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Comparative Study for the Simultaneous Estimation of Azithromycin Dihydrate and Metronidazole Benzoate using analytical techniques

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ABSTRACT

Azithromycin Dihydrate and Metronidazole Benzoate have been reported as an effective and safe combination therapy for the management of Crohn's disease. The present study was undertaken to develop and validate simple, precise, accurate, analytical methods using first order derivative UV spectrophotometry and RP-HPLC for the simultaneous estimation of Azithromycin Dihydrate and Metronidazole Benzoate in synthetic mixture. For the UV spectrophotometric method, Methanol was used as the solvent for analysis. The first order derivative method was selected using 226 nm and 289 nm wavelengths for measurement of Azithromycin Dihydrate and Metronidazole Benzoate, respectively. The zero-crossing point (ZCP) of Azithromycin dihydrate was found to be 289 nm and 226 nm for Metronidazole benzoate. Chromatographic separation was carried out under isocratic conditions using a C_{18} column. The optimized mobile phase consisted of Phosphate Buffer (pH 4 adjusted with 10% ortho phosphoric acid): Acetonitrile: Tetrahydrofuran (50:40:10 %v /v /v) to achieve well-resolved and symmetrical peaks of both analytes. The flow rate was maintained at 1.0 mL/min, and detection was performed at 225 nm wavelength. The linearity of Azithromycin dihydrate and Metronidazole benzoate for both methods were established in the range of 7.5-37.5 µg/ml and 20-100 µg/ml, respectively. The developed methods were validated for parameters including specificity, linearity, range, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), assay, system suitability. All validation results were found to be within the acceptable limits specified by ICH guideline Q2 (R2). All validation results were found to be within acceptable limits. Statistical comparison using Student's t-test indicated no significant difference between the UV and RP-HPLC methods with respect to recovery and assay results. The proposed methods were simple, sensitive, precise, accurate, and reproducible, and were suitable for routine quality control analysis and quantitative determination of Azithromycin Dihydrate and Metronidazole Benzoate in synthetic mixture.

1. INTRODUCTION:

Crohn's disease is a chronic, relapsing inflammatory disorder of the gastrointestinal tract characterized by transmural inflammation and microbial dysbiosis, leading to significant morbidity and reduced quality of life. Increasing evidence implicates altered intestinal microbiota in disease pathogenesis, supporting the use of antimicrobial agents as adjuncts to conventional immunosuppressive therapy [1]. Combination antibiotic regimens, such as azithromycin and

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metronidazole, have shown therapeutic potential in selected patient subgroups by targeting mixed aerobic and anaerobic bacterial populations [2, 3]. The combination of Azithromycin Dihydrate and Metronidazole Benzoate is particularly effective in patients with colonic or ileocolonic Crohn's disease, perianal involvement, and mild to moderate active disease. This regimen is also beneficial in postoperative patients and those with recurrent flares linked to microbial dysbiosis, where broad aerobic and anaerobic coverage enhances disease control and remission rates [4]. The combination administration of azithromycin dihydrate and metronidazole benzoate has been shown to be particularly effective in specific subgroups of patients with Crohn's disease. Clinical evidence suggests that this therapeutic approach is most beneficial in patients with colonic and ileocolonic involvement, where microbial dysbiosis and mixed aerobic-anaerobic bacterial populations play a prominent role in disease activity [5, 6]. Additionally, patients with perianal Crohn's disease, including those presenting with fistulas, abscesses, or inflammatory perianal lesions, demonstrate improved clinical outcomes due to the potent anaerobic coverage of metronidazole benzoate combined with the broad-spectrum and anti-inflammatory properties of azithromycin dihydrate [6, 7]. The combination is also effective in patients with mild to moderate active disease, particularly those who are intolerant to or unsuitable candidates for aggressive immunosuppressive or biologic therapies [5, 8]. Furthermore, this dual antimicrobial regimen has shown benefits in postoperative Crohn's disease patients, where it may help reduce bacterial-driven inflammation and lower the risk of disease recurrence [7, 9]. Patients exhibiting recurrent disease flares associated with suspected bacterial overgrowth or infectious complications may also experience enhanced remission rates with combination therapy. Overall, the synergistic action of azithromycin dihydrate and metronidazole benzoate makes this combination especially effective in patients where microbial imbalance and inflammatory burden are key contributors to disease persistence and progression [6, 8]. Clinical trial [10] has shown that antibiotic (Azithromycin and Metronidazole) therapy can induce and maintain remission, particularly in patients with colonic involvement or perianal disease. Accordingly, combination antimicrobial therapy has gained attention for its broader microbial coverage, synergistic effects, and potential to reduce antimicrobial resistance compared with monotherapy [10-12]. Azithromycin Dihydrate is a macrolide antibiotic known not only for its broad-spectrum antimicrobial activity but also for its anti-inflammatory and immunomodulatory properties. It has been shown to suppress pro-inflammatory cytokine production and inhibit neutrophil activation, which may provide additional therapeutic benefits in inflammatory bowel diseases [10, 11]. Metronidazole Benzoate is a nitroimidazole antimicrobial agent effective against anaerobic bacteria and certain protozoa. It is widely utilized in the management of Crohn's disease, particularly in cases involving perianal lesions and fistulizing complications, due to its efficacy against anaerobic organisms implicated in intestinal inflammation [12].

