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Research Article

Development and validation of HPLC-UV analytical method for active ingredient content of Carbendazim in technical and wettable powder (WP) formulation

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ABSTRACT

A robust, sensitive, precise, economic and reliable analytical method for the analysis of Carbendazim in Technical and Wettable Powder (WP) formulation was developed and validated with conditions like C18 analytical column of specifications 250 mm length, 4.6 mm internal diameter and 5µm particle size, Mobile Phase: Acetonitrile: Double Distilled water (pH adjusted to 3.00 with H_3PO_4) in 20: 80 ratio respectively. The method is validated in terms of Specificity, Linearity, Accuracy, Precision (in terms of Repeatability, Intermediate Precision and Reproducibility), Limit of Detection (LOD), Limit of Quantification (LOQ), Ruggedness and Robustness. Linearity of the instrument was observed over the concentration range of 250 µg/mL-750 µg/mL with Correlation Coefficient of 0.9999. LOD of the method was observed to be 0.01 µg/mL which was 0.004 % of the lower limit of the range of the analytical method and LOQ was found to be 0.04 µg/mL. Purity profile of a Technical Sample with PDA illustrated the Carbendazim peak as Purity profile shows spectral homogeneity and Purity Angle is less than Purity Threshold. Results from the criteria of System Suitability Test supports chromatographic behaviour is acceptable.

Key words : Diluent, Wettable Powder, Technical material, System Suitabil Test, 1, 4-Dioxane apoptosis.

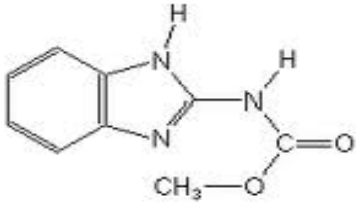
INTRODUCTION

Carbendazim or MBC [methyl-2-benzimidazole carbamate] is a broad spectrum benzimidazole carbamate fungicide with systemic activity that controls a wide range of pathogens on a broad range of field crops like paddy, cereals, fruits, vegetables (Reference No. 19). It is formulated as Wettable powder (500 g/kg and 600 g/kg), Oil Dispersion (200 g/litre), Aqueous Dispersion (500 g/litre), Aqueous Suspension (200 g/litre), Water Soluble Liquid (as phosphate salt, 7 g/litre) (Reference No.19). The existing collaborative analytical method which was reported in CIPAC (Ref: CIPAC Volume H, *263/TC/M/- , Carbendazim Technical , Section 3.2, HPLC method), meant for Active Ingredient analysis, was developed with C18 analytical column for which the specifications were 300 mm x 10 µ x 4.6 mm, is a less frequently used analytical column. Another disadvantage of the collaborative analytical method was, it was developed by employing Sulphuric Acid, a strong mineral acid, of 1 M strength (pH<1.90) which is detrimental for the life of most of analytical columns. There were several methods which were reported for Carbendazim analysis in low level concentrations (Reference No. 8). But there is no appropriate analytical method for the Active Ingredient analysis of Carbendazim in technical and WP formulations which can be carried out with more frequently available HPLC columns. So there is a need for the reliable and cost effective analytical method which can be applied with the routinely available HPLC columns in almost all the analytical laboratories like C18 (250 mm x 4.6 mm i.d. x 5µm). Based on the validation results it was confirmed that this analytical method can support high concentration analysis of like Active Ingredient analysis both in Technical grade and Wettable Powder Formulation.

MATERIALS AND METHODS

Chemicals and Trial for selection of suitable diluent

Trials for the solubility and stability of Carbendazim were done with solvents like acetonitrile and Methanol and 1, 4-Dioxane. The molecule has less solubility in Methanol. Even though Carbendazim is soluble in Acetonitrile, it was found unstable. More than 40 % of degradation was found after 24 hours of time in Acetonitrile. As the Carbendazim molecule consists of imidazole and carbamate structures in it, hence it was found soluble in 0.5 N H_2SO_4 by forming water soluble salts (Reference No.19). It is sparingly soluble and found stable in 1, 4 Dioxane. Hence, the trial for its solubility was carried out in the variable mixtures of 1, 4- Dioxane and 0.5 N H_2SO_4.



Carbendazim Structure

The trial for its solubility was carried out in variable diluents of 95:5, 90:10, 85:15, 80:20 and 75:25 of 1, 4-Dioxane and 0.5 N H_2SO_4 . The pH of the aliquot of each diluent was measured which showed 1.65, 1.79, 1.90, 1.98 and 2.25 respectively. Finally, the diluent of ratio, 75:25 (1, 4-Dioxane and 0.5 N H_2SO_4 respectively), which showed pH at 2.25, was selected for Method development of Carbendazim. The diluent of 1, 4-Dioxane and 0.5 N H_2SO_4 in 75:25 ratio, was preferably chosen keeping in view the safety of the C18 column (for which the lowest limit of pH is 2.00) and to minimise the interference from the 1, 4 -Dioxane solvent at the same time.

