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SIMULTANEOUS ESTIMATION OF NEW ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DAPALIFLOZIN AND VILDAGLIPTIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract:

A simple, rapid, accurate, precise, and economical Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Azithromycin and Metronidazole in pharmaceutical dosage forms. The chromatographic separation was achieved using a suitable C18 column with an optimized mobile phase composition under isocratic conditions. Detection was carried out at an appropriate wavelength, providing satisfactory sensitivity for both analytes. The developed method resulted in well-resolved peaks with acceptable retention times, good peak symmetry, and satisfactory system suitability parameters. The method was validated according to International Council for Harmonisation guidelines for specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), limit of quantification (LOQ), and system suitability. The calibration curves for both Azithromycin and Metronidazole showed good linearity within the selected concentration ranges, with correlation coefficients close to 1. Accuracy studies demonstrated satisfactory percentage recoveries, while precision studies showed low percentage relative standard deviation (%RSD), indicating the reliability and reproducibility of the method. The developed method was found to be specific, sensitive, and unaffected by common pharmaceutical excipients. The validated RP-HPLC method was successfully applied for the simultaneous quantitative estimation of Azithromycin and Metronidazole in pharmaceutical formulations. The results confirmed that the method is suitable for routine quality control analysis and can be effectively employed in pharmaceutical industries and analytical laboratories for the assay of these drugs.

Keywords: *Azithromycin, Metronidazole, RP-HPLC, Method Development, Method Validation, Simultaneous Estimation, Pharmaceutical Dosage Forms, Quantitative Analysis, ICH Guidelines, Quality Control Analysis*

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INTRODUCTION:

Analytical chemistry1 is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparation.

Measurements of physical properties of analytes such as conductivity, electrode potential, light absorption or emission, mass to charge ratio, and fluorescence, began to be used for quantitative analysis of variety of inorganic and biochemical analytes. Highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior to their qualitative or quantitative determination. These newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis. Most of the instrumental methods fit into one of the three following categories viz spectroscopy, electrochemistry and chromatography

Advantages of instrumental methods

- Small samples can be used
- High sensitivity is obtained
- Measurements obtained are reliable
- Determination is very fast
- Even complex samples can be handled easily

Limitations of instrumental methods

- An initial or continuous calibration is required
- Sensitivity and accuracy depend on the instrument
- Cost of equipment is large
- Concentration range is limited
- Specialized training is needed
- Sizable space is required

Principle types of chemical instrumentation:

Spectrometric techniques

1. Ultraviolet and visible Spectrophotometry
2. Fluorescence and phosphorescence Spectrophotometry.
3. Atomic Spectrometry (emission and absorption)
4. Infrared Spectrophotometry
5. Raman Spectroscopy
6. X-Ray Spectroscopy
7. Radiochemical Techniques including activation analysis
8. Nuclear Magnetic Resonance Spectroscopy
9. Electron Spin Resonance Spectroscopy

Electrochemical techniques

1. Potentiometry
2. Voltametry
3. Voltametric Techniques
4. Amperometric Techniques
5. Colorimetry
6. Electrogravimetry
7. Conductance Techniques

Chromatographic techniques

1. Gas Chromatography
2. High Performance Liquid Chromatography
3. High Performance Thin Layer Chromatography

Miscellaneous techniques

1. Thermal Analysis
2. Mass Spectrometry
3. Kinetic Techniques
4. Hyphenated techniques
5. GC-MS (Gas Chromatography – Mass Spectrometry)
6. GC-IR (Gas Chromatography – Infrared Spectroscopy)
7. MS-MS (Mass Spectrometry – Mass Spectrometry)

EXPERIMENTAL METHODS

INSTRUMENTS USED

Table-: Instruments used

S.No.	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Lab man

CHEMICALS USED:

Table: chemicals used

S.No.	Chemical	Brand names
1	Azithromycin	Sura labs
2	Metronidazole	Sura labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT:

