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Analytical method development and validation for the determination of Flibanserin using reverse phase HPLC method

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A B S T R A C T

Flibanserin (Addyi) is a medication used to treat women who have not gone through menopause who have low sexual desire. Specific and simple stability indicating method was developed and validated by high-performance liquid chromatography (RP-HPLC) for the determination of Flibanserin. Separation was carried out by using a mobile phase consisting of 0.01M Potassium phosphate monohydrate buffer (KH_2PO_4) pH 3.50 buffer: Acetonitrile in the ratio of 60:40. The column used was Agilent C18, (150×4.6) mm, 5µm with a flow rate of 1.0ml/min and temperature at 30°C. The detection was carried out at 248 nm and Flibanserin was eluted at around 2.8min. This method was validated as per ICH guidelines and validation included specificity, precision, linearity, accuracy, forced degradation, and robustness. Forced degradation was conducted under the conditions of hydrolysis, oxidation, thermal, acid, base, and peroxide hydrolysis. The calibration curve was linear over the concentration range from 20 to 200µg/mL and the coefficient of determination (r^2) was observed as 0.9998. The precision and accuracy of the method were within the acceptable range. The results of this study showed that the validated method is simple and accurate, which confirmed that the method is suitable for the determination of Flibanserin.

1. Introduction

Flibanserin (Fig. 1) is a 5-HT_{1A} receptor agonist and a 5-HT_{2A} receptor antagonist that is indicated for the treatment of hypoactive sexual desire disorder (HSDD) in premenopausal women.[1] The chemical name of Flibanserin is 2H-Benzimidazol-2-one, 1, 3-dihydro-1-[2-[4-[3-(trifluoromethyl) phenyl]-1-piperazinyl] ethyl]. Its empirical formula is $C_{20}H_{21}F_3N_4O$ and its molecular weight is 390.41.[1]

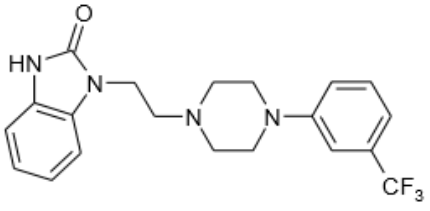

Fig. 1. Chemical structure of Flibanserin

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Flibanserin is non-hygroscopic. It has no chiral centers and therefore does not form stereoisomers. It has good aqueous solubility at acidic pH values but is practically insoluble at neutral and basic pH.[1] Flibanserin is a white to off-white powder that is insoluble in water, sparingly soluble in ethanol, methanol, toluene, and acetonitrile, soluble in acetone, freely soluble in chloroform, and very soluble in methylene chloride.[1] According to U.S. Food and Drug Administration (FDA), all pharmaceutical drugs must be tested with the stability-indicating analytical method before release. This study aims to develop and validate rapid and simple stability indicating reverse-phase high-performance liquid chromatographic method for the determination of Flibanserin. To date, many analytical techniques had been published for quantitation of Flibanserin in biological fluids[2,3,4,5,6,7,8,9] and spectrophotometric techniques were also described.[10,11,12,13] Very few methods[14,15] are reported in the literature, and no stability-indicating methods are available in the official compendia using RP-HPLC for analysis of Flibanserin. The advantages of current research work have shorter run time, information about degradation study, and complete validation performed following the international guidelines when compared with published journals.[14] In quantitative analysis, the advantages of short run time in chromatography are test results are available faster which saves time, fewer resources are used such as buffers, organic solvents, instrument utilization time and it helps in cost reduction of the test. The advantages of the forced degradation study propose shelf life of the product without real-time stability data and support identification of root cause during out of specification, out of trend, and lab investigations.

2. Results and Discussion

Flibanserin was developed and validated with RP-HPC-UV and PDA detection in a bulk product as per ICH guidelines for validation of the analytical method, Q2 (R1).

2.1 Method Development

For the estimation of Flibanserin drug content, a variety of mobile phases were tried in the development of the HPLC method. Sensitivity, pKa, and eluting efficiency were considered for selecting the mobile phase. To develop an effective stability-indicating method, different compositions of mobile phases were evaluated (Acetonitrile with 1% Phosphoric Acid, Acetonitrile with potassium phosphate monobasic pH 3.5, and Acetonitrile with Disodium Phosphate). After performing multiple chromatographic runs with several solvent mixtures, the mobile phase consisted of a mixture of 10mM Potassium Dihydrogen Phosphate buffer (pH 3.50) and Acetonitrile (60:40 v/v) was selected because it provided good symmetrical peaks.

