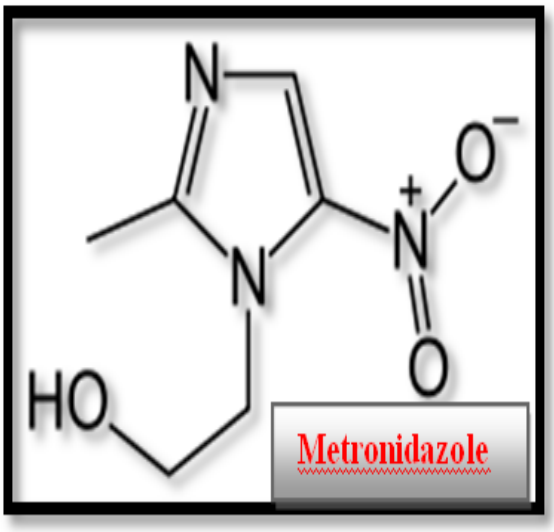
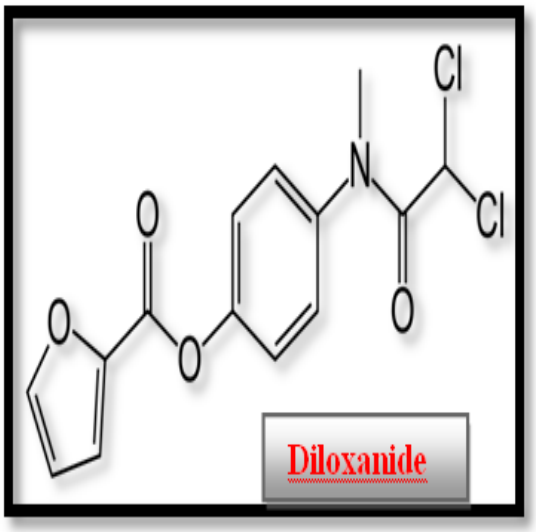
CHAPTER 2





Simultaneous Analysis of Metronidazole and Diloxanide Furoate in Human Plasma (In-vivo) by LCMS-MS

2.1 Introduction

2.1.1 Metronidazole

Metronidazole is an antibiotic, amoebocide and anti protozoan [1].The drug is first line treatment of mild-to-moderate *Clostridium difficile* infection [2].

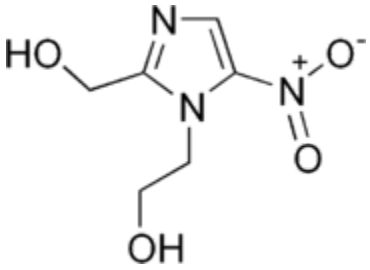


Figure 2.A: Structure of Metronidazole

The IUPAC name of the drug is 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol. The chemical formula is represented by $C_6H_9N_3O_3$ and the molecular weight being 171.15 g/mol. It is structurally nitro imidazole antibiotic used especially for bacteria and protozoa. It is marketed by Pfizer and globally by Sanofi under the brand name Flagyl in the USA. In Bangladesh it is marketed by Beximco Pharma under the brand names of Filmet. Metronidazole was developed in 1960. Metronidazole is used also as a gel preparation in the treatment of the dermatological conditions such as rosacea (Rozex and MetroGel by Galderma) and fungating lesions (Anabact, Cambridge Healthcare Supplies).

The drug is available in tablet as well as injectable form with different dosages. The drug is widely used in the treatment of vaginal infections, in inhibits the overgrowth of Gardnerella species and Co-infective anaerobes (Mobiluncus, Bacteroides) which causes vaginosis. In order to treat pelvic inflammatory disease Metronidazole is tabulated with other Antibiotics such as cefriaxone, levofloxacin and ofloxacin.The drug is extensively used in the treatment of anaerobic infections such as Bacteroid esfragilis spp, Fusobacterium spp, Clostridium spp, Pepto streptococcus spp, Prevotella spp, or any other anaerobes in intra-abdominal abscess, peritonitis, diverticulitis, empyema, pneumonia, aspiration pneumonia, lung abscess, diabetic foot ulcer, meningitis and brain abscesses, bone and joint infections, septicemia, endometritis, or endocarditis Pseudo membranous colitis due to Clostridium difficile

Helicobacter pylori. Metronidazole is used in combination with other drug such as Amoxicillin for the treatment of Dental infection which is caused due to bacterial infection, periapical abscess, periodontal abscess, acute pericoronitis were some predominately occurred diseases of dental cavity. The most common side effects by intake of Metronidazole are nausea; occasionally abdominal cramps, vomiting, diarrhea or constipation, headache, and anorexia.

2.1.2 Diloxanide Furoate

Diloxanide furoate acts as luminal amebicide in the treatment of amebiasis [3].The IUPAC name of the drug is 4-[(dichloroacetyl) (methyl) amino] phenyl furan-2-carboxylate. The chemical formula of the drug is $C_{14}H_{11}Cl_2NO_4$. The molecular weight was 328.147 g/mol. The drug is available in tablet formulation in 250 and 500mg dosage. It is considered the luminal agent of choice for amebiasis. It was first discovered by by The Boots Company Plc in the year 1956 and introduced as Furamide. Abbott Laboratories now own the brand of Furamide in india it is available in Amicline by Franco-Indian. It is not available in United States of America.A research study conducted by Center for Disease control between 1977 to 1990 revelaed that the drug had low incidence of side effects and proved to be very successful in the treatment of asymptomatic carriers of Entamoeba histolytica [4].Some of the common side effects of Diloxanide Furoate are flatulence, itching, urticaria and vomiting.

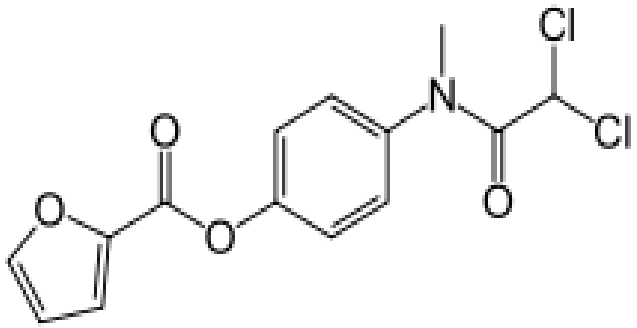


Figure 2.B: Structure of Diloxanide Furoate

Table 2.1: Properties of Metronidazole

Molecular formula	***2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol***
Formula`	$C_6H_9N_3O_3$
Mol. mass	171.15 g/mol
Routes	oral, topical, rectal, IV, vaginal
Excretion	Urine (77%), faeces

Table 2.2: Commercial Formulations of Metronidazole

S.No	Brand Name	Manufacturer	Form	Dosage
1	Aldezol	Albert David	Capsule/ Tablet	200mg
2	Aldezol	Albert David	Capsule/ Tablet	400mg
3	Aldezol	Albert David	Liquid	200mg per 5ml
4	Aristogyl	Aristo Pharmaceutical	Capsule/ Tablet	200mg
5	Aristogyl	Aristo Pharmaceutical	Capsule/ Tablet	400mg
6	Aristogyl	Aristo Pharmaceutical	Liquid	100mg per ml
7	Avimet	Moraceae Lab	Liquid	100mg per 5ml
8	Balgyl Gel	BalPharma	Cream/ Gel/ Ointment	0.01
9	Compeba	IDPL	Capsule/ Tablet	200mg
10	Flagyl	Nicholas Piramal	Capsule/ Tablet	200mg
11	Flagyl	Nicholas Piramal	Capsule/ Tablet	400mg
12	Flagyl	Nicholas Piramal	Liquid	200mg per 5ml
13	Flagyl	Nicholas Piramal	Liquid	200mg per 5ml
14	Largyl	Lark Laboratories	Capsule/ Tablet	200mg
15	Largyl	Lark Laboratories	Capsule/ Tablet	400mg
16	Largyl Gel	Lark Laboratories	Cream/ Gel/ Ointment	0.5%w/w
17	Metgyl	Jagsonpal Pharmaceuticals	Capsule/ Tablet	200mg
18	Metgyl	Jagsonpal Pharmaceuticals	Capsule/ Tablet	400mg
19	Metrogyl	J.B Chemicals & Pharmaceuticals	Capsule/ Tablet	200mg
20	Metrogyl	J.B Chemicals & Pharmaceuticals	Capsule/ Tablet	200mg
21	Metrogyl	J.B Chemicals & Pharmaceuticals	Capsule/ Tablet	400mg
22	Metrogyl	J.B Chemicals & Pharmaceuticals	Infusion	500mg per 5ml
23	Metrogyl	J.B Chemicals &	Liquid	200mg per

		Pharmaceuticals		5ml
24	Metrogyl	J.B Chemicals & Pharmaceuticals	Liquid	200mg per 5ml
25	Metrogyl V Gel	LekarPharma	Cream/ Gel/ Ointment	0.01
26	Metron	Alkem Laboratories	Capsule/ Tablet	200mg
27	Metron	Alkem Laboratories	Capsule/ Tablet	400mg
28	Metron	Alkem Laboratories	Infusion	5mg per 5ml
29	Metron	Alkem Laboratories	Liquid	200mg per 5ml
30	Pdzole - D	Parenteral Drugs	Infusion	500ml
31	Sprot - P	Lark Laboratories	Capsule/ Tablet	400mg
32	Sprot - P	Mapra Labs	Liquid	100mg per 5ml

Table 2.3: Properties of DiloxanideFuroate

IUPAC name	***4-[(dichloroacetyl)(methyl)amino]phenyl furan-2-carboxylate***
Formula`	$C_{14}H_{11}Cl_2NO_4$
Mol. mass	328.147 g/mol
Routes	Oral
Excretion	Renal (90%), fecal (10%)

Table 2.4: Commercial Formulations of Metronidazole

S.NO	Brand Name	Combined Drug	Form	Manufacturers
1	Amibactin DS (300+500)	DiloxanideFuroate, Tinidazole	Tablet	CFL Pharmaceuticals Ltd
2	Tinifur	DiloxanideFuroate, Tinidazole	Tablet	Vostok & Wilcure Remedies
3	Abdogyl	DiloxanideFuroate, Dicyclomine (Dicycloverine), Methyl Polysiloxane, Metronidazole	Tablet	Altar Healthcare (P) Ltd.
4	Ambilan (300+250)	DiloxanideFuroate, Tinidazole	Tablet	Swastik Formulations (P) Ltd.

