"RP-HPLC ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR NEWLY SYNTHESIZED 4-{[(6-METHOXY-1,3-BENZOTHIAZOLES-2-YL)IMINO]METHYL}BENZENE-1,4-DIOL"

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Abstract:
The analytical method for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol (MBTIMB) was developed and validated using reverse-phase high-performance liquid chromatography (RP-HPLC). The determination was carried out on a HYPERSIL column with a mobile phase consisting of acetonitrile and water in an 80:20v/v ratio, flowing at a rate of 0.5 ml/min, and UV detection at 254nm. The retention time for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was measured at 8.142 minutes. The method exhibited a linear response for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol in the concentration range of 4-24 ppm, with a high correlation coefficient (r-value) of 0.9993. Validation of the developed method included assessments of precision, accuracy, linearity, selectivity, range, and force degradation study. The results indicated that the method was specific, accurate, linear, and precise. The relative standard deviations (RSD) for injector repeatability and inter-assay precision were found to be greater than 2%. The percentage recoveries for 4-{[(6-methoxy-1,3-benzothiazole-2-yl)imino]methyl}benzene-1,4-diol ranged from 98.87% to 100.65%, with an overall mean recovery of 100.02%

Keywords: Method validation, linearity, accuracy, precision, inter-assays precision.

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INTRODUCTION:

In the realm of analytical chemistry within the scientific domain, the assessment of composition relies heavily on cutting-edge technologies encompassing analytical techniques [1-5]. These advanced methodologies play a pivotal role in the utilization of analytical instruments, ensuring the attainment of dependable and superior-quality analytical data in the investigative process.A critical aspect of analytical method development involves the meticulous selection of an accurate assay procedure, integral for determining the composition of formulations. This process is indispensable in crafting a reliable analytical method that facilitates the precise identification and quantification of various components within a given sample.Furthermore, the analytical method validation process assumes a paramount significance, as it involves substantiating the method's acceptability for measuring concentrations in laboratory settings, particularly in the analysis of subsequent samples. The validation process serves as a rigorous confirmation of the analytical method's efficacy and suitability for use in obtaining accurate and meaningful results [6-7].In the specific context of instrumental reverse-phase high-performance liquid chromatography (RP-HPLC), adherence to Good Laboratory Practices (GLP) and Good Manufacturing Practices (GMP) is imperative. The development of such analytical methods within these controlled environments [8-11] ensures not only the precision and accuracy of the analyses but also compliance with established quality standards. This underscores the need for rigorous adherence to protocols, guaranteeing the reliability of analytical results and the credibility of the entire analytical process.

EXPERIMENTAL:

The experiment utilized HPLC/AR grade solvents. The standard sample of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was prepared in-house using a double purification process. Additionally, in-house manufacture of the 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol technical grade was also used.

Chromatographic conditions

The study utilized an HPLC system consisting of a Shimadzu LC-20AD with a PDA detector. The system employed a HYPERSIL column (C18, 5.0 µ x 250 x 4.6mm) for the analysis. The Empower software was utilized for data processing on an HPLC system. Isocratic elution was performed using a mobile phase consisting of acetonitrile and water in a ratio of 80:20 (v/v). The flow rate was set at 0.5 ml/min and detection was conducted at a wavelength of 254 nm.

A stock solution of standard

A stock solution of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol standard was made by accurately weighing 100mg of the standard compound. 5ml of acetonitrile was added, and the solution was then diluted to a final volume of 20mL with acetonitrile (stock solution I). Transfer 5 ml of the solution mentioned above into a 50 ml volumetric flask and dilute it with acetonitrile up to the mark. Label this solution as stock solution II.

Stock solution of sample

The 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol sample was measured to be 100 mg and then diluted to a volume of 10 mL using acetonitrile. Transfer 5 ml of the solution into a 50 ml volumetric flask and dilute it up to the mark with acetonitrile.

Calibration curve

The 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol is measured by pipetting a reference solution into a 100 ml volumetric flask. The concentration range of 4, 8, 12, 16, 20, and 24 parts per million (ppm) was prepared by diluting 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol with acetonitrile up to the mark. Individual dilutions of each medication were made independently for each concentration. Each concentration of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was independently injected into the RP-HPLC system using 20µl injections from the duplicate solutions. The chromatography was conducted under the conditions stated above. The evaluation of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was conducted using a UV detector at a wavelength of 340nm, as described [14-15].

Method Validation:

The approach is validated for several aspects including robustness, force degradation, system appropriateness studies, precision, selectivity, accuracy, range, and linearity.

