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Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS QUANTITATIVE 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 C18 column, ZODIAC (250 X 4.6 mm; 5µ), mobile phase consisting of mixed phosphate buffer (KH_2PO_4 and K_2HPO_4): acetonitrile in the proportion of 30:70 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 279 nm.

Results: The developed method resulted in Diloxanide furoate eluting at 4.293 min and Ornidazole at 3.34 min. Diloxanide furoate exhibited linearity in the range 90-210µg/ml, while Ornidazole exhibited linearity in the range 60-140µg/ml. The precision is exemplified by relative standard deviations of 0.97% for Diloxanide furoate and 0.3% for Ornidazole. Percentage Mean recoveries were found to be in the range of 98102, during accuracy studies. The limit of detection (LOD) for Diloxanide furoate and Ornidazole were found to be 8.80µg/ml and 6.68µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Ornidazole were found to be 26.6µg/ml and 20.25µg/ml respectively.

Conclusion: A simple, accurate, precise, linear and rapid RP-HPLC method was developed for simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in Amicline plus 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 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].

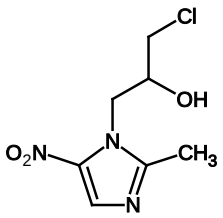

Fig. 1: Structure of Ornidazole

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].

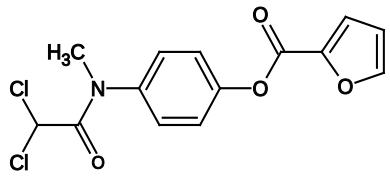

Fig. 2: Structure of Diloxanide furoate

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. While there is hardly any literature reported on RP-HPLC method development and validation for the simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in pharmaceutical dosage forms.

Hence we have explored in developing a new, accurate, precise, linear and a rapid isocratic RP-HPLC method for the simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in Amicline plus tablets and validate as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure sample of Diloxanide furoate and Ornidazole with purities greater than 99% 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 375mg and 250mg of Diloxanide furoate and Ornidazole respectively. Acetonitrile (HPLC grade) was obtained from Sigma aldrich (Hyderabad, India), water (HPLC grade), potassium dihydrogen ortho phosphate (KH_2PO_4) and dipotassium hydrogen ortho phosphate (K_2HPO_4) (AR grade), ortho phosphoric acid (AR Grade) were obtained from SD Fine chemicals (Hyderabad, India), 0.45µm Nylon membrane filters were obtained from Spincotech Private Limited, Hyderabad, India.

Instrument

HPLC analysis was performed on Shimadzu LC-20AT VP Liquid Chromatograph comprising a LC-20AT pump, Shimadzu SPD-20A UV-VISIBLE detector and a reverse phase C18 column, ZODIAC (250 X 4.6 mm; 5µ). A manually operating Rheodyne injector with 20 µL sample loop was equipped with the HPLC system. The HPLC system was controlled with "Spinchrom" software. A double beam UV-visible spectrophotometer Nicolet evolution 100 having two matched quartz cells with 1 cm light path was used for recording of spectra and measuring absorbance. An electronic analytical weighing balance (1mg sensitivity, Shimadzu BL220H), digital pH meter (Global digital) and sonicator (Citizen) were used in this study.

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 279 nm (Fig. 3-4).

