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Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS QUANTITATIVE ESTIMATION OF DILOXANIDE FUROATE AND TINIDAZOLE 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 Tinidazole 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 (pH 6.5; KH_2PO_4+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 270 nm.

Results: The developed method resulted in Diloxanide furoate eluting at 4.70 min and Tinidazole at 3.45 min. Diloxanide furoate exhibited linearity in the range 30-70µg/ml, while Tinidazole exhibited linearity in the range 36-84µg/ml. The precision is exemplified by relative standard deviations of 0.266% for Diloxanide furoate and 0.35% for Tinidazole. 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 Tinidazole were found to be 0.32µg/ml and 0.40µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Tinidazole were found to be 0.96µg/ml and 1.21µg/ml respectively.

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

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

INTRODUCTION

Tinidazole (fig. 1) chemically is 1-[2-(ethylsulphonyl) ethyl]-2-methyl-5-nitro-1H-imidazole. Tinidazole is a prodrug and the anti-protozoal action of tinidazole results from reduction of nitro group of tinidazole in *Trichomonas* by a ferredoxin-mediated electron transport system. As a result of this reduction, a free nitro radical is generated and is believed to be responsible for the antiprotozoal activity. This toxic free radical covalently binds to DNA, resulting in DNA damage and leads to cell death [1]. It has a molecular formula of $C_8H_{13}N_3O_4S$ and a molecular weight of 247.272 g/mol.

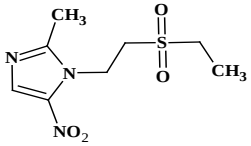

Fig. 1: Structure of Tinidazole

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 [2]. It is an effective drug for the treatment of asymptotic persons who are passing cysts of *Entameba histolytica* [3]. 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* [3] and is excellent amoebicide for cyst passers [4-5].

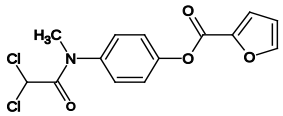

Fig. 2: Structure of Diloxanide furoate

A detailed literature survey reveals that there exists literature on chromatographic methods for Tinidazole in combination with other drugs [6-12] and similarly Diloxanide furoate in combination with other drugs [13-17] in various matrices. While there is only one literature reported on RP-HPLC method development and validation for the simultaneous quantitative estimation of Diloxanide furoate and Tinidazole in pharmaceutical dosage forms [18]. 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 Tinidazole in tablets and validate as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure sample of Diloxanide furoate and Tinidazole with purities greater than 99% were obtained as gift samples from Chandra Labs, Hyderabad, India and tablet formulation [Metroquin] was procured from Medplus pharmacy, Hyderabad, India with labelled amount 250mg and 300mg of Diloxanide furoate and Tinidazole 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, Inertsil ODS 3V (150X4.6 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 Tinidazole and Diloxanide furoate. The resulting spectra (fig. 3-5) shows characteristic absorption maxima at 305 nm for Tinidazole, 257 nm for Diloxanide furoate and 270 nm for the combination.

