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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CLOTRIMAZOLE AND METRONIDAZOLE IN FORMULATION BY RP-HPLC METHOD

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ABSTRACT

A Reverse Phase High pressure liquid chromatographic method has been developed for simultaneous estimation of Mertonidazole and Clotrimaole in its Soft gelatin Capsule form. Acolumn C8-250 mm x4.6 mm, 5µm in isocratic mode, with mobile phase A 80 VOLUMES OF Buffer and 20 volumes of Methanol mixed well. The flow rate was 1.0ml per minute and effluent was monitored at 210 nm. The approximate retention time for Metronidazole and Clotrimazole were 7.16 min and 17.36 min respectively. The linearity for Metronidazole and Clotrimazole was in the range of 50% to 150% respectively. The proposed method is precise and rapid for simultaneous estimation of Metronidazole and clotrimazole in Soft gelatin Capsule formulation.

INTRODUCTION

Analytical chemistry is a branch of chemistry that deals with the separation, identification and determination of components in a sample. It is the science of making quantitative measurements, which requires background knowledge of chemical and physical concepts.

Analytical chemistry may be defined as the science and art of determining the composition of material in terms of elements or compounds contained in it.

Importance of analytical methods

The newly developed analytical methods having their importance in different fields

- Research & Development Centre
- Quality control Department
- Approved Testing Laboratories

- Chemical Analysis Laboratories

High performance liquid chromatography

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures up to 400 atmospheres. That makes it much faster.

It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing through it. This allows a much better separation of the components from the mixture.

The switch to the use of small particles requires much higher pressure instruments to pump mobile phase through a column. The later developed technique i.e., Ultra-high pressure liquid chromatography (UHPLC) uses the sub 2 μm particles which results in the system pressure exceeding 6,000 psi pressure limit, all the way up to 19,000 psi. However the introduction of fused core silica particles of sub 3 μm reduced the system pressure and with increased efficiency.

HPLC instruments consist of a reservoir of mobile phases, pumps, an injector, a separation column, a detector and a data control and processor. Solvents must be degassed to eliminate formation of bubbles. The pumps are provided with pulse dampers, so that to attain a steady high pressure with pulse free flow and can be programmed to vary the composition of the solvent during the course of the separation and deliver the desired flow and composition of the mobile phase through the column. The liquid sample is introduced into a sample loop of an injector with a syringe or an auto-sampler. The presence of analytes in the column effluent is detected by detectors and amplified into a signal by data processor. The various types of detectors used in HPLC are UV/visible detectors, refractive index detector, fluorescence detector, electrochemical detector, conductivity detector, light scattering detector. Spectrometric detectors are used in hyphenated HPLC methods such as LC/MS, LC/NMR, and LC/GC etc.

Modes of separation in liquid chromatography

Normal-Phase Chromatography

Normal-phase chromatography, or NP, is the classic form of liquid chromatography using

polar stationary phases and non-polar mobile phases. The analyte is retained by the interaction of its polar functional groups with the polar groups on the surface of the packing. Analytes elute from the column starting with the least polar compound followed by other compounds in order of their increasing polarity. Normal-phase chromatography is useful in the separation of analytes with low to intermediate polarity and high solubility in low-polarity solvents. Water-soluble analytes are usually not good candidates for normal-phase chromatography.

Reverse-Phase Chromatography

Reverse-phase chromatography, or RP, has become the most common mode of liquid chromatographic separation. In RP the stationary phase is non-polar and the mobile phase is polar. The analytes are attracted to the surface by their non-polar functional groups. The most polar analyte elutes from the RP column first followed by other analytes in order of decreasing polarity. RP chromatography is useful for the separation of compounds having high to intermediate polarity.

