METHOD DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CLIDINIUM BROMIDE, CHLORDIAZEPoxide AND DICYCLOMINE HYDROCHLORIDE IN BULK AND COMBINED TABLET DOSAGE FORMS

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ABSTRACT
The study describes method development and subsequent validation of RP-HPLC method for simultaneous estimation of Clidinium bromide (CDB), Chlordiazepoxide (CDZ) and Dicyclomine hydrochloride (DICY) in bulk and combined tablet dosage forms. Chromatographic separation was achieved on a Kromasil C18 (250 mm x 4.6 mm id, 5µm) column using a mobile phase ratio consisting of (40:30:30) Methanol: Acetonitrile: Potassium di hydrogen phosphate buffer (0.05M, pH 4.0 adjusting with 0.5% Ortho phosphoric acid) at flow rate 1.0 ml/min. The detection wavelength is 270 nm. The retention times of Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride were found to be 7.457 min, 4.400 min and 3.397 min respectively. The developed method was validated as per ICH guidelines using the parameters such as accuracy, precision, linearity, LOD, LOQ, ruggedness and robustness. The developed and validated method was successfully used for the quantitative analysis of Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride in bulk and combined tablet dosage forms.

KEY WORDS
Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride, Normaxin tablet dosage forms, HPLC, Method validation.

INTRODUCTION
Clidinium bromide (3-[(2-hydroxy-2,2diphenylacetyl)-oxy]-1-methyl-1-azoniabicyclo[2.2.2] octan-1-ium bromide) is used for anticholinergic and antisecretory agent which exerts its action by inhibiting the action of parasympathetic innervations thus reducing the secretions of stomach acid and is also a mild antispasmodic [1]. Chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3H-1, 4-benzodiazepene-4-oxide) is a benzodiazepine. It has GABA facilitator action. It is used as anxiolytic, sedatives, hypnotics, skeletal muscle relaxants. The drug may inhibit monosynaptic and polysynaptic reflexes by acting as inhibitory neuronal transmitters or by blocking excitatory synaptic transmission [2]. Dicyclomine hydrochloride (1, 1-bicyclohexyl-1-carboxilicacid-2-diethyl amino ethyl ester) is an anticholinergic drug (tertiary amine). It has direct smooth muscle relaxant action in addition to weak anticholinergic effect by slowing the natural movements of the gut and by relaxing the muscles in the stomach and intestine so it is used for the antispasmodic agent [3-5]. This Combination of three drugs (Normaxin tablet) is highly effective and used in the treatment of peptic ulcer, nervous dyspepsia, gastritis, irritable spastic colon, mucous colitis, acute Enterocolitis. Literature survey reveals that some analytical methods have been used for the estimation of Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride individually or combination with other drugs. The United States Pharmacopoeia (USP) stated the non-aqueous
titration method for the assay of Clidinium bromide and chlordiazepoxide [6]. Very few methods for the determination of Clidinium bromide and chlordiazepoxide in combined dosage forms including RP-HPLC [7-9], derivative spectroscopy [10] and spectrophotometry using multivariate calibration techniques [11] was reported. Chlordiazepoxide has been determined alone or with other compound in pharmaceutical formulations using first-derivative spectrophotometry [12], spectrophotometry [13], High-Performance Liquid Chromatography [14-24], HPTLC [25-26], Voltammetry [27] and flow-injection Potentiometry [28]. Stability indicating gas-liquid chromatography [29-30], RP-HPLC [31] methods was reported for the estimation of Dicyclomine hydrochloride alone or in combination with other drugs. Since, there is no HPLC method has been reported till date for the simultaneous estimation of CDB, CDZ and DICY in bulk and combined tablet dosage forms. Therefore the present research work, our aim is to develop a new analytical RP-HPLC method and validate according to the ICH guidelines [32] to estimate CDB, CDZ and DICY containing bulk drugs and combined tablet dosage forms in routine analysis.