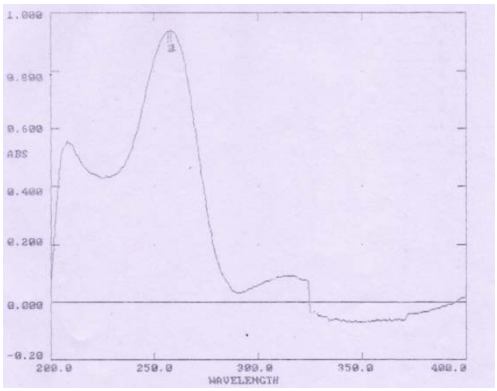

Fig. 3: UV spectrum of standard Diloxanide furoate

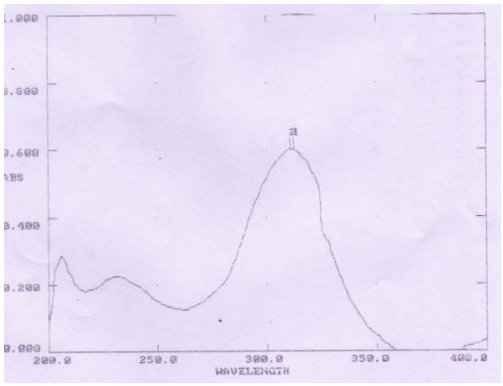

Fig. 4: UV spectrum of standard Ornidazole

Chromatographic conditions

The developed method uses a reverse phase C18 column, ZODIAC (250 X 4.6 mm; 5µ), mobile phase consisting of buffer: acetonitrile in the proportion of 30:70 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 279 nm.

Buffer preparation

1.625 gm of potassium dihydrogen ortho phosphate (KH_2PO_4) and 0.3 gm of dipotassium hydrogen ortho phosphate (K_2HPO_4) was weighed and dissolved in 100 ml of water and volume was made up to 1000 ml with water. Adjust the pH to 6.0 using ortho phosphoric acid. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

Mobile phase preparation

The mobile phase was prepared by mixing acetonitrile and buffer in the ratio of 70:30 v/v and later it was sonicated for 10 minutes for the removal of air bubbles.

Diluent

Diluent used is the mobile phase itself.

Preparation of stock and working standard solution for Diloxanide furoate

15mg of Diloxanide furoate was accurately weighed and taken in 10 ml clean and dry volumetric flask containing 8 ml of solvent and then the solution was made up to the mark using the solvent. This is considered as standard stock solution (1500µg/ml). 1 ml of the stock solution was pipetted out and made up to 10 ml to get a concentration 150µg/ml, treated as working standard, 100% target concentration.

Preparation of stock and working standard solution for Ornidazole

10mg of Ornidazole was accurately weighed and taken in 100 ml clean and dry volumetric flask containing 80 ml of solvent and then the solution was made up to the mark using the solvent. This is considered as standard stock solution (1000µg/ml). 1 ml of the stock solution was pipetted out and made up to 10 ml to get a concentration 100µg/ml, treated as working standard, 100% target concentration.

Preparation of stock and working sample solution

10tablets 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 dissolving weight equivalent to 100 mg of Ornidazole and 150 mg of Diloxanide furoate and made up to 100 ml with mobile phase. Sonicated for 5 min and later filtered the solution using 0.45micron syringe filter. 1 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 100µg/ml for Ornidazole and 150µg/ml for Diloxanide furoate.

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 3.34 min and Diloxanide furoate at 4.293 min. Fig.5-6 represents chromatograms of blank solution and mixture of standard solutions respectively. The total run time is 8 minutes. 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 six replicate injections of the 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 were chromatographed at working concentration and it is shown in Fig.7. The sample peaks were identified by comparing the relative retention times with the mixture of standards solution (Fig.6-7).

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.

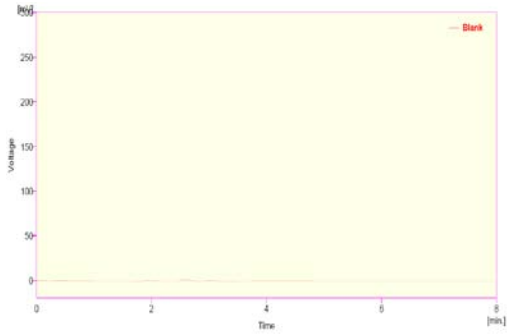

Fig. 5: Typical Chromatogram of Blank solution

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 [23] for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, intra-day precision, limit of detection (LOD) and limit of quantitiation (LOQ).