Metronidazole: 2-(2-Methyl-5-nitro-1H-imidazol-1-yl)ethanol, Molecular Formula :C6H9N3O3

Molecular weight: 171.15 g/mol,

Clotrimazole: 1-[(2-chlorophenyl) (diphenyl) methyl]-1H-imidazole, Molecular Formula: C22H17ClN2, Molecular Weight: 344.837

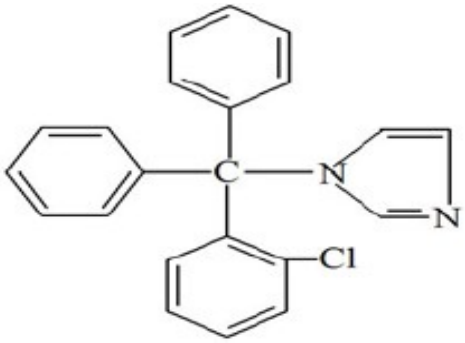

Figure 1: Structure of clotrimazole.

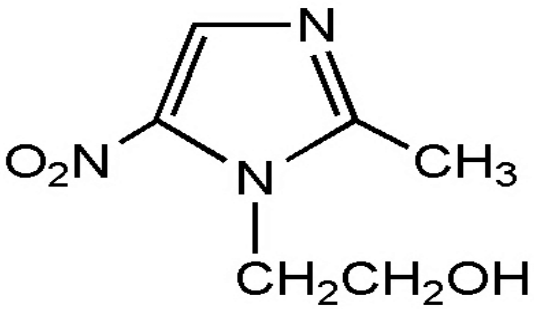

Figure 2: Structure of metronidazole.

Methods and Reagents

All The Chemicals and reagents used in the investigation were of analytical grade, which are Metronidazole Working Standard, Methanol obtained from Rankem Chemicals, Mumbai., Acetonitrile obtained from Rankem Chemicals, Mumbai, Potassium dihydrogen phosphate

obtained from Rankem Chemicals, Mumbai. Commercially available marketed dosage forms, Metronidazole and Clotrimazole Soft gelatin Capsules manufactured by Softgel Health Care pvt Ltd., it contains 500.0 mg of Metronidazole, 100.0 mg of Clotrimazole.

Instrument & Chromatographic conditions

Instrument: HPLC with UV/PDA detector, Column: C8, 250 X 4.6 mm, 5µm, Flow rate: 1.0 mL/min Column Temperature: 40°C, Injection Volume: 10µlWave length : 210nm, Run time: 30.0 minutes

Preparation of solutions

Preparation of Buffer solution: Weigh about 1.0 g of potassium dihydrogen phosphate and 0.5 g of tetrabutylammonium hydrogen sulfate in a 1000 mL volumetric flask, dissolve, sonicate for 5 minutes and make up with water.

Mobile phase A: Mix 80 volumes of Buffer and 20 volumes of Methanol**.**

Mobile Phase B: Mix 50 volumes of buffer and 50 volumes of Acetonitrile.

Preparation of diluent solution: Mix 50 volumes of Buffer, 25 volumes of Methanol and 25 volumes of Acetonitrile.

Preparation of standard solution: (Metronidazole-600 ppm, Clotrimazole-120 ppm)

Solution A: Weigh accurately 60 mg of Clotrimazole WS/RS into a 100 mL volumetric flask, dissolve in diluent and make up to 100 mL with diluent.

Solution B: Weigh accurately 30 mg of Metronidazole WS/RS into a 50 mL volumetric flask, add 10 mL of Solution A, dissolve and make up the volume with diluent.

Preparation of sample solution: (Metronidazole-600 ppm, Clotrimazole-120 ppm)

Transfer 3 capsules into a 250 mL volumetric flask, add 70 mL diluent, sonicate for 15 minutes and make up the volume with diluent. Filtered through 0.45µ filter. Pipette out 5 mL of above solution in to 50 mL volumetric flask and make up the volume with diluent.

Method validation

The HPLC method's validation was done for the simultaneous estimation of Metronidazole and clotrimazole in capsule in accordance with ICH criteria.

Optimized method validation parameters performed:

System suitability

Standard Solution of Metronidazole and Clotrimazole is prepared and injected in six replicates into HPLC System. The system suitability parameters were calculated as below.

Table 1: System suitability results.

