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Original Article

A stability indicating RP-HPLC method for the simultaneous estimation of metronidazole, clindamycin and clotrimazole in bulk and their combined dosage form

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ABSTRACT

A simple, specific, and precise stability indicating reverse phase high performance liquid chromatography method was developed and validated as per the ICH guidelines for the simultaneous determination of Metronidazole, Clindamycin and Clotrimazole in bulk and combined dosage forms. The quantification was carried out by using Hypersil BDS C18 (4.6*250mm, 5µm) column at 30^0c with Phosphate Buffer pH 4.5: Methanol: Acetonitrile in the ratio of 30:20:50 % V/V as mobile phase, pH 4.5 adjusted by using 0.1M ortho phosphoric acid. The flow rate is 1 mL/min and the estimation is carried out by using PDA detector at 210 nm. The retention time of Metronidazole, Clindamycin and Clotrimazole were 1.636, 2.289 and 4.928 minutes respectively. The linearity was observed from 25-150µg/mL with correlation coefficient 0.999 for Metronidazole, Clindamycin and Clotrimazole. The LOD and LOQ of Metronidazole, Clindamycin and Clotrimazole were found to be 1.77 & 5.35µg/mL, 2.55 & 7.77µg/mL and 1.28 & 5.25µg/mL respectively and the statistics data for the MNZ, CDM and CTM were concluded that the method was found to be simple, reliable, selective, reproducible and accurate. The method was successfully used for quality control analysis of Metronidazole, Clindamycin and Clotrimazole.

Keywords: Metronidazole (MNZ), Clindamycin (CDM) and Clotrimazole (CTM), RP-HPLC, Stability and Validation.

INTRODUCTION

Metronidazole is used as an anti-protozoal and it is 2-methyl-5-nitroimidazole-1-ethanol [1]. It is widely used for antibacterial activity against gram-negative aerobes and gram-positive bacteria, including bacteriode fragilis that produces β-lactamases [2] and also used in the treatment of amoebiasis, trichomoniasis, lambliasis, and anaerobic infections [3, 4]. It is one of the most promising agents in combination with antimicrobial agents used in the eradication of helicobacter pylori, a recognized cause of gastritis and duodenal ulcers [5, 6, 7]. Clindamycin is methyl 7-chloro-6,7,8-trideoxy-6-{[(4*R*)-1-methyl-4-propyl-L-prolyl]amino}-1-thio-L-threo-α-D-galacto-octopyranoside [8, 9] and it is a semi synthetic derivative of lincomycin. It is active against gram-positive aerobes and highly active against both gram-positive and negative anaerobes [10]. Clindamycin inhibits bacterial protein synthesis at the level of the bacterial ribosome. Clotrimazole is an antifungal agent and it is used for primarily in the treatment of superficial fungal infections [11]. Chemically it is known as 1-[(2-chlorophenyl) di phenyl methyl]-1H-imidazole [12, 13]. Clotrimazole is used to treat skin infections such as athlete's foot, jock itch, ringworm, and other fungal skin infections like lightening or darkening of the skin of the neck, chest, arms, or legs. This medication is also used to treat a skin condition known as pityriasis (tinea versicolor) and Small amounts this drug also used to treat yeast infections in pregnant women.

Few analytical methods were reported in the literature, such as determination of Metronidazole, Clindamycin, and Clotrimazole present in individual or in combination with other drugs. However literature survey reveals that, there is no method for the simultaneous estimation of Metronidazole, Clindamycin, and Clotrimazole in bulk and their combined dosage form by reversed phase-HPLC. Chemical structure of Metronidazole,

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Clindamycin and Clotrimazole are shown in Figure No. 1, 2 & 3 respectively.

MATERIALS AND METHODS

Materials: Metronidazole, Clindamycin and Clotrimazole pure drugs were obtained as a gift sample from Dewcare Concept (P) Ltd, Gujarat, India. HPLC grade Acetonitrile, Methanol and water [filtered through 0.2µ filters] were purchased from Merck, Mumbai, India. Potassium dihydrogen phosphate and ortho phosphoric acid were purchased from Rankem, RFCL limited, New Delhi, India.

Preparation of Solutions

Stock and Standard solution: The stock solution prepared from pure drugs of 50mg of Metronidazole, Clindamycin and Clotrimazole were taken in 50mL volumetric flask and dissolved in 10mL of HPLC grade methanol, and diluted up to the mark with diluent.

The standard solution prepared from 10mL of stock solution was taken in 100mL volumetric flask and diluted up to the mark with diluent to get a concentration of 100µg/mL of Metronidazole, Clindamycin and Clotrimazole.

Phosphate Buffer pH 4.5: Dissolve 6.8g of potassium di-hydrogen phosphate in 1000mL of HPLC grade water (filtered through 0.2µ filters) and degassed. Adjust the pH to 4.5 by 0.1M ortho phosphoric acid.

Sample solution: 20 tablets (Shekit-V) of Metronidazole, Clindamycin and Clotrimazole were powdered and an amount of the powder equivalent to 50mg of Metronidazole, Clindamycin and Clotrimazole was accurately weighed and transferred to the 50mL volumetric flask, made up to the volume with Diluent. The solution was placed in an ultrasonicator for 30 minutes and filtered through a 25 mm, 0.45 µm nylon syringe filter. 10mL of this solution was taken and diluted to 100mL by using a diluent to get a final concentration of 100µg/mL. Five replicate sample solutions were prepared in similar manner.