5	Amebis Forte (300+500)	DiloxanideFuroate, Tinidazole	Tablet	ZydusCadila Healthcare Ltd
6	Amequin (375+300+75)	DiloxanideFuroate, Furazolidone, Tinidazole	Tablet	Shinto Organics Pvt Ltd.
7	Amibactin BD (600+750)	DiloxanideFuroate, Tinidazole	Tablet	CFL Pharmaceuticals Ltd
8	Amodil (300+25+250)	DiloxanideFuroate, Simethicone, Tinidazole	Tablet	Agron Remedies Pvt Ltd
9	Amodil DS	DiloxanideFuroate, Dicyclomine (Dicycloverine), Simethicone, Tinidazole	Tablet	Agron Remedies Pvt Ltd
10	Aristogyl Plus (400+100+500)	DiloxanideFuroate, Metronidazole, Simethicone	Tablet	Aristo Pharmaceuticals Pvt Ltd.
11	Dialox	DiloxanideFuroate, Methylpolysiloxane, Tinidazole	Tablet	Aglowmed Ltd
12	Diclotin	DiloxanideFuroate, Dicyclomine Hydrochloride, Simethicone, Tinidazole	Tablet	Pasteur Laboratories Pvt Ltd
13	Dilomet	DiloxanideFuroate, Metronidazole, Simethicone	Tablet	Adcco Limited
14	Dlotin MPS	DiloxanideFuroate, Methyl Polysiloxane, Tinidazole	Tablet	Bombay Tablet Mfg Co
15	Dlotin MPS (60ml)	DiloxanideFuroate, Methylpolysiloxane, Tinidazole	Suspension	Bombay Tablet Mfg Co
16	Dycos	DiloxanideFuroate, Tinidazole	Tablet	Dynamic Laboratories Pvt. Ltd.
17	Dyranil	DiloxanideFuroate, Tinidazole	Tablet	Alicon Pharmaceuticals Pvt. Ltd
18	Eldazole	DiloxanideFuroate, Activated Dimethicone, Tinidazole	Tablet	Elder Pharmaceuticals Pvt Ltd

19	Eltid	DiloxanideFuroate, Tinidazole	Tablet	Aurochem Labs (India) Pvt Ltd
20	Emib Forte	DiloxanideFuroate, Homatropine Methyl Bromide, Methylpolysiloxane, Tinidazole	Tablet	Synokem Pharmaceuticals Ltd
21	Enterosulphazyme Plus	DiloxanideFuroate, Simethicone, Tinidazole	Tablet	Adcco Limited
22	Enterozol	DiloxanideFuroate, Dicyclomine Hydrochloride, Simethicone, Tinidazole	Tablet	Union Drug Company Ltd
23	Entrolate	DiloxanideFuroate, Tinidazole	Tablet	StadmedPvt Ltd
24	Gastronorm	Diloxanide, Tinidazole	Tablet	Talent Laboratories
25	Gastronorm MPS	Diloxanide, MPS, Tinidazole	Tablet	Talent Laboratories
26	Kumagyl Forte	DiloxanideFuroate, Metronidazole, Simethicone	Tablet	Kumayun Drugs Private Limted
27	Lumigyl Plus	DiloxanideFuroate, Dimethicone, Tinidazole	Capsule	Plethico Pharmaceuticals
28	Mebio	DiloxanideFuroate, MPS, Tinidazole	Tablet	Briskon Laboratories
29	Mobitide	DiloxanideFuroate, Tinidazole	Tablet	Jenburkt Pharmaceuticals Ltd.
30	Mylone	DiloxanideFuroate, Metronidazole, PolydimethylSiloxane	Tablet	Perk Pharmaceuticals Ltd.
31	Nidalox (250+300)	DiloxanideFuroate, Tinidazole	Tablet	Indamed Pharmaceuticals Pvt. Ltd
32	Nidalox MPS (60 ml)	DiloxanideFuroate, Simethicone, Tinidazole	Suspension	Indamed Pharmaceuticals Pvt. Ltd
33	Novogyl MPS	DiloxanideFuroate, Dimethicone, Metronidazole	Tablet	Mandar Pharmaceuticals
34	Paat -4	DiloxanideFuroate, HomotropineMethylbromide, Methyl Polysiloxane,	Tablet	Pans Laboratories

		Metronidazole		
35	Patina M P S	DiloxanideFuroate, Simethicone, Tinidazole	Tablet	Patson Laboratories Pvt.Ltd.
36	Protorid Forte	DiloxanideFuroate, Dicyclomine (Dicycloverine), Simethicone, Tinidazole	Tablet	Duckbill Drugs Pvt Ltd
37	Sprot - Plus	DiloxanideFuroate, Metronidazole, Simethicone	Tablet	Mapra Laboratories Pvt Ltd.
38	Sprot Plus (60ml)	Diloxanide, Metronidazole, Simethicone	Suspension	Mapra Laboratories Pvt Ltd.
39	T.D.F Forte	DiloxanideFuroate, Tinidazole	Tablet	Cadila Healthcare (ZydusCadila Healthcare Ltd)
40	Tagrid	DiloxanideFuroate, Tinidazole	Tablet	Core Healthcare Ltd.
41	Tdf	DiloxanideFuroate, Tinidazole	Tablet	The Pharmed Research Lab Pvt Ltd
42	TDF Forte	DiloxanideFuroate, Tinidazole	Tablet	ZydusAlidac (ZydusCadila Healthcare Ltd)
43	Threogyl (250+600)	DiloxanideFuroate, Tinidazole	Tablet	Pharma Synth Formulations Ltd.
44	Tinamax MPS	DiloxanideFuroate, Activated Dimethyl Polysiloxane, Metronidazole	Tablet	Bestochem Formulations (India) Ltd.
45	Tinazole - DF	Diloxanide, Tinidazole	Tablet	Talent Laboratories
46	Tinco (300+250)	DiloxanideFuroate, Tinidazole	Tablet	Pans Laboratories
47	Tiniba DF	DiloxanideFuroate, Tinidazole	Tablet	Cadila Healthcare (ZydusCadila Healthcare Ltd)
48	Tinicide Forte	DiloxanideFuroate, Simethicone, Tinidazole	Tablet	Blue Cross Laboratories Ltd.
49	Tinidafyl Plus	DiloxanideFuroate, Methylpolysiloxane, Tinidazole	Tablet	JagsonpalPharmaceuticals Ltd
50	Tinidil	DiloxanideFuroate, Dimethicone, Tinidazole	Tablet	IntermedPharmaPvt Ltd

51	Tinil	DiloxanideFuroate, Tinidazole	Tablet	Sayona (Zota Healthcare Pvt Ltd)
52	Tinilox -DS	DiloxanideFuroate, Dicyclomine (Dicycloverine), PolydimethylSiloxane , Tinidazole	Tablet	YashPharma Laboratories Limited
53	Tinilox MPS	DiloxanideFuroate, Simethicone, Tinidazole	Suspension	Yash Vision
54	Tinilox - MPS	DiloxanideFuroate, Dimethyl Polysiloxane, Homatropine Methyl Bromide, Tinidazole	Tablet	YashPharma Laboratories Limited
55	Tinilox MPS (Liquid)	DiloxanideFuroate, Simethicone, Tinidazole	Syrup	YashPharma Laboratories Limited
56	Tinizol D	DiloxanideFuroate, Activated Methyl Polysiloxane, Dicyclomine (Dicycloverine), Tinidazole	Tablet	Libra Drugs (India)
57	Tinorid Plus	DiloxanideFuroate, Dicyclomine (Dicycloverine), Simethicone, Tinidazole	Tablet	Brussels Laboratories Pvt. Ltd.
58	Tonid DF	DiloxanideFuroate, Tinidazole	Tablet	Hinglaj Laboratories of India
59	Tozonil DF	DiloxanideFuroate, Activated Dimethicone, Tinidazole	Tablet	KlarSehen Pvt. Limited
60	Wotinex	DiloxanideFuroate, Tinidazole	Tablet	Wockhardt Ltd.

2.2 Review of Literature

PratibhaVermaet al [5] described the simple, economic, selective, precise, and stability-indicating HPLC analytical method to determine Metronidazole and its impurities, as well as gives degradation profile for a formulation by using of Solvent-X (Propylene Carbonate: methanol 60:40) as a Eco-friendly mobile phase component in place of acetonitrile (ACN).

The method was efficacious by phosphate buffer (HPLC grade water to which 10µL of 10% H3PO4 pH 4.1) and Solvent-X in ratio 90:10 (v/v) on 5µm Phenyl Column (250X4.6mm), with a flow rate of 1ml/min at 310nm of detector wavelength. Linear regression analysis data for the calibration plot showed there was a good linear relationship between response and concentration in the range 1.0–2.4µg/ ml. The regression coefficient was 0.9997. Metronidazole was found to degrade significantly in alkaline conditions. Mild degradation of the drug occurred in acidic conditions and oxidative stress. The drug was stable to dry heat. Their method was validated for accuracy, precision, reproducibility, specificity, robustness, detection and quantification limits, in accordance with ICH guidelines. Statistical analysis proved the method was precise, reproducible, selective, specific and accurate for analysis of Metronidazole. The wide linearity range, sensitivity, accuracy, short retention time, and simple mobile phase imply the method is suitable for routine quantification of Metronidazole with high precision and accuracy. The efficiency of method was compared with solvent Acetonitrile in terms of linearity, Accuracy, Precision, Specificity and Robustness.

