Development and validation of RP-HPLC-PDA method for the simultaneous estimation of clonazepam and paroxetine hydrochloride in bulk and tablet dosage forms.

Aziz Unnisa1,2*, Santosh Kumar. S1, Yogesh Babu. A1, Siva Chaitanaya. K1 and Mrudula. B1
1 KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada-520010, AP, India.
2 Sunrise University, Alwar; Rajasthan, India.

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ABSTRACT
Objective: The aim of the present study is to develop RP-HPLC-PDA method for the simultaneous estimation of Clonazepam (CLP) and Paroxetine hydrochloride (PAR). Method: The method uses a Agilent C18 reverse phase column (150 x 4.6mm, 5µm) with mobile phase consisting of 15mM Ammonium acetate : Methanol (40:60 v/v) at isocratic mode with an injection volume of 10µL and the eluents were monitored at 254nm. Results and Discussion: The retention times of CLP and PAR were 4.01 and 5.42 min respectively and showed a good linearity in the concentration range of 1-5 µg/mL for CLP and 25-125µg/mL for PAR with a good correlation coefficient for both the drugs. The validation parameters like specificity, system suitability, linearity, LOD, LOQ, precision, robustness were all within the limits as per ICH guidelines. Conclusion: The proposed RP-HPLC-PDA method is specific, accurate, precise and economic and can be successfully applied for the simultaneous estimation of CLP and PAR in bulk and tablet dosage forms.

KEYWORDS: Paroxetine hydrochloride, Clonazepam, Simultaneous estimation, RP-HPLC-PDA, Method validation.

INTRODUCTION:
Paroxetine hydrochloride (PAR) Chemically, (3S,4R)-3-[(2H-1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluoro phenyl) piperidine hydrochloride hemihydrate (fig.1) belong to a class of antidepressant agents known as selective serotonin-reuptake inhibitor (SSRIs). It is used to treat major depressive disorder (MDD). Paroxetine likely inhibits the reuptake of serotonin at the neuronal membrane, enhances serotoninergic neurotransmission by reducing turnover of the neurotransmitter, therefore it prolongs its activity at synaptic receptor sites and potentiates 5-HT in the CNS.

Clonazepam (CLP) chemically, 5-(2-chlorophenyl)-7-nitro-2,3-dihydro-1H-1,4-benzodia zepin-2-one (fig.2) belongs to the drug class benzodiazepines. It is prescribed for the treatment of anxiety and seizure disorders. Mechanism of action involves Allosteric interactions between central benzodiazepine receptors and gamma amino butyric acid (GABA) receptors potentiate the effects of GABA. As GABA is an inhibitory neurotransmitter, this results in increased inhibition of the ascending reticular activating system.

Literature survey states that Paroxetine hydrochloride and Clonazepam are official in IP, BP and USP with HPLC methods for their assay individually. Combined dosage form of Paroxetine and clonazepam is not official in any monographs. Numerous UV, Spectrophotometric, HPLC, GCMS, HPTLC, LCMS based methods have been reported for estimation of these drugs alone or in combination with other drugs in pharmaceutical dosage forms and biological fluids. Only one RP-HPLC method was reported for the estimation of Paroxetine hydrochloride and Clonazepam combination, however this method used costly and LC-MS incompatible solvents so the aim of the author was to develop an economic, efficient and LC-MS compatible method for simultaneous determination of clonazepam and paroxetine hydrochloride in bulk and tablet dosage forms.
age form. The developed method was validated in accordance with ICH guidelines.

MATERIAL AND METHODS

Materials
CLP and PAR were generously gifted by Dr. Reddy’s Laboratories, Hyderabad, India. Ammonium Acetate, water, methanol and acetonitrile of HPLC grade were used. All the other chemicals and reagents were of AR grade.

Instrumentation
A Shimadzu Prominence HPLC system provided with DGU-20A3 degasser, LC-20AD binary pumps, SIL-20AHT auto sampler, and SPD-M20A PDA detector was used. Data acquisition was carried out using LC solutions software. The chromatographic analysis was performed on Agilent C18 reverse phase column (150 x 4.6mm, 5µ).

