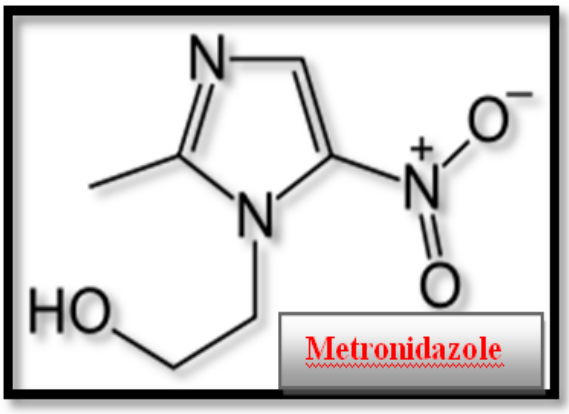
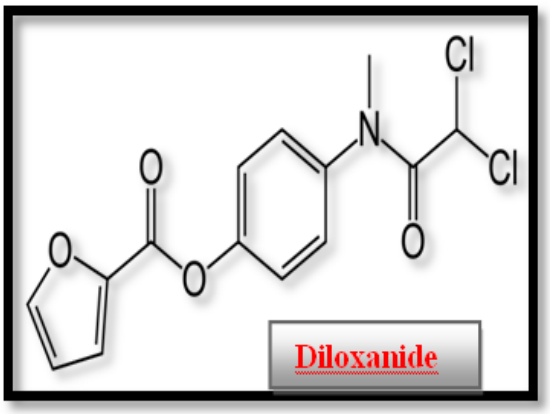
CHAPTER 5





RP - HPLC Method Development and Validation for Simultaneous Estimation of Metronidazole and Diloxanide Furoate in Formulation Dosage Forms

5.1: Introduction:

Detailed description of drugs Metronidazole and Diloxanide Furoate is given in chapter 2.1

5.2 Literature Review

Detailed description of available literature for the estimation of Metronidazole and Diloxanide Furoate using different analytical methods were given in chapter 2.2

5.3 Materials and Methods

5.3.1 Chemicals and Solvents:

HPLC grade Methanol, Acetonitrile and water were purchased form Merck chemicals, Mumbai. Laboratory reagent grade Potassium dihydrogen fisher scientific products, Mumbai. The working standard drugs Metronidazole and Diloxanide Furoate were obtained from Cipla pharmaceutical private limited, Hyderabad. The market available brand QUGYL (Metronidazole – 200mg and Diloxanide Furoate – 250mg) was purchased from local pharmacy.

5.3.2 Instrumentation:

Chromatographic separation was performed on a PEAK chromatographic system with kromasil C18 column (250mm×4.6mm; 5µm particle size) equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µl fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L) Ultrasonicator was used to sonicate the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

5.3.3 Chromatographic Conditions:

The mobile phase consisted of Methanol: Acetonitrile: 0.01M KH_2PO_4 in 60:20:20 (v/v). Separation was carried in kromasil C18 column (250mm×4.6mm; 5µm particle size. Injections were carried out using a 20µl loop at ambient temperature and the flow rate was 1.0 ml/min. Detection was performed at 245nm with 10min runtime.

5.3.4 Preparation of Standard Solutions:

In order to prepare stock solutions of both the drugs Metronidazole and Diloxanide Furoate accurately weigh and transfer 10mg of each of the into 10ml separate volumetric flasks. Both the flasks were sonicate for 2min to dissolve the drug completely. An individual concentration of 1000µg/ml solutions was obtained. These solutions were used as standard stock solutions. From theses standard stock solutions, required dilutions were prepared by proper dilution. From the prepared standard

dilutions, 1ml from both of the solutions were mixed separately to get a combined dilutions for construction of calibration curve and for simultaneous analysis of sample.

5.3.5 Preparation of Sample Solutions:

10 tablets of QUGYL (Metronidazole – 200mg and Diloxanide Furoate – 250mg) were purchased from local market. The tablets were crushed and grounded using clean motor and piston. From the tablet powder an amount equal to 10mg of standard drug Metronidazole was weighed accurately and was dissolved in little amount of methanol in a 10ml volumetric flask. Sonicate the flask for 5min to dissolve the drug in the solvent. Then make up to the mark with same solvent. A concentration of 1000µg/ml of Metronidazole was obtained. This was filtered through 0.45µm nylon membrane filter paper. Then from this solution a concentration of 100µg/ml was prepared by selected dilution. As per the label claim of the two drugs, a concentration of 100µg/ml of Diloxanide Furoate was obtained in the combined tablet solution. The obtained solution was used as sample solution for the estimation of drugs in tablet dosage forms.

