EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 9
Issue: 8
239-244
Year: 2022

METHOD DEVELOPMENT, VALIDATION AND SIMULTANEOUS ESTIMATION OF OFLOXACIN AND TINIDAZOLE APIS AND TABLET FORMULATION IN HUMAN PLASMA USING RP-HPLC

Ravichandran S.* and Gowtham Prabakaran

Department of Pharmaceutical Analysis, PSG College of Pharmacy, Coimbatore, Tamil Nadu.

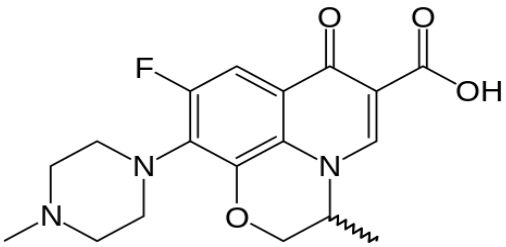
***Corresponding Author: Dr. Ravichandran S.**
Department of Pharmaceutical Analysis, PSG College of Pharmacy, Coimbatore, Tamil Nadu

Article Received on 16/06/2022 **Article Revised on 06/07/2022** **Article Accepted on 26/07/2022**

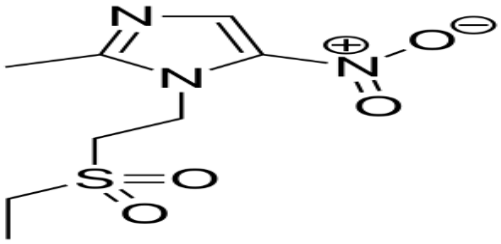
ABSTRACT
A superficial, selective, precise, and rapid isocratic reverse phase high performance liquid chromatography method with UV detection at 293nm was developed and validated for simultaneous estimation of Ofloxacin and Tinidazole as APIs and combined formulation from spiked human plasma. The column was equilibrated for at least 30min and separation was achieved by using Shimadzu-shim pack solar C8 (250×4.6mm, 5µm). The column was maintained at ambient temperature (25˚C). The chromatographic separation was performed with a mobile phase of acetonitrile: potassium dihydrogen orthophosphate buffer pH 4.5 (30:70v/v). The flow rate was 1mL/min, and the injector volume was 20µL. Ofloxacin and Tinidazole were well resolved from plasma constituents. The retention times was found to be 1.9 min for Ofloxacin and 3.2 min for Tinidazole. The method was found to be linear in the range of 1-5 µg/mL for Ofloxacin and 3-15 µg/mL for Tinidazole. The developed method was validated as per ICH guidelines. The developed precise method was validated in respect to precision, range, limit of detection, limit of quantitation, robustness as per ICH Q2B guidelines.

KEYWORDS: Ofloxacin, Tinidazole, Plasma analysis, Bio-analytical method, RP-HPLC.

1. INTRODUCTION
Ofloxacin is an antibiotic. It is chemically 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 a quinolone antibiotic useful for the treatment of a number of bacterial infections. When taken by mouth or injection into a vein, these include pneumonia, cellulitis, UTI, and certain types of infectious diarrhoea. Mechanism of action of the drug is by inhibiting two enzymes involved in bacterial DNA synthesis, both of which are DNA topoisomerases that human cells lack and that are essential for bacterial DNA replication.[3,4]



Tinidazole is an antiamoebic, antiprotozoal drug. It is chemically 1- (2-ethylsulfonylethyl) -2 -methyl -5 -nitro-imidazole.[2] Antimicrobial used to treat bacterial infections of the vagina, stomach, liver, skin, joints. It belongs to a class of nitroimidazole antimicrobials that works by stopping the growth of bacteria. The mechanism of action of the drug is that it diffuses into an organism, inhibits protein synthesis by interacting with DNA and cause a loss in helical DNA structure. This drug is used to treat trichomoniasis, amoebiasis, and bacterial infections.[3,4]



A bio-analytical method is intended to develop a simple, precise, and new validated method of analysis. The analytical techniques are available for the estimation of individual drugs and in combination with other drugs. It consists of UV spectrophotometry,[5] HPTLC,[6] and HPLC.[7] Already a method has been developed for the estimation of Ofloxacin[8] and Tinidazole[9] in human plasma. Since no method was developed for simultaneous estimation of Ofloxacin and Tinidazole

from spiked human plasma. Hence explored in developing a precise, and a rapid isocratic RP–HPLC method for the simultaneous estimation of Ofloxacin and Tinidazole from spiked plasma as per ICH guidelines