Chromatographic Conditions
Mobile phase consisting of 15mM Ammonium acetate: methanol (40:60 v/v) was used in isocratic mode and the mobile phase was filtered through nylon disc filter of 0.45µm (Millipore) and sonicated for 3 min before use. The flow rate was set at 1.2 mL/min and the injection volume was 10 µL. PDA detection was performed at 254 nm and the separation was achieved at ambient temperature.

Preparation of stock solutions
Accurately weighed quantities (10mg each) of CLP and PAR were dissolved separately in sufficient quantity of methanol in a 10mL volumetric flask. The volume was adjusted up to the mark with methanol to obtain a stock solution of mg/mL each of CLP and PAR.

Method validation

Linearity
A linear relationship was evaluated across the range of the analytical procedure with five concentrations. A series of combination standard dilutions were prepared over a concentration range of 1-5µg/mL for CLP and 25-125µg/mL for PAR from stock solutions of both the drugs and injected on to the column in triplicate. Linearity is evaluated by a plot of peak areas as a function of analyte concentration, and the test results were evaluated by appropriate statistical methods where by slope, intercept, regression (R²) and correlation coefficient (R) were calculated and the data was given in Table 1.

Precision
Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Repeatability was assessed by using a minimum of six determinations at 100 % of the test concentration (2µg/mL of CLP and 50µg/mL of PAR). The standard deviation and the relative standard deviation (RSD) were reported for precision and the data was given in Table 2.

Accuracy
Accuracy was established across the specified range of the analytical procedure. To ascertain the accuracy of the proposed method recovery studies were performed by the standard addition method by spiking 80%, 100%, 120% of the known quantities of standards within the range of linearity to the synthetic solution of drug product (2µg/mL of CLP and 50µg/mL of PAR), these mixture solutions were analyzed by developed method in triplicate. The % recovery and the %RSD were calculated for both the drugs at each level of addition and the data was given in Table 3.

Limit of Detection and Limit of Quantification
LOD and LOQ were calculated based on calibration curves. They were expressed as LOD = (3.3 ×σ/m); LOQ= (10.0×σ/m) (Where, σ is the standard deviation of the y-intercepts of the three regression lines and m is mean of the slopes of the three calibration curves).

Robustness
To determine the robustness of the method developed, the experimental conditions were deliberately altered and the chromatographic parameters viz., capacity factor, tailing factor, no. of theoretical plates and % assay were recorded. The flow rate of the mobile phase was 1.2mL/min. To study the effect of flow rate, the flow rate was changed by ±0.2 mL/min and the effect of wavelength was studied by changing wavelength by ±1 nm and the data was given in Table 5.

System suitability
System suitability was carried out by injecting a mixed standard concentration at different injection volumes in the range of 10-50±L. The system suitability test parameters were noted and % RSD was calculated. The data was given in Table 6.

Assay
Twenty tablets were weighed and finely powdered, the powder blend equivalent to 12.5mg of PAR and 0.5 mg of CLP was accurately weighed, transferred into a 5 mL volumetric flask, dissolved in methanol, vortexed for 5 min and volume was adjusted up to the mark with methanol to get 2.5mg/mL of PAR and 0.1mg/mL of CLP. The above stock solution was centrifuged, filtered using Nylon disposable syringe filter (13 mm, 0.45 µm) and 400µL of filtrate was diluted with water to 5mL to get a solution containing 2µg/mL of CLP and 50µg/mL of PAR. The solution was injected in triplicate and analyzed. The
amount present in the each tablet was quantified by comparing the area of standard TS with that of the sample. The values were shown in Table 4.

RESULTS AND DISCUSSION

Few HPLC, UV,MS methods were published for the estimation of CLP and PAR in combination with other drugs in bulk and pharmaceutical dosage forms. However, no economic and efficient method was reported for the simultaneous estimation of CLP and PAR in bulk and tablet dosage forms. Hence, the present investigation was aimed to develop a simple, economic, specific and efficient RP-HPLC-PDA method for the simultaneous determination of CLP and PAR in bulk and in combined tablet dosage forms.