Standard Preparation

The 100.11 mg of Carbendazim reference material of 99.7 % supplied by Sigma Aldrich, was accurately weighed into 50 ml standard flask, added the diluent of 1, 4-Dioxane and 0.5 N H_2SO_4 in 75:25 ratio respectively followed by sonicating the contents for 10 minutes for proper dissolution. Sonication is of critical importance for proper dissolution. The concentration of Carbendazim Stock solution of reference material prepared was 2002.20 µg/mL. Additional precautions were taken while preparing the sample with Wettable Powder (WP) Formulation. Sample solution of WP formulation was sonicated for 15 minutes and allowed the sediment to settle down before analysis.

Instrumentation and the chromatographic conditions

The HPLC system consisted of LC 20A pumps, Model: LC-20A, Detector: SPD-20A, Column Oven: CTO-20 A, Analytical Column: Enable C-18 G (250 mm x 4.6 mm x 5 µ), Mobile Phase: Acetonitrile: Double Distilled water (pH adjusted to 3.00 with H_3PO_4) in 20: 80 ratio respectively, Flow rate: 1 ml/min, Injection Volume: 5 µl, Oven temperature was maintained at 30°C. Retention Time was found to be 4.6 minutes (Approximately). The detection wavelength was 280 nm.

Method Validation

Linearity

The linear solutions for calibration were prepared by serial dilution of the stock solution. Linearity of the analytical method was checked at three points from 250 µg/mL to 750 µg/mL in triplicate. The care was taken to maintain the constant aliquot volume of the diluent which was with composition of 1, 4-Dioxane and 0.5 N H_2SO_4 in 75:25 ratio constantly in all the linear solutions to get rid of interference caused by 1, 4- Dioxane. Final make up to mark was carried out with Double Distilled water (pH adjusted to 3.00 with H_3PO_4).

Table 1. Sequence of Serial dilution for Detector Linearity

S No	Stock solution Concentration (µg/mL)	Concentration of the Linear solution Prepared (µg/mL)	Aliquot volume of Stock solution (ml)	Aliquot Volume of Additional Diluent (ml)	Final Volume (ml)
1	2002.20	750	1.87	-	5
2		500	1.25	0.62	5
3		250	0.62	1.25	5

Table 2. Results of Detector Linearity

S. No	Concentration (µg/mL)	Area	Mean Area
1	750	12600031	12516519
		12404880	
		12544645	
2	500	8634949	8650582
		8622234	
		8594563	
3	250	4550201	4572119
		4565532	
		4600623	
Slope		15889	
Intercept		635340	
CC		0.9999	

Specificity

Aliquots of diluent (1, 4-Dioxane and 0.5 N H_2SO_4 in 75:25 ratio), Standard 500 µg/mL, Technical sample and Wettable Powder Formulation were analysed.

Precision

Repeatability and System precision were carried out with Standard solution of 500 µg/mL.

A. Repeatability: System Precision (Repeatability) was carried out by 5 replicate injections.
B. Intermediate Precision: Intermediate precision was carried out on two different HPLC instruments in two different days by 2 different analysts within the same

laboratory. Different samples were analysed on 2 different days.

C. Method Precision: Method precision (Reproducibility) was carried out for both Technical (TC) and Wettable Powder (WP) material by five replicate samples at the level of 10 mg/5ml. And calculated the % RSDr from results as per modified Horwitz Equation.

Modified Horwitz equation RSDr < $2^{(1-0.5 \log C)}$ x 0.67

Whereas C = Nominal Concentration of analyte in the sample as a decimal fraction.

Table 3. Results of System Precision (Injection repeatability)

S. No.	Concentration (µg/mL)	Response	Retention Time (RT)
1	500	8550201	4.645
2	500	8683434	4.624
3	500	8610923	4.635
4	500	8574556	4.670
5	500	8708754	4.633
Mean		8625574	4.641
Standard Deviation		68493	0.02
Relative Standard Deviation		0.79	0.38

Table 4. Intermediate Precision (Repeatability) - Day- I by Analyst- I on Instrument - I.

