APPLICATION OF HPTLC-DENSITOMETRIC ANALYSIS FOR SIMULTANEOUS DETERMINATION OF CLIDINIUM BROMIDE, CHLORDIAZEPoxide AND PANTOPRAZOLE SODIUM IN THEIR COMBINED CAPSULE DOSAGE FORM

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DOI: 10.7897/2230-8407.04432
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ABSTRACT

A simple, precise, accurate and reliable HPTLC method has been developed and validated for analysis of Clidinium Bromide (CLBr), Pantoprazole Sodium (PNT) and Clidinium (CDZ) in their combined dosage form. Identification and analysis were performed on 100mm x 100mm layer thickness 0.2 mm, pre-coated silica gel 60F254 aluminum sheet, prewashed with methanol and dried in an oven at 50°C for 5 min. Chloroform: Acetone: 0.5M Ammonium acetate in Methanol: Formic acid (1.0:5:0.3:0.1:0) (v/v/v/v) was used as mobile phase. Calibration plots were established showing the dependence of response (peak area) on the amount chromatographed. The validated calibration ranges were 600-1600 ng/spot, 4800-12800ng/spot and 1200-3200 ng/spot for CLBr, PNT and CDZ respectively. The spots were scanned at 220nm in a reflectance mode. The proposed method has been validated as per ICH guidelines and successfully applied to the estimation of CLBr, PNT and CDZ in their combined Capsule dosage form.

Key Words: Clidinium Bromide, Clidinium, Pantoprazole Sodium, HPTLC, Validation.

INTRODUCTION

Ultran Capsule contains CLBr, CDZ and PNT in combination. It is useful to treat Irritable bowel syndrome which is a brain gut disorder characterized most commonly by cramping, abdominal pain, bloating, constipation, and diarrhea, which is the most common and fatal disease. Chloridiazepoxide is chemically 3-[(2-hydroxy-2,2-diphenylacetoxy)-1-methyl-1 azabicyclo [2.2.2] octan-3-yl]methyl sulfinyl]benzofuran-2-yl)methyl sulfoxide, which inhibits muscarinic actions of acetylcholine at postganglionic parasympathetic neuroeffector sites. It is used for the treatment of peptic ulcer disease and also to help relieve abdominal or stomach spasms or cramps due to colicky abdominal pain, diverticulitis, and irritable bowel syndrome. CLBr is official in United state pharmacopoeia XXX. Chemically PNT is, (RS)-(Difluoromethoxy) -2-[3,4dimethoxyphenyl -2 yl]methyl sulfanyl]-1H-benzo[d]imidazole sodium (Figure 1b), a benzimidazole derivative blocks the proton pump by reacting with (H+/k+) ATPase enzyme by causing it’s inhibition of action resulting long lasting inhibition of gastric acid secretion. CDZ is chemically 7-Chloro-N-methyl-5-phenyl-1H-1,4-benzodiazepin-2-amine 4-oxide (Figure 1c), which is an anxiolytic & agonist at specific benzodiazepine receptor, which are inhibitory in nervous system, Clidiazepoxide and GABA forms complex with chloride ion leads to stimulation of benzodiazepine receptors and potentiate the action of GABA which in turn controls the flow of chloride ions across neuron membrane, thereby relieves the patient from anxiety. PNT and CDZ are official in British Pharmacopoeia, United States Pharmacopoeia and Indian Pharmacopoeia. The review of literature revealed that various analytical methods involving spectrophotometry, HPLC, HPTLC have been reported for CLBr, PNT and CDZ in single form and in combination with other drugs while few analytical methods have been reported for CLBr and CDZ in combination with each other including HPLC, and HPTLC. To the best of our knowledge, there is no published chromatographic method for these three combinations of drugs. So, the present paper describes a simple, accurate and precise method for simultaneous estimation of CLBr, PNT and CDZ in combined capsule dosage form by HPTLC method. The developed method was validated in accordance with ICH Guidelines and successfully employed for the assay of CLBr, PNT and CDZ in their combined dosage form.

