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# AN ECONOMICAL STABILITY INDICATING RP HPLC METHOD FOR THE SIMULTANEOUS ANALYSIS OF OFLOXACIN AND TINIDAZOLE

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**ABSTRACT**

The present manuscript describes the development of a simple, economical and LC-MS compatible stability indicating high performance liquid chromatographic (HPLC) method for the simultaneous analysis of ofloxacin (OFL) and tinidazole (TNZ) under different stresss conditons as specified by ICH. For the analysis, an isocratic method was chosen which used Phenomenex (250 x 4.6mm, 5µm particle size) ODS column and a SPD 20 A UV detector set at 298 nm. The mobile phase is a combination of organic phase and aqueous phase, where the former is a mixture of methanol and acetonitrile in the ratio of 3 : 7 and the latter is 0.1% trifluoroacetic acid solution (pH adjusted to 3.5 with dil ammonia solution). The ratio of aqueous and organic phase was 80 : 20, at a flow rate of 1.5 mL $min^{-1}$.The linear regression analysis data for the calibration plots showed good linear relationship with $r^2$ = 0.9998 for OFL in the linearity range of 10 – 60 µg $mL^{-1}$ and $r^2$ = 0.9996 for TNZ and the linearity range is 15 – 90 µg $mL^{-1}$. The assay results were found tobe in the range of 98.35 – 100.71% and 99.81-101.35% respectively for OFL and TNZ.The recovery level of OFL and TNZ was found to be in the range of 97.57–104.37% for the two formulations analysed and the %RSD was found to be < 2.0%. The stress degradation studies were performed using acid, alkali, water, hydrogen peroxide, uv light and heat. Since there is no coelution of the degradation products' peaks with the drug peaks, it can be employed as a stability indicating method.Further the structure of the degradants can be studied by LC-MS,as the optimised mobile phase is LC-MS compatible.

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## INTRODUCTION

Ofloxacin (OFL),a fluorinated carboxyquinolone, is a racemate and (±)- 9-fluro-2, 9-fluro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid )[1]. It is used as an antibiotic for the treatment of urinary tract infection and sexually transmitted diseases.

Tinidazole (TNZ) [1-(2-ethylsulfonylethyl)-2-methyl- 5-nitroimidazole] is a 5-nitroimidazole derivative, an anti parasitic drug used against protozoan infections.[2] It is used in the treatment of a variety of amoebic and parasitic infections [3]. Chemical structure of ofloxacin and tinidazole is given in figure 1 and 2 respectively.

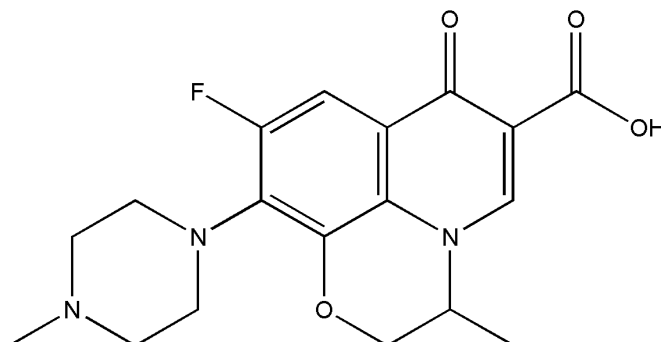


**Figure 1.Chemical structure of ofloxacin.**

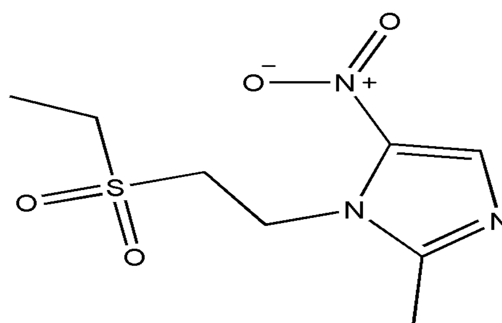


**Figure 2.Chemical structure of tinidazole.**

OFL was determined by different methods like spectrophotometry [4-7] and chemiluminescence [8,9]. The drug is analysed along with other drugs by HPLC [10-16], spectrophotometric [17,18] and capillary electrophoretic method [19,20]. TNZ was determined by spectrophotometry[21,22] , HPTLC [23,24] and electrochemical method [25]. Along with other drugs, TNZ was analysed by uv spectroscopy [26-28] capillary electrophoresis [29] and polarography[30]

