Development And Validation of Rp-Hplc Methods for Simultaneous Estimation of Sildenafil Citrate and Tramadol Hydrochloride in Synthetic Mixture

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Abstract: It is simple, sensitive and accurate RP-HPLC method was developed for simultaneous estimation for Sildenafil citrate and Tramadol hydrochloride. A reversed-phase high-performance liquid chromatography method is developed and validated for the determination of both drugs with the help of RP-HPLC it gives us to good resolution and better separation for the both drugs. The separation was conducted by using Shim pack C18 column (250 mm×4.6 mm×5 µm) with mobile phase consisting Acetonitrile: Methanol: Water (5:75:20 v/v/v) Ph 4.5 Orthophosphoric acid. The mobile phase was delivered at the flow rate of 1.0 ml/min. The eluent was monitored at wavelength 274 nm and found shorter retention time of Drug and peak shape was proper, resolution good of Sildenafil citrate and Tramadol hydrochloride were found to be 6.17 min and 9.05 min respectively. The method was validated for linearity, accuracy, precision, system suitability, and stability. The method was found to be linear over the concentration range for SIL was 5-25 µg/ml and for TRA was 10-50 µg/ml with coefficient R² for SIL 0.9991 and TRA 0.9996. Therefore, proposed method can be successfully used for routine analysis of Sildenafil citrate and Tramadol hydrochloride in bulk as well as synthetic mixture.

Keywords: Sildenafil citrate (SIL), Tramadol hydrochloride (TRA), Reversed-Phase-High performance Liquid Chromatography (RP-HPLC).

1. INTRODUCTION
Sildenafil was the first phosphodiesterase-5 (PDE5) inhibitor approved for use in erectile dysfunction by US Food and Drug Administration on March 27, 1998. Method for determination of sildenafil citrate official methods. Sildenafil is a selective inhibitor of cGMP-specific phosphodiesterase (PDE-5). Penile erection involves relaxation of the corpus cavernosum, an event mediated by NO and cGMP. The biological actions of cGMP are terminated by phosphodiesterase enzymes and PDE-5 is the major cGMP metabolizing enzyme in this tissue. Tramadol is a centrally-acting opioid agonist and SNRI (serotonin/norepinephrine reuptake inhibitor) used for the management of moderate to severe pain in adults. It is considered a class IV drug by the FDA in July 7th, 2014. Method for determination of tramadol hydrochloride official methods. Tramadol is an opioid and, like other opioids, selectively bind to different opiate receptors in the central nervous system. The liver enzyme, CYP2D6, converts tramadol to its active metabolite M1, which has a stronger affinity for the μ receptor compared to the inactive form. Tramadol does not bind to the μ receptor as much as morphine. Unlike other opioids, tramadol does not reverse its course completely after the administration of naloxone. Along with the partial agonist activity on the opioid receptors, it also inhibits the reuptake of serotonin and norepinephrine.

![Figure 1: Structure formula of SIL](image1.png)  
![Figure 2: Structure formula of TRA](image2.png)

2. Material and methods
2.1 Instrument
- Instrument use for the development and validation for SIL and TRA are RP-HPLC (LC-20 AD from Shimadzu Lab).

2.2 Reagents and Material
- Sildenafil Citrate API (Globela Pharma Pvt.Ltd)
- Tramadol Hydrochloride (Globela Pharma Pvt.Ltd)/Methanol HPLC grade (Finar)
- Acetonitrile HPLC grade (Rankem)
- Double distilled Water
- Orthophosphoric acid (Chemthink Lab)

2.3 Preparation of Standard Solutions
- Standard solution of Sildenafil Citrate (SIL)
Preparation of stock solution of SIL (1000 μg/ml)
Accurately weighed quantity of Sildenafil Citrate 10 mg was transferred to 10 ml volumetric flask, add some methanol and sonicate for 10min and diluted up to the mark with methanol to give a stock solution having strength of 1000μg/ml.

Preparation of stock solution of SIL (100 μg/ml)
Aliquot of 1 ml from above standard stock solution was pipette out into 10 ml of volumetric flask and diluted up to the mark with methanol to give a stock solution having strength of 100μg/ml.

Standard solution of Tramadol Hydrochloride (TRA)
Preparation of stock solution of TRA (1000 μg/ml)
Accurately weighed quantity of Tramadol Hydrochloride 10 mg was transferred to 10 ml volumetric flask, dissolved and diluted up to mark with methanol to give a stock solution having strength of 1000μg/ml.

Preparation of stock solution of TRA (100 μg/ml)
Aliquot of 1 ml from above standard stock solution and transferred to 10 ml of volumetric flask and diluted up to the mark with methanol to give a stock solution having strength of 100μg/ml.