5.4 Method Development

Method development plays crucial role in effective estimation of drugs in tablet and pharmaceutical formulation. The present method has undergone various developmental trails before the appropriate conditions for the determination of the drug were devised. First of all the mode of chromatographic condition was chosen, basing upon the polarity of the drug the isocratic mode of elution was used. The mobile phase for the analysis is stated after many trails by using different solvents such as methanol, acetonitrile and water in varying proportions. They were taken in the ratio 50:30:20, 60:30:10 and 80:10:10v/v. But the peaks were obtained with poor resolution. Then use of buffer and acidic solutions as the constituent of mobile phase was considered. Therefore, methanol, acetonitrile and potassium dihydrogen phosphate of 0.01M was chosen as mobile phase after carrying out different trials. The mobile phase composition of methanol: acetonitrile: 0.01M KH_2PO_4in 60:20:20 (v/v) gave sharp peaks with acceptable retention time. The two drugs were eluted with different retention time is 4.46min for Metronidazole and 6.16min Diloxanide Furoate. The column selected was kromasil C18 column. The pH of the system was varied in the range 4.0 to 5.2 using Ortho phosphoric. The pH of 4.1 was well suited for the analysis. So as to find the suitable wavelength for the estimation the mixed

solution of both the drugs were scanned in UV-Spectrophotometer and it was found that at 245 nm both the drugs showed maximum absorbance. Thus, it was taken as suitable wavelength for the analysis. The development studies also satisfy the system suitability conditions. All the parameters were verified for the system suitability. Tailing factor, Theoretical plates, resolution etc were satisfied in acceptable criteria. The optimized chromatographic conditions were given in table 5.1

Table 5.1: Optimized Chromatographic Conditions for the Estimation of Metronidazole and Diloxanide Furoate

S.NO	Parameter	Results
1	MP	methanol: acetonitrile: 0.01M KH_2PO_4 in 60:20:20 (v/v)
2	Wavelength	245nm
3	Stationary Phase	RP- C18 Column
4	pH of MP	4.1 with OPA
5	Flow Rate	1.0 ml/min
6	Pump Mode	Isocratic
7	Pump Pressure	12.5 ± 5MPa
8	Api Concentration	Metronidazole– 80 µg/ml Diloxanide Furoate- 100 µg/ml
9	RT	Metronidazole– 4.46 min Diloxanide Furoate- 6.16 min
10	Resolution	Metronidazole-------- Diloxanide Furoate- 7.12
11	Area	Metronidazole– 295655 Diloxanide Furoate-171735
12	Theoretical Plates	Metronidazole– 9018 Diloxanide Furoate- 9329
13	Tailing Factor	Metronidazole– 1.57 Diloxanide Furoate- 1.59