A detailed literature survey revealed the presence of very less analytical methods for the combination of OFL and TNZ in pharmaceutical formulations. There are two spectrophotometric [31,2] and two HPLC[32,33] methods, available for the estimation of the aforementioned combination. Upto date not even a single stability indicating method has been reported for this combination and hence there is an urgent need to develop one method for this combination as per the ICH requirement.

## MATERIALS AND METHODS

### Drugs and Reagents

Gratis samples of ofloxacin(OFL) and tinidazole (TNZ) API were available from Dr.Reddys Lab, Hyderabad. Tablet dosage form was purchased from a local pharmacy. Methanol, acetonitrile and trifluoroacetic acid were of HPLC grade. Hydrochloric acid, sodium hydroxide, hydrogen peroxide and ammonia solution were of A.R grade.All chemicals were purchased from S.D Fine Chemicals (Mumbai, India). HPLC grade water was prepared in house by the triple distillation of water followed by filtration through millipore filter paper of 0.45 µm pore size.

### HPLC Instrumentation and Chromatographic Conditions

The HPLC system consists of a Shimadzu LC 20 AD binary pump and SPD 20 A UV detector. The column consists of Phenomenex (250mm X 4.6 mm, 5µm) Luna ODS column. Data acquisition was done with LC Solution software. Rheodyne manual Injector was used and each time 20 µL of sample solution was injected. Several trials with different mobile phase compositions were performed for a satisfactory separation for the drug and its degradation products and finally arrived at a suitable mobile phase. For the detection, a single wavelength was selected, were both drugs are having considerable absorbance and the peak areas integration was performed using LC solution software.

**Preparation of solutions for injection**

20 mg of the reference substance of OFL and 30 mg of TNZ were dissolved in 10 mL of methanol so as to give 2000 and 3000 µg/mL of OFL and TNZ respectively. Initially single standard solutions were prepared from the stock solutions and injected separately so as to distinguish the retention time of individual drugs. Then mixed standard solution cotaining both drugs was prepared by suitable dilution of the stock solution using mobile phase. Similarly sample solution was prepared using the tablets conatining 200mg of OFL and 300 mg of TNZ. Tablets were accurately weighed and powdered and a quantity of powder equivalent to 20mg of OFL was dissolved in methanol by keeping in an ultrasonic bath for 10 min. Later the solution was filtered and diluted suitably with the mobile phase to get a mixed sample of 20 µg/mL of OFL and 30 µg/mL of TNZ.

**Validation study**

The developed method was validated as per ICH guidelines [34]. The parameters included are linearity, accuracy, precision, LOD, LOQ, specificity, robustness etc[35,36]. For the linearity studies, standards were prepared in the range of 10, 20, 30, 40, 50 and 60 µg/mL for ofloxacin and 15, 30, 45, 60, 75 and 90 µg/mL for tinidazole. Standard curve was obtained by plotting peak area against concentration and the evaluation of linearity was done by linear regression analysis using least square method. Each concentration was analysed in triplicate.

Accuracy of the developed method was assessed in triplicate, as % recovery of each drug after spiking the standard solutions at three percentage levels ie 80, 100 and 120 to the preanalysed sample solutions of 20 µg/mL of OFL and 30 µg/mL of TNZ. The % recovery of each drug was calculated as (OFL found/OFL spiked) X100 and (TNZ found/ TNZ spiked) X100. The amount of OFL or TNZ found, were calculated from the linear regression equation obtained in the linearity studies.

