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# DoE-ORIENTED ISOCRATIC HPLC OPTIMIZATION OF AZOLE ANTIFUNGALS IN COMBINED PHARMACEUTICAL DOSAGE FORM

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## ABSTRACT

A simple, consistent and reasonable isocratic RP-high performance liquid chromatography (RP-HPLC) approach was developed and optimized for the concurrent determination of Ornidazole and Miconozole in bulk and tablet dosage forms. HPLC analysis illustrated satisfactory separation of Ornidazole and Miconozole and optimal resolution was attained with C18 column using methanol, 5mM Ammonium acetate in the ratio 40:60 v/v at pH 4.8 as the mobile phase and UV detection at 276 nm. A unique chromatogram and spectra for Ornidazole and Miconozole were able to be envisioned by developed methods, which are analogous to the sample results. The calibration curve's observed linearity for ORZ and MCZ at selected concentration range. Recovery studies investigated the approach's accuracy with percentage recovery ranges between 97.33% and 98.33%. The developed techniques were verified in terms of precision, accuracy and linearity. Quality by design (QbD) was employed to optimize the accurate ratio mobile phase, pH, flow rate and its impact on retention time and peak area. The validation outcomes met the acceptance criteria as per ICH recommendations and found to be reliable for the routine examination of drugs in therapeutic formulations. The developed methodology can be used for quantitative determination of Ornidazole and Miconozole combination in pharmaceutical formulations without the immersion of additional diluents or excipients.

**KEYWORD:** Miconozole, Ornidazole, HPLC, Box Behnken Design, validation, Greenness approach.

## INTRODUCTION

Ornidazole, 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole, 5-nitroimidazole drug typically used for the treatment of both anaerobic-bacterial and protist infections. It is effective in infections caused by *Entamoeba histolytica*, *Giardia lamblia*, and *Trichomonas vaginalis*. The structural nitro group gets reduced to more reactive amine resulting in the destruction of DNA and induces cytotoxic effect. Miconozole, chemically 1-[2,4-Dichloro-b-(2,4-dichlorobenzyloxo) phenylethyl]-1H-imidazole, employed for treating intestinal candidiasis and oropharyngeal candidiasis.[1,2] The chemical structure was exemplified in figure 1.

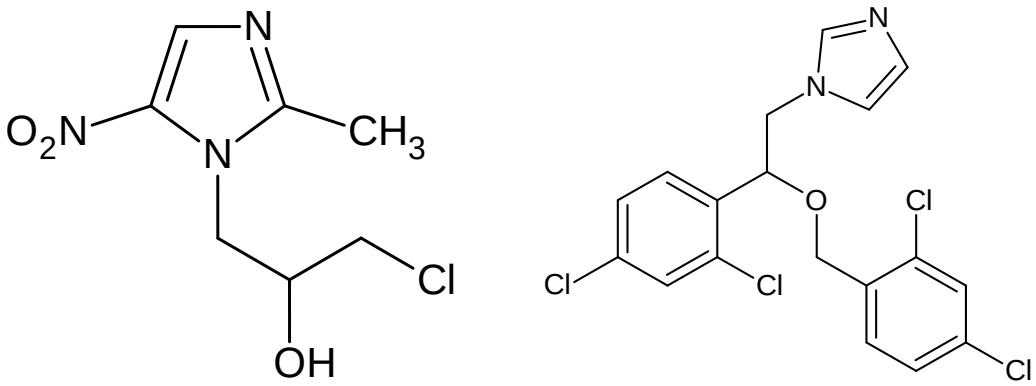



**Figure 1: Chemical structure of Ornidazole and Miconozole.**

Through the use of quality-by design (QbD), method development is viewed as a holistic assurance of product quality throughout its lifespan, rather than just a legal necessity. QbD provide in-depth knowledge of how changes in pH, temperature and preparation of analyte may profoundly affect the overall performance of the analytical procedure. This methodology guarantees that analytical procedure is both compliant and resilient enough to endure changes in sample matrices, apparatus and other practices. The analytical quality-by-design (AQbD) is a superior-quick way approach to create new analytical method, which reduces the fluctuations and improves the efficacy of the method. Design of Experiments software (DoE) can be utilized to optimize the development of HPLC technique and the best tool offers Box-Behnken design (BBD). Using Box Behnken-aided surface design, the mobile phase, pH, and flow rate of the HPLC procedure were chosen and adjusted.[3,4]

