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Analytical Method Development and Validation Description for Quantitative Assessment of Chlorbenzuron by HPLC Procedure

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Abstract: ***A consistent, systematic, exact and precise high performance liquid chromatographic method for the analysis of pesticide Chlorbenzuron in its formulations was developed and validated in the present study. The mobile phase consists of a mixture of acetonitrile, water and 1, 4 dioxane in the proportion 55: 45: 0.03 respectively (v/v/v). This was found to give sharp peak of Chlorbenzuron at a run time of 20 min. HPLC analysis of Chlorbenzuron was carried out at a wave length of 250 nm with a flow rate of 1.4mL/min. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.999 in the concentration range of 25% to 125%. The linear regression equation was y= 1866x+760.7. The developed method was employed with a high degree of precision and accuracy for the analysis of Chlorbenzuron. The method was validated for accuracy, precision, robustness, ruggedness and specificity. The precision, accuracy, sensitivity, short retention time and composition of the mobile phase stipulated that this method is applicable to the evaluation of Chlorbenzuron.***

Keywords: Chlorbenzuron, HPLC Method, Development and analysis

1. Introduction

Chlorbenzuron is chemically 2-chloro-N-[(chlorophenyl) carbmoyl]benzamide, in (fig-1), Empirical formula: $C_{14}H_{10}Cl_2N_2O_2$ and Molecular Weight: 309.147 g/mol^{-1}. Chlorbenzuron is an odourless, appearance as white solid. Its melting point is 190-201^{o}c. It is insoluble in water. The original solubility in acetone is 10g/L, soluble in N, n-dimethyl formamide, pyrrole and other organic solvents, Chlorbenzuron is easy to decompose in the presence of alkali or strong acid, stable storage under normal conditions, stable for light and heal. It controls insect pests, particularly forest tent caterpillar moths, boll weevils, gypsy moths, and other types of moths.

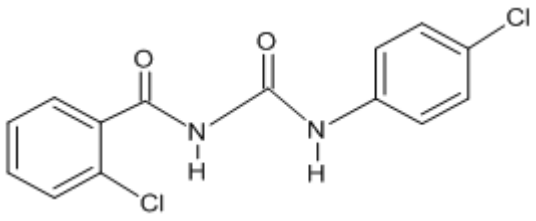


Figure 1: Chemical Structure of Chlorbenzuron

Chlorbenzuron is chitin synthesis inhibitor that affect reproduction that act as an insect growth regulatory agent, bring on to death of insecticide eggs [1], additionally the most typical morphological defects of insecticides treated; therefore, abnormal eggs died during embryonic incomplete development and the lethal caused by its lead to large number of abnormal eggs to have apparently fully developed inside the eggshell but died at the moment that normal exclusion would occur[2]. Chlorbenzuron also used for the prevention and treatment of a variety of lepidoptera pests, such as beet armyworm, pine moth, leach,pear etc. [3].

Literature review stipulates that Chlorbenzuron has been analyzed by varied analytical instrumental techniques like GC [4]. Chlorbenzuron residues determination in tea at tropical and subtropical temperature zones, where high throughput methods for the residue determination has been developed [5], have been formerly discussed in the methods [6&7], wide range of its residues in various forms such as, fruit and vegetables, fish, honey and infant foods [8] tracing by HPLC methods [9 & 10,]. Super Critical Fluid variant [11]. HPLC analytical method for Chlorbenzuron, recommended by CIPAC [12],.

Initially this analysis, an analytical method was developed and validated for the determination of Chlorbenzuron in its formulations. To the best of author knowledge, there is no one can report the determination of Chlorbenzuron in its formulations. An effort has been made to develop and validate to establish their accuracy, precision and other analytical method validation parameters as mentioned in various gradients for pesticide formulation, the proposed method is suitable for their analysis with virtually no interference of the standard additives presented in pesticide preparations.

Equipments and Chemical

Equipments and Chemical used for the validation of Chlorbenzuron. We used High performance liquid chromatography, with UV / PDA detector, HPLC Analytical column of C18- Inertsil-3, 250mm x 4.6mm x 5µ and Analytical weighing balance - Mettler Toledo AB204, Sigma Aldrich Nylon 0.2µm and experimental appurtenances. Chlorbenzuron working Standard, BOSMAN Chlorbenzuron , Acetonitrile – Analytical Reagent**,** 1:4 dioxane - Analytical Reagent and Sigma Aldrich Water [13].

2. Analytical Method

The quantitative determination is carried out by HPLC system equipped with UV/VIS detector.