HPLC Instrumentation and Conditions

Instrumentation: Waters HPLC system consisting of WATERS 2695 separation module, an inbuilt auto sampler, column oven and WATERS 2996 (PDA) detector was employed throughout the analysis. Chromatography was performed on a Hypersil BDS C18 column. A sonerex sonicator was used for sonication and the data was acquired by using the EM Power^2 software.

Optimized chromatographic conditions: Chromatography was performed on a Hypersil BDS C18 column using mobile phase containing mixture of Phosphate Buffer pH 4.5: Methanol: Acetonitrile in the ratio of 30:20:50 % V/V. The mobile phase was filtered through membrane filter (0.45 µm), and vacuum degassed by sonication prior to use. The pump pressure and run time was maintained at 1500-2000 psi and 10 minutes respectively. Chromatography was performed at 30^0C with flow rate at 1 mL/min and detection was carried out at 210 nm. Instrumentation and optimized chromatographic conditions for proposed method details are shown in Table No 1.

RESULTS AND DISCUSSION

Validation study of Metronidazole, Clindamycin and Clotrimazole: The Method validation was performed as per ICH guidelines for the simultaneous estimation of Metronidazole, Clindamycin and Clotrimazole in bulk and combined dosage form. The method was validated with respect to parameters including accuracy, precision, linearity, robustness, specificity, system suitability, LOD and LOQ [14].

Assay of Metronidazole, Clindamycin and Clotrimazole: The developed method was applied to the assay of Metronidazole, Clindamycin and Clotrimazole in combined dosage forms. The drug content was estimated with an average of six determinations, and results were given in Table No 2. The results were similar to the labeled claim of market formulations. The standard and sample chromatograms of Metronidazole, Clindamycin and Clotrimazole were shown in Figure No 3 and 4 respectively.

Specificity: The specificity of the proposed method was established by injecting the placebo and mobile phase solution in triplicate and the chromatograms were recorded. Comparison of chromatograms revealed that there were no interactions between the placebo and sample peaks. Finally, it was indicated that the method was specific.

Accuracy: The accuracy was determined by calculating the recovery of Metronidazole, Clindamycin and Clotrimazole at 50, 100, & 150% and they were added to pre quantified sample solution. The recovery studies were carried out in the dosage form in triplicate each in the presence of placebo. The mean percentage recovery of MNZ, CDM and CTM at each level was not less than 99%, and not more than 102%. The percentage recovery of Metronidazole, Clindamycin and Clotrimazole was found to be in the range of 99 to

101%. The results are shown in the Table No 3, 4 and 5.

Precision: Precision should be investigated by using authentic and homogeneous samples. The Precision of this method was expressed as S.D and %RSD of series of repeated measurements. Precision of MNZ, CDM and CTM determination by proposed method were ascertained by repeated analysis of homogeneous samples of Metronidazole, Clindamycin and Clotrimazole standard solutions in the intraday under the similar conditions. The method precision result was shown in Table No 6.

Linearity: Linearity of the proposed method was established by using series of standard solutions of Metronidazole, Clindamycin & Clotrimazole, and these studies are repeated in triplicate with different stock solutions. The curve obtained by concentration on x-axis and peak area on y-axis against showed linearity in the concentration range of 25 to 150µg/mL for MNZ, CDM and CTM and its correlation coefficient is 0.999 and linearity graph is shown in Graph No 1. The regression equation of MNZ, CDM and CTM were found to be Y = 12285x + 7337 & Y = 3723x – 2718 and Y = 72477x + 37486 respectively. The Linearity and statistical analysis of data are shown in Table No 7, 8 and 9.

Robustness: The robustness was evaluated by the analysis of Metronidazole, Clindamycin and Clotrimazole under different experimental conditions such as slight changes in chromatographic conditions like change of flow rate (±0.2 mL/min), temperature (±5^{0}C), and mobile phase composition (± 5%). It was distinguished that there were no changes in the chromatograms, and the parameters were within the limits, which indicates that the method was robust and suitable for routine use. The complete results are shown in Table No 10, 11 & 12 and the method is having good system suitability.

Limit of Detection: The limit of detection (LOD) has established the minimum concentration at which the analyte can be reliably detected. LOD is determined by the signal to noise ratio and generally acceptable detection limit ratio is 3:1. It was found for Metronidazole, Clindamycin & Clotrimazole is 1.77, 2.55 and 1.28 µg/mL respectively.

Limit of Quantification: The limit of quantification (LOQ) has established the minimum concentration at which the analyte can be reliably quantified. LOQ is determined by the signal to noise ratio and a typical signal to noise ratio is 10:1 which is acceptable for estimating the quantification limit. It was found to be 5.35, 7.77 and 5.25 µg/mL for Metronidazole, Clindamycin & Clotrimazole respectively.

System suitability: This test was conducted on freshly prepared Metronidazole, Clindamycin and Clotrimazole standard solution and was used for the evaluation of the system suitability parameters such as retention time, area, USP tailing and theoretical plates, limit of detection and limit of quantification. Five replicate injections for a system suitability test were injected into the chromatographic system. Finally the proposed method is having good system suitability and its parameters are shown in Table No 13.

