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Development and validation of the HPLC method for the analysis of ametoctradinin bulk and commercial dosage forms

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ABSTRACT

A simple, economic, selective, precise, and accurate High Performance liquid Chromatographic method for the analysis of Ametoctradin in bulk and commercial formulations was developed and validated in the present study. The mobile phase consists of a mixture of Methanol and water in the proportion 70:30 and adjust the pH to 6.0 ± 0.05 with sodium hydroxide solution. This was found to give a sharp peak of Ametoctradin at a retention time of 7.865 min. HPLC analysis of Ametoctradin was carried out at a wavelength of 294 nm with a flow rate of 1.0 ml/min. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient of 0.999 in the concentration range of 50 µg ml^{-1} to 150 µg ml^{-1}. The linear regression equation was y =2160x-495.9. The developed method was employed with a high degree of precision and degradation for the analysis of Ametoctradin. The developed method was validated for precision, robustness, detection and quantification limits as per the ICH guidelines. The wide linearity range, sensitivity, short retention time and composition of the mobile phase indicated that this method is better for the quantification of Ametoctradin.

Keywords: Ametoctradin, HPLC, Validation

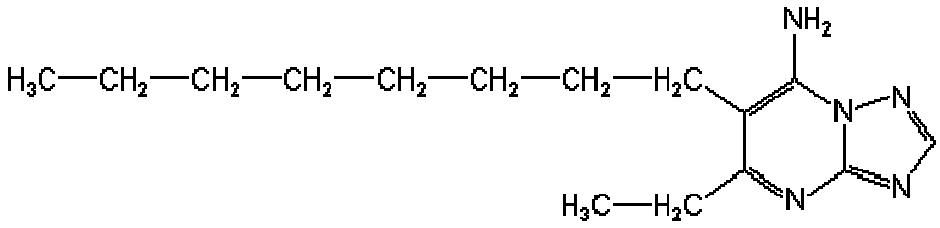
INTRODUCTION

A survey of the literature revealed that different analytical techniques for the assay of Ametoctradin have been reported. Voltametric detection of the herbicide Ametoctradin at a bismuth film electrode in no deaerated solution[1]Electroanalisis of Ametoctradin and metribuzen on lignin by Adsorption[2] ,Electrochemical reduction of Ametoctradin[3] , Identification of different products obtained by electrochemical and photochemical reduction of the Ametoctradin[4]Votametric determination of Ametoctradin with an elctrogenerated molecularity imprinted polymer microsencer[5] Electrochemical determination of the effect of lead(II) on the photochemical degradation of the pesticide Ametoctradin[6] Votametric determination of Herbicide Ametoctradin using Mercury and silver solid amalgam electrode[7]Preconcentration and voltametric determination of the herbicide Ametoctradin with a silica modified carbon paste electrode[8] Determination and method validation of Ametoctradin in soil by RP-HPLC[9] Electrochemical determination of the effect of Copper (II) on the photochemical degradation of the pesticide Ametoctradin[10] .

Early, analysis of Ametoctradin in Human plasma by HPLC with fluorescence detection , HPLC determination of Ametoctradin polyglutamates after Low-Dose Ametoctradin therapy in patients with Rheumatoid arthritis ,Quality control of Ametoctradin by HPLC and Polarographic and voltammetric methods for the quantitation of MTM in pharmaceuticals and plasma samples have been published.

There is however no reported HPLC method for the analysis of Ametoctradinin its technical grade and formulations. This is describes a validated HPLC method for the quantitative determination of Ametoctradin.

Structure:



Ametoctradin

Chemical name: 1,2,4] Triazolo [1,5-a] pyrimidin-7-amine, 5-ethyl-6-octyl (IUPAC)

Empirical formula: $C_{15}H_{25}N_5$

Molecular weight: 275.4

The HPLC method described here is simple, sensitive, and reproducible for Ametoctradin determination in formulations with low background interference. An attempt has been made to develop and validate to ensure here, precision and other analytical method validation parameters as mentioned in various gradients. One method reported for the HPLC determination for developed based on the use of a C-18column, with a suitable mobile phase, without the use of any internal standard. For formulation the proposed method is suitable for their analysis with virtually no interference of the usual additives presented in formulations.

