New RP-HPLC Method for Simultaneous Estimation of Desvenlafaxine and Clonazepam in Tablets

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ABSTRACT
A simple, specific and precise high performance liquid chromatographic method was developed and validated for simultaneous determination of Desvenlafaxine and Clonazepam in tablets. The chromatographic separation was performed using Hypersil ODS C18 Column (4.6 × 250 mm, 5μm particle size). The mobile phase consisted of a combination of acetonitrile and 0.05 M ortho phosphate buffer (pH 4.0) in the ratio of 60:40 v/v at a flow rate of 1.2 ml/min with the detection wavelength at 225 nm. Both the drugs showed good linearity in the concentration range of 60-140μg/ml and 0.6-1.4μg/ml respectively. The correlation coefficients were obtained as 0.996 and 0.997 respectively for Desvenlafaxine and Clonazepam. The retention times for Desvenlafaxine and Clonazepam were found to be 2.687 and 3.817 min respectively. The developed analytical method was validated for linearity, precision, accuracy, ruggedness, robustness, specificity and system suitability according to ICH guidelines. The developed method can successfully be employed for routine quality control of Desvenlafaxine and Clonazepam in combined tablet dosage forms.

Keywords: HPLC, Anti-Depressant, method development, validation.

INTRODUCTION
Desvenlafaxine, 4-[2- dimethylamino-1-(1-hydroxycyclohexyl) ethyl] phenol, is an antidepressant drug [1-2] called selective serotonin and nor epinephrine reuptake inhibitor (SNRI). It blocks the transporter reuptake proteins to key neurotransmitters affecting mood, thereby leaving more active neurotransmitters in the synapse [Fig. 1]. Clonazepam, chemically, 5-(O-chloro phenyl) -1, 3-dihydro-7-nitro - 2H -1, 4-benzodiazepine- 2-one, [Fig. 2] is a benzodiazepine derivative having anxiolytic, anti convulsant, muscle relaxant and hypnotic activity used to reduce the symptoms of anxiety. [3] The literature survey revealed that several HPLC [1-5] and UV [6-8] methods have been reported for the

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simultaneous estimation of Desvenlafaxine and Clonazepam in pharmaceutical dosage forms. However, HPLC is the most commonly used analytical method for the estimation of these drugs, either individually or in combination with other drugs. The present work was intended to develop and validate a simple, sensitive analytical method with optimum chromatographic conditions for simultaneous quantification of Desvenlafaxine and Clonazepam in pharmaceutical dosage forms. The developed method was validated as per ICH guidelines \[9-11\] and can be applied successfully in routine quality control of Desvenlafaxine and Clonazepam in bulk and in dosage forms.

MATERIALS AND METHODS

Equipment
A Shimadzu LC 20-AT VP high performance liquid chromatographic instrument with spin chrome software, Hypersil ODS (250 mm × 4.6 mm; 5µ) column and manual injector was used for separation. The detection was done using an UV-VIS SPD 20A Detector.

Reagents and Chemicals
Standard samples of Desvenlafaxine and Clonazepam were obtained from SD fine chemicals, Maharashtra. The commercial tablet, Zy Ven-OD plus containing 50 mg of Desvenlafaxine and 0.5 mg of Clonazepam were employed in the study. HPLC grade acetonitrile and millipore water were purchased from Merck specialties Pvt limited Mumbai. Analytical grade sodium hydroxide and o-phosphoric acid were purchased from Finar chemicals Limited, Ahmedabad.

Chromatographic Conditions
A freshly prepared 60:40 v/v mixture of acetonitrile and 0.05 M ortho phosphate buffer (pH 4.0) was used as the mobile phase. Both acetonitrile and ortho phosphate buffer were filtered through a 0.45µm membrane filter and sonicated before use. The flow rate was adjusted to 1.2 ml/min; the injection volume was 20µl at a detection wavelength of 225 nm.

Preparation of Analytical Solutions

Preparation of Mobile Phase
A mixture of acetonitrile and 0.05 M ortho phosphate buffer (pH 4.0) in the ratio of 60:40 v/v was taken. Then the solution was filtered through 0.45µ nylon membrane filter, degassed and used as the mobile phase.

Preparation of buffer
2.9 ml of o-phosphoric acid was pipetted out into a 1000 ml volumetric flask. It was diluted with distilled water and finally made up to 1000 ml. The pH of the buffer was adjusted to 4.0 with 0.5 M sodium hydroxide and finally filtered through 0.45µm nylon membrane filter.