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Analytically pure sample of Ofloxacin and Tinidazole were purchased from yarrow chemicals Pvt Ltd. Acetonitrile (HPLC Grade) purchased from Sigma Aldrich chemicals Pvt Ltd, Bangalore. Water for HPLC purchased from Thermo fisher scientific, Mumbai. Methanol (HPLC Grade) obtained from Himedia laboratory Pvt ltd, Mumbai. Potassium dihydrogen orthophosphate obtained from Himedia laboratory Pvt Ltd, Mumbai. Plasma samples are provided from PSG multi-speciality hospital, Coimbatore.

2.2. Instrumentation and Chromatographic condition

The chromatographic analysis was performed using Shimadzu HPLC equipped with Photo diode array detector and LC solution software for statistics acquisition. The analyte was separated using Shimadzu-shim pack solar C8 (250×4.6mm, 3µm). The phosphate buffer pH 24.5 and acetonitrile in the ratio (70:30 v/v) was used as a mobile phase. The flow rate was maintained at 1mL/min. The injection volume is kept at 20 µL and the detection was carried out at 293nm.

2.3. Preparation of standard stock solution

A standard stock solution of 100 for Ofloxacin and Tinidazole was prepared by using acetonitrile as diluent.

2.4. Preparation of standard calibration curves

Appropriate dilutions of Ofloxacin and Tinidazole standard stock solutions were done in 10 mL volumetric flask to get final concentrations of 1, 2, 3, 4, 5 µg/ml of Ofloxacin and 3, 6, 9, 12, 15 µg/ml of Tinidazole. A 10 µL of each mixture was injected into the column and the chromatogram was obtained at 293nm. A graph was plotted as concentration of drugs against peak area response.

2.5. Preparation of blank solution

0.5 mL of plasma was added to a polypropylene tube (Torson's). Then the tube was cyclomixed for 5mins. Then 1mL of Methanol was added to the tube and centrifuged for 20mins at 3000rpm. Further supernatant liquids were collected in another Eppendorf tube and supernatant was injected into the analytical column.

2.6. Preparation of sample

0.5 mL of Ofloxacin in the concentration of 1, 2, 3, 4, 5 µg/ml and 0.5 mL of Tinidazole in the concentration of 3, 6, 9, 12, 15 µg/ml were separately pipetted out and spiked into 0.5 mL of plasma in a polypropylene tube (Torson's). Then the tube was cyclomixed for 5 minutes. Then 1ml of Methanol was added to the tube and centrifuged for 20 minutes at 3000 rpm. Further supernatant liquids were collected in another Eppendorf tube and the supernatant was injected into the analytical column.

2.7. Preparation of mobile phase

Prepare a mixture of potassium dihydrogen ortho phosphate buffer pH 4.5 and acetonitrile in the volume ratio 70: 30 v/v for optimization. Then sonicate for 15 minutes to degas the mixture.

2.8. Method validation

Analytical validation parameters for the analysis of the proposed method were determined according to ICH (Q2 R1) guideline. The parameters such as linearity, precision, LOD, LOQ, robustness was performed.

2.9. Linearity

The linearity response was determined by analyzing five level of calibration curve in the range of 1-5 (1, 2, 3, 4, 5 µg/ml) for Ofloxacin and 3-15 (3, 6, 9, 12, 15 µg/ml) for Tinidazole. The calibration curve obtained by plotting concentration against peak area was plotted. The correlation coefficient and regression line equations for Ofloxacin and Tinidazole were calculated.

2.10. Precision

Repeatability and intermediate precision are expressed in terms of %RSD. As %RSD was found to be < 2 indicating the presented method is precise. The results are summarized in table respectively for repeatability and intermediate precision.

2.11. LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated by formula as given in ICH (Q2R1).

LOD = 3.3 × Standard deviation/slope

LOQ = 10 × Standard deviation/slope

2.12. Robustness

The robustness of the methods was evaluated by making changes in the flow rate, and detection wavelength keeping the other chromatographic conditions constant. The changes made in flow rate ±0.2ml/min and detection wavelength ±2%.