MATERIALS AND METHODS

INSTRUMENTS:
The chromatographic separation was performed with a Shimadzu HPLC instrument (UFLC-20AD) equipped with Photo Diode Array (PDA) detector (SPD-M20A) and LC-solution software. The Kromasil Stainless steel C₁₈ G column (250 mm × 4.6 mm, 5μm) packed with ODS chemically bounded porous silica particles were used as stationary phase for analysis. BL-220H analytical balance (Shimadzu corporation, Japan), an ultrasonic cleaner (Frontline FS 4, Mumbai, India) and Digital pH meter (LI 612 pH analyzer, Elico Ltd., Ahmadabad), were used in the study.

MATERIALS AND REAGENTS:
Clidinium bromide and chlordiazepoxide were received as gift sample from MSN Laboratories Ltd., India. The Dicyclomine hydrochloride was received from Systopic pharmaceutical ltd, India. The pharmaceutical preparations of combination of Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride that is NORMAXIN tablet (Systopic) contains 2.5mg of Clidinium bromide, 5mg of chlordiazepoxide and 10mg of Dicyclomine hydrochloride was procured from local market. The solvents used was Methanol AR Grade, HPLC grade Methanol (S.D fine chemicals ltd, Mumbai, India), HPLC grade Acetonitrile and water for HPLC (Finar Chemicals Ltd., Mumbai, India). The analytical reagent grade potassium di hydrogen phosphate (Qualikems fine chemicals Pvt.ltd, vadodara) and orthophosphoric acid (Ranbaxy laboratories ltd) was used to prepare the mobile phase which is filtered through a nylon 0.45µm membrane filter paper (Gelman laboratories Mumbai, India).

CHROMATOGRAPHIC CONDITIONS:
Method was developed using a Kromasil Stainless steel C₁₈ G column (250 mm ×4.6 mm, 5μm). Mobile phase used was potassium dihydrogen phosphate buffer (0.05 M, pH 4.0adjusted with 0.5% orthophosphoric acid): methanol: acetonitrile (30:40:30, v/v/v) at flow rate is 1.0 mL/min. Samples were injected using Rheodyne injector with 20μl loop.

PREPARATION OF STANDARD STOCK SOLUTION:
An accurately weighed quantity of CDB 10mg, CDZ 20mg and DICY 40mg (Working standard drugs) were transferred to a 50ml volumetric flask and dissolved in bulk and combined tablet dosage forms. Therefore the present research work, our aim is to develop a new analytical RP-HPLC method and validate according to the ICH guidelines [32] to estimate CDB, CDZ and DICY containing bulk drugs and combined tablet dosage forms in routine analysis.

PREPARATION OF SAMPLE SOLUTION:
Twenty tablets were weighed and crushed to fine powder. The tablet powder equivalent to 10mg of CDB, 20mg of CDZ and 40 mg of DICY was transferred to a 50ml volumetric flask and dissolved in mobile phase and finally the volume was adjusted up to the mark with mobile phase. From this stock solution working standard solution having concentration 10µg/ml, 20µg/ml and 40µg/ml were prepared by appropriate dilution with mobile phase for CDB, CDZ and DICY respectively.

PREPARATION OF SAMPLE SOLUTION:
Twenty tablets were weighed and crushed to fine powder. The tablet powder equivalent to 10mg of CDB, 20mg of CDZ and 40 mg of DICY was transferred to a 50 ml volumetric flask and dissolved in mobile phase and the content was kept in ultrasonicator for 15 min. The flask was allowed to stand for 5 min at room temperature and the volume was adjusted up to the mark with mobile phase. The solution was filtered through a nylon 0.45 μm membrane filter paper. The solution was suitably diluted with mobile phase to get a final
concentration of 10µg/ml of CDB, 20µg/ml of CDZ and 40µg/ml of DICY respectively.

**METHOD VALIDATION:** The developed RP-HPLC method was validated as per ICH guidelines [32].

**ASSAY:**
Twenty tablets were weighed and crushed to fine powder. The tablet powder equivalent to 10mg of CDB, 20mg of CDZ and 40 mg of DICY was transferred to a 100 ml volumetric flask and dissolved in mobile phase and the content was kept in ultrasonicator for 15 min. The flask was allowed to stand for 5 min at room temperature and the volume was adjusted up to the mark with mobile phase. The solution was filtered through a nylon 0.45 µm membrane filter paper. The solution was suitably diluted with mobile phase to get a final concentration of 10µg/ml of CDB, 20µg/ml of CDZ and 40µg/ml of DICY respectively. The % assay of the drugs was calculated and the results are given in Table-1.

