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Statistical Evaluation of NP-HPTLC and RP-HPTLC Method for the Simultaneous Estimation of Cinitapride and Pantoprazole in Bulk and in Capsule Formulation

*Vijay K. Patil **Jineetkumar B. Gawad

*,** Research Scholar, Department of Pharmaceutical Chemistry, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, Dist: Dhule - 425 405 Maharashtra, India.

ABSTRACT

A simple, rapid, selective and sensitive NP- HPTLC and RP-HPTLC/ densitometry method was developed and validated for the simultaneous estimation of cinitapride and Pantoprazole. For normal phase Ethyl acetate: Methanol (7: 3: v/v) and methanol: water: triethylamine (8:2:0.5 v/v) as a mobile phase in normal and reverse phase respectively. Both analyses were scanned with a camag scanner-3 at 280 nm. In normal phase Rf values of cinitapride and Pantoprazole were found to be 0.50 ± 0.02 and 0.72 ± 0.02 respectively and for reverse phase Rf values of cinitapride and Pantoprazole were found to be 0.36 ± 0.02 and 0.63 ± 0.02 respectively Linearity was studied in the concentration range of 300 to 1800 ng band^{-1} for cinitapride and 4000 to 24000 ng band^{-1} for Pantoprazole for both the methods with correlation coefficient 0.999 and 0.999 cinitapride and Pantoprazole for both normal phase and 0.998 and 0.999 for reverse phase respectively. The result obtained shown that the method best fits for estimation of drug in capsule formulation and thus can be used for its routine analysis. The newly developed method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness.

Keywords : Cinitapride, Pantoprazole, NP- HPTLC, RP-HPTLC, ANOVA and Student's t- test.

1. Introduction

Cinitapride, (CIN) chemically 4-amino-*N*-[3-(Cyclohexan-1-yl-methyl)-4-piperidinyl]-2-ethoxy-5- nitrobenzamide (Figure 1), is a substituted benzamide gastroenteric prokinetic agent acting via complex, but synergistic effects on serotonergic 5-HT2 (inhibition) and 5-HT4 (stimulation) receptor and dopaminergic D2 (inhibition) receptors in the neuronal synapses of the myenteric plexi1-3. Detailed literature survey reveals various methods for its determination such as Polarographic method and LC-MS/MS methods for its determination in plasma UV-spectrometric method Stress induced HPLC method.

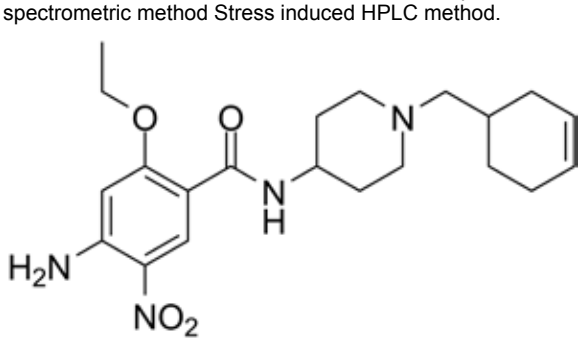

Figure 1. Structure of Cinitapride (CIN)

Pantoprazole (PAN), chemically as: sodium 5- (difluoromethoxy) - 2- [3,4 - dimethoxy - 2 - pyridyl) methylsulfinyl] - 1H benzimidazole sesquihydrate (Fig. 1). Pantoprazole sodium sesquihydrate (PAN) is widely used as anti-ulcer drugs (proton pump inhibitors) through inhibition of hydrogen-potassium adenosine triphosphatase (H+/ K+ - ATPase) in gastric parietal cells PAN reduces the gastric acid secretion regardless of the nature of stimulation. Methods for the determination of PAN in pharmaceutical formulations and biological materials which have been reported previously included high performance liquid chromatography (HPLC) capillary electrophoresis and spectrophotometric determination.

In present work, an effort has also been made to estimate CIN and PAN in bulk and in capsule dosage form by NP-HPTLC and RP-HPTLC method and validated according to ICH guidelines.

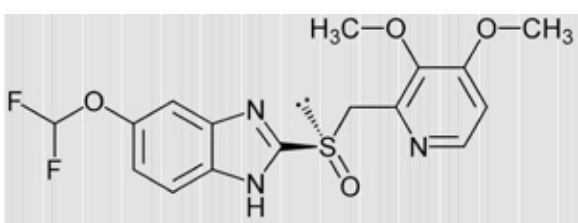

Figure 2. Structure of Pantoprazole (PAN)

2. Experimental

2.1. Materials and Reagents

Cinitapride and Pantoprazole were provided as a gift sample by Cadila Health care Ahmadabad, Gujarat (India). All other reagents and solvents utilized were of analytical (AR) grade and are from Merck Chemicals Ltd, Mumbai (India).

