Development and validation of new analytical method for the simultaneous estimation of amitriptyline and perphenazine in bulk and pharmaceutical dosage form by RP-HPLC

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ABSTRACT
A new, simple, precise, accurate and reproducible RP-HPLC method for simultaneous estimation of Amitriptyline and Perphenazine in bulk and pharmaceutical formulations was developed. Separation of Amitriptyline and Perphenazine was successfully achieved on Inertsil ODS (250x4.6mm) 5µm column in an isocratic mode utilizing Methanol: ACN: Water (50:30:20) at a flow rate of 1.0 ml/min and eluents were monitored at 253nm with a retention time of 2.440 and 5.503 minutes for Amitriptyline and Perphenazine respectively. The method was validated and it was found to be linear. The values of the correlation coefficient were found to be 0.992 for Amitriptyline and 0.9992 for Perphenazine respectively. The LOD for Perphenazine and Amitriptyline were found to be and 33.8µg/ml and 4.2 µg/ml. The LOQ for Perphenazine and Amitriptyline were found to be 20.88µg/ml and 12.12µg/ml respectively. The percentage recoveries for Amitriptyline and Perphenazine were found to be within the limit indicates that the proposed method is highly accurate. The method was extensively validated according to ICH guidelines.

Keywords: Amitriptyline, Perphenazine, RP-HPLC

INTRODUCTION
Analytical methods
The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias[1]. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs[2].

Figure 1: Amitriptyline

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International
Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behaviour. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients.

**MATERIALS AND METHOD**

**Apparatus**

The instrument used for the study was Shimadzu (LC20) HPLC, Separation module 2695, UV detector with Spin chrome software version 2.

**Reagents and Materials**

The solvents used were Methanol, Acetonitrile, Potassium dihydrogen ortho phosphate, Dipotassium hydrogen phosphate, Tri Ethyl Amine and HPLC Water.

**Selection of detection wavelength**

The sensitivity of method that uses UV-Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected.

Standard solutions of Amitriptyline and Perphenazine were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlay spectrum was recorded. From the overlay spectrum, 253 nm was selected as the detection wavelength for the present study.

**Selection of mobile phase**

Initially the mobile phase tried was Methanol and water, Methanol, Acetonitrile and water in various proportions. Finally, the mobile phase was optimized to Methanol: ACN: Water (50:30:20) v/v respectively.

Chromatographic trials for Simultaneous Estimation of Amitriptyline and Perphenazine by RP- HPLC.

**Trial 1: Chromatographic conditions**

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Methanol:ACN:Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Analytical(Hyperchrom) ODS</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
</tr>
<tr>
<td>Ratio</td>
<td>50:10:40</td>
</tr>
<tr>
<td>Column</td>
<td>Inertsil ODS 3V (250×4.6× 5µ)</td>
</tr>
<tr>
<td>Wavelength</td>
<td>253 nm</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml/min</td>
</tr>
</tbody>
</table>

These are polycyclic aromatic compounds containing a phenothiazine moiety, which is a linear tricyclic system that consists of a two benzene rings joined by a para-thiazine ring. It acts by binding to the dopamine D1 and dopamine D2 receptors and inhibits their activity. The mechanism of the antiemetic effect is due predominantly to blockage of the dopamine D2 neurotransmitter receptors in the chemoreceptor trigger zone and vomiting centre. Perphenazine also binds the alpha adrenergic receptor. This receptor’s action is mediated by association with G proteins that activate a phosphatidylinositol-calcium second messenger system.

**Ami**

**triptyl**

**Perphenazine**

This compound belongs to the class of organic compounds known as phenothiazines.

Figure 2: Perphenazine

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behaviour. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients.

