Application of Ratio Derivative Spectrophotometry for Simultaneous Determination of Clonazepam and Paroxetine Hydrochloride in Tablet Dosage Form

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ABSTRACT

Ratio derivative spectrophotometric method has been developed for the simultaneous estimation of Clonazepam and Paroxetine hydrochloride in pharmaceutical dosage form. In this method, the overlapping spectra of Clonazepam and Paroxetine hydrochloride were well resolved by making use of the first-derivative of the ratios of their direct absorption spectra. The derivative ratio absorbance of Clonazepam and Paroxetine hydrochloride were measured at 312.28 nm and 304.88 nm, respectively for their quantification. Clonazepam and Paroxetine hydrochloride were determined in the concentration range of 5-30 µg/ml and 10-60 µg/ml respectively. The linear regression analysis for the calibration plots produced r²= 0.9956 and r²= 0.9994 for Clonazepam and Paroxetine hydrochloride respectively. The precision, accuracy, limit of detection and limit of quantitation of the method were validated according to the ICH guidelines. The results indicate that the method is suitable for the routine quality control testing of marketed tablet formulations.

Keywords: Simultaneous determination, Clonazepam, Paroxetine hydrochloride, Ratio derivative spectrophotometric method
INTRODUCTION

Clonazepam (CLO) \([5-(2\text{-chlorophenyl})-7\text{-nitro-2, 3\text{-dihydro-1H-1,4-benzodiazepin-2-one}]\), [Fig. 1 (a)] is a benzodiazepine drug that has anxiolytic, anticonvulsant, muscle relaxant, sedative, and hypnotic properties. Clonazepam exerts its action by binding to the benzodiazepine site of the GABA receptors, which causes an enhancement of the electric effect of GABA binding on neurons, resulting in an increased influx of chloride ions into the neurons. These results in an inhibition of synaptic transmission across the central nervous system\(^1\text{-}^3\).

Paroxetine hydrochloride (PH) \([(-)\text{-}\text{Tr}ans\text{-}4R\text{-}(4'\text{-}fluorophenyl)\text{-}3S\text{-}[\text{3'}, 4'\text{-methyleneedioxygenoxo}]}\text{methyl], piperidine hydrochloride}, [Fig. 1 (b)] is a selective serotonin (5\text{-}HT) reuptake inhibitor (SSRI) and potentiates 5\text{-}HT in the CNS. PH is indicated for the treatment of major depressive disorder, social anxiety disorder, obsessive-compulsive disorder, panic disorder, generalized anxiety disorder, and post traumatic stress disorder\(^1\text{-}^5\). It exerts its antidepressant effect through a selective inhibition for the reuptake of the neurotransmitter serotonin by the presynaptic receptors.

Depression and anxiety disorders are distinct illnesses that often coexist. Patients with co-morbid depression and anxiety are more debilitated than patients with either condition alone. Mixed anxiety-depression is gaining recognition as a separate diagnosis and has been included in the 10\text{th} edition of the International Classification of Diseases and in the appendix of the 4\text{th} edition of the Diagnostic and Statistical Manual of Mental Disorders. Currently, a fixed dose combination of an antidepressant, such as PH, and an anti-anxiety drug, such as CLO, is an available option for the treatment of co-morbid depression and anxiety\(^5\text{-}^6\).

Scientific literature reports that CLO and PH are official in IP, USP and BP when used individually\(^1\text{-}^7\text{,}^8\), but the combination of CLO and PH is not official in any Pharmacopoeia. Various analytical methods, such as spectrophotometry \(^9\text{-}^\text{13}\), spectrofluorimetry\(^14\text{-}^15\), HPLC\(^16\text{-}^26\) and HPTLC\(^27\text{-}^30\) have been reported to detect CLO and PH alone and in combination with other drugs in pharmaceutical dosage forms. Spectrophotometric by simultaneous equation and colorimetric method\(^31\text{-}^32\), stability-indicating HPLC method\(^33\text{-}^34\), HPTLC\(^35\) and UPLC\(^36\) have been reported for the simultaneous estimation of CLO and PH in combined pharmaceutical formulations. To the best of our knowledge, there is no any method reported for resolving the spectra of both drugs without prior separation of them in a combined dosage form. Therefore, the goal of present work is to develop a simple Ratio derivative method that could be applied in quality control laboratories for the simultaneous determination of both drugs particularly when the spectra of the two drugs overlap. This work aims to present simple, accurate and precise ratio derivative spectrophotometric method for the simultaneous determination of CLO and PH in pharmaceutical dosage form.