OlajireAremuAdegoke et al [6] developed a new approach to the spectrophotometric determination of metronidazole (MZ) and tinidazole (TZ). The procedure involves coupling of diazotized nitro imidazoles with p-dimethylamino benzaldehyde(DMAB) to form a greenish-yellow solution. Optimal temperature and time were 0 °C (iced) and 3 minutes for diazotization and 30 °C and 2 minutes for coupling for both MZ and TZ. Coloured adducts of MZ and TZ showed shoulders at 406 nm and 404 nm, respectively, which were selected as analytical wavelengths. The reaction with p-DMAB occurred in a 1:1 mole ratio. Beer's law was obeyed within the 4.8–76.8 mg ml–1 concentration range with low limits of detection. The azo adducts were stable for over a week. Molar absorptivities were 1.10,103 (MZ) and 1.30 ´ 103 l mol–1 cm–

1 (TZ). Overall recoveries of MZ and TZ from quality control samples were 103.2 ± 1.3 and 101.9 ± 1.3 % over three days. There was no interference from commonly utilized tablet exceipients. No significant difference was obtained between the results of the new method and the BP titrimetric procedures. Theazo approach using the p-dimethyl amino benzaldehyde procedure described in this paper is simple, fast, accurate and precise. It is the first application of DMAB as a coupling component in the diazo coupling reaction.

Bassam M. Tashtoush et al [7] proposed a rapid and simple method using an isocratic high-pressure liquid chromatography (HPLC) and UV detection for the determination of metronidazole in dermatological formulations is presented. Metronidazole samples were extracted with a solution composed of 60% methanol and 40% mobile phase by a procedure that can be completed in less than 10 min. Subsequent separation and quantification was accomplished in less than 20 min using reversed-phase HPLC with isocratic elution with 0.01% trifluoroacetic acid/acetonitrile (85:15%, vol/vol). Validation experiments confirmed the precision and accuracy of the method. When applied to a commercial metronidazole cream and gel formulation, recoveries of 100.4% for cream formulations and 102.3% for gel formulations were obtained. Their method facilitated studies of the formulation compatibility of metronidazole topical formulations with agents that may improve its clinical tolerability for treatment of rosacea.

Li Qun Ouyang et al [8] developed a method using HPLC-DAD coupled with second-order calibration was developed to simultaneously determine metronidazole and tinidazole in plasma samples in this paper. The second-order calibration method based on APTLD (alternating penalty trilinear decomposition) algorithm was proposed to analyze the three-way HPLC-DAD data from both standard and prediction samples, which makes it possible that calibration can be performed even in the presence of unknown interferences with a simple and green chromatographic condition and short analysis time. Their results showed that good recoveries were obtained although the chromatographic and spectral profiles of the analytes of interest as well as background were partially overlapped with each other in plasma samples.

Tashtoush BM et al [9] proposed a rapid and simple method using an isocratic high-pressure liquid chromatography (HPLC) and UV detection for the determination of metronidazole in dermatological formulations is presented. Metronidazole samples were extracted with a solution composed of 60% methanol and 40% mobile phase by a procedure that can be completed in less than 10 min. Subsequent separation and quantification was accomplished in less than 20 min using reversed-phase HPLC with isocratic elution with 0.01% trifluoroacetic acid/acetonitrile (85:15%, vol/vol). Validation experiments confirmed the precision and accuracy of the method. When applied to a commercial metronidazole cream and gel formulation, recoveries of 100.4% for cream formulations and 102.3% for gel formulations were obtained. Their method facilitated studies of the formulation compatibility of metronidazole topical formulations with agents that may improve its clinical tolerability for treatment of rosacea.

EssamEzzeldinet al [10] developed a new sensitive, accurate, rapid and reproducible high performance liquid chromatography (HPLC) method to determine metronidazole levels in human plasma and to apply the method in a bioequivalence study. Metronidazole was extracted from human plasma through one step of protein precipitation by methanol using carbamazepine as internal standard (IS). After centrifugation of the plasma sample, the supernatant layer was separated and injected into HPLC system using Eclipse XDB-phenyl column. The mobile phase consisted of phosphate buffer (pH 4.5): acetonitrile (95:5, v/v). The UV detector was set at 320nm. The bioavailability of the test metronidazole product (Brand A) was compared to a commercial metronidazole brand as reference product in 24 healthy volunteers who received a single dose equivalent to 500 mg of the test and reference products in a randomized balanced two-way cross-overdesign separated by two-week wash-out period. Mean standard calibration curves of metronidazole over the concentration range of 0.05 – 30µg/ml were linear. No significant differences were found based on analysis of variance of the pharmacokinetics parameters required for the assessment of bioequivalence of test and reference formulations. The mean value and 90 %CI of test/reference ratios for the derived parameters were: C max, 9.64 vs. 8.38 (0.93 – 1.10), AUC0-24, 124.6 vs.122.3 µg/ml (0.973 – 1.051) and AUC0-∞, 140.9vs. 128.4 h/ml (1.15 – 1.23).The test metronidazole product was bioequivalent

to the reference. The method is suitable for bioequivalence and pharmacokinetic studies in humans with a low limit of quantification of 0.05μg/ml.

Hesham Salemet al [11] gave two sensitive and precise chromatographic methods were developed and validated for simultaneous determination of metronidazole (MTR) and diiodohydroxyquin (DIQ) in pharmaceutical preparation. The techniques adopted for quantification are coupled TLC-densitometry and HPLC. A mixture of chloroform, toluene, ethanol and acetic acid (9:9:1:1, v/v/v/v) was used as the developing solvent for TLC-densitometry. A mixture of methanol and acetonitrile, (96:4, v/v) was used as a mobile phase for HPLC at 0.6 ml min-1 flow rate and UV detection at 254nm. Linearity was obtained in concentration range of 0.5-10 μg spot-1 for DIQ and 1-20 μg spot-1 for MTR applying TLC-densitometry and 0.005-0.5 mg mL-1 for DIQ and 0.01-0.5 mg mL-1 for MTR applying HPLC. The selectivity of the proposed methods was checked using laboratory prepared mixtures. The proposed methods were successfully applied to the analysis of MTR and DIQ in their mixture and in pharmaceutical dosage forms without interference from other additives.

S. M. El-Gizawy et al [12] developed a high performance liquid chromatographic method for the simultaneous determination of metronidazole and diloxanidefuroate in single and combined dosage form. Each drug acted as internal standard for the other. Both of the drugs eluted from the cyclobond column (β-cyclodextrin stationary phase) without retardation effects, using methanol-0.05 M NaH_2PO_4 (pH 7) in ratio 35:65, flow rate 1 ml min^{-1} and detection at 254 nm. Metronidazole and diloxanidefuroate gave rectilinear calibration graphs in the ranges 1–10 μg and 1–5 μg respectively. Recoveries were in the range 99.6 to 101.3% and their corresponding coefficient of variation are 0.7 and 1.05% (n=6).

P. Thulasamma et al [13] proposed two simple, precise, rapid, sensitive and accurate Spectrophotometric methods for the estimation of metronidazole either in pure form or in tablet dosage forms. The proposed methods are based on the reduction of metronidazole was carried out with Zinc powder and 5N HCl at room temperature in methanol. The resulting amine was used to two methods. Method A is based on oxidation Coupling with 1,10- Phenanthrolin to form Orange red colored chromogen exhibiting absorption maxima at 510 nm with apparent molar absorptivity of2.32x103(Lm-1cm-1) and obeyed beer's law in the concentration range 5-55 μg/ml.

Method B is based on diazotization and coupling reaction with NaNo2 and 4-chloro3-nitro Aniline to form Yellow colored chromogene exhibiting absorbance maximum at 480 nm with apparent molar absorptivity of 2.71x103(Lm-1cm-1) and obeyed beer's law in the concentration range of 5-60 μg/ml. The assay of results was found to be in good agreement with label claim. The proposed methods were Simple, Sensitive, Precise, Accurate, quick and useful for routine quality control.

Nandipura Dyavegowda Dineshet al [14] developed a simple, sensitive and rapid spectrophotometric method for the determination of tinidazole (TZ) and metronidazole (MZ) in pure as well as in dosage form is described. The method is based on the reduction of the nitro group of drugs using a novel and versatile reduction system comprising 10% Pd-C and formic acid. The resulting amine was then subjected to a condensation reaction with sodium1,2-naphthaquinone-4-sulfonate (NQS) to form red Schiff base with an absorption maximum at 510 nm. Beer's law was obeyed in the concentration ranges 2.0 to 45.0 _g mL−1 and 1.5 to 37.0 _g mL−1 with a limit of detection (LOD) of 0.44 _g mL−1 and 0.36_g mL−1 for TZ and MZ, respectively. Other statistical analyses such as Student's t test and F test values are included. The sensitivity of the method surpasses that of the reported spectrophotometric methods. The method was successfully applied for the assay of different tablets, suspensions and injections of TZ and MZ.

2.3 Materials and Methods

2.3.1 Instrumentation:

An HPLC system (Shimadzu, Kyoto, Japan) consisting of an advance C18 column, a binary LC-20AD prominence pump, an auto-sampler (SIL-HTc) and a solvent degasser (DGU-20A3) was used for the study. Aliquots of the processed samples (20 mL) were injected into the column, which was kept at 30 1 C. The isocratic mobile phase was delivered into the electro-spray ionization chamber of the mass spectrometer. Quantitation was achieved with MS–MS detection in positive ion mode for both the analytes using an MDS Sciex API-4000 mass spectrometer equipped with a Turboionspray TMinterface at 500 1 C. The ionspray voltage was set at 5500 V. The source parameters, viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas were set at 45, 20, 45 and 10 psi, respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM)

2.3.2 Chemicals and standard drugs

The working standard drug Metronidazole having a purity of 99.62% and Diloxanide Furoate with 99.15% purity were kindly provided by Perk Pharmaceuticals Ltd; New Delhi, India. All the chemicals used were of laboratory reagent grade and were purchased from Merck chemicals private limited, Mumbai; Maharashtra, India.