Ami**triptyl**ne hydrochloride is a dibenzocycloheptene-derivative tricyclic antidepressant (TCA). TCAs are structurally similar to phenothiazine. They contain a tricyclic ring system with an alkyl amine substituent on the central ring. In non-depressed individuals, amitriptyline does not affect mood or arousal, but may cause sedation. In depressed individuals, amitriptyline exerts a positive effect on mood. TCAs are potent inhibitors of serotonin and norepinephrine reuptake. Tertiary amine TCAs, such as amitriptyline are more potent inhibitors of serotonin reuptake than secondary amine TCAs, such as nortriptyline.

Perphenazine is an antipsychotic phenothiazine derivative with actions and uses similar to those of chlorpromazine. This compound belongs to the class of organic compounds known as phenothiazines.

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Preparation of mixed standard solution

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10 ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Amitriptyline and Perphenazine is prepared by diluting 1 ml to 10 ml with mobile phase. This solution is used for recording chromatogram.

Observation: The Efficiency was not satisfactory for Perphenazine and peak response of Amitriptyline was very less. Hence it was not taken for optimization.
Trial-2: Chromatographic conditions

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Methanol: ACN: Phosphate buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.5</td>
</tr>
<tr>
<td>Ratio</td>
<td>50:30:20</td>
</tr>
<tr>
<td>Column</td>
<td>Inertsil ODS 3V (250×4.6×5µ)</td>
</tr>
<tr>
<td>Wavelength</td>
<td>253nm</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml/min</td>
</tr>
</tbody>
</table>

Preparation of mixed standard solution

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 μg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.
**Observation:** Efficiency of both the drugs was good. The run time is very more. The peaks of Amitriptyline and Perphenazine showed tailing. Hence it was not taken for optimization.

**Trial- 3: Chromatographic conditions**
- Mobile phase: Phosphate buffer : ACN : Methanol
- pH: 4.0
- Ratio : 30:30:40
- Column : Inertsil ODS 3V, (250×4.6× 5µ)
- Wavelength : 253nm
- Flow rate : 1ml/min

**Preparation of mixed standard solution**
Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

**Observation:** Asymmetry factor for Perphenazine does not meet the system suitability requirements.
### Table 3: Showing accuracy results for Amitriptyline

<table>
<thead>
<tr>
<th>Recovery level</th>
<th>Amount taken (µg/ml)</th>
<th>Area</th>
<th>Average Area</th>
<th>Amount recovered (mcg/ml)</th>
<th>% Recovery</th>
<th>Average % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>240</td>
<td>4662.113</td>
<td>4671.027</td>
<td>254.21</td>
<td>99.30</td>
<td>100.023</td>
</tr>
<tr>
<td>100%</td>
<td>320</td>
<td>5595.271</td>
<td>5589.477</td>
<td>321.69</td>
<td>100.53</td>
<td>100.023</td>
</tr>
<tr>
<td>120%</td>
<td>400</td>
<td>6296.468</td>
<td>6313.257</td>
<td>384.94</td>
<td>100.24</td>
<td>100.023</td>
</tr>
</tbody>
</table>

### Table 4: Showing accuracy results for Perphenazine

<table>
<thead>
<tr>
<th>Recovery level</th>
<th>Amount taken (µg/ml)</th>
<th>Area</th>
<th>Average Area</th>
<th>Amount recovered (mcg/ml)</th>
<th>% Recovery</th>
<th>Average % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>45</td>
<td>558.057</td>
<td>560.812</td>
<td>48.45</td>
<td>100.94</td>
<td>100.61</td>
</tr>
<tr>
<td>100%</td>
<td>60</td>
<td>659.972</td>
<td>657.722</td>
<td>60.02</td>
<td>100.04</td>
<td>100.61</td>
</tr>
<tr>
<td>120%</td>
<td>75</td>
<td>751.964</td>
<td>755.928</td>
<td>72.64</td>
<td>100.88</td>
<td>100.61</td>
</tr>
</tbody>
</table>