2.13. System suitability

The typical values for evaluating the system suitability of a chromatographic procedure includes the RSD < 2%, symmetry factor < 2, and theoretical plates > 2000. The determination of the system suitability of the analytical method was accomplished by assaying the samples of the same strength as Ofloxacin and Tinidazole. The sample concentration of Ofloxacin and Tinidazole used in this analysis was 3 µg/ml and 12 µg/ml respectively. The retention time, peak area, theoretical plates, and asymmetry factor were evaluated for system suitability

3. RESULTS

3.1. Optimized condition for method development

The chromatographic detection was performed at 293nm as the appropriate wavelength using PDA detector. The method was performed on a Shimadzu shim-pack solar C8(4.6×250mm, 3µm). Furthermore, under severe trials of mobile phase optimization regarding its composition ratio and pH, it was observed that the optimized mobile phase was determined as a mixture of 0.5mM potassium dihydrogen orthophosphate adjusted to pH 4.5 using ortho-phosphoric acid and acetonitrile (70:30v/v) at a flow rate of 1mL/min. Under these conditions Ofloxacin and Tinidazole can be separated and eluted at 1.9 and 3.2 min respectively with a total run time of 10 min. The optimized chromatogram and optimized conditions are mentioned in Fig:3 and Table 1

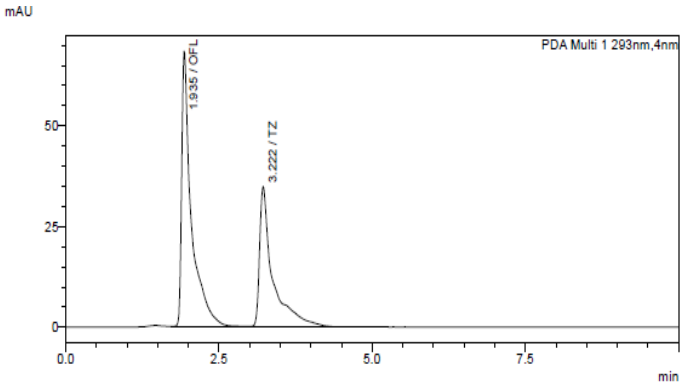


Fig. 3: Chromatogram of ofloxacin and tinidazole.

Table 1: Optimised chromatographic conditions.

Stationary phase	Shimadzu C8, (250X4.6mm) 3µm
Mobile phase	Acetonitrile: Potassium dihydrogen orthophosphate buffer
Solvent ratio	30 :70
Detection wavelength	293 nm
Flow rate	1.0 ml/min
Injection volume	20µl
pH	4.5
UV-Detector	Photodiode array detector
Retention time	
Ofloxacin	1.9 min
Tinidazole	3.2 min

3.2. Method validation

The chromatographic method was developed and validated for simultaneous estimation of Ofloxacin and Tinidazole in human plasma. The summary of the validation data is represented in Table 2

Table 2: Result of validation parameters

Parameters	**Ofloxacin**	**Tinidazole**
Intraday precision(%RSD)	1.25	1.27
Interday precision(%RSD)	1.29	1.27
Repeatability	1.25	1.28
Linearity(µg/mL)	1-5	3-15
Correlation coefficient	0.999	0.994
LOD(µg/mL)	5.21	19.1
LOQ(µg/mL)	15.8	57.9
Robustness(%RSD)	1.28	1.30

3.3. Linearity

The calibration curve was obtained for Ofloxacin and Tinidazole in the range of 1-5 µg/ml and 3-15 µg/ml and the correlation coefficient were found to be 0.999 and 0.994 respectively. The linearity graph is revealed in Fig: 6, 7 and regression data are shown in Table 3.