Both drugs are available individually in various oral dosage forms and have been extensively studied for gastrointestinal infections. However, their combined administration of azithromycin dihydrate and metronidazole benzoate has demonstrated superior therapeutic outcomes compared with individual agents, including improved disease control, reduced relapse rates, and enhanced mucosal healing, without a significant increase in adverse effects. This therapeutic synergy is attributed to their complementary mechanisms of action and their ability to target both aerobic and anaerobic bacterial populations, thereby promoting restoration of microbial balance and attenuation of intestinal inflammation [11]. Clinical investigations further suggest that this combination may contribute to higher remission rates in patients with Crohn's disease, reinforcing its clinical relevance [10-12].

A comprehensive review of the literature reveals numerous analytical methods for the individual estimation of Azithromycin Dihydrate and Metronidazole Benzoate, including chromatographic techniques such as High-Performance Liquid Chromatography (HPLC), High-Performance Thin-Layer Chromatography (HPTLC), spectroscopic methods such as UV-Visible spectrophotometry, and hyphenated techniques like LC–MS/MS. These methods have been reported for single drugs and in combination with other pharmaceutical agents.

However, no analytical method has been reported for the simultaneous estimation of Azithromycin Dihydrate and Metronidazole Benzoate in combined dosage forms. Therefore, the present study aims to develop and validate a simple, accurate, and precise UV spectrophotometric method using the simultaneous equation approach, along with a RP-HPLC method for the concurrent determination of Azithromycin Dihydrate and Metronidazole Benzoate in bulk and pharmaceutical formulations.

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Several analytical methods have been reported for the estimation of azithromycin, including a UV spectrophotometric method for azithromycin in API and stress degradation studies [13], UV spectrophotometric estimation of ambroxol dihydrochloride and azithromycin [14], a liquid chromatographic method for confirmation of drug stability of azithromycin in bulk samples, tablets, and suspensions [15], HPLC method for azithromycin and cefixime in bulk and pharmaceutical formulations [16], HPTLC method for azithromycin [17], and RP-UPLC techniques [18]. In contrast, metronidazole benzoate has been analyzed using UV spectrophotometry [19]. Additionally, UV spectrophotometric methods have been developed with albendazole [20], for combination drug products [21], and HPLC method has been reported for the simultaneous in vitro determination of amoxicillin and metronidazole [22].

However, no validated method has been reported for the simultaneous estimation of Azithromycin Dihydrate and Metronidazole Benzoate in a combined synthetic mixture, highlighting the need for the present study.

Recent studies demonstrate that combined administration of these drugs produces synergistic therapeutic effects, enhancing overall treatment efficacy. However, the lack of a validated analytical method for their simultaneous estimation necessitates the development of a precise, accurate, and reliable analytical methods on the basis of the present study. Therefore, the present study aimed to develop and validate a sensitive, precise, and robust RP-HPLC and first order derivative UV spectrophotometric methods for the simultaneous estimation of Azithromycin Dihydrate and Metronidazole Benzoate in a synthetic mixture, in accordance with ICH guidelines [23].

2. MATERIALS AND METHODS:

2.1 Compounds and Components:

Azithromycin Dihydrate and Metronidazole Benzoate were bought from Cadila pharmaceuticals and Intas pharmaceuticals from Ahmedabad as gift samples. Finar Chemicals, Ahmedabad provided the HPLC-grade methanol, acetone, and water that were employed. Ortho-phosphoric acid (75%, AR grade) and potassium dihydrogen phosphate were procured from Astron Chemicals Ltd., India. All solutions were freshly prepared each day.

2.2 Scientific conditions with instrumentation:

The RP-HPLC process was successfully executed using the Clarify software, a Systronic RP-HPLC (LC-20-AD), a UV Detector SPD-20 A, and a Rheodyne injector equipped with a 20 µL loop. Reversal aspect strategies were employed during the technique's execution. Applying Phosphate Buffer (pH 4.0 adjusted with 10% ortho phosphoric acid): Acetonitrile: Tetrahydrofuran (50:40:10 %v/v/v) with a flow rate of 1 ml/min, both medicines had been isocratically eluted. The pH of the buffer solution was measured using Chemi-Line digital pH meter. The UV-Vis Detector's detecting wavelength has been configured at 225 nm. Every day, preparations containing mobile phases had been made and passed through 0.45 µm Millipore membrane filters and sonicate with sonicator (Equitron, India) prior usage. Both a pH meter and a Kromstar C_{18} (250 × 4.6 mm, 5 µm) Column consisted utilised. At 25°C, the high-performance LC system was run at ambient temperature. Shimadzu UV Visible double beam spectrophotometer (Model 1900) along with UV probe 2.7 version software and 1.0 cm quartz cells were used for the UV Spectrophotometric technique. Balancing was carried out throughout on a Scale-Tec Analytical balance.

2.3 Preparation of stock solution:

An accurately weighed quantity of Azithromycin dihydrate (10 mg) and Metronidazole benzoate (10 mg) were separately transferred into two different 100 ml volumetric flasks. Each flask was then made up to the mark with the mobile phase to prepare standard stock solutions, resulting in a final concentration of 100 µg/ml for both Azithromycin dihydrate and Metronidazole benzoate.