MATERIALS AND METHODS

Reagents and Chemicals

Analytically pure CLBr and PNT were kindly provided by Mission Research Laboratories (I) Pvt. Ltd., Chandigarh-160019, India and Vasudha Pharma Chem Limited, Hyderabad, AP, India respectively as gratis samples while CDZ was provided by Aum Research Laboratories, Ahmedabad, Gujarat, India. Analytical grade chloroform, methanol, ammonium acetate and formic acid were purchased from SD Fines chemicals, Bombay, India. Capsule of CLBr, PNT and CDZ in combined dosage form, ULRAX, with a 2.5 mg CLBr, 5mg CDZ and 20 mg PNT label claim, manufactured by Mission Research Laboratories (I) Pvt. Ltd., Chandigarh, India was procured from local market.

Instrumentation and conditions

Chromatography was performed on 100mm x 100mm on pre-coated silica gel 60F254 aluminum sheet (E. Merck, Mumbai, India). Before use the plates were washed with methanol then dried at room temperature. Samples were applied as 6mm bands by means of a Camag Linomat V (Muttenz, Switzerland) sample applicator equipped with 100µL syringe; operated with settings of band length, 6 mm; distance between bands, 5 mm; distance from the plate edge, 10 mm; and distance from the bottom of the plate, 10 mm. The constant application rate was 15 s µL−1 and a nitrogen aspirator was used. Ascending development of plate, migration distance 70mm was performed at ambient temperature with Chloroform: Acetone: 0.5M Ammonium
acetate in Methanol: Formic acid (1.0:5.0:3.0:1.0) (v/v/v/v), as mobile phase in a 10 cm × 10 cm Camag twin-trough chamber previously saturated for 15 min. After development the plates were dried with hot-hair dryer and viewed in a CAMAG UV cabinet. Densitometric scanning at 220nm was then performed with a Camag TLC Scanner 3 equipped with WinCATS 3.2.1 software. The scanning rate was 20 mm s⁻¹. The source of radiation used was the deuterium lamp.

**Calibration**

**Clidinium bromide (200 μg/ml), Chlordiazepoxide (400 μg/ml), and Pantoprazole sodium (1600 μg/ml) standard stock solution**

Standard CLBr 12.5 mg, CDZ 25mg and PNT 100 mg were weighed and transferred to a 25 ml volumetric flask and dissolved in methanol. The flask was shaken and volume was made up to the mark with methanol to give a solution containing 500 μg/ml CLBr, 1000 μg/ml CDZ and 4000 μg/ml PNT. 10 ml of this aliquot was added to 25 ml volumetric flask and Volume was made up to the mark with methanol to give a solution containing 200 μg/ml CLBr, 400 μg/ml CDZ and 1600 μg/ml PNT.

**Calibration curve for CLBR, CDZ and PNT**

Semi automatic spotter was used containing a syringe having capacity of 100 μl. Mixed stock solution having concentration of 200 μg/ml of CLBr, 400 μg/ml CDZ and 1600μg/ml PNT was filled in the syringe and under nitrogen stream, it was apply in form of band of desired concentration. Resulting solution was filtered through whatman filter paper (0.45μ) in to a 25ml volumetric flask. The band was applied on TLC plate with that of standard drug and sample. The scanning rate was 20 mm s⁻¹ with retention factor (Rf) values of 0.54 for CLBr, 0.56 for CDZ and 0.64 for PNT. The calibration graphs were developed by plotting peak area vs concentrations (n = 6) with the help of the winCATS software.

**Accuracy (Recovery)**

Known amounts of standard solution of CLBr (600,1200, 1800 ng/spot), CDZ (1200,2400, 3600 ng/spot) and PNT (4800, 9600, 12800 ng/spot) for the HPTLC method were added to prequantitated sample solutions of capsule dosage forms. The amounts of CLBr, CDZ and PNT were estimated by applying values of peak area to the regression equations of the calibration graph.