**SPECIFICITY:**
The specificity of the RP-HPLC method was determined by comparison of the chromatogram of mixed standards and sample solutions. The parameters like retention time (Rt), resolution (Rs), and asymmetry factor (As) and number of theoretical plates were calculated. Good correlation was found between the results of mixed standards and sample solutions.

**ACCURACY:**
The accuracy of the method was determined by calculating the recovery studies at three levels (80%, 100% and 120%) by standard addition method. Known amounts of standard CDB, CDZ and DICY were added to the pre quantified samples and they were subjected to proposed HPLC method. The results of the recovery studies are given in Table-3.

**PRECISION:**
Precision study was performed to find out intra-day and inter-day variations. In this process the combined solution (10µg/ml, 20µg/ml and 40µg/ml of CDB, CDZ and DICY respectively) analyzed by same day (Intra-day precision) and on three different days (Inter-day precision). The %relative standard deviation (RSD) for intra-day precision was 1.135% of CDB, 1.252% of CDZ and 0.262% of DICY and for inter-day precision was 1.40% of CDB, 1.54% of CDZ and 0.65% of DICY respectively, which is less than 2% indicating high degree of precision.

**LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ):**
The LOD and LOQ for CDB, CDZ and DICY were separately determined by based on calculating the signal-to-noise ratio (S/N is 3.3 for LOD and 10 for LOQ) and from the calibration curves the standard deviation of the y-intercepts and slope of the regression lines were used. Results of LOD and LOQ are given in Table. The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by using the following equations designated by International Conference on Harmonization (ICH) guidelines (32).

\[
\text{LOD} = 3.3 \times \sigma/S \\
\text{LOQ} = 10 \times \sigma/S
\]

Where, \(\sigma\) = the standard deviation of the response \\
\(S\) = slope of the calibration curve.

**LINEARITY:**
An accurately weighed quantity of CDB 10mg, CDZ 20mg and DICY 40mg (Working standard drugs) were transferred to into a separate 50mL clean and dry volumetric flasks and dissolved in mobile phase and finally each volumetric flask volume was adjusted up to the mark with mobile phase respectively. From this stock solution prepare 12, 16, 20, 24 and 28µg/ml of CDB, 24, 32, 40, 48 and 56µg/ml of CDZ and 48, 64, 80, 96 and 112µg/ml of DICY concentrations respectively. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results were shown in Table-4.

**ROBUSTNESS:**
The robustness study was done by making small changes in the optimized method parameters like changing in pH of the mobile phase by ± 1%, mobile phase ratio by ±2%, column oven temperature by ± 2°C and flow rate by ± 1 ml/min and the chromatographic characteristics were evaluated. No significance change was observed.
Figure 1: Chromatogram of CDB, CDZ and DICY in Bulk analysis

Figure 2: Chromatogram of CDB, CDZ and DICY in tablet analysis
Table 1: Assay Parameters

<table>
<thead>
<tr>
<th>Marketed formulation</th>
<th>Drugs</th>
<th>Label claim (mg)</th>
<th>Amount found (mg)</th>
<th>%Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normaxin-Tab</td>
<td>CDB</td>
<td>2.5</td>
<td>2.49</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>CDZ</td>
<td>5</td>
<td>4.99</td>
<td>99.80</td>
</tr>
<tr>
<td></td>
<td>DICY</td>
<td>10</td>
<td>9.91</td>
<td>99.10</td>
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</table>

Table 2: Results from validation and system suitability studies

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>CDB</th>
<th>CDZ</th>
<th>DICY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates</td>
<td>4101</td>
<td>3186</td>
<td>2415</td>
</tr>
<tr>
<td>Resolution</td>
<td>7.834</td>
<td>3.350</td>
<td>-</td>
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<tr>
<td>Asymmetry factor</td>
<td>1.365</td>
<td>1.244</td>
<td>1.541</td>
</tr>
<tr>
<td>Intra-day precision (%RSD)</td>
<td>1.135</td>
<td>1.252</td>
<td>0.262</td>
</tr>
<tr>
<td>Inter-day precision (%RSD)</td>
<td>1.40</td>
<td>1.54</td>
<td>0.65</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.83</td>
<td>4.58</td>
<td>1.39</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>2.52</td>
<td>13.89</td>
<td>4.22</td>
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</table>