1. Instrumentation
HPLC Analytical column Chromolith Zorbax Extended - C18, 250mm x 4.6mm x 5µ

Table- 1: Chromatographic conditions for Ametoctradin

Stationary phase	Mobile phase	Flow rate (ml min^{-1})	Run time (min)	Column Temp (0^c)	Volume of injection loop (µl)	Detection wavelength (nm)	Retention time (min)
Zorbax Extended - C18, 250mm x 4.6mm x 5µ	Methanol and water in the proportion 70: 30	1.0	15	25	20	294	7.865

II. Analytical Methodology
1. Preparation of Mobile phase
For isocratic system, prepare a mixture of Methanol and water in the proportion 70:30 respectively. Mix well, adjust the pH to 6.0 ± 0.05 with sodium hydroxide pellets. Filter through 0.2 µ Nylon membrane filter paper and degas prior to use.

2. Chromatographic conditions
Separation was performed on Zorbax Extended - C18, 250mm x 4.6mm x 5µ Column. Dimethyle Sulfoxide used as a Diluent and Mobile phase consists of mixture of Methanol and water in the proportion 70:30. Injection volume of 20 µl was used. Mobile phase was filtered before use through 0.5 µm Nylon membrane filter paper and degassed with helium purge for 10 min. The components of the mobile phase were pumped from solvent reservoir to the column at flow rate 1.0 ml min^{-1} and wavelength was set to 294 nm. The column temperature was set at 25^oC.

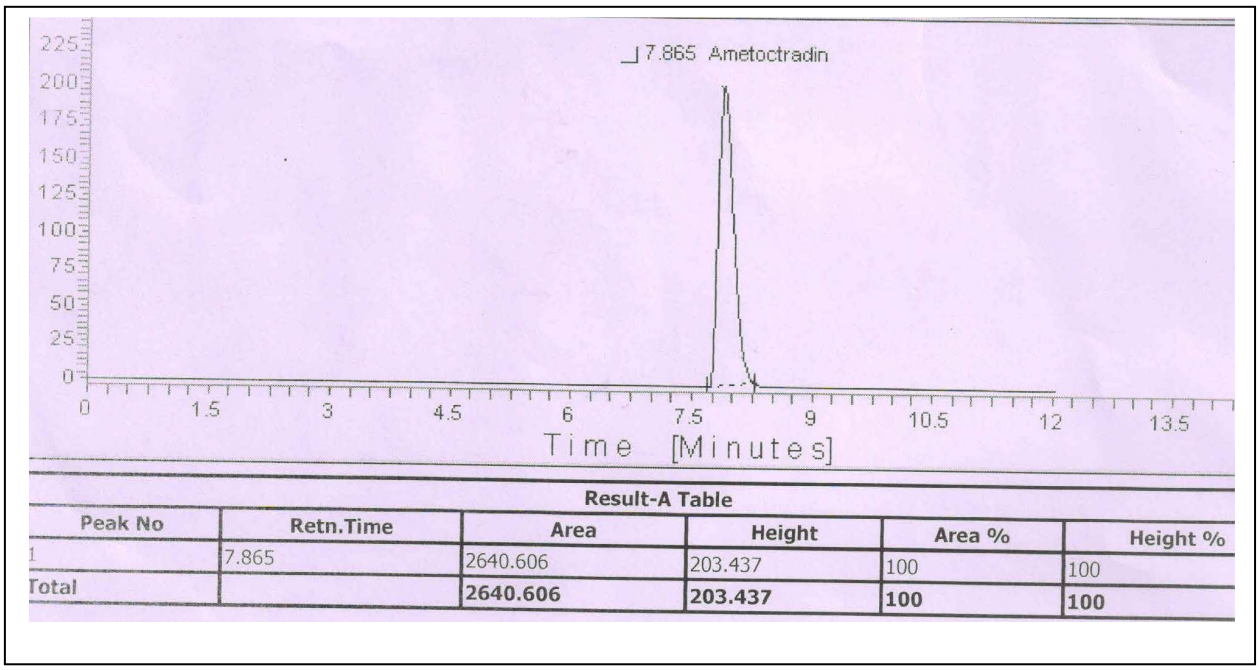
III. Analytical methodology:
❖Preparation of Ametoctradin Standard Solution:
Weigh accurately about 20 mg of Ametoctradin working Standard and transfer to a 25 ml volumetric flask. Add 10 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix.(Dilution scheme: 25mg → 25.0 ml → 1 ml /10.0 ml)

❖Preparation of Test Solution:
Weigh accurately about 80 mg of sample and transfer to a 25 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.(Dilution scheme: 80mg → 25.0 ml → 1 ml /10.0 ml)

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

❖Procedure:
Separately inject equal volumes of blank, five replicate injections of system suitability solution (Ametoctradin 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 (Ametoctradin Standard working solution). Check tailing factor and theoretical plates of the peak in the chromatogram obtained with 5th injection of system suitability solution (Ametoctradin Standard working solution).