FORCED DEGRADATION STUDY

Forced degradation studies were conducted to evaluate the stability and specificity of the method. The acceptable limit for consideration in the present study is between 5 to 20% for chromatographic assays [15, 16]. The specificity of the developed method was evaluated by using different ICH prescribed stress conditions like acidic, basic, oxidative, and thermal. Acidic degradation studies are performed by taking 10 mL of stock solution in 50 mL volumetric flask. 10 mL of 5N HCL was added to the stock solution and these solutions were kept at reflux for 4 hours. Finally this solution was neutralized with 5 N NaOH. Alkali degradation studies are performed by taking 10 mL of stock solution in 50 mL volumetric flask. 10 mL of 5 N NaOH was added to the stock solution and these solutions were kept at reflux for 4 hours. Finally this solution was neutralized with 5N HCL. Oxidative degradation studies are performed by taking 10 mL of stock solution in 50 mL volumetric flask and 10 mL of 3% hydrogen peroxide added to the flask. These mixtures were kept for up to 3 days in the dark. Thermal degradation studies are performed by taking 10 mL of stock solution in 50 mL volumetric flask and then sample solution were heated to 80^{0}c for 15-60 minutes. Photo degradation studies are performed by taking 10 mL stock solution in 50 mL volumetric flask and this solution exposing to the ultraviolent light by keeping this flask in UV chamber for 7days or 200 Watt hours/m^2 in photo stability chamber.

Neutral degradation studies are performed by taking 10 mL stock solution in 50 mL volumetric flask and this solution refluxing the drug in water for 6 hours at 60ºC. Finally forced degradation studies of Metronidazole, Clindamycin and Clotrimazole concluded that purity of angle less than purity of threshold and forced degradation

chromatogram were shown in Figure No 6 to 11. All the Degradation summary results were shown in Table No: 14

CONCLUSION

A stability indicating RP-HPLC method for simultaneous estimation of Metronidazole, Clindamycin and Clotrimazole in bulk and pharmaceutical dosage forms is established. The method is simple, accurate, linear, sensitive and reproducible as well as economical for the effective quantitative analysis of Metronidazole, Clindamycin and Clotrimazole in bulk and combined dosage forms. The method was validated, and all the method validation parameters were tested and shown to produce satisfactory results. The method is free from interactions of the other ingredients and excipients used in the formulations. Finally, concluded that the method is suitable for use in the routine quality control analysis of Metronidazole, Clindamycin and Clotrimazole in active pharmaceutical ingredients and in pharmaceutical dosage forms.

ACKNOWLEDGEMENTS

The authors would like to thank the management of Dewcare Concept (P) Ltd, Gujarat, India for the gift sample of drugs used in this investigation.

Table No 1: Instrumentation and Optimized chromatographic conditions for proposed method

S. No	Instrumentation	Optimized Chromatographic Conditions
1	HPLC	Waters: 2695 Separation Module
2	Detector	Waters: 2996 PDA
3	Column	Hypersil BDS C_{18} (4.6*250mm, 5µm)
4	Column temperature	30^0C
5	Flow rate	1 mL/min
6	Injection volume	10µL
7	Wavelength	210 nm
8	Run time	10 minutes
9	Diluent	First drug dissolved in methanol and then made up with Buffer and Methanol (50:50)
10	Mobile phase composition	Phosphate Buffer: Methanol: ACN in ratio of 30:20:50 %V/V

Table No 2: Assay results of Metronidazole, Clindamycin and Clotrimazole formulations

S. No	Formulation		Labeled Amount(mg)	Amount Found(mg)±S.D	%Assay ±RSD
1	Shekit-V	Metronidazole	100	99.97±0.141	0154
2		Clindamycin	100	99.94±0.127	0.132
3		Clotrimazole	100	99.98±0.104	0.112

Table No 3: Recovery data for the proposed RP-HPLC method for Metronidazole

S. No	Concentration level (%)	Amount added (µg/mL)	Amount found (µg/mL)	Area obtained	Mean %Recovery ± S.D*	%RSD*
1	50	5	4.97	528051	99.667±0.64	0.645
			5.02	529345		
			4.96	526367		
2	100	10	10.03	1056118	10.033±0.03	0.305
			10.01	1061449		
			9.97	1053689		
3	150	15	14.99	1580858	99.977±0.33	0.335
			14.95	1578657		
			15.05	1587862		

Table No 4: Recovery data for the proposed RP-HPLC method for Clindamycin

S. No	Concentration level (%)	Amount added (µg/mL)	Amount found (µg/mL)	Area obtained	Mean %Recovery ± S.D*	%RSD*
1	50	5	4.98	155687	99.86±1.02	1.02
			4.95	156382		
			5.05	156952		
2	100	10	10.01	311932	99.90±0.2	0.200
			9.97	315265		
			9.99	313683		
3	150	15	14.97	467338	99.466±0.88	0.886
			15.02	471819		
			14.77	472123		