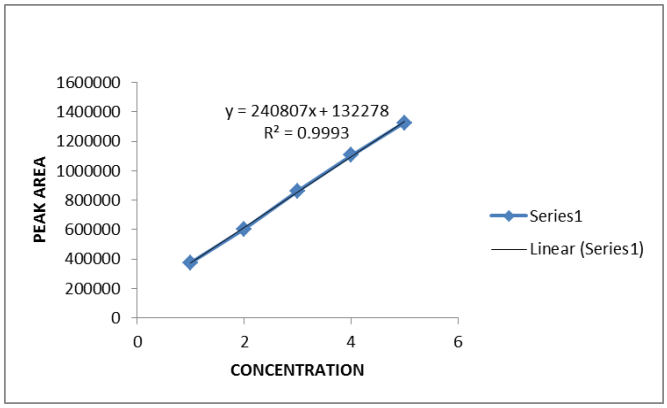


Fig. 6: Linearity graph of ofloxacin.

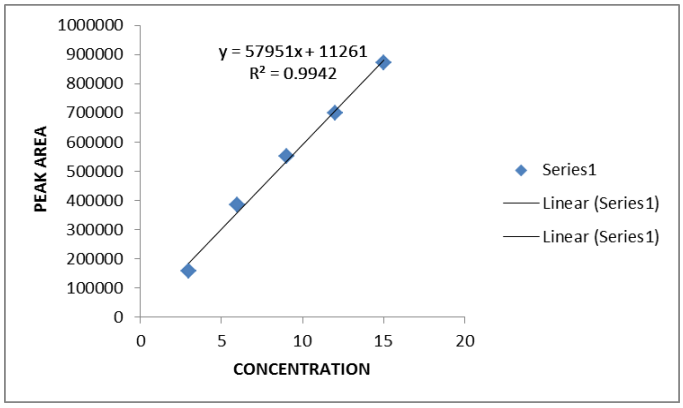


Fig. 7: Linearity graph of tinidazole.

Table 3: Linearity data.

Parameters	Ofloxacin	Tinidazole
Linearity and range (µg/ml)	1-5	3-15
Coefficient of correlation	0.999	0.9994
Slope	240807	57951
Y-Intercept	132278	11261

3.4. Precision

Repeatability and intermediate precision are expressed in terms of %RSD. As %RSD was found to be < 2 indicating the presented method is precise. The results are summarized in Table 4 respectively for repeatability study and intermediate precision.

Table 4: Precision data.

Parameters	Intra-day precision		Inter-day precision		Repeatability	
	OFL	TZ	OFL	TZ	OFL	TZ
Concentration	3	9	3	9	3	9
Standard deviation	10688	6926.2	10982.	6918.1	10758	6965.4
%RSD	1.25	1.27	1.29	1.27	1.25	1.28

3.5. LOD and LOQ

LOD and LOQ were found to be 5.2 µg/ml and 15.8 µg/ml for Ofloxacin and 19.1 µg/ml and 57.9 µg/ml for Tinidazole respectively.

3.6. Robustness

Making changes in flow rate were taken place and %RSD was found to be less than 2, specifying that the method is robust. Table 5 indicates that the selected factors remained unaffected by a small variation of these parameters.

Table 5: Robustness data.

Parameters	Changes done	Peak area	
		OFL	TZ
Changes in flow rate	0.8 mL	854220	549130

	1.0 mL	863060	550907
	1.2 mL	859150	534670
Detection wavelength	291 nm	843280	540150
	293 nm	873060	543680
	295 nm	868280	543680
%RSD		1.28	1.30

3.7. System suitability parameter

The optimized method is acceptable as system suitability parameters are valid as it passed the criteria of acceptability, as shown in Table 6.

Table 6: System suitability.

Parameters	OFL	TZ
Retention time	1.9min	3.2min
Peak area	863030	550907
Theoretical plate	5172	1749
Tailing factor	2.2	2.7

3.8. Recording of blank chromatogram

A study of baseline was recorded with the fixed chromatographic conditions. Blank plasma was injected, and the chromatogram was recorded and shown in Fig: 4

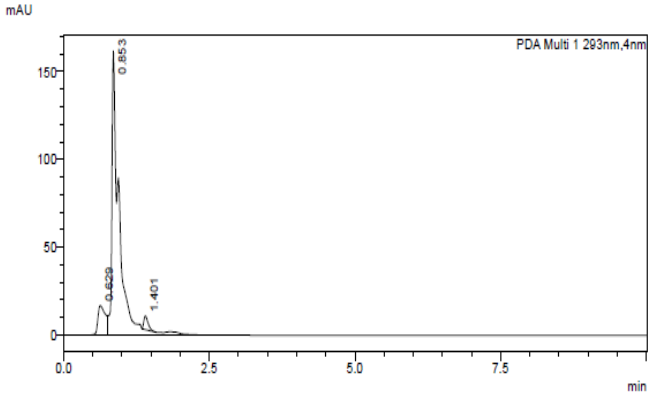


Fig. 4: Chromatogram of blank plasma.

