Development and Validation of RP-HPLC method for Lorazepam in Tablet Dosage Form

Dammalapati Srikantha and Rudra Raju Ramesh Raju

1Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar-522 510, Guntur, Andhra Pradesh, India

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Abstract

A reversed phase-high performance liquid chromatography (RP-HPLC) method was developed for determination of Lorazepam in the tablet dosage form. The developed method was validated by measuring the parameters such as linearity range, precision, limit of detection (LOD), robustness, ruggedness, drug recovery and for the system suitability. In the HPLC method, acetonitrile: methanol (65:35 v/v) was the mobile phase, C_{18} column (250 mm x 4.5 mm x 5 µm), pH 4.3 and detection wavelength was 224 nm. The measured retention time of Lorazepam was found to be 4.68 minutes, which is the shortest time compared to the values reported so far. The limit of detection was 0.3µg/ml and the linearity was found to be in the range 10-100 µg/ml. The correlation coefficient value was 0.9998 and a low relative standard deviation (RSD < 1%) was obtained for linearity, precision and robustness. From the above observed parameters it can be concluded that the developed method satisfies to be a powerful tool in determination of Lorazepam drug in tablet dosage forms.

Keywords
Lorazepam; RP-HPLC; UV detection; Retention time; Tablet dosage form.

INTRODUCTION

In this modern world, there have been instances and reports of ever increasing stress, anxiety, sleep disorders and neurological disorders. Benzodiazepines have been in wide use to treat these disorders, which act on the central nervous system (CNS) through gamma-amino butyric acid (GABAₐ) receptors^{1-3}. Of the family of benzodiazepines, Lorazepam was an anticonvulsant/sedative and hypnotics drug. It was mainly used for the treatment of severe anxiety and status epilepticus^{3}. Its systematic (IUPAC) name is (RS)-7-chloro-5-(2-chlorophenyl)-4-hydroxy-2, 5-diazabicyclo [5.4.0] undeca- 5, 8, 10, 12-tetraen-3-one and the chemical formula was C_{15}H_{10}Cl_{2}N_{2}O_{2}. It has poor solubility in water, high protein binding (85-90%) and has a half-life of 6-12 hours. Because of these characteristics, a faster release of the
drug when administered is desirable during the treatment\[^1\]. From the literature it was found that benzodiazepines were mostly analyzed in biological samples such as blood, urine and gastric content. To mention, various analytical techniques have been employed to determine the benzodiazepines in biological fluids \[^2, 3\]. These methods include gas chromatography (GC) and high performance/pressure liquid chromatography (HPLC) \[^2-6\], GC-mass spectrometry (MS) method \[^7\], Liquid Chromatography (LC)-MS/MS method \[^8\] and Voltammetry \[^9\]. However, now-a-days, HPLC has become the most chosen method compared to the other methods because of its versatility and cost effectiveness. In particular, Lorazepam has been determined in the samples of urine\[^7\], saliva \[^8\] and blood \[^10-12\]. Pharmacokinetic and pharmacodynamic analysis of Lorazepam was reported \[^13-15\]. In addition to the chromatographic methods \[^15-20\] other methods which have been developed include fluorescence immunoassay \[^21\], luminescence method \[^22\] and micellar electrokinetic capillary chromatography \[^23\], for determination of Lorazepam. In this paper, we present the development of a method for estimation of Lorazepam and carry out its validation based on RP-HPLC, by checking various parameters such as linearity, precision, robustness, ruggedness, limit of detection, and system stability. The reported retention time for Lorazepam was one of the fastest among the reported results without adding buffer to the mobile phase to the best of our knowledge.

**MATERIALS AND METHODS**

**Chemicals and Reagents**
All the chemicals and reagents used were of HPLC grade for the presented study. Methanol, water (pH between 5 and 8) and acetonitrile were procured from Merck Specialties private Ltd., Mumbai, India.