The buffer was prepared by accurately weighing and dissolving 1.36g of KH_2PO_4 in 1000 mL of HPLC grade water and adjusting the pH to 3.50 with 85% Ortho-Phosphoric acid. A mixture of 0.01M Potassium Dihydrogen Phosphate buffer (pH 3.50) and Acetonitrile (60:40 v/v) was used as the mobile phase. The mobile phase was filtered and degassed by sonication. The chromatographic separation was achieved by using an Agilent C18, (150x4.6) mm, 5µm column. The flow rate was set at 1.0mL/min and the column temperature was set at 30°C. The UV detector wavelength was set at 248nm. The injection volume was 10µL and the total run time was 6.0min. The mobile phase was used as the sample solvent.

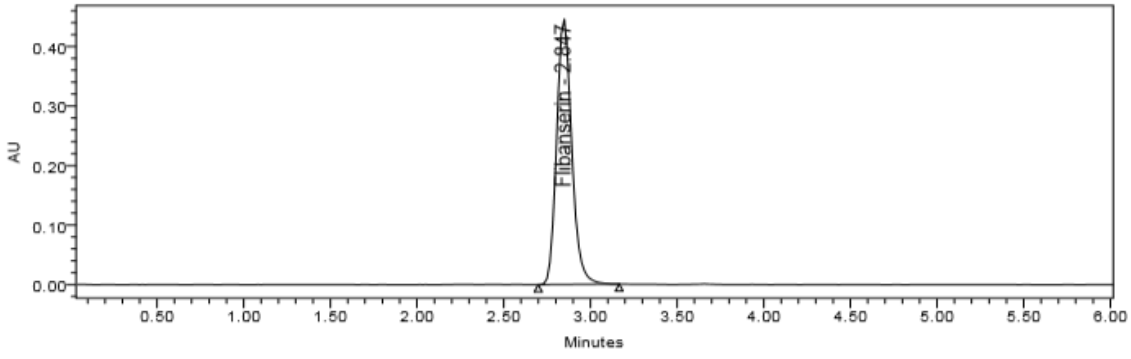


Fig.2. HPLC chromatogram of Flibanserin. Final chromatographic conditions: Agilent C18, (150 mm x 4.6 mm i.d., particle size 5µm); mobile phase: mixture of 0.01M Potassium Dihydrogen Phosphate buffer (pH 3.50) and Acetonitrile (60:40 v/v); flow rate 1.0ml/min; and UV detection at 248 nm.

2.2 Validation of the proposed method

The method was validated as per FDA and ICH guidelines. This method was validated for the following parameters: system suitability, specificity, precision, linearity, accuracy, stress study, and robustness.

2.2.1 System Suitability

The following system suitability parameters were monitored and recorded during the method validation. The retention time for Flibanserin was 2.8 min. The RSD of the Flibanserin peak area from five consecutive injections of the working standard solution was not more than 2.0%. The system suitability results met the acceptance criteria during the method validation. The results of system suitability are presented in **Table1**.

Table 1. System suitability results

Sr. No	Peak Name	RT	Area	USP Plate Count	USP Tailing
1	Flibanserin	2.845	2850690	5114	1.15
2	Flibanserin	2.846	2893873	5160	1.16
3	Flibanserin	2.846	2886119	5098	1.15
4	Flibanserin	2.847	2895628	5165	1.15
5	Flibanserin	2.849	2888667	5076	1.16
Mean			2882995		
STDEV			18462.6		
%RSD			0.6		

Limit for %RSD: Not more than 1.0 % ($n \geq 5$)
Limit for USP Tailing: Not more than 2.0
Limit for USP Plate count: >2000

2.2.2 Specificity

To ensure method specificity, the sample solvent and standard solution were injected. Peak purity was determined by the photodiode array detector. No interference or influence of the UV spectrum of the analyte was observed and therefore the method is specific for the determination of the chromatographic purity of the Flibanserin drug substance.

2.2.3 Limit of Detection and Limit of Quantitation

To determine the sensitivity of the analytical method, the LOQ and LOQ for Flibanserin were estimated based on the standard deviation of the response and the slope.

$$\text{The Limit of Detection (LOD)} = \frac{3.3 \times \sigma}{S}$$

$$\text{The Limit of Quantitation (LOQ)} = \frac{10 \times \sigma}{S}$$

where σ = the standard deviation of the response and S=the slope of the calibration curve

The results of LOD=2.45µg/ml and LOQ=7.42µg/ml

2.2.4 Precision

Method precision (intraday) and Intermediate precision (inter-day) were performed by injecting six replicates of 100µg/mL sample solution. The %RSD values for method precision and Intermediate precisions were 0.4% and 0.3% respectively. The % assay for method precision was 100.0% and intermediate precision was 100.3% which indicates that the method is suitably precise. The results of % assay and % RSD are presented in **Table 2**.

