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Analytical method development and validation analysis for quantitative assessment of vinclozolin by HPLC procedure

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Abstract
The definite, systematic, precise, particular, linear, proper and robust scientific method was developed and validated for the assay of Vinclozolin in Ronilan DF fungicide. Presently utilized Vinclozolin as a working standard having limit for assay of Vinclozolin in Ronilan DF fungicide are not less than 95.0% for method development and validation. The quantitative determination bring to accomplished by HPLC- Waters - Alliance 510 system equipped with UV/PDA detector. Methanol, Phosporic Acid and water in the ratio (55:45:0.1 v/v/v) used as mobile phase and flow rate 1.0 ml / min. with 15 minutes run time. The detection was carried at 220 nm with Newcrom R1 - 100mm x 3.2mm x 3µ column and ambient column temperature was maintained. In this connection, method uses the 20 µl injection volume and diluent as a blank solution. The linearity of this method was found to be linear in the range of 50% to 150% of the working concentration and the range for the analytical Method is 25 ppm to 75 ppm. The accuracy and precision of the method were within acceptable. The present developed HPLC method is detected to be suitable. The analytical solution was detected to be stable up to 48 Hrs at room temperature.

Keywords: vinclozolin, robust, precision, linearity and stability

Introduction
Vinclozolin was introduced at late 1970s, it acts like a systematic anti-fungal compound [1], which it is used to eradicate fugal plagues on vegetables and fruits like lettuce, cauliflowers, beans and grapes [2], it leads to terminate life of fungal contamination. It inhibits biosynthesis of triglyceride in forming of sclerotic fungi including Botrytis cinerea [3].
Vinclozolin is oxazole type fungicides, during the periodical applications of Vinclozolin fungicide Botrytis cinerea can be controlled in North America region which was investigated by USDA forest services. It is used in ornamentals and as turfs as in golf courses. During the course of scientific experiments Vinclozolin cannot affect the lactic acid bacreria. Experiments articulates mammalian attacks with chronic toxicity and it involving in the hormone of male like androgene receptor and affects feminization of cellular and a lest on of its metabolites is carcinogenic [4], furthermore adverse effects determined in the male fetuses in the mammalian species, testicular effects in additionally reported in the species of avians. Its solubility in water is 3.4mg/L, it is also soluble in ethanol, benzene, xylene and cyclohexane. Experimental results determined that Vinclozolin is pragmatically non-toxic to birds, honey bees and mammals on an acute level, and it is moderately toxic to freshwater fish, invertebrates on an acute level [5]. The structure of Vinclozolin was as follows.

Chemical name: ((RS)-3-(3, 5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2, 4-dione. Molecular formula is C12H9Cl2NO3 and Molecular weight is 286.11g/mol [6].

Early investigations expels that, there was accurate and reliable HPLC method has developed for using stability indicating method for the determination of Vinclozolin spontaneous deferments [7]. Subsequential literature survey, found chromatographic methods of stability validation studies for the resolvation of Vinclozolin by using RP-HPLC [8] and for LC-MS for Vinclozolin samples in cucumber, grapefruit, wheatflour, and kiwi fruits [9]. In the same manner cited in the compositions, method was detected to be reproducible and convenient for

the quantitative analysis of this dichlorophenyl dicarboximide fungicides [10]. Many authors were investigated the highly sensitive and more productive chromatographic methods. These methods represents biotransformation and non pharamaco dynamics of vinclozolin which resolute the relationship between toxicity and tissue dose of vinclozolin and its metabolites [11]. The limits for Assay of Vinclozolin for 20ml are not less than 95.0%. This analytical method verification report is intended to summarize the results obtained during the verification of HPLC method for the assay of Vinclozolin in Ronilan DF fungicide. A High Performance Liquid Chromatography-UV Detection (HPLC- UV/PDA) method for the quantitative determination of analytical method of assay of Vinclozolin in Ronilan DF fungicide, 10ml was developed and validated in the present study. The validation parameters such as Specificity or Selectivity, linearity, Method of precision, Intermediate Precision, Robustness and stability were studied according to the International Conference on Harmonization Guidelines with numbers: Q2A & Q2B of CPMP/ICH/281/95 and non pharmacopoeial method developed in- house [12].