2.3.3 Preparation of solutions

2.3.3.1 Preparation of mobile phase

Tetrahydrofuran, Methanol and Acetonitrile in the ratio of 200:500:300 (v/v) respectively and sonicated the solution for ten minutes to ensure the homogeneous mixing using ultrasonicater, and then it was filtered through 0.45 µ nylon membrane filter paper using vacuum filtration set. The solution was stored at room temperature and used within 7 days from the date of preparation. Later the mixture was degassed.

2.3.3.2 Preparation of diluent:

An equal ratio of methanol and acetonitrile was used as diluent in the analysis. For the preparation of diluent, 50mL of methanol was transferred into a 100 mL reagent bottle and 50mL of acetonitrile was added, mixed and sonicated for 5

minutes. The solution was stored at room temperature and used within 7 days from the date of preparation.

2.3.3.3 Rinsing solution:

8:2 ratios of methanol and Water mixture were used as rinsing solution. To prepare this 80ml of methanol was mixed with 20 ml of water in a 100ml beaker. Resultant solution was mixed well and then it was filtered through a membrane filter paper. The solution was used as rinsing solution to rinse useful things. The solution was stored at room temperature and used within 7 days from the date of preparation.

2.3.3.4 Preparation of extraction solution:

Diethyl ether and dichloromethane in the ratio of 60:40 (v/v) was used for the extraction of drugs from the biological matrix. 60 ml of Diethyl ether was added to 40 ml of dichloromethane. The solution was mixed well and then it was filtered and used for the extraction. The solution was stored at room temperature and used within 7 days from the date of preparation.

2.3.3.5 Preparation of standard stock solution for Metronidazole:

A stock solution of mg/ml (1000 mcg/ml) was prepared by accurately weighing 25.00mg of the standard drug Metronidazole and was dissolved in 25ml of methanol. The standard stock solution was prepared as per the potency of Metronidazole. A standard concentration of 996.20mcg/ml was obtained. The solution was filtered and was used as a standard stock solution. The solution was preserved safely and was used when it is required.

2.3.3.6 Preparation of standard stock solution for Diloxanide Furoate:

A stock solution of mg/ml (1000mcg/ml) was prepared by accurately weighing 25mg of the standard drug Diloxanide Furoate and was dissolved in 25ml of methanol. The standard stock solution was prepared as per the potency of Diloxanide Furoate. A standard concentration of 991.50mcg/ml was obtained. The solution was filtered and was used as a standard stock solution. The solution was preserved safely and was used when it is required.

2.3.3.7 Plasma spiked calibration curve for Metronidazole:

The prepared aqueous dilutions were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 49.81ng/ml - 298.86ng/ml. The preparation of solution was given in table 2.5.

Table 2.5: Preparation of plasma spiked calibration curve dilutions for Metronidazole

S. No	Concentration of Metronidazole (ng/ml)	Volume taken (ml)	Volume of plasma added (ml)	Final volume (ml)	Final concentration (ng/ml)	Vial code
1	996.20	0.5	9.5	10	49.81	PS-CC 1
2	996.20	1.0	6.0	10	99.62	PS-CC 2
3	996.20	1.5	8.5	10	149.43	PS-CC 3
4	996.20	2.0	8.0	10	199.24	PS-CC 4
5	996.20	2.5	7.5	10	249.05	PS-CC 5
6	996.20	3.0	7.0	10	298.86	PS-CC 6

2.3.3.8 Plasma spiked calibration curve for Diloxanide Furoate:

The prepared aqueous dilutions of Diloxanide Furoate were used to spike the screened blank human plasma matrix to prepare plasma calibration curve standards. The plasma spiked calibration curve was prepared with in the concentration range of 19.83ng/ml – 118.98ng/ml. The preparation of solution was given in table 2.6

Table 2.6: Preparation of plasma spiked calibration curve dilutions for Diloxanide Furoate:

S. No	Concentration of Diloxanide (ng/ml)	Volume taken (ml)	Volume of plasma added (ml)	Final volume (ml)	Final concentration (ng/ml)	Vial code
1	991.50	0.2	9.8	10	19.83	PS-CC 1
2	991.50	0.4	9.6	10	39.66	PS-CC 2
3	991.50	0.6	9.4	10	59.49	PS-CC 3
4	991.50	0.8	9.2	10	79.32	PS-CC 4
5	991.50	1.0	9.0	10	99.15	PS-CC 5
6	991.50	1.2	8.8	10	118.98	PS-CC 6

2.3.3.9 Extraction of drugs from plasma:

Prior to sample analysis, 100µL of each solution was extracted using 300µL of diethyl ether: dichloromethane (60:40% v/v) mixture for protein precipitation. Further, each of the mixtures was vortex separately for a period of 5 min in a vortex mixer with subsequent centrifugation at 10000 rpm, for a period of 10 min at 4°C using a centrifuge. For each sample, an aliquot of a supernatant was isolated and subjected to dryness. The residue was reconstituted in 100µL of mobile phase and subsequently centrifuged at 10000 rpm for10 min at 4°C in a centrifuge. The supernatant was finally collected and directly injected for analysis. This procedure was followed for the construction of calibration curve for plasma spiked dilutions and plasma spiked samples.

2.4 Method Development

The goal of this work is to develop and validate a simple, rapid, sensitive method for simultaneous extraction and quantification of Metronidazole and Diloxanide Furoate suitable for pharmacokinetic study of drugs in Human Plasma by High Performance Liquid Chromatography with Tandem Mass Spectrometry.

The chromatographic conditions particularly the composition of mobile phase, flow-rate of mobile phase, choosing of suitable column, injection volume, column oven temperature, auto-sampler temperature, splitting of sample in to ion source, as well as a short run time were optimized through several trials to achieve good resolution and symmetric peak shapes for the Metronidazole and Diloxanide Furoate. It was found that a mixture of Tetrahydrofuran, Methanol and Acetonitrile in the ratio of 200:500:300 (v/v) could achieve this purpose and this was finally adopted as the mobile phase.

The formic acid was found to be necessary in order to lower the pH and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in the mass spectrometer. The high proportion of organic solvent eluted both the Metronidazole and Diloxanide Furoate at retention times of 2.82min and Diloxanide Furoate elutes at 5.13min at a flow rate of 0.80mL/min, produced good peak shapes, and permitted a run time of 10min.

The instrumental parameters for mass spectroscopy were optimized. The source temperature was 600°C. The gas pressures of nebulizer, heater, curtain, and CAD were 40, 30, 20, and 4 psi, respectively. The ion spray voltage, entrance potential, declustering potential, collision energy, and collision cell exit potential were optimized at 5500, 10, 50, 32, and 12 V, respectively. The dwell time was 400 milliseconds for both ME and MED6.

Mass spectrometry parameters, fragmentation pattern, and mode of ionization are the main task in mass spectrometry tuning to obtain respective fragmented ions and response for both ME and MED6 which were shown in Figures 2G and 2H. ESI-

LC-MS/MS is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Multiple-reaction monitoring (MRM) technique was chosen for the assay development. The MRM parameters were optimized to maximize the response for the analyte.

Liquid-liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful to clean the samples. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS analyses. Several organic solvents and their mixtures in different combinations and ratios were evaluated. Finally, diethyl ether/dichloromethane (60 : 40) was found to be optimal, which produced a clean chromatogram for a blank plasma sample and yielded the highest recovery for the separation and identification of Metronidazole and Diloxanide Furoate from the plasma. Memantine-D6 hydrochloride was used as internal standard for the present purpose. Clean chromatograms were obtained, and no significant direct interferences in the MRM channels at the relevant retention times were observed.

At the optimized conditions, the standard drug Metronidazole elutes at a retention time of 2.82min and Diloxanide Furoate elutes at 5.13min. The separation was found to be accurate and symmetric peaks with high resolution was observed. The optimized chromatographic and mass parameters were given in table 2.7.

Table 2.7: Method conditions of the estimation of Metronidazole and Diloxanide Furoate

S. No	Parameters	Conditions
1	API	Metronidazole and Diloxanide Furoate
2	Mobile phase	Tetrahydrofuran, Methanol and Acetonitrile in the ratio of 20:50:30 (v/v)
3	Retention time	Metronidazole - 2.82min Diloxanide Furoate -5.13min
4	Flow rate	0.80mL/min
5	Run time	10min