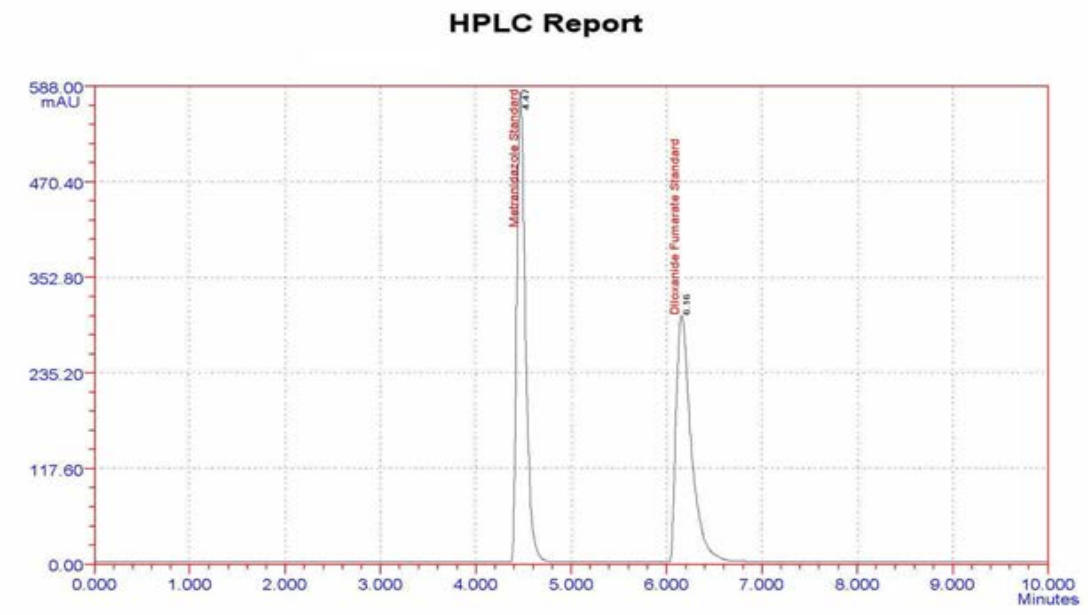


Figure 5.A: Standard chromatogram of Metronidazole and Diloxanide Furoate

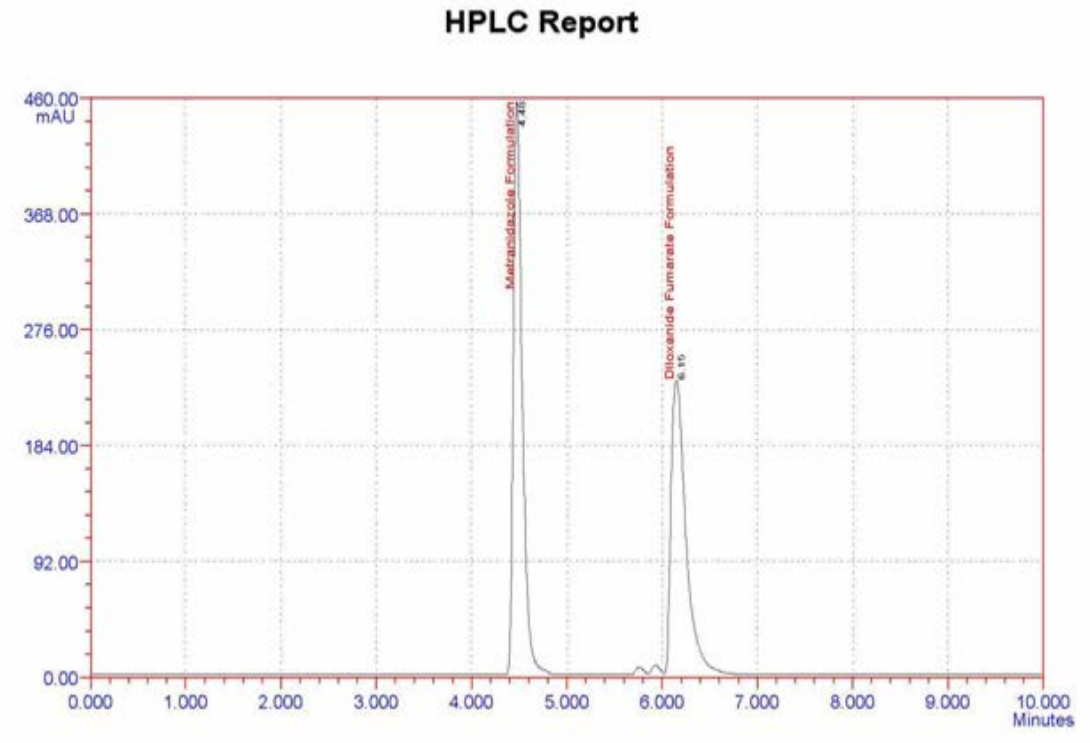


Figure 5.B: Formulation Chromatogram of Metronidazole and Diloxanide Furoate

5.5 Method Validation

The proposed method has been validated for the assay of Metronidazole and Diloxanide Furoate in pharmaceutical formulations using parameters system suitability conditions, Linearity, Precision, Recovery, Ruggedness, Robustness, LOD & LOQ and assay of the tablet. All these parameters were verified using ICH guidelines.