2.4 Preparation of standard solution:

Azithromycin Dihydrate and Metronidazole Benzoate standard, individually and precisely measured at 1.5 mg and 4 mg, were then added to a 100 ml volumetric flask, and then submerged in 100 ml of methanol. This solution is sonicated till the drug dissolves and was made up the mark with Methanol.

2.5 Preparation of std working solution:

The concentration ranges of 7.5-37.5 µg/mL of AZI and 20-100 µg/mL of METRO produced from each stock solution, AZI (0.75, 1.5, 2.25, 3 and 3.75 ml) and METRO (2, 4, 6, 8 and 10 ml) were pipetted out in ten different 10 ml volumetric flasks and made up to mark with Methanol to obtained 7.5, 15, 22.5, 30 and 37.5 µg/mL of AZI and 20, 40, 60, 80 and 100 µg/mL for METRO, respectively. Under the optimized spectrophotometric conditions, the samples were analyzed using a 1 cm quartz cuvette in the UV spectrophotometer. Similarly, the optimized chromatographic conditions, 20 µL of each standard working solution was injected into the RP-HPLC system.

2.6 Preparation of 10% Orthophosphoric acid:

10% orthophosphoric acid was prepared by diluting

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1.0 ml of concentrated ortho phosphoric acid in 10 ml HPLC grade water.

2.7 Preparation of 10mM Phosphate Buffer:
Accurately weighed 1.36 g of potassium dihydrogen phosphate (KH_2PO_4) was transferred to a 1000 mL volumetric flask, dissolved in approximately 800 mL of purified water, and sonicated until completely dissolved. The pH of the solution was adjusted to the acidic value 4 using 10% Orthophosphoric acid. The final volume was made up to 1000 mL with purified water and mixed well. The buffer solution was filtered through a 0.45 µm membrane filter prior to use.

3. ANALYTICAL TECHNIQUES:
3.1 Method development:
3.1.1 Method I: UV Spectrophotometric Method:
A first-order derivative spectrophotometric method was used for the simultaneous quantification of Azithromycin dihydrate and Metronidazole Benzoate in a synthetic mixture. Separate working standard solutions were scanned in the 200-400 nm range to obtain derivative spectra and determine suitable zero-crossing wavelengths for analysis.

Standard stock solutions of both drugs were prepared in methanol at 100 µg/mL. Aliquots of 1.5 mL of Azithromycin dihydrate and 4 mL of Metronidazole Benzoate were transferred into separate 10 mL volumetric flasks and diluted to volume with methanol to obtain concentrations of 15 µg/mL and 40 µg/mL, respectively.

The zero-order spectra were recorded and converted to first-derivative spectra. Upon overlaying, Azithromycin dihydrate showed a zero-crossing at 289 nm, while Metronidazole Benzoate showed a zero-crossing at 226 nm. Quantification was performed at 226 nm for Azithromycin dihydrate (ZCP of Metronidazole Benzoate) and at 289 nm for Metronidazole Benzoate (ZCP of Azithromycin dihydrate). The zero- and first-order overlaid spectra are presented in Figure 1 (a) and (b), respectively.

3.1.2 Method II: Reverse Phase High Performance Liquid Chromatography:
The isocratic analysis was carried out using Reverse phase chromatographic technique because of its recommended use for ionic and moderate to non-polar compounds using a mobile phase comprised Phosphate Buffer (pH 4 adjusted with 10% ortho phosphoric acid): Acetonitrile: Tetrahydrofuran (50:40:10 %v/v/v) at a flow rate of 1 ml/min found better separation of both the drug peaks. Prior to usage, the solvents were filtered through a 0.45 µ filter and sonicated for 10 min. The stationary phase was a Kromstar C_{18} (250 mm × 4.6 mm, 5 µm), and the eluent was observed by a U.V Detector at 225 nm showed in Figure 1 (a).

3.2 Method Validation:
According to ICH guideline Q2 (R2) [23], the approaches have been verified and confirmed. The technique has undergone rigorous validation through a variety of evaluations for System compatibility, Specificity, Linearity and range, Accuracy, Precision, Detection limit, and Quantification limit [24-25].

3.2.1 Specificity:
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. It was proved by comparing the chromatogram of mobile phase, test preparation solution to show that there was no interference of mobile phase and excipients peaks with peak of Azithromycin dihydrate and Metronidazole benzoate shown in figure 5.

3.2.2 Linearity and Range (n=6):
Azithromycin dihydrate and Metronidazole benzoate exhibited good linearity within the concentration ranges of 7.5-37.5 µg/ml and 20-100 µg/ml, respectively. In the UV spectrophotometric method, calibration graphs were generated by plotting absorbance against concentration (µg/ml). For the HPLC method, calibration plots were constructed by correlating peak area with the respective concentrations of Azithromycin dihydrate and Metronidazole benzoate. The regression equations were then determined, and linearity was evaluated using parameters such as slope, intercept, and correlation coefficient (R^2).

3.2.3 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. Precision may be considered at three levels: Intermediate (Intraday) precision, reproducibility (Interday precision), repeatability.

3.2.3.1 Intraday Precision (n=3): Solutions containing 7.5, 15, 22.5 µg/ml of Azithromycin dihydrate and 20, 40, 60 µg/ml of Metronidazole benzoate were analyzed three times on the same day and % R. S. D was calculated.