Preparation of standard mixture solution:
From the above standard stock solution(100μg/ml) of TRA take 3 ml and from stock solution of SIL take 1.5 ml and transferred in to 10ml volumetric flask and diluted up to mark with methanol to give a solution having strength of SIL was 15 μg/ml and TRA was 30 μg/ml.

Preparation of test solution
Take synthetic mixture equivalent to 50 mg SIL and 100 mg TRA in 100ml volumetric flask and add methanol up to the mark give solution strength (500, 1000μg/ml) and sonicate for 10min. Take 1ml from above solution and transferred in 10ml volumetric flask and make the volume up to mark with methanol give solution strength (50, 100 μg/ml). Take again 3 ml from above solution and transferred in 10ml volumetric flask and diluted up to the mark so, final Concentration of SIL was 15 μg/ml and TRA was 30 μg/ml.

2.4 Procedure for Determination of Wavelength for Measurement
1 ml of stock solution of SIL (100 μg/ml) and 1 ml of stock solution of TRA (100 μg/ml) were pipette out into two separate 10 ml volumetric flasks and volume was adjusted to the mark with methanol to get 10μg/ml of SIL and 10 μg/ml of TRA. Each solution was scanned between 200-800 nm against methanol as a blank reagent. The spectrum of each solution was obtained. The finalized detection wavelength was 274 nm. It was selected based overlay spectra of TRA and SIL as both drugs were showing good absorbance.

2.5 Chromatographic condition
Column: Shim pack ODS C18 column (250 mm x 4.6 mm, 5 μm)
Mobile phase: Acetonitrile: Methanol: Water (5:75:20 v/v/v) pH 4.5 with Orthophosphoric acid.
Flow Rate: 1 mL/min
Run Time: 15 min
Volume of Injection: 10 μl
Detection of Wavelength: 274 nm

Result and Discussion
3.1 Linearity:
The concentration range of SIL and TRA was in the range of 5-25 μg/ml and 10-50 μg/ml respectively. The regression coefficient of determination is 0.9991 and 9996 for SIL and TRA respectively.
FIGURE 3: Overlays Chromatogram of SIL (5-25 µg/ml) and TRA (10-50 µg/ml)

Table 1: linearity of SIL (5-25 µg/ml)

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Concentration (µg/ml)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0.83</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1.40</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>1.27</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>0.64</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>1.19</td>
</tr>
</tbody>
</table>

FIGURE 4: Calibration curve of SIL

Table 2: linearity of TRA (10-50 µg/ml)

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Concentration (µg/ml)</th>
<th>Mean peak area ± S.D. (n=6)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>83946.00±704.95</td>
<td>0.84</td>
</tr>
</tbody>
</table>
3.2 Precision

Precision was evaluated by injecting 3 replicate injections of sildenafil citrate and tramadol hydrochloride of sample solution under the same chromatographic conditions and calculated by the % RSD. The intraday and Interday precision study were conducted for both sildenafil citrate and tramadol HCl. The % RSD indicates that the developed method is repeatable. The results are shown in Table 3 & 4. Both inter-day and intra-day R.S.D. were less than 2 %, indicating a sufficient precision of the developed method.

**Table 3: Intra day**

<table>
<thead>
<tr>
<th>Conc. (μg/ml)</th>
<th>Mean peak area ±SD</th>
<th>% RSD</th>
<th>Mean peak area ±SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIL</td>
<td>TRA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>48003.33±219.55</td>
<td>0.46</td>
<td>83981.00±271.83</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>153244.33±1214.91</td>
<td>0.79</td>
<td>237930.00±2078.21</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>241516.67±1327.68</td>
<td>0.55</td>
<td>402169.33±1694.96</td>
</tr>
</tbody>
</table>

**Table 4: Inter day**

<table>
<thead>
<tr>
<th>Conc. (μg/ml)</th>
<th>Mean peak area ±SD</th>
<th>% RSD</th>
<th>Mean peak area ±SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIL</td>
<td>TRA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>48375.00±378.10</td>
<td>0.78</td>
<td>84419.33±411.37</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>154609.67±1721.28</td>
<td>1.11</td>
<td>240336.33±1296.34</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>242703.33±2496.47</td>
<td>1.03</td>
<td>404627.67±4907.67</td>
</tr>
</tbody>
</table>

**Repeatability:**

The Solution of SIL and TRA containing 15 μg/ml and 30 μg/ml respectively and same solution were analyzed seven times. The % RSD was found to be 0.98% for SIL and 1.18% for TRA. These %RSD value was found to be less than ±2.0 indicated that the method is precise.
### 3.3 Accuracy
In order to judge the quality and applicability of method the recovery analysis was performed at three levels 50 %, 100 %, and 150 % by standard addition method. The % recoveries for Sildenafil citrate and Tramadol Hydrochloride were calculated and it was found to be within the limits; the results are given in Table 6.