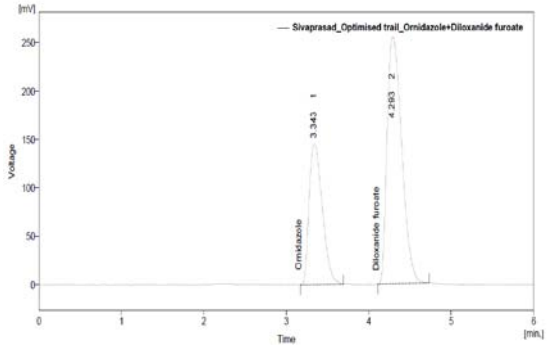

Fig. 6: Typical chromatogram of mixture of standards solution

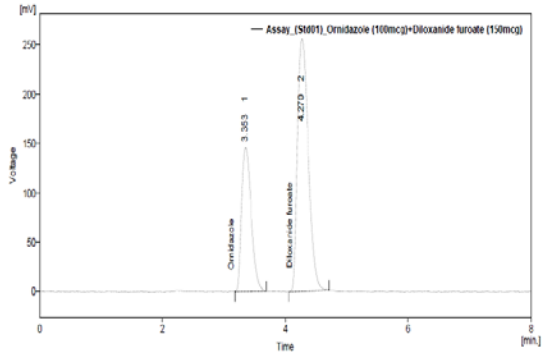

Fig. 7: Typical chromatogram of sample solution

Table 1: System suitability studies results

Parameters	Acceptance Limits	Ornidazole	Diloxanide furoate
Retention time (min)	-	3.34	4.293
Resolution factor (Rs)	Not less Than 2	2.917	
Number Of Theoretical plates (N)	Not less Than 2000	3911	4470
Peak Asymmetry (A)	Not More Than 2	1.765	1.811

Specificity

Fig. 5-7 for blank, mixture of standards drug solution and sample chromatogram reveal that the 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 (Intra day 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 (Table 4).

Linearity

Standards solution of Diloxanide furoate and Ornidazole at different concentrations was prepared. Calibration curves (Fig.8-9) 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 60-140µg/ml for Ornidazole and 90-210µg/ml for Diloxanide furoate (Tables 5-6). The correlation coefficients were greater than 0.99 for both the drugs, which meet the method validation acceptance criteria and hence the method is said to be linear for both the drugs.

Accuracy

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of both the drugs at three different levels (80-120%)..

Table 2: System precision results of Ornidazole

Injection	Retention time (min)	Peak area
1	3.353	1572.949
2	3.35	1580.092
3	3.347	1572.007
4	3.343	1547.302
5	3.343	1584.774
6	3.34	1580.024
Mean	3.346	1572.858
SD	0.0049	13.413
%RSD	**0.15**	**0.85**

At each level, three determinations were performed. Percent mean recovery is calculated as shown in Tables 7-8. The accepted limits of mean recovery are 98% -102% and all observed data were within the required range, which indicates good recovery values and hence the accuracy of the method developed

Table 3: System precision results of Diloxanide furoate

Injection	Retention time (min)	Peak area
1	4.3	3110.948
2	4.3	3138.373
3	4.297	3105.906
4	4.297	3050.692
5	4.3	3113.749
6	4.297	3127.232
Mean	4.299	3107.817
SD	0.002	30.406
%RSD	0.04	0.98

Table 4: Intraday precision results of Diloxanide Furoate and Ornidazole

S. No.	% Assay (Ornidazole)	% Assay (Diloxanide furoate)
1	100.16	99.96
2	100.95	100.84
3	101.04	99.8
4	100.628	98.02
5	100.86	100.05
6	100.729	100.48
Mean	100.72	99.85
SD	0.31	0.97
%RSD	0.307	0.97

Table 5: Calibration data for Ornidazole

Concentration (µg/ml)	Peak Area
60	1189.618
80	1488.806
100	1799.722
120	2099.537
140	2446.73
Regression equation	y=15.625x+242.41
Regression coefficient	0.9992