Figure 2C: Standard LC chromatogram of Metronidazole and Diloxanide Furoate

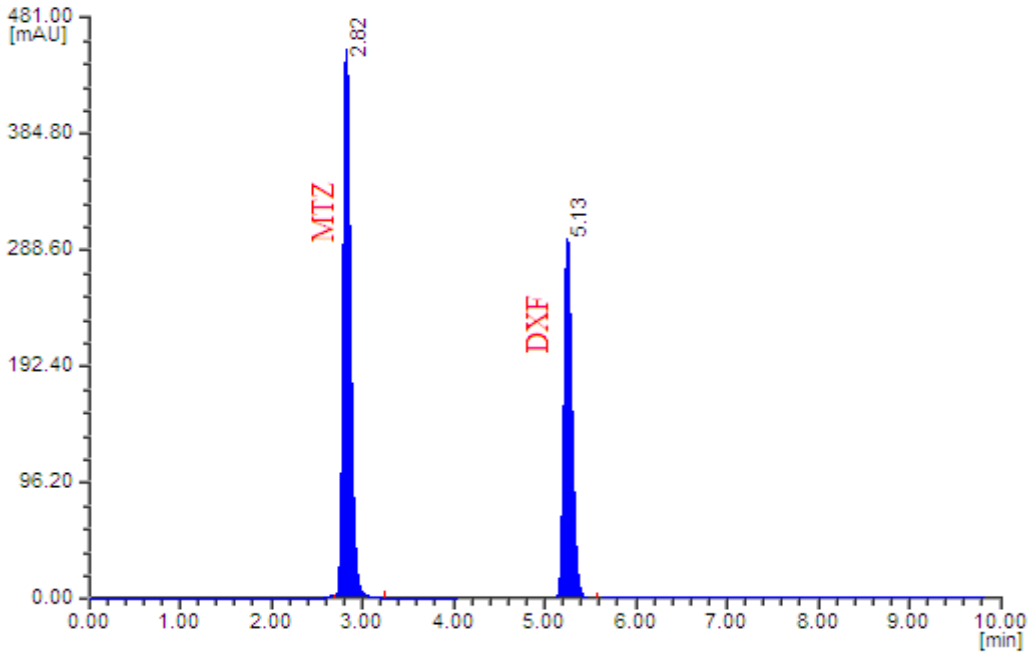


Figure 2D: Blank chromatogram of Metronidazole and Diloxanide Furoate

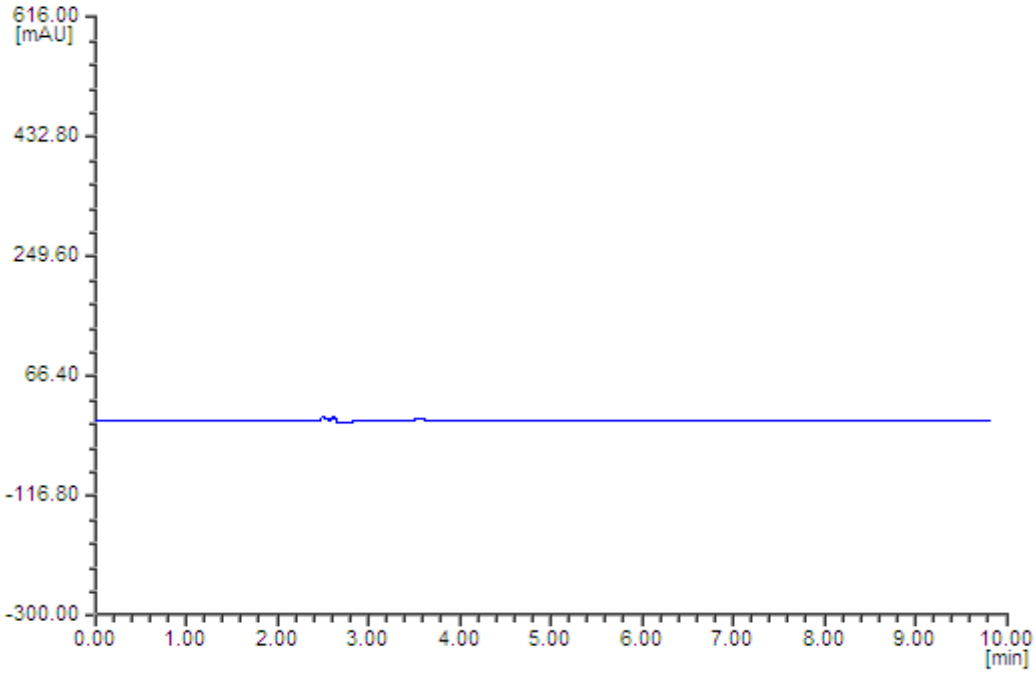


Figure 2E: Standard LC chromatogram of Metronidazole

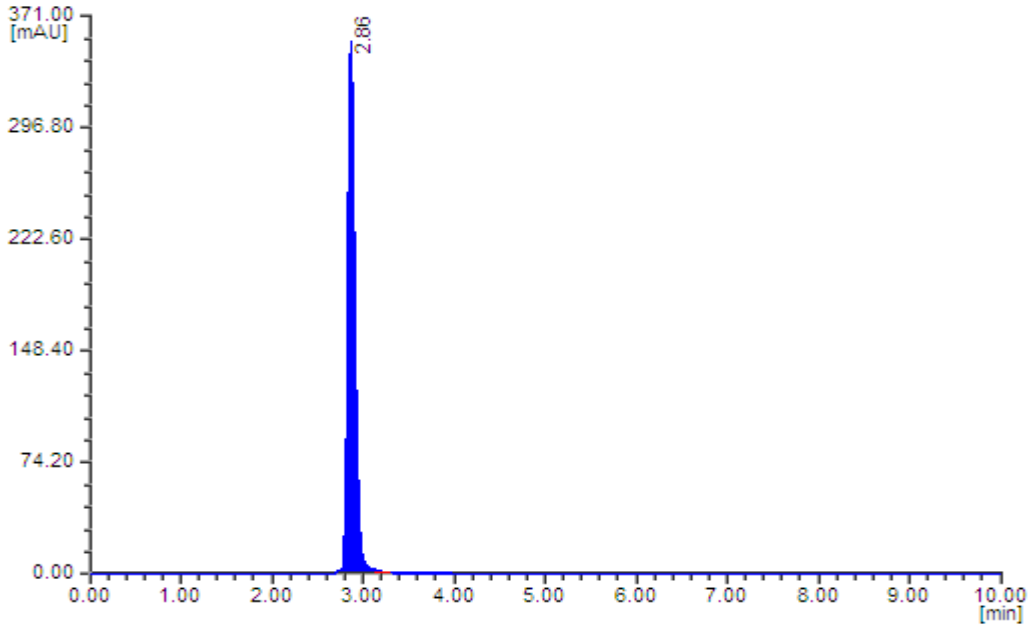


Figure 2F: Standard LC chromatogram of Diloxanide Furoate

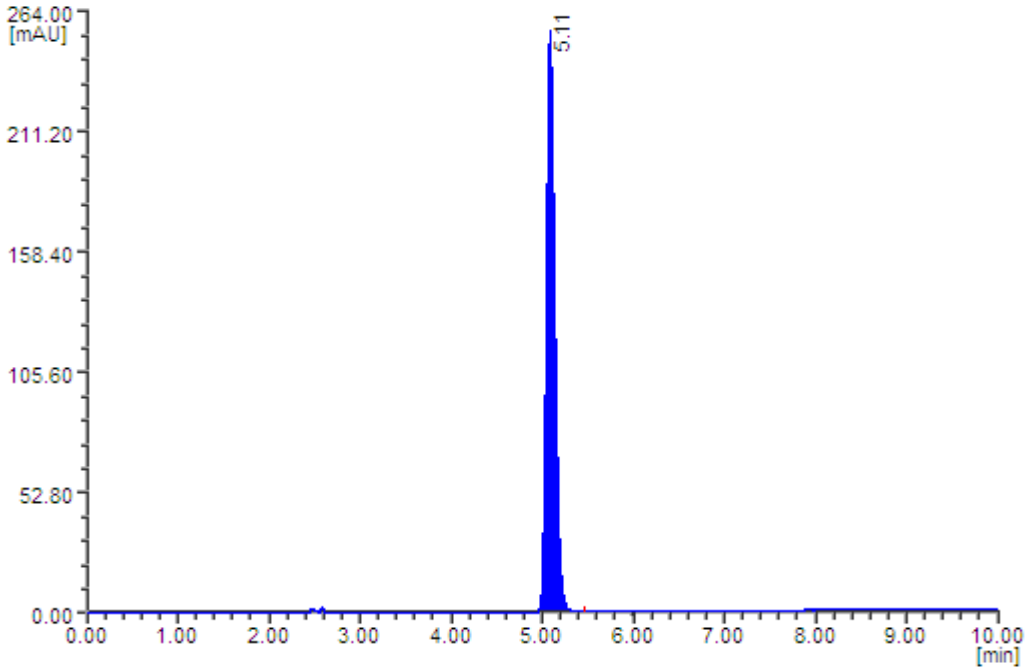


Figure 2G: Mass spectrum of Metronidazole

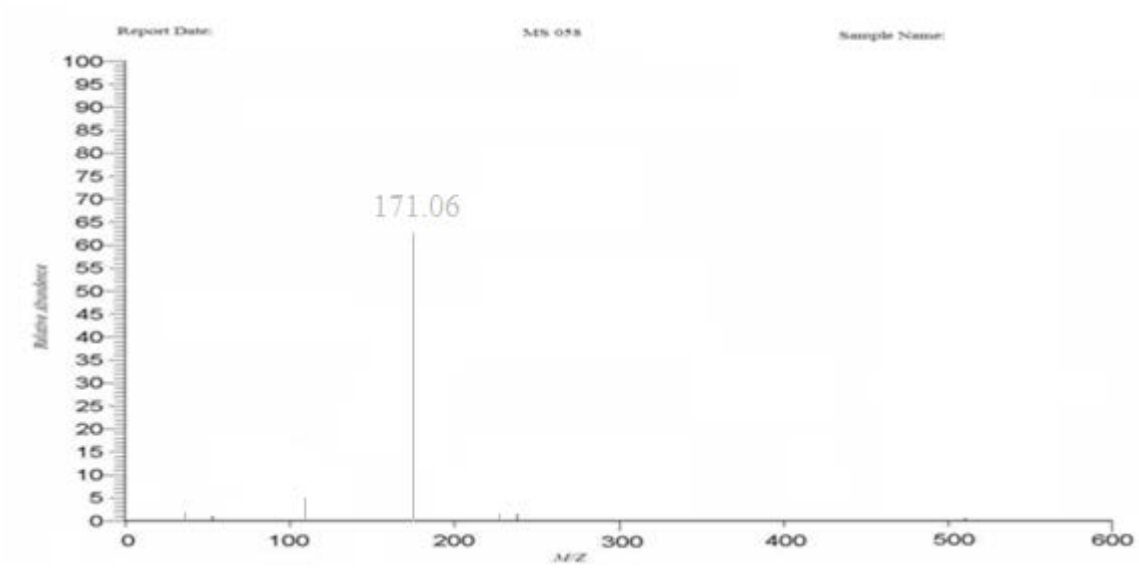
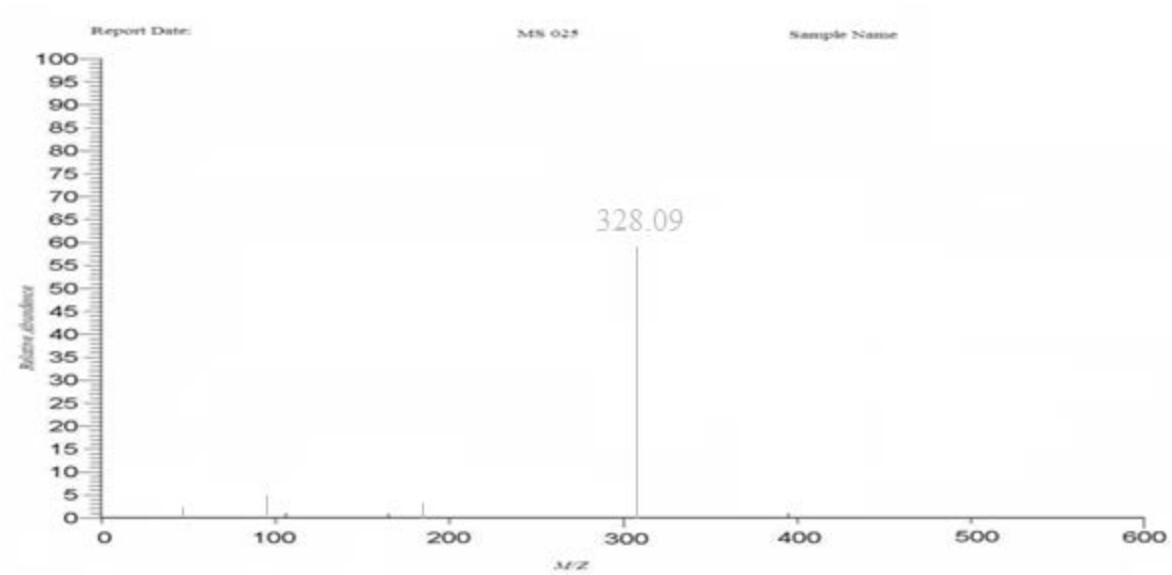