TRAILS

Preparation of standard solution:
Accurately weigh and transfer 10 mg of Azithromycin and Metronidazole working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.
Further pipette 2.25ml of the above Azithromycin and 0.45ml of the Metronidazole stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:
Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:
Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: TEA buffer pH 4.8 in proportion 32:68 v/v respectively.
Optimization of Column:
The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Gemini C18 (4.6mm×150mm, 5.0 µm) particle size was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:
Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.
Column : Phenomenex Gemini C18 (4.6mm×150mm, 5.0 µm) particle size
Column temperature : 38˚C
pH : 4.8
Mobile phase : Methanol: TEA buffer pH 4.8 (32:68v/v)
Flow rate : 1ml/min
Wavelength : 248nm
Injection volume : 20µl
Run time : 7 min

METHOD VALIDATION

PREPARATION OF MOBILE PHASE:

Preparation of mobile phase:
Accurately measured 320ml (32%) of HPLC Methanol and 680ml of TEA buffer (68%) were mixed and degassed in a digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION:
(Optimized Chromatogram):
Column : Phenomenex Gemini C18 (4.6mm×150mm, 5.0 µm) particle size
Column temperature : 38˚C
Wavelength : 248nm
Mobile phase ratio : Methanol: TEA buffer pH 4.8 (32:68v/v)
Flow rate : 1ml/min
Injection volume : 20µl
Run time : 7minutes

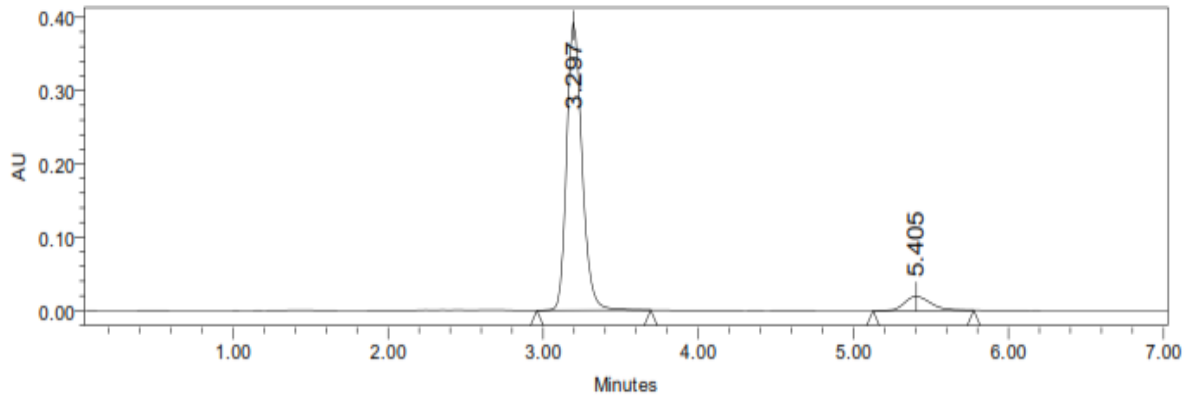

Figure-: Optimized Chromatogram (Standard)

Table-: Optimized Chromatogram (Standard)

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Azithromycin	3.297	859856	42569	1.24	7896	
2	Metronidazole	5.405	5698	3652	1.36	6582	6.8

Observation: From the above chromatogram it was observed that the Azithromycin and Metronidazole peaks are well separated and they show proper retention time, resolution, peak tail and plate count. So, it's optimized trial.

Optimized Chromatogram (Sample)

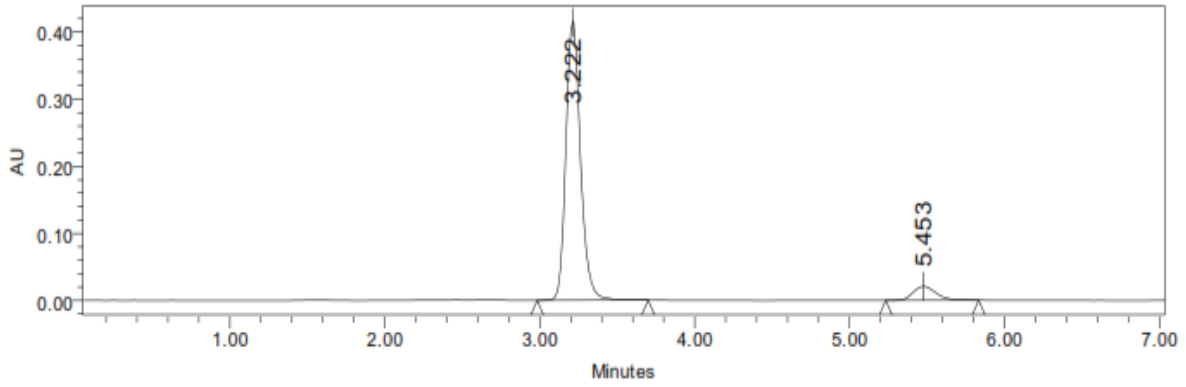

Figure-: Optimized Chromatogram (Sample)

