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Research Article

SIMULTANEOUS ESTIMATION OF METRONIDAZOLE, FURAZOLIDONE AND LOPERAMIDE BY HPTLC IN VETERINARY FORMULATION

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ABSTRACT

A simple and accurate High Performance Thin Layer Chromatography (HPTLC) method has been developed for the simultaneous estimation of Metronidazole (MTZ), Furazolidone (FZD) and Loperamide (LOP) in veterinary formulations. The chromatographic separation was achieved on aluminium plates precoated with silica gel 60GF-254 with mobile phase Methanol: Ethyl acetate: Acetonitrile: Chloroform: Benzene in the ratio of 2: 2.9: 0.8: 2.9: 1.4%v/v/v/v/v measured at a wavelength of 230nm. The R_f values was found to be 0.61, 0.32 and 0.48 for MTZ, FZD and LOP respectively. The method was validated according to International Conference on Harmonization (ICH) Guidelines and the reliability of the method was assessed by the evaluation of Linearity range from 1600 – 2400 ng/spot for MTZ and FZD and 400-600ng/spot for LOP, Recovery studies include 99.27 – 101.12% w/w. This method is simple, accurate, precise and sensitive. This method can be used for the routine analysis of these drugs in pharmaceutical and veterinary formulations.

Keywords: HPTLC, Metronidazole, Furazolidone, Loperamide, Veterinary formulation.

INTRODUCTION

Metronidazole (MTZ) is a nitroimidazole antibiotic chemically 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol, is used particularly for anaerobic bacteria and protozoa to treat parasitic infections, certain sexually transmitted diseases, stomach ulcers, gastro intestinal infections and vaginitis. It acts by Inhibiting Nucleic acid synthesis by binding to DNA and also inhibits Electron transport proteins.

Furazolidone (FZD) is nitro furan derivative chemically 3-{[(5-nitro-2-furyl)methylene] amino} 1,3-oxazolidin -2-one which is a broad spectrum antiprotozoal and antibacterial agent used to treat diarrhoea and enteritis caused by bacteria or protozoan infections and Helicobacter pylori infections. The mode of action is, it binds to DNA and induces cross-links and also acts as a monoamine oxidase inhibitor (MAOI) which prevents the inactivation of tyramine by neither hepatic and gastrointestinal monoamine oxidase that releases nor epinephrine from sympathic nerve terminals.

Loperamide hydrochloride (LOP) is a Long-acting synthetic opioid antidiarrhoeal drug used against diarrhoea resulting from gastro enteritis or inflammatory Bowel disease. It is chemically 4-[4-(4-Chlorophenyl) -4- hydroxypiperidin -1-yl]- N,N- dimethyl- 2,2-diphenylbutane amide hydrochloride. It decreases the activity of Myenteric Plexus, tone of the longitudinal smooth muscles, colonic mass movements and suppresses the gastro colic reflex.

The combined drug formulation (Tablet) was branded as Triogyl containing MTZ, FZD and LOP at a concentration of 2mg, 100mg and 200mg respectively which was manufactured by Pharmatech Healthcare private Limited.

RP-HPLC methods individually and with Ofloxacin[1,2] and Diloxadine[3], HPTLC method with Miconazole[4], Spectroscopic methods with Secnidazole[5] and Nalidixic Acid[6] were reported for MTZ. Spectroscopic and HPLC estimation methods individually[7,8] and with Tinidazole[9] and Nifuroxime[10] were reported for FZD. HPLC[11,12] and HPTLC[13] estimation of Loperamide[14] were obtained from Literature survey. Recently validated spectroscopic method for the simultaneous estimation of MTZ and FZD were reported[15,16]. As no method was developed for the combination of these three drugs an attempt was made to develop simple, sensitive, accurate and reproducible method for the simultaneous estimation of these drugs in veterinary formulation. The method was validated according to ICH guidelines.