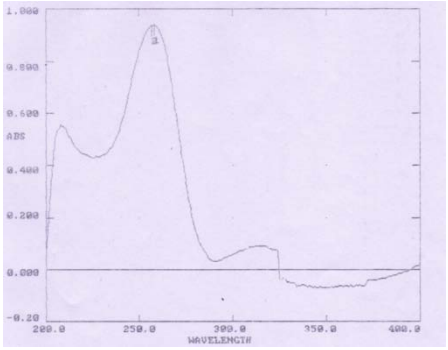

Fig. 3: UV spectrum of standard Diloxanide furoate

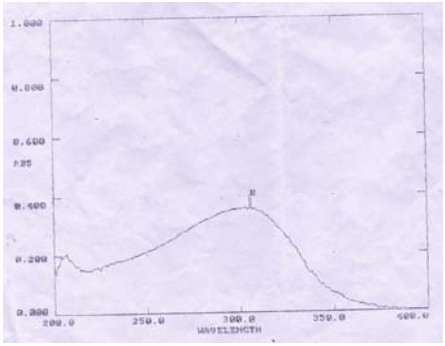

Fig. 4: UV spectrum of standard Tinidazole

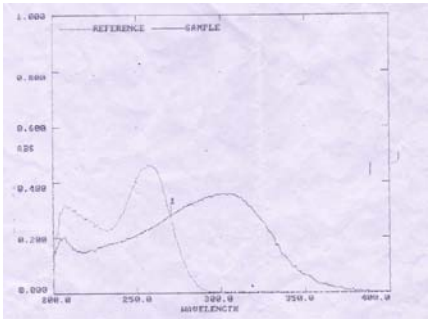

Fig. 5: UV spectrum of Tinidazole and Diloxanide furoate

Chromatographic conditions

The developed method uses a reverse phase C18 column, Inertsil ODS 3V (150X4.6 mm; 5µ), mobile phase consisting of mixed phosphate buffer (pH 6.5): 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 270 nm.

Preparation of mixed buffer solution

1.625g of potassium dihydrogen ortho phosphate (KH_2PO_4) and 0.3g 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 6.5 using ortho phosphoric acid and potassium hydroxide solution. 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 mixed phosphate buffer and acetonitrile in the ratio of 30:70 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 mixed standard solution

Weigh accurately 60 mg of Tinidazole and 50 mg of Diloxanide furoate in 100 ml of volumetric flask and dissolve in 10 ml of mobile phase and make up the volume with mobile phase. From above stock solution 60 µg/ml of Tinidazole and 50 µg/ml of Diloxanide furoate is prepared by diluting 1 ml to 10 ml with mobile phase. This is treated as mixed working standards solution, 100% target concentration.

Preparation of stock and working sample solution

10 tablets were weighed and taken into a mortar, crushed and then uniformly mixed. Test stock solutions of Tinidazole (3000µg/ml) and Diloxanide furoate (2500µg/ml) were prepared by dissolving average of 10 tablets, equivalent to 300 mg of Tinidazole and 250 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. 0.2 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 60µg/ml for Tinidazole and 50µg/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, Tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of Tinidazole at 3.45 min and Diloxanide furoate at 4.70 min. Fig. 6-7 represent chromatograms of mixture of standard solutions and sample solution respectively. The total run time is 7 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 Tailing factor (T) were evaluated for six replicate injections of the standards at working concentration. The results given in table 1 were within acceptable limits.

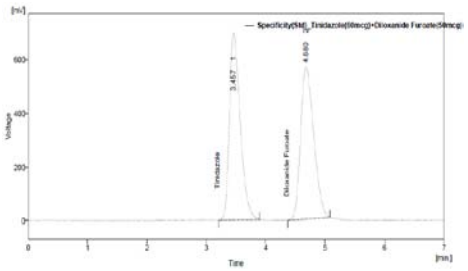

Fig. 6: Typical chromatogram of mixture of standards solution

Table 1: System suitability studies results

Parameters	Acceptance Limits	Tinidazole	Diloxanide furoate
Retention time (min)	-	3.45	4.70
Resolution factor (Rs)	Not less Than 2	3.36	
Theoretical plates (N)	Not less Than 2000	3818	2493
Tailing factor (T)	Not More Than 2	1.88	1.71

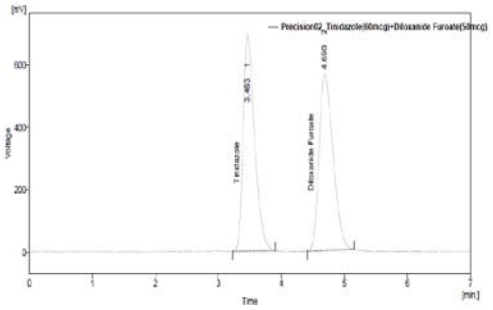

Fig. 7: Typical chromatogram of sample solution

In order to test the applicability of the developed method to a commercial formulation, 'Metroquin' 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.

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 [19] 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).

Specificity

As there were no interferences found between peaks in Blank and mixture of standards drug solution, we could conclude that the method optimized is sad 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 (Intra day 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).