3.2.3.2 Interday Precision (n=3): Solutions containing 7.5, 15, 22.5 µg/ml of Azithromycin dihydrate and 20, 40, 60 µg/ml of Metronidazole benzoate were analyzed on three different successive days and % R. S. D was calculated.

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3.2.3.3 Repeatability (n=6): Solutions containing 15 µg/ml of Azithromycin dihydrate and 40 µg/ml of Metronidazole benzoate were analyzed for six times and %R.S.D. was calculated. % R.S.D was not more than 2%.

3.2.4 Limit of Detection (LOD)

Limit of detection can be calculated using following equation as per ICH guidelines.

$$\mathrm{LOD} = 3.3 * \frac{\sigma}{S}$$

Where, σ = standard deviation of the calibration curve

S = slope of the calibration curve

3.2.5 Limit of Quantification (LOQ):

Limit of quantification can be calculated using following equation using the standard deviation of the Y-intercept (σ) and the mean slope (S) of the calibration curve according to ICH Q2 (R2) guideline.

$$\mathrm{LOQ} = 10 * \frac{\sigma}{S}$$

3.2.6 Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50 %, 100 %, 150 % and the values were measured for Azithromycin dihydrate (15 µg/ml) and Metronidazole benzoate (40 µg/ml). This performance was done in triplicate.

3.2.7 Assay:

A synthetic mixture of Azithromycin dihydrate and Metronidazole benzoate was prepared in a 15:40 ratio along with common excipients and mixed thoroughly. Common excipients such as Micro Crystalline Cellulose (9 mg), Lactose (11 mg), Magnesium Stearate (8 mg), Talc (7 mg), and Croscarmellose Sodium (10 mg) were added in the motor pestle along with Azithromycin dihydrate (15 mg) and Metronidazole benzoate (40 mg). This mixture was transferred to a 100 ml volumetric flask, sonicated, and diluted to volume with methanol. The solution was filtered, yielding final concentrations of 150 µg/ml and 400 µg/ml, respectively. This solution was filtered through Whatmann filter paper. The filtrate was diluted to the mark with Methanol. The mixture contains 150 µg/ml of Azithromycin dihydrate and 400 µg/ml of Metronidazole benzoate. The final concentration of Azithromycin Dihydrate and Metronidazole Benzoate were 15 µg/ml and 40 µg/ml, respectively, it required to scoop out 1.0 ml from the aforesaid combined solutions into a 10 ml volumetric flask from mixture solution and to correct the quantity up towards the target using moving phase. With the purpose of detecting spectrum, Azithromycin Dihydrate (15 µg/ml) and Metronidazole Benzoate (40 µg/ml) been utilised. The resulting mixtures were scrutinised and the electromagnetic UV spectrum of their 200-400 nm wavelengths were captured over a blank reagent composed of methanol as well as in RP-HPLC system.

3.2.9 Robustness

Robustness refers to the ability of an analytical method to produce consistent and reliable results despite small, intentional changes in experimental conditions. It demonstrates the method's dependability during routine use. In liquid chromatography, robustness is evaluated by slightly varying parameters such as mobile phase pH, composition, column type or batch, temperature, and flow rate. If these minor modifications do not significantly affect results like retention time, resolution, or assay values, the method is considered robust.

3.2.9 System suitability test

A system suitability test is a crucial component of liquid chromatography, performed to ensure that the chromatographic system is functioning properly before analysis. It confirms that the system provides adequate resolution and consistent reproducibility for accurate results. Key parameters evaluated in this test include resolution, column efficiency, tailing factor, and the number of theoretical plates, all of which collectively indicate whether the system's performance meets the required standards for reliable analysis.

3.2.10 Statistical Comparison of Methods by Student's t-test:

Statistical analysis was performed to identify significant differences among the developed analytical methods. A statistical analysis was conducted using the student's t-test [24] to compare the results of accuracy and assay for proposed UV spectrophotometric and HPLC methods. A statistical test (Student's *t*-test) [24] was applied to evaluate the significance of difference between the two methods. The calculated *t*-value was compared with the theoretical *t*-value at a 95% confidence level. The student's *t*-test was calculated using the following formula.

$$t = \frac{|\bar{X}_1 - \bar{X}_2|}{Sp\sqrt{(\frac{1}{n_1} + \frac{1}{n_2})}}$$

where, $\bar{X}_1$ and $\bar{X}_2$ were Mean value obtained from UV and RP-HPLC methods as two groups, n1 and n2 were number of observations for both methods,

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respectively and Sp was pooled standard deviation. The pooled standard deviation was calculated using the formula.

$$\mathrm{Sp} = \sqrt{\frac{(n_1 - 1)(S_1)^2 + (n_2 - 1)(S_2)^2}{n_1 + n_2 - 2}}$$

Where, S_1 and S_2 were standard deviation of the proposed methods as two groups.

4. RESULTS AND DISCUSSION:

4.1 Method I: UV Method:

Among various UV-spectrophotometric techniques, the first-order derivative method offers distinct advantages over conventional zero-order UV methods for the simultaneous estimation of multiple components. This technique enhances spectral resolution by minimizing spectral overlap and background interference, thereby improving selectivity and accuracy. Unlike the simultaneous equation and absorbance ratio methods, first-order derivative spectrophotometry allows quantification at zero-crossing wavelengths, enabling precise estimation of each component without mutual interference. Additionally, the method demonstrates improved sensitivity, better baseline correction, and reduced matrix effects. Owing to its simplicity, rapid analysis, and ability to resolve overlapping spectra, first-order derivative UV spectrophotometry is highly beneficial for routine analysis of combined pharmaceutical formulations.