MATERIALS AND METHODS

Chemicals and Reagents

The raw materials (Metronidazole (98.90%), Furazolidone (98.75%), and Loperamide (99%)) were obtained from Pond Chy Pharmaceuticals, Pondicherry as gift samples and used as reference materials throughout the experiment without any prior treatment. The Reagents used were of analytical grade and are purchased from RANKEM and SRL chemicals Pvt Ltd.

Preparation of Stock Solution

The standard stock solution was prepared by dissolving 100mg of MTZ, FZD and LOP in Methanol individually in 100ml volumetric flask. Then the solution was made up to 100ml with Methanol to get a concentration of 1mg/ml. (1000µg/ml).

Preparation of Sample Solutions

Twenty tablets (Triogyl tablets-consisting of 200mg MTZ, 100mg FZD and 2mg LOP from Pharmatech Healthcare Pvt. Ltd.) were weighed and the average weight was found and the tablets were powdered. From the powdered mixture a weight equivalent to the label claim of Loperamide was accurately weighed and dissolved in 50ml Methanol. Shake well and sonicate for 20min and make up the volume to 100ml with methanol. Then filter the solution and the filtrate was used to carry out the further analysis.

Instrumentation and Chromatographic Conditions

The samples were spotted as bands of width 5mm using Camag 100 µl sample (Hamilton, Bonaduz, Switzerland) syringe on a precoated silica gel 60 F-254 aluminium Plate (20 cm×10 cm) with 250 µm thickness using a Camag Linomat V (Switzerland). The rate of flow from the Syringe was maintained at 5µl and the distances between two spots were 8mm.

The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and the baseline correction was used. Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20-30 min at room temperature (25°C±2) at relative humidity of 60±5%. The mobile phase consisted of Methanol: Ethyl acetate: Acetonitrile: chloroform: benzene in the ratio of 2:2.9:0.8:2.9:1.4%v/v/v/v/v and 10 ml of mobile phase was used per chromatography. The length of chromatogram run was 8 cm. Subsequent to the development, HPTLC plates were dried in current of air. Densitometric scanning was performed on a Camag HPTLC

scanner III in the reflectance absorbance mode at 230 nm and operated by WINCATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting continuous UV spectrum between 200 and 400 nm. Concentrations of the compound on chromatographic plate were determined from the intensity of Reflected light. Evaluation was via peak areas with linear regression.

Method validation[21,22]

Linearity

Linearity 1: [**80%** - (MTZ)**320**µg, (FZD)**320**µg,(LOP)**80**µg]

3.2ml of Metronidazole, 3.2ml of Furazolidone and 0.8ml of Loperamide standard stock solutions were pipetted out in to a 10ml standard flask and final volume was made with water.

Linearity 2: [**90%**- (MTZ)**360**µg, (FZD)**360**µg, (LOP)**90**µg]

3.6ml of Metronidazole, 3.6ml of Furazolidone and 0.9ml of Loperamide standard stock solutions were pipetted out in to a 10ml standard flask and final volume was made with water.

Linearity 3: [**100%**- (MTZ)**400**µg,(FZD)**400**µg, (LOP)**100**µg]

4.0ml of Metronidazole, 4.0ml of Furazolidone and 1.0ml of Loperamide standard stock solutions were pipetted out in to a 10ml standard flask and final volume was made with water.

Linearity 4: [**110%**- (MTZ)**440**µg,(FZD)**440**µg,(LOP)**110**µg]

4.4ml of Metronidazole, 4.4ml of Furazolidone and 1.1ml of Loperamide standard stock solutions were pipetted out in to a 10ml standard flask and final volume was made with water.

Linearity 5: [**120%**- (MTZ)**480**µg,(FZD)**480**µg,(LOP)**120**µg]

3.2ml of Metronidazole, 3.2ml of Furazolidone and 0.8ml of Loperamide standard stock solutions (1500µg/ml) were pipetted out in to a 10ml standard flask and final volume was made with water.