**Instrumentation**
The HPLC system employed for the current study was from PEAK HPLC operated in isocratic mode. It was equipped with a LC 20AT pump and variable wavelength programmable UV-Visible detector (SPD-10AVP). A Chromosil C18 column (250 x 4.6 mm, 5 μm) with a 20 μL Hamilton syringe was used for injecting the samples. Degassing of the mobile phase was done by using an ultrasonic bath sonicator (Loba). A Denver (SI234) balance was used for weighing the materials. Chromatograms were recorded and integrated on PEAK software. The obtained data were analyzed.
using Microsoft Excel software. A UV-Visible spectrophotometer (Techcomp UV 230D6) with HITACHI software was used for determining the wavelength of Lorazepam. The mobile phase consisted of acetonitrile: methanol in the ratio (65:35, v/v).

**Preparation of Standard Solution**
The standard (stock) solution for the present study was prepared from a 10 mg of Lorazepam drug. It was weighed and dissolved in 10 ml of methanol in a 10 ml volumetric flask. The solution was then sonicated for two minutes to dissolve the drug completely and was cooled. Then it was filtered through a 0.45 µm nylon membrane ultipore filter paper to obtain a 1000 µg/ml stock solution. From the above solution, 2 ml was further diluted to 20 ml to get a stock concentration of 100 µg/ml solution. From the stock solution required concentrations were prepared by selective dilution.

**METHOD DEVELOPMENT**

**Optimization of Chromatographic conditions**
The RP-HPLC conditions like mobile phase composition, flow rate, wavelength were optimized for a sharper peak and to fulfill the ICH guidelines. Firstly, mobile phase volume ratio was developed and standard organic solvents methanol and acetonitrile in pure form were tested separately as a mobile phase. From the observation, acetonitrile showed better result hence it was more in volume than methanol. Addition of methanol gave broader peak but lower theoretical plates. Then different volume ratios of acetonitrile and methanol were tried and the ratio of 65:35 gave a sharper chromatogram, high theoretical plates and low tailoring factor. After several iterations (trials) and chromatographic runs, it was concluded that acetonitrile and methanol at a pH of 4.3 resulted in a better peak symmetry and good signal to noise (S/N) ratio. The active pharmaceutical ingredient (API) concentration chosen was 60 µg/ml. This was also used as standard concentration because it was the optimum concentration from the Beer Lambert’s law obtained from the linearity range measurements. The pump pressure was noted down during the development phase and the optimum value was 5.2 MPa for the standard solution, which was dependent on the mobile phase and flow rate. The flow rate used for the reported results was 1.0 ml/min. The runtime of 10 minutes was chosen such that it was not too short or too long while checking for the interferences from the excipients. The optimum conditions...
obtained during the method development and that were used for validation of various parameters were: mobile phase - acetonitrile:methanol (65:35 v/v); detection wavelength - 224 nm; stationary phase column - C\textsubscript{18} column (250 mm x 4.6 mm, 5 µm); pH of the mobile phase - 4.3; API concentration - 60 µg/ml; flow rate - 1.0 ml/min; pump pressure - 5.2 ± 0.5 Mpa and runtime - 10 minutes.

**Table 1** Intraday, inter-day precision and ruggedness for Lorazepam.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration (µg/ml)</th>
<th>Normalized area Mean ± SD(^1) (n=6)</th>
<th>RSD</th>
<th>Recommended values of RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day precision</td>
<td>60</td>
<td>0.993 ± 0.007</td>
<td>0.66</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Inter-day precision</td>
<td>60</td>
<td>1.005 ± 0.005</td>
<td>0.49</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Ruggedness</td>
<td>60</td>
<td>1.013±0.011</td>
<td>1.10</td>
<td>&lt;2%</td>
</tr>
</tbody>
</table>

**METHOD VALIDATION**

The parameters described below have been validated for the developed method in accordance with the ICH guidelines\(^{[24-25]}\). The general acceptance criteria was that residual standard deviation (RSD) of peak areas should be less than 2%. Further, the system suitability was evaluated from the theoretical plate numbers and tailing factor. The theoretical plate (TP) numbers should be at least 2500 for each peak and in the present study it was 6075, a factor of 2.4 more than the recommended value. The tailing factors have to be less than 2, which was 1.37 for the presented results. For Lorazepam these conditions were fulfilled in the present study. The linearity of the peak areas was determined for 10 different concentrations of Lorazepam in the 10-100 µg/ml range. The repeatability of the sample of Lorazepam for the application was studied for six samples at 60 µg/ml concentration each. It was evaluated by comparing the RSD obtained from the peak area of the six measurements. The precision of the method was evaluated for both intraday and inter-day precision. Intraday precision was studied at 60 µg/ml of Lorazepam. For inter-day precision the same concentration was used but its peak area variation was studied for three consecutive days (Table 1).