Table-: Optimized Chromatogram (Sample)

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Azithromycin	3.222	865898	43659	1.26	7985	
2	Metronidazole	5.453	5789	3785	1.38	6659	7.0

Acceptance Criteria: Azithromycin and Metronidazole

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

METHOD VALIDATION

Blank:

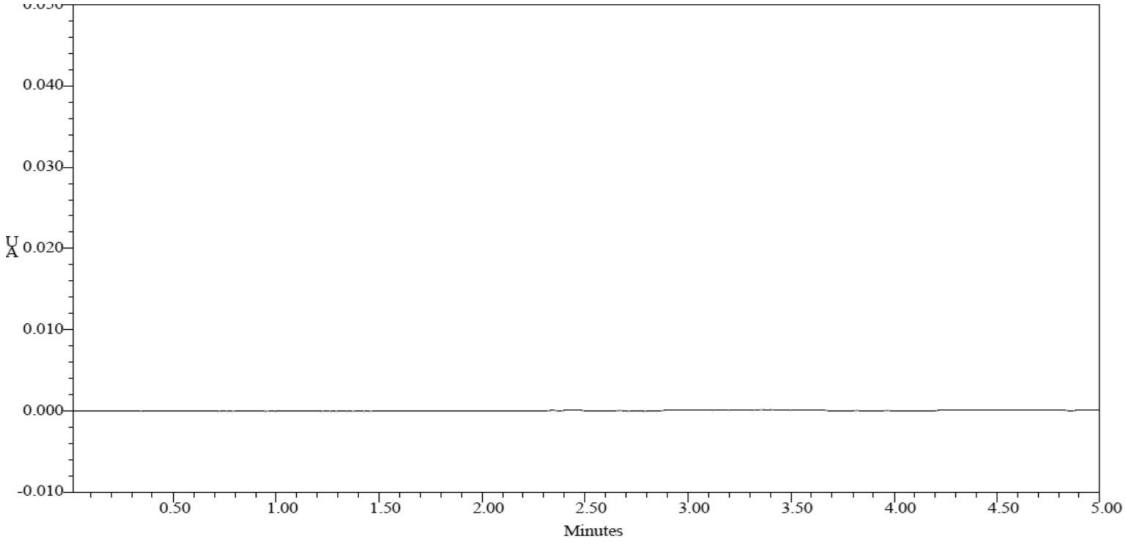

Fig: Chromatogram showing blank (mobile phase preparation)

System Suitability:

Table-: Results of system Suitability for Azithromycin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Azithromycin	3.200	859865	42568	7895	1.24
2	Azithromycin	3.248	859788	42587	7859	1.24
3	Azithromycin	3.299	857984	42659	7869	1.24
4	Azithromycin	3.297	854879	42875	7849	1.24
5	Azithromycin	3.297	857896	42487	7859	1.23
Mean			858082.4			
Std. Dev.			2024.409			
% RSD			0.235922			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Table-: Results of System Suitability for Metronidazole

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Metronidazole	5.413	5689	3659	6583	1.36
2	Metronidazole	5.484	5687	3648	6592	1.37
3	Metronidazole	5.405	5682	3698	6549	1.37
4	Metronidazole	5.405	5649	3675	6571	1.36
5	Metronidazole	5.409	5674	3649	6529	1.36
Mean			5676.2			
Std. Dev.			16.2696			
% RSD			0.286628			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitate Azithromycin and Metronidazole in drug product.