Table No 5: Recovery data for the proposed RP-HPLC method for Clotrimazole

S. No	Concentration level (%)	Amount added (µg/mL)	Amount found (µg/mL)	Area obtained	Mean %Recovery ± S.D*	%RSD*
1	50	5	5.02	3861928	100.06±1.13	1.136
			4.94	3840516		
			5.05	3866126		
2	100	10	9.94	7675605	100.16±0.70	0.708
			10.08	7653968		
			10.03	7725564		
3	150	15	15.08	11587757	100.17±0.31	0.315
			15.01	11506666		
			14.99	11503537		

***S.D & %RSD** is Standard Deviation and percentage of Relative Standard Deviation

Table No 6: Method Precision results of the proposed RP-HPLC method

S. No	Injections	METRONIDAZOLE		CLINDAMYCIN		CLOTRIMAZOLE	
		RT	Peak Area	RT	Peak Area	RT	Peak Area
1	1	2.231	1060387	3.662	311277	5.085	7690249
2	2	2.234	1049952	3.663	312715	5.086	7672098
3	3	2.236	1056703	3.668	310564	5.096	7682066
4	4	2.238	1065894	3.668	312459	5.097	7643794
5	5	2.242	1057403	3.672	315557	5.100	7722332
6	6	2.245	1050708	3.680	314154	5.127	7731967
7	**MEAN**	2.237	1056842	3.668	312788	5.098	7690418
8	**SD**	0.005	5998.963	0.006	1837.181	0.015	32631.356
9	**%RSD**	**0.230**	**0.567**	**0.179**	**0.587**	**0.298**	**0.424**

*** RT is Retention Time**

Table No 7: Linearity and Statistical analysis data for Metronidazole

S. No	Concentration (µg/mL)	Area	Average Area	Statistical Analysis		
				Slope	Y-Intercept	Correlation Coefficient
1	25	320770	1083487	12285	7337	**0.999**
2	50	623918				
3	75	933278				
4	100	1231009				
5	125	1541966				
6	150	1849976				

Table No 8: Linearity and Statistical analysis data for Clindamycin

S. No	Concentration (µg/mL)	Area	Average Area	Statistical Analysis		
				Slope	Y-Intercept	Correlation Coefficient
1	25	90163	322663	3723	2718	**0.999**
2	50	180540				
3	75	275080				
4	100	370938				
5	125	461161				
6	150	558093				

Table No 9: Linearity and Statistical analysis data for Clotrimazole

S. No	Concentration (µg/mL)	Area	Average Area	Statistical Analysis		
				Slope	Y-Intercept	Correlation Coefficient
1	25	1921943	6385509	72477	37486	**0.999**
2	50	3638642				
3	75	5435830				
4	100	7316584				
5	125	9100014				
6	150	10900039				

Table No 10: Robustness results of the proposed RP-HPLC method for Metronidazole

S. No	Parameters			Peak Area	RT*	USP	
	Optimized		Used			Plate Count	Tailing Factor
1	Flow rate (±0.2)	**1 mL/min**	0.8	1168256	2.449	2549	0.93
			1.2	1064695	2.273	2605	0.89
2	Temperature (±5^{0}c)	**30^{0}c**	25	1168256	2.449	2549	0.93
			35	1072359	2.273	2603	0.89
3	Mobile phase composition (± 5%)	**30:20:50**	25:30:45	988254	2.276	2471	0.92
			35:10:55	1075190	2.445	2275	0.93

Table No 11: Robustness results of the proposed RP-HPLC method for Clindamycin

S. No	Parameters			Peak Area	RT*	USP	
	Optimized		Used			Plate Count	Tailing Factor
1	Flow rate (±0.2)	**1 mL/min**	0.8	334147	3.975	5010	1.39
			1.2	307212	3.678	4723	1.38
2	Temperature (±5^{0}c)	**30^{0}c**	25	337974	3.975	4979	1.42
			35	310739	3.678	4698	1.40
3	Mobile phase composition (± 5%)	**30:20:50**	25:30:45	245773	3.689	4890	1.38
			35:10:55	267727	3.906	5036	1.39

Table No 12: Robustness results of the proposed RP-HPLC method for Clotrimazole

S. No	Parameters			Peak Area	RT*	USP	
	Optimized		Used			Plate Count	Tailing Factor
1	Flow rate (±0.2)	**1 mL/min**	0.8	8421299	5.510	4990	1.03
			1.2	7675106	5.099	4958	1.07
2	Temperature (±5^{0}c)	**30^{0}c**	25	8433990	5.510	4988	1.03
			35	7690710	5.099	4954	1.07
3	Mobile phase composition (± 5%)	**30:20:50**	25:30:45	6190578	5.114	6601	1.14
			35:10:55	6837097	5.318	6477	1.13

*** RT** is Retention Time

Table No 13: System Suitability Parameters of the proposed RP-HPLC method

Parameters	METRONIDAZOLE	CLINDAMYCIN	CLOTRIMAZOLE
Linearity range(µg/mL)	25-150	25-150	25-150
Regression equation	Y = 12285x + 7337	Y = 3723x - 2718	Y = 72477x + 37486
Correlation coefficient(r^2)	0.999	0.999	0.999
Retention time (minutes)	1.636	2.289	4.928
Theoretical plates	3605	5178	7880
Tailing factor	0.95	1.40	0.93
Limit of Detection (µg/mL)	1.77	2.55	1.28
Limit of Quantitation (µg/mL)	5.35	7.77	5.25
Capacity factor (k)	1.045	1.018	1.025
Wavelength-Isosbestic point	210		