2.2. Instrumentation and chromatographic condition

In normal phase, chromatography was performed on 20 cm ×10 cm aluminium-backed TLC plates coated with 0.20 mm layers of silica gel 60 F254S (Merck, Darmstadt, Germany, supplied by Merck India, Mumbai, India) while in reverse phase, 20 cm ×10 cm aluminium-backed RP-TLC plates coated with 200-µm layers of silica gel 60 RP-18 F254S were used. Before chromatography the plates were prewashed with methanol and activated at 105^{0}C for 5 min in oven. The samples were applied as 6 mm wide bands with the help of Linomat 5 sample applicator (Muttenz, Switzerland) fitted with a 100-µl sample syringe (Hamilton, Bonaduz, Switzerland). The plate was developed in a pre-saturated Camag twin trough glass chamber (20 cm × 10 cm). In normal and reverse, Ethyl acetate: Methanol (7: 3 *v/v*) and Methanol: Water: Triethylamine (6: 4: 0.5 *v/v*), respectively were used as mobile phases and for optimized chamber saturation time was 15 min and

30 min, respectively. The plates were developed to a distance of 8.0 cm and scanned densitometrically using Camag TLC Scanner 3 equipped with win CATS software version 1.3.0 at 280 nm for both method. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 200- 400 nm. Evaluation was performed using peak area with linear regression.

2.3. Preparation of standard and sample solutions
Indepandent stock solution of 1000 µg/ml of CIN and 10000 µg/ml of PAN were prepared in methanol.

2.4. Preparation of calibration curves
From each stock standard solution, 0.3 – 1.8 ml of CIN and 0.4 – 2.4 ml of PAN were transferred into six 10 ml volumetric flasks separately and volume was made up to the mark with methanol From each volumetric flask a volume 1 µl was applied on TLC plate to obtain series of concentration 300 - 1800 ng/band of CIN and 4000 - 24000 ng/band of PAN. The plates were developed and scanned as described. Each standard in six replicates was analyzed and peak areas were recorded Calibration curves of CIN and PAN were plotted separately of peak area *vs.* respective concentration.

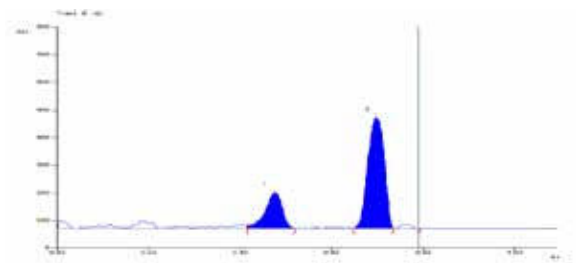

Figure 3. Densitogram of Standard CIN and PAN R_f 0.50 and 0.72 respectively (NP)

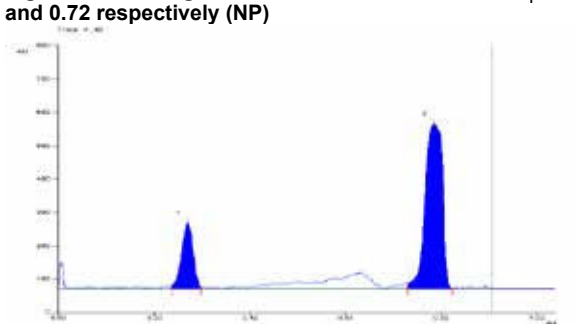

Figure 4. Densitogram of Standard CIN and PAN R_f0.36 and 0.76 respectively (RP)

2.5. NP-HPTLC versus RP- HPTLC
Two test differences between the proposed NP- HPTLC and RP- HPTLC method statistical tests were performed for the level of confidence 95% (P = 0.05) . Two way ANOVA was applied to test both method–sample interactions (interaction variation) and differences in the method precision since the within cell variation (residual variation) is greater than interaction variation as well as plate variations, the method–sample interaction and the differences between the methods are not significant.