Figure-1.: Chromatogram of Ametoctradin



Result-A Table

Peak No	Retn.Time	Area	Height	Area %	Height %
1	7.865	2640.606	203.437	100	100
Total		**2640.606**	**203.437**	**100**	**100**

Figure-2: Linearity graph of Ametoctradin standard

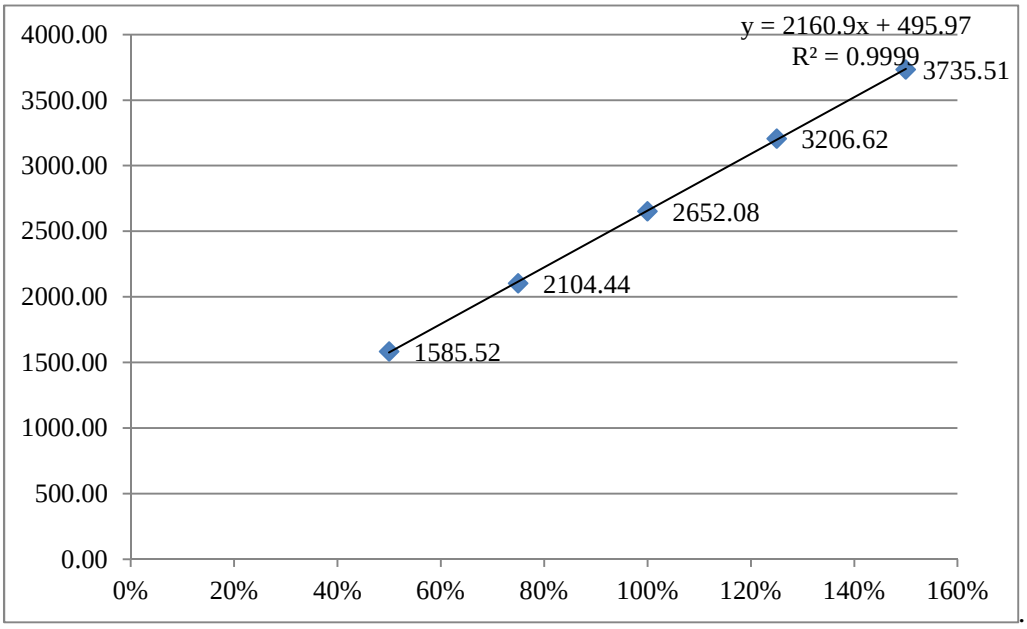


Figure-3: Precision graph of Ametoctradin standard

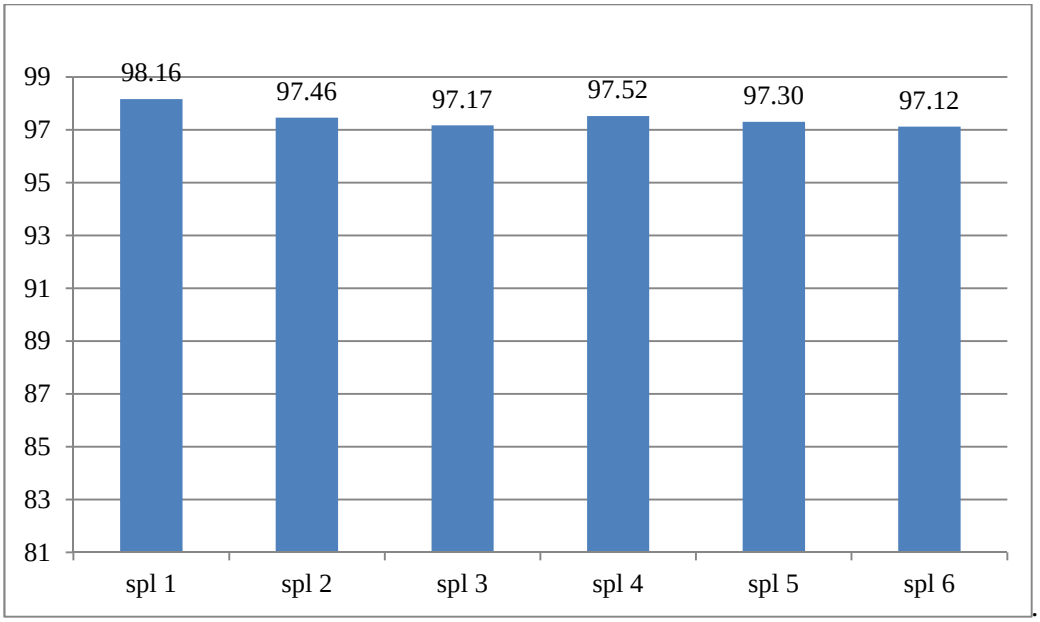


Figure-3: Intermediate precision graph of Ametoctradin standard

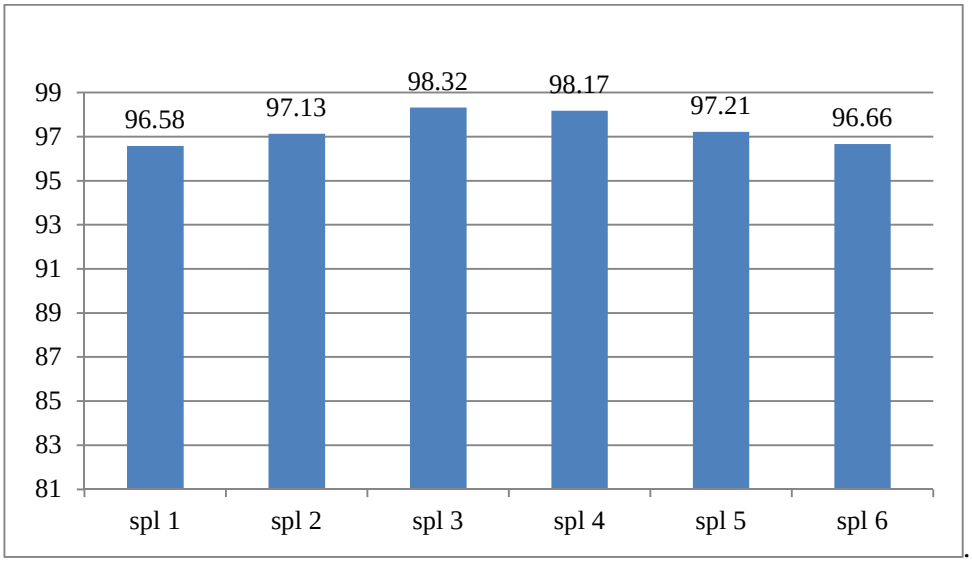


Table -2.: Performance calculations, detection characteristics precision and accuracy of the proposed method for Ametoctradin

Parameter	HPLC Method
Wavelength (nm)	294
Retention time (t) min	7.865
Linearity range (µg ml^{-1})	50-150
LOD(µg ml^{-1})	0.002031
LOQ(µg ml^{-1})	0.006773
Regression equation (y=bc+a)	
Slope (b)	2160
Intercept (a)	495.9
Standard deviation (SD)	1.463
Correlation coefficient(r^2)	0.999
Relative Standard deviation (%RSD)*	0.05508
Intermediate Precision (%RSD)	0.56
Range of errors	
Confidence limits with 0.05 level	2.867
Confidence limits with 0.01 level	3.768

**RSD of five independent determinations*

Table - 3: System suitability – Selectivity

Sr. No.	Area of Ametoctradin
1	2661.70
2	2602.34
3	2640.22
4	2652.85
5	2668.58
Mean	**2645.14**
Standard Deviation (±)	**26.17**
(%) Relative Standard Deviation	**0.99**

IV. Validation parameters

1. Forced Degradation

Table – 4: System suitability – Forced Degradation

Sr. No.	Area of Ametoctradin
1	2380.87
2	2355.66
3	2363.46
4	2373.96
5	2368.70
Mean	**2368.53**
Standard Deviation (±)	**9.66**
(%) Relative Standard Deviation	**0.41**

Table – 5 Conditions – Forced Degradation

Sample stress condition	Description of stress condition
Acid degradation	5N HCl heated at about 60°C for 10 min on a water bath.
Alkali degradation	5N 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

