Stability-Indicating HPLC Method for Simultaneous Determination of Clidinium Bromide and Chlordiazepoxide in Combined Dosage Forms

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Abstract

The study describes development and subsequent validation of a stability indicating reverse-phase high-performance liquid chromatography method for the simultaneous estimation of clidinium bromide (CLI) and chlordiazepoxide (CHLOR) from their combination drug product. Chromatographic separations are performed at ambient temperature on a Phenomenex Luna C18 (250 mm × 4.6 mm, i.d., 5 µm) column using a mobile phase consisting of potassium dihydrogen phosphate buffer (0.05 M, pH 4.0 adjusted with 0.5% orthophosphoric acid)–methanol–acetonitrile (40:40:20, v/v/v). The flow rate is 1.0 mL/min, and the detection wavelength is 220 nm. The method is validated with respect to linearity, precision, accuracy, system suitability, and robustness. The utility of the procedure is verified by its application to marketed formulations that were subjected to accelerated degradation studies. The method distinctly separated the drug and degradation products even in actual samples. The products formed in marketed tablet dosage forms are similar to those formed during stress studies.

Introduction

Chemically, CLI (3-[[hydroxy-diphenylacetyl]-oxy]-1-methyl-1-azoniabicyclo-[2.2.2]) octane bromide is reported to be effective for anxiety-related conditions including spastic colon (1). CHLOR (7-chloro-N-methyl-5-phenyl-3H-1,4-benzodiazepine-2-amaia-4-xide) is used as an anxiolytic, sedative-hypnotic, tranquilizer, and antidepressant (2). It shares the actions of other benzodiazepines and is used for the management of anxiety disorders or for short-term relief of symptoms of anxiety and for the management of agitation associated with acute alcohol withdrawal (3). Combination of CLI and CHLOR in the ratio 1:2, respectively, produce antispasmodic effects, antianxiety action, and also help in treatment of peptic ulcer disease and irritable bowel syndrome.

There are several assay methods for the determination of CLI (4) and CHLOR (5–11) individually in formulations, biological fluids, and in combination with other drugs. Also a spectrophotometric (1) and a simple high-performance liquid chromatography (HPLC) (12) method have been developed for the combination of the two drugs. However, the sensitivity and resolution of related drug components is not sufficient for assay. Further, to our present knowledge, no stability-indicating assay method has been reported for the simultaneous determination of CLI and CHLOR in the presence of their degradants using the ICH approach of stress testing. The aim of this study was to develop a simple, rapid, precise, and accurate isocratic reversed-phase stability-indicating HPLC method for simultaneous determination of CLI and CHLOR in the tablet dosage form. Stress testing under various conditions like hydrolysis (i.e., acid, base, and water), oxidation, heat, and photolytic degradation was carried out as per ICH guidelines (13). Validation of developed analytical method was carried out as per ICH guidelines (14,15). The developed method was applied to their two marketed tablet dosage forms.

Experimental

Materials and chemicals

Pure CLI and CHLOR were procured as gift samples from Torrent Pharmaceutical (Gandhinagar, India). Sugar-coated tablets (Ulcon-P, Unimarck Healthcare Limited, New Delhi, India and Equirex, Jagsonpal Pharmaceuticals Limited, New Delhi, India) of CLI (2.5 mg) and CHLOR (5.0 mg) in combination were purchased from a local pharmacy store. HPLC-grade acetonitrile (ACN), methanol (MEOH), and water were purchased from Spectrochem (Hyderabad, India). Potassium dihydrogen phosphate (KH2PO4), ortho phosphoric acid, hydrochloric acid, sodium hydroxide pellets, and hydrogen peroxide solution (all analytical reagent-grades) were purchased from Rankem (Mumbai, India).

Instrumentation

Chromatography was performed on Shimadzu (Kyoto, Japan) chromatographic system equipped with an isocratic HPLC pump (Shimadzu LC-20AT), a UV-visible detector (Shimadzu SPD-20AV) with a Rheodyne syringe-loading sample fixed loop (20 µL) injector (7725). The LC separation was performed at ambient temperature on a Phenomenex Luna C18 (250 mm × 4.6 mm,
Preparation of standard and sample solutions
Individual stock solutions of CLI (500 µg/mL) and CHLOR (1000 µg/mL) were prepared by dissolving appropriate amount of pure drugs in mobile phase in separate volumetric flasks. A stock solution of CLI (and 500 µg/mL) and CHLOR (1000 µg/mL) in combination was also prepared in mobile phase. Suitable dilutions were made from the previously mentioned stock solutions to obtain the optimum concentration for degradation study.

