DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF PROPRANOLOL HCI AND CLONAZEPAM IN BULK AND PHARMACEUTICAL DOSAGE FORM

Tanikella Sai Annapurneswari1,2, Sakinala Shilpa2, Chodavarapu Bala Tripura Sundari1*, Vaidya Jayathirtha Rao2, Anisetti Ravinder Nath1

1Department of Pharmacy & Biotechnology, University College of Technology, Osmania University, Hyderabad, India
2Crop Protection Chemicals Division, CSIR- Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad, India

ABSTRACT

The present work describes a reverse phase high performance liquid chromatographic method (RP-HPLC) for the simultaneous estimation of Propranolol HCl (PRH) and Clonazepam (CNZ) in bulk and in pharmaceutical dosage form. Chromatographic separation was performed on Agilent Eclipse xdb C18 (150 mm × 4.6 mm i.d., 5 μm) column, with a mobile phase comprising of a mixture of methanol, acetonitrile and 20 mM potassium dihydrogen phosphate buffer in the ratio of 27.5:27.5:45 v/v. The pH of buffer was adjusted to 3.0 with orthophosphoric acid. The flow rate was 1.0 ml/min with detection at 266 nm. Retention times of Propranolol HCl and Clonazepam were found to be 2.400 and 4.492 min respectively. As per International Conference on Harmonisation (ICH) guidelines the method was validated for linearity, accuracy, precision, limit of quantitation, limit of detection, and robustness. Linearity of PRH was found to be in the range of 20-120 μg/mL and that for CNZ was found to be 1-6 μg/mL. The correlation coefficients were 0.9994 and 0.9995 for PRH and CNZ respectively. The mean recoveries obtained for PRH and CNZ were 100.6% and 100.1%. This demonstrates that the developed method is simple, precise, accurate, reproducible and rapid for simultaneous estimation of these drugs in bulk and in tablet dosage forms. 

Key words: Propranolol HCl, Clonazepam, RP-HPLC, Simultaneous determination, Validation.

INTRODUCTION

Propranolol hydrochloride (PRH): PRH (Figure 1) is chemically 1-naphthalen-1-yl-oxy-3-(propan-2-ylamino) propan-2-ol hydrochloride. It is a non-selective beta blocker mainly used in the treatment of hypertension. PRH is used in the treatment or prevention of many disorders including acute myocardial infarction, arrhythmias, angina pectoris, hypertension, hyperthyroidism, migraine, pheochromocytoma, menopause and anxiety.

Clonazepam (CNZ): CNZ (Figure 2) is 5-(o-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one. It is a Benzodiazepine drug which act on the brain and central nervous system to produce a calming effect. It is used to treat the panic and anxiety symptoms associated with panic disorder. Further it is also used to treat seizures, anxiety, muscle spasms and insomnia.

The combination of PRH and CNZ is useful in the treatment of pulsatile tinnitus1 and in management of chronic anxiety. Propranolol hydrochloride is official in IP2, USP3 and BP4. IP and BP describe potentiometric titration methods while USP describe liquid chromatographic method for its estimation. Clonazepam is official in IP3, BP4 and USP3, all of which describes liquid chromatographic method for its estimation. Their combination is not official in any pharmacopoeia, so no official method is available for the estimation of these two drugs in combination.

Literature survey reveals a few spectrophotometric5-12, HPLC13-16 and bioanalytical methods17-24 for the estimation of both drugs as a single component and in combination with other drugs. However, thorough literature survey revealed that there are no analytical methods reported for the analysis of these drugs in combined dosage form. The objective of the present work is to develop a highly sensitive, simple, rapid, and precise RP HPLC method for the estimation of propranolol hydrochloride and clonazepam in combined tablet dosage form.

EXPERIMENTAL:

Instrumentation:

JASCO 2080 model chromatograph equipped with an Agilent eclipse xdb-reverse phase C18 column (150 x 4.6 mm i.d: particle size 5 μm) was employed for the study. Sample injection was done with a Rheodyne 7725 injection valve via a 20 μL loop. Detection of the drug was done by using a UV-2075 detector (JASCO) and the output signal was monitored and integrated by JASCO BORWIN software. Solubility of the compound was enhanced by sonication on an ultrasonicator. A JASCO V-550 UV-Visible spectrophotometer was used to record the UV spectra of Propranolol HCl and Clonazepam combination to select the working wavelength for detection of the drugs. A Digisol Electronic analytical balance (model DI 707) was used for preparation of all samples and buffer solutions required.