Assay (Standard):

Table-: Peak Results for Assay Standard

Azithromycin

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Azithromycin	3.211	859785	42598	1.25	7856
2	Azithromycin	3.222	859865	42895	1.24	7859
3	Azithromycin	3.254	857849	42578	1.25	7869

Metronidazole

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Metronidazole	5.414	5699	3685	1.36	6598	6.9
2	Metronidazole	5.453	5687	3659	1.37	6537	6.9
3	Metronidazole	5.424	5689	3649	1.36	6582	7.0

Assay (Sample):

Table-: Peak Results for Assay sample

Azithromycin

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Azithromycin	3.297	865985	43659	1.26	7985
2	Azithromycin	3.294	865798	43875	1.26	7925
3	Azithromycin	3.295	865456	43659	1.27	7946

Metronidazole

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Metronidazole	5.435	5789	3659	1.37	6659	6.9
2	Metronidazole	5.417	5798	3684	1.38	6689	7.0
3	Metronidazole	5.434	5749	3695	1.38	6648	6.9

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Azithromycin and Metronidazole in pharmaceutical dosage form was found to be 99.82%.

LINEARITY

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:

Azithromycin

Concentration µg/ml	Average Peak Area
20	164436
30	255571
40	348687
50	439024
60	534830

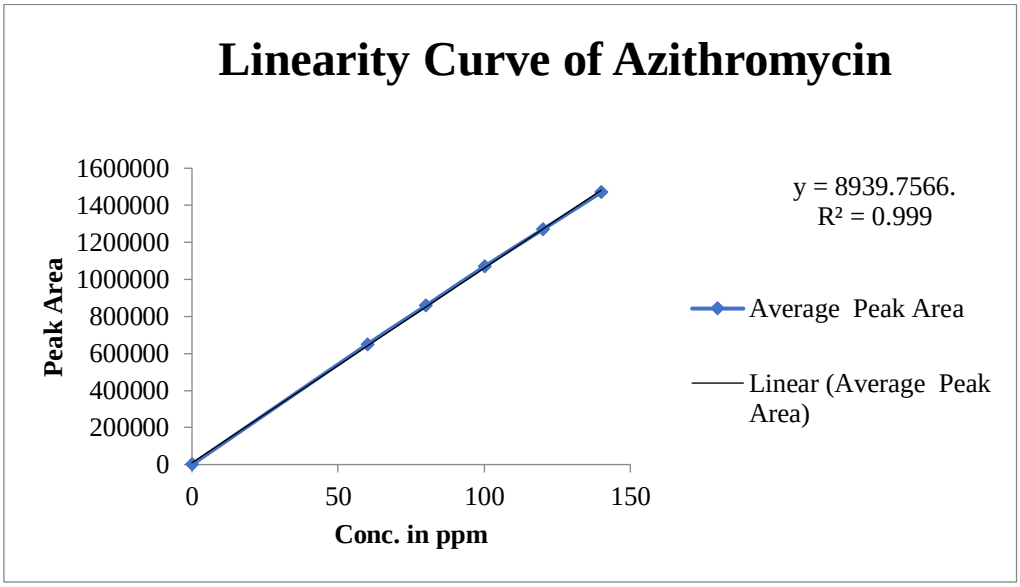

Fig-: Calibration Curve of Azithromycin

LINEARITY PLOT:
The plot of Concentration (x) versus the Average Peak Area (y) data of Azithromycin is a straight line.

Y = mx + c
Slope (m) = 8939
Intercept (c) = 9566
Correlation Coefficient (r) = 0.999

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 7566. These values meet the validation criteria.

Metronidazole

Concentration µg/ml	Average Peak Area
25	1782454
37.5	2728974
50	3688678
62.5	4658022
75	5592695

