Original Article

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

D. VIJAY KUMAR[1], P. SWETHA[1], G. SAI PRASAD[2], A. ASHOK KUMAR[1]*

[1]Department of Pharmaceutical Analysis and Quality Assurance, Vijaya College of Pharmacy, Munaganur (village), Hayathnagar (Mandal), Hyderabad 501511, India, [2]Chandra Labs, 5-5-35/173, Plot No-10, 1st Floor, IDA Prasanthi Nagar, Kukatpally, Hyderabad, India 500090
Email: ashok576@gmail.com

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 tri ethyl ammonium 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 4.31 min and Ornidazole at 2.64 min. Diloxanide furoate exhibited linearity in the range 46.87-140.62μg/ml, while Ornidazole exhibited linearity in the range 31.25-93.75μg/ml. The precision is exemplified by relative standard deviations of 1.02% for Diloxanide furoate and 0.514% 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 122.54μg/ml and 81.54μg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Ornidazole were found to be 371.33μg/ml and 247.11μg/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 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].
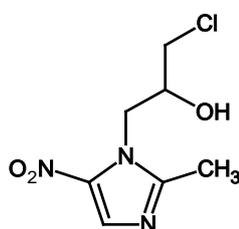

Fig. 1: Structure of Ornidazole

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].
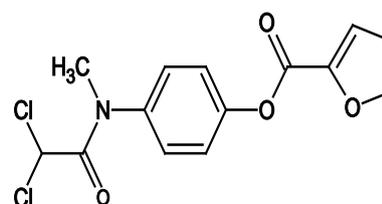

Fig. 2: Structure of Diloxanide furoate

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 only one literature on RP-HPLC assay methods for the simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in pharmaceutical dosage forms using mixed phosphate buffer at pH 6.0[23]. As there is no literature reported using triethylammonium 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 Ornidazole in tablets using triethylammonium phosphate 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), Triethylamine (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 Chromatograph comprising a 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).

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 270 nm (fig. 3-4).
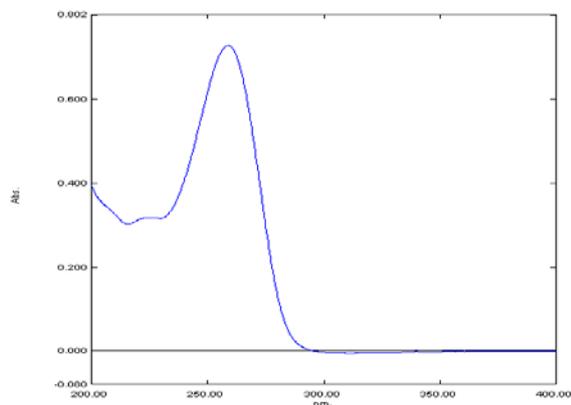

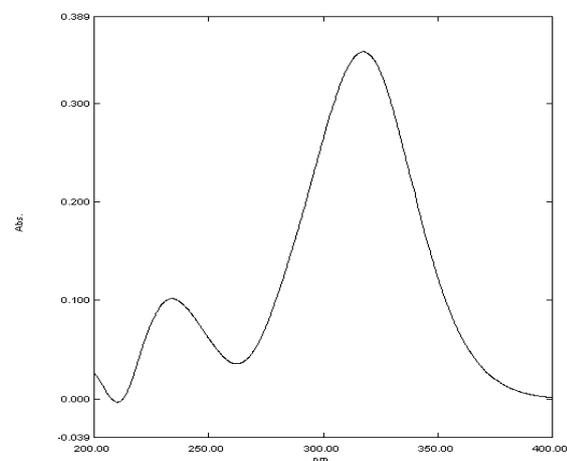**Fig. 3: UV spectrum of standard Diloxanide furoate**

Fig. 4: UV spectrum of standard Ornidazole

Chromatographic conditions

The developed method uses a reverse phase C18 column, Waters Symmetry C18 (250 X 4.6 mm; 5μ), mobile phase of triethylammonium phosphate 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 270 nm.

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 ortho phosphoric 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

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 62.5μg/ml of Ornidazole and 93.75μg/ml of Diloxanide furoate were prepared further by by diluting 6.25 ml to 10 ml with mobile phase. This is treated as working standards solution, 100% target concentration.