Figure 2H: Mass spectrum of Diloxanide Furoate



2.5 Method Validation

A thorough validation of the method was carried out as per the US FDA guidelines. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability.

2.5.1 Selectivity

Six lots of blank plasma were screened and interference at retention time of both analytes was evaluated. Only those lots which are under the acceptance criteria (<20 % in comparison to the spiked LOQ) were selected. No significant interference was observed at the RT and m/z of both the drugs in all the batches screened. Hence the proposed method was found to be selective for Metronidazole and Diloxanide Furoate.

2.5.2 Linearity

For the determination of linearity, standard calibration curves of six points (nonzero standards) were used. Six non-zero points 49.81, 99.62, 149.43, 199.24, 249.05 and 298.86ng/ml for Metronidazole and 19.83, 39.66, 59.49, 79.32, 99.15 and 118.98ng/ml for Diloxanide Furoate. In addition, a blank plasma sample were also analyzed to confirm absence of interferences, these sample was not used to construct the calibration function. The data from three precision and accuracy batches were subjected to goodness of fit analysis using 1/x and $1/x^2$ weighing factor. Deviation from nominal concentration should be within +20% for LLOQ and within +15% for the other concentrations.

A calibration curve was established on each validation day. The calibration curve was linear over the concentration range of 49.81ng/ml - 298.86ng/ml for Metronidazole and 19.83ng/ml – 118.98ng/ml for Diloxanide Furoate respectively in human plasma with a coefficient of relation (r) ≥0.99. The slope and intercept of regression equations were 5270 and -47375 for Metronidazole and 8243.x - 22905 for Diloxanide Furoate respectively. Linearity was found to be satisfactory and reproducible with time. The determination coefficients (r^2) for Metronidazole and Diloxanide Furoate were greater than 0.999 and 0.998 respectively for all curves. The

results were given in table.2.8.and linearity graphs were given in figure 2I and 2.J for Metronidazole, and Diloxanide Furoate respectively.

Table 2.8: Plasma spiked calibration curve

S.NO	Metronidazole		Diloxanide Furoate		Sample vial code
	Concentration (ng/ml)	Area at the retention	Concentration (ng/ml)	Area at the retention time	
1	49.81	225851	19.83	142597	PSCC 001
2	99.62	462683	39.66	296593	PSCC 002
3	149.43	751269	59.49	479864	PSCC 003
4	199.24	998566	79.32	614692	PSCC 004
5	249.05	1245867	99.15	809568	PSCC 005
6	298.86	1543965	118.98	952143	PSCC 006
	Slope Intercept r^2	5270 - 47375 0.999	Slope Intercept r^2	8243 – 22905 0.9984	

Figure 2I: Linearity graph for Metronidazole

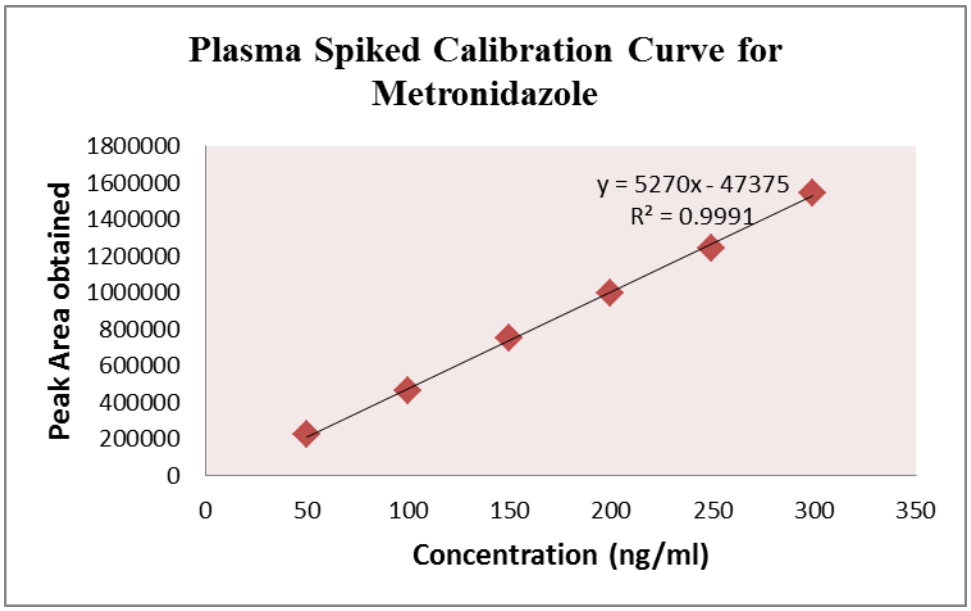
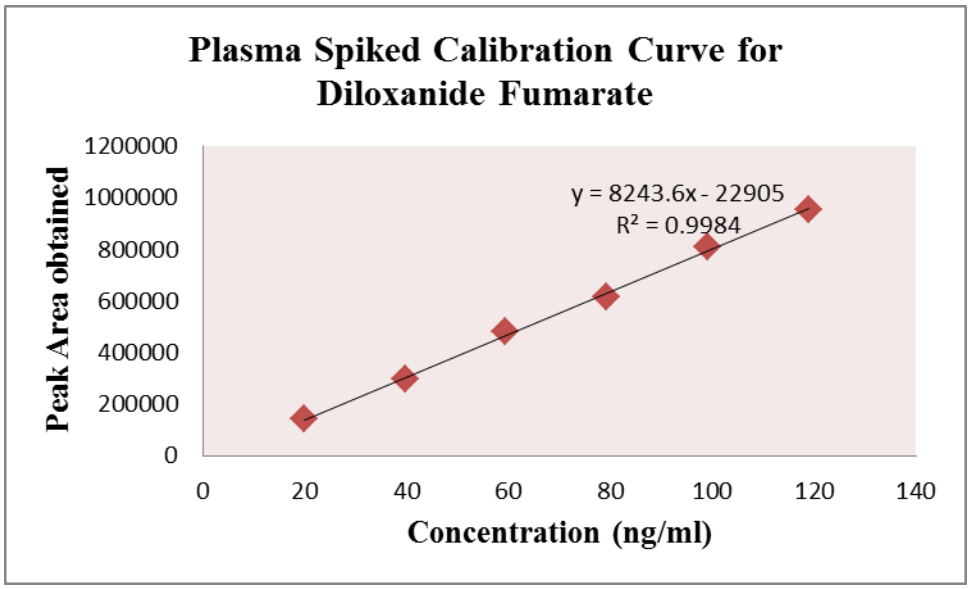


Figure 2 J: Linearity graph for Diloxanide Furoate



2.5.3 Precision and Accuracy

Precision and accuracy for this method was controlled by calculating the intra and inter-day batch variations of QC samples in six replicates at four concentrations of HQC, QC, LQC and LLOQC of 298.86, 199.24, 99.62 and 49.8ng/ml for Metronidazole and 118.98, 79.32, 39.66 and 19.83ng/ml for Diloxanide Furoate. Detector response at the retention time of both the drugs in each level was determined and the %CV (coefficient of variance) of the response was calculated. The acceptance criteria included accuracy within ±15% deviation (SD) from the nominal values, except for LLOQ QC, where it should be ±20%. The %CV was found to be 0.464 and 0.355 at HQC, 0.215 and 0.356 at MQC, 0.253 and 0.566 at LQC, 0.543 and 0.750 at LLOQC for Metronidazole and Diloxanide Furoate respectively. More than 98% accuracy was observed for all the recovery levels for both the drugs in each recovery level. These results indicated that the method was reliable, reproducible and accurate as all the parameters were within the acceptance limit of < 15 % and ± < 15 % for

precision and accuracy respectively for LQC, MQC and HQC and ≤ 20 % for LLOQQC. Results were given in table 2.9 and 2.10.