Although there are a few papers on the quantification of ODZ and MCZ,[5-8] none of them mention the application of statistical design in parallel approaches to create and verify an appropriate stability-indicating analytical method for the quantification of medicines in dose and bulk forms. The process is difficult, nonetheless, because developing the analytical method necessitates a thorough analysis of a number of factors that might affect the sensitivity and specificity. The purpose of current work is to determine if using BBD may improve the simultaneous HPLC approach for the estimation of ODZ and MCZ in pharmaceutical formulations.

## MATERIALS AND METHODS

### Instrumentation

Electronic Balance (Shimadzu AUX220, New Delhi, India), Membrane Filter (Merck Millipore, SMWP04700, Haryana, India), HPLC (Agilent 1220 Infinity G4288C, New Delhi, India), Rheodyne injector (7725 D2-0, Scientific Laboratory Supplies, New Delhi), Double Beam Spectrophotometer (Cary 5000 Version 2.24, United States).

**Chemicals and Reagents**

Ornidazole and Miconozole reference standard was kindly provided by Sigma Aldrich Laboratories, (Delhi, India). By evaluating melting point and IR spectra, its purity was assessed, and no more purification was necessary. HPLC-Grade Methanol and Water, ammonium acetate and Glacial Acetic Acid was procured from Yarrow Chem laboratories, (Mumbai, India). The commercial formulation Candifem tablet (AstraZeneca, Bangalore, India) was pic up from local retailer.

**Preparation of Standard Drug Solution**

50 mg of Ornidazole and Miconozole working standards were perfectly weighed and transferred into a 50ml clean dry standard flask, about 25 mL of methanol was added and sonicated to dissolve it wholly and final volume was increase up to the mark with methanol, the solution was then filtered through 0.45µ under vacuum filtration (Stock solution A). 5 mL of the above solution was pipetted into a 50mL standard flask and diluted up to the mark to obtain a concentration of 100 µg/ml of the two resultant solutions. The final solution was again filtered through 0.45µ under vacuum filtration (Standard solution).

**Preparation of Sample Solution**

Accurately weighed twenty Candifem tablets (500 mg of ODZ and 100 mg of MCZ) were first extracted using 15 ml of methanol by sonicating for ten minutes, and it was then filtered through Whatman No. 1 filter paper into a 50 ml standard flask. A simple two-step extraction was employed using 10mL of methanol before being passed through the same filter paper into the same standard flask. Dilute the mixture until 50µg/mL of ODZ and 10µg/mL of MCZ are present. The reference standard was used to concoct a standard drug combination with the same composition as that of the sample solution.[9]

**Method Optimization by BBD**

The AQbD approach starts with defining method objectives, evaluating risks, and identifying critical parameters. Before using BBD, OFAT trials were conducted on HPLC to select independent parameters and understand their impact on dependent variables. BBD is a response surface design that strategically places midpoints on 3D cube edges. The study used randomized experimental trials to optimize independent variables like ethanol content in mobile phase, pH, and flow rate, while focusing on dependent variables like retention time, and peak area based on BBD disability. The upper and lower limits were set using the OFAT method. The lowest and maximum bounds for the mobile phase ratio were chosen at 30% and 50%, respectively. In a similar vein, pH 4.0 was chosen as the lower bound and pH 5.0 as the upper bound for the buffer. Between 0.8 mL/min (low level) and 1.0 mL/min (high level), the flow rate range was fixed. The data generated were statistically analyzed using Design Expert (version 9.0.0.1, Stat-Ease Inc.) software. The significance of the corresponding factors was computed using ANOVA (Fischer's statistical test for the Analysis of variance) model. The DoE software recommended 17 runs of independent variables, including 5 center points and RPHPLC trials were conducted for these predicted runs.[10,11]