3. Results and Discussion
3.1. Development of optimum mobile phase
An NP- HPTLC and RP-HPTLC methods was optimized with a view to develop an accurate and reproducible method so as to resolve drugs. Optimization of method was done by altering almost all the chromatographic conditions and the effect on Rf and peak shape were monitored for the drugs selected i.e. CIN and PAN. The samples were applied on the plates as bands, under continuous flow of nitrogen, by means of a CAMAG (Muttenz, Switzerland) Linomat-5 sample applicator fitted with a 100-µL syringe. Finally, for normal phase, Ethyl acetate: Methanol (7: 3 *v/v*) showed well- defined and resolved peaks when the chamber was saturated with mobile phase for 15 min at room temperature. Both the peaks were well resolved and no tailing observed when plate was scanned at 280 nm. And for reverse phase Methanol: Water: Triethylamine in the ratio (8: 2: 0.5 *v/v*). The chamber saturation time was 30 min. The R_f for CIN and PAN were found to be 0.36± 0.02 and 0.63 ± 0.02, respectively.

3.2. Calibration curves
The linear regression data for the calibration curves (n=5) as shown in table 1 showed a good linear relationship over the concentration range 300 - 1800 ng/band of CIN and 4000 - 24000 ng/band of PAN. The plates were developed and scanned as described. No significant difference was observed in the slopes of standard curves (ANOVA, P > 0.05)

3.3. Validation
The method was validated by establishing linearity, accuracy, inter - day and intra - day precision of measurement of sample application. The limit of detection and limit of quantification were also determined.

3.3.1. Linearity
Linearity was studied in the concentration range from 300 – 1800 ng/band for CIN and 4000 – 24000 ng/band for PAN for both methods. The drugs showed good linearity in the tested range. The regression co-efficient values for CIN and PAN were found to be r^2 = 0.999 and 0.999 in normal and 0.998 and 0.999 in reverse phase respectively Table 1.

Table 1 Linearity of CIN and PAN for proposed method (n=6)

Parameters	Normal phase		Reverse phase	
	CIN	PAN	CIN	PAN
Linearity range (ng/band)	300 – 1800	4000 - 24000	300 – 1800	4000 – 24000
Slope	2.064	0.778	1.566	0.539
Intercept	621.2	991.9	766.4	4975
Correlation Coefficient (r^2)	0.999	0.999	0.998	0.999

3.3.2. Precision
For quantitative estimation of the mixture, three series (600, 900, 1200 ng/band and 8000, 200, 16000 ng/band for CIN and PAN respectively) were prepared for both methods and %RSD was found to be <2% Table 2.

Table 2 Precision of CIN and PAN for proposed method (n=3)

Drugs	Conc. [ng/spot]	Intra-day precision		Inter-day precision	
		Mean ± S.D.	% RSD [n = 3]	Mean ± S.D.	% RSD [n = 3]
Normal phase CIN	600	1846 ± 8.71	0.47	1867 ± 21.65	1.15
	900	2451 ± 21.70	0.88	2474 ± 9.84	0.39
	1200	3138± 34.23	1.09	3136 ± 24.11	0.76
PAN	8000	7200 ± 54.99	0.76	7230 ± 64.83	0.89
	12000	10173 ± 41.50	0.40	10193 ± 41.50	0.40
	16000	13627± 94.56	0.69	13637 ± 98.65	0.72
Reverse phase					

CIN			0.86	9158 ± 60.00	0.65
			0.89	11235 ± 80.72	0.71
			1.15	13476 ± 56.32	0.41
PAN			0.50	7230 ± 64.30	0.89
			0.71	10193 ± 41.50	0.40
			1.22	13637 ± 98.65	0.72

3.3.3. Accuracy

The accuracy of the experiment was established by spiking pre-analyzed sample with known amounts at three different concentration levels i.e. 80, 100 and 120 % of the drug in the tablet. The spiked samples were then analyzed for three times. The mean recovery is found to be within acceptable limits, indicating the method is accurate for both methods Table 3.

