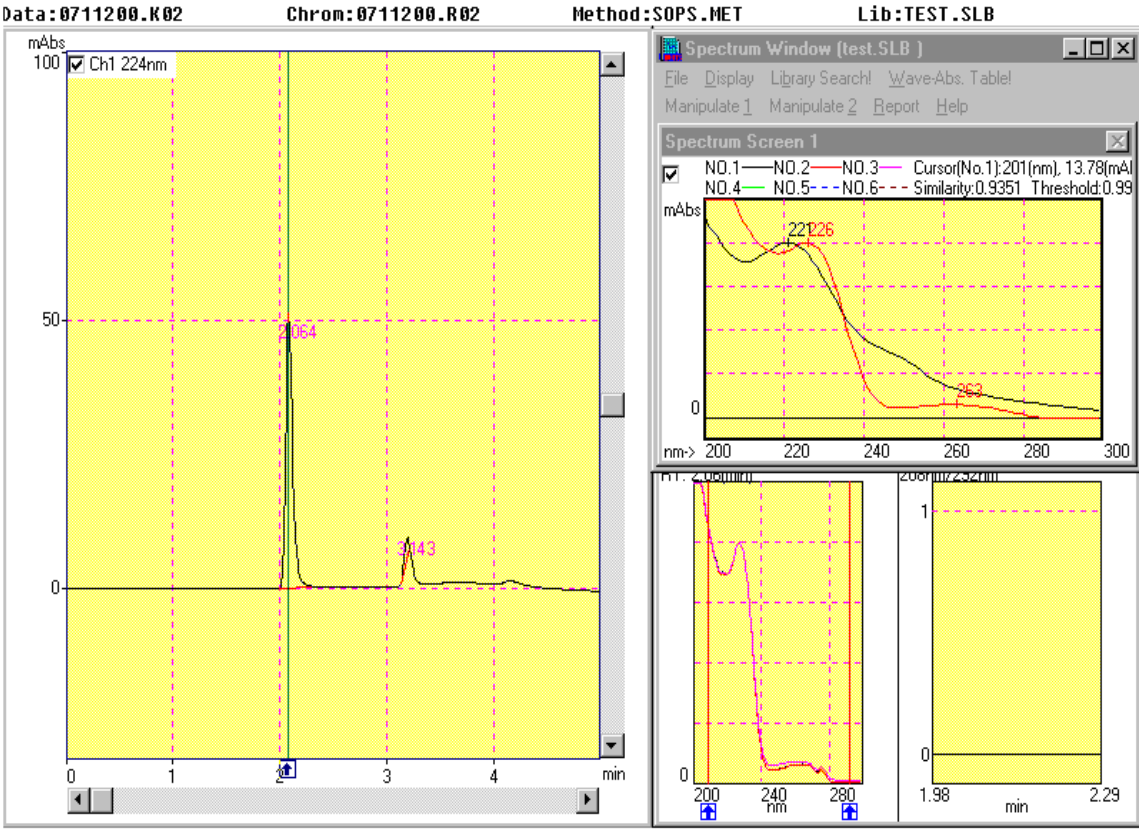

Fig:1. 2D Chromatogram of Flx (RT =2.06 min) and Alp (RT =3.14 min)

2.8. Analysis of Laboratory Sample

FIDEN-AZ (Condor Biotech Ltd, Solan) and FLUWEL (Bestochem Formulation Ltd, Delhi), each tablet was labelled to contain 0.25 mg of Alp and 20 mg of Flx. Twenty tablets were weighed accurately and grind to a fine powder. Powder with equivalent weight of 10mg Fluoxetine and 0.125 mg of Alprazolam was taken in 10ml volumetric flask. It was dissolved

in 3 mL of Acetonitrile, filtered through 0.45 µ filter and sonicated for 10 min. finally volume was made up to 10 mL with Acetonitrile. From this solution, further dilutions were made by using Acetonitrile to get a final concentration of 3 µg mL^{-1} of Alprazolam and 240 µg mL^{-1} of Fluoxetine hydrochloride. Twenty micro liters of solution was injected into HPLC system to obtain chromatogram for standard drug solution (six replicates) and sample solution (six replicates). Concentrations of Alprazolam and Fluoxetine hydrochloride in the formulation were calculated by comparing AUC of sample with that of standard.