4.1.1 Selection of detection wavelength for Azithromycin Dihydrate and Metronidazole Benzoate:

The remarkable absorbance of Azithromycin Dihydrate exhibited an absorption maximum at 226 nm, while Metronidazole Benzoate showed an absorption maximum at 289 nm shown in Figure 1 (b). The zero-order and First Order UV absorption spectra of Azithromycin Dihydrate (15 µg/ml) and Metronidazole Benzoate (40 µg/ml) in Methanol were showed in Figure 1 (a) and 1 (b), respectively.

4.1.2 First order derivative UV Method Development:

The overlapping absorption of Azithromycin dihydrate and Metronidazole Benzoate in the 200–400 nm range is evident from the spectra, which makes direct quantification by conventional UV spectrophotometry difficult without compensating for spectral interference. The total absorbance of a mixture at a specific wavelength represents the sum of the individual absorbances of both drugs. When the absorption bands overlap, the concentration of each drug can be determined using their zero-order spectra [figure 1(a)].

To eliminate interference from overlapping components, the absorption spectra were converted into first-derivative spectra using Δλ = 2 nm and a scaling factor of 4. The amplitude values were measured at 226 nm (λ_1) for Azithromycin dihydrate and at 289 nm (λ_2) for Metronidazole Benzoate, as shown in Figure 1 (b).

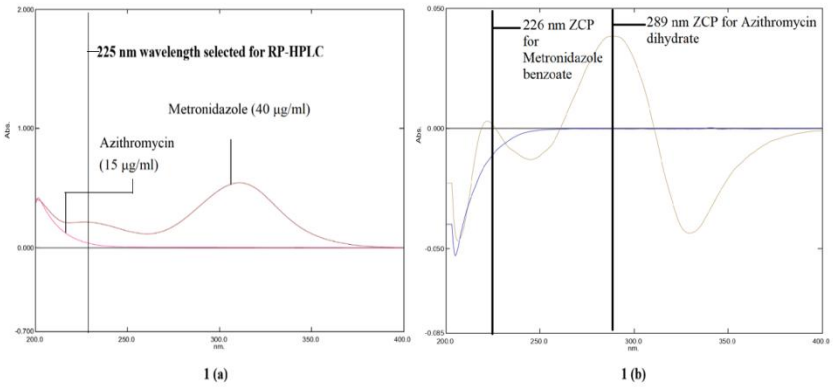

Figure 1: Overlain UV Spectra of Azithromycin Dihydrate (15 µg/ml) and Metronidazole Benzoate (40 µg/ml) in Methanol 1 (a) Zero Order 1 (b) First order

4.2 Method II: RP-HPLC Method:

Pharmaceutical analysis commonly uses simultaneous estimation using RP-HPLC. It enables the use of RP-HPLC to determine the presence of many chemicals in a sample. For the simultaneous estimate of various components, including medications and their contaminants, in pharmaceutical formulations, a number of techniques have been devised and proven effective. Utilizing an appropriate column, mobile phase, and detection equipment, the simultaneous estimation technique by HPLC allows for the separation and quantification of the target substances. In pharmaceutical analysis, Reverse Phase high-performance liquid chromatography (RP-HPLC) is a great instrument for simultaneous estimation that offers confidence and specificity for the identification of chemical entities in Synthetic Mixture.

Reverse phase chromatography was chosen because of its recommended use for ionic and

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moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also performs better in terms of efficiency, stability and reproducibility. C_{18} column was selected because it is least polar compare to C_4 and C_8 columns. C_{18} column allows eluting polar compounds more quickly compare to non-polar compounds. In addition to this UV detector is used which allows easy detection of the compounds in UV transparent organic solvents. Hence, C_{18} (250×4.6 mm) column of 5µm particle packing was selected for separation of Azithromycin Dihydrate and Metronidazole Benzoate.

4.2.1 Selection of detection wavelength:

The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 225 nm both drugs give good peak height and shape. So, 232 nm was selected for simultaneous estimation of Azithromycin Dihydrate and Metronidazole Benzoate in synthetic mixture. Overlay UV spectra of Azithromycin Dihydrate (15 µg/ml) and Metronidazole Benzoate (20 µg/ml) in Methanol have been shown in Figure 1 (a).

4.2.2 RP-HPLC Method Development:

Liquid chromatography coupled with UV detection was used to develop a way for simultaneously measuring Azithromycin Dihydrate and Metronidazole Benzoate. Achieving acceptable peak symmetry and theoretical plates within a realistic time period was the aim. The chromatographic conditions were optimized by experimenting with various stationary and mobile phases. The mobile phase Phosphate Buffer (pH 4 adjusted with 10% ortho phosphoric acid): Acetonitrile: Tetrahydrofuran (50:40:10 %v /v /v) was selected because it was found to ideally resolve the peaks with retention time 2.2 min and 4.7 min for Azithromycin Dihydrate and Metronidazole Benzoate, respectively showed in figure 2 and table 1. Kromstar C_{18} (250×4.6 mm, 5 µm) column was used for separation of Azithromycin Dihydrate and Metronidazole Benzoate with flow rate of 1.0 ml/min at 225 nm.