Table 3: Accuracy

<table>
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<tr>
<th>Drug</th>
<th>Label claim (mg)</th>
<th>Sample concentration (µg/ml)</th>
<th>Amount added (µg/ml)</th>
<th>Amount recovery (mg)</th>
<th>% Recovery</th>
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</thead>
<tbody>
<tr>
<td>CDB</td>
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<td>16</td>
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<td>4</td>
<td>24.4274</td>
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<tr>
<td>CDZ</td>
<td>5</td>
<td>32</td>
<td>8</td>
<td>39.44</td>
<td>98.61</td>
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<tr>
<td></td>
<td></td>
<td>40</td>
<td>8</td>
<td>48.08</td>
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<td></td>
<td>48</td>
<td>8</td>
<td>55.81</td>
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<tr>
<td>DICY</td>
<td>10</td>
<td>64</td>
<td>16</td>
<td>80.71</td>
<td>100.89</td>
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<td>96</td>
<td>16</td>
<td>109.81</td>
<td>98.05</td>
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</table>
Table 4: Linearity

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Concentration(µg/ml)</th>
<th>Area</th>
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<tbody>
<tr>
<td></td>
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<td>CDZ</td>
</tr>
<tr>
<td></td>
<td>CDB</td>
<td>CDZ</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>226.682</td>
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<tr>
<td></td>
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<td>315.477</td>
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<td>4</td>
<td>523.759</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>624.446</td>
</tr>
</tbody>
</table>

Linearity of CDB, CDZ and DICY

![Graph showing linearity of CDB, CDZ and DICY](image)

\[ y = 25.09x - 77.03 \]
\[ R^2 = 0.998 \]
RESULTS AND DISCUSSION

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for CDB, CDZ and DICY was obtained using Kromasil Stainless steel C18 G column (250 mm ×4.6 mm, 5μm) with a mobile phase consisting of potassium dihydrogen phosphate buffer (0.05 M, pH 4.0) adjusted with 0.5% orthophosphoric acid: methanol: acetonitrile (30:40:30, v/v/v) at flow rate is 1.0 mL/min, PDA detection was performed at y = 9.109x - 63.67
R² = 0.996

CDZ

y = 59.37x - 338.8
R² = 0.995

DICY

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270nm. The retention times of Clidinium bromide, Chlordiazepoxide and Dicyclomine hydrochloride were found to be 7.457 min, 4.400 min and 3.397 min respectively (Figure). The amount of CDB, CDZ and DICY present in the sample solutions were determined respectively and the results obtained were comparable with the corresponding labeled claim (Table 1). The results of system suitability testing are given in Table 2. The %RSD of CDB, CDZ and DICY for intra-day precision and inter-day precision was less than 2% it reveal that the proposed method is precise (Table 2). The sensitivity of method LOD and LOQ is shown in Table 2. The % recovery was found to be 98-102% within the limits for CDB, CDZ and DICY (Table 3) which indicates high degree of accuracy of developed method. Linear correlation was obtained between concentration versus peak area of CDB, CDZ and DICY in the concentration ranges of 12-28(µg/ml), 24-56(µg/ml) and 48-112(µg/ml) respectively (Table 4). The correlation co-efficient (‘r’ value) for CDB, CDZ and DICY was 0.998, 0.996 and 0.995 respectively. The results of the robustness study also indicated that the method is robust and is unaffected by small variations in the chromatographic conditions.

CONCLUSION

The proposed study describes RP-HPLC method for the estimation of CDB, CDZ and DICY in bulk drugs as well as in tablet formulation. The method was validated according to the ICH guidelines. Hence, it can be concluded that the developed RP-HPLC method is accurate, precise, and selective and it can be employed successfully for the estimation of CDB, CDZ and DICY in their bulk drugs and tablet formulation in routine analysis.

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