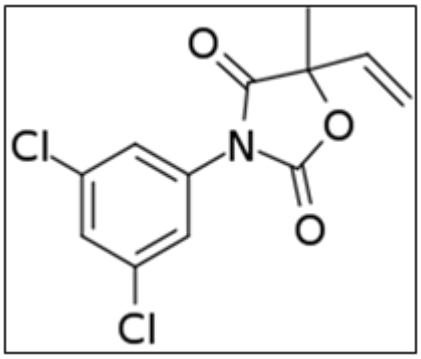

Fig 1: Structure of Vinclozolin

Chemical name: ((RS)-3-(3, 5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2, 4-dione. Molecular formula is C12H9Cl2NO3 and Molecular weight is 286.11g/mol [6].

Materials and Methods Chemicals and Reagents

All chemicals and reagents used for present study were of high quality and purity procured from various sources. Vinclozolin working standard and Ronilan DF fungicide, Phosphoric Acid-AR, Methonal-AR were purchased from Merck. Millipore water (HPLC- Grade) were procured from SD Fine chemicals, India. All the materials used were within the expiry date and stored at recommended storage conditions.

Preparation of Vinclozolin Standard Solution

Weigh accurately about 50 mg of Vinclozolin working Standard and transfer to a 50 ml volumetric flask. Add 20 ml of diluent and sonicate to deliquesce. Dilute to volume with diluent and mix. 1.0 ml of this solution transfer into a 10 ml of volumetric flask and then diluted to volume with the diluent and mix. (Scheme of Dilution: 50mg □□50.0 ml □□1 ml /10.0 ml)

Preparation of Sample Solution

Take 200mg weight of sample and then transfer into 50 ml volumetric flask. To dissolve, sonicate and augment 20ml of diluent [13]. Dilute to volume with diluent and mix. In 10ml of volumetric flask 1.0 ml of this solution is transfer and diluted to volume with the diluent and then mix.

(Scheme of Dilution: 100mg □□50.0 ml □□1 ml /10.0 ml) System Suitability Solution Preparation

Used Vinclozolin working standard solution as system suitability solution.

Procedure

Separately inject equal volumes of blank, five replicate injections of system suitability solution (Vinclozolin working standard solution). Subsequently inject two injections of test solution and record the chromatograms.

Ignore any peak due to blank in the test solution. Calculate % RSD of five replicate injections of system suitability solution (Vinclozolin standard working solution). Check tailing factor and theoretical plates of the peak in the chromatogram obtained with 5th injection of system suitability solution (Vinclozolin working standard solution).

The limits are as below,

1. Theoretical plates should be greater than or equal to 2000.
2. Tailing factor should be not more than 2.0. and
3. % RSD should be below 2.0%.

No options while fixing limits mention 2 or 3 not 2 and 3. 2 is enough. Everything in same manner.

Instrumentation and Chromatographic Conditions

For the current analysis, the HPLC - Agilent 1100 Series and HPLC- Waters - Alliance 510 pump with UV- 484 detector was used. The Chromeleon software and Data Ace softwares were utilized for data acquirement. Sample injection was done by auto injector which was coupled with instrument itself. System was equipped with HPLC Analytical column-Nucleosil R1 (100 mm × 3.2 mm x 3-µm dimensions) and column was maintained at ambient temperatures for quantification. Mettler Toledo-B204S as analytical weighing balance was employed for weighing the working substances [14].

Mobile Phase Preparation

Prepare a mixture of Methanol, water and Phosphoric acid in the ratio 55:45:0.1 respectively used as diluent which was blank sample. Mix well. The rate of flow has been 1.0 ml/min. with 15 minutes run time and uses the 20 µl injection volume for testing sample quantity. The detection was carried at 220 nm with ambient chromatographic conditions. Then Filter through 0.2 µm Nylon membrane filter paper and degas prior to use.

HPLC Method Validation

According to non pharmacopoeial method and the International Conference on Harmonization Guidelines, the method was validated in terms of Specificity or selectivity, linearity, method of precision, intermediate precision, robustness and stability studies of the samples.

Results and Discussion Specificity /Selectivity

In accordance of the analytical method the system suitability criteria were detected to converge with the pre-established acceptance criteria. The results of system suitability corresponding selectivity were shown in the Table 1 and standard chromatogram was given in the following Figure 1.