5.5.1: System Suitability Parameters:

System-suitability tests are an integral part of method development. System suitability conditions were verified to ensure whether the developed chromatographic conditions give the best results or not. Important parameters like numbers of theoretical plates (N), retention time (RT) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 80 µg/ml Metronidazole and 100 µg/ml of Diloxanide Furoate. In the optimized conditions, a well resolved peak with a resolution factor of 7.12 was observed at retention of 4.46 min for Metronidazole and 6.16 min for Diloxanide Furoate. Very high theoretical plates (9018, 9329) and very less tailing factor (1.57, 1.59) were observed respectively for both the drugs. Hence the developed method obeys the system suitable criteria.

5.5.2 Specificity:

The specificity of the method was determined by comparing the base line and retention times obtained when standard, blank and sample solutions were injected in to HPLC system using the optimized conditions. The results confirmed that the no spectral and chromatographic detection were observed at the retention of both the drugs in blank injection. A retention time of 4.46 min for Metronidazole and 6.16 min for Diloxanide Furoate were observed for standard solution and similar retention times were observed for samples also. Hence the developed method was specific for Metronidazole and Diloxanide Furoate.

5.5.3: Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample. It is essential to determine the useful range at which the instrumental response is proportional to the analyte concentration. Different concentration ranges as per the dosage of two drugs in formulation were prepared and were analyzed in the optimized

conditions. Individual calibration curve was constructed for both the drugs using concentration against the area at the retention of drug. The linearity range was found to be 25-150g/ml for Diloxanide Furoate and 20-140g/ml for Metronidazole. The regression equation was found to be y = 1840x -11288 with coefficient of correlation R^2 = 0.999 in case of Diloxanide Furoate and y=3750x-6491 and co-relation coefficient as 0.999 for Metronidazole. Results of the linearity results were given in table 5.2 and calibration curve was shown in figure 5.C for Diloxanide Furoate and 5.D for Metronidazole.

Table 5.2: Table Showing Linearity Results

S.NO	Diloxanide Furoate		Metronidazole	
	Concentration (µg/ml)	Peak Area	Concentration (µg/ml)	Peak Area
1	25	35362	20	72434
2	50	79645	40	139583
3	75	126788	60	216342
4	100	171735	80	295655
5	125	221932	100	367075
6	150	263096	120	445132

