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Original Article

RP-HPLC ASSAY METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF DILOXANIDE FUROATE AND ORNIDAZOLE IN TABLETS

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ABSTRACT

Objective: To develop an accurate, precise and linear Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in tablets and validate as per ICH guidelines.

Methods: The optimized method uses a reverse phase column, Waters Symmetry C18 (250 X 4.6 mm; 5µ), a mobile phase of potassium dihydrogen ortho phosphate buffer (20 mM, pH2.3): acetonitrile in the proportion of 40:60 v/v, flow rate of 1.0 ml/min and a detection wavelength of 230 nm using a UV detector.

Results: The developed method resulted in Diloxanide furoate eluting at 5.85 min and Ornidazole at 2.85 min. Diloxanide furoate exhibited linearity in the range 22.5-67.5µg/ml, while Ornidazole exhibited linearity in the range 15-45µg/ml. The precision is exemplified by relative standard deviations of 1.58% for Diloxanide furoate and 1.53% for Ornidazole. Percentage Mean recoveries were found to be in the range of 95-105 by percentage method during accuracy studies.

Conclusion: A rapid, simple, accurate, precise and linear RP-HPLC method was developed for simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in tablets and validated as per ICH guidelines. Hence it can be used for the routine analysis of Diloxanide furoate and Ornidazole in tablets in various pharmaceutical industries.

Keywords: RP-HPLC, Diloxanide furoate, Ornidazole, Method development, Validation.

INTRODUCTION

Ornidazole (fig. 1) chemically is 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole. It has a molecular formula of $C_7H_{10}ClN_3O_3$ and a molecular weight of 219.625 g/mol. Ornidazole is a derivative of 5-nitro imidazole used as an anti-infective agent [1]. Ornidazole is converted into an active form by reduction of its nitro group to amine that binds to microbial DNA and prevents nucleic acid formation, belonging to the class of bacteriostatic [2]. Ornidazole is used for the treatment of bacterial vaginosis, trichomoniasis, genitourinary infections in women and men, amoebiasis, giardiasis. It is also used in infections against anaerobic bacteria and in the treatment of prophylaxis during surgical interventions, particularly those involving the colon, and in gynaecological operations [2]. Ornidazole has been successfully employed in combination with other drugs for peptic ulcers, few types of gastritis, stomach cancers, rheumatoid arthritis [3] and in the prophylaxis of Crohn's disease [4].

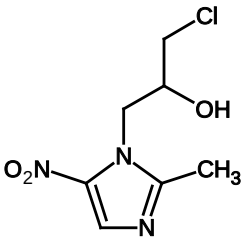

Fig. 1: Structure of ornidazole

Diloxanide furoate (fig. 2) chemically is 4-(N-methyl-2,2-dichloroacetamido) phenyl-2-furoate having the molecular formula as $C_{14}H_{11}Cl_2NO_4$ and the molecular weight as 328.147 g/mol [5]. It is an effective drug for the treatment of asymptotic persons who are passing cysts of *Entameba histolytica* [6]. It acts principally in the bowel lumen and is used in the treatment of the intestinal amoebiasis. Diloxanide furoate has been used in the treatment of the asymptotic carriers of *Entameba histolytica* [6] and is excellent amoebicide for cyst passers [7, 8].

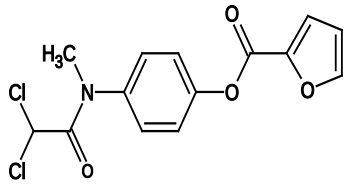

Fig. 2: Structure of Diloxanide furoate

A detailed literature survey reveals that there exists literature on chromatographic methods for Ornidazole alone and in combination with other drugs [9-16] and similarly Diloxanide furoate in combination with other drugs [17-22] in various matrices. Only one literature on RP-HPLC assay method for the simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in pharmaceutical dosage forms using mixed phosphate buffer at pH 6.0 is reported, while there is one more research work from our analysis lab is accepted using triethyl ammonium phosphate buffer (pH 2.3) [23,24]. As there is no literature reported using only potassium diydrogen orthophosphate buffer at acidic pH, as aqueous media along with acetonitrile as mobile phase, the objective of our study to understand the feasibility as a new and a rapid RP-HPLC validated method for the simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in tablets using potassium diydrogen orthophosphate buffer (pH 2.3) as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure sample of Diloxanide furoate and Ornidazole with purities greater than 95% were obtained as gift samples from