Table 2. Precision (Method & Intermediate) Results

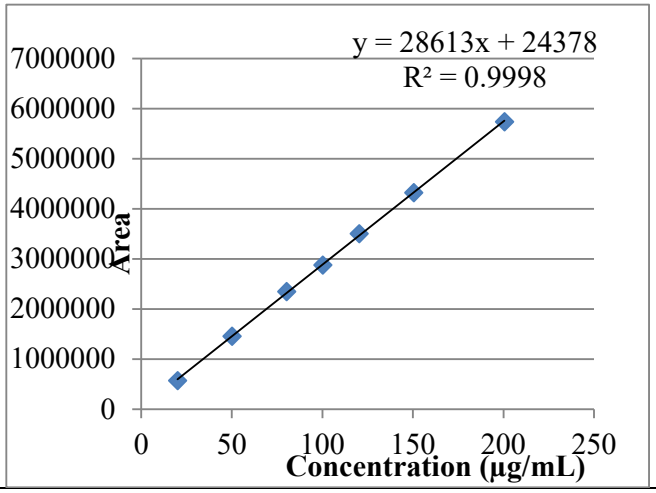
Sample	Method Precision %Assay	Intermediate Precision %Assay
1	100.1	100.2
2	99.6	100.2
3	99.6	100.4
4	100.2	100.3
5	100.1	100.9
6	100.5	99.9
Average	**100.0**	**100.3**
%RSD	**0.4**	**0.3**
Limit for % Assay	99.0%-101.0%	
Limit for %RSD	Not more than 1.0%	

2.2.5 Linearity

A linear response was observed in the concentration range from 0.02 mg/mL to 0.20 mg/mL (about 20-200% of the test concentration). A calibration curve was obtained by plotting the peak areas versus the concentration of active in solution. The equation of the calibration curve line was found to be y=28613x +24378 and the coefficient of determination (R^2) was found to be 0.9998. The linearity results are presented in **Table 3**.

Table 3. Linearity of Flibanserin

% Target Level	Flibanserin Concentration µg/ml	Average Peak Area
20%	20.052	572223
50%	50.130	1456956
80%	80.208	2348249
100%	100.260	2878952
120%	120.312	3504791
150%	150.390	4324218
200%	200.520	5740004
Slope	28613	
STEYX	26964	
Intercept	24378.3858	
R^2	**0.9998**	
Limit for R^2	0.997	



2.2.6 Accuracy

The accuracy of the method was determined by spiking Flibanserin Drug substance in triplicate at concentrations equivalent to 50%, 100%, and 150% of the target concentration. The % recovery results are shown in **Table 4** and the results demonstrated that the method has met all the acceptance criteria. Therefore, the method is accurate for the determination of Flibanserin.

Table 4. Recovery summary for Flibanserin Drug substance

Recovery Level	% Recovery	Avg % Recovery	STDEV	% RSD	Limit for % Recovery
50%	99.4	99.3	0.2082	0.21	99.0 %-101.0%
	99.5				
	99.1				
100%	99.9	99.8	0.1000	0.10	
	99.7				
	99.8				
150%	99.8	99.9	0.1000	0.10	
	100.0				
	99.9				

2.2.7 Stress Study

Stress studies were performed on Flibanserin drug substances using the conditions outlined below. A summary of the results obtained during the stress study is presented in **Table 5**. The chromatograms were recorded using a Photodiode Array Detector to determine the spectral purity of the peak.

