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Research Article

Analytical method development and validation of diloxanide furoate and ornidazole in its combined pharmaceutical dosage form

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Abstract: A simple, rapid, economical, precise and accurate Stability indicating RP-HPLC method for simultaneous estimation of Diloxanide Furoate and Ornidazole in Their Combined Dosage Form has been developed. The separation was achieved by LC- 20 AT C18 (250mm x 4.6 mm x 2.6 µm) column and Buffer (pH 4.5): Acetonitrile (40:60) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 277 nm. Retention time of Ornidazole and Diloxanide Furoate were found to be 4.620 min and 7.633 min, respectively. The method has been validated for linearity, accuracy and precision. Linearity observed for Ornidazole 5-15 µg/ml and for Diloxanide Furoate 7.5-22.5 µg/ml. The percentage recoveries obtained for Ornidazole and Diloxanide Furoate were found to be in range of 100.88 ± 0.60 and 100.85± 0.20 respectively. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial combined dosage form.
Keywords: Ornidazole (ORN), Diloxanide Furoate (DIF), Stability indicating RP-HPLC Method, Validation.

INTRODUCTION

The number of drugs introduced into the market is increasing every year [1]. DIF: DIF is an anti-protozoal drug used in the treatment of Entamoeba histolytica and some other protozoal infections. DIF is used alone as a primary agent in the treatment of asymptomatic (cyst passers) intestinal amoebiasis caused by Entamoeba histolytica this medication may also be used concurrently, or sequentially, with other agents such as the nitroimidazoles in the treatment of invasive or extra intestinal forms of amoebiasis. DIF alone is not effective in the treatment of invasive or extra intestinal amoebiasis [2]. ORN: ORN is a nitroimidazoles antiprotozoal agent used in amoeba and trichomonas infections. It is partially plasma-bound and also has radiation sensitizing action. ORN is primarily indicated in conditions like Amoebiasis, Amoebic dysentery, Bacterial vaginosis, Giardiasis, Trichomoniasis. Plasma half life is 12-14 hrs[3].

Several HPLC methods are available in the literature for individual drugs and for a combination with other drugs for determination of ORN and DIF, but no stability-indicting assay method (SIAM) has been reported. Accordingly, the present study was planned to develop stability-indicating assay RP-HPLC method for the simultaneous determination of ORN and DIF in presence of interaction/degradation product. The method was validated with respect to linearity, precision, accuracy, specificity, and robustness. Statistically designed experiments were performed by varying different method parameters such as buffer concentration, pH of mobile phase, flow rate, mobile phase composition, and column temperature, to study the effect of these method parameters on system suitability criteria of all two drugs as a part of the robustness study.

EXPERIMENTAL:

Materials and Reagents:

Pure ORN and DIF were obtained as gratis sample from Alembic pharmaceuticals (Baroda, India). Acetonitrile, potassium hydrogen phosphate, methanol were purchased from Samir tech chem (Baroda, India).All reagents used were at least of analytical grade except acetonitrile, methanol, water which was HPLC grade. High purity water was prepared by passage through a samir tech chem. (Baroda, India) and was used to prepare all solutions.

Preparation of Standard Solutions: [4, 5]

(A) ORN standard stock solution: (100 µg/mL) A 10 mg of ORN was weighed and transferred to a 100 mL volumetric flask. Volume was made up to the mark with methanol.

(B) DIF standard stock solution: (150 µg/mL) A 15 mg of DIF was weighed and transferred to a 100 mL volumetric flask.

(C) Preparation of standard solution of binary mixtures of ORN (10 µg/mL) and DIF (15 µg/mL) 1 mL from the

ORN stock solution and 1mL from DIF stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

HPLC Instrumentation & Chromatographic Conditions:

The analysis was carried out on a HPLC system (shimadzu) equipped with U.V detector. Other apparatus and instruments used were a micro analytical balance (shimadzu, type-BL-22OH), Ultra sonicator (EIE instruments), pH meter (welltronixPM100).All instruments and glassware's were calibrated. Chromatographic analysis was carried out on an Hypersil BDS C-18 column,(25cm× 0.46cm).The mobile phase consisted of Buffer(pH 4.5):Acetonitrile (40:60).The mobile phase was filtered through Millipore filter paper type HV (0.45 µm) and degassed by sonication, was pumped at 1 ml/min flow rate. The column was thermo stated at room temperature. Under these conditions the run time was 9 min.