**METHOD VALIDATION**

The analytical method development and their validations are ongoing-interdependent process utilized to analyze newly developed drug formulations. Validation assures that an analytical method yields repeatable findings without error under a certain set of predetermined parameters. In accordance with ICH guidelines, analytical validation parameters were established for the examination of proposed technique.[12]

The system suitability was evaluated by retention time, peak area, theoretical plates and asymmetry factor. When assessing a chromatographic procedure's system suitability, typical values to examine are RSD less than 1%, asymmetric factor less than 2 and theoretical plates >2000. By assaying three samples with the same strength as Ornidazole and Miconazole, the system suitability is evaluated. the sample concentration for ODZ and MCZ were 50µg/mL and 10µg/mL respectively.

The linearity response was determined by analyzing 05 independent level of calibration curve in the range of 50-250µg/mL (50,100,150, 200, 250µg/mL) for both ODZ and MCZ. A linear relationship was drawn against mean peak area and concentration. Slope, intercept and correlation coefficient (r2) were tabulated for regression analysis of ODZ and MCZ. Detection and quantification limit were also calculated by using subsequent formula.

Precision and accuracy were evaluated using three independent QC samples in six replicates on 3 consecutive validation days. The outcomes were expressed as percent relative standard deviation (%RSD) and relative error (RE). The results for accuracy and precision falls within 15%.

The recovery study was evaluated by spiking the sample after the addition of an extra amount of standard drug (80, 100, 120 %). The analysis was carried out using an optimized mobile phase, flow rate and UV wavelength in triplicate.

## RESULTS

### Method development

The isocratic RP-HPLC method was developed for the simultaneous quantification of ODZ and MCZ and optimized by AQbD. The mobile phase, which was proven to be methanol, 5mM Ammonium acetate in the ratio 40:60v/v at pH 4.8, was delivered using a flow velocity gradient of 1.0 ml/min through a $C_{18}$ column (stationary phase) in the HPLC method, which was created using chemometric factorial design based on dependent and independent factors. A defining peak can be seen in the HPLC chromatogram at 3.721 (ORZ) and 7.450 (MCZ), as seen in the **Figure 2.**

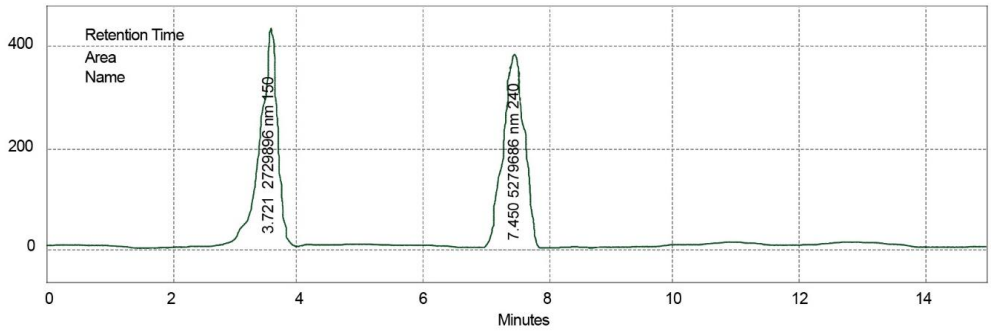



**Figure 2: HPLC chromatogram of ODZ and MCZ.**

### Assay

The optimized chromatogram Ornidazole and Miconozole showed resolved peak at retention time 3.75 and 7.45 min when performed assay from tablets. The % assay of drug content was found to be 493.95 ± 0.7411 (n = 6) for label claim of Ornidazole and 99.76 ± 0.8801 (n = 6) for Miconozole. The assay result indicated the method's ability to measure accurately and specifically in presence of excipients presents in tablet powder.