The precision study was performed as the repeatability of the method and intermediate precision of the sample solutions. It was done at three concentration levels for OFL (20, 40 and 60 µg/ml) and TNZ (30, 60 and 90 µg/ml). Repeatability (intra day precision) was calculated by analysing the samples prepared on the same day. Intermediate precision (inter day precision) was calculated by analysing the samples on 3 consecutive days. Precision of the method was estimated by calculating the percentage relative standard deviation of the concentrations obtained by the method.

Limit of detection (LOD) and limit of quantitation (LOQ) were estimated at a signal to noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions of known concentration. The values can be calculated by equation 1 and 2 respectively.

$LOD = 3.3\ \sigma/S$ equation 1

$LOQ = 10\ \sigma/S$ equation 2

$\sigma$ = standard deviation of the response and S= slope of calibration curve

Experimental conditions were deliberately altered, in order to determine the robustness. From the different experimental conditions such as flow rate (1.50 mL/min), wavelength of measurement (298 nm), pH of the aqueous phase (3.5) and percentage of methanol (20), each selected factor was changed at three levels (-1, 0, +1) one by one so that the impact of the imposed change on the assay results can be analysed. Change in the peak area (ie concentration) and the retention time were noted for each change in the optimised parameters.

**Specificity**

Specificity is the ability of a method to measure analytical response in presence of its potential impurities. It was proved by the deliberate degradation of the drug by oxidation, heat, hydrolysis (acidic, alkaline, and neutral) and photolysis, followed by its analysis using the developed method. The method can be called as a stability indicating method if there is no co elution of the degradants with the pure drugs along with good resolution between the drug peak and that due to the degradant. The stress degradation studies were done on individual drugs and later extended to binary drug combination. A mixed stock solution of 2000 µg/mL of OFL and 3000 µg/mL of TNZ, prepared in methanol was used for stress degradation. The procedure followed for each degradation is given below in detail.

**Hydrolytic degradation**

Hydrolytic degradation was performed under neutral, alkaline and acidic pH for which 2mL of each reagent (water, 1N NaOH, 1N HCl) was added to 2mL of stock solution and allowed the reaction to proceed for the stipulated time. For neutral hydrolysis, the mixture of solution was kept for 24hrs at room temperature for the possible degradation. Since the degradation reaction was faster in alkaline media for tinidazole the reaction mixture was kept for 10min at room temp. Acidic degradation was allowed to proceed for 5hrs. at room temp. For acidic and alkaline degradation the solutions were neutralised with 1N NaOH, 1N HCl respectively, before dilution.

**Oxidative degradation**

To 2mL of stock solution, 2mL of 3% w/v $H_2O_2$ solution was added and the reaction was allowed to proceed for 24hrs at room temperature. Then it was diluted with the mobile phase.

**Thermal degradation**

Sample powders were heated at $100^0$C for 20 hrs. in a hot air oven and these samples were used for preparing the stock solution in methanol and sample solution was prepared by diluting with the mobile phase.

**Photolytic degradation**

Sample powders exposed to short UV radiation (wavelength of 253nm) for 48hrs in a UV chamber was used for the preparation of stock solution by dissolving it in methanol and from this dilution was prepared using mobile phase.

**System suitability test**

System suitability tests were done to verify the system performance and was done by measuring the parameters such as retention time, tailing factor, number of theoretical plates, peak area etc. after performing three injections of a standard solution containing 20 µg/ml of OFL and 30 µg/ml of TNZ. The %RSD values were calculated for each parameter.

**RESULTS AND DISCUSSION**

An isocratic LC- UV method was developed for the concurrent analysis of OFL and TNZ and at the same it can reveal the presence of forced degradation products. Different trials were performed by using different buffers in combination with methanol, acetonitrile or methanol and acetonitrile in different ratios. Either only one drug used to elute or both drugs used to elute with poor resolution. Finally arrived at the optimized method by considering the various chromatographic parameters such as tailing factor, theoretical plate etc.

**UV Spectral Analysis**

In order to fix the wavelength of measurement, both the standard solutions prepared in the mobile phase were scanned separately and the overlaid spectra showed that 298nm can be used as both drugs are having considerable absorbance at this wavelength.