**Precision**

Precisions of the proposed HPTLC methods were determined by analyzing mixed standard solution of TOL and DFS at 3 different concentrations (800,1000, 1200 ng/spot for CLBr, 1600,2000, 2400 ng/spot for CDZ and 6400,8000, 9600 ng/spot for PNT) 3 times on the same day and on 3 different days. The results are reported in terms of coefficient of variance (CV).

**Repeatability**

Repeatability of method was assessed by applying the same sample solution 6 times on a plate with the automatic spotter using the same syringe and by taking 6 scans of the sample spot for triple drugs CLBr, CDZ and PNT (1000 ng/spot of CLBr, 2000ng/spot of CDZ and 8000 ng/spot of PNT) without changing the positions of the plate.

**Specificity**

The specificity of the method was ascertained by analyzing standard drug and sample. The band of CLBr, CDZ and PNT in sample was confirmed by comparing the Rf and spectra of the band with that of standard. The peak purity of three drugs were assessed by comparing the spectra at 3 different level, i.e. peak start (S), peak apex (M) and peak end (E) position of the band.

**Limit of Detection & Limit of Quantification**

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by using the following equations as per International Conference on Harmonization (ICH) guidelines which is based on the calibration curve.

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

Where \(\sigma\) = the standard deviation of y-intercepts of regression lines, \(S = \text{Slope of calibration curve.}\)

**Robustness**

Sample solution was prepared and then analyzed with change in the typical analytical conditions like amount of mobile phase, proportion of mobile phase, saturation time, plate pretreatment and Stability of analytical solution.

**RESULTS**

**Method Optimization**

Several mobile phases were tried to accomplish good separation of CLBr, CDZ and PNT. Using the mobile phase Chloroform: Acetone: 0.5M Ammonium acetate in Methanol: Formic acid (1.0:5.0:3.0:1.0) (v/v/v/v) and 10×10 cm HPTLC silica gel 60 F254 aluminum-backed plates, good separation was attained with retention factor (Rf) values of 0.54 for

**Validation of the method**

**Linearity and range of the HPTLC method**

Calibration graphs were constructed by plotting peak areas vs concentrations of CLBr, CDZ and PNT, and the regression equations were calculated. The calibration graphs were plotted over 6 different concentrations in the range of 600-1600 ng/spot for CLBr, 1200-3200ng/spot for CDZ and 4800-12800 ng/spot for PNT by applying different volumes stock solution containing CLBr, CDZ and PNT (200 μg/ml of CLBr, 400 μg/ml of CDZ and 1600 μg/ml of PNT). The calibration graphs were developed by plotting peak area vs concentrations (n = 6) with the help of the winCATS software.
CLBr, 0.66 for PNT and 0.76 for CDZ. A wavelength of 220 nm was used for the quantification of the drugs. Figure 2 shows the detection of three drugs in their combined dosage form at 220nm by HPTLC method. Resolution of the peaks with clear baseline separation was found. Figure 3 shows the densitogram of mixture which has a clear baseline. Figure 4 showed a good linearity when overlapped and scanned between 200nm to 400nm. Figure 5 shows a 3D overlapped spectrum of both the drugs which has good linearity. The system suitability test parameters for the developed method are shown in Table 1.

Table 1: System Suitability Test Parameters

<table>
<thead>
<tr>
<th>System suitability Parameter</th>
<th>CLBr</th>
<th>PNT</th>
<th>CDZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Purity</td>
<td>0.9999</td>
<td>0.9998</td>
<td>0.9993</td>
</tr>
<tr>
<td>Rf value</td>
<td>0.54</td>
<td>0.66</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Table 2: Result of calibration readings for CLBr by HPTLC method

<table>
<thead>
<tr>
<th>Concentration (ng/spot)</th>
<th>Area Mean ± S.D. (n=6)</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>600</td>
<td>1063.38 ± 22.515</td>
<td>0.22</td>
</tr>
<tr>
<td>800</td>
<td>1564.68 ± 32.027</td>
<td>0.20</td>
</tr>
<tr>
<td>1000</td>
<td>2133.70 ± 5.1571</td>
<td>0.24</td>
</tr>
<tr>
<td>1200</td>
<td>2678.48 ± 11.1965</td>
<td>0.41</td>
</tr>
<tr>
<td>1400</td>
<td>3138.50 ± 7.0279</td>
<td>0.22</td>
</tr>
<tr>
<td>1600</td>
<td>3786.03 ± 4.3292</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 3: Determination of Accuracy