Specificity

The specificity was determined by scanning the diluent solution and the standard solution of 4-{[(6-

methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol, which had concentrations of 20µg/ml. Derivatized solutions containing 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol were injected into the chromatographic system, along with a solvent blank, reagent blank, and sample blank. This was done to confirm that there is no interference from any reagent or solvent blank at the retention time of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol.

Linearity:

The linearity test solutions were prepared by diluting a stock solution to different concentrations, specifically 4, 8, 12, 16, 20, and 24 ppm. The assay method was used to determine the concentration of these solutions. A volume of 20µl from each solution was injected into the HPLC system, and the peak area was recorded from the resultant chromatogram. The data on peak area and concentration was analyzed using the method of least squares linear regression. The calibration curve was reported to have a y-intercept and slope.

Precision

The precision of the proposed method, including intra-day precision and injector repeatability, was determined by analyzing six replicates of a fixed concentration of the 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol mixture. The linearity range of the mixture was determined on different days and under different conditions, such as with different analysts and columns.

Accuracy (Recovery studies)

The percent recovery was determined by comparing the region before and following the introduction of the operating standard. Both medications underwent the same method of recovery. The standard addition procedure was conducted at levels of 20%, 60%, 80%, 100%, and 120%, and the percentage recovery was determined.

RESULTS AND DISCUSSION:

The RP-HPLC technique was used to develop and validate the separation of the molecule 4-{[(6-methoxy-1,3-benzothiazol-2-
is specific. This specificity was confirmed in previous studies [20-22].

zl)imino]methyl}benzene-1,4-diol. The separation was achieved using a HYPERSIL RP C18 column and a mobile phase consisting of acetonitrile and water at a ratio of 80:20 (v/v). The flow rate was set at 0.5 ml/min and the detection wavelength was 254 nm. In summary, the presence of excipients did not affect the detection of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol peaks,
confirming the method's selectivity. The analytes were completely separated within a time frame of less than 10 minutes.

HPLC method optimization and development:

The initial analysis involved examining the mobile phase, which consisted of a mixture of water and acetonitrile at a ratio of 20:80 (v/v), with a flow rate of 0.5 mL/min. Under these circumstances, peaks are clearly distinguished with excellent clarity and symmetry. Hence, a mobile phase consisting of a mixture of acetonitrile and water in a volumetric ratio of 80:20 was selected for the entire investigation due to its optimal chromatographic performance.

System suitability studies of method validation:

The system suitability tests were conducted to confirm that the system is suitable for the intended purpose. Upon conducting measurements, it was observed that the peak at 8.142 minutes had an average retention duration and a peak area variation of less than 2. The tailing factor was also less than 2, and more than 2000 theoretical plates were observed for the 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol peak. The proposed approach gives a high level of sensitivity, allowing for reliable detection of the peak. The 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was
successfully isolated from the peak in all cases, along with the excipients.

Specificity:

The 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol compound had a retention time of 8.142 minutes. There were no interfering peaks observed from the blank at the same retention period, indicating that the proposed method for determining 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol

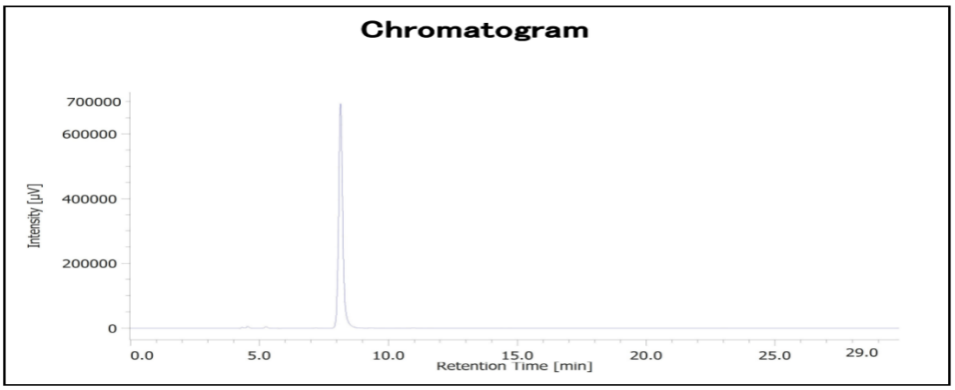

Figure-1: *Specificity peak purity chromatogram of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol*

Linearity

The linear calibration curve for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was seen within the concentration range of 4.036–24.216ppm. The peak area data for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol in the treated samples was analyzed using linear regression (Table-1) and calibration curves (Figure-2). The regression equation for the calibration curve was found to be Y = 338,743.39X + (446173.300) (Figure-2), with a coefficient of correlation of 0.9993, indicating a strong positive relationship.

