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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ABSTRACT

A simple, precise, accurate and reliable HPTLC method has been developed and validated for analysis of Clidinium Bromide (CLBr), Pantoprazole Sodium (PNT) and Chlordiazepoxide (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 with correlation coefficient (R^2) 0.998, 0.994 and 0.998 respectively. Average % recovery was between 99.63 – 99.89%, 99.87 – 100.05% and 99.77-99.93% 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, Chlordiazepoxide, Pantoprazole Sodium, HPTLC, Validation.

INTRODUCTION

Ultrax 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.¹ CLBr is chemically 3-[(2-hydroxy-2,2-diphenylacetyl)oxy]-1-methyl-1-azabicyclo [2.2.2] octan-1-ium bromide (Figure 1a), 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.⁶ CLBr is official in United state pharmacopoeia XXX⁶. Chemically PNT is, (RS)-5-(Difluoromethoxy) -2[(3,4dimethoxypyridin -2 yl)methyl sulfinyl]-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.² and CDZ is chemically 7-Chloro-N-methyl-5-phenyl-3H-1,4-benzodiazepin-2-amine 4-oxide (Figure 1c), which is an anxiolytic & agonist at specific benzodiazepine receptor, which are inhibitory in nervous system, Chlordiazepoxide 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 Pharmacopoeia⁵, United States Pharmacopoeia⁷ and Indian Pharmacopoeia.^{3,4}

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.⁸⁻⁵⁵

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 Guidelines⁵⁶ 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, ULTRAX, 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 (Muttentz, 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 range for each of drug on a single plate having concentration of 600 to 1600 ng/spot for CLBr, 1200 to 3200 ng/spot for CDZ & 4800 to 12800 ng/spot for PNT. Plate was developed using above mentioned conditions. Plots of peak area vs. concentration for three drugs were obtained.

Analysis of marketed formulation

Commercially marketed formulations of ULRAX were purchased. Twenty capsules were weighed carefully, equivalent amount of solid content was weighed accurately. A quantity equivalent to 12.5 mg CLBr, 25 mg CDZ and 100 mg was accurately weighed and transferred to volumetric flask of 25 ml capacity. 15 ml of methanol was transferred to this volumetric flask and sonicated for 20 min to dissolve the drug. Resulting solution was filtered through whattman filter paper (0.45µ) in to a 25ml volumetric flask. The flask was shaken and volume was made up to the mark with methanol to give a solution containing 500 µg/ml of CLBr, 1000 µg/ml of CDZ & 4000 µg/ml of 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 of CLBr, 400 µg/ml of CDZ & 1600 µg/ml of PNT. 5 µl of this aliquot was applied on TLC plate, developed, dried in air and photometrically analyzed as described above which containing 1000 ng/spot of CLBr, 2000ng/spot CDZ and 8000 ng/spot of PNT. From the peak area obtained in the densitogram, the amounts of three drugs were calculated.

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.

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 R_f 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 σ = the standard deviation of y-intercepts of regression lines, S = 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 (R_f) values of 0.54 for

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

System suitability Parameter	CLBr	PNT	CDZ
Peak Purity	0.9999	0.9998	0.9993
Rf value	0.54	0.66	0.76

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

Concentration (ng/spot)	Area Mean $\pm$ S.D. (n=6)	C.V.
600	1003.38 $\pm$ 2.2515	0.22
800	1564.68 $\pm$ 3.2027	0.20
1000	2133.70 $\pm$ 5.1571	0.24
1200	2678.48 $\pm$ 11.1965	0.41
1400	3138.50 $\pm$ 7.0279	0.22
1600	3786.03 $\pm$ 4.3292	0.11

Result of calibration readings for PNT by HPTLC method

Concentration (ng/spot)	Area Mean $\pm$ S.D. (n=6)	C.V.
4800	2971.66 $\pm$ 7.5425	0.25
6400	4132.60 $\pm$ 6.4149	0.15
8000	6254.66 $\pm$ 5.4825	0.087
9600	8190.41 $\pm$ 5.7422	0.070
11200	10472.95 $\pm$ 10.1699	0.097
12800	12136.5 $\pm$ 7.8467	0.064

Result of calibration readings for CDZ by HPTLC method

Concentration (μ g/spot)	Area Mean $\pm$ S.D. (n=6)	C.V.
1200	1157.91 $\pm$ 6.9127	0.59
1600	2086.1 $\pm$ 5.7720	0.27
2000	2861.26 $\pm$ 8.4582	0.29
2400	3715.08 $\pm$ 9.8692	0.26
2800	4530.9 $\pm$ 7.1484	0.15
3200	5591 $\pm$ 6.0246	0.10

Table 3: Determination of Accuracy

% Level	Amount Added (ng/spot)			Mean of Amount Recovered (ng/spot) (n=3)			% Mean Recovery $\pm$ S.D (n=3)		
	CLBr	PNT	CDZ	CLBr	PNT	CDZ	CLBr	PNT	CDZ
50	600	4800	1200	597.8	1797.8	1197.367	99.63 $\pm$ 0.3064	99.87 $\pm$ 0.2030	99.77 $\pm$ 0.2705
100	1200	9600	2400	1196.4	4802.8	2397.267	99.70 $\pm$ 0.3538	100.05 $\pm$ 0.1011	99.88 $\pm$ 0.1934
150	1800	12800	3600	1798.2	9598.2	3597.767	99.89 $\pm$ 0.2289	99.97 $\pm$ 0.0602	99.93 $\pm$ 0.1588

Table 4: Summary of validation Parameters of HPTLC

Parameters	CLBr	PNT	CDZ
Recovery %	99.63 – 99.89	99.87 – 100.05	99.77–99.93
Repeatability (C.V., n=6)	0.60	0.11	0.29
Precision (C.V.)			
Intra - day (n=3)	0.17 – 0.18	0.025 – 0.107	0.019–0.039
Inter - day (n=3)	0.12 – 0.31	0.012 – 0.074	0.039–0.043
Limit of Detection (μ g/ml)	4.6725	30.81165	12.61327
Limit of Quantitation (μ g/ml)	14.1583	93.36863	38.22203
Specificity	Specific	Specific	Specific
Robustness	Robust	Robust	Robust
Solvent suitability	Suitable for 24hr	Suitable for 24hrs	Suitable for 24hr

Table 5: Assay result of marketed formulation

Formulation	Drug	Amount Taken (ng/spot)	Amount Found (ng/spot) (n = 3)	Labeled Claim (mg)	Amount found per Capsule (mg)	% Label claim $\pm$ SD
Ultrax (capsule)	CLBr	1000	1000.72	2.5	2.59	101.63 $\pm$ 0.8946
	CDZ	2000	2000.58	5	5.19	100.19 $\pm$ 0.5431
	PNT	8000	8000.61	20	20.25	100.29 $\pm$ 0.4441

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 220nm

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

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

Linearity

Linear correlation was obtained between peak areas and concentrations of CLBr in the range of 600-1600 ng/spot with $R^2=0.998$, 1200-3200ng/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, 12.61327ng/spot and 30.81165 ng/spot respectively. LOQ values for CLBr, CDZ and PNT were found to be 14.1583 ng/spot, 38.22203ng/spot and 93.36863ng/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.

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