Table No 14: Forced Degradation results of proposed RP-HPLC method

Degradation conditions	METRONIDAZOLE		CLINDAMYCIN		CLOTRIMAZOLE	
	Purity of		Purity of		Purity of	
	Angle	Threshold	Angle	Threshold	Angle	Threshold
Control sample	--	--	--	--	--	--
Acidic Degradation	0.222	0.292	0.636	1.442	0.157	0.627
Alkali Degradation	0.133	0.282	0.257	0.434	0.111	0.659
Oxidative Degradation	0.133	0.282	0.257	0.434	0.111	0.659
Thermal Degradation	0.149	0.288	0.178	0.357	0.124	0.883
Photo Degradation	0.883	0.283	0.239	0.430	0.121	0.694
Neutral Degradation	0.145	0.284	0.245	0.245	0.152	0.731

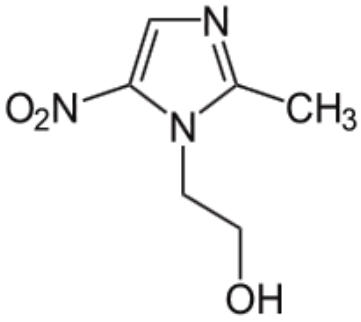


Fig No 1: Metronidazole

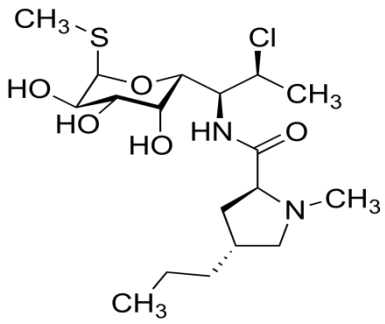


Fig No 2: Clindamycin

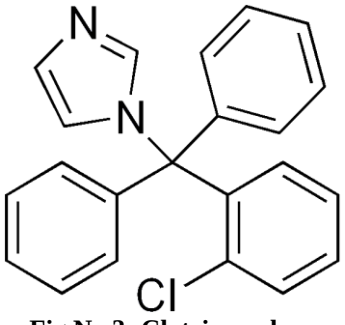
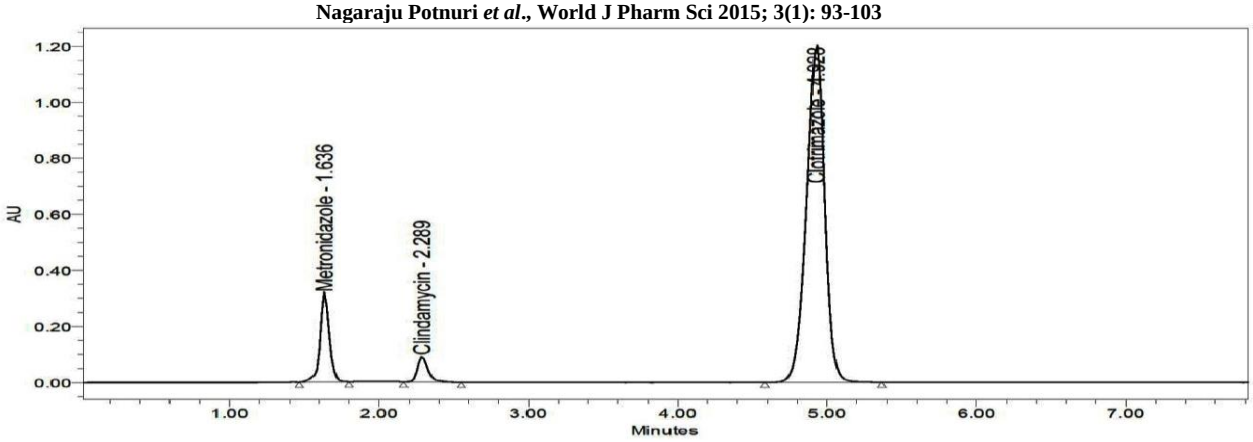


Fig No 3: Clotrimazole



	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing
1	Metronidazole	1.636	1370049	11.44	3605	0.95
2	Clindamycin	2.289	440189	3.68	5178	1.40
3	Clotrimazole	4.928	10166134	84.88	7880	0.93