5µl of each of the solutions were spotted on precoated TLC plate and kept for development. After development the plate was dried and scanned at a wavelength of 230nm and the densitogram was recorded. The results were tabulated in Table 2 and 3 and the densitogram and calibration graphs were shown in (Fig. 1-4).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for MTZ, FZD and LOP by the proposed method were calculated using the following formula

$$LOD = \frac{3.3 \text{ X SD}}{S}$$

$$LOQ = \frac{10 \text{ X SD}}{S}$$

Where, S – Slope of the calibration curve.

SD – Standard Deviation of response.

Method Precision

5µl of the sample solution was spotted six times on the precoated TLC plates and the development was carried out. The densitogram was recorded and shown in (Fig.5), the % RSD was calculated which was found to be within the limits (>2.0) according to ICH guidelines. The results were tabulated in Table 4.

System precision

5µl of the standard linearity solution of 100% concentration was spotted six times on the precoated TLC plates and the development was carried out. The densitogram was recorded and shown in (Fig.6) and the results were tabulated in Table 5.

Accuracy

An accuracy study was carried out by Standard Addition method. To ensure accuracy of the developed method, known quantity of standard stock solution was mixed with unknown sample and the % recovery was calculated.

Preparation of solutions for Accuracy

Solution 1: (80%)

To 10ml of sample solution 7.8ml of standard Loperamide solution, 12ml of standard Metronidazole solution and 22ml of standard Furazolidone solution were added and the volume was made up to 100ml with distilled water.

Solution 2: (100%)

To 10ml of sample solution 9.8ml of standard Loperamide solution, 20ml of standard Metronidazole solution and 28ml of standard Furazolidone solution were added and the volume was made up to 100ml with distilled water.

Solution 3: (120%)

To 10ml of sample solution 11.8ml of standard Loperamide solution, 30ml of standard Metronidazole solution and 38ml of standard Furazolidone solution were added and the volume was made up to 100ml with distilled water

5µl of the solution was spotted six times on the precoated TLC plates and the development was carried out. After development the plates was dried and scanned at 230nm. The % recovery was calculated and the densitogram were shown in (Fig.7) and the results were tabulated in Table 6.

$$\% \text{ Recovery} = \frac{\text{Amount Received} - \text{Amount Added}}{\text{Amount Present}} \text{X } 100$$

RESULTS

Table 1: Data showing Retention factor

Drug	R_f Value
Metronidazole(MTZ)	0.61
Furazolidone(FZD)	0.32
Loperamide(LOP)	0.48

Table 2: Linearity data

S. No.	Drugs	Concentration (ng/spot)	Peak area
		1600	6520
1	MTZ	1800	7248
		2000	8145
		2200	8874
		2400	9852
		1600	6784
2	FZD	1800	7521
		2000	8324
		2200	9236
		2400	10005
		400	4227
3	LOP	450	4795
		500	5438
		550	6039
		600	6706