Forced Degradation Study: [6, 7]

(A) Acid degradation: One ml of stock solution was transferred in to 10 ml of volumetric flask. Two ml of 0.1 N HCl solutions was added and mixed well and put for 4 hrs at 70 ºC. After time period the content was cooled to RT. Then the volume was adjusted with diluents to get 10µg/ml for ORN and 15 µg/ml for DIF.

(B) Base degradation: One ml of stock solution was transferred in to 10 ml of volumetric flask. Two ml of 0.1 N NaOH solutions was added and mixed well and put for 3 hrs at 70 ºC. After time period the content was cooled to RT. Then the volume was adjusted with diluents to get 10µg/ml for ORN and 15 µg/ml for DIF.

(C) Oxidative degradation: One ml of stock solution was transferred in to 10 ml of volumetric flask. Two ml of 3% H_2O_2 solutions was added and mixed well and put for 30 min at 70 ºC. After time period the content was cooled to RT. Then the volume was adjusted with diluents to get 10µg/ml for ORN and 15 µg/ml for DIF.

(D) Photo Degradation: One ml of stock solution was transferred in to 10 ml of volumetric flask. The volumetric flask was kept in Sunlight for 1 hrs. Then the volume was adjusted with diluents to get 10µg/ml for ORN and 15 µg/ml ford IF.

(E) Thermal degradation: One ml of stock solution was transferred in to 10 ml of volumetric flask. The volumetric flask was stored in oven at 105°C for 5 hrs. Then the volume was adjusted with diluents to get 10µg/ml for ORN and 15 µg/ml for DIF.

Separation Studies and Development of Stability-Indicating Method: [8, 9]

Satisfactory separations were achieved by gradient elution using mobile phase of different composition of Buffer (pH 4.5): Acetonitrile (40:60) at a flow of 1.0mL/min. The detection wavelength was 277nm. Mobile phase was filtered through 0.45µm Chrom Tech Nylon-66 filter and degassed prior to use by sonication in all HPLC runs. The injection volume was 20µL and a mixture of mobile phase A and B (40:60v/v) was taken as diluent. The column used for the entire study was hypersil BDS (25cm × 4.6 mm i.d., particle size 5µm).

Method Validation [10, 11]

The method was validated for linearity, precision (interday, intraday, and repeatability), accuracy, and robustness as per the ICH guideline Q2 (R1). Precision: Results should be expressed as Relative standard deviation (RSD) or coefficient of variance. A. Repeatability: Standard solution containing ORN (10µg/ml) and DIF (15 µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated. B. Intra-day precision Standard solution containing (5, 10, 15 µg/ml) of ORN and (7.5,15,22.5 µg/ml) of DIF were analyzed on the same day in triplicate and Inter-day precision were analyzed three times on the different day and % R.S.D was calculated of both .The accuracy was determined by spiking the mixture of stressed samples with three concentration of drug corresponding to 80%, 100%, and 120% of ORN and DIF (80µg/mL, 100µg/mL, 120µg/mL) in combination in triplicate. The mean percentage recoveries for the proposed method were calculated then determining percent recovery of the added drug. LOD and LOQ were determined from the set of 3 calibration curves used to determination method linearity.

Robustness Experiments:

Robustness of the method was checked by deliberate changes in the chromatographic conditions ratio of Mobile phase was changed (±2) Buffer: Acetonitrile (38:62) and Buffer: Acetonitrile (42:58), flow rate (0.3 and 1.2mL/min), pH (4.3 and 4.7), gradient flow (in composition), and column temperature (25°C and 30°C).