Twenty tablets from each brand of one batch were accurately weighed, their mean weight determined, and powdered in a glass mortar. An amount of the tablet mass equivalent to five tablets content was dissolved in 20 mL MEOH followed by sonication for 15 min. These samples were filtered using a 0.45-µm nylon filter paper, and dilutions were made with MEOH to obtain solution of CLI (500 µg/mL) and CHLOR (1000 µg/mL). Suitable dilutions were made to achieve the optimum concentration for analysis.

Forced degradation studies
From the previously mentioned stock solutions of standard drug and sample, 5 mL of aliquots were diluted separately up to 10 mL with 3% H₂O₂ (v/v), distilled water, 0.1 M HCl, and 0.1 M NaOH to achieve a concentration of 250 µg/mL and 500 µg/mL each of CLI and CHLOR, respectively. Solutions in water, 0.1M HCl, and 0.1 M NaOH were heated at 80°C for 24 h. For oxidative degradation, drugs were stored at room temperature (r.t.) in 3% H₂O₂ (v/v) for 48 h. Degradation was also carried out in solid state by exposing pure drugs and drug product to dry heat at 80°C for 48 h. Photolytic studies were carried out by exposing pure drugs to dry heat at 80°C for 48 h. Photolytic studies were performed on the HPLC system (Figure 1). The peak area versus concentration data was performed by least-squares linear regression analysis.

Method validation
Linearlity
Linearity was studied by injecting eight concentrations of the standard CLI (2.5, 5, 10, 50, 100, 150, 200, and 250 µg/mL) and CHLOR (5, 10, 20, 100, 200, 300, 400, and 500 µg/mL) in triplicate into the HPLC system (Figure 1). The peak area versus concentration data was performed by least-squares linear regression analysis.

Sensitivity
The limit of detection (LOD) and limit of quantitation (LOQ) values were calculated from kSD/b where k = 3 for LOD and 10 for LOQ. SD is the standard deviation of the responses of the minimum detectable drug concentration, and b is the slope of the calibration curve (17).

Precision
For intra-day precision, three different concentrations of sample solutions of CLI (5, 50, and 200 µg/mL) and CHLOR (10, 100, and 400 µg/mL) were analyzed three times on the same day whereas for inter-day precision same drug concentrations were analyzed on three different days, and the percentage RSD of area was calculated. Intermediate precision was established through separation studies on two different columns (column I Phenomenex Luna–RP C₁₈ and column II-Waters XTerra – RP C₁₈).

Accuracy
Accuracy was evaluated by spiking the mixture of degraded solutions with three different levels of standard solutions of CLI (40, 50, and 60 µg/mL) and CHLOR (80, 100, and 120 µg/mL) and calculating the percent recovery from the differences between the peak areas obtained for the fortified and unfortified solutions.
Specificity
The specificity of the method was established through study of resolution factors (Rs) of the drug peaks from the nearest resolving peak and also among all other peaks. Specificity of the method towards the drugs was also established through determination of purity of CLI and CHLOR peak in a mixture of stressed samples through study of purity plots using a PDA detector.

Robustness
Robustness of the method was determined by deliberately varying certain parameters like flow rate (0.9, 1.0, and 1.1 mL/min), volume of ACN (15, 20, and 25 mL) in the mobile phase and the pH of the mobile phase by ± 0.1. Each parameter was studied at three levels (–1, 0, and 1). One factor at a time was changed to estimate the effect. The assay was carried out in triplicate (n = 3) at three different concentration levels of CLI (10, 50, and 100 µg/mL) and CHLOR (20, 100, and 200 µg/mL).

System suitability
In the system suitability tests, six replicate injections of freshly prepared working standard solutions of CLI and CHLOR (50 µg/mL each) and two injections of the solutions prepared for the specificity procedure were injected into the chromatograph, and the % relative standard deviation (% RSD) of peak areas, resolution factor, tailing factor, and theoretical plates were determined.

Results and Discussion
The optimized composition was used for the analysis of all the reaction solutions individually as well as in combination of all the samples in which decomposition was observed. The mobile phase used initially was composed of potassium dihydrogen phosphate (KH$_2$PO$_4$) buffer (0.05 M) and MEOH. However, to achieve the optimum resolution, small portions of ACN was added in the mobile phase. The chromatographic conditions were optimized for separation of drugs and degradation products by varying MEOH, strength of buffer solution, pH, proportion of ACN–buffer solution, and flow rate. Good separation (Figure 1) was obtained in mobile phase composed KH$_2$PO$_4$ buffer (0.05 M, pH 4.0 adjusted with 0.5% orthophosphoric acid) MEOH–ACN (40:40:20, v/v/v).

Degradation studies
Acidic conditions
Both the drugs were found to be labile to acid hydrolysis in 0.1M HCl at 80°C. It was observed that CLI gradually degraded on heating at 80°C in 0.1M HCl for 24 h, forming degradation products showing retention time ($t_R$) 7.62 and 12.32 min. CHLOR showed higher degradation as compared to CLI. At the end of 12 h, around 25% fall in CHLOR peak area was observed. After refluxing for 24 h, drug was degraded by 60% with corresponding increase in concentration of the degradation products. The degradation products showed $t_R$ of 9.03, 10.08, and 11.11 min (Figure 2A).