**Validation of Analytical Methods**

**Linearity and Range**

The outcomes of the linearity study (**Figure 5**) gave linear relationship over the concentration range of 50.0-250.0µg/mL for ODZ and MCZ. From the regression analysis, the linear equation was obtained: y = 23716x+2006 for Ornidazole and y =11214x+497408 for Miconozole, and the range coefficient of determination $R^2$ was 0.9-1.00 for both analytes, indicating a linear relationship between the concentration of analyte and area under the peak.

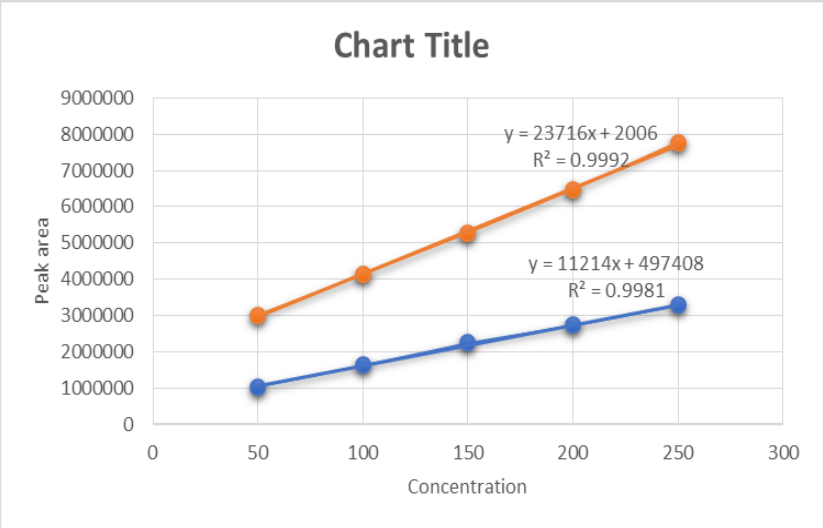



**Figure 5: Linearity Plot of ODZ and MCZ Concerning Peak Area and Concentration (HPLC).**

**LOD and LOQ**

The method showed detection limit and quantification limit as 0.2181 and 0.6609 ng/mL for ODZ, 0.1225 and 0.3711 ng/mL for MCZ, and thus indicating very high sensitivity of the developed method.

**Accuracy and Precision**

Accuracy data showed good percent recovery between 97.33 and 98.33% and % RSD value NMT 2%, confirming high degree of accuracy of the developed method. Table IV illustrates the accuracy data for various quality control samples of ODZ and MCZ. The intraday and inter-day precision showed higher values of percent recovery of ODZ and MCZ, ranging between 99.56 and 99.85 %. Further, the %RSD for assay as per repeatability and intermediate precision were well within 1%. These conclusions verified high degree of precision.

**System suitability**

The system suitability results authenticated lack of significant difference in the peak area, retention time, theoretical plates and peak tailing of ODZ and MCZ following six replicate injections. The values of %RSD and SEM were found to be <1%, thus corroborating high degree of accuracy of the chromatographic instrument.

The whole validation results are given in **Table 3.**

**Table 3: Validation Outcomes of Developed Methods.**

| Method parameter | Ornidazole | Miconozole |
|---|---|---|
| Linearity | 50-150 µg/ml | 50-150 µg/ml |
| Slope | 11214 | 23716 |
| Intercept | 49740 | 2006 |

| | | |
|---|---|---|
| $R^2$ value | 0.9981 | 0.9992 |
| LOD (ng/ml) | 0.2181 | 0.1225 |
| LOQ (ng/ml) | 0.6609 | 0.3711 |
| RSD (%) | 0.0020 | 0.0907 |
| CV | 0.0019 | 0.0090 |
| Amount obtained(mg) | 493.95 | 99.76 |
| Recovery (%) | 98.32 | 99.15 |

**DoE assisted optimization**

Method optimisation using Box-Behnken design (BBD) was carried out in order to provide a chromatographic method that was both optimised and verified. In this study, three-level BBD was employed to optimize independent variables such as mobile phase ratio (A), pH (B), and flow rate (C). The final model was chosen for further research based on the F-value, P-value, coefficient of correlation (R), predicted error sum of squares (PRESS) and lack of fit.