<table>
<thead>
<tr>
<th>Level</th>
<th>Conc. of SIL from Synthetic mixture (µg/ml)</th>
<th>Amount of Std. SIL added (µg/ml)</th>
<th>Total amount of SIL (µg/ml)</th>
<th>Total amount of SIL Recovered (µg/ml) Mean ± SD</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>10</td>
<td>5</td>
<td>15</td>
<td>15.06 ± 0.01</td>
<td>100.37 %</td>
</tr>
<tr>
<td>100%</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>20.18 ± 0.26</td>
<td>100.89 %</td>
</tr>
<tr>
<td>150%</td>
<td>10</td>
<td>15</td>
<td>25</td>
<td>24.79 ± 0.09</td>
<td>99.14 %</td>
</tr>
</tbody>
</table>

### 3.4 Robustness:
The small deliberate change in HPLC conditions were used for determines robustness. In this method two changes were measured for both SIL 15 µg/ml and TRA 30 µg/ml. Effect of flow rate 0.9 ml/min, 1.1 ml/min and effect of change in detection wavelength 272 nm and 276 nm was observed.

### 3.5 LOD (Limit of Detection) and LOQ (Limit of Quantification)
The Limit of detection (LOD) and Limit of Quantification (LOQ) of the developed method was calculated from the five-calibration curve.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Change in condition</th>
<th>Mean Peak Area ± S.D. (n=3)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SIL</td>
<td>TRA</td>
<td>SIL</td>
</tr>
<tr>
<td>Change in detection wavelength</td>
<td>272 nm</td>
<td>152193.0±241.60</td>
<td>235529.7±2593.85</td>
</tr>
<tr>
<td></td>
<td>276 nm</td>
<td>156774.0±751.84</td>
<td>238099.7±2306.62</td>
</tr>
</tbody>
</table>
The LOD and LOQ were calculated by using this formula.

\[
LOD = 3.3 \times \frac{\sigma}{\text{Slope}}
\]

\[
LOQ = 10 \times \frac{\sigma}{\text{Slope}}
\]

Where, \( \sigma \) = standard deviation of intercept of 6 calibration curves
\( \text{Slope} \) = the mean slope of the 6 calibration curves

The obtained LOD and LOQ results are presented in Table 8.

<table>
<thead>
<tr>
<th>SIL (µg/ml)</th>
<th>TRA (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>0.53</td>
</tr>
<tr>
<td>LOQ</td>
<td>1.61</td>
</tr>
</tbody>
</table>

4. Assay of Synthetic mixture
Preparation for synthetic mixture
Take synthetic mixture equivalent to 50 mg SIL and 100 mg TRA in 100ml volumetric flask and add methanol up to the mark give solution strength (500, 1000µg/ml) and sonicate for 10min. Take 1ml from above solution and transferred in 10ml volumetric flask and make the volume up to mark with methanol give solution strength (50, 100 µg/ml).

<table>
<thead>
<tr>
<th>Sr.no</th>
<th>Ingredient</th>
<th>Quantity (mg)</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sildenafil Citrate</td>
<td>50</td>
<td>API</td>
</tr>
<tr>
<td>2</td>
<td>Tramadol Hydrochloride</td>
<td>100</td>
<td>API</td>
</tr>
<tr>
<td>3</td>
<td>Microcrystalline cellulose</td>
<td>20</td>
<td>Disintegrate</td>
</tr>
<tr>
<td>4</td>
<td>Hydroxypropyl methylcellulose</td>
<td>15</td>
<td>Binder</td>
</tr>
<tr>
<td>5</td>
<td>Lactose monohydrate</td>
<td>45</td>
<td>Diluent</td>
</tr>
<tr>
<td>6</td>
<td>Magnesium stearate</td>
<td>10</td>
<td>Lubricant</td>
</tr>
<tr>
<td>7</td>
<td>Talc</td>
<td>10</td>
<td>Glidant</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>250</td>
<td></td>
</tr>
</tbody>
</table>

5. Conclusion
- In RP-HPLC method, linearity was observed in the concentration range of 5-25 µg/ml for SIL and 10-50 µg/ml for TRA the regression coefficient (R2) was found to be 0.9991 and 0.9996 for SIL and TRA at 247 nm respectively.
- Limit of detection for SIL and TRA was found to be 0.53 µg/ml and 1.29 µg/ml and limit of quantification for SIL and TRA was found to be 1.61 µg/ml and 3.90 µg/ml respectively.
- The % assay was found to be 99.98 % w/w and 100.35 % w/w for SIL and TRA respectively. Further % R.S.D. was found to be less than 2% for precision, repeatability, intraday and Interday study.
- The % recovery for SIL and TRA were found to be 99.14-100.89 and 99.93-101.18% respectively.
- Thus, overall result obtained for both drugs suggested that all proposed methods are specific for estimation of SIL and SIL.
- So, the develop method is accurate, sensitive, and precise.
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