Table 3 Recovery studies

Method	Drugs	Initial amount (ng per band)	Amount added (%)	% recovery	%RSD [n=3]
Normal Phase	CIN	600	80	100.07	0.95
		600	100	100.81	0.40
		600	120	99.02	0.38
	PAN	8000	80	100.31	0.51
		8000	100	100.78	0.45
		8000	120	100.65	0.68
Reverse Phase	CIN	600	80	100.03	0.92
		600	100	98.97	0.85
		600	120	100.93	1.28
	PAN	8000	80	100.93	0.43
		8000	100	101.59	0.58
		8000	120	99.51	0.68

3.3.4. Analysis of capsules formulation

Twenty capsules (Cintodac) (each contained 3 mg CIN and 40 mg PAN) were accurately weighed and finely powdered. A quantity of the powder containing weight equivalent to 7.5 mg CIN and 100 mg PAN was transferred to a 100 ml volumetric flask and methanol (75 ml) was added followed by ultrasonication for 10 min, volume was adjusted to mark and filtered using 0.45 µm filter (Mill filter, Milford, MA) and 6 ml of filtrate was further diluted to 10 ml with methanol. Appropriate volume 6 µl, was spotted for assay of CIN and 80 µl MS. The plates were developed and scanned. The low % R.S.D. value indicated the suitability of this method for routine analysis of cinitapride and Pantoprazole in pharmaceutical dosage forms. For normal and reverse phase the % amount found were 100.15, 100.70 for cinitapride and 101.02, 100.45 for Pantoprazole respectively. And % RSD found to be ≤ 2.

3.3.5. Specificity

3.3.5.1 Normal phase

The specificity of the method was ascertained by analyzing standard drug and sample. The mobile phase resolved both the drugs very efficiently. The peak purity of CIN extracted from capsule and standard CIN was tested at the peak - start (S), peak - apex (A) and at the peak - end (E) position. The peak purity of PAN was tested by correlating the spectra's of PAN at the peak - start (S), peak - apex (A) and at the peak - end (E) positions (Fig. 5).

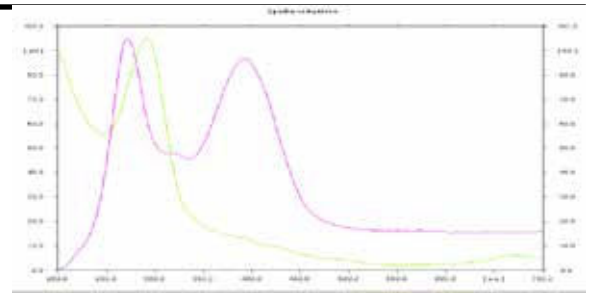

Figure 5. The peak purity of CIN extracted from capsule and standard CIN was tested at the peak - start (S), peak - apex (A) and at the peak - end (E) position (NP)

3.3.5.2. Reverse phase

The mobile phase designed for the method resolved both the drugs very efficiently. The Rf value of CIN and PAN was found to be 0.36 and 0.63, respectively. The peak purity of CIN extracted from capsule and standard CIN was tested at the peak - start (S), peak - apex (A) and at the peak - end (E) positions. The peak purity of PAN was tested by correlating the spectra's of PAN at the peak - start (S), peak - apex (A) and at the peak - end (E) positions (Fig. 6).

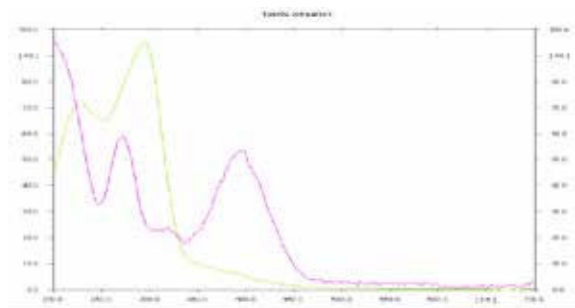

Figure 6. The peak purity of CIN extracted from capsule and standard CIN was tested at the peak - start (S), peak - apex (A) and at the peak - end (E) positions (RP)

3.3.6. Ruggedness and Robustness

Ruggedness of the both method was performed for CIN and PAN by two different analysts maintaining similar experimental and environmental conditions. Robustness was performed by introducing various small deliberate changes in the existing chromatographic conditions and effects on the results were examined for both methods Table 4.