Entire injections were processed at the wavelength furnished in the method. There was no interference observed from diluent blank solution, placebo with Vinclozolin peak. From the Table 1, it was evident that the % of Relative standard lesser than one (0.25).

Result

The method is selective.

Linearity

In the theoretical concentration of preparation of assay, the linearity evaluation of five standard blends of Vinclozolin were developed in the span of initiating from 50% to 150%. As per the protocol the linearity solutions and the system suitability solutions were injected. The linearity graph of concentration in respect of peak performances was plotted and the correlation coefficient was detected. The average peak area of Vinclozolin peak at each concentration level was identified and the linearity graph was plotted against the sample concentration in percentage. The outcomes of linearity study are as given in Table 3. Below Figure 2 interprets, observation of a linearity graph of the average area at every level against the concentration (%) was plotted and was detected to be a straight line graph.

Table 1: System suitability – Selectivity

Sr. No.	Area of Vinclozolin
1	2174.32
2	2162.76
3	2174.21
4	2170.70
5	2164.25
Mean	2169.25
Standard Deviation(±)	5.47
(%) Relative Standard Deviation	0.25

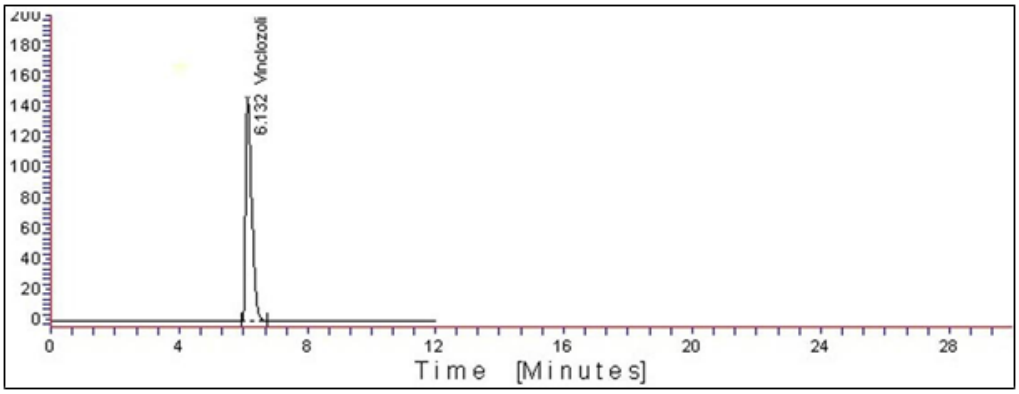

Fig 2: Standard chromatogram of Vinclozolin

Table 2: Result-A Table

Peak No	Retn.Time	Area	Height	Area %	Height %
1	6.132	2027.719	147.251	100	100
Total		2027.719	147.251	100	100