Table 2: System precision results of Tinidazole

Injection	Retention time (min)	Peak area
1	3.45	8773.21
2	3.46	8733.27
3	3.46	8713.98
4	3.45	8868.77
5	3.45	8773.21
6	3.46	8733.27
Mean	3.45	8765.95
SD	0.0049	55.704
%RSD	0.14	0.64

Table 3: System precision results of Diloxanide furoate

Injection	Retention time (min)	Peak area
1	4.68	8815.58
2	4.83	8708.39
3	4.68	8510.45
4	4.67	8553.08
5	4.68	8815.58
6	4.69	8708.39
Mean	4.70	8685.25
SD	0.064	128.893
%RSD	1.36	1.48

Table 4: Intraday precision results of Diloxanide Furoate and Tinidazole

S. No.	% Assay (Tinidazole)	% Assay (Diloxanide furoate)
1	98.77	99.95
2	99.82	99.51
3	99.46	100.13
4	99.18	99.5
5	99.48	99.51
6	99.35	99.72
Mean	99.35	99.72
SD	0.35	0.266
%RSD	0.35	0.266

Linearity

Standards solutions of Diloxanide furoate and Tinidazole at different concentrations were 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 36-84µg/ml for Tinidazole and 30-70µg/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.

Table 5: Calibration data for Tinidazole

Concentration (µg/ml)	Peak Area
36	4996.42
48	7059.64
60	9041.83
72	10709
84	12615.3
Regression equation	y=157.3x-559.1
Regression coefficient	0.998

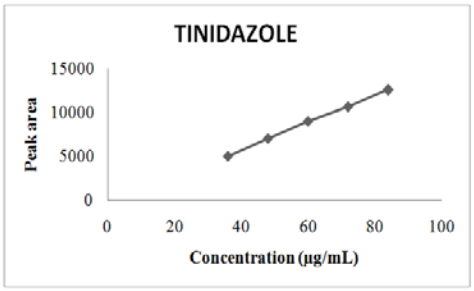

Fig. 8: Linearity graph of Tinidazole

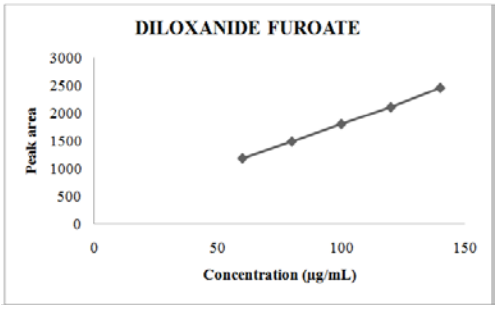

Fig. 9: Linearity graph of Diloxanide furoate

Table 6: Calibration data for Diloxanide furoate

Concentration (µg/ml)	Peak Area
30	5461.510
40	7087.548
50	8551.583
60	10219.607
70	12135.206
Regression equation	y=164.7x+451.3
Regression coefficient	0.997

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%). 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 7: Results of recovery studies for Tinidazole

% Spiking	Amount taken (µg/ml)	Mean Amount recovered (µg/ml)	% Mean Recovery
80	60	59.23	98.72
	60		
	60		
100	72	71.26	98.98
	72		
	72		
120	84	84.89	101.06
	84		
	84		

Table 8: Results of recovery studies for Diloxanide furoate

% Spiking	Amount taken (µg/ml)	Mean Amount recovered (µg/ml)	% Mean Recovery
80	50	49.43	98.86
	50		
	50		
100	60	59. 65	99.41
	60		
	60		
120	70	71.17	101.67
	70		
	70		

Sensitivity

The sensitivity of measurement of Diloxanide furoate and Tinidazole 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 Tinidazole were found to be 0.32µg/ml and 0.40µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Tinidazole were found to be 0.96µg/ml and 1.21µg/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 Tinidazole in Metroquin tablets. The developed method resulted in Diloxanide furoate eluting at 4.70 min and Tinidazole at 3.45 min. Diloxanide furoate exhibited linearity in the range 30-70µg/ml, while Tinidazole exhibited linearity in the range 36-84µg/ml. The precision is exemplified by relative standard deviations of 0.266% for Diloxanide furoate and 0.35% for Tinidazole. 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 Tinidazole were found to be 0.32µg/ml and 0.40µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Tinidazole were found to be 0.96µg/ml and 1.21µg/ml respectively.

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CONFLICT OF INTERESTS

Declared None

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