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Original Article

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

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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 column, Waters Symmetry C18 (250 X 4.6 mm; 5µ), a mobile phase of triethylammonium phosphate buffer (pH 2.3):acetonitrile in the proportion of 40:60 v/v, flow rate of 1.0 ml/min and a detection wavelength of 270 nm using a UV detector.

Results: The developed method resulted in Diloxanide furoate eluting at 4.07 min and Tinidazole at 2.52 min. Diloxanide furoate exhibited linearity in the range 31.25-93.75µg/ml, while Tinidazole exhibited linearity in the range 37.5-112.5µg/ml. The precision is exemplified by relative standard deviations of 0.90% for Diloxanide furoate and 0.68% for Tinidazole. Percentage Mean recoveries were found to be in the range of 98102, during accuracy studies. The limit of detection (LOD) for Diloxanide furoate and Tinidazole were found to be 68.53µg/ml and 97.87µg/ml respectively, while the limit of quantitation (LOQ) for Diloxanide furoate and Tinidazole were found to be 207.677µg/ml and 296.6µg/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-(ethyl sulphonyl) ethyl]-2-methyl-5-nitro-1H-imidazole, (fig. 2). It is used as antiprotozoal agent. Tinidazole is a prodrug and the anti-protozoal action of tinidazole results from the 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.

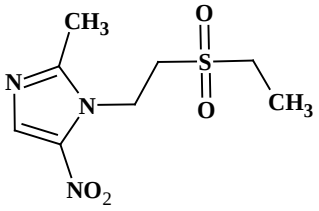

Fig. 1: Structure of Tinidazole

Diloxanide furoate (fig. 2) chemically is 4-(N-methyl-2, 2-dichloro-acetamido) 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].

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 are only two RP-HPLC assay methods reported for the simultaneous quantitative estimation of Diloxanide furoate and Tinidazole in pharmaceutical dosage forms using potassium dihydrogen orthophosphate as buffer (pH 5.0) and mixed phosphate buffer pH 6.5 [18-19].

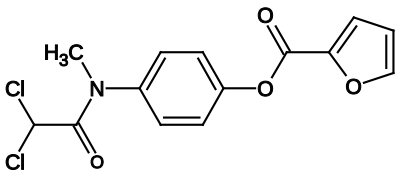

Fig. 2: Structure of Diloxanide furoate

As there is no literature reported on working using triethyl-ammonium phosphate buffer as aqueous media along with acetonitrile as mobile phase, we here report a new and a rapid RP-HPLC validated method for the simultaneous quantitative estimation of Diloxanide furoate and Tinidazole in tablets using triethyl-ammonium phosphate buffer (pH 2.3) as per ICH guidelines.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure sample of Diloxanide furoate and Tinidazole with purities greater than 95% were obtained as gift samples from Chandra Labs, Hyderabad, India and tablet formulation [Metroquin] was procured from Medplus pharmacy, Hyderabad, India with labelled amount 250 mg and 300 mg of Diloxanide furoate and Tinidazole respectively. Acetonitrile (HPLC grade) was obtained from Sigma Aldrich (Hyderabad, India), water (HPLC grade), Triethylamine (AR grade), orthophosphoric 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 an LC-20AD pump, Shimadzu SPD-20A Prominence UV-VISIBLE detector and a reverse phase C18 column, Waters Symmetry (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 "Lab solutions lite" 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).

Methods

Selection of wavelength

The 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. Suitable wavelength selected for simultaneous estimation is 270 nm (fig. 3-4).

Chromatographic conditions

The developed method uses a reverse phase C18 column, Waters Symmetry C18 (250 X 4.6 mm; 5µ), a mobile phase of triethylammonium phosphate buffer (pH 2.3):acetonitrile in the proportion of 40:60 v/v, flow rate of 1.0 ml/min and a detection wavelength of 270 nm using a UV detector.

Buffer preparation

The buffer solution was prepared by adding 5 ml of triethylamine to 1000 ml of HPLC grade water and later pH was adjusted to 2.3 using 30% v/v of orthophosphoric acid in water. The buffer was filtered through 0.45µ filter to remove all fine particles.