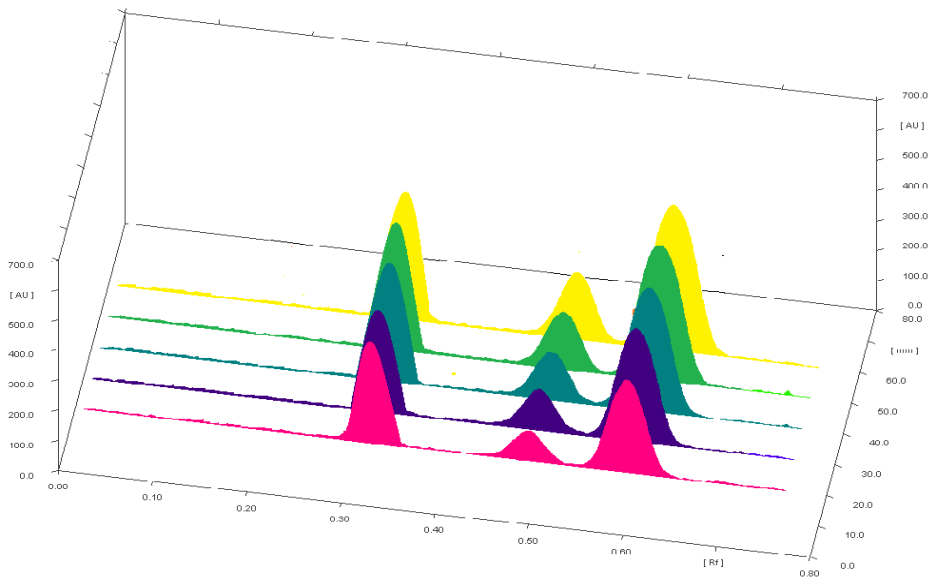

Fig. 1: The Densitogram showing linearity of MTZ, FZD and LOP at 230nm.

Table 3: Linearity parameters of Metronidazole, Furazolidone and Loperamide

Drug	Linearity range (ng/spot)	R^2	Slope	Intercept	LOD (ng)	LOQ (ng)
MTZ	1600-2400	0.9999	4.103	98.96	283.08	857.84
FZD	1600-2400	0.9998	4.078	217.9	284.82	863.10
LOP	400-600	0.9999	12.4	759.3	93.67	283.84