Table 2.9: Precision at HQC and MQC levels

Precision at HQC					
S.NO	Sample ID	Metronidazole		Diloxanide Furoate	
		Area obtained	Observed Concentration (ng/ml)	Area obtained	Observed Concentration (ng/ml)
P and A at HQC	PA001	1542965	99.93523	952343	100.021
	PA002	1545154	100.077	953067	100.097
	PA003	1542686	99.91716	950568	99.83458
	PA004	1554638	100.6913	956319	100.4386
	PA005	1558361	100.9324	958193	100.6354
	PA006	1541220	99.82221	958915	100.7112
Nominal Conc.		**298.86ng/ml**		**118.98ng/ml**	
N		**6**		**6**	
Average		**7177.677**	**0.464886**	**3395.35**	**0.356601**
SD		**1547504**	**100.2292**	**954900.8**	**100.2896**
%CV		**0.464**	**0.464**	**0.355**	**0.355**
Accuracy (%)		**100.229**		**100.290**	
Precision at MQC					
S.NO	Sample ID	Metronidazole		Diloxanide Furoate	
		Area obtained	Observed Concentration (ng/ml)	Area obtained	Observed Concentration (ng/ml)
P and A at MQC	PA007	993566	99.49928	615692	100.1627
	PA008	995978	99.74083	615018	100.053
	PA009	998794	100.0228	613224	99.76118
	PA010	997962	99.93951	615742	100.1708
	PA011	998654	100.0088	617045	100.3828
	PA012	998979	100.0414	619764	100.8251
Nominal Conc.		**199.24ng/ml**		**79.32ng/ml**	
N		**6**		**6**	
Average		**2145.233**	**0.214831**	**2193.283**	**0.35681**
SD		**997322.2**	**99.87544**	**616080.8**	**100.2259**
%CV		**0.215**	**0.215**	**0.356**	**0.356**
Accuracy (%)		**99.875**		**100.226**	

Table 2.10: Precision at LQC and LLOQC levels

Precision at LQC					
S.NO	Sample ID	Metronidazole		Diloxanide Furoate	
		Area obtained	Observed Concentration(ng/ml)	Area obtained	Observed Concentration (ng/ml)
P and A at LQC	PA013	462133	99.88113	296493	99.96628
	PA014	465073	100.5166	295364	99.58563
	PA015	462031	99.85908	295413	99.60215
	PA016	463598	100.1978	298341	100.5894
	PA017	462457	99.95115	294125	99.16788
	PA018	463574	100.1926	293745	99.03976
Nominal Conc.		**99.62ng/ml**		**39.66ng/ml**	
N		**6**		**6**	
Average		**1170.035**	**0.252881**	**1674.245**	**0.564492**
SD		**463144.3**	**100.0997**	**295580.2**	**99.65851**
%CV		**0.253**	**0.253**	**0.566**	**0.566**
Accuracy (%)		**100.10**		**99.66**	
Precision at LLQC					
S.NO	Sample ID	Metronidazole		Diloxanide Furoate	
		Area obtained	Observed Concentration(ng/ml)	Area obtained	Observed Concentration(ng/ml)
P and A at LLOQC	PA019	225831	99.99114	142097	99.64936
	PA020	225479	99.83529	143265	100.4685
	PA021	223058	98.76334	143258	100.4635
	PA022	224875	99.56786	140581	98.58623
	PA023	226598	100.3307	143045	100.3142
	PA024	224538	99.41864	141753	99.40812
Nominal Conc.		**49.8ng/ml**		**19.83ng/ml**	
N		**6**		**6**	
Average		**1220.664**	**0.540473**	**1067.033**	**0.748285**
SD		**225063.2**	**99.65117**	**142333.2**	**99.81498**
%CV		**0.543**	**0.543**	**0.750**	**0.750**
Accuracy (%)		**99.651**		**99.815**	

2.5.4 Recovery

Recovery of the analytes from the extraction procedure was determined by comparing the peak areas of the analytes in spiked plasma samples (six each of HQC, MQC, and LQC samples) with those of the analytes in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of the analytes at the step immediately prior to chromatography.

Table 2.11: Recovery of Metronidazole

S. NO	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	1497256	1755243	254.933	85.302	945846	1118564	126.3564	84.55895	449853	542463	82.61274	82.92787
2	1469852	1736257	253.00	84.656	953584	1125471	126.6084	84.72755	447586	541632	82.32253	82.63655
3	1494258	1762584	253.363	84.776	943256	1125214	125.2657	83.82903	442356	540165	81.58156	81.89275
4	1495786	1785328	250.391	83.782	941428	1125230	125.0212	83.66538	443592	545463	81.01491	81.32394
5	1492475	1785473	249.817	83.590	935402	1105476	126.4407	84.61532	448721	548934	81.43344	81.74407
6	1468523	1786775	245.628	82.188	942543	1132547	124.3606	83.2233	446257	549760	80.8646	81.17306
SD	**13401.62**	**20763.07**	**3.331**	**1.114**	**5964.06**	**9261.27**	**0.921**	**0.617**	**2931.75**	**3977.13**	**0.700018**	**0.703**
Mean	**1486358**	**1768610**	**251.189**	**84.049**	**943676.5**	**1122084**	**125.67**	**84.103**	**446394.2**	**544736**	**81.6383**	**81.950**
CV	**0.902**	**1.174**	**1.326**	**1.326**	**0.632**	**0.825**	**0.733**	**0.733**	**0.657**	**0.730**	**0.857462**	**0.857**
Standard Deviation					**1.228**							
Average recovery of three levels					**83.367**							
% Recovery					**1.473**							

Table 2.12: Recovery of Diloxanide Furoate

S. NO	Recovery at HQC level				Recovery at MQC level				Recovery at LQC level			
	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery	Extracted	Aqueous	recovered	% recovery
1	933568	1016840	109.236	91.81071	596234	722541	49.08233	82.51905	256984	312547	32.60945	82.22251
2	936521	1021624	109.069	91.66983	598426	726523	48.99278	82.36849	256321	316886	32.07996	80.88745
3	930450	1037452	106.708	89.68608	594261	724253	48.80428	82.05158	254167	319760	31.52447	79.4868
4	935247	1013652	109.777	92.2651	598710	728452	48.88623	82.18935	251470	312370	31.92784	80.50389
5	931854	1025214	108.145	90.89361	596243	729584	48.60925	81.72369	256843	314867	32.35142	81.5719
6	936521	1036453	107.508	90.35827	597211	728254	48.77709	82.00587	254170	319842	31.51676	79.46736
SD	**2516.19**	**9927.49**	**1.163**	**0.977552**	**1644.657**	**2722.49**	**0.168**	**0.281919**	**2141.088**	**3348.28**	**0.439**	**1.108377**
Mean	**934026**	**1025206**	**108.407**	**91.114**	**596847**	**726601**	**48.85866**	**82.143**	**254992**	**316045**	**32.002**	**80.690**
CV	**0.269**	**0.968**	**1.073**	**1.07289**	**0.275557**	**0.375**	**0.343**	**0.343**	**0.839667**	**1.059**	**1.374**	**1.374**
Standard Deviation					**5.646**							
Average recovery of three levels					**84.649**							
% Recovery					**6.670**							

The results of the recovery conforms that the % recovery was found to be 83.367 for Metronidazole and 84.679 for Diloxanide Furoate in three levels. The results of the recovery were given in table 2.11 and 2.12 for Metronidazole and Diloxanide Furoate respectively

2.5.5 Stability of the drug in solution

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the 7 intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

2.5.5.1Short term stability

The stock solution stability at room temperature for 8 hours was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Freshly prepared and stability stored solution at 298.86ng/ml for Metronidazole and 118.89ng/ml Diloxanide Furoate were analyzed and area response of the two conditions were compared and % stability was calculated. % stability was found to be within the range of more than 98% for both the drugs.

Table 2.13 Short term stability results for Metronidazole and Diloxanide Furoate

S. NO	Metronidazole			Diloxanide Furoate		
	Area obtained for		% Stability	Area obtained for		% Stability
	Fresh Stock	Stability stock		Fresh Stock	Stability stock	
1	1532965	1498751	97.76812	952343	936547	98.34135
2	1523352	1497214	98.28418	952142	934845	98.18336
3	1520554	1495746	98.36849	951865	943547	99.12614
4	1519243	1493862	98.32937	954262	932752	97.7459
5	1514475	1492423	98.54392	950423	931623	98.02193
6	1509247	1485479	98.42517	950241	927575	97.61471
SD	**8079.05**	**4712.065**	**0.269363**	**1467.401**	**5393.397**	**0.539229**
Mean	**1519973**	**1493913**	**98.28654**	**951879.3**	**934481.5**	**98.17223**
CV	**0.531526**	**0.315418**	**0.274059**	**0.154158**	**0.577154**	**0.549268**
% Stability	**98.28**			**98.17**		
% Change	**1.715**			**1.828**		

2.5.5.2 Long term stability:

Long term stability of the sample solution was studied by incubating the solution at a below 10^{o}C (stability samples) in a refrigerator for 11 days. Solution at 298.86ng/ml for Metronidazole and 118.89ng/ml Diloxanide Furoate was used for stability study. The solutions were analyzed in after the stability period. % stability and % assay were calculated. % stability was found to be 98.457 for Metronidazole with a % change of 1.543. % stability for Diloxanide Furoate was found to be 98.333 and % change was 1.667. Results were given in table 2.14

Table 2.14: Long term stability results for Metronidazole and Diloxanide Furoate

S. NO	Metronidazole			Diloxanide Furoate		
	Area obtained for		% Stability	Area obtained for		% Stability
	Fresh Stock	Stability stock		Fresh Stock	Stability stock	
1	1542965	1527594	99.0038	946572	938564	99.154
2	1542354	1524854	98.86537	943258	935513	99.179
3	1541423	1521879	98.73208	941257	931687	98.983
4	1540759	1526548	99.07766	938521	928973	98.983
5	1540214	1520984	98.75147	938142	923812	98.472
6	1539214	1522841	98.93628	936854	920479	98.252
SD	**1385.302**	**2645.561**	**0.137787**	**3671.237**	**6876.326**	**0.383**
Mean	**1541155**	**1524117**	**98.89444**	**940767.3**	**929838**	**98.837**
CV	**0.089887**	**0.17358**	**0.139327**	**0.390239**	**0.739519**	**0.388**
% Stability	**98.89**			**98.84**		
% Change	**1.105**			**1.162**		