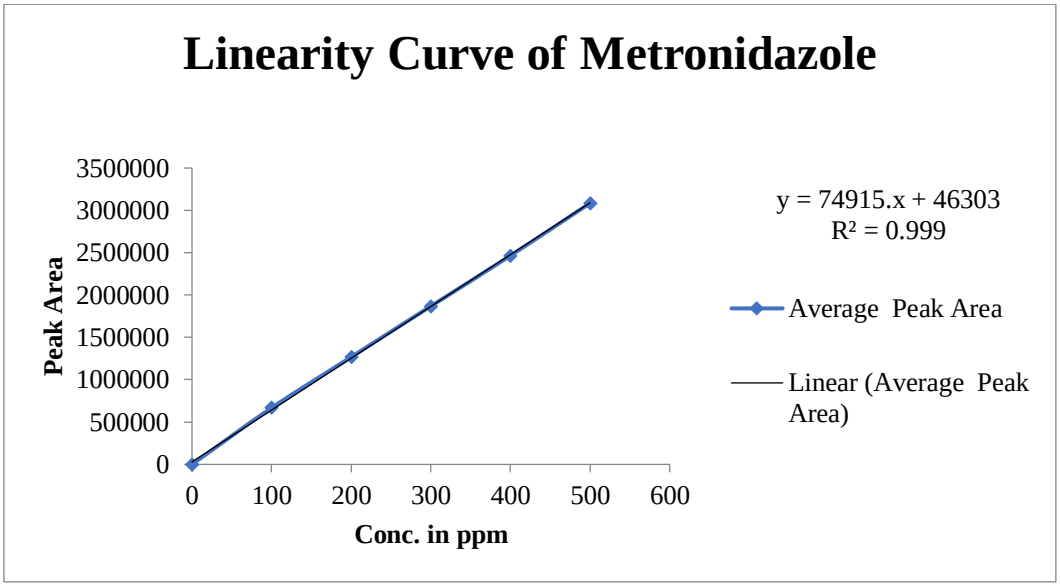

Fig-: Calibration Curve of Metronidazole

LINEARITY PLOT:
The plot of Concentration (x) versus the Average Peak Area (y) data of Metronidazole is a straight line.

Y = mx + c
Slope (m) = 74915
Intercept (c) = 46303
Correlation Coefficient (r) = 0.999

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 46303. These values meet the validation criteria.

PRECISION:
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

REPEATABILITY
Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table-: Results of Repeatability for Azithromycin:

S. No.	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Azithromycin	3.213	859856	42659	7859	1.24
2	Azithromycin	3.253	857985	42598	7869	1.24
3	Azithromycin	3.297	856984	42587	7846	1.25
4	Azithromycin	3.215	856987	42569	7819	1.25
5	Azithromycin	3.254	859878	42894	7856	1.24
Mean			858338			
Std.dev			1454.222			
%RSD			0.169423			

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table-: Results of repeatability for Metronidazole:

S. No.	Peak Name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Metronidazole	5.441	5697	3659	6592	1.36
2	Metronidazole	5.442	5689	3648	6539	1.36
3	Metronidazole	5.409	5698	3692	6584	1.37
4	Metronidazole	5.520	5639	3648	6579	1.36
5	Metronidazole	5.424	5688	3689	6549	1.36
Mean			5682.2			
Std.dev			24.57031			
%RSD			0.432408			

Intermediate precision:

Day 1:

Table-: Results of Intermediate precision for Azithromycin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Azithromycin	3.211	868956	43659	7985	1.26
2	Azithromycin	3.211	869857	43985	7954	1.27
3	Azithromycin	3.210	865983	43879	7946	1.26
4	Azithromycin	3.212	866587	43865	7963	1.27
5	Azithromycin	3.211	864256	43875	7964	1.26
6	Azithromycin	3.297	868974	43562	7942	1.26
Mean			867435.5			
Std. Dev.			2167.095			
% RSD			0.249828			

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2.

Table-: Results of Intermediate precision for Metronidazole

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Metronidazole	5.411	5785	3789	6659	1.37
2	Metronidazole	5.410	5798	3758	6625	1.38
3	Metronidazole	5.420	5766	3746	6649	1.38
4	Metronidazole	5.423	5746	3795	6675	1.37
5	Metronidazole	5.419	5782	3761	6653	1.38
6	Metronidazole	5.409	5786	3752	6627	1.37
Mean			5777.167			
Std. Dev.			18.40018			
% RSD			0.318498			

Acceptance Criteria:

- %RSD of six different sample solutions should not more than 2.

Day 2:

Table-: Results of Intermediate precision Day 2 for Azithromycin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Azithromycin	3.211	845985	44585	8025	1.27
2	Azithromycin	3.233	847895	44895	8069	1.28
3	Azithromycin	3.244	848985	44758	8046	1.27
4	Azithromycin	3.297	847859	44548	8094	1.28
5	Azithromycin	3.297	845984	44865	8042	1.28
6	Azithromycin	3.202	847898	44254	8076	1.27
Mean			847434.3			
Std. Dev.			1201.345			
% RSD			0.141763			

Acceptance Criteria:

- %RSD of six different sample solutions should not more than 2.