3. Result and Discussion

To develop a precise, accurate and suitable RP- HPLC method for the simultaneous estimation of Alp and Flx, different mobile phases were tried but the proposed chromatographic conditions were found to be appropriate for the quantitative determination (Table 1). The results obtained by the assay of marketed formulation are summarized in Table 2. System suitability tests were carried out as per USP XXIV and parameters are summarized in Table 3**.**

3.1. Validation

The proposed HPLC method was validated as per ICH guidelines [17].

3.2. Linearity

Linearity was studied by preparing standard solutions at different concentration levels. The linearity range for Alp and Flx were found to be 1.5-7.5 µg mL^{-1} and 120-600 µg mL^{-1}, respectively. The regression equations for Alp and Flx were found to be y = 153775x + 9130 and y = 21814x –77807 with coefficient of correlation, (r) 0.9989 and 0.9994, respectively.

3.3. Accuracy

Recovery studies were performed to validate the accuracy of developed method. To the preanalysed sample solution, a definite concentration of standard drug was added and then its recovery was analyzed. The percent recovery for Alprazolam was found to be 99.56 % and for Fluoxetine hydrochloride it was 99.96 %.(Table 4)

3.4. Precision

3.4.1. Repeatability

Dilutions of different concentrations were prepared and triplicates of each dilution were analyzed in same day for repeatability and the results were subjected to statistical analysis. The %RSD for Alp was 0.88 and for Flx it was 0.09 which is according to ICH norms.

3.4.2. Intermediate Precision

In these, triplicates of each dilution was analyzed in different days and by different analysts. In all the condition %RSD was near to1 which shows method is precise (Table.5).

3.5. Robustness

As per ICH norms, small but deliberate variations, by altering the pH or concentration of the mobile phase or flow rate were made to check the method's capacity to remain unaffected. The method found to be unaffected by changing the pH from 2.75 to 2.9 and 2.5.

it also does not show any changes due to alteration of mobile phase ratio from ACN:Water (75:25) to ACN:Water(70:30) and (80:20).

Table 2. Result of Alp and Flx in marketed formulation (n=6).

Marketed Formulation	Drug	% Amount found ± SD	% RSD
FIDEN-AZ	Alp	99.49 ± 0.93	0.94
	Flx	99.07 ± 0.40	0.41
FLUWEL	Alp	99.79 ± 0.89	0.90
	Flx	99.78 ± 0.46	0.47

Table 3. System suitability parameters

Parameter	Alp	Flx
Linearity range (μg mL^{-1})	1.5 – 7.5	120 – 600
Correlation Coefficient (r^2)	0.9989	0.9994
Slope (m)	153775	21814
Tailing factor	1.31	0.92
No. of theoretical plates	3186	6701
Retention time(min)	3.14	2.06

Table 4. Statistical data for accuracy

Statistical data	Alprazolam	Fluoxetine Hydrochloride
% Mean	99.56	99.96
SD	0.98	0.72
%R.S.D.	0.99	0.72

Table 5. Statistical data for precision

Statistical parameter	Alprazolam		Fluoxetine Hydrochloride	
	SD	%RSD	SD	%RSD
Repeatability	0.88	0.88	0.09	0.09
Intermediate Precision				
a. Day to day	1.26	1.27	0.82	0.83
b. Analyst to Analyst	1.22	1.23	0.90	0.90

Table 6. Statistical data for Robustness

Parameters	Alprazolam	Fluoxetine Hydrochloride
Change in pH of Mobile Phase		
S.D.	0.93	0.40
% R.S.D.	0.94	0.41
Change in Ratio of Mobile Phase		
S.D.	0.42	0.88
% R.S.D.	0.43	0.90

4. Conclusion

The developed method can be used for routine analysis because the linearity found in Alprazolam and Fluoxetine Hydrochloride is nearby to 1 i.e. not a single component lies below 0.9989 which shows the good regression for linearity. As system suitability parameters are concern the %RSD of each parameter lies below the limit of 2% as per the norms of ICH. Maximum recovery obtained by this developed method and the "% relative standard deviation" for each component is not more then 0.99 %. As the precision accuracy and robustness are concern the maximum %RSD found is 1.27 %. Hence there is not unfair result shown by the validated method. So, method can be used for the routine analysis and one most important reason is that the developed method does not require use of expensive reagent and also less time consuming. Due to these reasons this method can be performed routinely in industry for analysis of drug.

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