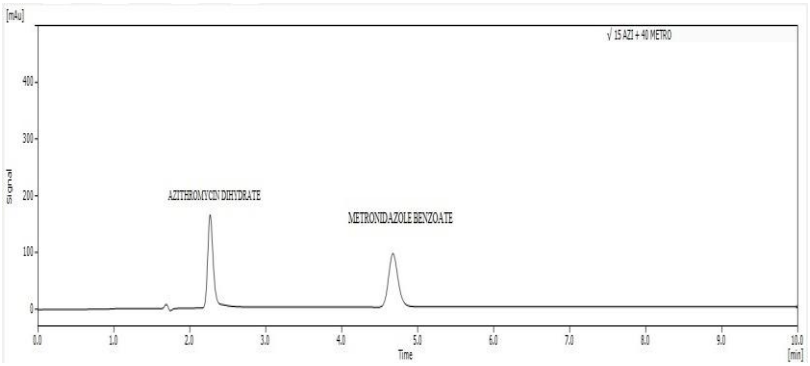

Figure 2: RP-HPLC chromatogram at Azithromycin Dihydrate (15 g/ml) and Metronidazole benzoate (40 g/ml) in Phosphate buffer (pH 4 adjusted with 10% ortho phosphoric acid): Acetonitrile: Tetrahydrofuran (50:40:10: % v/v/v) at 225 nm

Table 1: System suitability parameters

Parameters	Retention Time	Tailing Factor	Number of Theoretical plates	Resolution
Azithromycin Dihydrate	2.2 min	0.8	8554	2.8
Metronidazole Benzoate	4.7 min	1.0	6256	

4.3 Validation of the proposed methods:

4.3.1 Linearity and range:

For UV method, the mean absorbance was measured for linearity of Azithromycin Dihydrate (7.5-37.5 µg/ml) at 226 nm and Metronidazole Benzoate (20-100 µg/ml) at 289 nm showed in Figure 3 (a) and 3 (b), respectively. Linearity data of UV method showed in Table 2**.**

Table 2: Results of validation parameters for UV and RP-HPLC method

Sr. No	Validation parameters	UV Method		RP-HPLC Method	
	Parameters	Azithromycin Dihydrate	Metronidazole Benzoate	Azithromycin Dihydrate	Metronidazole Benzoate
1.	Detection Wavelength (nm)	226 nm	289 nm	225 nm	225 nm
2.	Linearity range (µg/ml)	7.5-37.5	20-100	7.5-37.5	20-100
3.	Regression Equation (y=mx + c)	y =0.0004x+0.0037	y = 0.0007x+0.0123	y = 89.947x + 650.06	y = 38.395x + 121.98
4.	Correlation Coefficient	0.9959	0.9951	0.9992	0.998

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5.	Intraday Precision (%RSD)	1.06-1.22	1.00-1.18	0.92-1.16	0.82-1.03
6.	Interday Precision (%RSD)	1.08-1.26	1.02-1.21	0.96-1.18	0.87-1.07
7.	Repeatability (%RSD)	1.15	1.08	0.99	0.89
8.	LOD (µg/ml)	0.82	1.60	0.67	1.06
9.	LOQ (µg/ml)	2.50	4.85	2.04	3.22

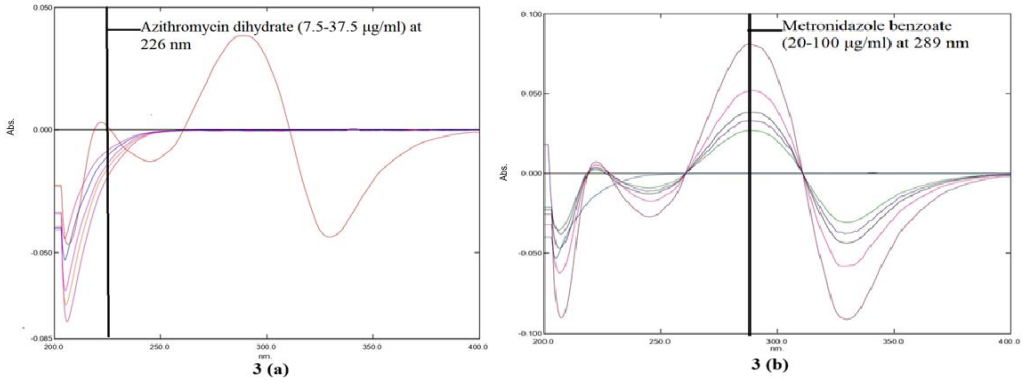

Figure 3: Overlain UV spectra of (a) Azithromycin Dihydrate (7.5-37.5 µg/ml) at 226 nm (b) Metronidazole Benzoate (20-100 µg/ml) at 289 nm

For RP-HPLC method, the linearity of Azithromycin Dihydrate and Metronidazole Benzoate was found to be 7.5-37.5 µg/ml and 20-100 µg/ml at 225 nm, respectively which showed in figure 4. Linearity data for RP-HPLC method showed in Table 2**.**