S. No	Metronidazole			Clotrimazole		
INJ	RT	NTP	TF	RT	NTP	TF
01	7.881	1.156	77069	17.320	0.884	502654
02	7.881	1.158	77040	17.309	0.884	500779
03	7.881	1.158	77072	17.315	0.884	502912
04	7.881	1.158	77062	17.314	0.884	502288
05	7.882	1.157	77053	17.315	0.884	501614
06	7.880	1.157	76902	17.311	0.884	503013

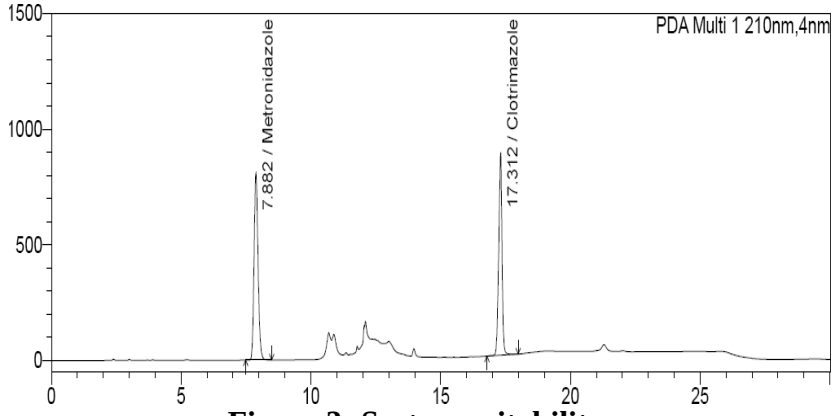

Figure 3: System suitability.

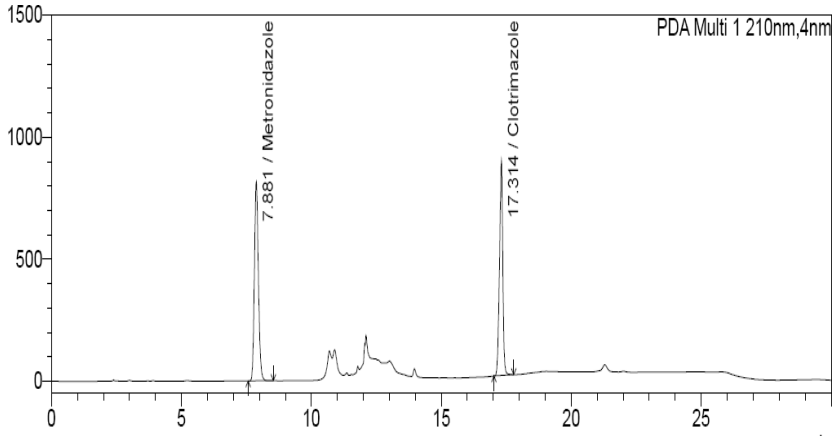

Figure 4: Specificity.

Table 2: Forced degradation.

Stressed conditions	Metronidazole			Clotrimazole		
	%Content	% Degradation	Peak purity index	% Content	% Degradation	Peak purity index
Unstressed Samples	99.7750	NA	0.999	98.8184	NA	0.999
Acid Hydrolysis (0.1M HCl) reflux for 10 minutes at 60°C Sample	93.3558	6.4192	0.999	90.1152	8.7032	0.999
Base Hydrolysis (0.1M NaOH) reflux for 10 minutes at 60°C Sample	92.7174	7.0576	0.999	89.2254	9.593	0.999
Oxidation reflux (1%H2O2) for 10 minutes at 60°C Sample	93.3595	6.4155	0.999	89.9599	8.8585	0.999
Water Hydrolysis reflux for 10 minutes at 60°C Sample	92.9501	6.8249	0.999	89.3329	9.4855	0.999
Exposed to heat for a period of 2 hours at 105^0C Sample	92.4644	7.3106	0.999	88.8903	9.9281	0.999
Exposed to humidity i.e. 90% RH and 25°C in a desiccator for 7 days Sample	93.3529	6.4221	0.999	89.2846	9.5338	0.999