Degradation in alkali
CLI was found to be highly labile to alkaline hydrolysis. Around 60% degradation of the drug was observed in 0.1M NaOH at 80°C within 2 h. The degradation peaks appeared at $t_R$ 7.62 and 12.32 min whereas mild degradation was seen in CHLOR in alkaline condition. It was observed that around 10–12% of the drug degraded on heating it in 0.1M NaOH for 24 h at 80°C. Two peaks were generated at 9.0 and 11.11 min in the chromatogram (Figure 2B).

Neutral (water) conditions
In neutral condition, CLI was found to be relatively stable. Upon heating the drug solution in water at 80°C for 24 h, only minor degradation product at $t_R$ 7.62 min was formed. On further heating up to 48 h, there was no rise in the proportion of the already degraded peaks. On the other hand, 10–15% degradation of CHLOR was seen after heating for 24 h at 80°C with the generation of three minor peaks at around 6.52, 10.08, and 11.11 min (Figure 2C).

Oxidative conditions
CLI was found to be relatively stable following exposure to oxidative condition (3% H$_2$O$_2$ at r.t. for 48 h) resulting in 4–5% degradation while CHLOR was found to degrade more than 25%. Mild degradation was seen in CLI with appearance of single peak at 7.62 min whereas the degradation products of CHLOR appeared at $t_R$ 9.08 and 11.11 min (Figure 2D).

Figure 2. HPLC chromatogram of CLI and CHLOR obtained from degradation studies: acid hydrolysis (0.1 M HCl, 80°C, 24 h) (A); alkaline hydrolysis (0.1 M NaOH, 80°C, 24 h) (B); neutral hydrolysis (water, 80°C, 24 h) (C); oxidative degradation (3% H$_2$O$_2$, r.t., 48 h) (D); thermal degradation (dry heat, 80°C, 48 h) (E); and photolytic degradation (photostability chamber, 30 d) (F). Chromatographic conditions: RP C$_{18}$ column (250 mm x 4.6 mm, 5 µm); flow rate 1.0 mL/min; mobile phase KH$_2$PO$_4$ buffer (0.05 M, pH 4.0 adjusted with 0.5% orthophosphoric acid–MEOH–ACN (40:40:20, v/v/v); and UV detection at 220 nm.
Thermal stress

Thermo labile property of CLI was clearly observed when it was exposed to dry heat at 80°C for 48 h. Profound degradation (15–20%) in CLI was seen with a single degradation peak at 12.32 min. On the other hand, CHLOR was found to be relatively stable in the study. However, minute degradation peak was observed at \( t_R \) 11.11 min (Figure 2E).

Photolytic conditions

Mild decomposition was seen on exposure of CLI and CHLOR solid drug powder and their tablets to light in the photostability chamber. The photolytic exposure (30 days) of CLI in 0.1M HCl and 0.1M NaOH resulted in 45% and 12% degradation, respectively. On the other hand standard CHLOR and its tablet were found to be more stable under acidic photolytic stress conditions, resulting in 25% decomposition. CLI and CHLOR API and the pharmaceutical tablets were found to be sufficiently stable under neutral photolytic degradation conditions. The major degradation peaks of CHLOR appeared at \( t_R \) 9.08 and 11.11 min while degradation peak was found at 7.62 min for the CLI (Figure 2F).

Validation of the method

The method was validated in respect to the following parameters.

### Linearity

The method was strictly linear in the concentration range of 2.5–250 µg/mL and 5–500 µg/mL for CLI and CHLOR, respectively. The mean (± % RSD) values of slope, intercept, and correlation coefficient were 13.32 (± 0.75), 16.102 (± 1.360), and 0.9995 for CLI and 38.40 (± 0.58), 38.30 (± 0.65), and 0.9997 for CHLOR, respectively. The results show that good correlation existed between the peak area and concentration of the analyte.

### Limit of detection and quantification

The LOD values for CLI and CHLOR were 0.087 µg/mL and 0.122 µg/mL, and the LOQ values for CLI and CHLOR were 0.291 µg/mL and 0.406 µg/mL, respectively.

### Precision

The low RSD (< 2%) values of intra-day and inter-day precision for CLI and CHLOR revealed that the proposed method is precise (Table I). An intermediate precision showed that similar resolution was possible in repeating the experiment on two different reversed phase HPLC columns. The % RSD (< 2%) values indicate that the method was sufficiently precise (Table II).

### Accuracy

Recovery of standard drugs added was found to be 97.58–100.0% for CLI and 99.47–100.93% for CHLOR with the value of % RSD less than 2, indicating proposed method is accurate (Table I).