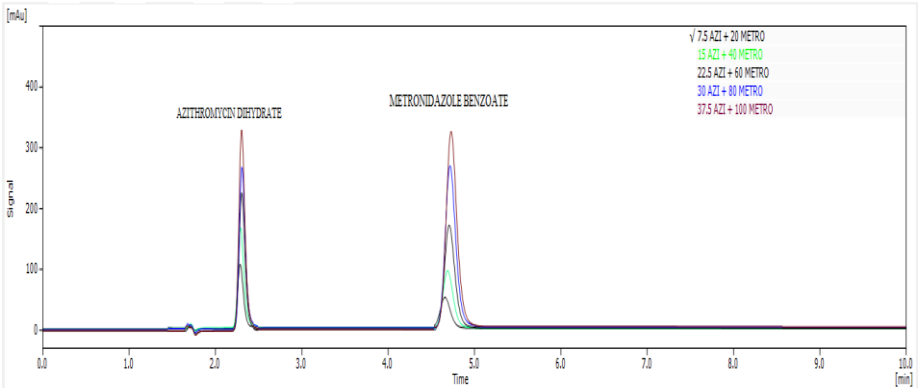

Figure 4: Overlain RP-HPLC chromatogram of Azithromycin Dihydrate (7.5-37.5 µg/ml) and Metronidazole Benzoate (20-100 µg/ml) at 225 nm

4.3.2 Precision:

Precision was assessed by intraday, interday, and repeatability studies. Azithromycin dihydrate (7.5, 15, 22.5 µg/ml) and Metronidazole benzoate (20, 40, 60 µg/ml) were analyzed in triplicate on the same day and on three consecutive days. Repeatability was evaluated at 15 µg/ml and 40 µg/ml, respectively. The %RSD values for Inter-day precision, Intraday, and Repeatability were showed in Table 2.

4.3.3 LOD and LOQ:

The limits of detection (LOD) for the UV method were found to be 0.82 µg/mL for Azithromycin Dihydrate and 1.60 µg/mL for Metronidazole Benzoate, while the corresponding limits of quantification (LOQ) were 2.50 µg/mL and 4.85 µg/mL, respectively. In comparison, the HPLC method exhibited lower LOD values of 0.67 µg/mL for Azithromycin Dihydrate and 1.06 µg/mL for Metronidazole Benzoate, with LOQ values of 2.04 µg/mL and 3.22 µg/mL, respectively, indicating higher sensitivity of the HPLC method. The results of LOD and LOQ for both methods are shown in Table 2.

4.3.4 Accuracy:

The accuracy of the method was evaluated by recovery studies using the standard addition method. Known amounts of Azithromycin Dihydrate and Metronidazole Benzoate were added to the pre-analyzed sample at 50%, 100%, and 150% levels. The studies were performed in triplicate, and accuracy was expressed as %

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recovery. For the UV method, azithromycin dihydrate showed mean recoveries ranging from 99.77 ± 1.50% to 99.95 ± 1.06%, while metronidazole benzoate exhibited recoveries between 99.93 ± 1.04% and 99.99 ± 1.03%. Similarly, the RP-HPLC method demonstrated mean recoveries of 99.91 ± 0.7412% to 100.06 ± 0.9253% for azithromycin dihydrate and 99.54 ± 0.9426% to 100.01 ± 0.4357% for metronidazole benzoate. These results (table 3) confirmed the high accuracy and reliability of both analytical methods.

Table 3: Recovery study data for UV and RP-HPLC Method

UV Method						
Name of Drug	**% Level of recovery**	**Test Amount (µg/ml)**	**Amount of drug taken (µg/ml)**	**Total Std Amt (µg/ml)**	**Total amount Recovered (µg/ml)**	**% Mean Recovery ± SD(n=3)**
Azithromycin Dihydrate	50	15	7.5	22.5	22.48	99.92± 1.13
	100	15	15	30	29.93	99.77± 1.50
	150	15	22.5	37.5	37.48	99.95± 1.06
Metronidazole Benzoate	50	40	20	60	59.96	99.93±1.04
	100	40	40	80	79.96	99.95±1.05
	150	40	60	100	99.99	99.99±1.03
RP-HPLC Method						
Azithromycin Dihydrate	50	15	7.5	22.5	22.48	99.91±0.7412
	100	15	15	30	29.98	99.94±0.6358
	150	15	22.5	37.5	37.52	100.06±0.9253
Metronidazole Benzoate	50	40	20	60	59.72	99.54±0 .9426
	100	40	40	80	79.89	99.87±0.7561
	150	40	60	100	100.01	100.01±0.4357

4.3.5 Assay as Analysis of Synthetic mixture:

From assay, Final concentration of Azithromycin Dihydrate was 15 µg/ml and Metronidazole Benzoate 40 µg/ml were run into UV and HPLC. Assay of the synthetic mixture by the UV method yielded assay values of 99.54 ± 1.5682% for Azithromycin Dihydrate and 99.85 ± 1.7134% for Metronidazole Benzoate (n = 3). Similarly, the RP-HPLC method showed assay values of 100.13 ± 0.443% and 99.92 ± 0.838% for Azithromycin Dihydrate and Metronidazole Benzoate, respectively, confirming the accuracy and precision of both methods. Its results showed in Table 4.