**Optimization of Chromatographic conditions**

For the analysis, different combinations of mobile phases were tried and the chromatographic method was finalised by giving special attention to the theoretical plate count, tailing factor and resolution. Isocratic mode was selected and the mobile phase was a combination of organic phase and aqueous phase, where the former was a mixture of methanol and acetonitrile in the ratio of 3: 7 and the latter was 0.1% trifluoroacetic acid solution (pH adjusted to 3.5 with dil ammonia solution) For the mobile phase, the ratio of aqueous and organic solution was 80: 20, pumped at a rate of 1.5 mL/min at room temp. The pH of the mobile phase was found to be significant as at lesser pH, OFL peak was tailing, though TNZ peak was good and at pH greater than 3.5 both peaks were getting eluted very closely with poor resolution. 20 µL of sample solution was injected each time using rheodyne manual injector. For the detection, the wavelength was set at 298nm and the peak areas integration was performed using LC solution software. To analyze the drugs together with its possible degradation products, RP - HPLC- UV instrument was employed and the developed method was validated. The retention time (in min) was found to be 11.45 for ofloxacin and 7.46 for tinidazole. The chromatograms obtained for standard and samples prepared from formulation are shown in Figure 3 and 4 respectively.

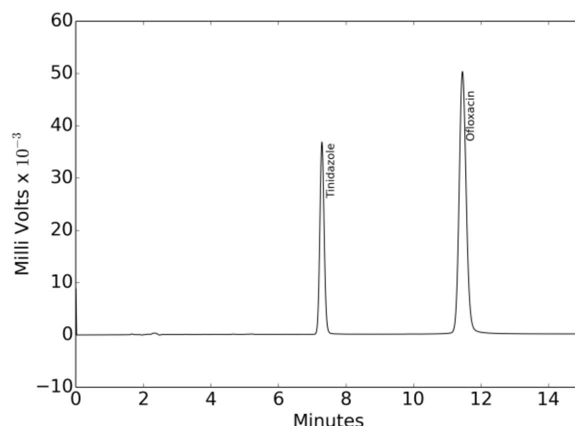


**Figure 3 HPLC chromatogram of TNZ and OFL(standard): Rt of TNZ=7.46 min and Rt of OFL= 11.45 min.**

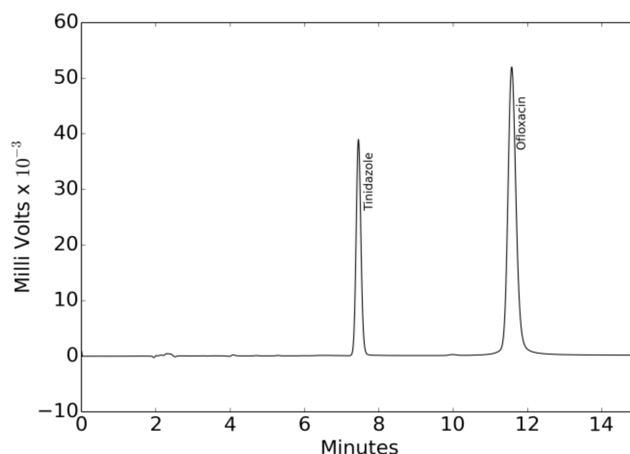


**Figure 4 HPLC chromatogram of TNZ and OFLsamples (prepared from tablet): Rt of TNZ=7.44 min and Rt of OFL= 11.46 min.**

**Results of Stress degradation studies**

Ofloxacin was found to degrade under acidic condition whereas tinidazole did not undergo degradation. This was proved by the injection of stress samples of single drugs after degradtion which showed the absence of extra peaks for TNZ. The extent of degradation was faster in alkaline condition for tinidazole as shown by the presence of extra peak and the area of tinidazole peak is reduced drastically. Studies were extended to photolytic, dry heat, oxidative and hydrolytic degradation and both drugs were found to be stable under these conditions.The chromatograms that indicate the stability indicating nature of the method are given in figure 5 and 6.