Preparation of 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 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. 0.625 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 62.5μg/ml for Ornidazole and 93.75μg/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 2.64 min and Diloxanide furoate at 4.31 min. Fig. **5-8** represents chromatograms of blank solution, standard solutions individually and mixture of standard solutions respectively.
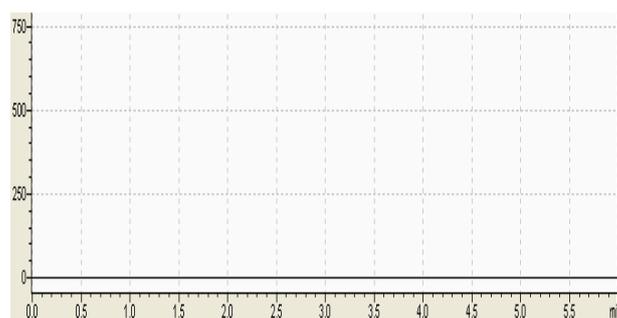

Fig. 5: Typical chromatogram of blank solution

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.
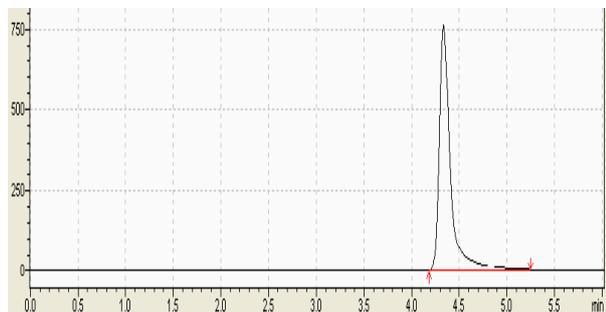

Fig. 6: Typical chromatogram of Diloxanide furoate standard solution

Fig. 7: Typical chromatogram of Ornidazole standard solution

In order to test the applicability of the developed method to a commercial formulation, 'Amicline plus' tablets were chromato graphed at working concentration and it is shown in fig. 9. 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 chromato graphed 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 [24] 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 quantitation (LOQ).
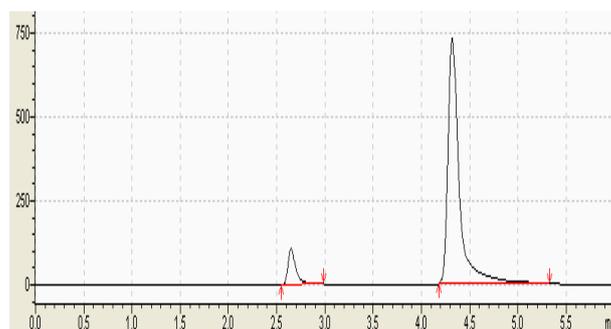

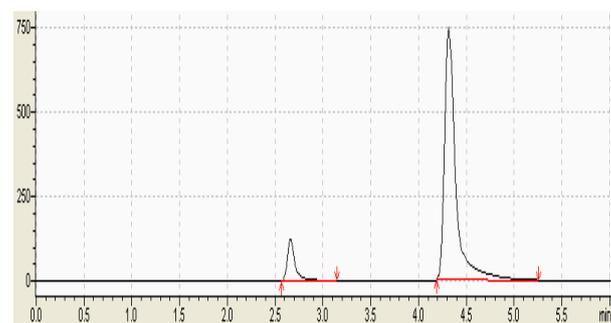**Fig. 8: Typical chromatogram of mixture of standard solutions**

Fig. 9: Typical chromatogram of sample solution

Table 1: System suitability studies results.

Parameters	Acceptance limits	Ornidazole	Diloxanide furoate
Retention time (min)	-	2.64	4.31
Resolution factor (Rs)	Not less Than 2	9.495	
Number Of Theoretical plates (N)	Not less Than 2000	4705	7748
Tailing factor (T)	Not More Than 2	1.942	1.928

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 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 table 2.

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 (table 3).

Table 2: System precision results of Diloxanide furoate and Ornidazole

n	Diloxanide furoate		Ornidazole	
	RT	Peak Area	RT	Peak Area
1	4.300	6313215	2.655	683654
2	4.317	6308980	2.646	676895
3	4.321	6318285	2.650	679536
4	4.319	6286694	2.650	677006
5	4.313	6306579	2.647	678444
Average 4.314		6306750.6	2.649	679107
SD 0.0083		12063.35	0.0035	2766.13
% RSD 0.193		0.191	0.132	0.407

Table 3: Intraday precision results of Diloxanide furoate and Ornidazole

n	Diloxanide furoate	Ornidazole
	% Assay	% Assay
1	99.9	102.8
2	99.9	101.7
3	101.1	103.6
4	101.0	104.5
5	100.4	104.4
6	100.5	103.9
Average	103.5	100.4
S. D.	1.064	0.516
% RSD	1.02	0.514

Linearity

Standard solutions of Diloxanide furoate and Ornidazole 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 31.25-93.75μg/ml for Ornidazole and 46.87-140.62μg/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.