Table-1: *Linearity data of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol standard*[24]

Linearity Sol Level	**Conc ppm**	**Replications**	**Peak Area Counts**		**Means Area**	
L1	4.036	R1	1768272		1768070	
		R2	1767868			
L2	8.072	R1	3206990		3204605	
		R2	3202220			
L3	12.108	R1	4631850		4630152.5	
		R2	4628455			
L4	16.144	R1	5907180		5907154.5	
		R2	5907129			
L5	20.18	R1	7183655		7179955	
		R2	7176255			
L6			24.216	R1	8697525	8697638
				R2	8697751	

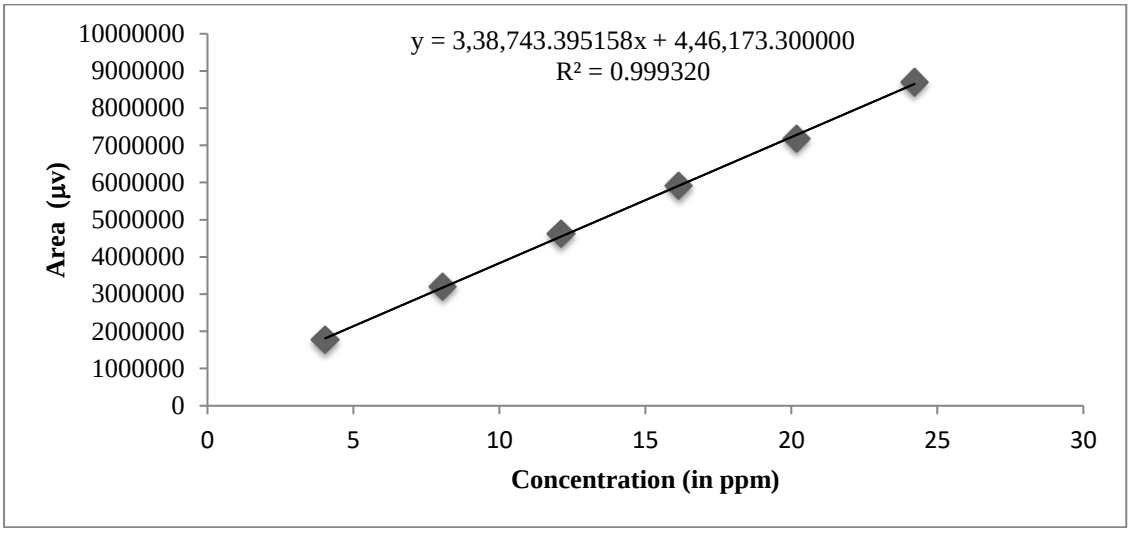

Figure-2: *Linearity graph of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol standard*

Precision

The precision of the procedure was determined by assessing the intra-assay and injector repeatability of the 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol standard solutions. The % RSD for repeatability and intra-assay precision was less than 2%, indicating a good level of precision.

Table 2: *Injection repeatability (precision) for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol.*

Sample no.	Conc in ppm	Area (mv)	% Content
Sample-1	20.09	7144269	99.79
Sample-2	20.12	7144538	99.64
Sample-3	19.95	7132033	100.32
Sample-4	20.02	7143383	100.12
Sample-5	20.06	7103493	99.37
Sample-6	20.12	7134097	99.50
Average	NA	NA	99.79
STDEV	NA	NA	0.37
% RSD	NA	NA	0.37

Table-3: *Intra-assay (precision) data of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol technical.*

Sample no.	Conc in ppm	Area (mv)	% Content
Sample-1	20.18	7144636	99.44
Sample-2	20.16	7144277	99.54
Sample-3	20.25	7144368	99.10
Sample-4	20.09	7144715	99.89
Sample-5	20.22	7144215	99.24
Sample-6	20.01	7134097	100.14
Average	NA	NA	99.56
STDEV	NA	NA	0.39
% RSD	NA	NA	0.40

Table-4: *Comparison between analyst-1 and 2*

	Mean % Content	Absolute Difference
Analyst 1	99.79	0.23
Analyst 2	99.56	

Accuracy

The recovery consequent % RSD for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was found to range between 98.87% to 100.65%, with an overall mean recovery of 100.02%. 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 is within the acceptable range, indicating the accuracy of the suggested method [26].