Table 1: Chromatographic conditions

Column	C18- Inertsil-3, 250mm x 4.6mm x 5µ
Mobile Phase	For isocratic system, prepare a mixture of Acetonitrile, water and 1:4 dioxane in the proportion 55: 45: 0.03(v/v/v) respectively. Mix well. Filter through 0.2 µ Nylon membrane filter paper and degas prior to use.
Wavelength	250 nm
Flow Rate	1.4 mL / minute
Injection volume	20 µl
Run time	20 minutes
Blank solution	Take diluent as blank
Diluent	Take mobile phase as diluent

Preparation of Chlorbenzuron Standard Solution
Precise heavyness of substance about 50 mg of Chlorbenzuron working Standard and collected to a 20 ml volumetric flask. Add 10 ml of diluent and sonicate to deliquesce. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix. (Mitigation strategy: 50mg → 50.0 ml → 1 ml /10.0 ml)

Formation of Test Solution
Heavyness of substance precisley about 102 mg of sample and change it into a 50 ml volumetric flask. Add 10 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix. (Mitigation strategy: 102mg → 50.0 ml → 1 ml /10.0 ml)

System Suitability Solution: Use Chlorbenzuron Standard working solution as system suitability solution.

Method Development [14]
Separately inject equal volumes of blank, five replicate injections of system suitability solution (Chlorbenzuron Standard working solution). Then inject two injections of test solution and record the chromatograms. Disregard any peak due to blank in the test solution. Calculate % RSD of five replicate injections of system suitability solution (Chlorbenzuron Standard working solution). Check tailing factor and theoretical plates of the peak in the chromatogram obtained with 5^{th} injection of system suitability solution (Chlorbenzuron Standard working solution). The limits are as below,

1) Theoretical plates should be not less than 2000.
2) Tailing factor should be less than 2.0.
3) % RSD should be not more than 2.0%.

Validation Parameters [15]

Specificity / Selectivity:
Selectivity was performed by injecting the diluent blank solution, excipient blend, system suitability solution, test solution. Acceptance criteria: The Chlorbenzuron peak should be well resolved from any other peak and from each other. The diluent blank solution, excipient blend solution should not show any peak at the retention time of the Chlorbenzuron. The system suitability criteria were found to meet the pre-established acceptance criteria consistent with the analytical method.

Table 2: System suitability - Selectivity

Sr. No.	Area of Chlorbenzuron
1	1992.67
2	1988.85
3	1975.57
4	1959.98
5	1977.95
Mean	1979.04
Standard Deviation (±)	12.83
(%) Relative Standard Deviation	0.65

Altogether the injections were processed at the wavelength stipulated in the method. There was no interference observed from diluent blank solution, excipient blend solution with Chlorbenzuron peak.

Linearity and Range for sample
For the linearity study five standard solutions of Chlorbenzuron were prepared from the range starting from 25% to 125% of the theoretical concentration of assay preparation. The system suitability solution and the linearity solutions were injected as per the protocol. The linearity graph of concentration against peak response was plotted and the correlation coefficient was determined. Acceptance criteria: Correlation coefficient should be greater than or equal to 0.999. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table-3 for system suitability results).

Table 3: System suitability - Linearity of standard

Sr. No.	Area of Chlorbenzuron
1	2043.36
2	2015.04
3	2087.17
4	2031.28
5	2045.89
Mean	2044.54
Standard Deviation (±)	26.77
(%) Relative Standard Deviation	1.31

The average peak area of Chlorbenzuron peak at each concentration level was determined and the linearity graph was plotted against the sample concentration in percentage. The result of linearity review refers in Table - 4.

Table 4: Results of linearity of sample

Linearity Level	Sample Concentration (in %)	Sample Concentration (in ppm)	Peak Area	Correlation Coefficient
Level – 1	25	25	1230.77	0.999
Level – 2	50	50	1705.15	
Level – 3	75	75	2129.35	
Level – 4	100	100	2640.89	
Level – 5	125	125	3095.56	