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.

Diluent

Diluent used is the mobile phase itself

Preparation of standard solution

12 mg of Tinidazole and 10 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 75 µg/ml of Tinidazole and 62.5 µg/ml of Diloxanide furoate were prepared further by appropriate dilution. This is treated as working standards solution, 100% target concentration.

Preparation of sample solution

10 tablets were weighed and taken into a mortar, crushed and then uniformly mixed. Test stock solution of Tinidazole (750µg/ml) and Diloxanide furoate (625 µg/ml) were prepared by taking tablet powder equivalent to 75 mg of Tinidazole and 62.5 mg of Diloxanide furoate to 80 ml of mobile phase which is sonicated for a min and later made up to 100 ml with mobile phase. This solution was filtered using 0.22 micron syringe filter. 1 ml of the stock solution was pipetted out and made up to 10 ml to get working sample solution equivalent to a concentration of 75µg/ml for Tinidazole and 62.5µg/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), the number of theoretical plates (N), runtime and the cost effectiveness.

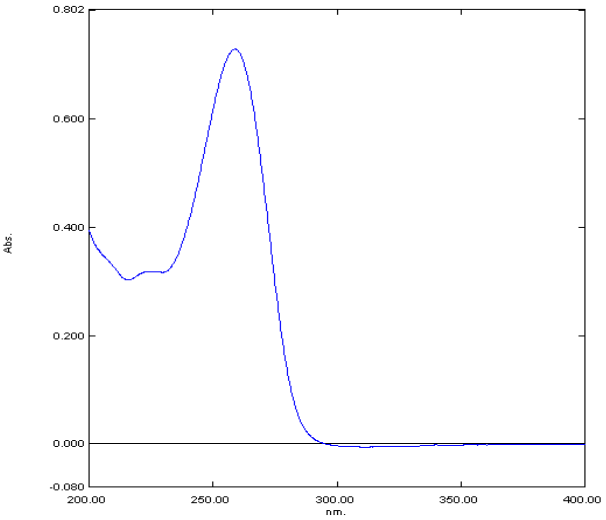

Fig. 3: UV spectrum of standard Diloxanide furoate

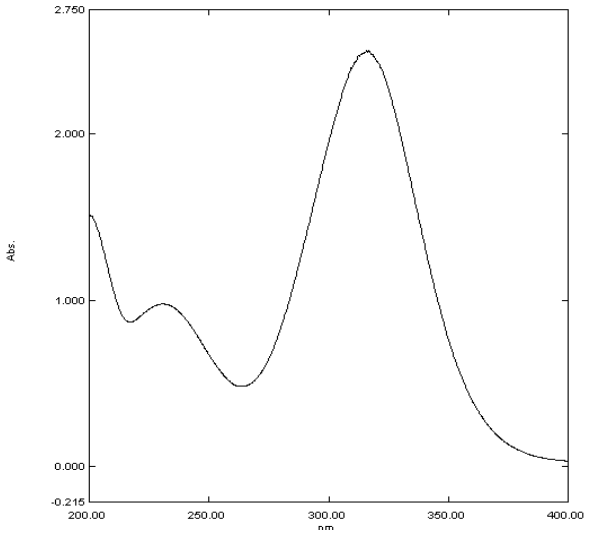

Fig. 4: UV spectrum of standard tinidazole

The optimized method developed resulted in the elution of Tinidazole at 2.52 min and Diloxanide furoate at 4.07 min. fig. 5-8 represents chromatograms of the blank solution, standard solutions individually and mixture of standard solutions respectively. The total run time is 6 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 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.

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. 9**.** The sample peaks were identified by comparing relative retention times with the standard solutions (Fig. 5-8).

System suitability parameters were within the acceptance limits, ideal for the chromatograph ed 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.