RESULTS AND DISCUSSION:

Development and Optimization of the Stability-Indicating HPLC Method:

The literature search indicated that many HPLC methods were available for individual and a combination of two drugs. Based on the literature search, attempts were made to develop a simple method which had less retention time and higher selectivity. But Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performing in terms of efficiency, stability and reproducibility. C18 column is least polar compare to C4 and C8 columns. Here, A 250 x 4.6 mm column of 5.0 µm particle packing was selected for separation of ORN and DIF. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

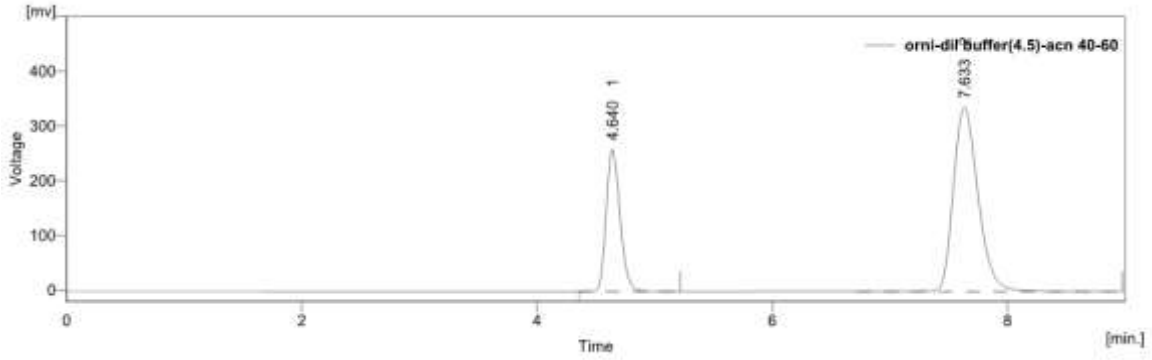

Fig-1: Chromatogram of ORN and DIF in Buffer (pH 4.5) : Acetonitrile (40:60).

Forced Degradation Study:

Forced degradation study was done on an individual and drug combination by Acid, Base, Photo Oxidative and thermal methods. Each of the degradation products of ORN and DIF. Labeling of all degradation products was done by a degrading individual drug with a similar condition as used for the combination. Retention time and wavelength of degradation product were useful parameters to label degradation products. Such labeling was very useful to identify common degradation products among different degradation conditions was found to be a common degradation product under thermal degradation condition. Preliminary trials on individual drugs and those in combination were conducted to optimize various stress conditions. Samples were withdrawn at 4 hours intervals, to monitor the rate of degradation and optimize the stress conditions. All drugs showed degradation in Acid, Base, Photo, Oxidative, and thermal methods at room temperature for 24 hours. DIF was easily susceptible to degradation in comparison of ORN in drastic condition. DIF was comparatively more prone to degradation under acid, basic, photo, oxidative and thermal conditions.