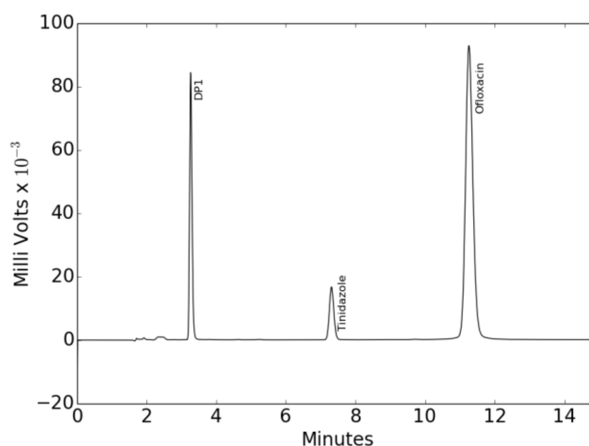


**Figure 5 HPLC chromatogram of TNZ and OFL after alkaline degradation: TNZ sample degraded with 1.0 N NaOH; Degradation product's peak appeared at 3.265 min.**

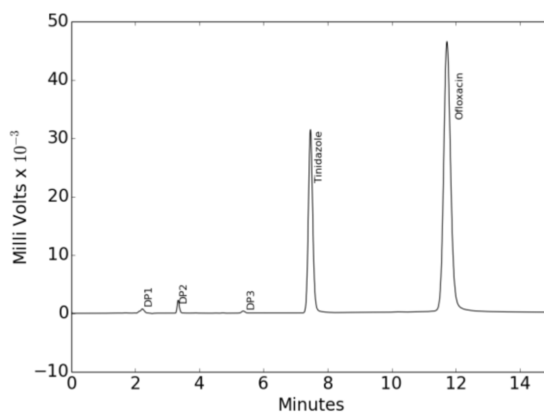


**Figure 6 HPLC chromatogram of TNZ and OFL after acidic degradation: OFL sample degraded with 1.0 N HCl; Degradation products' peaks appeared at 2.22, 3.33 and 5.36 min.**

**Validation results**

**Linearity results**

A linear calibration plot was obtained for OFL over the concentration range of 10 – 60 µg/ml. The linear regression equation was found to be y = 75190x - 42156 with a correlation coefficient of 0.9998. For TNZ, the linearity range was 15 – 90 µg/ml and the linear regression equation was found to be y = 17628x - 14593 with a correlation coefficient of 0.9996. The linearity results are given in detail in table 1 and the corresponding graphs are given in figure 7 and 8.

**Table 1 Linearity data.**

| Parameters | OFL | TNZ |
|---|---|---|
| Linearity range[a] | 10 – 60 | 15 – 90 |
| Slope | 75190 | 17628 |
| Intercept | - 42156 | - 14593 |
| Correlation coefficient | 0.9998 | 0.9996 |
| LOD ( ng/ml) | 283.60 | 164.40 |
| LOQ ( ng/ml) | 859.40 | 498.19 |