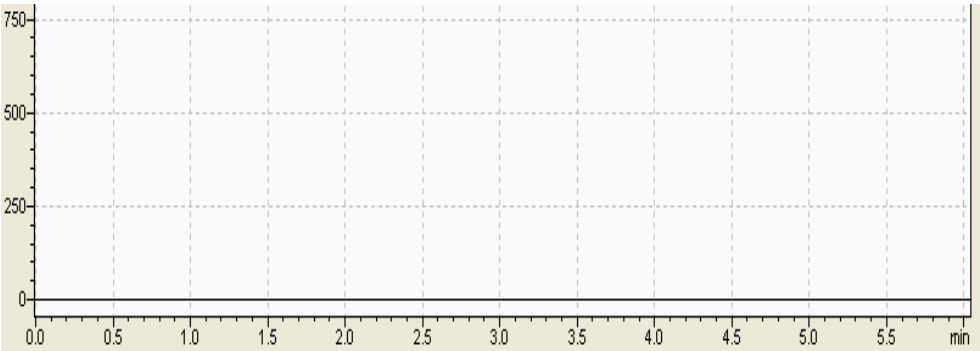

Fig. 5: Typical chromatogram of blank solution

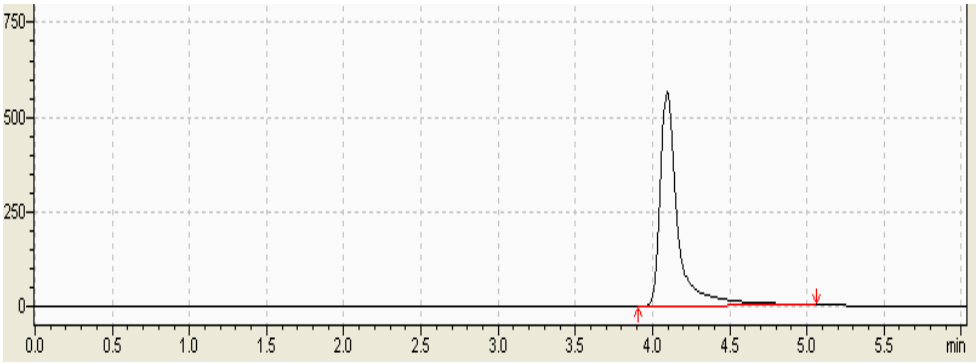

Fig. 6: Typical chromatogram of Diloxanide furoate standard solution

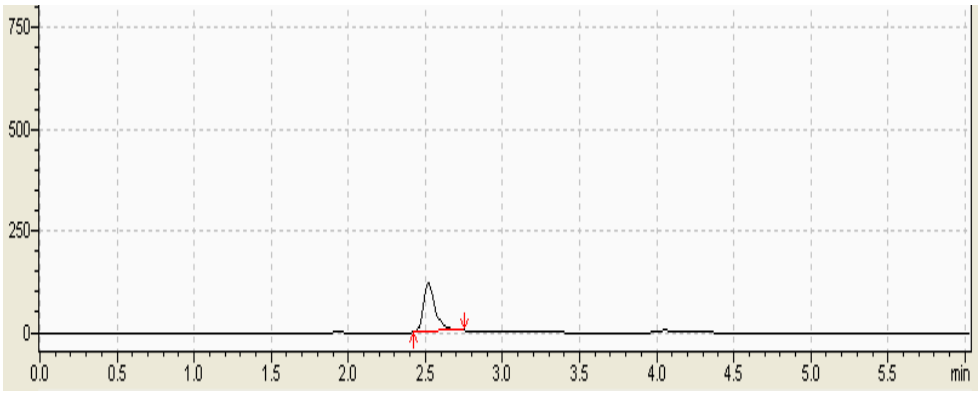

Fig. 7: Typical chromatogram of Tinidazole standard solution

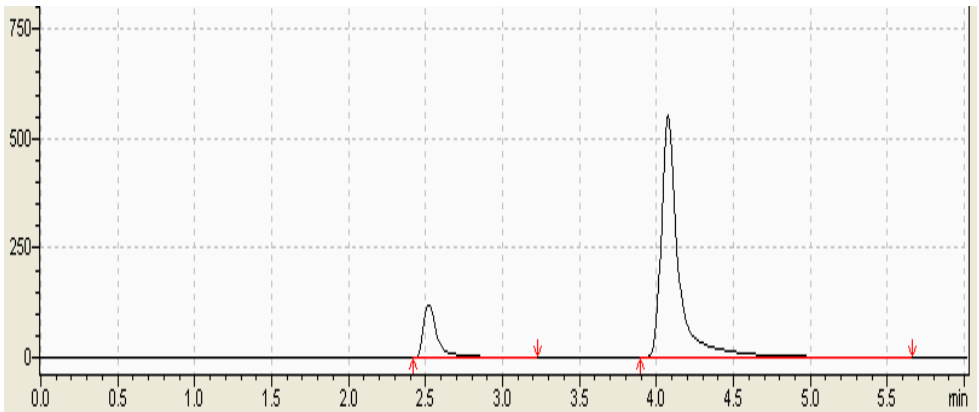

Fig. 8: Typical chromatogram of mixture of standard solutions

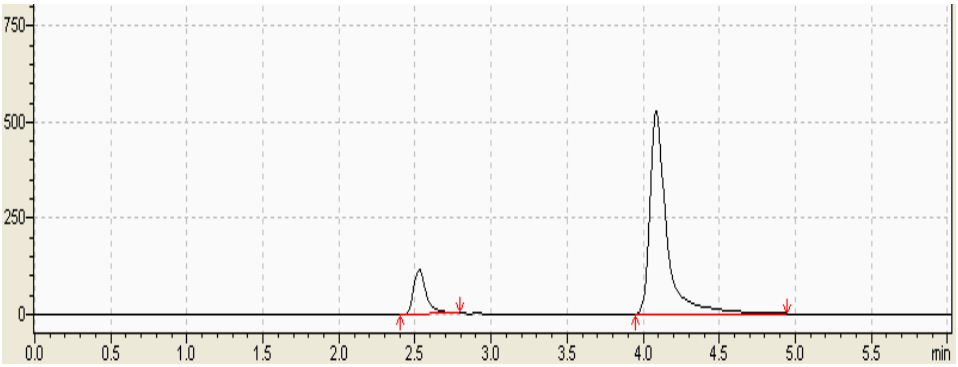

Fig. 9: Typical chromatogram of sample solution

Table 1: System suitability studies results

Parameters	Acceptance Limits	Tinidazole	Diloxanide furoate
Retention time (min)	-	2.52	4.07
Resolution factor (Rs)	Not less Than 2	9.027	
Number Of Theoretical plates (N)	Not less Than 2000	4076	7648
Tailing factor (T)	Not More Than 2	1.919	1.884

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 [20] 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

Fig. 5-9 for blank, individual and mixture of standards drug solutions and sample solution 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 Tinidazole 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 table 2**.**

Method precision

Method precision was determined by performing assay of the 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 (table 3).

Linearity

Standards solutions of Diloxanide furoate and Tinidazole at different concentrations were prepared. Calibration curves (fig. 10-11) 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 377.5-112.5µg/ml for Tinidazole and 31.25-93.75µg/ml for Diloxanide furoate (tables 4-5). 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.

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 table 7**.** 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 2: System precision results of Diloxanide furoate and Tinidazole

n	Diloxanide furoate		Tinidazole	
	RT	Peak area	RT	Peak area
1	4.075	4407555	2.521	764673
2	4.070	4439757	2.519	755950
3	4.078	4399433	2.527	763716
4	4.070	4380246	2.520	756354
5	4.069	4429097	2.519	754293
Average	4.072	4411217.6	2.521	758997
SD	0.0039	23694.41	0.0033	4818.79
% RSD	0.095	0.53	0.13	0.63

Table 3: Intraday precision results of Diloxanide furoate and Tinidazole

N	Diloxanide furoate	Tinidazole
	% Assay	% Assay
1	98.8	101.4
2	98.6	101.2
3	98.2	101.2
4	98.3	101.2
5	99.0	99.6
6	98.7	100.5
Average	98.6	100.8
SD	0.30	0.686
% RSD	0.304	0.68

