DEVELOPMENT AND VALIDATION OF STABILITY INDICATING
RP-HPLC AND SPECTROPHOTOMETRY FOR SIMULTANEOUS
ESTIMATION OF SILDENAFIL CITRATE AND FLUOXETINE IN
BULK & TABLET DOSAGE FORM.

Anjani Chaudhari, Bhargav Patel and Rajashree Mashru*

Faculty of Pharmacy, G.H. Patel Building, Donor’s Plaza, The M.S. University of Baroda,
Vadodara-390 002, India.

ABSTRACT
A simple, precise, sensitive and reproducible, rapid stability indicating
RP-HPLC method for the simultaneous estimation of Sildenafil citrate
and Fluoxetine HCl by forced degradation studies has been developed.
The developed method separates degradation products. Sildenafil
citrate and Fluoxetine and their combination drug product were
exposed to acid, base, oxidation, thermal and photostability conditions.
The proposed HPLC method utilizes the Shimadzu HPLC, Chrome
Budget C18 Column (particle Size 5μm: 250 mm × 4.6 mm internal
diameter,5μm particle size using mobile phase consisting of
Acetonitrile : Potassium Dihydrogen Phosphate buffer with 30 mM
Triethylamine pH adjusted to 6 with o-phosphoric acid: Methanol
(60:30:10 v/v) in a gradient at the flow rate of 1 ml/min, with a load of
20μL. The detection was carried out at 230 nm. The retention time of Sildenafil citrate and
Fluoxetine was found to be around 7.23 min and 9.33 min, respectively. The number of
theoretical plates and tailing factor for Sildenafil citrate and Fluoxetine were NLT 3000 and
should NMT 2 respectively. The proposed method was validated according to ICH guidelines
and the results were found to be within the acceptable range. Hence, the proposed method can
be used for the routine quality control of the drugs and can also be applied to
pharmacokinetic studies. UV spectrophotometric methods namely 1) Absorption factor
method (AFM) 2) Dual wavelength method 3) "zero-crossing"first derivative
spectrophotometry were developed and validated for the quantitative determination of
Sildenafil citrate (SC) and Fluoxetine hydrochloride (FT). Different analytical performance
parameters such as linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined according to International Conference on Harmonization ICH Q2B guidelines.

**KEYWORDS:** Sildenafil citrate[SC], Fluoxetine[FT], Reverse phase HPLC, Validation, stability indicating methods.

**INTRODUCTION**

Sildenafil citrate (SC) chemically is 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl1H-pyrazolo[4,3-d]pyrimidin-5-yl)phenylsulfonyl]-4-methylpiperazine.[1], structure of SC is shown in Figure 1(b). It acts as a Vasodilator Agents and Phosphodiesterase Inhibitors.

Fluoxetine (FT) chemically is (RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine[1] as shown in Figure 1(a). It is anti depressant.

SC and FT is a combination for selective serotonin reuptake inhibitor(SSRI) has been shown to be more effective than fluoxetine alone in delaying ejaculation and providing sexual satisfaction.[1] The stability of a drug dosage form refers to the ability of a particular form to maintain its physical, chemical, therapeutic, and toxicological specification presented in the monograph on identity, strength, quality, and purity.[2,3] The stability of a drug product should ordinarily be demonstrated by its manufacturer by methods appropriate for the purpose. Obviously, a stability testing problem is never simple. Stability testing is an important part of the process of drug product development. The purpose of the stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and sun light, and enables recommendation of storage conditions, retest period and shelf life to be established.[4]
The literature survey reveals that many analytical methods are reported for the determination of SC and FT individually and in combination. The literature survey revealed spectroscopic\textsuperscript{[5–12]} and chromatographic\textsuperscript{[13–18]} methods.

But review of literature reveals that there is no any method reported till now for simultaneous estimation of Sildenafil citrate and Fluoxetine by stability indicating RP-HPLC in the fixed pharmaceutical dosage forms.

The present paper describes a simple simultaneous method for the determination of Sildenafil citrate and Fluoxetine by stability indicating reverse phase HPLC in Isocratic mode in pharmaceutical API, which could be applied especially to the determination of drugs in clinical data monitoring, and in pharmacokinetic investigations. The method would help the assay of drugs in a single run which reduces the time of analysis and does not require stability indicating power of the method was established by comparing the chromatograms obtained under optimized conditions before forced degradation with those after forced degradation via acidic, basic, oxidative, thermal, and photolytic stress conditions.\textsuperscript{[19]}

**Different UV spectrophotometric multicomponent analysis methods**

1. **Absorption factor method (AFM):** This method describes the analysis of a binary mixture where the two components x and y have overlapped spectra. Y shows interference at \( \lambda_{\text{max}} \) of x, while x shows no interference with y at another wavelength (\( \lambda_{2} \)).