Acid Hydrolysis: Exposure to 0.1N HCl at room temperature for 24 hours

Base Hydrolysis: Exposure to 0.1N NaOH at room temperature for 24 hours

Oxidation: Exposure to 30% hydrogen peroxide at room temperature for 5 hours

Heat/Thermal: Exposure to 80°C for 5 hours

UV Photolytic Stress: Exposure to 1200 Lux hours

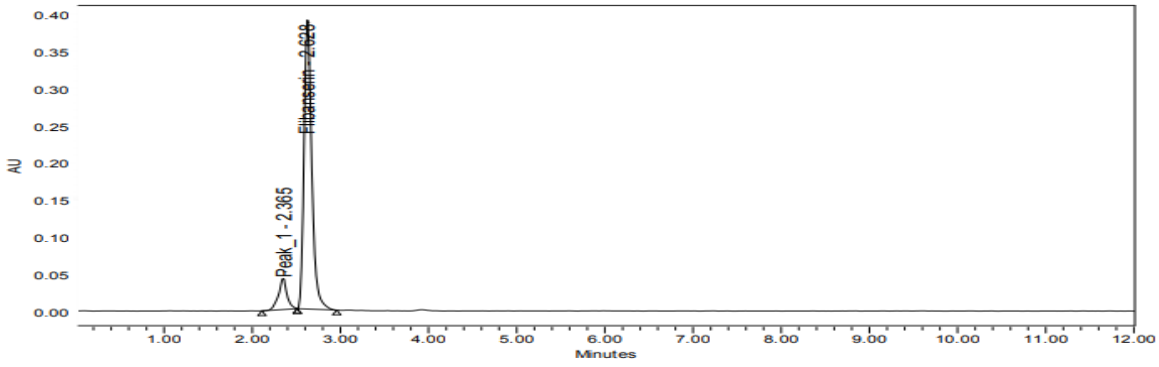


Fig. 3. Peroxide Stress Chromatogram of Flibanserin

Table 5. Results of stress study

Sr. No	Condition	Purity angle	Purity Threshold	% Assay	% Degradation
1	Unstressed	0.150	0.267	100.24	N/A
2	Acid degradation	0.102	0.264	96.71	3.52
3	Base Degradation	0.106	0.265	97.61	2.62
4	Peroxide Degradation	0.305	0.335	94.43	5.80
5	Thermal Degradation	0.344	0.410	99.74	0.50
6	UV Degradation	0.362	0.384	99.89	0.35

The results in **Table 5** demonstrate that % assay under stress conditions range from 94.43% to 100.24%. The active peak was subjected to peak purity analysis in each case, with unstressed samples serving as a control to determine spectrometric homogeneity. The results of the peak purity test are presented in **Table 5**. The active peak in all stress samples is spectroscopically pure, thus indicating that the method is specific and stability-indicating.

2.2.8 Robustness of HPLC Parameters

The robustness of HPLC parameters such as the flow rate, the column temperature, and mobile phase composition was verified. The analytical method remains unaffected by small variations in method parameters. The results of the robustness of the HPLC parameters are presented in **Table 6**.

Table 6. Results of Robustness of HPLC Parameters

Parameter	Condition	RT (min)	Peak area	Tailing Factor	Plate Count
Optimized (As per method)	Control	2.847	2888667	1.15	5123
Flow rate	0.8mL/min	3.117	2839098	1.14	4183
	1.2mL/min	2.618	2732226	1.16	4849
Mobile phase composition	5% Less	2.978	2894282	1.17	4642
	5% More	2.790	2849320	1.15	4711
Column temperature	25°C	2.858	2830762	1.17	4643
	35°C	2.785	2904403	1.15	4781

Note: RT (min), peak area, USP Plate counts, and USP Tailing factors are in average

3. Conclusion

A simple reverse phase HPLC isocratic method was developed and validated for the determination of Flibanserin Drug substance as per ICH guidelines. Method validation showed that the method is sensitive, precise, and accurate with a short analysis time. The Flibanserin drug is liable to degradation in Acid and Peroxide conditions by 3.52 and 5.80 percentages respectively. The method can be successfully applied for the routine analysis of Flibanserin in quality control laboratories.

4. Experimental

4.1 Material and chemicals

Flibanserin with purity (>99.0%), Potassium dihydrogen phosphate and Orthophosphoric acid (85%) used were of HPLC Grade, while Acetonitrile and water used were of HPLC grade used. Other chemicals used were of analytical grade throughout the study.

4.2 Instrumentation

The liquid chromatography (HPLC) system used was a Waters 2695 separation module 2996, PDA detector, which was controlled by Empower data acquisition and evaluation software. List of equipment: Electronic balance (Make: Mettler Toledo, Model: XPE205), sonicator (Make: Branson, Model: 8510), hot air oven (Make: Serve well Instruments, Model: H02436), digital pH meter (Make: Mettler Toledo) and UV-Visible chamber (Make: Mack Equipment, Model: MK-2).