Table 4: Calibration data for Diloxanide furoate

% Level	Concentration (µg/ml)	Peak area
50	31.25	2216411
75	46.87	3309655
100	62.5	4451303
125	78.12	5623684
150	93.75	6578781
Regression equation		y=70647.99x+20608.41
Regression coefficient		0.999

Table 5: Calibration data for Tinidazole

% Level	Concentration (µg/ml)	Peak area
50	37.5	391612
75	56.25	584953
100	75	784818
125	93.75	990979
150	112.5	1159699
Regression equation		Y=10358.29x+5536.2
Regression coefficient		0.999

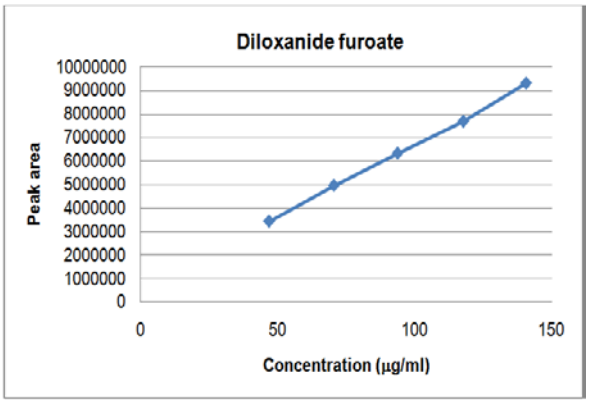

Fig. 10: Linearity graph of Diloxanide furoate

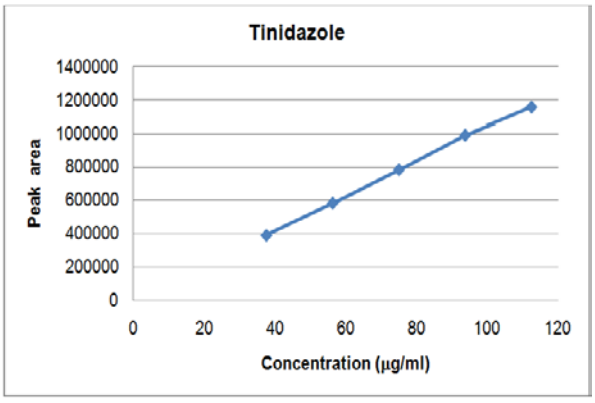

Fig. 11: Linearity graph of Tinidazole

Table 6: Results of Accuracy studies for Diloxanide furoate and Tinidazole

Level (%)	Diloxanide furoate		Tinidazole	
	% Recovery	% Mean	% Recovery	% Mean
50	101.7		99.5	
50	101.0	100.55	98.3	99.3
50	100.1		100.1	
100	98.2		98.8	
100	98.3	99.23	101.4	100.3
100	101.2		100.7	
150	98.1		99.7	
150	99.4	98.63	100.1	99.66
150	98.4		100.1	

Table 3: Ruggeness results of Diloxanide furoate and Tinidazole.

N	Diloxanide furoate	Tinidazole
	% Assay	% Assay
1	100.6	98.4
2	96.1	99.9
3	98.6	100.5
4	98.2	98.8
5	98.3	101.4
6	100.5	100.7
Average	98.4	99.9
SD	1.435	1.157
% RSD	1.16	1.157

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 68.53µg/ml and 97.87µg/ml respectively, while limit of quantitation (LOQ) for Diloxanide furoate and Tinidazole were found to be 207.677µg/ml and 296.6 µg/ml respectively.

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 7).

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 4.07 min and Tinidazole at 2.52 min. Diloxanide furoate exhibited linearity in the range 31.25-93.75µg/ml, while Tinidazole exhibited linearity in the range 37.5-112.5µg/ml. The precision is exemplified by relative standard deviations of 0.90% for Diloxanide furoate and 0.68% for Tinidazole. Percentage Mean recoveries were found to be in the range of 98 -102, during accuracy studiesThe limit of detection (LOD) for Diloxanide furoate and Tinidazole were found to be 68.53µg/ml and 97.87µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Tinidazole were found to be 207.677µg/ml and 296.6µg/ml respectively.

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CONFLICT OF INTERESTS

Declared None

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