Chemicals and reagents:

The reference samples of Propranolol HCl and Clonazepam were obtained from Pellets Pharma Ltd and Suraksha Pharma Pvt Ltd, Hyderabad, India. Purified water was obtained by using 0.22μ Millipore Milli-Q water purification systems. HPLC grade acetonitrile and methanol (Merck, Mumbai) were used for preparing the mobile phase and the diluent. Potassium dihydrogen orthophosphate and orthophosphoric acid are of analytical grade obtained from Sigma Aldrich. Clotas Plus-H, a commercial tablet containing a combination of CNZ (0.5 mg) and PRH (10 mg) manufactured by Tas Med (I) Pvt. Ltd., Chandigarh was purchased from local firms.

Mobile Phase: Methanol, acetonitrile and 20 mM KH₂PO₄ buffer (adjusted to pH 3.0 with orthophosphoric acid) in the ratio of 27.5:27.5:45 v/v was used for separation of these drugs. Prior to use, the mobile phase was filtered through a 0.22μ membrane filter after sonication of each solvent for 15 min.
As per the International Conference on Harmonization (ICH), Twenty tablets (CLOTAS PLUS H tabs) each containing 10 mg of propranolol hydrochloride and 0.5 mg of clonazepam were weighed and powdered. The contents were made up to the mark with methanol. Further dilutions were made from the stock solution with diluent in the required concentration range in 10 mL volumetric flasks for the calibration curve.

Preparation of standard stock solution
The stock solutions were prepared by dissolving a suitable quantity of CNZ and PRH to get the final concentration of 0.05 mg/mL. The solutions were standardized to 5 mg/mL and 1 mg/mL in standard volumetric flask and volume was made up with methanol.

Preparation of sample solution
Twenty tablets (CLOTAS PLUS H tabs) each containing 10 mg of propranolol hydrochloride and 0.5 mg of clonazepam were weighed and powdered. Tablet powder equivalent to 5 mg CNZ and 100 mg PRH was extracted with small amount of methanol in a 100 mL volumetric flask. The solutions were shaken well and allowed to stand for 15 min with intermittent sonication to ensure complete solubility of drug. The contents were filtered and supernatant was then centrifuged and supernatant was filtered using Whatman filter paper No. 41. From the filtrate, dilution was made in a 100 mL volumetric flask using methanol to get 3 μg/mL clonazepam and 60 μg/mL propranolol HCl. A 20 μL injection of the above sample was performed and chromatographed.

**METHOD VALIDATION**

As per the International Conference on Harmonization (ICH) guidelines^25-27^, the method validation parameters like linearity, precision, accuracy, limit of detection, limit of quantitation, robustness and specificity were experimentally determined and the method validated.

### Linearity and range
Series of mixed standard solutions of Propranolol HCl and Clonazepam were prepared in 10 mL volumetric flasks using diluent to get final concentration of 20-120 μg/mL of propranolol HCl and 1-6 μg/mL of clonazepam. Each of these drug solutions (20 μL) were injected into the chromatographic system for three times. The peak area and retention time were recorded and the mean values of peak areas were plotted against concentrations.

### Precision
The intra and inter day precision was determined by analyzing 60 μg/mL PRH and 3 μg/mL CNZ, six times each on same day (intra-day study). This was repeated on the second day (inter-day study).

### Accuracy
The accuracy of the method was determined by recovery studies. The recovery studies were performed by standard addition method; at 50%, 100%, 150% level for both the drugs i.e; three different levels corresponding to 30.0, 60.0 and 90.0 μg/mL for PRH and 1.5, 3.0 and 4.5 μg/mL for CNZ. The analysis was conducted in triplicate. Percentage recovery was calculated by comparing the area before and after the addition of the working standard.

### Limit of detection and limit of quantitation
The LOD and LOQ of PRH and CNZ by the proposed methods were determined on the basis of response and slope of the regression equation. LOD and LOQ values were calculated using the formula 3.3×s/S and 10×s/S, respectively, where S is the slope of the calibration curve and s is the standard deviation of y-intercept of regression equation.