S. No.	Concentration (µg/mL)	Response	Retention Time (RT)
1	500	8532240	4.654
2	500	8661234	4.671
3	500	8508723	4.635
4	500	8674556	4.629
5	500	8508754	4.666
Mean		8577101	4.651
Standard Deviation		83569	0.02
Relative Standard Deviation		0.97	0.40

Table 5. Intermediate Precision (Repeatability) - Day- I by Analyst- I on Instrument - II

S. No.	Concentration (µg/mL)	Response	Retention Time (RT)
1	500	8698876	4.610
2	500	8646535	4.603
3	500	8687653	4.589
4	500	8679870	4.605
5	500	8637611	4.569
Mean		8670109	4.595
Standard Deviation		26657	0.02
Relative Standard Deviation		0.31	0.36

Table 6. Intermediate Precision (Repeatability) - Day-II by Analyst- I on Instrument - II

S. No.	Concentration (µg/mL)	Response	Retention Time (RT)
1	500	8508873	4.635
2	500	8698730	4.622
3	500	8572234	4.659
4	500	8572274	4.676
5	500	8490763	4.700
Mean		8572495	4.658
Standard Deviation		82217	0.03
Relative Standard Deviation		0.96	0.67

Table 7. Intermediate Precision (Repeatability)-Day-II by Analyst- II on Instrument – II

S. No.	Concentration (µg/mL)	Response	Retention Time (RT)
1	500	8600984	4.613
2	500	8578986	4.604
3	500	8586735	4.659
4	500	8601232	4.702
5	500	8619875	4.687
Mean		8597562	4.653
Standard Deviation		15695	0.04
Relative Standard Deviation		0.18	0.94

Table 8. Method Precision (Reproducibility): Technical

Replication	Weight of sample taken in mg per 5 ml	Active Ingredient Observed	Limit of RSDr as per modified Horwitz equation	Observed RSDr as per modified Horwitz Equation
1	10.23	98.96	≤ 1.34	0.15
2	10.08	99.06		
3	10.11	98.79		
4	10.09	98.82		
5	10.06	99.14		

Table 9. Method Precision (Reproducibility): Wettable Formulation

Replication	Weight of sample taken in mg per 5 ml	Active Ingredient Observed	Limit of RSDr as per modified Horwitz equation	Observed RSDr as per modified Horwitz Equation
1	10.07	50.34	≤ 1.49	0.22
2	10.03	50.55		
3	10.06	50.50		
4	10.15	50.35		
5	10.15	50.29		

Recovery (Assay Accuracy)

As the blank matrix is not available, Recovery (Assay Accuracy) was carried out by Standard addition method and it was carried out at the level of nominal Active Ingredient concentration for both Technical (TC) and Wettable Powder (WP) formulation. Five replicate samples of Carbendazim technical and 50 % Wettable Powder(WP) formulation at 10 mg / 5 ml each were added 10 mg and 5 mg Carbendazim reference material respectively.

Table 10. Results of Recovery (Assay Accuracy) - Technical

Replication	Sample weight taken (per 5 ml)	Quantity of standard added (per 5 ml)	Recovered Quantity (mg)	% of Recovery	Mean Recovery (%)	SD
1	10.14	10.04	10.02	99.80	99.90	0.3

2	10.07	10.16	10.13	99.70		4
3	10.14	10.05	10.02	99.70		
4	10.16	10.11	10.09	99.80		
5	10.05	10.07	10.12	100.50		

Table 11. Results of Recovery (Assay Accuracy) – Wettable Powder (WP) Formulation

Replication	Sample weight taken (per 5 ml)	Quantity of standard added (per 5 ml)	Recovered Quantity (mg)	% of Recovery	Mean Recovery (%)	SD
1	10.08	5.08	5.10	100.39	100.40	0.82
2	10.02	5.02	5.08	101.20		
3	10.11	5.11	5.06	99.02		
4	10.08	5.08	5.12	100.79		
5	10.04	5.04	5.07	100.60		

Ruggedness

Evaluation of the Method in terms of Ruggedness was checked but it was limited to check instrumental variability on Shimadzu LC-20A and Waters HPLC systems 600 series (with PDA Model-2998, Auto sampler Model-2707 and Controller Model-600) and with 2 different columns, i.e. Enable-C18G (250 mm x 4.6 mm x 5 µ) which was manufactured and supplied by Spinco Biotech and Symmetry C-18 (250 mm x 4.6 mm x 5 µ) which was manufactured and supplied by Waters Corporation. The evaluation of the method was also carried out by the LC solution and Empower softwares.