Table 4: Calibration data for Diloxanide furoate

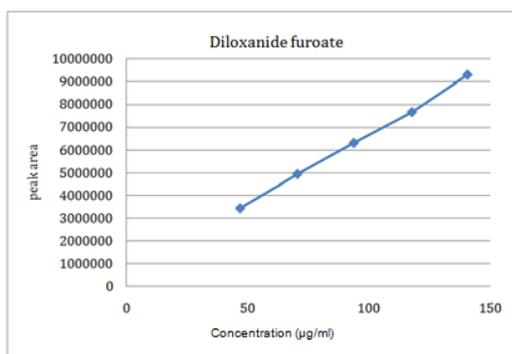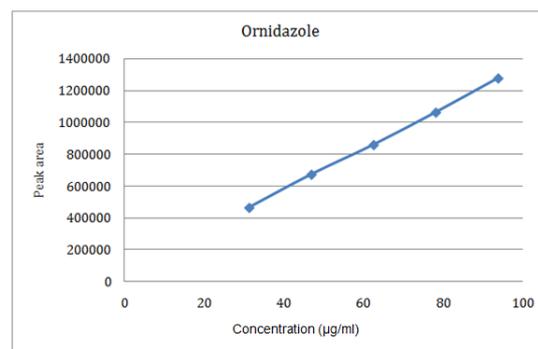| % Level	Concentration (μg/ml)	Peak area
50	46.87	3426733
75	70.30	4948667
100	93.75	6319280
125	117.7	7675938
150	140.62	9300420
Regression equation		y=61663.03x+544788.8
Regression coefficient		0.998

Fig.10: Linearity graph of Diloxanide furoate

Fig. 11: Linearity graph of Ornidazole

Table 5: Calibration data for Ornidazole

% Level	Concentration (μg/ml)	Peak area
50	31.25	463432
75	46.87	671353
100	62.50	857699
125	78.12	1061612
150	93.75	1275490
Regression equation		y=12892.13x+60181.14
Regression coefficient		0.999

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 6. 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 6: Results of accuracy studies for Diloxanide furoate and Ornidazole

Level (%)	Diloxanide furoate		Ornidazole	
	% Recovery	% Mean	% Recovery	% Mean
50	101.2		102.9	
50	98.7	100.2	102.2	102.5
50	100.7		103.5	
100	100.6		101.9	
100	100.3	100.3	101.5	102.3
100	100.1		103.5	
150	99.3		98.8	
150	99.2	99.2	99.7	99.5
150	99.2		100.2	

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 122.54µg/ml and 81.54µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Ornidazole were found to be 371.33µg/ml and 247.11µg/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).

Table 7: 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
SD	0.654	0.796
% RSD	0.654	0.77

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity, ruggedness, limit of detection and limit of quantitation, for simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in Amicline plus tablets. The developed method resulted in Diloxanide furoate eluting at 4.31 min and Ornidazole at 2.64 min. Diloxanide furoate exhibited linearity in the range 46.87-140.62µg/ml, while Ornidazole exhibited linearity in the range 31.25-93.75µg/ml. The precision is exemplified by relative standard deviations of 1.02% for Diloxanide furoate and 0.514% 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 122.54µg/ml and 81.54µg/ml respectively, while limit of quantitiation (LOQ) for Diloxanide furoate and Ornidazole were found to be 371.33µg/ml and 247.11µg/ml respectively.