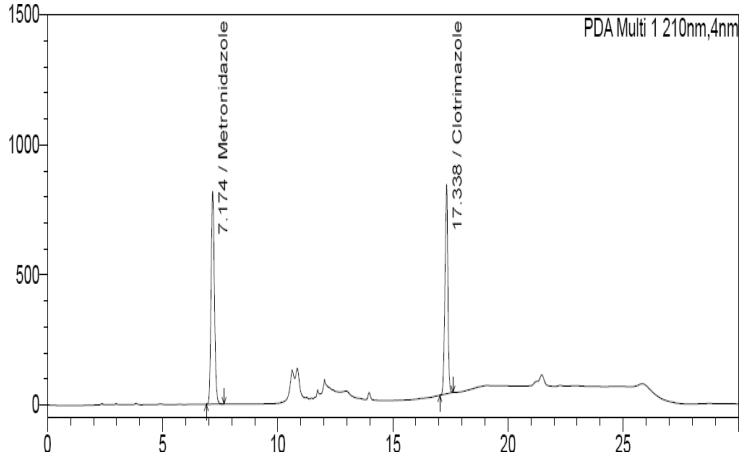

Figure 5: Acid hydrolysis.

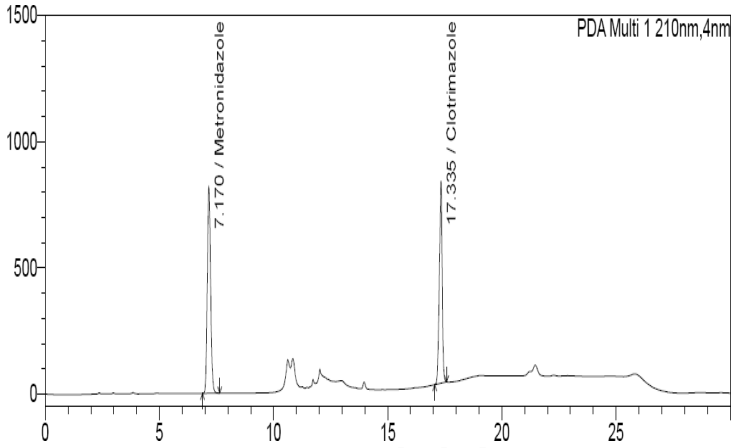

Figure 6: Base hydrolysis.

Figure 7: Oxidation.

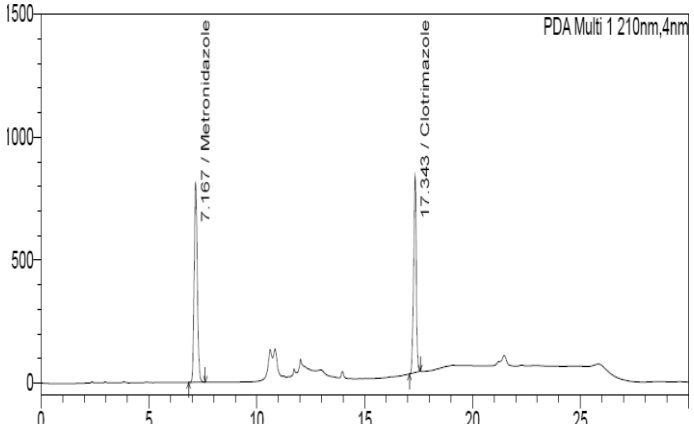

Figure 8: Water.

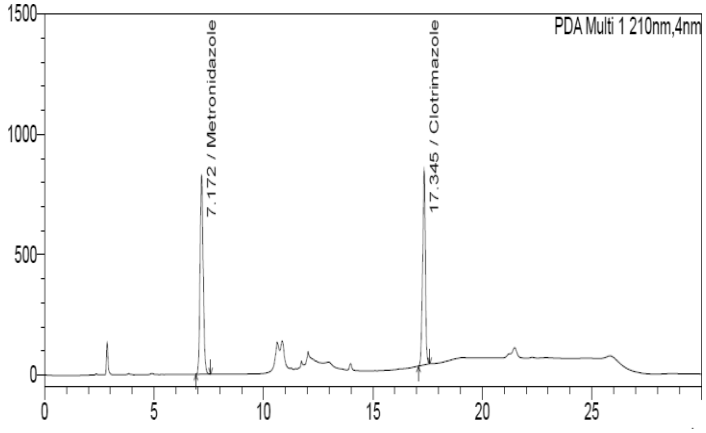

Figure 9: Heat.