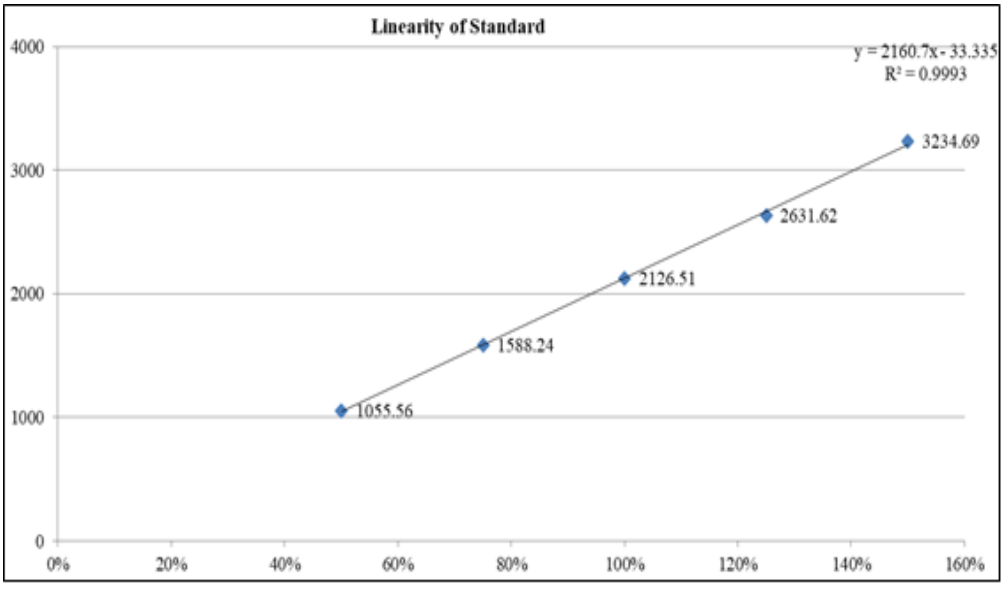

Fig 3: Linearity graph of Vinclozolin Standard

Table 3: System suitability - Linearity standard of Vinclozolin

Sr. No.	Area of Vinclozolin
1	2087.48
2	2065.75
3	2058.94
4	2074.90
5	2067.00
Mean	2070.81
Standard Deviation(±)	10.90
(%)Relative Standard Deviation	0.53

Results

1. A linearity graph of the average area at each level against the concentration (%) is plotted and is found to be a straight line graph. The correlation coefficient is detected to be greater than 0.999. Hence it is concluded that, the method is found to be linear in the range of 50% to 150% of the working concentration. The range for the analytical method is 50 ppm to 150 ppm.

Table 4: Results of linearity of standard

Linearity Level	Sample Concentration (in %)	Sample Concentration(in ppm)	Peak Area	Correlation Coefficient
Level – 1	50	50	1055.56	0.999
Level – 2	75	75	1588.24	
Level – 3	100	100	2126.51	
Level – 4	125	125	2631.62	
Level – 5	150	150	3234.69	

Precision System Precision

The system precision performed by injecting ten replicate injections of system suitability solution and the chromatograms are studied 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 have to pass as per analytical method. By the inference of analytical method, the system suitability criteria were detected to coincide with the pre-established acceptance criteria. Analytical method outcomes of System Precision shown in Table 5.

Table 5: System Suitability-System Precision

Sr. No.	Area of Vinclozolin
1	2046.86
2	1972.77
3	2045.54
4	2031.03
5	2027.72
6	1966.66
7	2034.95
8	2057.32
9	2044.02
10	1994.13
Mean	2022.10
Standard Deviation (±)	32.40
(%) Relative Standard Deviation	1.60

Result

Inference of the above data resolved that the system precision is well established.

Method Precision and Intermediate Precison

Six test solutions of Vinclozolin in Ronilan DF Fungicide was prepared as per the analytical method on different day. These test solutions were analyzed by a distinct analyst using distinct HPLC column of same preparation but having distinct serial number and distinct HPLC system.

Table 6: Results of twelve test solutions of Vinclozolin in (each of six samples from method precision & intermediate precision)

Analysis performed during method precision study By first Analyst on system 1 and on column 1 on day 1	
Same column	% Assay of Vinclozolin
1	99.96
2	98.70
3	99.86
4	98.99
5	100.09
6	99.79
Analysis performed during intermediate precision study By second Analyst on system 2 and on column 2 on day 2	

Column sr. no.	015132560136 02
Test Solution	% Assay of Vinclozolin
7	102.98
8	100.98
9	101.41
10	99.27
11	100.77
12	99.20
Mean of twelve samples	100.17
Standard Deviation (□)	1.21
(%) Relative Standard Deviation	1.21

The percentage of RSD of % assay outcomes of twelve test solutions (each of six samples from method precision and intermediate precision) was calculated. % RSD of the results of twelve test solutions (each of six samples from method precision and intermediate precision) should not be more than 2.0%. The system suitability criteria were detected to coincide the pre-established acceptance criteria as per the analytical method. The results of assay obtained from six test solutions are presented in the above Table - 5. % RSD of assay results from method precision and intermediate precision (12 results) are presented in the above Table - 6.

Result

The analysis was carried out on six test solutions of the same lot of the drug product by two distinct analysts with two separate equipments within the same laboratory using two distinct columns of the same preparation but having distinct serial numbers on two distinct days. The % RSD of the twelve assay results (six samples from each of method precision and intermediate precision) is identified to be less than 2.0%.Thus, the method is determined to be rugged and precise.

