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RESEARCH ARTICLE

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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR NEWLY SYNTHESIZED 3,6-DICHLORO-6-{[(6-METHOXY-1,3-BENZOTHIAZOL-2-YL)IMINO]METHYL}PHENOL

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Abstract

This study presents the development and validation of a reverse-phase high-performance liquid chromatography (RP HPLC) method for the analysis of 3,6-dichloro-6-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}phenol (DCMBT). The analysis was conducted on a HYPERSIL column (250 x 4.6 mm, 5 µm) using a mobile phase consisting of Acetonitrile: Water (80:20v/v) at a flow rate of 0.8ml/min. The detection was performed using UV at a wavelength of 249 nm. The DCMBT had a retention time of 5.742 minutes. The compound DCMBT exhibited a direct relationship between its concentration and response within the range of 4-24 ppm. The correlation coefficient of DCMBT was 0.999. The created technique underwent validation for linearity, accuracy, precision, selectivity range, force degradation study, and robustness. The results of the validation confirmed that the method is exact, accurate, linear, and specific. The approach was verified by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) criteria. The relative standard deviation (RSD) for both intra-day and inter-day precision was determined to be below 2%. The percentage recoveries achieved for isoeugenol DCMBT fall within the range of 98.74 - 101.11%, with an overall mean recovery of 99.59%. These results demonstrate a high level of agreement with the labeled amount in the pharmaceutical formulations.

Keywords: Method Validation, Analytical method development, High-performance liquid chromatography, pharmaceutical formulation.

1. INTRODUCTION:

Analytical Chemistry is a scientific discipline that uses advanced technologies to determine the composition of substances through analytical techniques. Researchers can attain outcomes that are both of high quality and measurable in quantity. Analytical devices are crucial in the pursuit of obtaining accurate and dependable analytical results of superior quality. Therefore, it is imperative for all individuals working in the analytical laboratory to priorities the quality assurance of equipment. The analytical method may include spectroscopic, chromatographic, electrochemical, hyphenated, or other techniques. Analytical method development involves the careful selection of a precise test approach to accurately assess the composition of a formulation. It is the process of

validating an analytical method to ensure its suitability for measuring the concentration of successive samples in a laboratory setting. The utilization of analytical techniques is necessary for GMP and GLP settings, and their development should adhere to the protocols and acceptance criteria outlined in the ICH recommendations Q2(R1).

This paper details the efforts made to establish a novel analytical technique that maintains the gradient programmed and column but employs acetonitrile and water as the mobile phase rather than methanol, and the experimental section reports the most important data.

2. MATERIALS AND METHODS

A. Chemicals and reagents

The solvents used were of high-performance liquid chromatography/analytical reagent grade. The in-house preparation uses a standard sample of DCMBT that has undergone double purification. Additionally, the in-house production also uses a technical grade of DCMBT.

B. Chromatographic conditions

The study used a High-Performance Liquid Chromatography (HPLC) system, namely the Shimadzu LC-20AD with a PDA detector. The HPLC system was equipped with a HYPERSIL column (C18, 250 mm x 4.6 mm x 5.0 µ) for the analysis. The HPLC system was outfitted with Empower software for data processing. The elution process was carried out using a mixture of acetonitrile and water in a ratio of 80:20 (v/v), with a flow rate of 0.8 ml/min. Measurement was conducted at a wavelength of 249 nm.

C. Standard stock solution

A stock solution was made by precisely weighing approximately 50 mg of DCMBT standard. Each component was then put into individual 20 ml volumetric flasks. 5 ml of acetonitrile was added to each flask, followed by sonication for 10 minutes. The volume was then adjusted to 20 ml using acetonitrile, resulting in stock solution-I. Take 5 ml of the initial stock solution and transfer it to a 50 ml volumetric flask. Fill the remaining volume of the flask with acetonitrile, which will be labelled as stock solution II.

D. Sample stock solution

A total of twenty tablets were measured and pulverized into a fine powder. The tablet powder containing 50 mg of DCMBT was placed into a 10 ml volumetric flask and dissolved in 5 ml of acetonitrile. The suspension underwent sonication for a duration of 15 minutes. Ultimately, the volume was adjusted to the desired level by adding acetonitrile. The solution underwent filtration using a 0.4 µm membrane filter paper. Dispense 5 ml of the aforementioned solution into a 50 ml volumetric flask and fill the remaining amount with acetonitrile.