Fig No 4: RP-HPLC Chromatogram of Metronidazole, Clindamycin and Clotrimazole

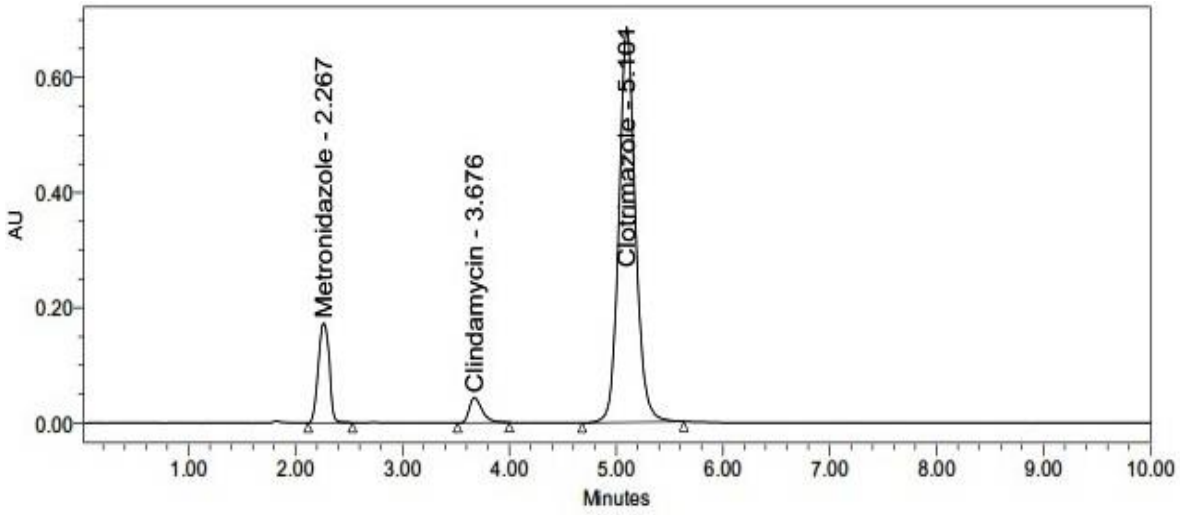


Fig No 5: RP-HPLC Chromatogram of Metronidazole, Clindamycin and Clotrimazole formulation

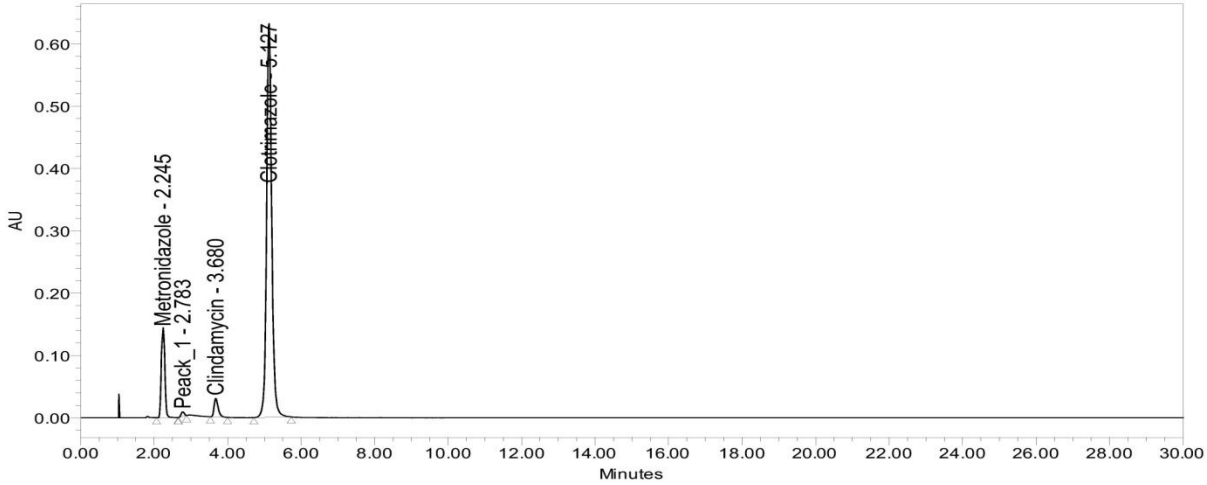


Fig No 6: Chromatogram of Metronidazole, Clindamycin and Clotrimazole for Acidic Degradation

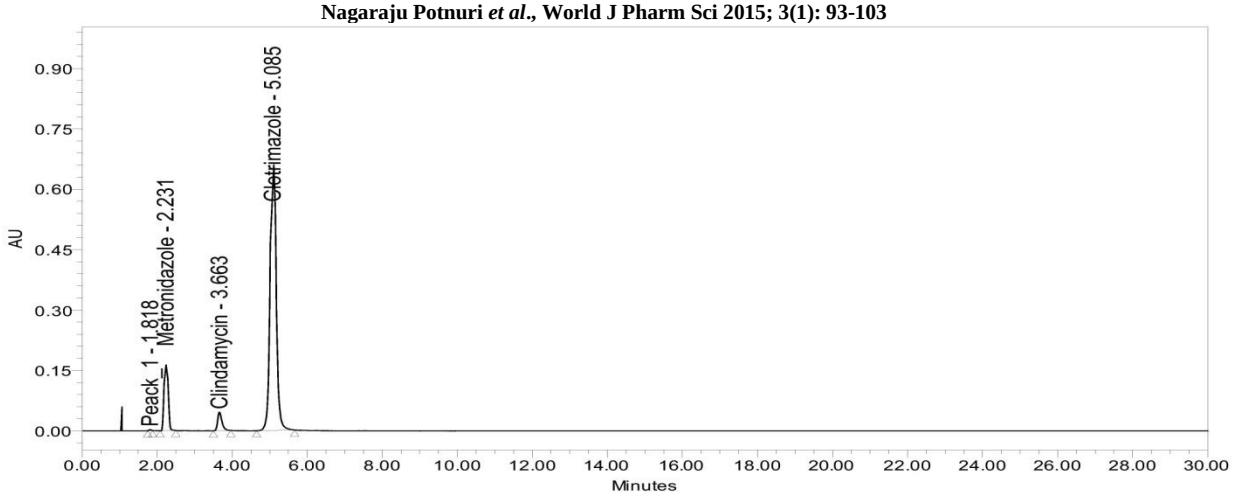


Fig No 7: Chromatogram of Metronidazole, Clindamycin and Clotrimazole for Alkali Degradation