<table>
<thead>
<tr>
<th>% Level</th>
<th>Amount Added (ng/spot)</th>
<th>Mean of Amount Recovered (ng/spot) (n=3)</th>
<th>% Mean Recovery ± S.D. (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLBr</td>
<td>PNT</td>
<td>CDZ</td>
</tr>
<tr>
<td>50</td>
<td>600</td>
<td>4800</td>
<td>1200</td>
</tr>
<tr>
<td>100</td>
<td>1200</td>
<td>9600</td>
<td>2400</td>
</tr>
<tr>
<td>150</td>
<td>1800</td>
<td>12800</td>
<td>3600</td>
</tr>
</tbody>
</table>

Table 4: Summary of validation Parameters of HPTLC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CLBr</th>
<th>PNT</th>
<th>CDZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery %</td>
<td>99.63 – 99.89</td>
<td>99.87 – 100.05</td>
<td>99.77-99.93</td>
</tr>
<tr>
<td>Repeatability (C.V., n=6)</td>
<td>0.60</td>
<td>0.11</td>
<td>0.29</td>
</tr>
<tr>
<td>Precision (C.V.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra - day (n=3)</td>
<td>0.17 – 0.18</td>
<td>0.025 – 0.107</td>
<td>0.019-0.039</td>
</tr>
<tr>
<td>Inter - day (n=3)</td>
<td>0.12 – 0.31</td>
<td>0.012 – 0.074</td>
<td>0.039-0.043</td>
</tr>
<tr>
<td>Limit of Detection (pg/ml)</td>
<td>4.6725</td>
<td>30.81165</td>
<td>12.61327</td>
</tr>
<tr>
<td>Limit of Quantitation (μg/ml)</td>
<td>14.1583</td>
<td>93.36863</td>
<td>38.2203</td>
</tr>
<tr>
<td>Specificity</td>
<td>Specific</td>
<td>Specific</td>
<td>Specific</td>
</tr>
<tr>
<td>Robustness</td>
<td>Robust</td>
<td>Robust</td>
<td>Robust</td>
</tr>
<tr>
<td>Solvent suitability</td>
<td>Suitable for 24hr</td>
<td>Suitable for 24hrs</td>
<td>Suitable for 24hr</td>
</tr>
</tbody>
</table>

Table 5: Assay result of marketed formulation

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug</th>
<th>Amount Taken (ng/spot)</th>
<th>Amount Found (ng/spot) (n = 3)</th>
<th>Labeled Claim (mg)</th>
<th>Amount found per Capsule (mg)</th>
<th>% Label claim ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulrax (capsule)</td>
<td>CLBr</td>
<td>1000</td>
<td>1000.72</td>
<td>2.5</td>
<td>2.59</td>
<td>101.63 ± 0.8946</td>
</tr>
<tr>
<td></td>
<td>CDZ</td>
<td>2000</td>
<td>2000.58</td>
<td>5</td>
<td>5.19</td>
<td>100.19 ± 0.5431</td>
</tr>
<tr>
<td></td>
<td>PNT</td>
<td>8000</td>
<td>8000.61</td>
<td>20</td>
<td>20.25</td>
<td>100.29 ± 0.4441</td>
</tr>
</tbody>
</table>
Figure 1: Chemical structure of (a) CLBr, (b) PNT and (c) CDZ

Figure 2: Photograph of developed HPTLC plate of CLBr, PNT and CDZ at 220 nm

Figure 3: Densitogram of standard solution of market formulation containing CLBr, PNT and CDZ 1000 ng/spot, 8000 ng/spot, and 2000 ng/spot, respectively

Figure 4: Overlain spectrum of 600-1600 ng/spot CLBr, 4800-12800 ng/spot PNT, and 1200-3200 ng/spot CDZ at 220 nm

Figure 5: 3D overlain spectra of CLBr, PNT, and CDZ by HPTLC method

**Linear correlation**
Linear correlation was obtained between peak areas and concentrations of CLBr in the range of 600-1600 ng/spot with $R^2=0.998$, 1200-3200 ng/spot for CDZ with $R^2=0.998$ and 4800-12800 ng/spot for PNT with $R^2=0.994$, respectively and data are shown in table 2.