[a] in µg/ml

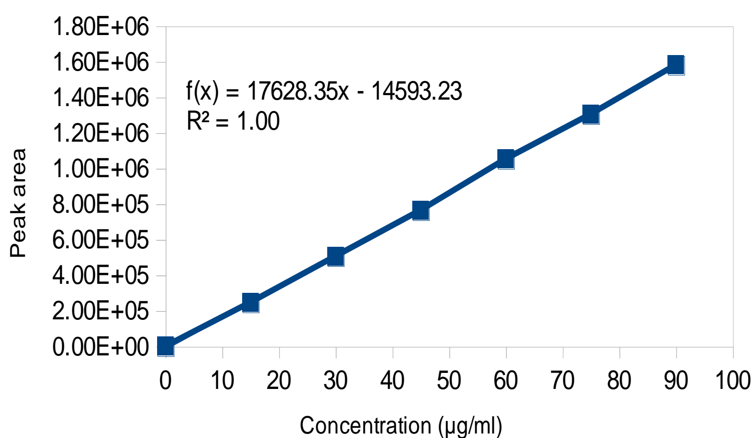


**Figure 7 Linearity graph of Tinidazole.**

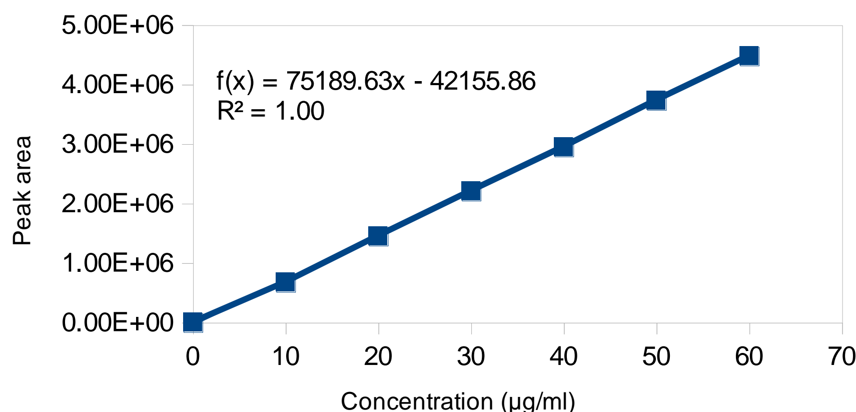


**Figure 8 Linearity graph of Ofloxacin.**

**Precision**

Intraday and interday precision studies were evaluated by calculating the % RSD values. The drug amount were calculated using the regression equation from linearity studies. The data obtained from precision experiments are given in table 2 for intraday and interday precision studies. The % RSD values for intraday and interday studies were < 2.0%, confirming that the method was precise.

**Table 2 Precision data.**

| Drug | Theoretical amount µg/ml | Amount found[a] ± SD[b], % RSD[c] intraday | interday |
|---|---|---|---|
| OFL | 20 | 20.17 ± 0.14, 0.72 | 20.29 ± 0.26, 1.29 |
| | 40 | 39.79 ± 0.38, 0.95 | 41.02 ± 0.76, 1.85 |
| | 60 | 61.05 ± 0.51, 0.83 | 60.59 ± 0.49, 0.81 |
| TNZ | 30 | 30.08 ± 0.51, 1.70 | 30.65 ± 0.60,1.94 |
| | 60 | 61.12 ± 1.05, 1.73 | 59.94 ± 1.05, 1.76 |
| | 90 | 89.34 ± 1.75, 1.96 | 89.72 ± 1.54, 1.72 |

[a] Mean of three determinations (n=3) for intraday precision and mean of six determinations for interday(n=6)precision and the amounts in µg/ml

[b] SD= Standard deviation

[c] RelativeStandard deviation = (Standard deviation/arithmetic mean)X100

**Accuracy (Recovery studies)**

The recovery level of OFL and TNZ was found to be in the range of 97.57–104.37% for the two formulations and the %RSD was found to be < 2.0%. The data is given in detail in *Table 3*.

**Table 3 Data for Recovery studies.**

| Brand | Spiking level (%) | Drug | Theoretical content (mg) | Amount found[a] (mg) | Recovery (%)[b] | %RSD[c] |
|---|---|---|---|---|---|---|
| ZANOCIN -TZ | 80 | OFL | 16 | 16.25 | 101.58 | 0.81 |
| | | TNZ | 24 | 24.94 | 103.90 | 0.06 |
| | 100 | OFL | 20 | 20.39 | 101.95 | 1.89 |
| | | TNZ | 30 | 29.36 | 97.87 | 2.04 |
| | 120 | OFL | 24 | 24.49 | 102.04 | 1.01 |
| | | TNZ | 36 | 36.36 | 100.99 | 1.57 |
| NITDIN-TZ | 80 | OFL | 16 | 15.61 | 97.57 | 0.73 |
| | | TNZ | 24 | 25.05 | 104.37 | 1.06 |
| | 100 | OFL | 20 | 20.73 | 103.67 | 1.42 |
| | | TNZ | 30 | 30.13 | 100.43 | 1.40 |
| | 120 | OFL | 24 | 25.04 | 104.32 | 1.46 |
| | | TNZ | 36 | 36.70 | 101.94 | 1.09 |

[a] Mean of three determinations (n=3)
[b] (drug found/spiked amount)X100
[c] (Standard deviation/arithmetic mean)X100.