### Table 5: Result of Robustness study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Amitriptyline</th>
<th>Perphenazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>Tailing factor</td>
<td>Retention time (min)</td>
</tr>
<tr>
<td>Flow Rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 ml/min</td>
<td>3.033</td>
<td>1.784</td>
</tr>
<tr>
<td>1.0 ml/min</td>
<td>2.447</td>
<td>1.710</td>
</tr>
<tr>
<td>1.2 ml/min</td>
<td>2.053</td>
<td>1.704</td>
</tr>
<tr>
<td>Wavelength</td>
<td>240nm</td>
<td>2.450</td>
</tr>
<tr>
<td></td>
<td>258nm</td>
<td>2.443</td>
</tr>
<tr>
<td></td>
<td>253nm</td>
<td>2.447</td>
</tr>
</tbody>
</table>

### Table 6: Results for method precision of amitriptyline and perphenazine

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Amitriptyline</th>
<th>Perphenazine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rt</td>
<td>Area</td>
</tr>
<tr>
<td>1</td>
<td>2.487</td>
<td>4181.754</td>
</tr>
<tr>
<td>2</td>
<td>2.450</td>
<td>4143.434</td>
</tr>
<tr>
<td>3</td>
<td>2.477</td>
<td>4162.886</td>
</tr>
<tr>
<td>4</td>
<td>2.493</td>
<td>4199.596</td>
</tr>
<tr>
<td>5</td>
<td>2.493</td>
<td>4161.196</td>
</tr>
<tr>
<td>6</td>
<td>2.460</td>
<td>4190.188</td>
</tr>
<tr>
<td>Avg</td>
<td>2.476667</td>
<td>4173.176</td>
</tr>
<tr>
<td>Stdev</td>
<td>0.018052</td>
<td>20.95232</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.728874</td>
<td>0.502071</td>
</tr>
</tbody>
</table>
The run time is 8 minutes and hence it was not taken for optimization.

**Trial 4: Chromatographic conditions**

Mobile phase: Mixed phosphate buffer: Methanol : ACN
pH : 4.5
Ratio : 30:50:20
Column : Inertsil ODS (250×4.6×5µ)
Wavelength : 253 nm
Flow rate : 1ml/min

**Preparation of mixed standard solution**

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

**Observation:** Peak Asymmetry factor for Amitriptyline and Perphenazine does not meet the system suitability requirements. The run time is very more hence it was not taken for optimization.

**Trial 5: Chromatographic conditions (Optimized Method)**

Mobile phase : METHANOL: ACN: WATER
Ratio : 50:30:20
Column : Inertsil ODS (250×4.6×5µ)
Wavelength : 253 nm
Flow rate : 1ml/min

**Preparation of mixed standard solution**

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

**Observation:** All the system suitability requirements were met. The peak Asymmetry factor was less than 2 for both Amitriptyline and Perphenazine. The efficiency was more than 2000 for both Amitriptyline and Perphenazine Resolution between two peaks >1.5. Hence this method was for optimized.

**Procedure**

**Preparation of mixed standard solution**

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

**Table 7: Results for Ruggedness**

<table>
<thead>
<tr>
<th></th>
<th>Analyst 01</th>
<th>Analyst 02</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>100%</td>
<td>99.54%</td>
</tr>
<tr>
<td>Perphenazine</td>
<td>100%</td>
<td>99.93%</td>
</tr>
</tbody>
</table>

**Table 8: LOD**

<table>
<thead>
<tr>
<th>Drug name</th>
<th>LOD(µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>33.8</td>
</tr>
<tr>
<td>Perphenazine</td>
<td>4.2</td>
</tr>
</tbody>
</table>

**Table 9: LOQ**

<table>
<thead>
<tr>
<th>Drug name</th>
<th>LOQ(µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>20.88</td>
</tr>
<tr>
<td>Perphenazine</td>
<td>12.12</td>
</tr>
</tbody>
</table>

**Tablet sample**

10 tablets (each tablet contains Amitriptyline 150 mg and Perphenazine 8 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of Amitriptyline and Perphenazine (µg/ml) were prepared by dissolving weight equivalent to 8 mg of Perphenazine and 150 mg of Amitriptyline and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 50ml with mobile phase. Further dilutions are prepared in 5 replicates of 10 µg/ml of Amitriptyline and Perphenazine was made by adding 1 ml of stock solution to 10 ml of mobile phase.