3.9. Analysis in human plasma

A standard drug sample solution containing (1-5 µg/ml of Ofloxacin and 3-15 µg/ml of Tinidazole) was injected and chromatograms were recorded and shown in Fig: 5. The retention times of Ofloxacin and Tinidazole were found to be 1.9 min and 3.2 min respectively, this was followed by the injection of sample solution extracted from the plasma.

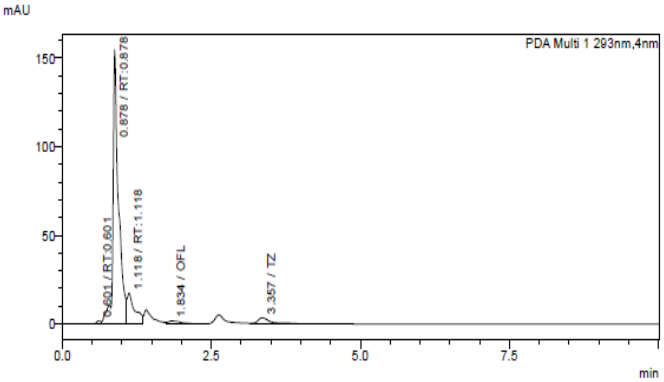


Fig. 5: Chromatogram of Ofl and Tz in plasma.

4. DISCUSSION

Ofloxacin and Tinidazole were estimated in plasma by RP-HPLC method. Optimisation of chromatographic parameters were carried out by RP- HPLC. The parameters such as selection of chromatographic method for separation, effect of ratio of mobile phase, effect of pH of mobile phase and effect of flow rate were optimized.

The linearity showed a good linear relationship over a concentration range of 1-5 µg/mL of Ofloxacin and 3-15 µg/mL in Tinidazole, the correlation coefficient was 0.999 and 0.994 respectively. From the results of validation parameters, it is evident that the proposed method validation as per ICH guidelines. Relative standard deviation (%RSD) was less than 2% which shows the method was precise.

As per the reported data the separation was carried out using mobile phase of triethylamine buffer of pH 3.0 and Acetonitrile in the ratio of 73:27.The column used was Kromasil C8, 5µ, 15cm x4.6mm i.d, with flow rate of 1.2ml/min using PDA detection at 303nm.the retention time of Ofloxacin and Tinidazole was found to be 2.3 min and 4.1min repectively.[7]

Analytical method was also developed by using Acetonitrile: Water: Tri ethylamine buffer with a pH 3.0 at the ratio of 75:25.The column used was Phenomenex C18,(250mm x4.6mm i.d,5m) with flow rate of 1ml/min and both the peaks eluted and the retention time observed for ofloxacin and tinidazole were 3.5 and 4.5 respectively,[10]

But, as per the newly developed Bioanalytical method, the Mobile phase was Acetonitrile: Potassium Dihydrogen orthophosphate buffer (30:70v/v). The retention time was found to be 1.9min, 3.2 min for Ofloxacin and Tinidazole respectively by using the flow rate of 1.0 ml/min, and elution was completed within 10 minutes.

5. CONCLUSION

From the forgoing it is concluded that the methods developed are simple, accurate, rapid, selective and precise. Hence it is suitable for the application in pharmaceutical analysis of drug in plasma. The method was validated as per ICH guidelines. The developed HPLC method was used for the estimation of bulk drug and its combination form in human plasma by *in-vitro* method. Extraction efficiency was determined by significant recovery studies. The method can be applied for toxicological, bio-equivalence, therapeutic drug monitoring studies. The developed HPLC method was used for the estimation of the drugs *in- vitro* method. We tried with different extraction solvents and Methanol was selected as it yields good quality.

6. ACKNOWLEDGEMENT

The authors are thankful to the PSG Sons` and Charities and PSG College of Pharmacy, Coimbatore for providing necessary facilities to the research work.

7. Conflict of interest

The authors declare no conflict interest.

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