Desvenlafaxine standard solutions

Stock solution: 100 mg of standard Desvenlafaxine was accurately weighed and transferred to a 100 ml volumetric flask. 50 ml of mobile phase was added, sonicated for 15 minutes and cooled to room temperature. The solution was diluted and made up to 100 ml with mobile phase to get a 1000µg/ml solution.

Working standard solutions: The standard stock solution was suitably diluted with the mobile phase to obtain concentrations ranging from 0.6-1.4µg/ml of Desvenlafaxine.

Clonazepam standard solutions

Stock solution: 1 mg of standard Clonazepam was accurately weighed and transferred to 100 ml volumetric flask. 50 ml of mobile phase was added, sonicated for 15 minutes and cooled to room temperature. The solution was diluted and made up to 100 ml with the mobile phase to obtain a 10µg/ml solution.

Working standard solutions: The standard stock solution was suitably diluted with the mobile phase to obtain concentrations ranging from 0.6-1.4µg/ml of Clonazepam.

Preparation of mixed standard solutions

Mixed standard stock solution: 100 mg of Desvenlafaxine and 1 mg of Clonazepam were weighed accurately into a 100 ml volumetric flask and dissolved in 50 ml of mobile phase. The solution was finally made up to 100 ml with the mobile phase to get a concentration of 1000µg/ml of Desvenlafaxine and 10µg/ml of Clonazepam.

Mixed working standard solutions: Mixed standard stock solution was suitably diluted with the mobile phase to obtain concentrations ranging from 60-140µg/ml of Desvenlafaxine and 0.6-1.4µg/ml of Clonazepam.

Calibration of the method

Working standard solutions of Desvenlafaxine and Clonazepam in the concentration ranges of 60-140µg/ml and 0.6-1.4µg/ml respectively were taken. Five replicates of each dilution were injected into the column. The calibration curve was constructed by plotting their concentrations against their respective peak areas. The corresponding regression equation was obtained and the values of slope-a, intercept-b and correlation coefficient (R²) were determined as shown in Table 1.

Preparation of working sample solution
20 tablets were weighed accurately and crushed to fine powder. Each tablet contained 50 mg of Desvenlafaxine and 0.5 mg of Clonazepam. The quantity of powder equivalent to 100 mg of Desvenlafaxine was weighed and dissolved by using sufficient quantity of mobile phase in a 100 ml volumetric flask. The volume was made up with mobile phase to give a concentration of 1000µg/ml of Desvenlafaxine and 10µg/ml of Clonazepam. The solution was filtered through 0.45µ nylon membrane filter. The amount of Desvenlafaxine and Clonazepam present in tablet formulation was calculated by comparing the peak area of the standard with that of the sample. The amount of each drug in the tablet was calculated by using the given formula:
HPLC Method Development and Validation
The developed analytical method was validated according to ICH guidelines. Analytical variable parameters such as linearity, precision, accuracy (percent recovery), specificity and system suitability were tested using the optimized chromatographic conditions and instruments.

Linearity
Mixed working standard solutions of concentrations ranging from 60-140μg/ml of Desvenlafaxine and 0.6-1.4μg/ml of Clonazepam were injected into the column and the corresponding chromatograms were recorded. The regression equations and correlation coefficients for Desvenlafaxine and Clonazepam were found to be \( y = 483.9x + 1414.1; 0.997 \) and \( y = 50.49x + 122; 0.998 \) respectively. The results were shown in Table 1 and Figs. 3, 4 & 5.

![UV overlain absorption plot of Desvenlafaxine and Clonazepam](image)

![Calibration curve for Desvenlafaxine](image)

![Calibration curve for Clonazepam](image)

**Accuracy (percent Recovery)**
The accuracy study was performed on 80 %, 100 % and 120 % of the analytical method target concentrations of Desvenlafaxine and Clonazepam. Standard and sample preparations were injected into HPLC system and three determinants for each concentration level were obtained. The percentage recoveries of Desvenlafaxine and Clonazepam were calculated using standard at the same concentration at each concentration level as shown in Table 2.

**Precision**

**System Precision:** System precision was checked by injecting five replicate preparations of the standard drug solutions of Desvenlafaxine (80μg/ml) and Clonazepam (0.8μg/ml). The corresponding peak areas were measured and % RSD calculated.

**Method Precision:** The method precision study was performed for five replicate sample preparations of marketed formulation containing Desvenlafaxine (80μg/ml) and Clonazepam (0.8μg/ml). The corresponding peak areas were measured and % RSD calculated as exhibited in Table 3.