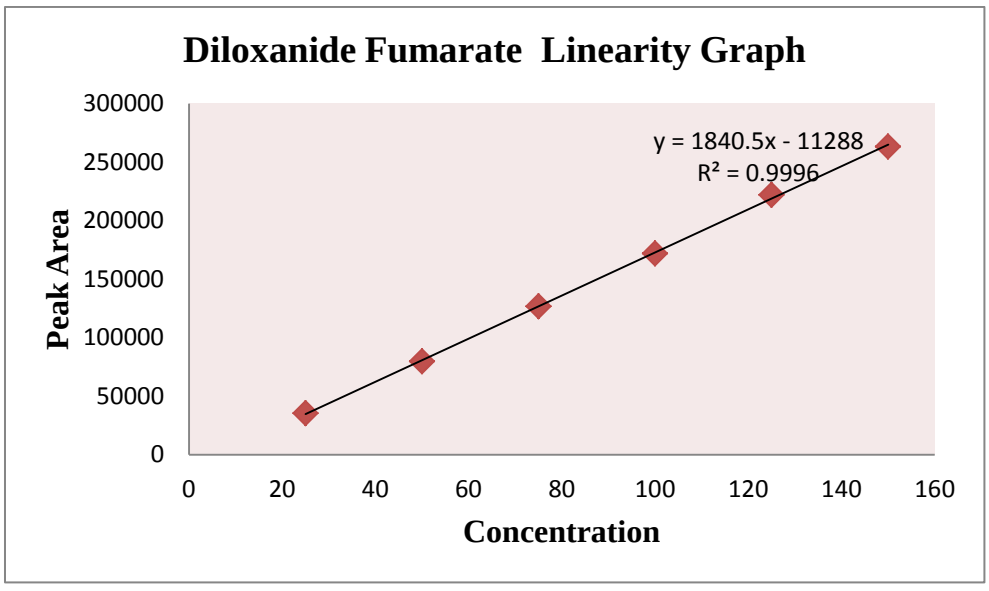


Figure 5.C: Calibration Curve of Diloxanide Furoate

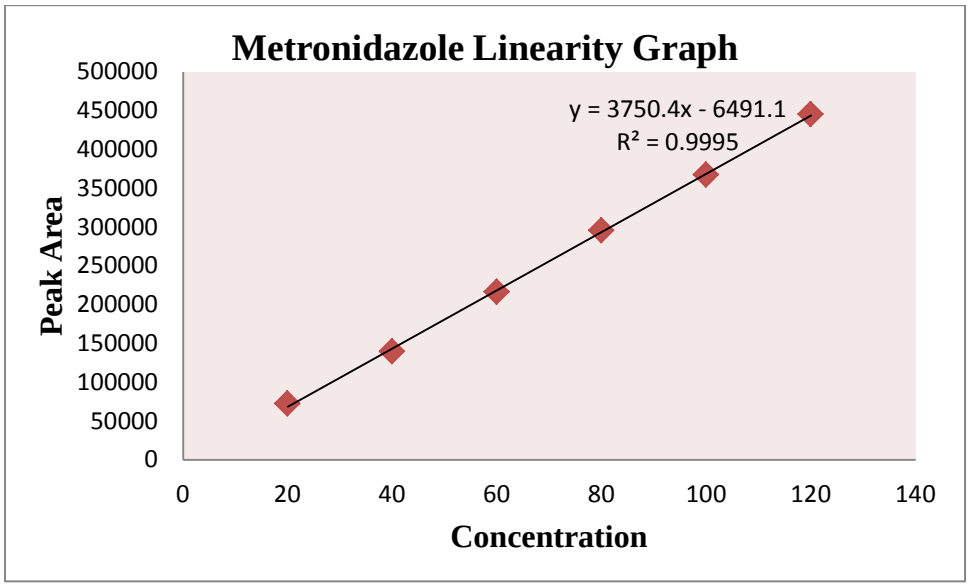


Figure 5.D: Calibration Curve of Metronidazole

5.5.4: Precision:

Precision was carried at a concentration of 80µg/ml of Metronidazole and 100µg/ml of Diloxanide Furoate. Precision expresses closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at two levels: repeatability, intermediate precision. Repeatability is also referred to as intra-assay precision. It is a measure of precision of analysis in one laboratory by one operator using one piece of equipment over a relatively short time-span. Intermediate precision was performed by same analyst in similar laboratory conditions and the analysis was carried in different times over a period of three days. Precision is the degree of agreement of results when experimental conditions are maintained as constant as possible, and expressed as RSD of replicate. The %RSD of the intraday precision was found to be 0.75 and 0.40 for Metronidazole and Diloxanide Furoate respectively. In case of interday precision the %RSD were found to be 0.77 for Metronidazole and 0.60 for Diloxanide Furoate. Table 5.3 and 5.4 showing the intraday and inter day precision results respectively for the optimized method.

Table 5.3: Table Showing Intraday Precision Results

S.NO	Metronidazole at 80µg/ml	Diloxanide Furoate at 100µg/ml
1	295184	171213
2	298306	171875
3	297163	170678
4	293936	171324
5	293962	172687
6	292326	171767
RSD	**0.75**	**0.40**

Table 5.4: Table Showing Interday Precision Results

S.NO	Metronidazole at 80µg/ml	Diloxanide Furoate at 100µg/ml
1	291530	172169
2	296540	173464
3	290722	173731
4	293630	171324
5	295116	172510
6	291940	171324
RSD	**0.77**	**0.60**

5.5.5: Recovery:

Accuracy is the closeness of agreement between a measured quantity value and a true quantity value of a measure and it is a qualitative characteristic that cannot be expressed as a numerical value. The accuracy of the method was determined by calculating recovery of Diloxanide Furoate and Metronidazole 50%, 100% and 150% was added to a pre-quantified sample solution. The recovery studies were carried out three times over the specified concentration range and the percentage recovery of Diloxanide Furoate and Metronidazole was found to be in the range of 98.11% to 101.87%, 98.22 to 101.64% and the results are presented in the table 5.5 and 5.6.