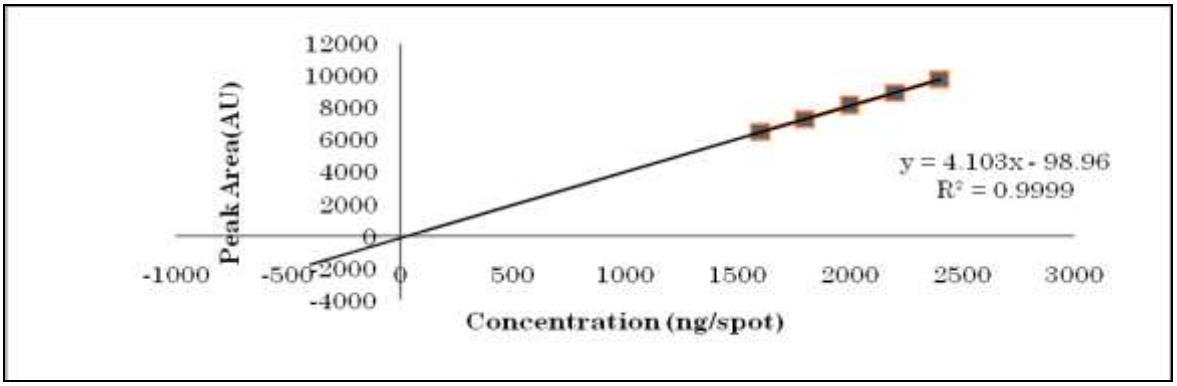

Fig. 2: Calibration graph of Metronidazole

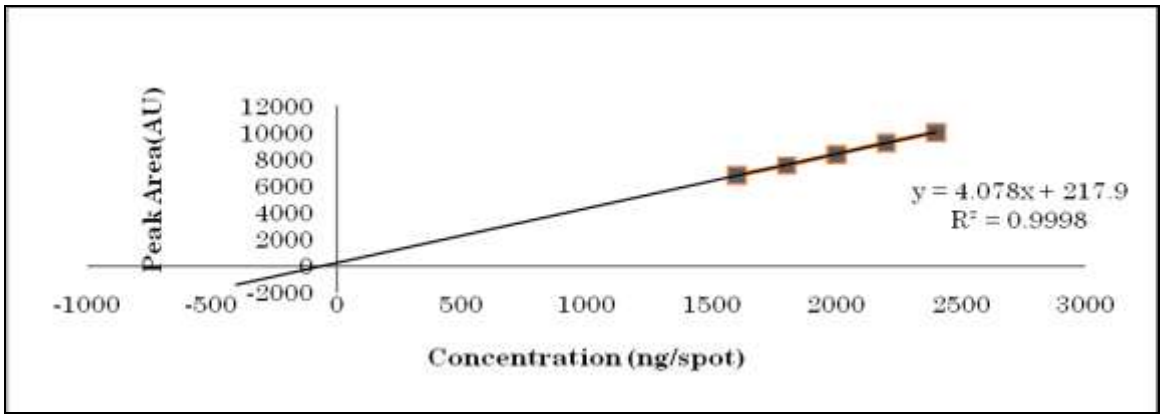

Fig. 3: Calibration Graph of Furazolidone

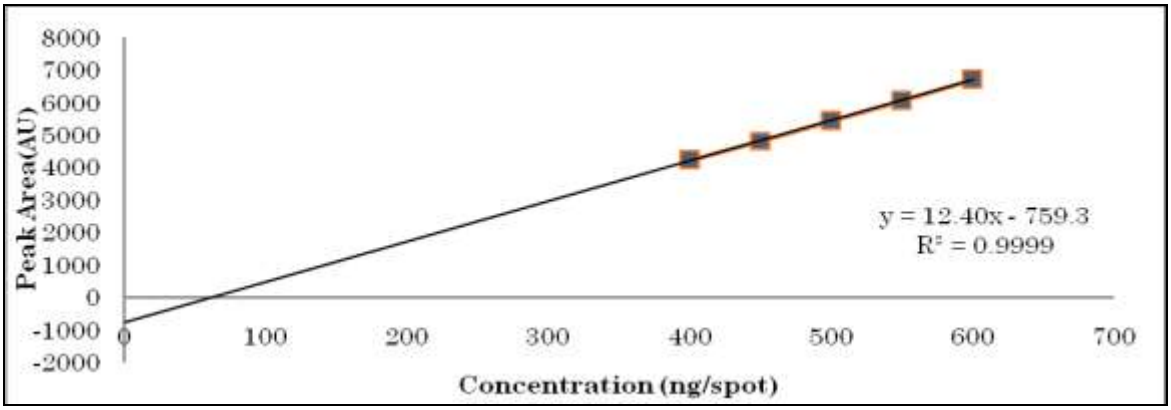

Fig. 4: Calibration Graph of Loperamide

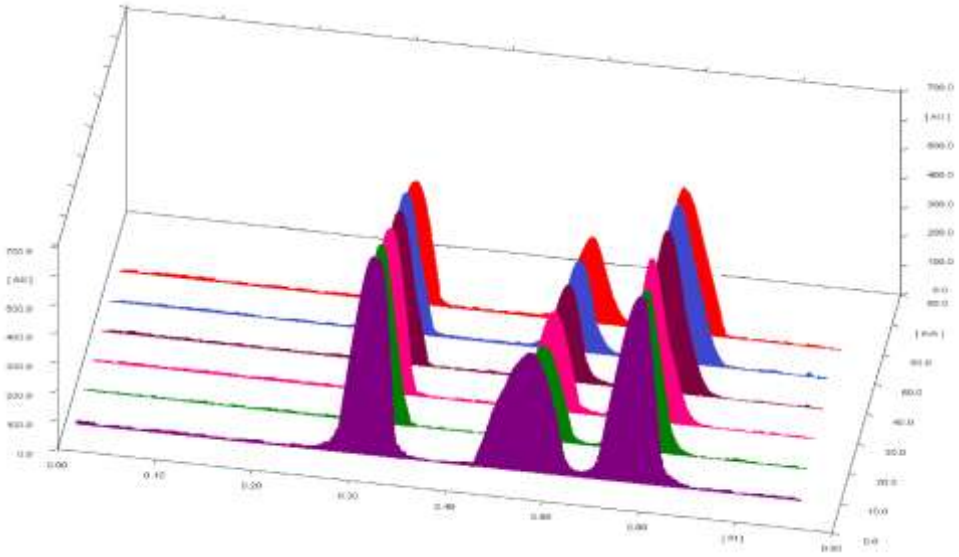

Fig. 5: Densitogram showing the precision of the method.