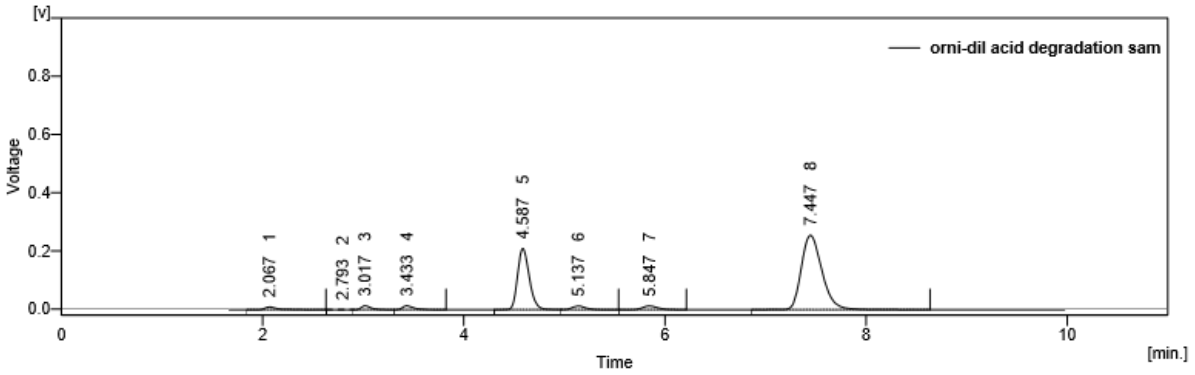

Fig-2: ORN and DIF Acid Degradation Sample

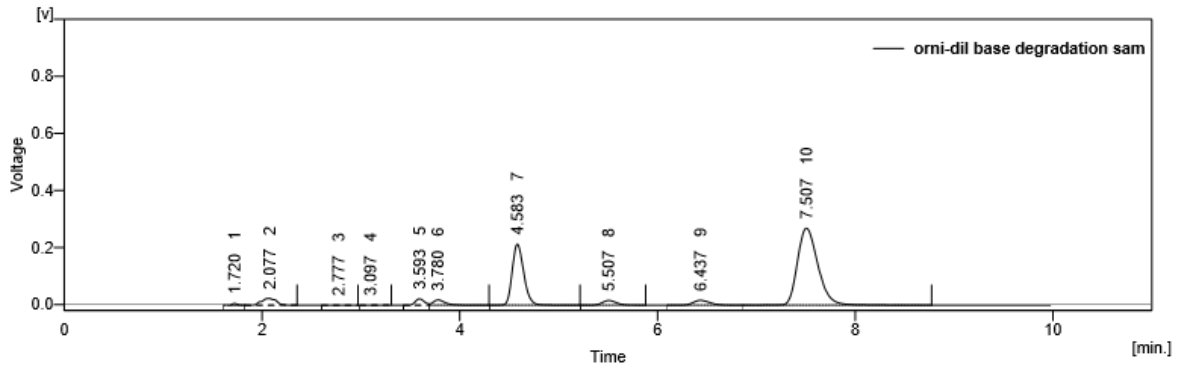

Fig-3: ORN and DIF Base Degradation Sample

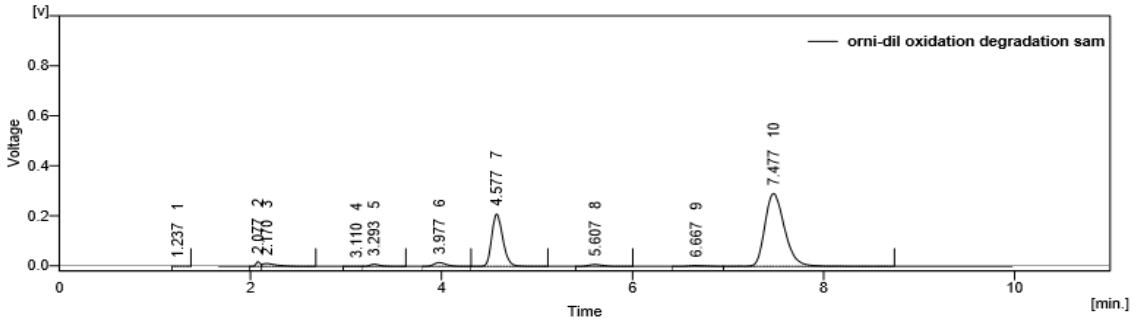

Fig-4: ORN and DIF Oxidation Degradation sample

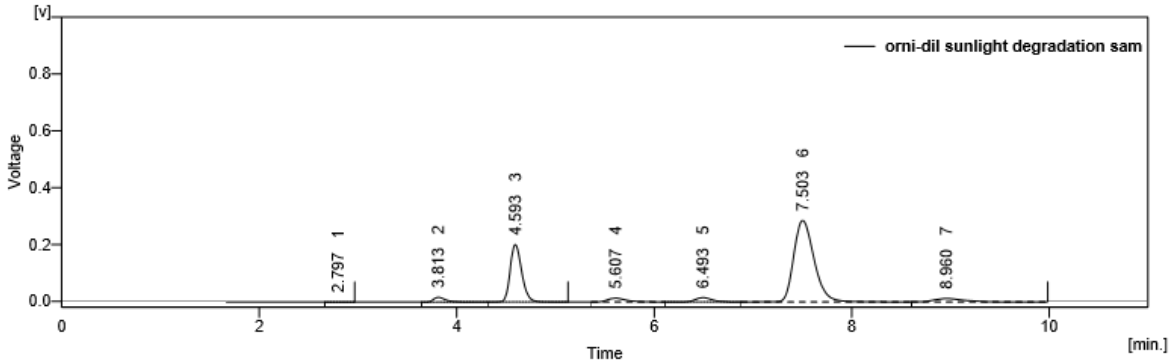

Fig-5: ORN and DIF Photo Degradation sample

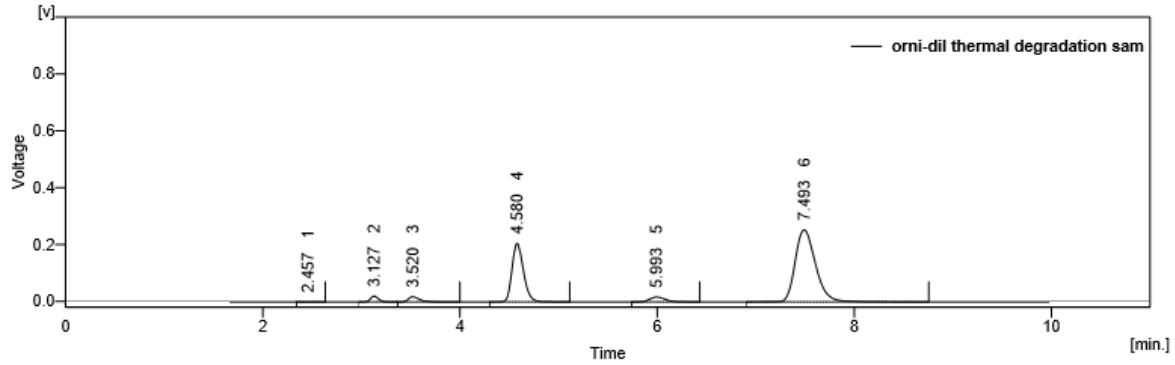

Fig-6: ORN and DIF Thermal Degradation sample

Table 1: Degradation sample data of ORN and DIF

	ORN				DIF			
PARAMETER	**STANDARD**		**SAMPLE**		**STANDARD**		**SAMPLE**	
	Area	**% Degradation**	**Area**	**% Degradation**	**Area**	**% Degradation**	**Area**	**% Degradation**
Acid	1700.76	20.82	1695.82	21.05	3646.18	25.03	3620.76	25.55
Base	1730.48	19.44	1724.49	19.72	3876.99	20.29	3858.37	20.67
Oxidation	1693.43	21.163	1682.95	21.651	4180.21	14.05	4146.79	14.74
Photo	1638.89	23.70	1630.77	24.080	4125.41	15.183	4102.35	15.65
Thermal	1669.83	22.26	1658.41	22.79	3619.79	25.57	3612.59	25.72

Method Validation:

Linearity, LOD, LOQ, and Specificity:

A linear response was obtained in the concentration range 5–15 µg/mL & 7.5–22.5 µg/mL for ORN and DIF respectively. The results of the system suitability tests assure the adequacy of the proposed HPLC method for routine analysis of ORN and DIF alone or in combination. Characteristic parameters are given in Table-2.

Table 2: linearity data for the proposed method

STATISTICAL PARAMETER	ORN	DIF
Average peak area* ± SD	2410.334 ±0.95	1047.249 ±0.95
	3573.12 ±0.97	1557.559 ±0.94
	4794.82 ±0.95	2079.666 ±0.96
	6020.38 ±0.94	2608.285 ±0.95
	7541.73 ±0.95	3023.098 ±0.96
Concentrate Range(µg/ml)	5–15	7.5–22.5
Regression equation	Y = 508.4x-215.9	Y= 33.4x+62.20
Correlation coefficient(R^2)	0.997	0.998
LOD(µg/ml)	0.790	0.925
LOQ(µg/ml)	2.394	2.804