**Robustness**
Robustness of the developed analytical method was tested by evaluating the affect of small variations in analytical method parameters such as change in flow rate from 1.2 ml/min by ±0.2 ml/min and change in wavelength by ±2 nm. The chromatograms were recorded and the results are shown in Table 4.

**Ruggedness**
Ruggedness was determined by injecting the standard and sample solutions into two different instruments by different analysts. The retention times and peak areas were obtained. The mean and % RSD were found to be within the acceptance criteria as shown in Table 5.

**Limit of Detection (LOD) and Limit of Quantification (LOQ)**
The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). LOD and LOQ were calculated as \( 3\sigma/S \) and \( 10\sigma/S \) respectively as per ICH guidelines, where \( \sigma \) is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot. The results are presented in Table 1.

**Specificity**
The specificity was determined to check whether there is any interference due to presence of excipients, impurities or other components with the retention times of the analytical peaks. The HPLC chromatograms were recorded for the drug-matrix (mixture of the drug and excipient) which showed almost no interfering peaks within retention time ranges.

**System suitability**
Five replicates of mixed working standard solutions were injected and the parameters like theoretical plate number (N), tailing factor (K) and resolution were calculated to check the system suitability. The results are presented in Table 1.
Table 1: Optimized chromatographic conditions of the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Desvenlafaxine</th>
<th>Clonazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration range (µg/ml)</td>
<td>60-140</td>
<td>0.6-1.4</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>2.687</td>
<td>3.817</td>
</tr>
<tr>
<td>Slope</td>
<td>483.9</td>
<td>50.49</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>+141.4</td>
<td>+122</td>
</tr>
<tr>
<td>LOD</td>
<td>0.22</td>
<td>0.02</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.65</td>
<td>0.06</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.997</td>
<td>0.998</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>4331</td>
<td>3586</td>
</tr>
<tr>
<td>Symmetry factor</td>
<td>1.09</td>
<td>1.51</td>
</tr>
<tr>
<td>Resolution</td>
<td>3.80</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Recovery studies and Assay results of the proposed method

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount of standard drug added to pre-analyzed formulation (µg/ml)</th>
<th>Amount recovered</th>
<th>Mean % recovery ± S.D (n=3)</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desvenlafaxine</td>
<td>20</td>
<td>18.96</td>
<td>99.15 ± 0.92</td>
<td>99.81</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>18.62</td>
<td>99.15 ± 0.92</td>
<td>99.81</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>0.2</td>
<td>0.20</td>
<td>99.64 ± 0.67</td>
<td>100.20</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.20</td>
<td>99.64 ± 0.67</td>
<td>100.20</td>
</tr>
</tbody>
</table>

Table 3: System and Method Precision of the proposed method

<table>
<thead>
<tr>
<th>Drug</th>
<th>Speck level (µg/ml)</th>
<th>System precision</th>
<th>Method precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=3)</td>
<td>S.D % R.S.D</td>
<td>S.D % R.S.D</td>
</tr>
<tr>
<td>Desvenlafaxine</td>
<td>80</td>
<td>4.87</td>
<td>0.17</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>0.8</td>
<td>1.693</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Table 4: Robustness of the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adjusted to</th>
<th>RT (min)</th>
<th>Peak Area (mV²/min)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Desvenlafaxine</td>
<td>Clonazepam</td>
<td>Desvenlafaxine</td>
<td>Clonazepam</td>
<td></td>
</tr>
<tr>
<td>Flow rate (mL/min)</td>
<td>1.0</td>
<td>3.230</td>
<td>4.573</td>
<td>3100.676</td>
<td>282.165</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>2.683</td>
<td>3.803</td>
<td>2826.021</td>
<td>270.189</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>2.313</td>
<td>3.270</td>
<td>2172.152</td>
<td>210.660</td>
<td></td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>223</td>
<td>2.683</td>
<td>3.803</td>
<td>2587.734</td>
<td>264.478</td>
<td></td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>2.683</td>
<td>3.803</td>
<td>2826.021</td>
<td>270.189</td>
<td></td>
</tr>
<tr>
<td></td>
<td>227</td>
<td>2.680</td>
<td>3.797</td>
<td>2449.827</td>
<td>224.713</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Ruggedness of the proposed method