Table 5.5: Table Showing Recovery Results of Diloxanide Furoate

S.NO	%Recovery	Concentration in µg/ml			Amount Found	% recovery
		Target	Spiked	Total		
1	50%	50	25	75	75.25	100.34
2		50	25	75	76.21	101.62
3		50	25	75	73.58	98.11
4	100%	50	50	100	101.87	101.87
5		50	50	100	101.05	101.05
6		50	50	100	98.25	98.25
7	150%	50	75	125	125.49	100.40
8		50	75	125	125.14	100.11
9		50	75	125	123.82	99.06

Table 5.6: Table Showing Recovery Results of Metronidazole

S.NO	%Recovery	Concentration in µg/ml			Amount Found	% recovery
		Target	Spiked	Total		
1	50%	40	20	60	58.93	98.22
2		40	20	60	60.86	101.43
3		40	20	60	61.00	101.67
4	100%	40	40	80	80.76	100.95
5		40	40	80	80.03	100.03
6		40	40	80	78.62	98.27
7	150%	40	60	100	98.64	98.64
8		40	60	100	101.64	101.64
9		40	60	100	100.29	100.29

5.5.6: Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For determination of method robustness, a number of chromatographic parameters, for example, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition are varied within a realistic range and quantitative influence of the variables is determined. Robustness of the method was studied by changing the wavelength from 245 to 249 and 259, the mobile phase composition changed by ± 5% and pH ± 2. The results showed that the retention time and peak area of Diloxanide Furoate and Metronidazole were remained almost unchanged and no significant degradation was observed. Results of the robustness were given in table 5.7

Table 5.7: Table Showing Robustness Results of Diloxanide Furoate and Metronidazole

S.NO	Condition	Change	Metronidazole		Diloxanide Furoate	
			Area	% Change	Area	% Change
1	Standard	……..	295655	……..	171735	…….
2	MP 1	65:15:20 (v/v)	301145	1.86	172940	0.70
3	MP 2	55:25:20 (v/v)	296540	0.30	173464	1.01
4	WL 1	249nm	297163	0.51	173915	1.27
5	WL 2	259nm	298206	0.86	173861	1.24
6	pH 1	4.0	300074	1.49	172687	0.55
7	pH 2	4.2	299550	1.32	174782	1.77

5.5.7: Ruggedness:

The ruggedness of the developed methods was expressed as % RSD of the same procedures applied in different laboratories by different instruments on different days or carrying out analysis by different analyst, for same standard and tablet dosage forms of Metronidazole and Diloxanide Furoate. The %RSD of Diloxanide Furoate was found to be 1.47 and that of Metronidazole was 1.38. Both the values were obtained within acceptable range below 2, which illustrate the ruggedness of the

method. Table 5.8 shows the results of ruggedness for Diloxanide Furoate and Metronidazole.

Table 5.8: Ruggedness Results of Diloxanide Furoate and Metronidazole

S.NO	Metronidazole	Diloxanide Furoate
1	286960	170680
2	291505	174905
3	293872	171720
4	291179	176618
5	298928	172687
6	289996	176674
RSD	**1.38**	**1.47**

5.5.8: LOQ and LOD:

LOD of an analytical procedure is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantitated as an exact value whereas LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ values of the proposed method were estimated using the standard formulae S/N Signal to noise ratio. It was found that LOD of the Diloxanide Furoate and Metronidazole were 0.25 and 0.20µg/ml. LOQ was found to be 0.825 and 0.66µg/ml.

Table 5.9: Table Showing Sensitivity Results of Diloxanide Furoate and Metronidazole

Drug	LOD	LOQ
Diloxanide Furoate	0.25µg/ml	0.825µg/ml
Metronidazole	0.20µg/ml	0.66µg/ml

5.5.9 Estimation of Drugs in Pharmaceutical Formulations:

The standard and sample solutions were injected separately; chromatograms and the peak areas were recorded. Representative chromatograms of sample have been given in Fig 5.B. The amount of drug present per tablet formulation was determined. Thus obtained results were presented in the table 5.10.