Chandra Labs, Hyderabad, India and tablet formulation [Amicline plus] was procured from Medplus pharmacy, Hyderabad, India with labelled amount 375 mg and 250 mg of Diloxanide furoate and Ornidazole respectively. Acetonitrile (HPLC grade) was obtained from Sigma aldrich (Hyderabad, India), water (HPLC grade), Potassium dihydrogen orthophosphate (AR grade), ortho phosphoric acid (AR Grade) were obtained from SD Fine chemicals (Hyderabad, India), 0.22 and 0.45µm Nylon membrane filters were obtained from Spincotech Private Limited, Hyderabad, India.

Instrument

HPLC analysis was performed on Shimadzu LC-20AD Prominence Liquid Chromatography comprising a LC-20AD pump, Shimadzu SPD-20A Prominence UV-VISIBLE detector and a reverse phase C18 column, Waters Symmetry (250X4.6 mm; 5µ). A manually operating Rheodyne injector with 20µL sample loop was equipped with the HPLC system. The HPLC system was equipped with "SPINCHROM" software. A double beam UV-visible spectrophotometer (Shimadzu, model UV-1800) having two matched quartz cells with 1 cm light path and loaded with UV probe software (version 2.41) was used for recording of spectra and measuring absorbance. An electronic analytical weighing balance (0.1 mg sensitivity, Shimadzu AY 220), digital pH meter (DELUX model 101), a sonicator (sonica, model 2200 MH).

Method

Selection of wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of Ornidazole and Diloxanide furoate. Suitable wavelength selected for simultaneous estimation is 230 nm as both the drugs were more sensitive at 230 nm (fig. 3-4).

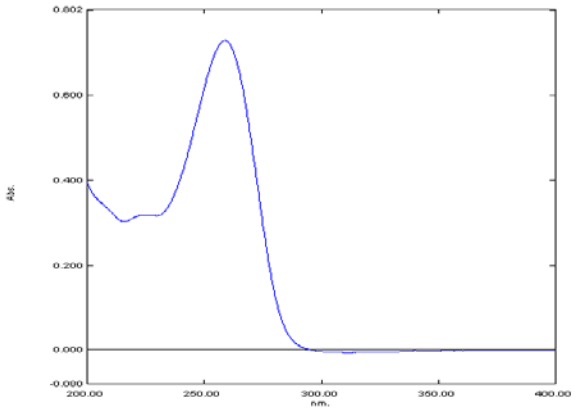

Fig. 3: UV spectrum of standard Diloxanide furoate

Chromatographic conditions

The developed method uses a reverse phase C18 column, Waters Symmetry C18 (250 X 4.6 mm; 5µ), mobile phase of potassium dihydrogen orthophosphate buffer (pH 2.3): acetonitrile in the proportion of 40:60 v/v. The mobile phase was set at a flow rate of 1.0 ml/min and the volume injected was 20µl for every injection. The detection wavelength was set at 230 nm.

Buffer preparation

The buffer solution was prepared by weighing 2.72g of potassium dihydrogen orthophosphate (KH_2PO_4) and transferring to 1000 ml of HPLC grade water to get 20 mM buffer strength, which was adjusted to pH 2.3 using 30% v/v ortho phosphoric acid. Later the buffer was filtered through 0.45 µm nylon membrane filter.

Mobile phase preparation

The mobile phase was prepared by mixing buffer and acetonitrile in the ratio of 40:60 v/v and later it was sonicated for 10 min for the removal of air bubbles.