**Limit of detection (LOD) and limit of quantification (LOQ)**

The LOD values of OFL and TNZ were found to be 283.60 ng/ml and 164.40 ng/ml and LOQ values were found to be 859.40 and 498.19 ng/ml respectively which indicate adequate sensitivity of the method.

**Robustness**

Analysis of the robustness results indicated that the small changes in chromatographic parameters such as flow rate (1.40 mL/min, 1.60 mL/min), wavelength of measurement (296 nm, 300nm), pH of the aqueous phase (3.3, 3.6) and percentage of methanol (18, 22) didnot cause a significant change in peak area and tailing factor, when each factor selected was changed one by one to estimate the effect of change on the assay results.

**System suitability test**

The results of system suitability tests indicate that the developed method can be utilised for the routine quality control of OFL and TNZ. The %RSD values calculated for each parameters such as retention time, tailing factor, number of theoretical plates, peak area etc after performing three injections of a standard solution containing 20 µg/ml of OFL and 30µg/ml of TNZ was found to be less than 2%. The tailing factor was found to be less than 1.2 and the number of theoretical plates was found to be greater than 2000 for both the drugs. The data regarding system suitability tests are given in detail in table 4.

**Table 4 System suitability studies[a].**

| Param eters | TNZ | OFL |
|---|---|---|
| Retention time (min) | 7.46 | 11.45 |
| Tailing factor | 1.106 | 1.179 |
| Resolution | - | 12.048 |
| Number of theoretical plates | 14354 | 12931 |

[a] average of three determinations.

**Analysis of the marketed formulations**

The method was validated by performing the assay of two tablet dosage forms namely, ZANOCIN -TZ with ofloxacin 200mg and tinidazole 300mg and NITDIN-TZ with ofloxacin 400mg and tinidazole 600mg. The results with the corresponding labeled amounts are reported in table 5. These amounts were within the limits. For both brands, the %RSD was less than 2, which indicated the accuracy of the proposed method.

**Table 5 Assay for tablet formulations.**

| Formulation | Drug, Labelled Claim (mg) | Amount found (mg) ± SD[a] | Assay (%)[b] | %RSD[c] |
|---|---|---|---|---|
| ZANOCIN -TZ | OFL, 200 | 201.41± 0.79 | 100.71 | 0.39 |
| | TNZ, 300 | 302.35± 1.74 | 101.35 | 0.57 |
| NITDIN-TZ | OFL, 400 | 393.42 ± 0.99 | 98.35 | 0.25 |
| | TNZ, 600 | 598.88± 5.45 | 99.81 | 0.91 |

[a]Mean of three determinations (n=3)
[b](Amount of drug found/labelled claim)X100
[c](Standard deviation/amount found)X100

**CONCLUSION**

A simple, accurate, precise and economical HPLC method has been developed and validated for the determination of OFL and TNZ in pharmaceutical formulations. The proposed method can be used as a stability indicating method for the drug combination as it can differentiate the pure drugs from the forced degradation products. It can be used for the routine quality control of OFL and TNZ. For the aqueous phase, 0.1% trifluoroacetic acid (80 parts) and the organic phase, a mixture of acetonitrile and methanol mixed at a ratio of 7:3 (20 parts) were selected. Since the organic phase required is less, it yields an economical method. Since there is no need of any tedious buffer preparation steps, it enhances the column life and also reduces the overall time required for the analysis. Due to the avoidance of any non volatile buffers as part of the optimised mobile phase, the degradation products can be characterised by LC-MS instrument.

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