Acid Stress	% Degradation
Standard	0.213
Sample	0.218
Alkali Stress	**% Degradation**
Standard	0.139
Sample	0.115
Thermal Stress	**% Degradation**
Standard	0.252
Sample	0.006
UV Stress	**% Degradation**
Standard	0.012
Sample	0.017

2. Linearity

Table 6: System suitability - Linearity of standard

Sr. No.	Area of Ametoctradin
1	2600.09
2	2638.11
3	2681.67
4	2697.00
5	2640.61
Mean	2651.50
Standard Deviation (±)	38.48
(%) Relative Standard Deviation	1.45

Table 7: Results of linearity of standard

Linearity Level	Sample Concentration (in %)	Sample Concentration (in ppm)	Peak Area	Correlation Coefficient
Level – 1	50	50	1585.52	**0.999**
Level – 2	75	75	2104.44	
Level – 3	100	100	2652.08	
Level – 4	125	125	3206.62	
Level – 5	150	150	3735.51	

3. Precision:

3.1 System Precision:

Table 8: System precision

Sr. No.	Area of Ametoctradin
1	2382.56
2	2374.39
3	2397.79
4	2395.86
5	2355.66
6	2369.11
7	2358.48
8	2319.38
9	2346.18
10	2391.16
Mean	**2369.06**
Standard Deviation (±)	**24.78**
(%) Relative Standard Deviation	**1.05**

3.2 Method Precision:

Table - 9: System suitability - Method precision

Analyst – 1HPLC No.: EH/R&D/HPLC-024

Sr. No.	Area of Ametoctradin
1	2471.77
2	2522.39
3	2493.08
4	2494.58
5	2514.37
Mean	**2499.24**
Standard Deviation (±)	**19.87**
(%) Relative Standard Deviation	**0.80**

Table - 10: Results of Method precision

Test Solution	% Assay of Ametoctradin
1	98.16
2	97.46
3	97.17
4	97.52
5	97.30
6	97.12
Mean	**97.45**
Standard Deviation (±)	**0.38**
(%) Relative Standard Deviation	**0.39**

3.3 Intermediate Precision:

Table - 11: System suitability-Intermediate precision

Analyst – 2 HPLC No.: EH/R&D/HPLC-023

Sr. No.	Area of Ametoctradin
1	2407.55
2	2434.58
3	2436.08
4	2439.16
5	2418.61
Mean	**2427.20**
Standard Deviation (±)	**13.57**
(%) Relative Standard Deviation	**0.56**

Table - 12: Results of Intermediate precision

Test Solution	% Assay of Ametoctradin
1	96.58
2	97.13
3	98.32
4	98.17
5	97.21
6	96.66
Mean	**97.34**
Standard Deviation (±)	**0.74**
(%) Relative Standard Deviation	**0.76**

Table - 13: Results of twelve test solutions of Ametoctradin in Zampro™ Fungicide (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 Ametoctradin**
1	98.16
2	97.46
3	97.17
4	97.52
5	97.30
6	97.12
Analysis performed during intermediate precision study By Analyst 2 on system 2 and on column 2 on day 2	
Column sr. no.	**015337030136 01**
Test Solution	**% Assay of Ametoctradin**
7	96.58
8	97.13
9	98.32
10	98.17
11	97.21
12	96.66
Mean of twelve samples	**97.40**
Standard Deviation (±)	**0.56**
(%) Relative Standard Deviation	**0.58**

5.0 Robustness:

5.1.1 Change in Column Lot:

Normal Experimental Condition: Zorbax Extended - C18, 250mm x 4.6mm x 5µ

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method. (Refer to Table - 13 for system suitability results).

Table - 14: System suitability - Robustness with Change in Column Lot

Sr. No.	Area of Ametoctradin	
	Same column	Diff column
1	2961.76	2637.87
2	2973.53	2665.15
Mean	**2967.64**	**2651.51**
Standard Deviation (±)	**8.33**	**19.29**
(%) Relative Standard Deviation	**0.28**	**0.73**

Table - 15: Results for Change in Column Lot

Flow rate →	Same column	Diff column
Sample	**% Assay**	
Test solution	97.96	97.94
Average assay result from Method precision	97.45	97.45
Mean	**97.71**	**97.70**
Standard Deviation (±)	**0.36**	**0.35**
(%) Relative Standard Deviation	**0.37**	**0.35**

5.1.2 Change in Flow Rate (± 0.2 mL/minute):

(Normal Experimental Condition: 1.0ml/minute)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method. (Refer to Table - 15 for system suitability results).