The data was statistically analysed. The standard error graph in **Figure 3** was used to assess the validity of the proposed design. The standard error of prediction values for each section of the design space were displayed in this graph. Getting comparatively low standard error values near 1 or less throughout the region of interest was adequate. The findings showed that the standard error varied from 0.447 to 0.866, suggesting that the design may be predicted with efficiency.

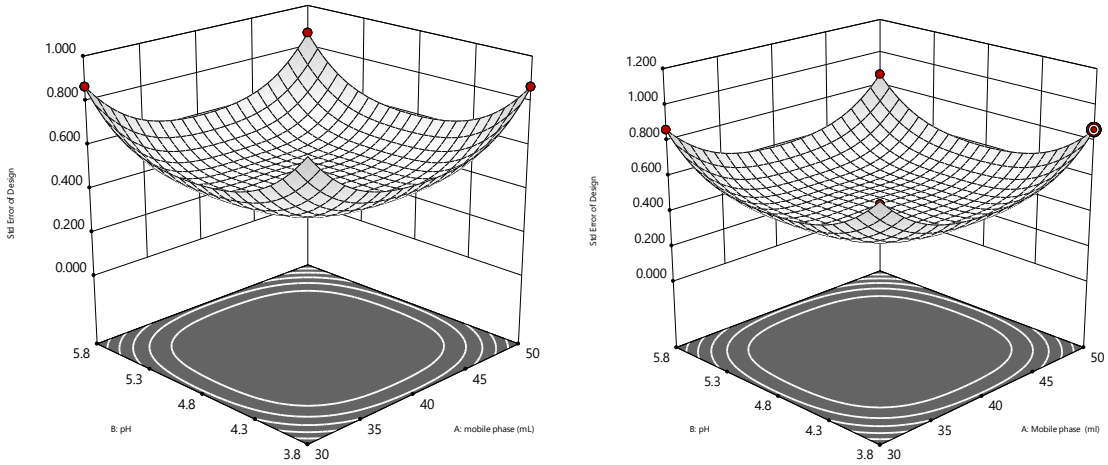



**Figure 3: Standard error graph of BBD in 3D view of Ornidazole and Miconozole.**

By applying multivariate regression analysis, a fitted quadratic model was shown to be significant for both responses, namely retention time (R1) and %recovery (R2). ANOVA analysis for both analytes also proved that the regression model statistically significantly predicts the outcome variable ($P < 0.05$). The values of P and F in ANNOVA results were shown in **table 2.**

**Table 1: ANNOVA F and P values of Ornidazole.**

| | P values | | F-Values | |
|---|---|---|---|---|
| | **R1 ODZ** | **R3 ODZ** | **R1 ODZ** | **R3 ODZ** |
| **Model** | 0.0102 | 0.0310 | 5.70 | 4.05 |
| **A-mobile phase** | 0.0014 | 0.0427 | 16.38 | 5.04 |
| **B-pH** | 0.6971 | 0.0196 | 0.1584 | 7.08 |
| **C-Flow rate** | 0.4630 | 0.9245 | 0.5719 | 0.0093 |
| **Lack of Fit** | 0.8185 | 0.8664 | 0.5053 | 0.4280 |