ACKNOWLEDGEMENT

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Rege PV, Ramesh M. Simultaneous quantification of Ofloxacin and Ornidazole from combined pharmaceutical drug formulation by HPLC. Int J Pharm Biol Sci 2011;2:51-8.
2. Khattab FI, Ramadan NK, Hegazy MA, Ghoniem NS. Stability indicating methods for the determination of Ornidazole in the presence of its degradate according to ICH guidelines. Pharm Anal Acta 2012;3:179:1-8.
3. Ogrendik M. Treatment of rheumatoid arthritis with ornidazole: a randomized, double-blind, placebo-controlled study. Rheumatol Int 2006;26:1132-7.
4. Faubion WA, Bousvaros JA. Medical therapy for refractory pediatric Crohn's disease. Clin Gastroenterol Hepatol 2006;4:1199-213.
5. Danao KR, Hiradeve SM, Moon RS, Kasture AV, Yeole PG. RP-HPLC Simultaneous estimation of metronidazole and diloxanide furoate in combination. Int J Pharm Life Sci 2010;1:82-5.
6. McAuley JB, Herwaldt BL, Stokes SL, Becher JA, Roberts JM, Michelson MK, *et al.* Diloxanide furoate for treating

asymptomatic entamoeba histolytica cyst passers: 14 y experience in the United States. Clin Infect Dis 1992;15:464-8.

7. Marsden PD. Treatment of Cyst Passers. Clin Infect Dis 1992;15:559.

8. Ata AH, El-Haieg MO, Aboul-Magd LA, Mansour AH. A comparative study on some anti-amoebic drugs in cyst-passers. J Egypt Soc Parasitol 1984;14:213-8.

9. Soma SM, Vidyasagar V, Narsaiah N, Anandkumar R, Krishna DR. Validated HPLC method for the determination of Ornidazole in human serum and urine. Indian J Pharm Sci 2005;67:302-6.

10. Groppi A, Papa P, Montagna M, Carosi G. Determination of Ornidazole in human plasma and RBCs using HPLC. J Chromatogr 1986;380:437-42.

11. Nagavallai D, Sankar ASK, Ananda kumar K, Karunambigai K, Raju MSSN. RP-HPLC method for simultaneous estimation of Gatifloxacin and Ornidazole in tablets. Indian J Pharm Sci 2007;69:333-5.

12. Gandhimathi M, Ravi TK, Nilima S. A validated HPLC method for simultaneous estimation of Ofloxacin and Ornidazole in tablet dosage form. Indian J Pharm Sci 2006;68:838-40.

13. Dhandapani B, Thirumoorthy N, Shaik Harun R, Rama kotaiah M, Anjaneyalu N. Method development and validation for the simultaneous estimation of Ofloxacin and Ornidazole in tablet dosage form by RP-HPLC. Int J Pharm Sci Res 2010;1:78-83.

14. Paramane S, Kothapalli L, Thomas A, Despandey AD. Simultaneous RP-HPLC Estimation of Gatifloxacin and Ornidazole in tablet dosage forms. Indian J Pharm Sci 2007;69:525-8.

15. Ranjane PN, Gandhi SV, Kadukar SS, Ranher SS. Simultaneous determination of cefuroxime axeitil and ornidazole in tablet dosage form by using RP-HPLC. Chin J Chromatogr 2008;26:763-5.

16. Natarajan S, Raman B. Development and validation of a stability indicating HPLC method for simultaneous estimation of Ofloxacin and Ornidazole. Indian Pharm 2005;4:79-84.

17. Pai P, Rao GK, Srinivas B, Puranik S. RPLC determination of Tinidazole and Diloxanide furoate in tablets. Indian J Pharm Sci 2008;70:670-2.

18. Satyabrata R. HPLC determination of Metronidazole and Diloxanide furoate in single and combined formulation. East Pharm 1994;2:131-3.

19. Rao GR, Murthy SSN, Mohan K. HPLC assay of Diloxanide furoate and Metronidazole in combined dosage forms. Indian Drugs 1983;21:455-7.

20. Gizawy EISM. HPLC analysis of Metronidazole and Diloxanide furoate in its dosage forms. Anal Lett 1995;28,83-92.

21. Mishal A, Sober D. Stability indicating reversed-phase liquid chromatographic determination of Metronidazole benzoate and Diloxanide furoate as bulk drug and in suspension dosage form. J Pharm Biomed Anal 2005;39:819-23.

22. Shaalan Al, Nora H. Determination of diloxanide furoate and metronidazole in binary mixture using first derivative of the ratio-spectra and high-performance liquid chromatography-UV methods. Am J Appl Sci 2007;4:66-72.

23. Sivaprasad P, Yaswanth kumar S, Ashok Kumar A. RP-HPLC method development and validation for simultaneous quantitative estimation of Diloxanide furoate and Ornidazole in tablets. Int J Pharm Pharm Sci 2015;7:515-9.

24. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use. Validation of Analytical Procedures: Text and Methodology ICH Q2 (R1); 2005.