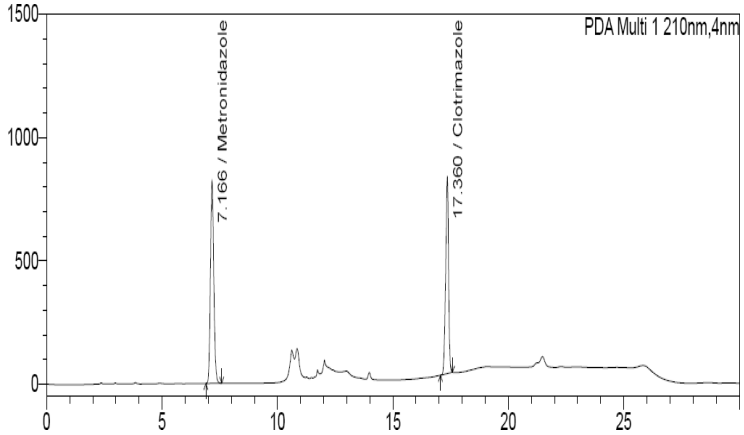

Figure 10: Humidity.

Linearity

The linearity of the method is established by performing 5 test concentrations from 50.0% to 150% of working concentrations as per protocol. The standard solutions were prepared with the concentrations of 50%, 75%, 100%, 125% and 150% with respect to 100% working concentration. For each concentration 3 replicates and injections are given into HPLC system. Based on the average area obtained with each concentration, a graph is plotted between Area and Concentration.

Table 3: Metronidazole and Clotrimazole linearity.

Concn. in %	Peak area	
	Metronidazole	Clotrimazole
50	4324454	4346161
75	6489275	6415737
100	8938297	8590273
125	10714150	10177294
150	13281583	12350404

Linearity curve of metronidazole

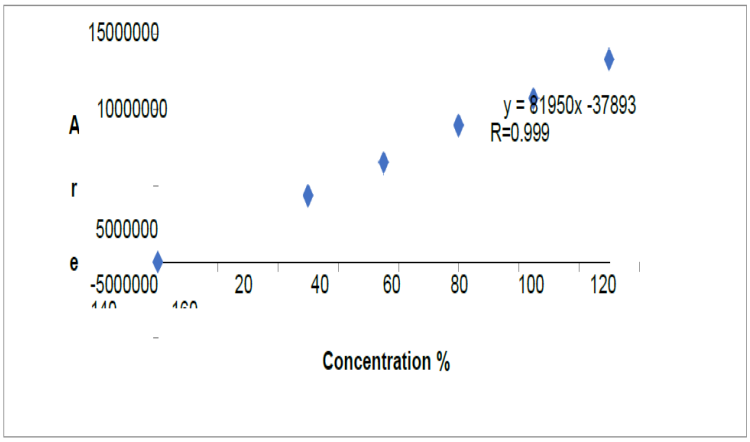

Figure 11

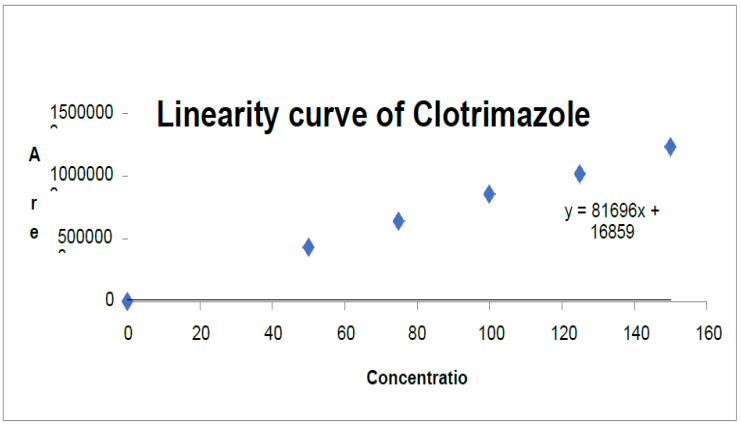

Figure 12

Table 4: Recovery data.