Table 4: Method Precision

Drug	Label claim (mg/tablet)	Amount estimated (mg)	Drug content (%)	SD	%RSD
FZD	100	101.34	101.34	0.5	0.49
MZD	200	199.58	99.79	0.3	0.30
LOP	2	1.96	98	0.7	0.78

Table 5: System Precision showing Mean S.D, % RSD

Drug	concentration (ng/spot)	Peak area	Average	SD	%RSD
MTZ	2000	8124	8124	47.89	0.58
		8145			
		8095			
		8059			
		8119			
		8201			
		8477			
FZD	2000	8324	8408	109.51	1.30
		8365			
		8269			
		8441			
		8569			
		7499			
LOP	500	7438	7485	42.96	0.57
		7481			
		7496			
		7554			
		7441			

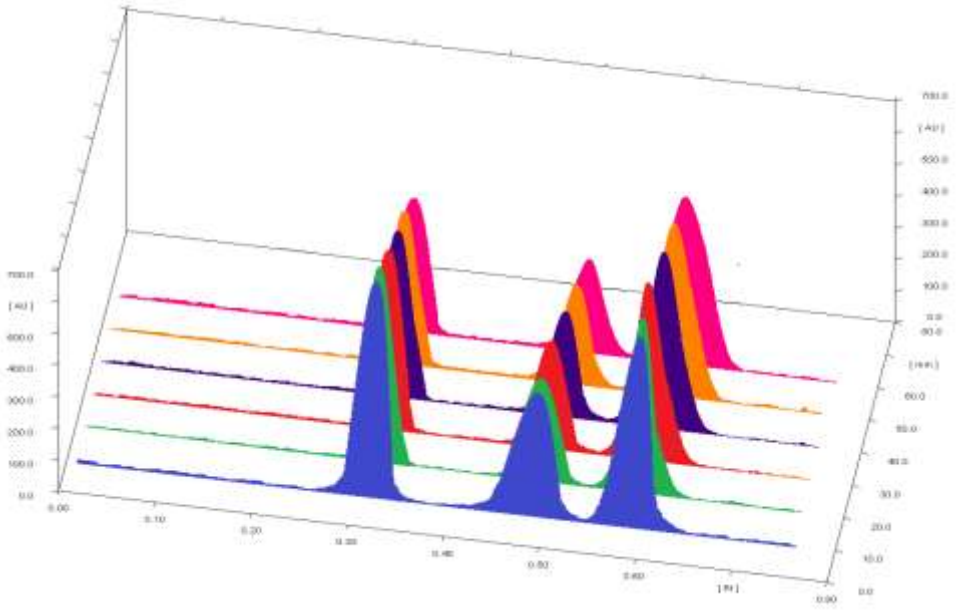

Fig. 6: Densitogram showing the system precision.