### Specificity

The drugs and all the degradation products resolved from each other with a resolution factor (Rs) of ≥ 1.9, which ensured complete separation of CLI and CHLOR from its degradation products (Figure 2A–2F). Studies performed to determine the purity of CLI and CHLOR peaks using a PDA detector showed purity angle (PA) values of 0.079 and 0.086 and purity threshold (TH) values of 0.251 and 0.279 for CLI and CHLOR, respectively (Table II). The PA value was found to be less than TH value, indicating that the CLI and CHLOR were free from any co-eluting peaks.

### Robustness

Insignificant difference in peak areas and retention time were observed upon slight variation in the selected parameters, and
also the resolution between CLI and CHLOR and its major degradation products was found to be ≥ 2.0, indicating the robustness of the LC method (Table III).

System suitability
The parameters, tR, resolution factor, tailing factor, and theoretical plates were evaluated. The results (Table II) obtained from system suitability tests are in agreement with the USP requirements. The variation in retention times among six replicate injections of CLI and CHLOR standard solutions was very low, rendering an RSD of 0.36 and 0.51%, respectively.

Application to the pharmaceutical products
The developed method was successfully applied to analyze CLI and CHLOR in marketed tablet formulations. A clear separation of the drugs and degradation products was achieved in tablet with no interference from excipients (Figure 3). The assay results are shown below for the average of six determinations of the two tablets, Ulcon-P and Equirex (2.5 mg of CLI and 5.0 mg CHLOR). Assay of Ulcon-P tablet gave the mean assay values of 2.45 ± 0.51; 0.53 (SD; RSD %) mg and 4.95 ± 0.59; 0.61 (SD; RSD %) mg for CLI and CHLOR, respectively, whereas in Equirex tablet mean assay values was found to 2.47 ± 0.53; 0.54 (SD; RSD %) mg and 5.02 ± 0.65; 0.66 (SD; RSD %) mg for CLI and CHLOR, respectively. The difference in the label claim and the results was very low, and the RSD value was less than 1%. The results of the quantitative analysis of tablets indicate that the proposed assay method can be used for quantitation and routine quality control analysis of CLI and CHLOR in pharmaceutical dosage forms.

Conclusion
The present study envisages the stability behavior of CLI and CHLOR individually and in combination as per the ICH guidelines. CHLOR was found to be more susceptible under stress conditions in comparison to CLI.

The method was found to be accurate and precise with good and consistent recoveries at all levels studied. In addition, simple isocratic elution and easy extraction procedure offered rapid and cost-effective analysis of CLI and CHLOR. Application of this method of the analysis of CLI and CHLOR in tablet dosage form shows that neither the degradation products nor the excipients interfere with the analytical determination. This indicates that the proposed method could be used as a stability indicating method for the simultaneous determination of CLI and CHLOR in bulk drug and in pharmaceutical formulations.

Acknowledgments
The authors are thankful to the Analytical Department of Alembic, (Vadodara, India) for providing the facility for photostability studies. Also thanks are extended to Torrent Pharmaceutical (Gandhinagar, India) for supplying gift samples of clidinium bromide and chlordiazepoxide.

Table III. Robustness Studies of CLI and CHLOR (n = 3)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level</th>
<th>Retention time (Mean ± SD)</th>
<th>Asymmetric factor (Mean ± SD)</th>
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<tr>
<td></td>
<td></td>
<td>CLI</td>
<td>CHLOR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retention time (Mean ± SD)</td>
<td>Asymmetric factor (Mean ± SD)</td>
</tr>
<tr>
<td>A: flow rate (mL/min)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.9</td>
<td>-1</td>
<td>5.37 ± 0.026</td>
<td>13.39 ± 0.019</td>
</tr>
<tr>
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<td>0</td>
<td>5.34 ± 0.013</td>
<td>13.36 ± 0.012</td>
</tr>
<tr>
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<td>1</td>
<td>5.32 ± 0.013</td>
<td>13.34 ± 0.013</td>
</tr>
<tr>
<td>B: ml of ACN in mobile phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>-1</td>
<td>5.37 ± 0.013</td>
<td>13.39 ± 0.008</td>
</tr>
<tr>
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<td>0</td>
<td>5.33 ± 0.014</td>
<td>13.36 ± 0.008</td>
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<tr>
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<td>1</td>
<td>5.31 ± 0.008</td>
<td>13.34 ± 0.005</td>
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<tr>
<td>C: pH of mobile phase</td>
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<td></td>
<td></td>
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<td>13.35 ± 0.008</td>
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References
15. ICH Guidance on analytical method validation, International convention on quality for the pharmaceutical industry, Toronto, Canada, September 2002.

Manuscript received May 2, 2008; revision received November 9, 2008.