% Level	% Recovery			
	Metronidazole		Clotrimazole	
	Amount found	% Recovery	Amount found	% Recovery
50%	292.1181	98.8756	61.5365	99.3166
	292.7923	98.7295	62.6493	99.1914
	292.2244	98.9115	62.6205	100.6761
100%	592.7211	98.0611	123.5340	98.9222
	582.1416	98.4944	119.6614	99.3205
	585.0737	98.1008	121.4394	99.0856
150%	879.4879	99.5842	176.7472	98.7414
	887.4421	98.2509	177.5172	98.3583
	872.5532	98.4245	175.0479	99.2560
Mean%		98.6		99.2

Method precision

Repeatability of solution is demonstrated as per protocol by preparing in 6 replicates the sample from the homogeneous sample of the product "Metronidazole 500mg and

Clotrimazole 100mg Capsules". The Assay values of 6 replicate preparations and % RSD shows that the method is precise and repeatable.

Intermediate precision

Intermediate precision is demonstrated as per protocol by preparing in 6 replicates the sample (Which is used by Analyst-1) of the product "Metronidazole 500mg and Clotrimazole 100mg Vaginal Capsules" by a different Analyst, using a different Instrument, Column and on a different day.

Table 5: Metronidazole precision data.

Parameter	Method precision	Intermediate precision
Content	Metronidazole	Metronidazole
Preparation	% assay	% assay
1	100.9331	101.3734
2	100.7170	101.5273
3	101.9946	103.8453
4	101.2770	101.3468
5	100.8926	102.0165
6	101.1960	101.5090
Average	101.2	101.9
%rsd	0.4	0.9
Confidence limit	0.4	0.8
Overall %rsd	0.8	

Table 6: Clotimazole precision data.

Parameter	Method precision	Intermediate precision
Content	Clotrimazole	Clotrimazole
Preparation	% assay	% assay
1	98.3687	98.2280
2	97.6465	98.1720
3	98.5773	100.4662
4	98.1843	98.0855
5	97.1051	98.6958
6	98.2895	98.0636
Average	98.0	98.6
%rsd	0.6	0.9
Confidence limit	0.4	0.8
Overall %rsd	0.8	

Table 7: Robustness results.

Chromatographic condition	Metronidazole (%RSD)	Clotrimazole (%RSD)
Flow	1.1	0.7
Temp	0.9	0.5
Wavelength	0.0	0.0

Stability of analytical solutions

The solution stability is demonstrated by injecting standard and sample solution for every 12 hours interval up to 48 hours. The % RSD of area of standard and sample solutions are calculated as shown below.

Table 8.

Time Intervals	Metronidazole		Clotrimazole	
	Standard	Sample	Standard	Sample
Initial	9037138	8734516	8339412	7794610
12 Hour	9123581	8676629	8635722	7699131
24 Hour	9310331	8715683	8638871	7679025
36 Hour	9407915	8776900	8672973	7678854
48 Hour	9418095	8898309	8639044	7743497
Average	9259412	8760407	8585204	7719023
Std dev	171524	85115	138246	49803
RSD (%)	1.9	1.0	1.6	0.6

CONCLUSION

An exact accurate, and long lasting reverse phase RP-HPLC technique has been developed and validated for the simultaneous quantification of Metronidazole and Clotrimazole in formulation. The other active ingredients and common excipients present in the dosage forms of Metronidazole and Clotrimazole did not interfere, when added in the mentioned concentration ranges to the drug and estimated by the proposed methods. The methods reported here are found to be simple, sensitive, accurate and economical can be used in the determination of Metronidazole and Clotrimazole from pharmaceutical formulation in a routine manner.

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