***R1- Retention time, R3-%responșe**

**Table2: ANNOVA F and P values of MCZ.**

| | P values | | Fvalues | |
|---|---|---|---|---|
| | **R2 ODZ** | **R4 ODZ** | **R2 MCZ** | **R4 MCZ** |
| **Model** | 0.0076 | 0.0454 | 7.40 | 3.82 |
| **A-mobile phase** | 1.000 | 0.0239 | 0.0000 | 8.25 |
| **B-pH** | 1.000 | 0.1208 | 0.0000 | 3.12 |
| **C-Flow rate** | 0.0026 | 0.0449 | 20.93 | 05.94 |
| **AB** | 0.0254 | 0.7227 | 8.01 | 0.1365 |
| **AC** | 0.6980 | 0.5488 | 0.1636 | 0.3968 |
| **BC** | 0.2644 | 0.3036 | 1.47 | 1.23 |
| **$A^2$** | 0.0007 | 0.0264 | 32.31 | 7.85 |
| **$B^2$** | 0.3717 | 0.0243 | 0.9107 | 8.18 |
| **$C^2$** | 0.1686 | 0.07591 | 2.36 | 0.1017 |
| **Lack of Fit** | 0.8789 | 0.9703 | 0.2174 | 0.0750 |

***R2- Retention time, R4-%recovery**

The R1 and R2 responses for ODZ and MCZ had model p-values in the range of 0.0075 and less than 0.0001, respectively. This indicated that, regardless of experimental mistakes or chance, the independent variables had a substantial impact on the tested responses. Furthermore, higher F-ratio values would support this instance, but lower values would indicate more model error. Additionally, non-significant values of lack of fit were good and tailored the suitable model, which could be used to examine the model's efficiency based on their p-values. Fit statistics values are detailed in table 2. The adjusted R2 values predicted the goodness of fit of model. The adequate precision value is a measure of signal to noise ratio which should be greater than 4. In the present study, the ratio was found between 7.1089 and 58.3538, indicating an adequate signal and significant model.

**Table 4: ANNOVA Fit statistics.**

| | **R1 ODZ** | **R2 MCZ** | **R3 ODZ** | **R4 MCZ** |
|---|---|---|---|---|
| **Model** | Linear | Quadratic | Linear | Quadratic |
| **Std. Dev.** | 0.0266 | 0.0012 | 4.72 | 2.84 |
| **Mean** | 3.77 | 7.45 | 7.51 | 89.30 |
| **C.V. %** | 0.7076 | 0.0166 | 0.0281 | 3.18 |
| **$R^2$** | 0.5682 | 0.9049 | 0.9986 | 0.8309 |
| **Adjusted $R^2$** | 0.4685 | 0.7825 | 0.9968 | 0.6136 |
| **Predicted $R^2$** | 0.3904 | 0.6588 | 0.9821 | 0.6059 |
| **Adeq Precision** | 7.0014 | 9.4913 | 57.4501 | 9.1172 |
| **PRESS** | 0.0130 | 0.0000 | 0.0004 | 0.0068 |

The polynomial equations for responses R1 and R2 in terms of coded factors are as follows:

$$R1\ ODZ = +3.61688 + 0.003812 * mobile\ phase + 0.007500 * pH - 00035625 * flow\ rate$$