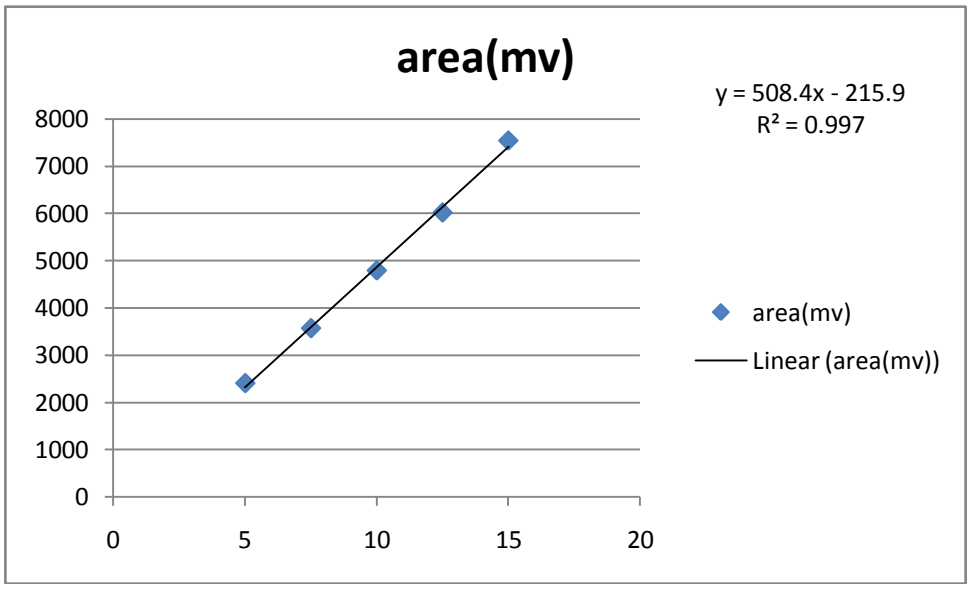

Fig-7: Calibration Curve of ORN (5-15 µg/ml).

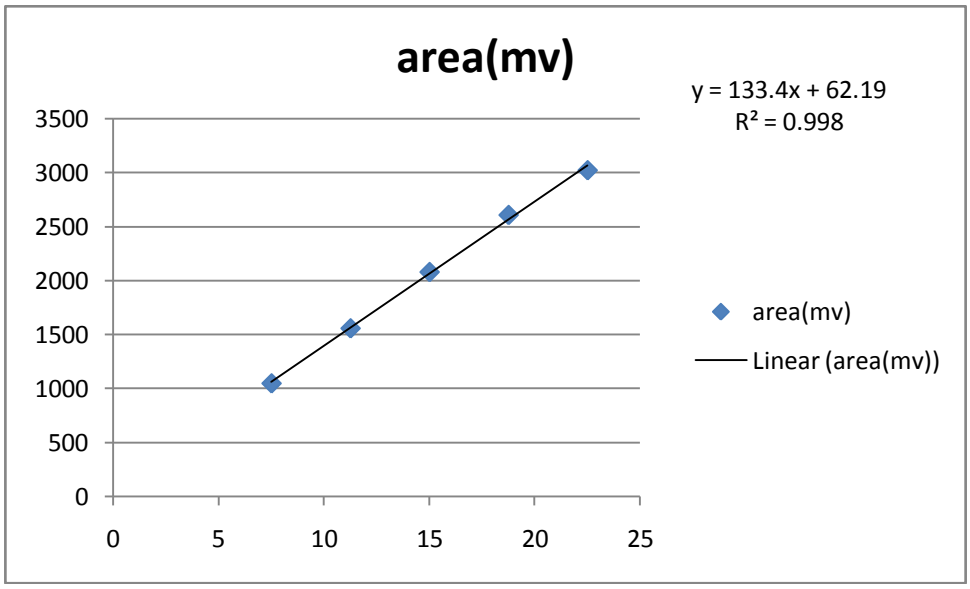

Fig-8: Calibration Curve of DIF (7.5-22.5 µg/ml).

Precision:

Interday and intraday precision studies data obtained on the analysis of sample from precision experiments are given in Table. The % relative standard deviation (RSD) values for inter- and intraday precision were less than 1%. Intermediate precision was determined by carrying out the experiment by a different analyst on a different system .Almost similar retention time was observed for all the two drugs.