Table 5.10: Table Showing Sensitivity Results of Diloxanide Furoate and Metronidazole

S.NO	Drug	Brand	Dosage	Amount Prepared	Amount Found	%Assay
1	Diloxanide Furoate	QUGYL	250mg	100µg/ml	98.79µg/ml	98.79
2	Metronidazole		200mg	80µg/ml	79.26µg/ml	99.07

5.6 Discussion of the Results

RP-HPLC method developed for simultaneously estimation Diloxanide Furoate and Metronidazole in fixed Dose. Developed RP-HPLC method was validated according to ICH guideline. RP-HPLC method has shown adequate separation for Diloxanide Furoate and Metronidazole. Separation was achieved on Inertsil C18 (250 x 4.6mm) 5µm column by using methanol: acetonitrile: 0.01M KH_2PO_4in the ratio of 60:20:20 (v/v) (4.1 with OPA) as a mobile phase at a flow rate of 1.0 ml/min, and UV detection was carried out at 245nm.

In the present study the specificity of the method was determined by assessing interference from the placebo & diluents. There was no other co eluting, interfering peaks from excipient's, impurities found and the method was specific for estimation of Diloxanide Furoate and Metronidazole.

Comparing the chromatograms obtained from standard drugs, with the chromatogram obtained from tablet solutions, the specificity of the method was assessed. As the retention time of standard drugs and the retention time of the drugs in sample solution was same, so the method was specific. The developed method was found specific and selective, as there was no interference of excipients found.

The method was validated in terms of linearity, precision, accuracy, specificity, System Suitability. The linearity of the proposed method was investigated in the range

of 25-150µg/ml of concentration for Diloxanide Furoate and 20-120µg/ml of concentration for Metronidazole (Table 5.2). Accuracy was determined by recovery study and it was found to be 98.25-101.62 for Diloxanide Furoate (Table 5.5) and 98.22-101.67% for Metronidazole (Table 5.6). The low RSD values of interday (0.75 and 0.40 for Metronidazole and Diloxanide Furoate respectively) and intraday (0.77 for Metronidazole and 0.60 for Diloxanide Furoate), reveal that the proposed method is precise. The LOD and LOQ values of the proposed method were estimated using the standard formulae S/N Signal to noise ratio. It was found that LOD of the Diloxanide Furoate and Metronidazole were 0.25 and 0.20µg/ml. LOQ was found to be 0.825 and 0.66µg/ml. the method can successfully estimate the amount of drug in tablet dosage forms more than 98% assay. The summery results were shown in table 5.11.

Table 5.11: Summary Results of Diloxanide Furoate and Metronidazole

	Parameter	Metronidazole	Diloxanide Furoate
Method Developed	Elution	Isocratic	
	Mobile Phase	methanol: acetonitrile: 0.01M KH_2PO_4in 60:20:20 (v/v)	
	pH	4.1 with OPA	
	Column	kromasil C18 column (250mm×4.6mm; 5µm particle size)	
	Wave Length	245 nm	
	Flow	1.0 ml/min	
	Runtime	10 min	
	Temperature	Ambient	
Method validation	Retention Time	4.46 min	6.16 min
	Tailing factor	1.57	1.59
	Theoretical plate	9018	9329
	Resolution	…….	7.12
	Linearity range	20-120 µg/ml	25-150 µg/ml
	Slope	3750.4	1840.5
	Intercept:	- 6491.1	-11288
	r^2	0.9996	0.9996
	Intraday Precision	0.75	0.40
	Interday Precision	0.77	0.60
	Ruggedness	1.38	1.47
	Recovery	98.22-101.67	98.11-101.87
	Robustness difference	0.30-1.86	0.55-1.77
	LOQ	0.66 µg/ml	0.825 µg/ml
	Limit of Detection	0.20 µg/ml	0.25 µg/ml
	Formulation assay	99.07 %	98.79 %

5.7 Conclusion

Metronidazole in combination with Diloxanide Furoate was useful in the treatment of intestinal and extra-intestinal amoebic infections. A simple HPLC method was developed for the simultaneous estimation of Metronidazole and Diloxanide Furoate in pharmaceutical formulations. The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Metronidazole and Diloxanide Furoate. A mobile phase composed of Methanol: acetonitrile: 0.01M KH_2PO_4in 60:20:20 (v/v) with a short run time (10 min) and isocratic elution used are advantageous and made the routine analysis easy. Among the significant advantages of this method are simplicity, selectivity, accuracy and precision ensuring that it is suitable for determining the content of Metronidazole and Diloxanide Fumarat in dosage form.

5.8 References

The references available for the drugs Metronidazole and Diloxanide Furoate were given in chapter 2.8