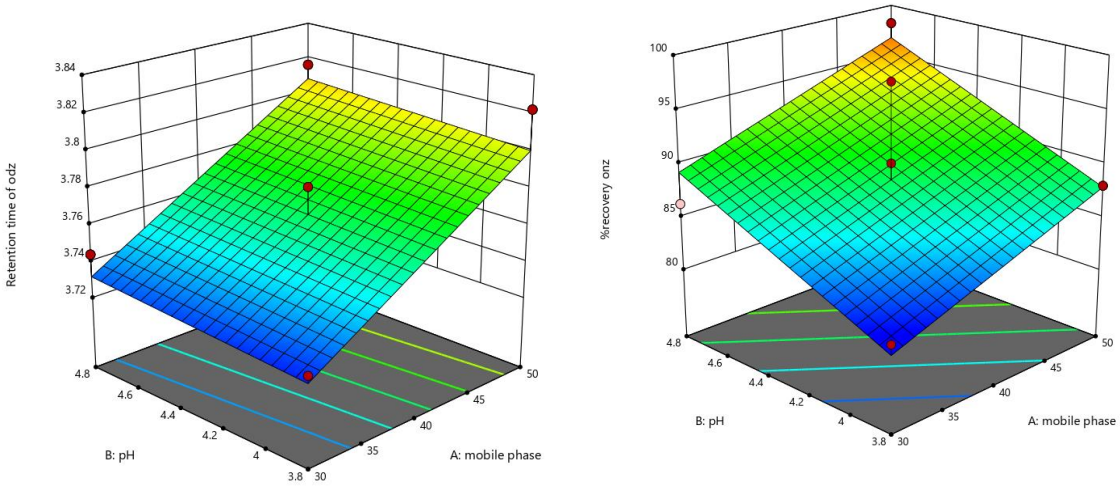
$$R2\ MCZ = +7.53075 - 0.004370 * moble\ phase + 0.013280 * pH - 0.029000 * flow\ rate + 0.000350 * mobile\ phase * pH + 0.000125 * mobile\ phase * flow\ rte - 0.007500 * pH * flow\ rate + 0.000034 * mobile\ phase2 - 0.002300 * pH2 + 0.023125 * flow\ rate2$$

$$R3\ ODZ = +36.18869 + 0.34875 * mobile\ phase + 8.88500pH - 0.806250 * flow\ rate$$

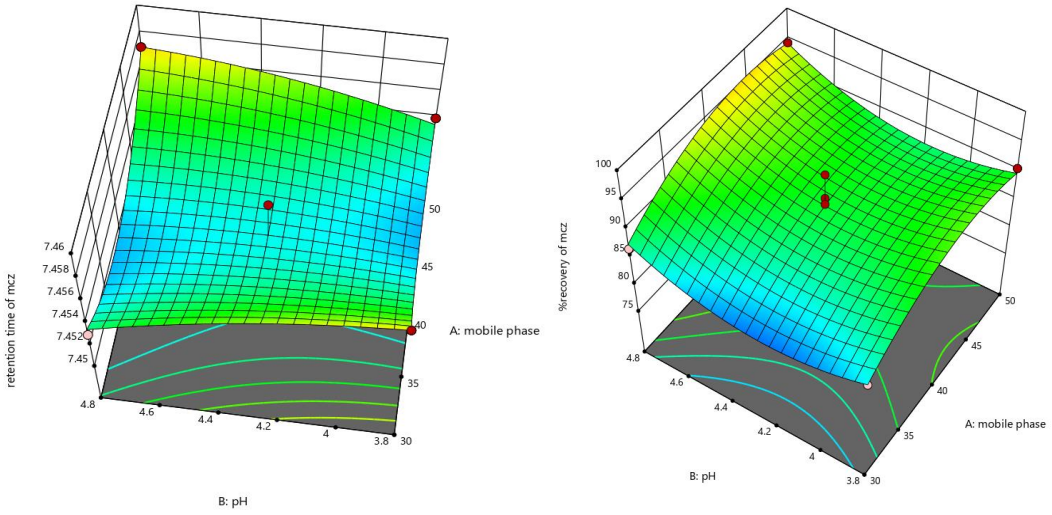
$$R4\ MCZ = +359.97128 + 3.38910 * mobile\ phase - 152.71170 * pH - 59.77625 * flowrate + 0.105000 * mobile\ phase * pH - 0.447500 * mobile\ phase * pH + 15.7500 * pH * flow\ rate - 0.038807 * mobile\ phase2 + 15.84700pH2 + 11.04375 * flow\ rate2$$

According to the regression equation, the positive sign in the equation indicates synergistic effects and the negative sign means antagonistic effects on the studied response.