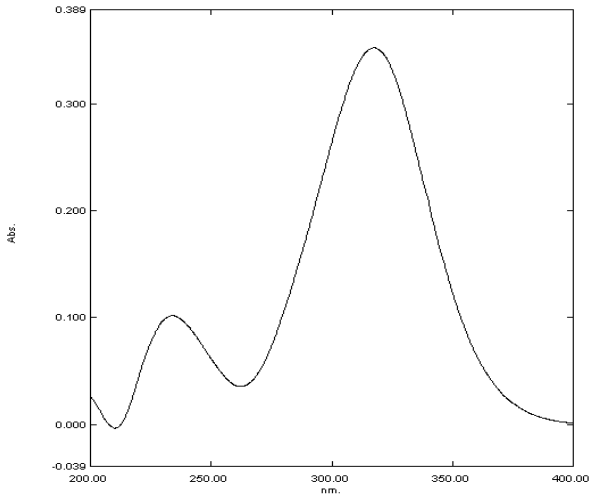

Fig. 4: UV spectrum of standard Ornidazole

Diluent

Diluent used is the mobile phase itself.

Preparation of standard solution

10 mg of Ornidazole and 15 mg of Diloxanide furoate were weighed accurately in 100 ml of volumetric flask and dissolved, in 80 ml of mobile phase and volume was made up with mobile phase. From stock solution 30µg/ml of Ornidazole and 45µg/ml of Diloxanide furoate were prepared further by pipetting out 3 ml and making up the solution to 10 ml with mobile phase. This is treated as working standards solution, 100% target concentration.

Preparation of sample solution

Ten tablets were weighed and taken into a mortar, crushed and then uniformly mixed. Test stock solutions of Ornidazole (1000µg/ml) and Diloxanide furoate (1500µg/ml) were prepared by transferring weight equivalent to 100 mg of Ornidazole and 150 mg of Diloxanide furoate to 80 ml of mobile phase which is sonicated for 10 min and later made up to 100 ml with mobile phase. This solution was filtered using 0.22micron syringe filter. 0.3 ml of the above stock solution was pipetted out and made up to 10 ml to get working sample solution equivalent to a concentration of 30µg/ml for Ornidazole and 45µg/ml for Diloxanide furoate, concentrations equal to 100% target concentration.

RESULTS AND DISCUSSION

Method development

A Reverse phase HPLC method was developed keeping in mind the system suitability parameters i.e. resolution factor (Rs) between peaks, Peak Asymmetry (A), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of Ornidazole at 2.85 min and Diloxanide furoate at 5.85 min. Fig. 5-6 represents chromatograms of standards solution mixture and sample respectively. The total run time is 8 min. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (RT), number of theoretical plates (N), peak resolution (Rs) and Peak Asymmetry (A) were evaluated for standards at working concentration. The results given in table 1 were within acceptable limits.

In order to test the applicability of the developed method to a commercial formulation, 'Amicline plus' tablets was chromatographed at working concentration and it is shown in fig. 6. The sample peaks were identified by comparing the relative retention times with the standard solutions (fig. 5-8). System suitability parameters were within the acceptance limits, ideal for the chromatographed sample. Integration of separated peak area

was done and each drug concentration was determined by using the peak area concentration relationship obtained in the standardization step. The protocol affords reproducible quantification of the two drugs with error less than 10%, which is the standard level in any pharmaceutical quality control.