Table 3: Repeatability Data for ORN and DIF

	ORN			DIF		
Sr. No.	**Conc. (µg/mL)**	**Mean ± S.D (n=6)**	**% R.S.D**	**Conc. (µg/mL)**	**Mean ± S.D(n=6)**	**% R.S.D**
1	10	4804.76±26.3	0.548	15	2086.99±4.74	0.227

Table 4: Intraday Precision data for ORN and DIF

	ORN			DIF		
Sr. No.	**Conc. (µg/mL)**	**Area Mean ± S.D. (n=3)**	**%R.S.D**	**Conc. (µg/mL)**	**Area Mean ± S.D. (n=3)**	**%R.S.D**
1	5	2355.53±7.69	0.326	7.5	1015.66±16.02	1.577
2	10	4764.78±25.77	0.541	15	2071.14±7.88	0.380
3	15	7098.57±20.85	0.294	22.5	3079.39±14.51	0.471

Recovery:

Accuracy was checked by the standard addition method, by spiking standard drugs at three different concentration levels to the marketed formulation containing ORN & DIF in triplicate. The mean percentage recoveries for the proposed method were calculated. Recovery of individual components from the pharmaceutical dosage form ranged from 99.83 to 101.30%. Based on the results, it can be concluded that the excipients used do not interfere in the analysis of ORN and DIF furoate in their pharmaceutical formulation (Table-5).

Robustness:

Robust analytical methods are required in quality control laboratories for routine use (Table-6, Table-7).

Table 5: Recovery Studies Data for ORN and DIF

		ORN				DIF			
Sr. No.	**Level (%)**	**Sample amount (µg/ml)**	**Conc. Added (µg/ml)**	**Conc. recovered (µg/ml)**	**Mean Recovery ± S.D (% , n=3)**	**Sample amount (µg/mL)**	**Conc. added (µg/mL)**	**Conc. Recovered (µg/mL)**	**Mean Recovery ± S.D (%, n=3)**
1	80%	5	4	4.02	100.69±0.3	7.5	6	6.07	101.3±0.5
2	100%	5	5	5.04	100.88±0.6	7.5	7.5	7.56	100.8±0.2
3	120%	5	6	5.97	99.55±1.3	7.5	8.93	8.98	99.83±0.8

Table -6: Robustness Data for ORN

Sr. No	Area at Flow rate (0.8ml/min)	Area at Flow rate (1.2ml/min)	Area at pH (4.3)	Area at pH (4.7)	Area at Mobile phase (38:62)	Area at Mobile phase (42:58)
1	5006.524	4722.541	4953.302	4617.008	4918.783	4755.571
2	4951.739	4719.342	4978.179	4598.266	4938.49	4717.82
3	5026.353	4692.616	4926.533	4621.396	4948.528	4713.105
% R.S.D	0.773	0.349	0.521	0.266	0.307	0.492

Table-7: Robustness Data for DIF

S.No	Area at Flow rate (0.8ml/min)	Area at Flow rate (1.2ml/min)	Area at pH (4.3)	Area at pH (4.7)	Area at Mobile phase (38:62)	Area at Mobile phase (42:58)
1	2171.467	2048.34	2148.416	2002.596	2133.425	2062.69
2	2197.553	2044.763	2159.204	2008.531	2134.462	2046.313
3	2180.013	2038.381	2148.456	2004.452	2146.3	2044.245
% R.S.D	0.609	0.247	0.289	0.151	0.334	0.493

CONCLUSION:

A simple, accurate, precise, rugged, economic and rapid stability indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed which could separate as well as accurately quantify ORN and DIF. Analysis of marketed formulation containing ORN and DIF showed no interference from the common additives and excipients. At the same time, same method shows specificity for stressed conditions. It can be successfully adopted for routine quality control analysis of ORN and DIF in combined Tablet dosage form without any interference from common excipients and impurity.ORN and DIF in combined Tablet dosage form without any interference from common excipients and impurity.

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