The difference between the variables were further elucidated by constructing three-dimensional surface response plots using the responses that were acquired after completing the aforementioned trial runs **(figure 4).** The impact of critical method parameters on the critical analytical attributes was demonstrated by these graphs. These plots were analysed in order to determine which procedure parameter produced the most satisfactory results. As a result, the method's final critical parameters and optimal chromatographic conditions were established based on these data.




**4a** **4b**




**4c** **4d**

**Figure 4: 3D- surface response plats illustrate how the independent variable affects (4a, 4b) the retention time and %recovery of ODZ, (4c,4d) the retention time and %recovery of MCZ.**

**Greenness assessment of the proposed method**

The DoE-based analytical approach promotes green analytical chemistry (GAC) since it requires fewer optimisation experiments. We developed a figure **(Fig.6)** that shows the environmental friendliness using AGREEprep software. For the detection of ODZ and MCZ, the recommended HPLC method with an overall AGREE score of 0.76 was found to be ecologically acceptable.


**Figure 6: AGREEprep pictogram of the developed HPLC.**

## DISCUSSION

An analysis of the correlation showed that, for every response, the difference between the adjusted and predicted R2 values was less than 0.2, falling within the bounds set by the software's approval requirements. This suggests that responses have a strong relationship. A signal-to-noise ratio greater than four suggested accurate model fitting. Strong correlation between experimental data and fitted models was demonstrated by low percentage coefficient of variance and high adjusted R2 values. The correctness of the prediction model was confirmed by the good alignment between the predicted and experimental R2 values. The relationship between the independent and dependent variables is displayed in the final optimised equation. Negative numbers represented the opposite effect, while positive ones indicated a favourable influence. Factor effects were shown by response surfaces and modification plots; nominal changes were displayed by perturbation plots, and the curvature of the plot indicated the degree of change.

The quality-by-design approach successfully developed the HPLC method for concurrent estimation of Ornidazole and Miconozole. The optimized RP-HPLC method for determination of ceftriaxone sodium used Phenomenex C18 column (250 × 4.6 mm, 5 µm particle size) and mobile phase consist of methanol and ammonium phosphate buffer, 60:40 v/v, pH adjusted to 4.8. The retention time for Ornidazole and Miconozole was found to be 3.75 and 7.450 min respectively. The method was linear in the range of 50–250 µg/ml with 0.9981 and 0.9992 correlation coefficient. The % RSD for repeatability, intraday, and inter day precision was found to be less than 2% indicating the optimized method was precise. The % recovery of spiked samples was found to be 97.33 and 98.33% as per the acceptance criteria of the ICH guidelines. The method was developed as per the ICH guidelines

## CONCLUSION

The study focusses on the analytical target product profile and outlines a quality-by-design approach to HPLC method development. The mobile phase, pH and flow rate, three essential HPLC technique components, are investigated and optimised. When the procedure was applied to concurrent quantification of Ornidazole and Miconozole, the validated

parameters were determined to be robust, rugged, linear, specific, and accurate. The risk of failure during validation and transfer is lessened by the QbD technique. In a shorter amount of time, the automated QbD method creation with Design Expert software yields an extra reliable technique. The QbD method hastes the performance and lowers the likelihood of failure. The technique is durable, precise, selective, and repeatable, making it appropriate for regular quality control in the pharmaceutical sector.

**Conflicts of Interest**

The authors declares that there are no conflicts of interest.

**ACKNOWLEDGE**

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**ABBREVIATIONS**

DoE- design of Experiment, QbD- Quality based design, BBD- Box Behnken Design, RP-HPLC- Reverse phase High Performance Liquid Chromatography, UV- Ultraviolet, R1, R2- Responses of retention time and peak area, ODZ-Ornidazole, MCA- Miconozole, ICH- International Council for Harmonization, LOD- Limit of Detection, LOQ-Limit of Quantification, NMT- Not More Than, %RSD- Percent Relative Standard Deviation, QC- Quality Control, SD-Standard Deviation, CV- Coefficient of Variance.

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