Fig. 5: Zero order spectra of x and y and their mixture.

As shown in Fig. 5, the absorption spectra of x and y show severe overlap in the wavelength region of 200–400 nm. So, the absorption spectra of the standard solutions of the y with different concentrations were recorded in the wavelength range of 200–400 nm, and the average value of absorption factor was calculated.

\[
\text{absorption factor} = \frac{\Delta A_{\lambda_{1}}}{\Delta A_{\lambda_{2}}}
\]

Since the absorbance of the mixture (x + y) at \( \lambda_{2} \) nm is equal to that of pure y due to lack of contribution of x at this wavelength, the absorption of x at could be calculated using the following equation

\[ A_{x,\lambda_{1}} = A_{x,\lambda_{1}}(x + y) - \frac{\Delta A_{\lambda_{2}}}{\Delta A_{\lambda_{2}}} A_{y,\lambda_{2}}(x + y) \]

Where; \( A_{x,\lambda_{1}}(x + y) \) and \( A_{y,\lambda_{2}}(x + y) \) are the absorbance values of mixture at \( \lambda_{1} \) and \( \lambda_{2} \) respectively, and \( \frac{\Delta A_{\lambda_{2}}}{\Delta A_{\lambda_{2}}} \) is the absorption factor of pure y.
The concentrations of x and y were calculated from the corresponding regression equation obtained by plotting the absorption values of the zero order spectra, 226 at and 291 against the corresponding concentrations, respectively.\[20]\n
2. **Dual wavelength method**: Dual wavelength method "also known as two wavelengths method" facilitates analyzing a component in presence of an interfering component by measuring the absorbance difference (delta A) between two points in the mixture spectrum. In this method (Fig. 4); one of the drugs is considered as a component of interest and the other drug is considered as an interfering component and vice-versa. The basis for such method is the selection of two wavelengths where the interfering component shows the same absorbance (delta A equals zero) whereas the component of interest shows significant difference in absorbance with concentration. delta A between two points on the mixture spectra is directly proportional to the concentration of the component of interest independent of interfering component. This method was used for simultaneous determination of different drugs determination.\[20]\n
3. The first derivative zero-crossing method is the most common for conducting analytical calibration in derivative spectro photometry, its first derivative amplitude at the zero crossing of 226.78 for sildenafil and 219.64 for fluoxetine.\[20]\n
**MATERIALS AND METHODS**

**Materials and Reagents**

Methanol, Water, Acetonitrile, and o-phosphoric acid and Tri-ethyl amine used were of HPLC grade (S.d. fine chem. Ltd.) Sildenafil citrate (SC) and Fluoxetine (FT) API were obtained as a gift sample from Alembic pharmaceutical, vadodara. Tablet dosage form containing SC 100mg and FT 60mg was used as the sample during the method development process.

**Instrument**

A high - performance liquid chromatograph (Shimadzu, Kyoto, Japan) was installed of a LC20AT prominence solvent delivery system, injector which was manually operated was fixed 20-μl loop and a SPD-20A Prominanace UV-visible detector. Separation was Optimized on a Chrome Budget C18 Column (particle Size 5μm: 250 mm × 4.6 mm internal daimeter,5μm particle size Torrance USA), at an ambient temperature chromatogram data
were recorded and observed using a Spinchrom chromatographic Station® CFR Version 2.4.195 (Spinchrom Pvt. Ltd., Chennai, India).

Spectrophotometric measurements were made on a Shimadzu 1700 double beam UV-VIS spectrophotometer with a fix slit width of 1 nm coupled with computer loaded with Shimadzu UV PC software (UV probe) version 2.31.

**Mobile Phase Preparation**

Mixture of ACN: Potassium Dihydrogen Phosphate buffer with 30 mM triethylamine pH adjusted to 6 with o-phosphoric acid and methanol (60:30:10 v/v) with 1.0 ml/min flow rate was selected as mobile phase. The mobile phase was filtered through a 0.2 μm Nylon membrane filter to remove any particulate matter and degassed by sonication for 10 minutes. The absorbance was taken at 230 nm. Before injecting solutions, the column was maintained at equilibrated for at least 30 min with the mobile phase flowing through the system.