**Assay**

**Preparation of samples for Assay: Standard solution**

Weigh accurately 8 mg of Amitriptyline and 1.5 mg of Perphenazine in 25 ml of volumetric flask and
dissolve in 10ml of mobile phase and make up the volume with mobile phase. This solution contains 320 μg/ml of Amitriptyline and 60 μg/ml of Perphenazine. This solution is used for recording chromatogram.

Tablet sample

10 tablets (each tablet contains Amitriptyline 150 mg and Perphenazine 8 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solution of Amitriptyline and Perphenazine (μg/ml) was prepared by dissolving weight equivalent to 800 mg of Amitriptyline and 150mg of Perphenazine dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 25 ml with mobile phase. This solution contains 320 μg/ml of Amitriptyline and 60 μg/ml of Perphenazine. This solution is used for recording chromatogram.

Calculation

The amount of Amitriptyline and Perphenazine present in the formulation by using the formula given below, and results shown in table.

Where,

\[ AS: \text{ Average peak area due to standard preparation} \]
\[ AT: \text{ Peak area due to assay preparation} \]
\[ WS: \text{ Weight of Amitriptyline/Perphenazine in mg} \]
\[ WT: \text{ Weight of sample in assay preparation} \]
\[ DT: \text{ Dilution of assay preparation} \]

RESULTS AND DISCUSSION

Method Validation Parameters

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Weigh accurately 16mg of Amitriptyline and 3 mg of Perphenazine in 50 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. This solution contains 160-480 μg/ml of Amitriptyline and 30-90 μg/ml of Perphenazine Acceptance criteria: Correlation coefficient should be not less than 0.999.

Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 160-480 ppm and 30-90 ppm for Amitriptyline and Perphenazine respectively.

Accuracy

Accuracy of the method was determined by recovery experiments. There are mainly 2 types of recovery studies are there.

Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.

Percentage method: For these assay method samples are prepared in three concentrations of 80%, 100%, and 120% respectively.

Acceptance criteria: The mean % recovery of the Amitriptyline and Perphenazine at each level should be not less than 95.0% and not more than 105.0%.

Assay procedure

10mL of the standard and sample solutions of Amitriptyline and Perphenazine were injected into the HPLC system and the chromatograms were recorded. Amount of drug present in the Tablets were calculated using the peak areas.

Precision

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions.

The % RSD of peak areas of six samples was calculated. The method precision was performed on Amitriptyline and Perphenazine formulation.

Acceptance criteria

The % RSD for the area of sample injections results should not be more than 2.

Selection of solvent

Solutions of Amitriptyline and Perphenazine were prepared by dissolving in mobile phase and UV spectrum of each was recorded by scanning between 200-400 nm.

VALIDATION OF THE METHOD

LINEARITY

Amitriptyline and Perphenazine: Serial dilutions of Amitriptyline and Perphenazine (160-480 ppm and
30-90 ppm) were injected into the column and detected at a wavelength set at 253 nm. The calibration curve was obtained by plotting the concentration vs. peak area and the correlation coefficient was found to be 0.992 and 0.992 respectively.

SUMMARY AND CONCLUSION

A new method was established for simultaneous estimation of Amitriptyline and Perphenazine by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Amitriptyline and Perphenazine by using C18 column (4.6×250mm) 5µ, flow rate was 1ml/min, mobile phase ratio was (50:30:20 v/v) Methanol: ACN: Water, detection wavelength was 253 nm. Precision and recovery studies were also found to be with the range. The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Amitriptyline and Perphenazine in pharmaceutical dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims.

Hence the suggested RP-HPLC method can be used for routine analysis of Amitriptyline and Perphenazine in API and Pharmaceutical dosage form

REFERENCES

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