### Robustness
The robustness of the developed method was determined according to ICH guidelines. Experimental conditions were deliberately altered one factor after the other. The effect of change in flow rate, buffer concentration, pH of buffer on the retention time, peak asymmetry and theoretical plate number were studied.

### System suitability
For system suitability, six replicates of the working standard sample were injected and the parameters like plate number (N), retention time, resolution and peak asymmetry of samples were calculated.

### Specificity and selectivity of the proposed method
Specificity is a procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix. Both these parameters were explored in the method development by using excipients.

**Table 1: RESULTS OF LINEARITY STUDY FROM CALIBRATION CURVE**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc.(μg/mL)</th>
<th>Equation of regression line</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRH</td>
<td>20-120</td>
<td>Y=11312x+20174</td>
<td>0.9994</td>
</tr>
<tr>
<td>CNZ</td>
<td>1-6</td>
<td>Y=41387x</td>
<td>0.9995</td>
</tr>
</tbody>
</table>

**Table 2: RESULTS OF PRECISION STUDY (n=6)**

<table>
<thead>
<tr>
<th>Drug</th>
<th>%RSD(Intraday)</th>
<th>%RSD(Interday)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol HCl</td>
<td>0.61</td>
<td>0.49</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>0.77</td>
<td>0.1</td>
</tr>
</tbody>
</table>

RSD is Relative Standard Deviation and n is the number of replicates.
Table 3: RESULTS OF ACCURACY STUDY (n=3)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Amount(%) of drug added to analyte</th>
<th>Theoretical conc. (µg/mL)</th>
<th>Measured conc. (µg/mL)</th>
<th>% Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol HCl</td>
<td>50</td>
<td>30</td>
<td>30.06</td>
<td>100.16</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>60</td>
<td>59.85</td>
<td>99.7</td>
<td>0.133</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>90</td>
<td>91.7</td>
<td>101.8</td>
<td>0.272</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>50</td>
<td>1.5</td>
<td>1.49</td>
<td>99.3</td>
<td>0.342</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.0</td>
<td>2.97</td>
<td>99.2</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>4.5</td>
<td>4.58</td>
<td>101.8</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Table 4: SYSTEM SUITABILITY PARAMETERS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PRH</th>
<th>CNZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>2.400</td>
<td>4.492</td>
</tr>
<tr>
<td>Resolution</td>
<td>-</td>
<td>9.66</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>2653</td>
<td>5248</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>1.31</td>
<td>1.09</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>1.21</td>
<td>0.75</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>3.68</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Table 5: EVALUATION OF ROBUSTNESS STUDY FOR PROPRANOLOL HCl AND CLONAZEPAM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Propranolol HCl</th>
<th>Clonazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate 0.9</td>
<td>2.66</td>
<td>4.98</td>
</tr>
<tr>
<td>Flow rate 1.1</td>
<td>2.17</td>
<td>4.07</td>
</tr>
<tr>
<td>pH 2.9</td>
<td>2.43</td>
<td>4.57</td>
</tr>
<tr>
<td>pH 3.1</td>
<td>2.40</td>
<td>4.52</td>
</tr>
<tr>
<td>Buffer conc.15mM</td>
<td>2.33</td>
<td>4.39</td>
</tr>
<tr>
<td>Buffer conc.25mM</td>
<td>2.39</td>
<td>4.39</td>
</tr>
</tbody>
</table>

Table 6: RESULTS OF ASSAY FROM TABLET DOSAGE FORM

<table>
<thead>
<tr>
<th>Drug</th>
<th>Labelled Amount (mg)</th>
<th>Amount taken for assay (µg/mL)</th>
<th>Amount found (mg)</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRH</td>
<td>10</td>
<td>60</td>
<td>59.05</td>
<td>98.4</td>
</tr>
<tr>
<td>CNZ</td>
<td>0.5</td>
<td>3</td>
<td>3.05</td>
<td>101.6</td>
</tr>
</tbody>
</table>