Table-5: *Accuracy data for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol technical.*

Level (%) / pptn	Smpl Wt (in mg)	Conc (in ppm)	Area (mv)	% Recovery	% Mean Recovery	STDEV	% RSD
20_1	4.09	4.09	1468000	100.46	99.87	0.52	0.52
20_2	4.13	4.13	1467868	99.48			
20_3	4.12	4.12	1467125	99.67			
60_1	12.11	12.11	4301851	99.43	99.25	0.26	0.26
60_2	11.96	11.96	4228455	98.96			
60_3	11.91	11.91	4228422	99.37			
80_1	16.11	16.45	5907180	100.51	100.65	0.54	0.54
80_2	16.09	16.33	5907159	101.25			
80_3	15.9	16.50	5907000	100.20			
100_1	20.13	20.13	7183655	99.88	99.80	0.08	0.08
100_2	20.14	20.14	7176255	99.73			
100_3	20.15	20.15	7183676	99.78			
120_1	24.12	24.12	8691325	100.86	100.50	0.50	0.50
120_2	24.17	24.17	8697461	100.72			
120_3	24.36	24.36	8697517	99.93			
Overall % Recovery					100.02		
Overall STDEV					0.38		
Overall % RSD					0.38		

Range:

The range of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol is assessed to be from 4 ppm (20% concentration) to 24 ppm (120% concentration).

Table-6: *Range for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol*

Solution	20% (4 ppm)	120% (24 ppm)
1	1768272	8697525
2	1767868	8697497
3	1767121	8697676
4	1742242	8697240
5	1768990	8697232

6	1767340	8697254
Average	**1763639**	**8697404**
STDEV	**10503.67**	**187.75**
% RSD	**0.6**	**0**

Force Degradation Studies:

In various conditions, 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol demonstrated stability as a drug substance. It remained stable under metallic conditions (0.05M FeCl3) at room temperature, basic conditions (1N NaOH) at room temperature, acidic conditions (1N HCl) at room temperature, oxidation conditions (3% H2O2) with exposure to light at room temperature, reduction conditions (1% Na2S), photolytic conditions (exposure to 1.2 million lux/hour), and thermal degradation conditions at 105°C. In every degradation scenario mentioned above, each degradant peak is distinguishable from both the blank and the main peak. The observed association between the increase in degradant impurities and the decrease in assay result for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol is deemed adequate. Based on the validation data provided, it can be stated that the HPLC technique for 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol is specific and serves as a stability indicating method [27].

Table 7: *Forced degradation calculation of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol technical*

Condition	Smpl Wt (in mg)	Conc (in ppm)	Area (mv)	% Assay	% Total Imp.	Mass Balance
As such	20.12	20.12	7134097	99.24	0.000	NA
0.05M_$FeCl_3$_24 Hrs	20.26	20.26	7176715	99.15	0.801	101.00
1N_NaOH_24 Hrs	20.35	20.35	7176005	98.70	0.810	100.00
1N_HCl_24 Hrs	20.19	20.19	7156987	99.22	0.280	100.00
3% H_2O_2_24 Hrs	20.11	20.11	7100326	98.82	0.675	100.00
1% Na_2S_24 Hrs	20.34	20.34	7191240	98.96	0.539	100.00
Photo @ 1.2 million lux/Hr	20.35	20.35	7179315	98.74	0.789	100.00
Thermal @ 105°C_24 Hrs	20.03	20.03	7163940	100.11	0.526	101.00

Table 8: *Force degradation of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol of impurity profile*

% impurity (by Area normalization)								
RT about -->	**Unk @ 2.90**	**Unk @ 3.15**	**Unk @ 3.26**	**Unk @ 3.45**	**Unk @ 3.99**	**Unk @ 6.82**	**Unk @ 8.32**	**Total Imp**
As such	ND	ND	ND	ND	ND	ND	0.000	ND
0.05M_$FeCl_3$_24 Hrs	0.280	ND	0.194	0.194	ND	0.133	0.801	0.280
1N_NaOH_24 Hrs	ND	0.142	0.274	0.274	0.12	ND	0.810	ND
1N_HCl_24 Hrs	ND	0.142	ND	ND	0.138	ND	0.280	ND
3% H_2O_2_24 Hrs	0.520	ND	ND	ND	0.155	ND	0.675	0.520
1% Na2S_24 Hrs	ND	ND	0.539	ND	ND	ND	0.539	ND
Photo @ 1.2 million lux/Hr	0.327	ND	ND	0.255	ND	0.207	0.789	0.327
Thermal @ 105°C_24 Hrs	ND	ND	0.526	ND	ND	ND	0.526	ND

CONCLUSION:

The compound 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol was verified,

created, and applied for the determination of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol. The technique was found to be specific, accurate, exact, and

robust. The compound 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol elutes quickly, in less than 10 minutes, and does not show any interference with the components of the pharmaceutical dosage form. The proposed approach is suitable due to the high reproducibility, accuracy, good selectivity, and sensitivity of 4-{[(6-methoxy-1,3-benzothiazol-2-yl)imino]methyl}benzene-1,4-diol for simultaneous determination.

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