Table-: Results of Intermediate precision Day 2 for Metronidazole

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Metronidazole	5.411	5898	3986	6852	1.39
2	Metronidazole	5.410	5884	3955	6864	1.39
3	Metronidazole	5.420	5863	3956	6829	1.40
4	Metronidazole	5.405	5845	3945	6874	1.39
5	Metronidazole	5.409	5896	3925	6829	1.39
6	Metronidazole	5.463	5874	3962	6825	1.40
Mean			5876.667			
Std. Dev.			20.39281			
% RSD			0.347013			

Acceptance Criteria:

- %RSD of six different sample solutions should not more than 2.

6.3.4: ACCURACY:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

Table-: The accuracy results for Azithromycin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	451144.3	25	24.998	99.992%	100.1873%
100%	897248.3	50	50.104	100.208%	
150%	1344562	75	75.278	100.362%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table-: The accuracy Results for Metronidazole

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	2895	15	15.084	100.560%	100.748%
100%	5685.333	30	30.282	100.940%	
150%	8449	45	45.335	100.744%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

LIMIT OF DETECTION

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$LOD = 3.3 \times \sigma / s$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Azithromycin:

Result:

=2.6µg/ml

Metronidazole:

Result:

=3.4µg/ml

Quantitation limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Azithromycin:

Result:

=7.8µg/ml

Metronidazole:

Result:

=10.2µg/ml

ROBUSTNESS

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Azithromycin and Metronidazole. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard sample of Azithromycin and Metronidazole were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor and plate count.

Variation in flow

Table-: Results for Robustness

Azithromycin

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0mL/min	859856	3.297	7896	1.24
Less Flow rate of 0.9mL/min	915847	3.639	7251	1.20
More Flow rate of 1.1mL/min	842564	2.859	7415	1.21
Less organic phase (about 5 % decrease in organic phase)	825498	3.460	7365	1.23
More organic phase (about 5 % Increase in organic phase)	814578	3.022	7258	1.22

Acceptance Criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Table-: Results for Robustness

Metronidazole

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1mL/min	5698	5.405	6582	1.36
Less Flow rate of 0.9mL/min	6452	6.250	6785	1.32
More Flow rate of 0.8mL/min	5254	4.863	6365	1.34
Less organic phase (about 5 % decrease in organic phase)	5487	6.196	6254	1.38
More organic phase (about 5 % Increase in organic phase)	5369	5.010	6298	1.33

Acceptance Criteria:
The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

SUMMARY AND CONCLUSION:

Summary
The present study was undertaken to develop and validate a simple, rapid, precise, accurate, and cost-effective RP-HPLC method for the simultaneous estimation of Azithromycin and Metronidazole in pharmaceutical dosage forms. Appropriate chromatographic conditions were optimized to achieve satisfactory separation of both drugs with well-resolved peaks, acceptable retention times, and good peak symmetry. The developed method was validated according to International Council for Harmonisation guidelines. Validation parameters including specificity, linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD), limit of quantification (LOQ), and system suitability were evaluated. The method exhibited excellent linearity within the selected concentration ranges for both Azithromycin and Metronidazole, with correlation coefficients close to unity. Accuracy studies demonstrated satisfactory recovery values, indicating the reliability of the method. Precision studies showed low percentage relative standard deviation (%RSD), confirming good repeatability and reproducibility. The developed RP-HPLC method was successfully applied to the analysis of pharmaceutical formulations containing Azithromycin and Metronidazole without interference from excipients. The results obtained confirmed the suitability of the method for routine quality control analysis.

Conclusion:
A simple, accurate, precise, specific, and robust RP-HPLC method was successfully developed and validated for the simultaneous estimation of Azithromycin and Metronidazole in pharmaceutical dosage forms. The method provided efficient separation of both drugs with satisfactory chromatographic performance and acceptable validation results as per ICH guidelines. The validation studies confirmed that the method is linear, accurate, precise, sensitive, and reproducible. The absence of interference from formulation excipients demonstrated its specificity, while robustness studies indicated that minor variations in analytical conditions did not significantly affect the results. Therefore, the developed RP-HPLC method can be reliably employed for routine quality control, assay determination, and quality assessment of pharmaceutical formulations containing Azithromycin and Metronidazole. Its simplicity, accuracy, and cost-effectiveness make it suitable for use in pharmaceutical industries and analytical laboratories.

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