Detection and quantification limits (LOD and LOQ) were calculated from the calibration equations obtained from the experiment. Determination of detection and
quantification limits was based on the signal to noise (S/N) ratio, repeatability and system suitability. The lowest concentration where the S/N ratio was better was chosen as the limit of detection (LOD). Then LOQ was determined from the following equation

\[ \text{LOQ} = 3.3 \times \text{LOD} \]  

Table 2 Robustness data of Lorazepam for three parameters (mobile phase ratio, flow rate and wavelength)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Peak Area (mAU)</th>
<th>% of change in peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>629853</td>
<td>---</td>
</tr>
<tr>
<td>Acetonitrile: Methanol (60:40)</td>
<td>631603</td>
<td>0.27</td>
</tr>
<tr>
<td>Acetonitrile: Methanol (70:30)</td>
<td>620178</td>
<td>1.53</td>
</tr>
<tr>
<td>Flow rate – 0.9ml/min</td>
<td>636735</td>
<td>1.09</td>
</tr>
<tr>
<td>Flow rate – 1.1ml/min</td>
<td>622376</td>
<td>1.18</td>
</tr>
<tr>
<td>Wavelength-220 nm</td>
<td>629013</td>
<td>0.13</td>
</tr>
<tr>
<td>Wavelength-228 nm</td>
<td>624972</td>
<td>0.77</td>
</tr>
</tbody>
</table>

To study the robustness of the method, deliberate changes were made to some parameters such as the mobile phase volume ratio, flow rate and wavelength. The details are given in Table 2. Lorazepam of concentration 60 µg/ml was applied to study the ruggedness of the method. The effect of these changes on the peak area was evaluated by calculating the percentage for each changed parameter.

Among the various methods to study the recovery, we used standard addition method in the present study. This was carried out on Lorazepam to which known amount of Lorazepam (standard addition method) was added. Finally, the percentage recovery of Lorazepam was compared with the actual amounts (Table 3).

RESULTS AND DISCUSSION

To determine the maximum absorption wavelength for Lorazepam spectrophotometric method was used. The wavelength was scanned in the range of 200 nm to 400 nm. The measured wavelength at maximum absorption was 224 nm. This value was in good agreement with the values reported in the literature within 10 nm corresponding to less than 5% deviation [18]. The difference was mainly due to the solvents used, instrumental conditions like resolution and the material used for cuvettes. The choice of the mobile phase was generally done by controlled trial and error method, which mainly depends on the suitability to the drug sample to be analyzed, cost-effectiveness and from the information available from the literature. In the present
work, we have chosen acetonitrile and methanol mobile phase in 65:35 v/v ratio. Fig. 1 shows the chromatogram of Lorazepam obtained after optimization of the standard solution. The measured retention time was 4.68 minutes. This value was much less than the values reported in the literature \cite{18}. This facilitates quick analysis of the sample in a shorter runtime.

**Figure 1** Chromatogram for Lorazepam (RT 4.68 min)

For Lorazepam the calibration graphs were obtained by plotting peak areas as ordinates and the corresponding ten concentrations (µg/ml) as abscissa (Fig. 2). The calibration graphs were constructed in the range 10-100 µg/ml.

From the ICH guidelines \cite{24}, validation of the analytical methods was done by fitting a linear function to the data. The linear regression was found to be precise from the correlation coefficient, $R^2 = 0.9998$. Another parameter that was widely validated for drugs under study was the repeatability of the drug application for over a period of time through the intraday and inter-day precision. The data obtained from intraday precision (on the same day) and inter-day precision (for three successive days) checks are given in Table 1.