MATERIALS AND METHOD

**Instrumentation**

A double beam UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) connected to computer loaded with spectra manager software UV Probe was employed with spectral bandwidth of 2 nm and wavelength accuracy of ±0.3 nm with a pair of 10 nm matched quartz cells was used for Spectrophotometric method. All weighing were done on electronic balance (Model Shimadzu AUW-220D). Ultrasonicator (Model 5.5 150H) was used for sample solution preparation.

**Reagents and chemicals**

Analytical pure samples of CLO and PH were procured as gratis samples from Vital Formulation and Torrent Pharmaceutical, India respectively. These samples were used without
further purification. Marketed tablet formulations of Pari-CR Plus manufactured by IPCA Laboratories, India (Label claim 0.5 mg of CLO and 12.5 mg of PH) were procured from the local market. Analytical grade methanol purchased from Merck, Mumbai was used throughout the study.

**Preparation of Standard Solutions and Calibration Curve**

Standard stock solutions each containing 1000 μg/ml of CLO and PH were prepared separately in the methanol. The working standard solutions (100 μg/ml) of mentioned drugs were obtained by dilution of the respective stock solution in methanol. For verification of Beer's law, a series of dilutions in the concentration range of 5-30 μg/ml for CLO and 10-60 μg/ml for PH were prepared separately to establish calibration curve.

**Ratio derivative Spectrophotometric method**

The method involves dividing of the absorption spectrum of mixture by the spectrum of standard solution of one of compound and first derivative of ratio spectrum is obtained, resulting spectra is dependent of concentration of divisor. The concentration of active compounds are then determined from calibration graph obtained by measuring amplitude at points corresponding to minima or maxima.

Different concentrations of CLO (5, 10, 15, 20, 25 and 30 μg/ml) and of PH (10, 20, 30, 40, 50 and 60 μg/ml) were tested as a divisor but the concentrations 10 μg/ml of CLO and 50 μg/ml of PH gave minimum noise in ratio spectra and maximum sensitivity. The wavelength increment over which the derivative is obtained (ΔΛ) was carefully tested, ΔΛ = 8 and scaling factor 20 with medium scanning speed was chosen to perform measurements to obtain minimum noise. Zero order spectra of CLO and PH of sample solution were taken [Figure 2(a) and 3(a)], spectra were divided by 50 μg/ml PH and 10 μg/ml CLO respectively [Figure 2(b) and 3(b)] and finally divided ratio spectra of CLO and PH were converted into first derivative as depicted in Fig. 2(c) and 3(c). 312.28 nm and 304.88 nm wavelength maxima (A_max) were selected for the simultaneous determination of CLO and PH in sample solutions respectively.

**Method Validation**

The methods were validated with respect to linearity, range, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision according to ICH Q2 (R1) guidelines. 37

1. **Linearity and range**

Linearity relationship between concentration and amplitude of both drugs were evaluated over the concentration range expressed in the concentrations range of 5-30 μg/ml for CLO while 10-60 μg/ml was selected for PH. The linearity ranges for the determination of CLO and PH by the proposed methods were repeated five times. Calibration plots were constructed by plotting the amplitude versus the concentration and treated using the method of ordinary least squares regression analysis.

2. **Limit of detection (LOD) and Limit of quantification (LOQ)**

The LOD and LOQ of the drugs was derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations as per ICH guideline.

Limit of detection=3.3 × σ/S

Limit of quantitation=10 × σ/S

Where, σ = Standard deviation of the response, S = Slope of calibration curve

3. **Accuracy**

Accuracy of method was ascertained by performing recovery at three levels (50%, 100% and 150%). Recovery studies for CLO and PH were carried out by spiking three different amount of both the standard to the dosage form by standard addition method. Recovery studies were performed in triplicate. The amounts of CLO and PH were estimated by applying obtained values to regression equation.

4. **Precision**
Figure 1 Chemical structure of (a) Clonazepam and (b) Paracetamol hydrochloride

Figure 2 (a) Zero order spectra of CLO (5–30 µg/ml)

Figure 2 (b) Division of the zero order spectra of CLO using PH (50 µg/ml) as divisor

Figure 2 (c) Ratio first derivative absorbance spectra of CLO (5–30 µg/ml)

Figure 3 (a) Zero order spectra of PH (10–60 µg/ml)

Figure 3 (b) Division of the zero order spectra of PH using CLO (1 µg/ml) as divisor

Figure 3 (c) Ratio first derivative absorbance spectra of PH (10–60 µg/ml)
Intraday and interday precision was studied by analyzing three replicates of standard solutions at three concentrations level.