Method Development and Optimization

Mobile phase optimization was initiated by using water and methanol (50:50 v/v) at 1 mL/min flow rate using Agilent C\textsubscript{18} column (150 x 4.6 mm, 5µm). CLP eluted before solvent peak and PAR at 10.8 min. In other, trial 10 mM ammonium acetate and methanol (40:60 v/v) were used at a flow rate of 1 mL/min using Agilent C\textsubscript{18} column (150 x 4.6 mm, 5µm). CLP eluted at 4.5 min and PAR eluted at 5.21 min but asymmetrical peak was observed for PAR. In the next trial mobile phase composition of 15 mM ammonium acetate and methanol (35:65 v/v) was used at flow rate of 1 mL/min, but peaks eluted were not of good shape. Finally, good peak shapes and resolution were obtained with a mobile phase composition of 15 mM ammonium acetate and methanol (40:60 v/v) at the flow rate of 1.2 mL/min using 15 mM ammonium acetate as diluent. CLP was eluted at 4.01 min and PAR at 5.42 min, tailing factors for both the drugs were within the limits. For quantitative purpose wavelength was set at 254 nm, which provided better reproducibility with minimum or no interference. The method was validated as per ICH guidelines. The peak purity index was found to be greater than 0.9999 for CLP and PAR (fig.6. & fig.7.) respectively indicating the peak purity of the drug samples used in the analysis. The method developed shown in the following chromatogram (fig.3.)

Method validation

The method has been validated as per ICH-Guidelines for following parameters

Specificity

Specificity studies were carried for both pure drug and drug product by comparing the 3D plots (fig.4.) with blank and placebo (fig.5.). No interferences were detected at the retention times CLP and PAR in the sample solution.

![Figure 4. Overlaid base shift chromatograms of A-diluent, B-placebo, C-standard, D-sample.](image-url)

![Figure 5. 3D Image showing standard of A-diluent, B-placebo, C-sample, D-standard CLP (2µg/mL) and PAR(50µg/mL).](image-url)

![Figure 6. Peak purity curve of Clonazepam.](image-url)

![Figure 7. Peak purity curve of Paroxetine.](image-url)
The range of reliable quantification was set at the concentrations of 1-5µg/mL for CLP and 25-125µg/mL for PAR. This range was selected based on 80-120 % of the standard concentration (used for accuracy) and were analyzed in triplicate. Peak areas and concentrations were subjected to least square regression analysis to calculate regression equation. The data from the calibration curve was given in Table 1. The overlaid chromatogram was shown in the following (fig.8.)The correlation coefficient (R) was found to be 0.997 for CLP and 0.999 for PAR indicating a linear response over the ranges used.(fig.9, 10).

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### Table.3. Accuracy data of CLP and PAR.

<table>
<thead>
<tr>
<th>Level of Recovery</th>
<th>Amount (µg/mL)</th>
<th>% Recovery (Mean ± SD)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLP (%)</td>
<td>PAR (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>2</td>
<td>1.6</td>
<td>0.76</td>
</tr>
<tr>
<td>100%</td>
<td>2</td>
<td>2.5</td>
<td>0.52</td>
</tr>
<tr>
<td>120%</td>
<td>2</td>
<td>2.4</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Accuracy

Accuracy of the proposed method was ascertained by performing recovery studies by standard addition method by spiking the known quantities of standard at 80%, 100%, 120% to the mixture of drug product solution comprising of 2µg/mL of CLP and 50µg/mL of PAR, and these solutions were analyzed in triplicate in each level of addition. The %RSD and the %Recovery were within the acceptable limits in all cases. It is evident from the results of accuracy study given in Table 3, that the proposed method enables very accurate quantitative simultaneous estimation of CLP and PAR in tablet dosage form.
Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined based on statistical calculation from the calibration curves, where LOD = (3.3 ×σ/m; LOQ= (10.0×σ/m (σ is the standard deviation of the y-intercepts of the three regression lines and m is mean of the slopes of the three calibration curves). The limit of detection for CLP and PAR were found to be 0.0176µg/mL, 0.892µg/mL respectively. The limit of quantification for CLP and PAR were found to be 0.0532µg/mL, 2.512µg/mL respectively.