Robustness

Change in Column Lot

(Experimental Condition: Newcrom R1 - 100mm x 3.2mm x 3µ**)**

As per system suitability Change in Column Lot %RSD is 0.28 and 0.99 at different columns. Hence, the analytical method represents that the system suitability criteria were detected to coincide the pre-established acceptance criteria.

Table 7: Results of change column Lot

Flow rate □	**Same column**	**Different column**
Sample	**% Assay**	
Test solution	99.96	100.97
Average assay result from method precision	99.56	99.56
Mean	99.76	100.27
Standard Deviation (□)	0.28	1.00
(%) Relative Standard Deviation	0.28	0.99

Above Table 7 represents change in Column Lot results.

The assay results were obtained with different column conditions are as given in above Table 6.

Change in Flow Rate (□□0.2 mL/minute) (Normal Experimental Condition: 1.0ml/minute)

As per analytical method %RSD at different flow rates 0.8mL/min and 1.2mL/min is 0.72 and 0.38 respectively.

Hence, the analytical method represents that system suitability criteria were detected to coincide the pre-established acceptance criteria.

Change in Wavelength (± 2 nm)

(Normal Experimental Condition: 220nm)

As per analytical method %RSD at different wavelengths 218nm and 222nm is 0.33 and 1.67 respectively.

Hence, the analytical method represents that the system suitability criteria were detected to coincide the pre-established acceptance criteria.

Change in Composition of Mobile Phase

(Normal Experimental Condition: Methanol: water: Phosporic Acid = 550 ml:450 ml:1ml)

As per analytical method %RSD at different compositions 53MeOH: 47 W: 0.1 P and 57 MeoH: 43W: 0.1P is 0.23 and 0.56 respectively. Hence, the system suitability criteria were detected to coincide the pre-established acceptance criteria as per the analytical method.

Results

a. The analysis of the same lot of Vinclozolin in Ronilan DF Fungicide was carriried out at different conditions of column lot, flow rate, wave length and change in composition of mobile phase.

b. The system suitablity was detected to coincide the pre-established criteria at all the stipulations and the %RSD is not more than 2.0% in between results obtained with modified stipulation and average result of Method precision. The analytical Method meets the pre-established acceptance criteria for robustness study as per protocol.

Thus, the Method is robust.

Stability of Analytical Solution

System suitability solution and test solution of Vinclozolin in Ronilan DF Fungicide brought to developed on session 0th, 12th, 24th, 36th and 48th hour of experiment and stored

these solutions at normal storage temperature for every time period up to 48 hrs and analyzed these solutions on 48 hrs with newly prepared test solution. Results for Solution Stability shown in the below Table 7. During the analysis the system suitability solution was prepared afreshly. The assay of Vinclozolin in Ronilan DF Fungicide in the sample was calculated.

Table 8: Results for Solution Stability

% Assay results computed against the newly prepared system suitability standard	
Sample	**% Assay of Vinclozolin**
0th hr	100.04
12th hr	100.22
24 hr	99.79
36 hr	100.52
48 hr	98.92
Mean	99.90
Standard Deviation (□)	0.61
(%) Relative Standard Deviation	0.61

Result

The system suitability was detected to coincide the pre-established criteria and the % RSD between assay results obtained for afreshly prepared test solution and the stored test solutions is less than 2.0%. The Assay level observes there is no significant change up to 48Hrs of test solution at room temperature. Hence, consequently it can be concluded that the solution is stable up to 48Hrs at room temperature.

Conclusion

The HPLC-UV/PDA method for determination of Vinclozolin was completely validated by using specificity or selectivity, linearity, method of precision, intermediate precision, robustness and stability parameters. The approach was validated in accordance with ICH and non-pharmacopeia standards. A simple economic HPLC method has been developed for the quantitative estimation of Vinclozolin with good precision, linearity, and robust. The prepared method was detected to be specific and accurate for the assay of Vinclozolin. The analyte was considered stable if there is no significant change in % assay. Hence the solution was found to be stable up to 48 Hours at room temperature. For these reasons, hence, it is concluded that the analytical method was validated, can be used for routine analysis and for stability study. Consequently, the suggested method can be easily used for the quantitative quality control in agro pesticide industries, and future research also.

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Disclosure of Conflict of Interest

Among the authors no conflict of interest exist.

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