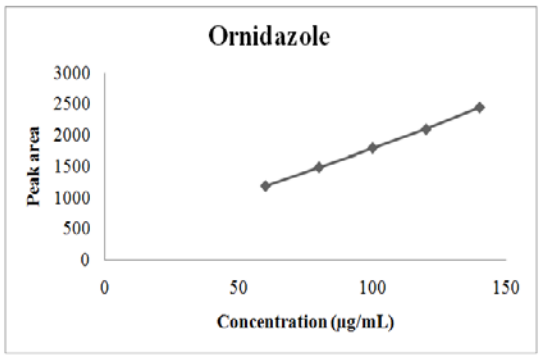

Fig. 8: Linearity graph of Ornidazole

Table 6: Calibration data for Diloxanide furoate

Concentration (µg/ml)	Peak Area
90	2257.974
120	2793.375
150	3309.193
180	3862.705
210	4392.083
Regression equation	y=17.792x+654
Regression coefficient	0.9999

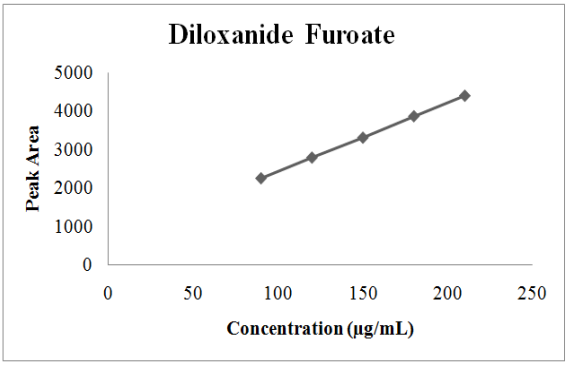

Fig. 9: Linearity graph of Diloxanide furoate

Table 7: Results of Accuracy studies for Ornidazole

% Recovery level	Amount taken (µg/ml)	Area	Average area	Amount recovered (µg/ml)	% recovery	% Mean recovery
80	100	1799.7				100.16
	100	1878.302	1825.9	98.12	98.12	
	100	1799.7				
100	120	2199.6				
	120	2199.7	2196.6	122.05	101.71	
	120	2190.537				
120	140	2499.1	2465.9	140.94	100.67	
	140	2498.9				
	140	2399.7				

Table 8: Results of Accuracy studies for Diloxanide furoate

% Recovery level	Amount taken (µg/ml)	Area	Average area	Amount recovered (µg/ml)	% Recovery	% Mean Recovery
80	144	3309.1				
	144	3519.6	3486.211	149.76	99.84	99.09
	144	3629.7				
100	180	3862.9				
	180	3864.6	3902.095	176.88	98.26	
	180	3978.7				
120	216	4438.9				
	216	4435.1	4469.728	208.29	99.18	
	216	4535.0				

Sensitivity

The sensitivity of measurement of Diloxanide furoate and Ornidazole by use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and limit of detection (LOD). LOQ and LOD were calculated by the use of the equations LOD = 3.3σ/S and LOQ = 10σ/S where σ is the standard deviation of response of calibration plots and S is the slope of the corresponding calibration plot. The limit of detection (LOD) for Diloxanide furoate and Ornidazole were found to be 8.80µg/ml and 6.68µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Ornidazole were found to be 26.6µg/ml and 20.25µg/ml respectively.

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity, limit of detection and limit of quantitation, for for simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in Amicline plus tablets. The developed method resulted in Diloxanide furoate eluting at 4.293 min and Ornidazole at 3.34 min. Diloxanide furoate exhibited linearity in the range 90-210µg/ml, while Ornidazole exhibited linearity in the range 60-140µg/ml. The precision is exemplified by relative standard deviations of 0.97% for Diloxanide furoate and 0.3% for Ornidazole. Percentage Mean recoveries were found to be in the range of 98-102, during accuracy studies. The limit of detection (LOD) for Diloxanide furoate and Ornidazole were found to be 8.80µg/ml and 6.68µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Ornidazole were found to be 26.6µg/ml and 20.25µg/ml respectively.

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CONFLICT OF INTERESTS

Declared None

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