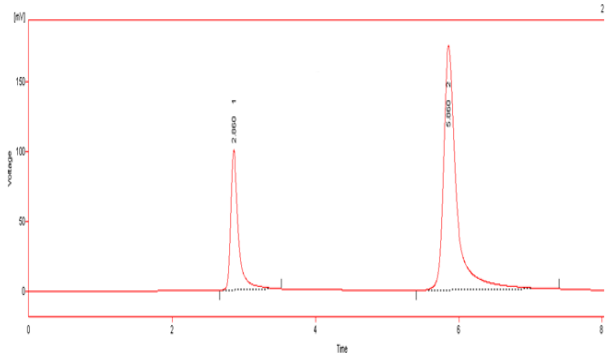

Fig. 5: Typical chromatogram of mixture of standard solutions

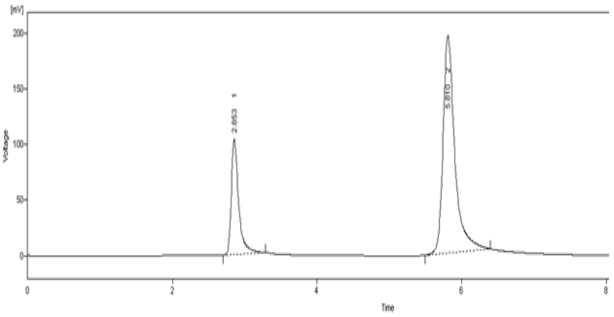

Fig. 6: Typical chromatogram of sample solution

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines [25] for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, intra-day precision, Ruggedness, limit of detection (LOD) and limit of quantitiation (LOQ).

Specificity

Peaks obtained in the standards solution and sample solution at working concentrations are only because of the drugs as blank has no peak at the retention time of Ornidazole and Diloxanide furoate standards. Accordingly it can be concluded that, the method developed is said to be specific.

Precision

System precision

Six replicate injections of the mixture of standards solution at working concentration showed % RSD (Relative Standard Deviation) less than 2 concerning peak area for both the drugs, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in tables 2-3.

Method precision

Method precision was determined by performing assay of sample under the test of repeatability (Intraday precision) at working concentrations.

Repeatability (Intraday precision)

Six consecutive injections of the sample from the same homogeneous mixture at working concentration showed % RSD less than 2 concerning % assay for both the drugs which indicate that the method developed is method precise by the test of repeatability and hence can be understood that the method gives consistently reproducible results (tables 4, 5).

Table 1: System suitability studies results

Parameters	Acceptance Limits	Ornidazole	Diloxanide furoate
Retention time (min)	-	2.85	5.85
Resolution factor (Rs)	Not less Than 2	-	11.8
Number Of Theoretical plates (N)	Not less Than 2000	4312	7117
Peak Asymmetry	Not More Than 2	1.82	1.83

Table 2: System precision results of ornidazole

n	RT	Peak Area
1	2.86	742.08
2	2.86	735.18
3	2.85	724.05
4	2.85	745.73
5	2.85	745.07
Average	2.85	738.426
SD	0.001	9.056
%RSD	0.035	1.22

Table 3: System precision results of Diloxanide furoate

n	RT	Peak Area
1	5.86	2218.673
2	5.86	2211.368
3	5.85	2211.358
4	5.84	2216.719
5	5.84	2200.011
Average	5.85	2211.6258
SD	0.007	7.256
%RSD	0.125	0.32

Table 4: Intraday precision results for Ornidazole

n	RT	Peak area	% Assay
1	2.86	754.466	100.6
2	2.86	737.589	98.88
3	2.85	747.825	100.26
4	2.85	719.373	96.93
5	2.85	738.269	98.09
Average	2.85		98.952
SD	0.001		1.52
% RSD	0.035		1.53

Table 5: Intraday precision results for Diloxanide furoate

n	RT	Peak area	% Assay
1	5.86	2239.353	100.24
2	5.86	2291.377	102.56
3	5.85	2299.476	102.93
4	5.84	2240.451	100.29
5	5.84	2205.504	99.24
Average	5.85	-	101.052
SD	0.007	-	1.6
%RSD	0.125	-	1.58

Linearity

Standard solutions of Diloxanide furoate and Ornidazole at different concentrations were prepared. Calibration curves (fig. 7-8) were constructed by plotting the concentration level versus corresponding peak area for both the drugs. The results show an excellent correlation between peak areas and concentration within the concentration range of 15-45µg/ml for Ornidazole and 22.5-67.5µg/ml for Diloxanide furoate (Tables 6-7). The correlation coefficients were greater than 0.995 for both the drugs, which meet the method validation acceptance criteria and hence the method is said to be linear for both the drugs.