Table 4: Analysis of synthetic mixture for UV and RP-HPLC Method

UV Method			
Name of Drug	**Amount in synthetic mixture (µg/ml)**	**Mean Amount found (µg/ml)**	**% Assay ± SD (n=3)**
Azithromycin Dihydrate	15	14.93	99.54±1.5682
Metronidazole Benzoate	40	39.94	99.85±1.7134
RP-HPLC Method			
Azithromycin Dihydrate	15	15.02	100.13 ±0.443
Metronidazole Benzoate	40	39.37	99.92± 0.838

4.3.6 Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. It was proved by comparing the chromatogram of mobile phase, test preparation solution to show that there was no interference of mobile phase and excipients peaks with peak of Azithromycin Dihydrate and Metronidazole Benzoate.

4.3.8 Robustness:

The robustness of the developed HPLC method was assessed by introducing small, intentional variations such as changes in analyst, slight modifications in flow rate, run time, and detection wavelength. The %RSD values were found to be within acceptable limits, confirming the method's robustness and reproducibility (Table 5).

Table 5: Robustness data

Condition	**Variation**	**Azithromycin dihydrate**	**Metronidazole benzoate**
		% Assay ± SD (n=3)	**% Assay ± SD (n=3)**
Flow rate (1 ml ± 0.2 ml/ min)	0.8 ml/min	99.79±6.5166	99.75±4.3730
	1.0 ml/min	99.36±6.2691	99.62±5.5545
	1.2 ml/min	99.87±8.4770	98.96±7.0286
Detection wavelength (225 nm ± 2 nm)	223	99.63±6.1268	98.98±1.0454
	225	98.98±5.4267	99.74±2.5055

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	227	99.83±9.0256	99.86±2.3762
Mobile Phase (Phosphate Buffer (pH 4): Acetonitrile: Tetrahydrofuran (50:40:10) ± 2 % v/v/v)	48:38:14	98.95±3.0784	99.85±4.1116
	50:40:10	99.83±2.9421	99.99±5.0552
	52:42:06	99.69±4.0143	99.95±6.1845

4.3.9 Statistical Evaluation of the UV Method with RP-HPLC:

The approach of statistical analysis was used to compare the proposed analytical approaches. The student's t-test was used, and the results did not reveal any discernible distinction among the values from the experiment reported in the data collection assessment through both approaches. At a 5% level of statistical significance, the t-value derived from the calculation ($t_{calculated}$) was lower than the critical value of t ($t_{tabulated}$ / $t_{critical}$) and it was found that the tabulated values were greater than calculated values. So, there was no significant difference between Recovery and Assay parameters obtained through U.V. method and RP-HPLC method. Results indicated that both developed and validated analytical methods were considered accurate, precise, and statistically insignificant. The results of Compared Recovery and assay data to compare Between U.V. and RP-HPLC Methods were showed in table 6.

Table 6: Statical Comparison data of developed methods by Student t-test Analysis

Analysis Type	Parameters	Azithromycin Dihydrate	Metronidazole Benzoate
Recovery Data	*t* calculated	0.96	0.54
	t tabulated	2.12	2.12
	Confidence level: 95% ($p \leq 0.05$, d.f. = 2)		
Assay Data	*t* calculated	0.66	0.10
	t tabulated	2.78	2.78
	Confidence level: 95% ($p \leq 0.05$, d.f. = 4)		

5. CONCLUSION:

The present study successfully demonstrated the development, optimization, and validation of first-order derivative UV spectrophotometric and RP-HPLC methods for the simultaneous estimation of Azithromycin Dihydrate and Metronidazole Benzoate in a synthetic mixture. The first-order derivative UV method effectively resolved the issue of overlapping spectra by employing zero-crossing wavelengths, enabling selective, accurate, and rapid quantification of both analytes. The RP-HPLC method provided efficient chromatographic separation with well-resolved peaks, acceptable retention times, good peak symmetry, and satisfactory system suitability parameters. Both analytical methods were validated in accordance with ICH Q2(R1) and Q2(R2) guidelines. Validation results confirmed excellent linearity within the studied concentration ranges, high accuracy with recoveries close to 100%, and precision demonstrated by low %RSD values for intraday, interday, and repeatability studies. The LOD and LOQ values indicated adequate sensitivity of both methods, with the RP-HPLC method exhibiting comparatively higher sensitivity. Specificity and robustness studies further confirmed that the methods are reliable and unaffected by minor variations in analytical conditions. Statistical comparison using Student's *t*-test showed no significant difference between the results obtained by the first-order derivative UV method and the RP-HPLC method for recovery and assay parameters at a 95% confidence level. This confirmed that both methods were statistically comparable in terms of accuracy and precision. Overall, the developed UV and RP-HPLC methods are simple, precise, accurate, reproducible, and suitable for routine quality control analysis and assay of Azithromycin Dihydrate and Metronidazole Benzoate in synthetic mixture.

6. FUTURE PERSPECTIVES:

The developed first-order derivative UV method can be effectively utilized as a rapid and cost-efficient technique for routine quality control analysis. The RP-HPLC method, owing to its higher sensitivity and specificity, may be further extended for stability studies, impurity profiling, and analysis of finished pharmaceutical dosage forms. Future investigations may also include application of these methods to marketed formulations and further enhancement toward stability-indicating and bioanalytical studies.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

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