Assay

Assay of CLP and PAR combination formulation was performed by the proposed method and the % assay of the formulation was calculated as an average of 3 determinations, which was about 101.7±0.098 for CLP and 99.17±1.02 for PAR. These results indicate that the proposed method and the % assay of the formulation was calculated as an average of 3 determinations, which was about 101.7±0.098 for CLP and 99.17±1.02 for PAR. These results indicate that the present HPLC method can be successfully used for the analysis of CLP and PAR.

Table 4. Assay data of CLP and PAR.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>CLP Peak Area</th>
<th>% Assay</th>
<th>PAR Peak Area</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33050</td>
<td>101.8</td>
<td>58113</td>
<td>98.01</td>
</tr>
<tr>
<td>2</td>
<td>32989</td>
<td>101.6</td>
<td>59323</td>
<td>99.9</td>
</tr>
<tr>
<td>3</td>
<td>33010</td>
<td>101.7</td>
<td>59121</td>
<td>99.6</td>
</tr>
<tr>
<td>Average</td>
<td>33016.333</td>
<td>101.7</td>
<td>58852.333</td>
<td>99.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Assay (Mean ± RSD)</th>
<th>CLP</th>
<th>PAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>101.7±0.098</td>
<td>99.17±1.02</td>
<td></td>
</tr>
</tbody>
</table>

Robustness

Method robustness was determined by analysing the same sample at normal operating conditions and also by changing the operating analytical conditions like wavelength of detection and flow rate of the mobile phase. Percent assay values were also estimated under these changed conditions and the results were given in Table 5. Changes in the flow rate slightly affected the retention times of the CLP and PAR. However, the parameters like capacity factor, theoretical plate number and assay were not changed and were within the limits. Similar results were obtained with the changed wavelength. These results indicated that the method is robust in terms of changed flow rate and wavelength.

Table 5. Robustness data of CLP and PAR.

<table>
<thead>
<tr>
<th>Robustness Parameters</th>
<th>Retention Time</th>
<th>Tailing factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLP</td>
<td>PAR</td>
</tr>
<tr>
<td>253nm</td>
<td>3.834</td>
<td>4.954</td>
</tr>
<tr>
<td>254nm</td>
<td>3.844</td>
<td>4.965</td>
</tr>
<tr>
<td>255nm</td>
<td>3.856</td>
<td>4.976</td>
</tr>
<tr>
<td>1mL/min</td>
<td>4.781</td>
<td>6.384</td>
</tr>
<tr>
<td>1.2mL/min</td>
<td>3.834</td>
<td>4.954</td>
</tr>
<tr>
<td>1.4mL/min</td>
<td>3.475</td>
<td>4.954</td>
</tr>
</tbody>
</table>

Stability of the analytical solution

The stability of the stock and standard solutions were determined by analyzing the samples under refrigeration (8±1°C) at different time intervals up to 48 hrs. The % variation in assay values at different time intervals were found to be less than 2% of the initial zero time interval solution, thus indicating that the solutions were stable for a period of 48 hrs when stored at 8±1°C. The results were shown in the Table 7.

Table 7. Stability data of CLP and PAR.

<table>
<thead>
<tr>
<th>Time interval</th>
<th>% Variation in peak area</th>
<th>CLP</th>
<th>PAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hrs</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48hrs</td>
<td>0.7</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

CONCLUSION

In this work, a simple, efficient and economic RP-HPLC-PDA method has been developed for the simultaneous determination of CLP and PAR from bulk and tablet dosage form. The method was validated fully as per International Conference on Harmonisation (ICH) Guidelines, and validation acceptance criteria were met in all cases. Application of this method for simultaneous determination of CLP and PAR from tablet dosage form showed that neither the degradation products nor the excipients interfered in the estimation of both the drugs, therefore this method was simple, specific and economic can be employed successfully for the simultaneous estimation of CLP and PAR in commercial tablet dosage forms.

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