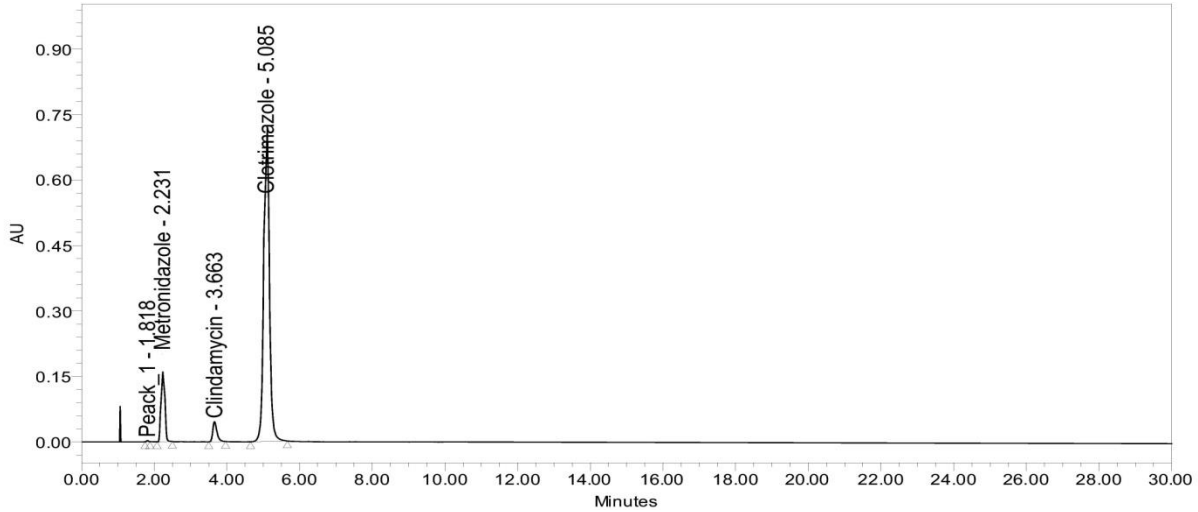


Fig No 8: Chromatogram of Metronidazole, Clindamycin and Clotrimazole for Oxidative Degradation

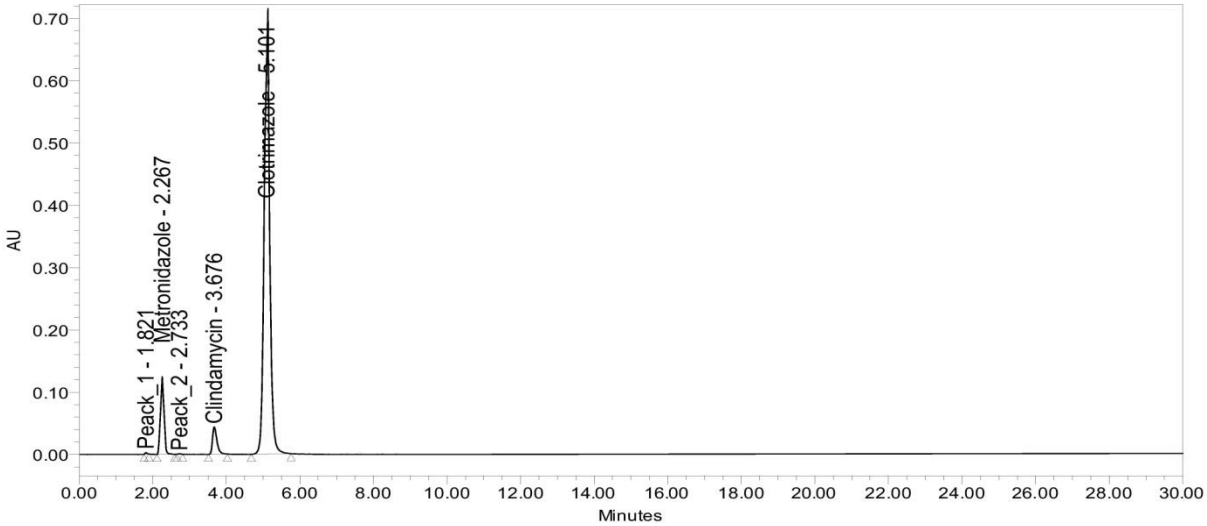


Fig No 9: Chromatogram of Metronidazole, Clindamycin and Clotrimazole for Thermal Degradation