Table 4 Validation parameters

Method	Normal		Reverse	
Parameters	CIN	PAN	CIN	PAN
Linearity (correlation coefficient)	0.999	0.999	0.998	0.999
Slope	2.064	0.778	1.566	0.529
Intercept	621.2	991.9	776.4	4968
Ruggedness [% RSD]				
Analyst I [n=6]	0.47	0.89	0.54	0.31
Analyst II [n=6]	0.54	0.76	0.30	0.59
Robustness [% RSD] [n=6]				
Mobile phase composition	0.41	0.84	0.67	0.49
Duration of saturation time	0.55	0.79	0.55	0.23
Mobile phase volume	0.49	0.96	0.66	0.62
Development distance	0.68	0.74	0.53	0.40
Sensitivity				
Limit of Detection (ng)	30.19	400.65	31.12	401.12
Limit of Quantitation (ng)	90.57	1201.95	93.36	1203.36
Precision [%RSD]				

Intra-day [n = 3]	0.47 – 1.09	0.39 – 1.15	0.86 - 1.15	0.41 – 0.75
Inter-day [n = 3]	0.40 – 0.76	0.40 – 0.89	0.50 - 1.22	0.44 – 0.67
Repeatability [n = 6]	1.00	0.77	0.52	0.40

3.3.7. Sensitivity

The sensitivity of measurements of CIN and PAN by the use of the proposed method was estimated in terms of the Limit of Quantitation (LOQ) and the lowest concentration detected under the chromatographic conditions as the Limit of Detection (LOD).

LOQ and LOD were calculated by the use of equation LOD = 3.3 x N/B and LOQ = 10 x N/B, where 'N' is standard deviation of the peak areas of the drugs (n=3), taken as a measure of noise, and 'B' is the slope of the corresponding calibration curve. For normal and reverse phase the LOD and LOQ were 30.19, 90.57 and 31.12, 93.36 for cinitapride and 400.65, 1201.95 and 401.12, 1203.36 for Pantoprazole.

3.4. NP-HPTLC versus RP- HPTLC

Two test means (averages) a paired *t*-test was applied. The test removes any variations between samples the obtained value of *t* stat is lower than two tail, which leads to the conclusion that there is no significant difference between the means. The results of two way ANOVA and paired *t*-test are given in (Table 5b and 5c) respectively.

Table 5 (a) Two-way ANOVA test of CIN and PAN determination in six indePANdent samples by NP-HPTLC and RP- HPTLC

Sample	NP- HPTLC		RP- HPTLC	
	CIN	PAN	CIN	PAN
1	98.78	98.90	101.57	101.11
2	100.51	98.57	101.05	101.93
3	99.93	100.83	101.26	98.98
4	99.10	100.19	98.98	100.56
5	101.15	102.37	100.02	98.79
6	100.41	100.03	101.36	101.32
Mean	99.98	100.15	100.70	100.87

Table 5 (b) ANOVA: Two factors with replications

Particulars	Normal (CIN)	Reverse (CIN)	Normal (PAN)	Reverse (PAN)
Count	6	6	6	6
Sum	600.92	606.17	604.2	602.05
Average	99.98	100.15	100.70	100.87
% of total variation	2.66	9.11	37.18	34.03
P – value	0.58	0.29	0.026	0.028
T	0.5726	1.09	2.66	2.48
Stand Deviation	1.3	0.84	0.82	0.80
Stand error	0.52	0.31	0.31	0.30
Significance	Not	Not	Not	Not
F value	1.20	1.17	1.72	2.15

Table 5 (c) Average results of CIN and PAN determination by NP- HPTLC and RP- HPTLC and their correlation by paired *t*-test

Particulars	CIN	PAN
Count	12	12
Sum	1207.09	1206.25
Average	100.59	100.52
% of total variation	6.95	9.83
P – value	0.36	0.27
T	0.94	1.14
Stand Deviation	1.25	0.62
Stand error	0.47	0.23
Significance	Not significant	Not Significant
F value	1.88	3.51

4. Conclusion

The modalities adopted in experiment were successfully validated as per ICH guidelines. The proposed normal and reverse phase was validated by preliminary analysis of standard sample and by recovery studies for the determination of CIN and PAN in bulk and in capsule dosage form. For normal the percentage of average recoveries for CIN and PAN was obtained 99.97 and 100.58 respectively and for reverse the percentage of average recoveries for CIN and PAN was obtained 100.31 and 100.68 respectively. The proposed normal and reverse phase provide simple, rapid, accurate, precise and specific. It was observed that all the values are within the limits. The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters and let us to the conclusion that it could be used for the rapid and reliable determination of CIN and PAN in capsule formulation.

Six real samples of capsule were determined simultaneously by NP - HPTLC and RP - HPTLC methods and the results were correlated. Statistical tests indicate that the proposed NP - HPTLC and RP - HPTLC methods reduce the duration of analysis and appear to be equally suitable for routine determination of CIN and PAN simultaneously in pharmaceutical formulation.

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