E. Calibration curve

To prepare DCMBT, measured amounts were transferred from a standard stock solution into a set of 100 ml volumetric flasks. The volume was adjusted with acetonitrile to get a variety of solutions with concentrations ranging from 4 to 24 ppm for DCMBT. Distinct dilutions of every concentration of each medication were made individually. Using the duplicate solutions, 20µl injections of each concentration of DCMBT were individually injected into the RP-HPLC system and subjected to chromatography under the specified conditions. The assessment of DCMBT was conducted using a UV detector set at a wavelength of 249nm. The regions corresponding to each peak were measured and plotted against the concentrations to generate the standard calibration curve.

F. Method Validation

The approach underwent validation for the parameters of system suitability, linearity, precision, accuracy, selectivity, range, robustness, and force degradation tests.

1) Specificity

Specificity refers to the capacity to accurately determine the analyte in the presence of other anticipated components. Commonly, these substances may encompass contaminants, degradants, matrix components, and so on. The specificity was determined by scanning the diluent solution and

the standard solution of DCMBT at a concentration of 20µg/ml. After injecting a solvent blank, reagent blank, and sample blank, derivatized solutions of the DCMBT standard and sample were injected into the chromatographic system. This was done to show that there is no interference from any reagent or solvent blank at the retention time of DCMBT.

2) Linearity

Solutions for the linearity test of the assay technique were prepared by diluting a stock solution to various concentration levels of the assay analyte (4, 8, 12, 16, 20, and 24 ppm). 20 microliters of each solution were injected into the HPLC apparatus, and the peak area of the resulting chromatogram was recorded. The data on peak area and concentration was analysed using the method of least squares linear regression. The provided values include the slope and y-intercept of the calibration curve.

3) Precision

The precision of the proposed method was evaluated by analyzing six replicates of a fixed concentration of the DCMBT within the linear range. This analysis was conducted on the same day and different days, with different analysts and columns.

4) Accuracy (Recovery studies)

The method's accuracy was assessed by recovery trials. A predetermined concentration of the reference standard was introduced into the constant concentration of the previously analyzed solution. The % recovery was determined by comparing the region before and following the introduction of the operating standard. Both medications underwent recovery using identical methods. The standard addition procedure was conducted at levels of 20%, 60%, 80%, 100%, and 120%, and the percentage recovery was determined.

5) Robustness

The robustness research was conducted by implementing minor alterations to the flow rates. There was no discernible effect on the duration of retention and the tailing factor.

3. RESULTS AND DISCUSSION

A reversed-phase chromatographic approach was devised for the isolation of DCMBT. The compound DCMBT was effectively separated on a HYPERSIL RP C18 column with dimensions of 250 x 4.6 mm and a particle size of 5 µm. The separation was achieved using a mobile phase consisting of acetonitrile and water at a ratio of 80:20 (v/v), as shown in Figure 1. The volumetric rate of fluid flow was 0.8 ml/min, and the detection was performed at a wavelength of 249 nm. In summary, the data showed that the excipients did not affect the DCMBT peaks, which indicates that the approach is selective. The analytes were fully separated in under 10 minutes.

A. HPLC method development and optimization

The samples were initially analyzed using a mobile phase consisting of a mixture of acetonitrile and water at a ratio of 80:20 (v/v). The analysis was conducted at a flow rate of 0.8 mL/min. Under these circumstances, the peaks exhibited clear separation with excellent symmetry and sharpness. Hence, the mobile phase consisting of a mixture of acetonitrile and water at a ratio of 80:20v/v was selected as the optimal choice for achieving the most favorable chromatographic results throughout the entire study.

B. Method validation System suitability

A system suitability test was conducted to verify the compatibility of the entire testing system for its intended purpose. The observed parameters included peak area, retention time, tailing factor, and theoretical plates. The peak area exhibited a variation of less than 2.0 in all measurements. The average retention duration was precisely 5.74 minutes. The theoretical plates exceeded 2000, and the tailing factor for the DCMBT peak was below 2. The proposed approach provides exceptional sensitivity and enables precise detection of the peak. The DCMBT peak was consistently distinct from the excipients in all instances.

C. Specificity:

The DCMBT had a retention time of 5.74 minutes. There were no interfering peaks from the blank at the same retention time, indicating that the suggested approach is specific for determining DCMBT [20-23].