2.5.5.3 Freeze Thaw Stability:

The stability studies of plasma samples spiked with Metronidazole and Diloxanide Furoate were subjected to three Freeze thaw cycles. The mean concentrations of the stability samples were compared to the theoretical concentrations. Freeze Thaw Stability was studied at HQC and MQC levels. Response at the retention time of the each drug was noted and the % stability was calculated. % stability was found to be 99.784 and 99.482 for Metronidazole; 99.638 and 97.528 for Diloxanide Furoate in HQC and MQC levels respectively. High stability was observed for the proposed method. Results were given in table 2.15

Table 2.15: Freeze Thaw Stability results for Metronidazole and Diloxanide Furoate

S.NO	Metronidazole				Diloxanide Furoate			
	At HQC (µg/ml)		At LQC (µg/ml)		At HQC (µg/ml)		At LQC (µg/ml)	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	Stability
1	298.81	298.79	99.68	99.14	118.14	118.02	39.47	39.08
2	298.54	298.25	99.25	98.58	118.68	118.57	39.54	37.58
3	298.11	297.85	99.68	99.68	118.19	118.14	38.58	38.14
4	298.36	297.58	99.58	99.57	117.98	118.25	38.91	38.05
5	298.81	297.66	99.14	98.41	118.96	117.69	38.14	37.26
6	298.47	297.1	99.25	98.11	118.54	117.25	38.74	37.58
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
SD	**0.270**	**0.585**	**0.243**	**0.645**	**0.374**	**0.461**	**0.537**	**0.644**
Mean	**298.512**	**297.871**	**99.43**	**98.915**	**118.415**	**117.987**	**38.897**	**37.948**
% CV	**0.090**	**0.196**	**0.245**	**0.652**	**0.316**	**0.391**	**1.3802**	**1.697**
Accuracy	**99.885**	**99.669**	**99.809**	**99.292**	**99.525**	**99.165**	**98.075**	**95.684**
Stability	**99.784**		**99.482**		**99.638**		**97.562**	

2.5.5.4 Bench-top stability:

Bench top stability, using six sets each of LQC and HQC was determined at 6hours. The quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy. Both the drugs were found to be stable over a period of time in the study. Results were found to be stable up to 6 hours as per the acceptance criteria. The percent nominal ranged from 99.708 at HQC and 98.602 at LQC for Metronidazole and 99.244 at HQC and 97.231 at LQC for Diloxanide Furoate. Results were given in table 2.16

Table 2.16: Bench-top stability results for Metronidazole and Diloxanide Furoate

S. NO	Metronidazole				Diloxanide Furoate			
	At HQC (µg/ml)		At LQC (µg/ml)		At HQC (µg/ml)		At LQC (µg/ml)	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	stability
1	298.54	297.58	99.14	97.85	118.05	117.96	39.47	38.25
2	298.99	297.55	99.47	97.58	118.69	117.85	39.01	38.47
3	297.85	297.08	99.25	97.55	117.85	117.25	38.58	37.14
4	297.55	297.14	98.74	98.69	117.58	117.01	39.11	37.67
5	297.14	296.69	98.58	96.69	117.57	116.69	38.54	37.58
6	297.66	296.47	98.47	96.99	118.96	116.58	38.57	37.71
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
SD	**0.684**	**0.447**	**0.402**	**0.69904**	**0.583**	**0.580**	**0.379**	**0.482**
Mean	**297.955**	**297.085**	**98.942**	**97.55833**	**118.117**	**117.223**	**38.88**	**37.803**
% CV	**0.229**	**0.150**	**0.406**	**0.716535**	**0.494**	**0.494**	**0.976**	**1.274**
Accuracy	**99.697**	**99.406**	**99.319**	**97.93047**	**99.274**	**98.523**	**98.033**	**95.318**
Stability	**99.708**		**98.602**		**99.244**		**97.231**	

2.5.5.5Auto-sampler stability:

The auto-sampler stability was evaluated by keeping the QC samples at 40^0C for 24 h in auto-sampler before analysis. Auto sampler stability was studied at HQC and LQC level. % stability was found to be 99.856 and 99.053 for Metronidazole and 98.281 and 97.780 for Diloxanide Furoate at HQC and LQC levels. Stability results were found to be accepted. Results were given in table.2.17.

Table 2.17: Auto-sampler stability results for Metronidazole and Diloxanide Furoate

S. NO	Metronidazole				Diloxanide Furoate			
	At HQC (µg/ml)		At LQC (µg/ml)		At HQC (µg/ml)		At LQC (µg/ml)	
	Fresh	stability	Fresh	stability	Fresh	stability	Fresh	Stability
1	297.58	296.87	99.58	98.84	118.96	116.69	39.36	38.36
2	297.66	297.85	99.14	98.69	118.04	116.25	39.47	38.96
3	298.89	297.96	99.47	98.25	118.47	116.74	39.25	38.19
4	297.85	297.55	98.57	97.51	118.36	116.36	39.47	38.99
5	298.09	297.01	98.69	97.36	118.14	116.01	39.63	38.69
6	298.74	298.99	98.07	97.25	118.99	116.69	39.74	38.47
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
SD	0.557	0.767	0.580	0.701	0.403	0.297	0.177	0.326
Mean	**298.1**	**297.70**	**98.92**	**97.983**	**118.493**	**116.46**	**39.487**	**38.61**
% CV	**0.187**	**0.258**	**0.587**	**0.715**	**0.340**	**0.255**	**0.449**	**0.845**
Accuracy	**0.557**	**0.767**	**0.580**	**0.700**	**99.5917**	**97.879**	**99.563**	**97.352**
Stability	**99.856**		**99.053**		**98.281**		**97.780**	

2.6 Discussion of the Results

A bioanalytical method has been developed using specific high-performance liquid chromatography combined with electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) method, operating in the positive ionization mode, for the simultaneous quantifying of Metronidazole and Diloxanide Furoate in human plasma was developed and validated.

The chromatographic conditions particularly the composition of mobile phase, flow-rate of mobile phase, choosing of suitable column, injection volume, column oven temperature, auto-sampler temperature, splitting of sample in to ion source, as well as a short run time were optimized through several trials to achieve good resolution and symmetric peak shapes for the Metronidazole and Diloxanide Furoate. It was found that a mixture of Tetrahydrofuran, Methanol and Acetonitrile in the ratio of 200:500:300 (v/v) could achieve this purpose and this was finally adopted as the mobile phase. The high proportion of organic solvent eluted both the Metronidazole and Diloxanide Furoate at retention times of 2.82min and Diloxanide Furoate elutes at 5.13min (Figure 3.C) at a flow rate of 0.80mL/min, produced good peak shapes, and permitted a run time of 10min. the mass spectra obtained for the standard components in the study were given in table 2.G and 2.H respectively for Metronidazole and Diloxanide Furoate.

Method validation is a process used to verify/confirm that an analytic method developed is suitable for its intended purpose, that it provides reliable and valid data for a specific analyte. Typical parameters to validate are; include selectivity, accuracy, precision, linearity and range, limit of detection, limit of quantification, recovery, robustness and stability. General recommendation for analytical method validation, i.e. for pharmaceutical methods, can be found in The US Food and Drug Administration (FDA) guideline.

Selectivity exercise is carried out to assess the ability of the bioanalytical method to differentiate and quantify the analyte(s) in presence of other components in the sample. For selectivity, analyses of blank samples of appropriate biological matrix (plasma, urine, or other matrix) obtained from at least six sources should be carried out. Each blank sample should be tested for interference and selectivity should be ensured at the lower limit of quantification.

A calibration curve is the relationship between instrument response and known concentration of the analyte. The calibration curve should be prepared in the same biological

matrix as the samples and a calibration curve should be generated for each analyte. The range of the method is the concentration interval where accuracy, precision and linearity have been validated. The used calibration curve should be the simplest model that adequately describes the concentration-response relationship. The deviation should not exceed more than 20% from the nominal concentration of the LLOQ and not more than 15% from the other standards in the curve. The calibration curve was linear over the concentration range of 49.81ng/ml - 298.86ng/ml for Metronidazole and 19.83ng/ml – 118.98ng/ml for Diloxanide Furoate respectively in human plasma with a coefficient of relation (r) ≥0.99. The slope and intercept of regression equations were 5270 and -47375 for Metronidazole and 8243.x - 22905 for Diloxanide Furoate respectively.

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, interbatch precision or repeatability, which measures precision with time and may involve different analysts, equipment, reagents and laboratories. Precision and accuracy for this method was controlled by calculating the intra and inter-day batch variations of QC samples in six replicates at four concentrations of HQC, QC, LQC and LLOQC of 298.86, 199.24, 99.62 and 49.8ng/ml

for Metronidazole and 118.98, 79.32, 39.66 and 19.83ng/ml for Diloxanide Furoate as shown in Table 2.9-2.12.

Matrix effect is investigated to ensure that selectivity and precision are not compromised within the matrix screened. Three blank samples from each of at least six batches of matrix under screening are extracted. For matrix effect LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control) spiking dilutions and internal standard dilution are spiked in the above extracted blank samples. Recovery comparison sample at LQC, MQC and HQC concentration level prepared and screened.

The results of stability tests obtained were well within the acceptable limit. Furthermore, they revealed that no significant degradation occurred during the chromatography, extraction and sample storage of plasma samples. Different stability experiments in plasma and the values for the precision and accuracy, expressed as percentage relative error (%RE) are shown in Table.2.13-2.17. The findings from these stability tests indicated that storage of both the drugs in plasma.

2.7 Conclusion

The LC–MS/MS validated method has proved to be very simple, sensitive and reliable and successfully applied for the pharmacokinetic study in human plasma. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The run time is within very less and only 0.250 mL of plasma was required for each determination of Metronidazole and Diloxanide Furoate. This method is very suitable and convenient for pharmacokinetics and bioavailability study of the simultaneous estimation of Metronidazole and Diloxanide Furoate.

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