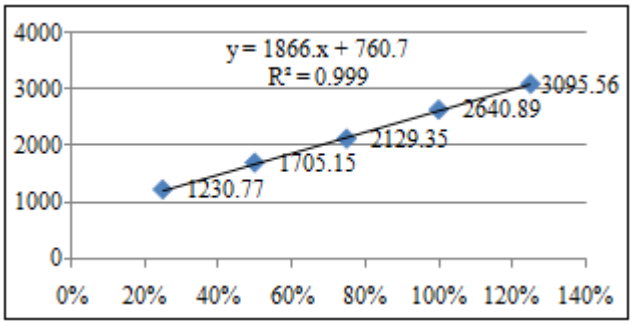
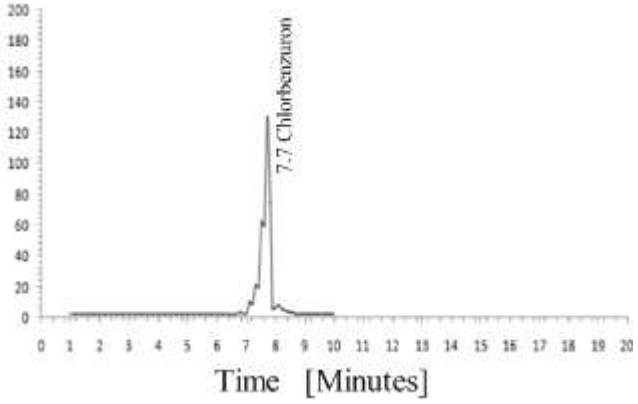


Figure 2: Linearity graph of Chlorbenzuron sample



Result- Table						
Peak No	Retn. Time	Area	Height	Area %	Height %	Width@50 %
1	7.7	1705.15	130.00	100	100	0.40

Figure 3: Chromatogram of Chlorbenzuron sample

Forced Degradation

The forced degradation learning's are performed to establish the stability indicating nature of the assay method and to observe any degraded compounds. Chlorbenzuron WS and Sample (BOSMAN Fungicide) are subjected to stress with 5.6N HCl, 5.6N NaOH, Thermal degradation and UV degradation. Altogether the above solutions are chromatographed and recorded the chromatograms. The following stress conditions are followed for degradation

Table 5: System suitability – Forced Degradation

Sr. No.	Area of Chlorbenzuron
1	2044.714
2	2025.953
3	2025.522
4	2028.262
5	2042.321
Mean	2033.35
Standard Deviation (±)	9.37
(%) Relative Standard Deviation	0.46

Table 6: Conditions – Forced Degradation

Sample stress condition	Description of stress condition
Acid degradation	5.6N HCl heated at about 60°C for 10 min on a water bath.
Alkali degradation	5.6N NaOH heated at about 60°C for 10 min on a water bath.
Thermal degradation	105ºC for 12 hours
UV degradation	expose to UV-radiation for 7 days

Table 7: % of degradation by applying different conditions

Acid Stress	**% Degradation**
Standard	0.016
Sample	0.026
Alkali Stress	**% Degradation**
Standard	0.003
Sample	0.034
Thermal Stress	**% Degradation**
Standard	0.020
Sample	0.003
UV Stress	**% Degradation**
Standard	0.246
Sample	0.016

Acceptance Criteria: The degradation peaks should be well separated from each other. The peak purity for Chlorbenzuron peak should pass. There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well separated from each other. The peak purity for Chlorbenzuron peak is passing. Hence, the method is very precise, selective and specific to the estimation of Assay of BOSMAN Chlorbenzuron by HPLC and the same method is stability indicating, as the degraded products are well separated from Chlorbenzuron and as well from each adjacent peaks.

Precision:

System Precision:

Procedure:

The system precision was performed by injecting 10 replicate injections of system suitability solution and the chromatograms are reviewed for the system suitability criteria. Acceptance criteria: % RSD of peak areas of ten replicate injections of system suitability solution should not be more than 2.0% and system suitability criteria should pass as per analytical method. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method.

Table 8: System precision

Sr. No.	Area of Chlorbenzuron
1	2065.14
2	2058.56
3	2047.84
4	2085.54
5	2033.14
6	2047.57
7	2042.17
8	2047.28
9	2053.57
10	2045.98
Mean	**2052.67**
Standard Deviation (±)	14.47
(%) Relative Standard Deviation	0.71

Method Precision

Procedure

Six test solutions of Chlorbenzuron in BOSMAN Chlorbenzuron and were prepared as per the analytical method. The % RSD of % assay of six test solutions was calculated. Acceptance criteria: % RSD of the results of six test solutions should not be more than 2.0%. The system

suitability criterion was found to meet the pre- established acceptance criteria as per the analytical method. The results of assay obtained from six test solutions preparations are presented in Table - 10

Table 9: System suitability - Method precision
Analyst – 1 HPLC No.: EH/R&D/HPLC-024

Sr. No.	Area of Chlorbenzuron
1	2055.94
2	2058.75
3	2051.36
4	2069.66
5	2052.69
Mean	2057.68
Standard Deviation (±)	7.28
(%) Relative Standard Deviation	0.35