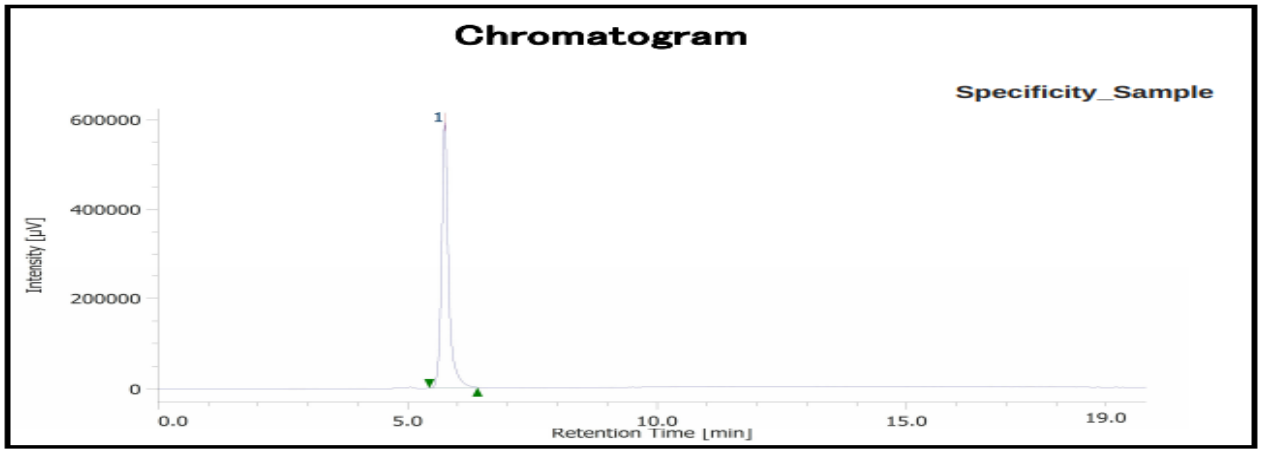

Figure 1: Specificity peak purity chromatogram of DCMBT

D. Linearity

The calibration curve for DCMBT exhibited linearity within the concentration range of 4-24ppm. The peak area data of the DCMBT was subjected to linear regression analysis (**Table 1**). The regression equations for the calibration curves (**Figure 2**) were determined to be y = 292132x - 52790 (**Figure 2**), with a correlation coefficient of 0.9999, which is close to unity.

Table 1: Linearity data of DCMBT standard [24]

Linearity Solution Level	Replications	Peak Area Counts	Means Area
L1	R1	1154876	1152441.5
	R2	1150007	
L2	R1	2284211	2283585.5
	R2	2282960	
L3	R1	3465205	3464652.5
	R2	3464100	
L4	R1	4618858	4618183.5
	R2	4617509	
L5	R1	5833185	5825213.5
	R2	5817242	
L6	R1	7030736	7025546.5
	R2	7020357	

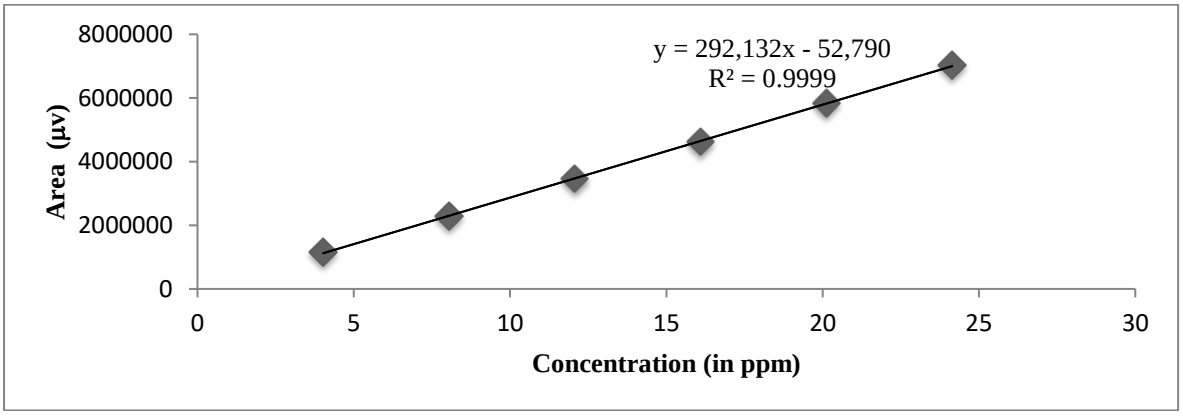

Fig.2: Linearity graph of DCMBT standard

E. Precision

The method's precision was assessed by examining the repeatability of the injector (intra-day precision) and the intermediate precision (inter-day precision) of the standard solutions containing DCMBT. Repeatability and intermediate precision were determined by analyzing six separate samples prepared from a single batch of formulation, both on the same day and on different days. The relative standard deviation (RSD) for both intra-day and inter-day precisions was determined to be less than 2%, demonstrating a high level of precision.