Table 12. Results of Ruggedness: (when used Waters HPLC system, Enable- C18G (250 mm x 4.6 mm x 5µ)

S. No.	Number of theoretical plates (N)	RSD of precision		Capacity factor (k′)	Tailing factor (10 %)	Resolution (Rs)	Degree of seperation
		Area	Retention Time (RT)				
1	3313.32	836704	4.305	0.906	1.265	1.905	1.346
2	3254.18	838642	4.275	0.908	1.274	1.913	1.349
3	3251.04	840853	4.334	0.902	1.282	1.917	1.353
4	3283.16	838735	4.342	0.912	1.279	1.915	1.318
5	3276.11	838785	4.354	0.918	1.274	1.932	1.318
Mean	3275.56	838524	4.322	0.909	1.275	1.916	1.337
SD	25.21	18480.54	0.032	0.006	0.006	0.010	0.017
RSD	0.77	0.22	0.738	0.671	0.507	0.513	1.297

Robustness

Robustness of the method was carried out with small and deliberate changes in method parameters. The method response was checked at 30.2°C, 30.0°C and 29.8 °C of Column Oven, and by changing pH of Phosphoric acid (Mobile Phase) at 3.05, 3.00 and 2.95. Composition of Mobile Phase was lightly changed from its original ratio of 80:20 (Acetonitrile: Double Distilled water (pH adjusted to 3.00 with H_3PO_4)) to 78:22 and also 82:18. At both changed Mobile Phase compositions, the instrument's response was checked.

Table 13. Results of Robustness

pH of H_3PO_4 of mobile phase	A	B	Mobile Phase Composition	A	B	Oven Temperature (°C)	A	B
3.05	6606545	4.656	78:22	6615674	4.586	29.5	6597465	4.614
2.95	6598753	4.599	82:18	6598745	4.697	30.5	6598875	4.633

A- Mean Observed Area

B- Mean Retention Time

Peak Purity Check

The purity of Carbendazim peak was checked after scanning the technical sample aliquot with PDA detector and the peak is found to be pure which was substantiated from the interpretation of purity profile i.e. Purity Angle < Purity Threshold and Spectral Homogeneity.

LOD and LOQ

Based on Signal to Noise ratio, 0.01 µg/mL concentration (S/N - 3 and) as LOD and 0.04 µg/mL (S/N - 9) as LOQ were determined. At LOQ level, five replicate samples were prepared and analysed. The reproducibility was found to be with 5.2 % RSD and 92 % mean recovery.

System Suitability Test

System suitability test was checked from 5 replicate injections of standard solution of concentration 500 µg/mL. As part of System Suitability Test, results were checked for Number of Theoretical Plates (N), Resolution (Rs), Capacity factor (K′), Relative Standard Deviation (RSD) of 5 replicate injections, tailing factor (10 %) and Degree of seperation. The results showed that all the values were well within the limits of acceptance criteria.

Table 14. Results of System Suitability Test

S. No.	**Number of theoretical plates (N)**	**RSD of precision**		**Capacity factor (k′)**	**Tailing factor (10 %)**	**Resolution (Rs)**	**Degree of seperation**
		Area	**Retention Time (RT)**				
1	3715.45	8690201	4.604	0.858	1.234	1.956	1.323
2	3654.41	8600982	4.635	0.863	1.245	1.975	1.340
3	3651.45	8598764	4.624	0.856	1.287	1.986	1.311
4	3543.13	8589887	4.620	0.893	1.245	1.988	1.309

5	3576.43	8623342	4.631	0.868	1.269	1.973	1.325
Mean	3628.17	8620635	4.623	0.868	1.256	1.976	1.322
SD	68.48	407954.54	0.01	0.01	0.02	0.01	0.01
RSD	1.89	0.47	0.26	1.72	1.72	0.65	0.94

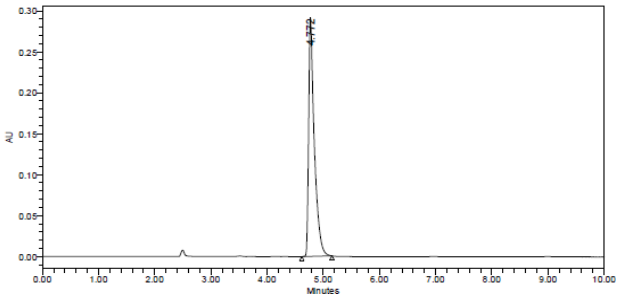


Figure 1. Representative Chromatogram of Carbendazim standard-Waters HPLC – Empower software