Table 10: Results of method precision

Test Solution	% Assay of Chlorbenzuron
1	99.34
2	99.05
3	98.66
4	99.09
5	99.77
6	99.01
Mean	99.15
Standard Deviation (±)	0.37
(%) Relative Standard Deviation	0.37

Intermediate Precision

Procedure:
Six test solutions of BOSMAN Chlorbenzuron were prepared as per the analytical method on different day. These test solutions were analyzed by a different analyst using different HPLC column of same make but having different serial number and different HPLC system. The % RSD of % assay results of twelve test solutions (six samples from method precision and six samples from intermediate precision) was calculated. Acceptance criteria: % RSD of the results of twelve test solutions (six of method precision and six of intermediate precision) should not be more than 2.0%. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method.(Refer to Table -10 for system suitability results). The results of assay obtained from six test solutions are presented in Table - 12. % RSD of assay results from method precision and intermediate precision (12 results) are presented in Table - 13.

Table 11: System suitability - Intermediateprecision Analyst – 2 HPLC No.: EH/R&D/HPLC-023

Sr. No.	Area of Chlorbenzuron
1	1884.33
2	1875.69
3	1858.66
4	1887.08
5	1866.40
Mean	1874.43
Standard Deviation (±)	11.96
(%) Relative Standard Deviation	0.64

Table 12: Results of Intermediate precision

Test Solution	% Assay of Chlorbenzuron
1	99.18
2	97.38
3	99.48
4	98.72
5	99.63
6	100.13
Mean	99.08
Standard Deviation (±)	0.95
(%) Relative Standard Deviation	0.96

Table 13: Results of Twelve Test Solutions of Chlorbenzuron in BOSMAN Chlorbenzuron (six of method precision & six of intermediate precision)

Analysis performed during method precision study By Analyst 1 on system 1 and on column 1 on day 1	
Same column	% Assay of Chlorbenzuron
1	99.25
2	99.01
3	98.78
4	99.12
5	99.65
6	99.05
Analysis performed during intermediate precision study By Analyst 2 on system 2 and on column 2 on day 2	
Column sr. no.	015337030137 01
Test Solution	% Assay of Chlorbenzuron
7	99.16
8	97.45
9	99.48
10	98.75
11	99.65
12	100.31
Mean of twelve samples	99.13
Standard Deviation (±)	0.68
(%) Relative Standard Deviation	0.68

Robustness:
Procedure
Prepare two test solutions of the same lot (as used in 7.0.a and 7.0.b) of Chlorbenzuron in BOSMAN Chlorbenzuron as per analytical method. Inject this solution along with diluent blank solution and system suitability solution along different chromatographic
conditions as shown below:
Change in flow rate (± 0.2 ml/minute)
Change in wavelength (± 2 nm)
Change in composition of mobile phase (± 20ml)
Change in Flow Rate (± 0.2 ml/minute): (Normal Experimental Condition:1.4ml/minute)
The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table - 14 for system suitability results).

Table 14: System suitability-Robustness with change in flow rate

S. No.	Area of Chlorbenzuron	
	1.1mL/ minute	1.5 mL/minute
1	2008.66	2006.89
2	2011.61	2003.39
Mean	2010.63	2005.14
Standard Deviation (±)	2.08	2.48
(%) Relative Standard Deviation	0.1	0.12

The assay results obtained with different flow rate conditions are as given in Table 15.

Table 15: Results for change in flow rate

Flow rate →	1.1mL/ minute	1.5 mL/minute
Sample	% Assay	
Test solution	100.11	100.25
Average assay result from method precision	99.06	99.12
Mean	99.58	99.68
Standard Deviation (±)	0.74	0.79
(%) Relative Standard Deviation	0.74	0.81

Change in Wavelength (± 2 nm):
(Normal Experimental Condition: 250nm)
The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method.(Refer to Table-16 for system suitability results).

Table 16: System suitability - Robustness with change in wavelength

Sr. No.	Area of Chlorbenzuron	
	248nm	252nm
1	2067.05	2081.49
2	2061.61	2074.08
Mean	2064.33	2077.78
Standard Deviation (±)	3.84	5.23
(%) Relative Standard Deviation	0.18	0.25

The assay results obtained with different wavelength conditions are as given in Table - 17.