**Preparation of standard solutions**

A stock solutions of SC(1000 μg/ml) and FT (1000 μg/ml) were prepared in Acetonitrile and were stored at 2-8 0C until used. Aliquots of these solutions were diluted stepwise with ACN to obtained calibration range for SC 5,10,15,20,25,30 (μg/ml) FL 3,6,9,12,15,18 (μg/ml).

**Stress Study**

All the stress decomposition studies were performed at a concentration of 100μg and 60 μg for SC and FT respectively in mobile phase. Acid hydrolysis was performed in 1M hydrochloric acid. The study in alkaline condition was carried out in 1M sodium hydroxide. Oxidative studies were carried out in 10% hydrogen peroxide. the drug powder was exposed to dry heat at 80ºC. Samples were withdrawn at appropriate time, cooled and neutralized by adding base or acid, and subjected to RP- HPLC analysis after suitable dilution.

**Preparation of Test Solution**

Five tablets were weighed, crushed and mixed in a mortar and pestle for 10min. A portion of powder equivalent to 10mg of Sildenafil citrate and 6mg of Fluoxetine weighed and transferred to 100ml volumetric flask. 1 ml from the volumetric flask and diluted with Acetonitrile as to final concentration 10μg/ml SC and 6μg/ml FT. Then the sample solution was filtered through 0.45μm cellulose acetate filter paper.
HPLC and UV Method Validation

Linearity
The linearity was tested for the concentration range of 5,10,15,20,25,30μg/ml for SC 3,6,9,12,15,18μg/ml for FT and the calibration curve was constructed and evaluated by its correlation coefficient. The correlation coefficient (R) for all the calibration curves was consistently greater than 0.999 ±0.0004. (Figure 2.1 diagram of linearity).

Precision
Three injections of same concentration of lower, middle and highest were given on the same day, and these studies were also repeated on different days to determine interday precision.

Accuracy
Accuracy may often be expressed as % Recovery by the assay of known, added amount of analyte. It’s measure of the exactness of the analytical method. The recovery experiments were carried out in triplicate by sparking previously analyzed samples of the SC (10μg/ml) & FT (6 μg/ml) solution with three different concentrations of standards at 50%, 100% and 150%. (Table no-2).

Limit of Detection (LOD) and Limit of Quantification (LOQ)
Limit of detection was calculated by using the formula
\[
LOD = 3.3 \frac{SD}{S}
\]
SD = standard deviation of the response
S = slope of calibration curve of the analyte.
Limit of quantification was calculated by using the formula:
\[
LOQ = 10 \frac{SD}{S}
\]
SD = standard deviation of the response
S= slope of calibration curve of the analyte.

Robustness
To determine robustness of the method the experimental conditions were deliberately changed. The flow rate of the mobile phase, pH of the mobile phase and mobile phase composition was varied parameters. The study was performed on same day. The area obtained from each variation was compared with that obtained under optimized conditions.
RESULTS AND DISCUSSION

2.1 OVERLAY GRAPH

Figure: 2.2 Linearity of Sildenafil and Fluoxetine Hplc Data

Table 1: Method Validation

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>HPLC</th>
<th>METHOD-1</th>
<th>METHOD-2</th>
<th>METHOD-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC</td>
<td>FT</td>
<td>SC</td>
<td>FT</td>
</tr>
<tr>
<td>$\lambda_{\text{MAX}}$</td>
<td>230</td>
<td>230</td>
<td>226</td>
<td>291</td>
</tr>
<tr>
<td>RANGE(µg/ml)</td>
<td>5-30</td>
<td>3-18</td>
<td>10-30</td>
<td>6-18</td>
</tr>
<tr>
<td>LOD(µg/ml)</td>
<td>0.020</td>
<td>0.016</td>
<td>0.263</td>
<td>0.163</td>
</tr>
<tr>
<td>LOQ(µg/ml)</td>
<td>0.712</td>
<td>0.621</td>
<td>0.683</td>
<td>0.583</td>
</tr>
<tr>
<td>REGRESSION</td>
<td>0.999</td>
<td>0.999</td>
<td>0.997</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Results of recovery study by HPLC and UV methods.