Table 6: Calibration data for Diloxanide furoate

% Level	Concentration (µg/ml)	Peak area
50	22.5	1071.768
75	33.75	1724.797
100	45	2206.195
125	56.25	2782.528
150	67.5	3220.462
Regression equation		y=47.601x+59.102
Regression coefficient		0.9959

Table 7: Calibration data for Ornidazole

% Level	Concentration (µg/ml)	Peak area
50	15	330.944
75	22.5	541.632
100	30	698.95
125	37.5	866.609
150	45	1009.345
Regression equation		y=22.42x+16.78
Regression coefficient		0.9952

Accuracy

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of both the drugs at three different levels (50-150%). At each level, three determinations were performed. Percent mean recovery is calculated as shown in tables 8-9. The accepted limits of mean recovery are 95%-105% by percentage method and all observed data were within the required range, which indicates good recovery values and hence the accuracy of the method developed.

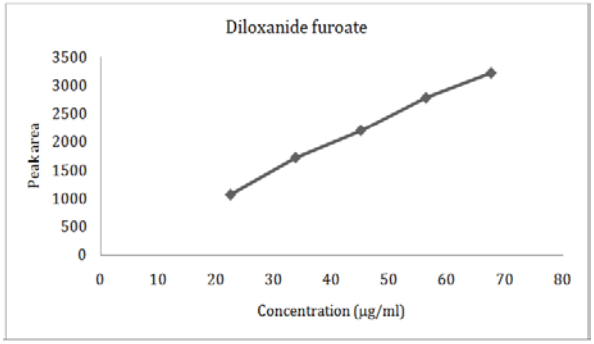

Fig.7: Linearity graph of Diloxanide furoate

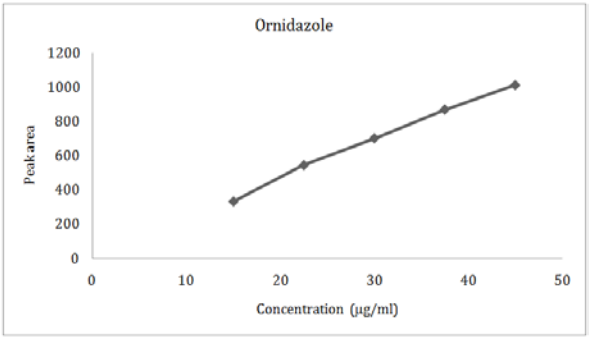

Fig. 8: Linearity graph of Ornidazole

Ruggedness

Ruggedness was evaluated by performing assay of the formulations by different analyst by injecting six consecutive injections of the sample at working concentration from the same homogeneous mixture of tablets. This study showed % RSD less than 2 concerning % assay for both the drugs which indicate that the method developed is rugged and hence can be understood that the method gives reproducible results irrespective of analyst (table 10).

Table 8: Recovery studies for Diloxanide furoate

% Level	% Recovery	% Mean recovery
50	102.23	
50	102.01	102.12
50	102.12	
100	100.24	
100	102.56	101.91
100	102.93	
150	101.28	
150	101.05	101.54
150	102.31	

Table 9: Recovery studies for Ornidazole

% Level	% Recovery	% Mean recovery
50	102.03	
50	100.56	101.05
50	100.56	
100	100.6	
100	98.88	99.91
100	100.26	
150	102.96	
150	102.05	102.65
150	102.96	

Table 10: Ruggedness results of Diloxanide furoate and Ornidazole

n	Diloxanide furoate	Ornidazole
	% Assay	% Assay
1	99.8	103.3
2	98.7	102.1
3	100.6	101.9
4	100.3	101.5
5	99.9	103.5
6	100.1	102.3
Average	99.9	102.4
S. D.	0.654	0.796
% RSD	0.654	0.77

CONCLUSION

A rapid, simple, accurate, precise and linear RP-HPLC method was developed for simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in tablets and validated as per ICH guidelines. Hence it can be used for the routine analysis of Diloxanide furoate and Ornidazole in tablets in various pharmaceutical industries.

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CONFLICT OF INTERESTS

Declared None

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