Table 2: Injection precision for DCMBT.

Sample no.	**Conc. in ppm**	**Area (mv)**	**% Content**
Sample-1	20.32	5791853	99.93
Sample-2	20.36	5796396	99.81
Sample-3	20.42	5817114	99.88
Sample-4	20.40	5830713	100.21
Sample-5	20.30	5780994	99.84
Sample-6	20.40	5805699	99.78
Average	**NA**	**NA**	**99.91**
STDEV	**NA**	**NA**	**0.16**
% RSD	**NA**	**NA**	**0.16**

Table 3: Intraday precision data of DCMBT technical.

Sample no.	**Conc in ppm**	**Area (□v)**	**% Content**
Sample-1	20.02	5854048	99.84
Sample-2	20.10	5878601	99.86
Sample-3	20.20	5869923	99.22
Sample-4	20.23	5894371	99.49
Sample-5	20.11	5832468	99.03
Sample-6	20.05	5867483	99.92
Average	**NA**	**NA**	**99.56**
STDEV	**NA**	**NA**	**0.37**
% RSD	**NA**	**NA**	**0.38**

Table 4: Comparison between analyst-1 and 2

	Mean % Content	**Absolute Difference**
Analyst 1	99.91	0.35
Analyst 2	99.56	

F. Accuracy

The method's accuracy was validated by the recovery test. An established quantity of DCMBT standard was introduced into portions of sample solutions and subsequently diluted to achieve the desired concentrations, as outlined in **Table 5**. The % relative standard deviation (RSD) was determined to be less than 2.0. The recovery rate for DCMBT ranged from 98.74% to 101.11%, with an overall mean recovery rate of 99.59%. This suggests that the approach is unaffected by any positive or negative interferences from the blank. Based on the aforementioned outcome, it was determined that the analyte's recovery data falls within the acceptable range, indicating the suggested method's accuracy.

Table 5: Accuracy data for DCMBT technical.

% Recovery					
Level (%) / pptn	**Smpl Wt (in mg)**	**Conc. (in ppm)**	**Area (mv)**	**% Recovery**	**% Mean Recovery**
20_1	4.02	4.02	1153876	98.74	99.26
20_2	3.96	3.96	1151152	100.00	
20_3	4.01	4.01	1154450	99.04	
60_1	12.04	12.04	3464265	98.98	99.31
60_2	12.01	12.01	3463840	99.22	
60_3	11.95	11.95	3464384	99.73	
80_1	16.02	16.02	4615098	99.10	98.92
80_2	16.15	16.15	4650942	99.07	
80_3	16.11	16.11	4617318	98.60	
100_1	20.05	20.05	5827585	99.99	99.92
100_2	20.14	20.14	5836985	99.70	
100_3	19.98	19.98	5812639	100.08	
120_1	24.31	24.31	7029200	99.47	100.43
120_2	24.47	24.47	7192099	101.11	
120_3	24.56	24.56	7190309	100.72	

G. Robustness

An analytical procedure's robustness is determined by its resistance to minor and intentional changes in technique parameters, which serves as an indicator of its reliability for regular analysis. The method's robustness was assessed by analyzing the same material using intentionally altered analytical circumstances, deviating from the original condition. The flow rate was adjusted to 0.75 and 0.85 mL/min with a tolerance of ± 0.05 mL/min. The assay results remained unaffected by the variation in circumstances and were consistent with the results obtained under the original settings. The relative standard deviation (RSD) value of the assay, assessed for the same sample under both original and robustness conditions, was found to be less than 2.0%. This suggests that the established method is robust.

4. CONCLUSION

The HPLC method that was suggested underwent validation according to the guidelines set by the ICH. This approach was then used to determine the presence of DCMBT. The approach was determined to possess accuracy, precision, robustness, and specificity. The chromatographic elution process is performed rapidly, typically taking less than 6 minutes. No interference was seen from any constituents of the medicinal dose form. Overall, the suggested approach exhibits excellent sensitivity, strong selectivity, precise precision, and consistent reproducibility, making it well-suited for the simultaneous detection of DCMBT.

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