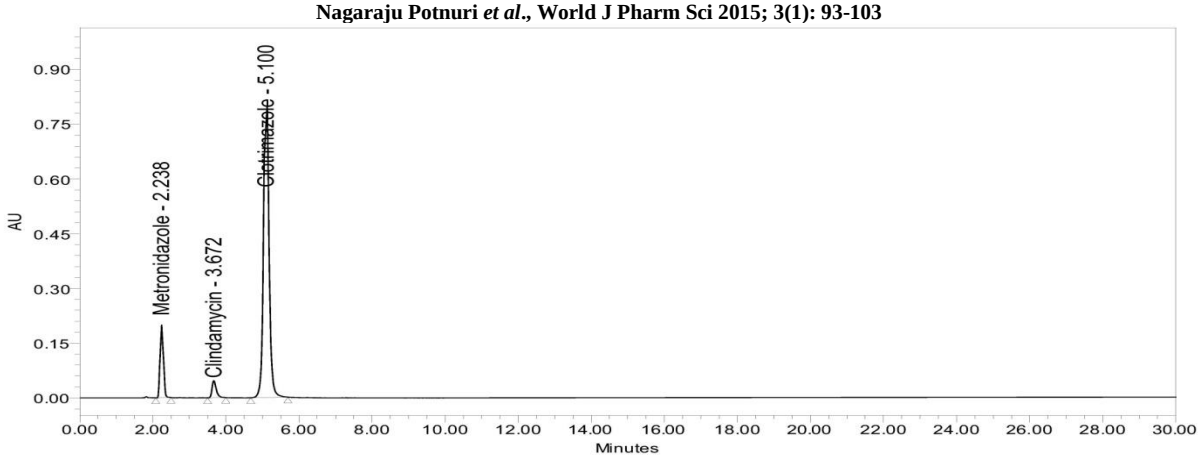


Fig No 10: Chromatogram of Metronidazole, Clindamycin and Clotrimazole for Photo Degradation

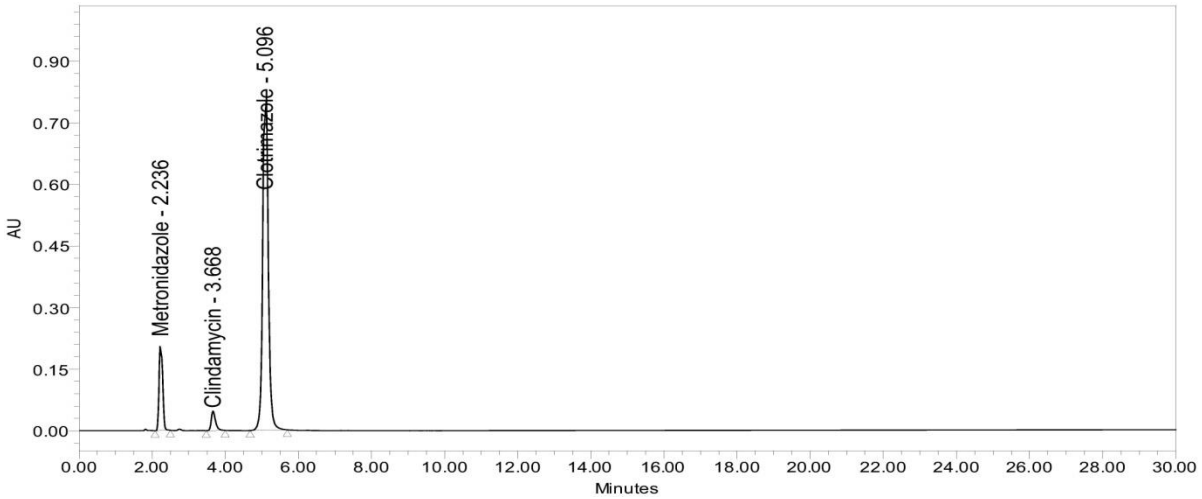
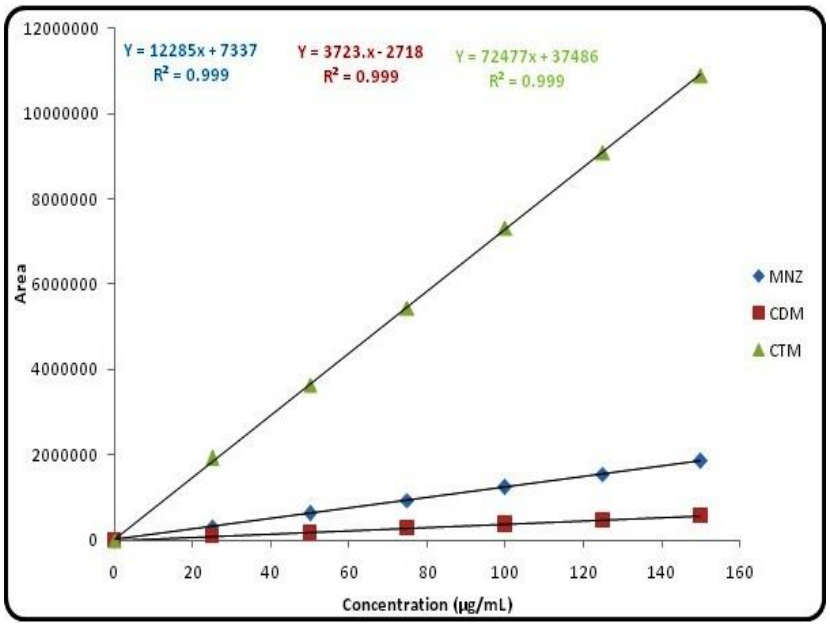


Fig No 11: Chromatogram of Metronidazole, Clindamycin and Clotrimazole for Neutral Degradation



Graph No 1:Linearity Graph of Metronidazole, Clindamycin & Clotrimazole

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