**Accuracy**
The recovery experiments were performed by the standard addition method. The HPTLC method was found to be accurate with % recovery of 99.63 – 99.89% for CLBr, 99.87 – 100.05% for PNT and 99.77-99.93% for CDZ respectively (Table 3). The high values indicate that the method is accurate.

**Repeatability**
The CV values for CLBr, CDZ and PNT were found to be 0.60, 0.29 and 0.11 respectively. The CV values were found to be <1%, which indicates that the proposed methods is repeatable.

**Precision**
The CV values were found to be <2%, which indicates that the proposed method is precise.

**Limit of Detection (LOD) and Limit of Quantification (LOQ)**
LOD values for CLBr, CDZ and PNT were found to be 4.6725 ng/spot, 12.6127 ng/spot and 30.81165 ng/spot respectively. LOQ values for CLBr, CDZ and PNT were found to be 14.1583 ng/spot, 38.22203 ng/spot and 93.36863 ng/spot respectively. These data shows that nanogram quantity of three drugs can be accurately determined. (Table 4)
Specificity
Excipients used in the specificity studies did not interfere with the estimation of either of the drugs by the proposed methods. Hence, the methods were found to be specific for estimation of CLBr, CDZ and PNT.

Robustness
Peak area and retention factor variation were found to be <1%. Also, no significant change in peak area was observed during 24 Hrs. No decomposition was observed in either the first or second direction of the 2-dimensional analysis for both drugs on the HPTLC plate. Hence, the method was found to be robust for estimation of CLBr, CDZ and PNT.

Assay of the capsule dosage form (CLBR 2.5mg, CDZ 5mg and PNT 20 mg per capsule)
The proposed validated method was successfully applied to determine CLBr, CDZ and PNT in their capsule dosage form (ULRAX). The results obtained for CLBr, CDZ and PNT was comparable with the corresponding labelled amounts (Table 5).

DISCUSSION
Thus, the objective of project work was development and comparison of analytical method of CLBr, CDZ and PNT in their combined dosage form. The developed and validated HPTLC method for CLBr, CDZ and PNT was found to be simple specific and cost effective and can be routine applied for analysis of CLBr, CDZ and PNT in their combined dosage form. We can say that HPTLC method is more sensitive giving precise results (interday, intraday) for three drugs & also HPTLC method is more sensitive in terms of LOD and LOQ. It also requires least solvents for analysis. The proposed method has the advantages of simplicity and convenience for the separation and quantitation of CLBr, CDZ and PNT in combination and can be used for the assay of their dosage form. Also, the low solvent consumption and short analytical run time lead to environmentally friendly chromatographic procedures. The additives usually present in the pharmaceutical formulations of the assayed analytes did not interfere with determination of CLBr, CDZ and PNT. The method can be used for the routine simultaneous analysis of CLBr, CDZ and PNT in pharmaceutical preparations.

ACKNOWLEDGEMENT
Authors are thankful to Mission Research Laboratories (I) Pvt. Ltd. (Chandigarh, India), Vasudha Pharma Chem Limited (Hyderabad, India) and Aum Research Labs (Ahmedabad, India) for providing gratis sample with the great pleasure. The authors also thankful to Indubhai Patel College of Pharmacy and Research Centre (Dharmaj, India) for providing the necessary facilities for research work and to all the staff members and friends for their guidance and help throughout the research work.

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Cite this article as: Dharmi Ram et al. Int. Res. J. Pharm. 2013, 4 (4)