Figure 1: Structure of Propranolol HCl

Figure 2: Structure of Clonazepam

Figure 3: Chromatogram of standard solution of Propranolol HCl and Clonazepam
RESULTS AND DISCUSSION
The goal of this present study was aimed at developing a sensitive, precise and accurate HPLC method for the analysis of Propranolol HCl and Clonazepam in its bulk and pharmaceutical combined dosage form. In order to achieve optimum separation of the component peaks, various proportions of buffer with acetonitrile and methanol were tested as mobile phase on an Agilent xdb C18 column. Mobile phase containing a mixture of methanol, acetonitrile and buffer in the ratio of 27.5:27.5:45 v/v was selected as it resulted in peaks with good symmetry and resolution. A flow rate of 1.0 mL/min was found to be optimum in the 0.5 to 1.0 mL/min range resulting in the short retention time, baseline stability and minimum noise. With the above optimised conditions, the retention times of PRH and CNZ were found to be 2.400 min and 4.492 min respectively showing the proposed method is time saving (Figure 3). The calibration curve showed linearity in the concentration range of 20-120 μg/mL for PRH and 1-6 μg/mL for CNZ (Figures 5 and 6). The regression equations of concentration over their peak areas were found to be $y=11312x$ ($R^2=0.9994$) and $y=41387x$ ($R^2=0.9995$) for PRH and CNZ respectively where $y$ is the peak area and $x$ is concentration of PRH and CNZ (μg/mL) (Table 1). The results of intraday and interday precision values are represented in (Table 2). The RSD % for assay of drugs during intra-day and inter-day were 0.61 and 0.77 for PRH and 0.49 and 0.1 for CNZ. Assay of the two drugs using the developed method showed acceptable relative error values that are less than 2 indicating that the method is highly precise. The percentage mean recovery at three different levels of study was 100.16, 99.7 and 101.8 for PRH and 99.3, 99.2 and 101.8 for CNZ (Table 3). The percentage mean recovery of individual analyte was high, satisfactory and indicates that the proposed method is accurate. The number of theoretical plates was determined to be 2653 and 5248 for PRH and CNZ respectively which indicate the efficient performance of the column. The LOD and LOQ were found to be 1.21 μg/mL and 0.075 μg/mL; 3.68 μg/mL and 0.22 μg/mL for PRH and CNZ respectively, which indicates the high sensitivity of the method (Table 4). The excipients used in formulation did not interfere with the drug peaks and thus the method is specific. The HPLC chromatograms recorded for the drug matrix (mixture of the drug and excipients) showed almost no interfering peaks within retention time ranges. Figures 3, 4 show the representative chromatograms for standard and the formulation. The chromatograms show that the selected drugs were clearly separated and thus the proposed HPLC method is selective. In robustness study,
three factors (flow rate, pH and concentration of buffer) were deliberately altered. Under all the above conditions specified above, asymmetric factor was less than 2.0 and theoretical plates were more than 2300 for PRH and CNZ peaks, which illustrates good robustness of the developed method (Table 5). The amount of PRH and CNZ present in the sample solutions were determined by fitting the responses into the regression equations of the calibration curve for PRH and CNZ respectively and the results obtained were comparable with the corresponding label claim (Table 6).

CONCLUSION

Proposed study describes a new isocratic RP-HPLC method for the estimation of Propranolol HCl and Clonazepam in combination using simple mobile phase. The method gives good resolution between the compounds with a short analysis time. The method was validated and found to be simple, sensitive, accurate, precise and can be used for analysis of regular quality control samples.

ACKNOWLEDGEMENTS

The authors wish to thank Pellets pharma & Suraksha pharma, Hyderabad, for providing the gift samples of propranolol hydrochloride and clonazepam for this work. The authors are also thankful to the Director & Head, Crop Protection Chemicals Division, CSIR andconstant encouragement. We are grateful to Dr. B. China Raju and Mr. A.B.N Nageshwarra Rao for their most valuable advice, guidance, support and keen interest.

REFERENCES

22. Elizabeth C. Kwong and Danny D. Shen; Versatile isocratic high-performance liquid chromatographic assay for propranolol and its basic, neutral and acidic metabolites in biological fluids; Journal of Chromatography B: Biomedical Sciences and Applications; 04/1987; 414(2):365-379.

Source of support: Nil, Conflict of interest: None Declared