Table 17: Results for change in wavelength

Wavelength →	248nm	252nm
Sample	% Assay	
Test solution	100.13	100.23
Average assay result from method precision	99.18	99.18
Mean	99.65	99.70
Standard Deviation (±)	0.67	0.74
(%) Relative Standard Deviation	0.67	0.74

Change in composition of Mobile Phase (± 20ml): (Normal Experimental Condition: Acetonitrile: water:1,4 dioxane = 55ml: 45ml: 0.03)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method (Refer to Table - 18 for system suitability results).

Table 18: System suitability - Robustness with change in composition of mobile phase

Sr. No.	Area of Chlorbenzuron	
	53:47:0.03	57:44:0.03
1	2084.04	2061.16
2	2095.90	2071.03
Mean	2089.97	2066.09
Standard Deviation (±)	8.38	6.97
(%) Relative Standard Deviation	0.40	0.33

The assay results obtained with change in composition of mobile phase are as given in Table- 19.

Table 19: Results for change in composition of mobile phase

Composition of methanol & water	53:47:0.03	57:44:0.03
Sample	% Assay	
Test solution	99.34	99.92
Average assay result from method precision	99.11	99.11
Mean	99.22	99.51
Standard Deviation (±)	0.16	0.57
(%) Relative Standard Deviation	0.16	0.57

Stability of Analytical Solution

Procedure
System suitability solution and test solution of BOSMAN Chlorbenzuron were prepared on 0^{th}, 12^{th}, 24^{th}, 36^{th} and 48^{th} hour of experiment and stored these solutions at room temperature for every time interval up to 48 hrs and analyzed these solutions on 48 hrs with freshly prepared test solution. The system suitability solution was prepared freshly at the time of analysis. The assay of BOSMAN Chlorbenzuron in the sample was calculated. Acceptance criteria: The analyte is considered stable if there is no significant change in % assay. The assay results obtained during solution stability experiment are as given in Table- 20.

Table 20: Results for solution stability

% Assay results calculated against the freshly prepared system suitability sample	
Sample	% Assay of Chlorbenzuron
0^{th} hr	100.01
12^{th} hr	99.45
24 hr	100.78
36 hr	99.23
48 hr	99.65
Mean	99.82
Standard Deviation (±)	0.606
(%) Relative Standard Deviation	0.606

3. Results and Discussion

System selectivity:
All the injections were processed at the wavelength provided in the Method. There was no interference observed from diluents blank solution, excipients blend solution with Chlorbenzuron peak. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method, hence this Method is selective**.**

Forced degradation:
There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well separated from each other. The peak purity for Chlorbenzuron peak is passing. Hence, the Method is very precise, selective and specific to the estimation of Assay of in test solution of BOSMAN Chlorbenzuron Fungicide as a Chlorbenzuron 99% by HPLC and the same method is stability indicating, as the degraded products are well separated from Chlorbenzuron and as well from each adjoining peak.

Linearity:
Linearity graph of the average area at each level against the concentration in Acetonitrile, water and 1:4 dioxane in the proportion 55: 45: 0.03 (v/v/v) is plotted and is found to be a straight line graph. The correlation coefficient is found to be

more than 0.999. Hence it is concluded that the method is found to be linear in the range of 25% to 125% of the working concentration.

Precision:
The analysis was carried out on six test solutions of the BOSMAN Chlorbenzuron Fungicide and by two different analysts using two different equipments within the same laboratory using two different columns of the same make but having different serial numbers on two different days. The % RSD of the twelve assay results which six of method precision and six from intermediate precision is found to be less than 2.0%. Thus, the method is found to be hardy and precise.
System precision=%RSD=0.64
Method precision=%RSD=0.35 Intermediate precision=%RSD=0.96

Robustness:
The analysis of the BOSMAN Chlorbenzuron Fungicide 99% was carried out at different conditions of column lot, flow rate, wavelength, and change in composition of mobile phase., The % RSD between results obtained with changed condition and average result of Method precision is not more than 2.0%.The analytical Method meets the reestablished acceptance criteria for robustness study.
Thus, the Method is robust.

Stability of Analytical Solution:
The %RSD between assay results obtained for freshly prepared test solution and the stored test solutions is less than 2.0%there is no significant change in assay level observed up to 48 hours for test solution at room temperature. The system suitability was found to meet the pre-established criteria and it can be concluded that the solution is stable up to 48 hours at room temperature

4. Summary and Conclusion

The above summary and the validation data summarized in this paper show the analytical method of assay of BOSMAN Chlorbenzuron Fungicide 99% by HPLC is found to be suitable, selective, specific, precise, linear, accurate and robust. The analytical solution is found to be stable up to 48 hours at room temperature. Hence, it is concluded that the analytical method is validated and can be used for routine analysis and for stability study.

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