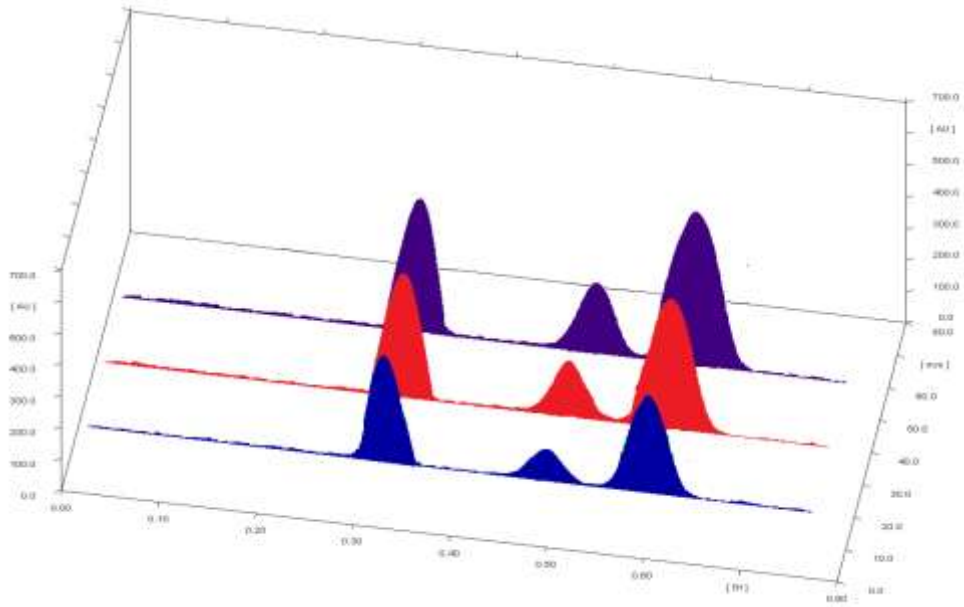

Fig. 7: Densitogram showing Recovery results.

Table 6: Results showing Recovery of the method

Drug	Recovery Level (%)	Initial amount (µg/ml)	Amount added (µg/ml)	Amount recovered (µg/ml)	Recovery (%)	SD	%RSD
MTZ	80	200	120	321.01	100.31		
	100	200	200	399.49	99.87	0.26	0.2
	120	200	280	479.25	99.84		
FZD	80	100	220	319.98	99.99		
	100	100	300	400.28	100.07	0.1	0.09
	120	100	380	480.96	100.2		
LOP	80	2	78	79.42	99.27		
	100	2	98	99.54	99.54	1.00	1.00
	120	2	118	121.35	101.12		

RESULT AND DISCUSSION

The mobile phase optimized containing Methanol: Ethyl acetate: Acetonitrile: chloroform: benzene, were showing sharp peaks with good resolution between MTZ, FZD and LOP at less retention time. Detection was carried out at 230nm as all the three drugs showed good response.

The Retention time was found to be 0.61, 0.32 and 0.48 respectively and the peak shapes of all the drugs were symmetrical. The Linearity

experiments were performed and the range was found to be 1600-2400ng/spot and the precision of the method was found to be 0.30 – 1.30 which was within the limits of ICH guidelines indicating that the method was precise for the estimation of these drugs. Accuracy of the method was calculated by recovery studies using standard addition method at three levels. The amount of drug recovered was found to be 99.27 % – 101.12 % which was within the range of standards according to ICH guidelines. The estimated % RSD for accuracy was found to be less than 2 which are within the limits according to ICH guidelines. The developed method was accurate, precise, reproducible and simple for the estimation of MTZ, FZD and LOP's in combined dosage formulations.

Table 7: Summary of Validation parameters and their limits

Parameters	Results			Acceptance Limits
	MTZ	FZD	LOP	
Retention time	0.61	0.48	0.32	--
Linearity Range (µg/ml)	1600-2400	1600-2400	400-600	--
Correlation coefficient (R^2)	0.9999	0.9998	0.9999	0.9990-1.0
Slope	4.103	4.078	12.4	--
Intercept	283.08	284.82	93.67	--
System Precision (% RSD)	0.58	1.30	0.57	< 2
Method Precision (% RSD)	0.49	0.30	0.78	< 2
Percentage recovery	99.84-100.31	99.99-100.20	99.27-101.12	97-103

CONCLUSION

The newly developed HPTLC technique was validated by evaluating various validation parameters. The results obtained for each parameter was tabulated below and all the results were found to be within the prescribed limits. The method developed in the study was found to be simple, accurate, precise and reproducible for determination of Metronidazole, Furazolidone and Loperamide in combined dosage formulation. Therefore, the developed method can be recommended for routine quality control analysis of these drugs in pharmaceutical formulations.

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