Table 2: Recovery study by HPLC and UV methods.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>DRUG</th>
<th>AMOUNT PRESENT.(µg/ml)</th>
<th>AMOUNT ADD.(µg/ml)</th>
<th>AMOUNT FOUND(µg/ml)</th>
<th>% RECOVERY</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>SC</td>
<td>10</td>
<td>5</td>
<td>14.981</td>
<td>99.83</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>19.925</td>
<td>98.34</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>24.963</td>
<td>99.45</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>6</td>
<td>3</td>
<td>8.984</td>
<td>99.98</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>11.977</td>
<td>99.32</td>
<td>1.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9</td>
<td>14.894</td>
<td>98.32</td>
<td>1.15</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Assay of dosage form by HPLC and UV methods

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>DRUG</th>
<th>LABEL CLAIMED(mg/tab)</th>
<th>AMOUNT FOUND(mg/tab)</th>
<th>% Label claimed</th>
<th>SD</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>SC</td>
<td>100</td>
<td>98.79</td>
<td>99.79</td>
<td>1.58</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>60</td>
<td>59.63</td>
<td>99.38</td>
<td>1.78</td>
<td>1.78</td>
</tr>
<tr>
<td>METHOD-1</td>
<td>SC</td>
<td>100</td>
<td>99.78</td>
<td>99.78</td>
<td>1.83</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>60</td>
<td>58.25</td>
<td>97.08</td>
<td>1.25</td>
<td>1.38</td>
</tr>
<tr>
<td>METHOD-2</td>
<td>SC</td>
<td>100</td>
<td>99.56</td>
<td>99.56</td>
<td>1.35</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>60</td>
<td>59.32</td>
<td>98.86</td>
<td>1.45</td>
<td>1.65</td>
</tr>
<tr>
<td>METHOD-3</td>
<td>SC</td>
<td>100</td>
<td>98.73</td>
<td>98.73</td>
<td>2.05</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>FT</td>
<td>60</td>
<td>59.72</td>
<td>99.53</td>
<td>2.12</td>
<td>1.37</td>
</tr>
</tbody>
</table>

- The product name mentioned below used as metrix and the drug was spiked in 10 and 6 µg/ml of SC and FT respectively and re-analysis results shows 100 % recovery ,that indicate if such sample would have been adultrated with this drug it would have been detected.results shows below:

Table 1: spiking studies

<table>
<thead>
<tr>
<th>PRODUCT NAME</th>
<th>SPIKING AMOUNT(µg/ml)</th>
<th>AMOUNT FOUND (µg/ml)</th>
<th>% OF RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>RED BULL</td>
<td>SC-10</td>
<td>9.98</td>
<td>99.98</td>
</tr>
<tr>
<td></td>
<td>FT-6</td>
<td>5.99</td>
<td>99.99</td>
</tr>
<tr>
<td>SUPRADYN</td>
<td>SC-10</td>
<td>9.97</td>
<td>99.78</td>
</tr>
<tr>
<td></td>
<td>FT-6</td>
<td>5.68</td>
<td>98.78</td>
</tr>
<tr>
<td>303 CAPSULES</td>
<td>SC-10</td>
<td>9.79</td>
<td>99.75</td>
</tr>
<tr>
<td></td>
<td>FT-6</td>
<td>5.87</td>
<td>97.25</td>
</tr>
</tbody>
</table>
Stability Studies

Sildenafil and fluoxetine acid degradation sample

Sildenafil and fluoxetine base degradation sample

Sildenafil and fluoxetine oxidative degradation sample.
Table-5: Summary of Stability study

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>% OF DEGRADATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC</td>
</tr>
<tr>
<td>ACIDIC</td>
<td>1.13</td>
</tr>
<tr>
<td>BASIC</td>
<td>2.23</td>
</tr>
<tr>
<td>OXIDATION</td>
<td>1.25</td>
</tr>
<tr>
<td>PHOTO STABILITY</td>
<td>0</td>
</tr>
</tbody>
</table>

CONCLUSION

The reported RP-HPLC method was proved to be simple, rapid, and reproducible. The validation data indicate good precision, accuracy and reliability of the method. The developed method is applied by spiking Sildenafil 10 ppm Fluoxetine 6 ppm to market samples of 303 capsules, red bull and supradyn tablet and reanalysed results shows nearity 100 % recovery that indicate if such sample were adultrated with sildenafil and fluoxetine they can also be analysed and this is an added advantage of this method.

REFERENCES

20. Rubesh kumar, Duganath N, Kiran CH, Sridhar c, Simultaneous estimation of Fluoxetine HCl and Olanzapine in bulk drug and pharmaceutical formulation by using UV-Visible

21. European Journal of Pharmacy and Medical Science, A REVIEW ON UV SPECTROPHOTOMETRIC METHODS FOR SIMULTANEOUS MULTICOMPONENT ANALYSIS ISSN 3294-3211 EJPM.