**Analysis of marketed formulation**

For the analysis of marketed formulation, twenty tablets were weighed and finely powdered. From tablet sample, an amount equivalent to 0.5 mg of CLO and 12.5 mg PH were accurately weighed and taken into the 100 ml volumetric flask. About 30 ml of methanol was added and the mixture was sonicated for 15 minutes. The mixture was diluted to volume with methanol, mixed well and filtered to obtain the sample stock solution 5 µg/ml of CLO and 125 µg/ml of PH. The resultant solution was used for the analysis of CLO and for analysis of PH; 1 ml from the above solution was withdrawn and made up the volume up to the 10 ml to make 12.5 µg/ml for PH. The resultant solutions were then used to estimate both the drugs at their particular λ\text{max} for both methods. The analysis was repeated in triplicate.

**RESULTS AND DISCUSSION**

**Ratio derivative spectrophotometric method**

The ratio spectra of different CLO standards at increasing concentrations in methanol obtained by dividing each with the stored zero order spectrum of standard solution of PH and the first derivative of these spectra traced with the interval of Δλ= 8 nm shown in fig. 2 (a, b, c). Similarly, the ratio derivative spectra of the solutions of PH in different concentrations in methanol traced with the interval of Δλ= 8 nm by using the zero order spectra of CLO as divisor by computer aid depicted in Fig. 3 (a, b, c). Here, the standard spectra of 10 µg/ml of CLO and 50 µg/ml of PH were considered as suitable for the determination of PH and CLO respectively, as divisor. From the Fig. 2 (c) and Fig. 3(c), several amplitude peaks were observed for both drugs spectra but at 312.28 nm for CLO and 304.88 nm for PH showed minimum noise and best recovery.

**Method validation**

Validation of the methods has been performed according to ICH recommendations.

1. **Linearity**

The calibration range for CLO and PH was established through considerations of the practical range necessary according to Beer–Lambert’s law. The linearity response was determined by analyzing 6 independent levels of concentrations in the range of 5-30 µg/ml and 10-60 µg/ml at 312.28 nm for CLO and 304.88 nm for PH respectively. The values of correlation coefficients of CLO and PH were close to unity indicating good linearity, the characteristic parameters for the constructed equations are summarized in Table 1.

2. **Limit of detection (LOD) and Limit of quantification (LOQ)**

The limit of detection and limit of quantitation were determined based on the standard deviation of response (y-intercept) and slope of the calibration curve according to ICH guideline. LOD and LOQ for CLO were found to be 0.087 µg/ml and 0.265 µg/ml and for PH were found to be 1.310 µg/ml and 3.971 µg/ml respectively as tabulated in Table 1.

3. **Accuracy**

Accuracy of the method was assured by applying the spiking method where good percentage recoveries were obtained, confirming the accuracy of the proposed method (Table 2). The recovery studies were carried out by adding known amount of standard to samples at 50, 100 and 150% level and analyzed by the proposed method, in triplicate.

4. **Precision**

The intraday and interday precision were determined by the analysis of three different concentrations of CLO and PH, within the linearity range, by three replicate analyses of three pure samples of both drugs on a single day and on three consecutive days respectively. As indicated in Table 3, data showed % RSD less than 2%. The precision of
Table 1 Linear regression parameters of CLO and PH for Ratio derivative method

\(n = 3\) concentration/3 replicates, SD = standard deviation, \(\alpha\) confidence interval at 95% confidence level and 5 degree of freedom \((t=2.571)\)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CLO</th>
<th>PH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg/ml)</td>
<td>5-36 µg/ml</td>
<td>10-60 µg/ml</td>
</tr>
<tr>
<td>Correlation Co efficient ((r^2))</td>
<td>0.9958</td>
<td>0.9992</td>
</tr>
<tr>
<td>Slope ± SD</td>
<td>0.721 ± 0.0080</td>
<td>0.059 ± 0.0008</td>
</tr>
<tr>
<td>Confidence limit of slope</td>
<td>0.654 to 0.798</td>
<td>0.057 to 0.061</td>
</tr>
<tr>
<td>Intercept ± SD</td>
<td>-1.963 ± 0.065</td>
<td>0.164 ± 0.029</td>
</tr>
<tr>
<td>Confidence limit of intercept</td>
<td>-3.277 to -0.679</td>
<td>0.072 to 0.246</td>
</tr>
<tr>
<td>Limit of detection (µg/ml)</td>
<td>0.087</td>
<td>1.310</td>
</tr>
<tr>
<td>Limit of quantification (µg/ml)</td>
<td>0.265</td>
<td>3.971</td>
</tr>
</tbody>
</table>