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter</th>
<th>Desvenlafaxine</th>
<th>Clonazepam</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Analyst - 01</td>
<td>99.02% w/w</td>
<td>104.32% w/w</td>
<td>90-110%w/w</td>
</tr>
<tr>
<td>2</td>
<td>Analyst - 02</td>
<td>100.27% w/w</td>
<td>99.06% w/w</td>
<td></td>
</tr>
</tbody>
</table>
| 3     | Acceptance     | 185

RESULTS AND DISCUSSION

The present study was aimed to develop a more sensitive, precise and accurate method for simultaneous estimation of Desvenlafaxine and Clonazepam in tablet dosage forms by RP-HPLC. A Hypersil ODS C18 Column (4.6 × 250 mm, 5µm) was chosen as the stationary phase for the separation and determination of Desvenlafaxine and Clonazepam. For optimization of the mobile phase, various mixtures consisting of acetonitrile, methanol and 0.05 M ortho phosphate buffer were examined at different ratios. The choice of the optimum composition is based on chromatographic response factor. A composition of 60:40 v/v of acetonitrile and 0.05 M ortho phosphate buffer provided an efficient separation of Desvenlafaxine and Clonazepam with sufficient retention times. The injection volume was set to 20µL and the Variable Wavelength Detector was set at 225 nm. The run time was 10 min. A flow rate of 1.2 ml/min was found to be optimum from the studied range 0.5-2.0 ml/min, which gave optimum retention time, base line stability and noise. The retention times were found to be 2.687 and 3.817 min respectively for Desvenlafaxine and Clonazepam.

The chromatograms for the validation studies were recorded and shown in Fig. 6-9. The quantitative estimation gave a satisfactory result for both Desvenlafaxine (99.81% w/w) and Clonazepam (100.2% w/w), as shown in Table 2. The linear dynamic range was 60-140µg/ml and 0.8-1.4µg/ml for Desvenlafaxine and Clonazepam respectively. The regression equations and correlation coefficients for Desvenlafaxine and Clonazepam were found to be y = 483.9x+1414.1; 0.997 and y = 50.49x+122; 0.998 respectively. The results were exhibited in Table 1. The recovery study was performed on 80%, 100% and 120% of the target concentrations of Desvenlafaxine and Clonazepam sample preparations. The percentage recoveries for Desvenlafaxine and Clonazepam were found to be 99.05% and 99.68% respectively as a mean % recovery of all determinants at three concentration levels as shown in Table 2, which indicates that the method is accurate and the commonly used excipients present in the tablet formulation did not interfere with the retention times of the analytical peaks. The precision of the method was determined from the peak areas of five homogeneous sample preparations. The % RSDs for system and method precision were reported as 0.17% (system), 0.34% (method) and 0.69% (system), 1.25% (method) respectively for Desvenlafaxine and Clonazepam as shown in Table 3, indicating that the method is quite precise.

The limit of detection and the limit of quantification for Desvenlafaxine and Clonazepam were found to be 0.22; 0.65µg/ml and 0.02; 0.06µg/ml respectively. The optimum HPLC conditions like detection wavelength, ratio of mobile phase and flow rate set for the proposed method have been slightly modified as a means to evaluate the robustness of the method. The results indicated that the selected factors remained unaffected by small variations in these quantities, as shown in Table 4. The result of ruggedness was found to be 99.02-100.27% for Desvenlafaxine and 99.06-104.32% for Clonazepam, which indicate the reproducibility of the developed method as presented in Table 5. The absence of additional peaks in the chromatograms showed non-interference of the common excipients used in the tablets indicating that the method is specific for the drugs under study. System suitability results such as theoretical plates, tailing factor and resolution were observed and found to be 4331 & 3586 (theoretical plates), 1.09 & 1.51 (tailing factor) and 3.80 (resolution) respectively for Desvenlafaxine and Clonazepam.
Fig. 6: Chromatogram for Mixed standard solution

Fig. 7: Chromatogram for Working sample solution

Fig. 8: Chromatogram for Specificity

Fig. 9: Chromatogram for System suitability
The proposed analytical method was developed and validated for system suitability, linearity, specificity, accuracy, robustness and ruggedness. All parameters tested were found to be within the acceptance limits of ICH guidelines. The proposed method was applied for the determination of Desvenlafaxine and Clonazepam in marketed formulation. The assay results confirm with the label claim of formulation. Hence, the proposed HPLC method is sensitive and reproducible for the routine analysis of Desvenlafaxine and Clonazepam in combined tablet dosage forms.

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