4.3 Chromatographic Conditions

The isocratic separation was performed on Agilent C18, 150 mm x 4.6 mm i.d., particle size 5µm with a mixture of 0.01M Potassium Dihydrogen Phosphate buffer (pH 3.50) and Acetonitrile (60:40 v/v) as a mobile phase at a flow rate 1.0ml/min. The analysis was conducted with UV detection at 248 nm and column temperature set at 30°C. The HPLC was controlled by Empower 2 software, which is installed with data collection and acquisition.

4.4 Solutions preparation

4.4.1 Mobile phase and sample solvent (Diluent)

The Potassium phosphate buffer (pH 3.50) was prepared by accurately weighing and dissolving 1.36g of KH_2PO_4 in 1000 mL of HPLC grade water and adjusting the pH to 3.50 with 85% Ortho-Phosphoric acid. A mixture of 0.01M Potassium Dihydrogen Phosphate buffer (pH 3.50) and Acetonitrile (60:40 v/v) was used as mobile phase. The mobile phase was filtered through a 0.45µm nylon filter and degassed by sonication. The mobile phase was used as the sample solvent.

4.4.2 Preparation of Stock and Working Standard solution

The stock solution was prepared by accurately weighing 50 mg of Flibanserin into a 50 mL volumetric flask. The standard was dissolved and diluted to volume with the mobile phase. The working standard solution was prepared by transferring 5.0mL of the above stock solution into a 50mL volumetric flask and diluted to volume with the mobile phase. The solution was mixed well.

4.5 Method Validation

The method was validated as per FDA and ICH guidelines. This method was validated for the following parameters: system suitability, specificity, precision, linearity, accuracy, stress study, and robustness.

4.5.1 Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix[16]. The active peak was checked for peak purity and interference of unknown peaks.

4.5.2 Limit of Detection and Limit of Quantitation

The detection limit is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated. Quantitation limit is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

4.5.3 Precision

Precision is the degree of agreement among individual test results when an analytical method is used repeatedly to evaluate the same homogeneous sample.[16] The determination of intra-and inter-day precision was done by analyzing six sample solutions at 100% concentration (100µg/mL) (n=6) by two different chemists.

4.5.4 Linearity

The ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range.[16] The test solutions were prepared at concentrations ranging

from 0.02-0.2mg/mL (about 20-200% of test concentration). The solutions were injected in triplicate. The slope and coefficient of determination were calculated.

4.5.5 Accuracy

Accuracy is the measure of how close the experimental value is to the true value.[17] It was evaluated at three different concentrations ranging from 50% to 150% of the sample concentration. The recovery of spiked drug amounts was determined.

4.5.6 Robustness

Robustness is the capacity of a method to remain unaffected by small, deliberate variations in method parameters (e.g., Flow, Organic composition, and column temperature variation). It is a measure of the reliability of a method.[16]

4.5.7 Stress Study

The stability of a drug substance is a critical parameter that may affect purity, potency, and safety. To monitor possible changes in a product over some time, the analytical method must be stability-indicating.[18] The experimental stress conditions are described below.

4.5.8 Acidic Stress

The drug substance was dissolved in 10.0 mL of 0.1N HCl and kept at room temperature for 24 hours. Then the sample solution was neutralized with 10.0 mL of 0.1N NaOH and the sample was diluted to 50mL with sample solvent and mixed. Further dilution was performed by adding 5.0ml of the solution to 50ml of sample solvent and mixing well.

4.5.9 Alkaline Stress

The drug substance was dissolved in 10.0 mL of 0.1N NaOH and kept at room temperature for 24 hours. Then the sample solution was neutralized with 10.0 mL of 0.1N HCl and the sample was diluted to 50mL with sample solvent and mixed. Further dilution was performed by adding 5.0ml of the solution to 50ml of sample solvent and mixing well.

4.5.10 Oxidative Stress

The sample was dissolved in 2.0 mL of 30% Hydrogen Peroxide and kept on the benchtop for 5 hours, then diluted to 50mL with sample solvent and mixed. This sample was further diluted by adding 5.0ml to 50ml of sample solvent and mixed. For preventing excess degradation, this sample solution was injected without delay.

4.5.11 Thermal Stress

A portion sample was kept in the oven at 80°C for 5 hours. After reaching room temperature, the sample solution was prepared with a concentration of 100µg/mL in sample solvent.

4.5.12 UV Photolytic Stress

Some amount of drug substance was stressed for 1200 Lux hours under UV light and sample solution was prepared with a concentration of 100µg/mL in sample solvent.

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