Table 2 Recovery study for CLO and PH by proposed method

\(n = 3\) concentration/3 replicates, SD = standard deviation, % RSD = relative standard deviation

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Taken (µg/ml)</th>
<th>% Level</th>
<th>Amount of std added (µg/ml)</th>
<th>Total amount of drug Found (µg/ml)</th>
<th>% Recovery ± SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLO</td>
<td>5</td>
<td>50%</td>
<td>2.5</td>
<td>7.521</td>
<td>100.28 ± 0.051</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100%</td>
<td>5.0</td>
<td>10.101</td>
<td>101.01 ± 0.030</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150%</td>
<td>7.5</td>
<td>12.521</td>
<td>101.69 ± 0.051</td>
<td>0.051</td>
</tr>
<tr>
<td>PH</td>
<td>15</td>
<td>50%</td>
<td>7.5</td>
<td>12.610</td>
<td>101.43 ± 0.069</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100%</td>
<td>15.0</td>
<td>30.132</td>
<td>100.44 ± 0.119</td>
<td>0.118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150%</td>
<td>22.5</td>
<td>37.891</td>
<td>101.04 ± 0.140</td>
<td>0.139</td>
</tr>
</tbody>
</table>
Table 3 Precision study
(n=3 concentration/3 replicates, SD = standard deviation, % RSD = relative standard deviation)

<table>
<thead>
<tr>
<th>Amount of drug (µg/ml)</th>
<th>Intraday precision</th>
<th>Interday precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of drug found ± SD (µg/ml)</td>
<td>%RSD</td>
</tr>
<tr>
<td>CLO</td>
<td>10  10.103 ± 0.028</td>
<td>0.324</td>
</tr>
<tr>
<td></td>
<td>20  20.207 ± 0.015</td>
<td>0.079</td>
</tr>
<tr>
<td></td>
<td>30  30.403 ± 0.018</td>
<td>0.054</td>
</tr>
<tr>
<td>PH</td>
<td>20  20.313 ± 0.008</td>
<td>0.603</td>
</tr>
<tr>
<td></td>
<td>40  40.419 ± 0.006</td>
<td>0.248</td>
</tr>
<tr>
<td></td>
<td>60  60.654 ± 0.031</td>
<td>0.754</td>
</tr>
</tbody>
</table>

Table 4
Analysis of marketed dosage form
(n=3 replicates, % RSD = relative standard deviation)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug</th>
<th>Label Claim</th>
<th>% Assay ± SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pari CR Plus</td>
<td>CLO</td>
<td>0.5 mg</td>
<td>101.19 ± 0.311</td>
<td>0.312</td>
</tr>
<tr>
<td></td>
<td>PH</td>
<td>12.5 mg</td>
<td>99.38 ± 0.332</td>
<td>0.331</td>
</tr>
</tbody>
</table>
the method was considered acceptable based on its intended use.

**Analysis of marketed dosage form**

The proposed method was applied for the simultaneous determination of CLO and PH in commercial tablet formulation and amount of CLO and PH were found to be 101.19 % and 99.38 % respectively as shown in Table 4. The percent recoveries of the amount of CLO and PH in tablet dosage form, expressed as a percentage assay were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients that normally present in tablets.

**CONCLUSION**

Ratio derivative method shows easy measurements on the separate peaks, higher values of analytical signals and there was no need to work on zero cross over point. The method does not need any mathematical calculations. The results demonstrate that the proposed UV spectrophotometric methods are simple, rapid, specific, accurate and precise. Hence, the proposed method could be regarded as useful alternative to the chromatographic techniques (HPLC) in the routine quality control of title drugs either alone or in combination with a relatively inexpensive instrumentation for simultaneous estimation of CLO and PH in their binary mixtures without interference with commonly used excipients and related substances.

**REFERENCES**

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17. Lambropoulos J, Spanos GA, Lazaridis NV. Method development and validation for the HPLC