**Figure 2** Linear regression results of concentration of the solution versus peak area of Lorazepam.

The normalization was done with respect to the area of one of the six samples. The RSD for all the samples for both intraday (0.66) and inter-day (0.49) measurements was less than 1%. From the data given it can be
concluded that the instrument has good precision and the results indicate the method was precise. Ruggeness was verified by two different analysts for the same concentration of Lorazepam. Six samples in total were analyzed. The results are given Table 1. The RSD value obtained was less than 2% indicating the ruggedness of the procedure. The limit of detection and limit of quantification were calculated based on the criteria \( \text{LOQ} = 3.3 \times \text{LOD} \). For Lorazepam, the respective value for limit of detection was 0.3µg/ml and for the limit of quantification was 1 µg/ml. These values were relatively lower than those reported in plasma and urine.

The robustness of the developed method was very important in determining the effects of variations to the instrumental parameters. This was done by deliberately changing the mobile phase volume ratio (<10%), flow rate (10%) and wavelength (<1%). The percentage change of peak areas was calculated for each changed parameter and was found to be less than 2%. These values are shown in Table 2. Though the method was robust at 10% variation of the chosen parameters; the difference between changed mobile phase compositions a factor of 5% change in peak areas was found to be a sensitive parameter. The above measurements indicate robustness of the developed method.

To check for the accuracy of the proposed method, recovery experiments were carried out by standard addition technique by adding a known amount of standard at three different levels (50%, 100% and 150%) to the sample. The analysis of each level was repeated three times \((n = 3)\). The results are presented in Table 3. Good recovery of the product in the range of 99.47% to 99.8% suggests the high accuracy of the method.

### Table 3 Recovery study results of lorazepam at three percentage levels.

| Percentage added | Target conc. 
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/ml)</td>
<td>Spiked concentration (µg/ml)</td>
<td>Final concentration (µg/ml)</td>
<td>Concentration Obtained Mean±SD</td>
<td>RSD or CV (%)</td>
<td>Recovery(%) Mean±SD</td>
</tr>
<tr>
<td>50%</td>
<td>40</td>
<td>20</td>
<td>60</td>
<td>59.73±0.97</td>
<td>2.0</td>
<td>99.57±1.65</td>
</tr>
<tr>
<td>100%</td>
<td>40</td>
<td>40</td>
<td>80</td>
<td>79.87±0.85</td>
<td>1.0</td>
<td>99.8±1.06</td>
</tr>
<tr>
<td>150%</td>
<td>40</td>
<td>60</td>
<td>100</td>
<td>99.5±0.83</td>
<td>0.8</td>
<td>99.47±0.83</td>
</tr>
</tbody>
</table>
The assay of the proposed method was applied to the determination of Lorazepam available in commercial tablets in fixed dosage form (Larpose-1 mg). The procedure was repeated three times, individually weighing the tablet in powder form each time. Twenty tablets of Lorazepam (Larpose-1 mg) were weighed and powdered. The average weight (28 mg) of the powder was noted. The powder equivalent to one mg of the drug was taken and dissolved in 10 ml of methanol. From this concentration of 100 µg/ml solution, 60 µg/ml was prepared and this was used for formulation assay studies. Assay results of Lorazepam expressed as a percentage of label claims were in good agreement within 90 to 100% of the label claims (Table 4). Only one peak in the chromatogram was observed in the drug sample, thereby suggesting that there was no interference from any of the excipients, normally present in the tablets. The low RSD showed that the method is suitable for routine analysis of the compound in pharmaceutical dosage form.

In conclusion, Lorazepam was determined in tablet dosage form using RP-HPLC method. The developed method provides a simple, precise, accurate and faster way for the determination of Lorazepam in pharmaceutical formulation. This method was validated on the basis of ICH guidelines. The retention time was 4.68 minutes, one of the fastest among the reported values in the literature. This facilitates faster analysis with reduced runtime of the sample.

Table 4 Assay of formulation of Lorazepam

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Form</th>
<th>Dosage</th>
<th>Concentration</th>
<th>Amount found</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larpose-1</td>
<td>Tablet</td>
<td>1 mg</td>
<td>60 µg/ml</td>
<td>59.35</td>
<td>98.92</td>
</tr>
</tbody>
</table>

The assayed formula was applied to the determination of Lorazepam available in commercial tablets in fixed dosage form (Larpose-1 mg). The procedure was repeated three times, individually weighing the tablet in powder form each
REFERENCES