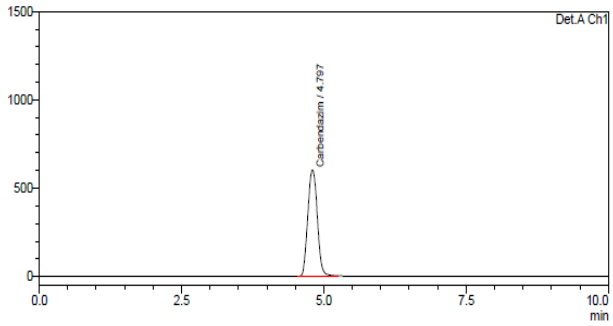


Figure 2. Representative Chromatogram of Carbendazim standard-Shimadzu HPLC – LC solution Software

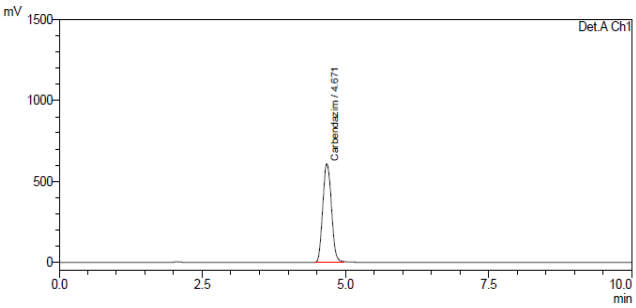


Figure 3: Chromatogram of Carbendazim Technical

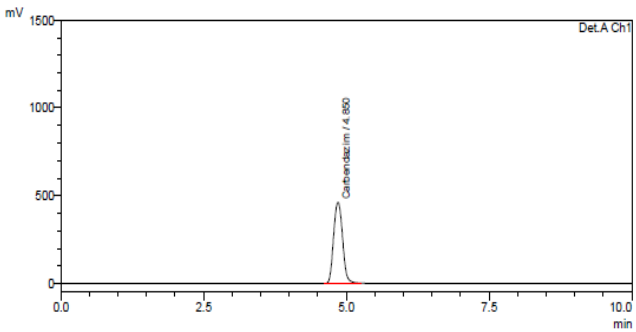


Figure 4: Chromatogram Carbendazim 50 % WP formulation

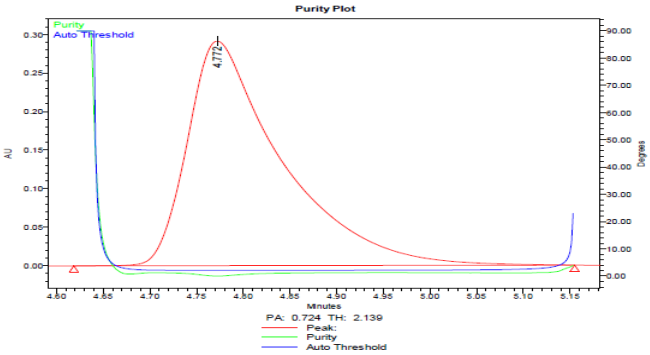


Figure 5. Representative Peak Purity data for Carbendazim

RESULTS AND DISCUSSION

The analytical method was developed and validated in terms of linearity, specificity, precision (repeatability and reproducibility), recovery, ruggedness and robustness. The correlation co-efficient for linearity was found to be 0.9999. Specificity of analytical method substantiates that there is no interference from mobile phase, diluent and the method is specific for the analysis of Carbendazim. The % RSD observed during System Precision (Repeatability), Intermediate Precision, Method Precision (Reproducibility) found to be within the limit, RSDr ≤ 1.34 for Technical material and RSDr ≤1.37 for 50% WP formulation, acceptance criteria established by Modified Horwitz equation). On Assay Accuracy, the percentage of recovery was found to be 99.90 ± 0.34(SD) for Technical material and 100.40 ± 0.82 (SD) which were within the range (98 % - 102 % for molecules with >10 % active (nominal)) set by 'SANCO/3030/99 rev.4, 11/07/00' guideline. The LOD and LOQ were found to be 0.01µg/mL and 0.04µg/mL respectively which reflect the sensitivity of analytical method for the target molecule. The data from ruggedness test, indicate that the results from analytical method are not altered significantly with change in instrument and with change in C18 column of 250mm length. The information from robustness parameter support that small and deliberate changes in method parameters like pH of mobile phase, temperature and mobile phase composition have least impact on performance of analytical method. On interpretation of purity profile which is indicated by 'Purity angle' is less than 'Purity Threshold' and spectral homogeneity, it is proved that peak is pure and well resolved from other possible interferences. Results from the criteria of System Suitability Test reveal chromatographic behaviour is acceptable.

CONCLUSION

The results showed that all the method validation parameters are within the acceptable limits of acceptance criteria established in the guidelines. From the observed results, it is demonstrated that, the validated HPLC method

is fit for the purpose of the active ingredient analysis of Carbendazim in technical and wettable powder formulation.

ACKNOWLEDGEMENT

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