Table - 16: System suitability - Robustness with change in flow rate

Sr. No.	Area of Ametoctradin	
	0.8mL/minute	1.2 mL/minute
1	2802.53	2641.68
2	2834.83	2681.67
Mean	**2818.68**	**2661.68**
Standard Deviation (±)	**22.84**	**28.27**
(%) Relative Standard Deviation	**0.81**	**1.06**

Table - 17: Results for change in flow rate

Flow rate →	0.8mL/minute	1.2 mL/minute
Sample	**% Assay**	
Test solution	98.25	98.92
Average assay result from Method precision	97.45	97.45
Mean	**97.85**	**98.19**
Standard Deviation (±)	**0.57**	**1.04**
(%) Relative Standard Deviation	**0.58**	**1.06**

5.1.3 Change in Wavelength (± 2 nm): (Normal Experimental Condition: 294nm)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method. (Refer to Table - 17 for system suitability results).

Table - 18: System suitability - Robustness with change in wavelength

Sr. No.	Area of Ametoctradin	
	292 nm	296 nm
1	2817.89	2841.40
2	2833.93	2831.47
Mean	**2825.91**	**2836.43**
Standard Deviation (±)	**11.34**	**7.02**
(%) Relative Standard Deviation	**0.40**	**0.25**

Table - 19: Results for change in wavelength

Wavelength →	292 nm	296 nm
Sample	**% Assay**	
Test solution	97.64	98.1
Average assay result from Method precision	97.45	97.45
Mean	**97.55**	**97.78**
Standard Deviation (±)	**0.13**	**0.46**
(%) Relative Standard Deviation	**0.14**	**0.47**

5.1.4 Change in composition of Mobile Phase (± 20ml):

Table - 20: System suitability - Robustness with change in composition of mobile phase

Sr. No.	Area of Ametoctradin	
	720ml:280ml	680ml:320ml
1	2646.17	2768.32
2	2678.22	2743.04
Mean	**2662.20**	**2755.68**
Standard Deviation (±)	**22.66**	**17.87**
(%) Relative Standard Deviation	**0.85**	**0.65**

Table- 21.Results for change in composition of mobile phase

Composition of Methanol & water	720ml:280ml	680ml:320ml
Sample	**% Assay**	
Test solution	96.75	96.44
Average assay result from Method precision	97.45	97.45
Mean	**97.10**	**96.95**
Standard Deviation (±)	**0.49**	**0.71**
(%) Relative Standard Deviation	**0.51**	**0.74**

Table-22: Stability of Analytical Solution

TIME	Std Area	Avgstd area	Spl area	AvgSpl area
0^{th}hr	2796.948	2812.38	2849.965	2858.44
	2827.809		2866.922	
12^{th}hr	2861.187	2819.95	2800.308	2812.45
	2778.718		2824.583	
24 hr	2806.371	2821.41	2836.101	2834.38
	2836.443		2832.648	
36 hr	2844.437	2851.07	2858.411	2819.41
	2857.704		2780.411	
48 hr	2782.763	2785.62	2831.091	2821.92
	2788.479		2812.754	
Mean	**2818.09**	**2818.09**	**2829.32**	**2829.32**
Standard Deviation (±)	**31.29**	**23.39**	**26.41**	**18.11**
(%) Relative Standard Deviation	**1.11**	**0.83**	**0.93**	**0.64**

Table - 23: Results for solution stability

% Assay results calculated against the freshly prepared system suitability standard	
Sample	**% Assay of Ametoctradin**
0^{th}hr	97.49
12^{th}hr	95.67
24 hr	96.36
36 hr	94.86
48 hr	97.19
Mean	96.31
Standard Deviation (±)	1.08
(%) Relative Standard Deviation	1.12

RESULTS AND DISCUSSION

The appropriate wavelength in UV region has been selected for the measurement of active ingredient in the proposed method. This method was validated by linear fit curve and all the other parameters were calculated.

Parameters fixation:

In developing methods, systematic study of the effects of various parameters was undertaken by varying one parameter at a time controlling all other parameters. The following studies were conducted for this purpose.

a. **Mobile phase characteristics**

In order to get sharp peaks and baseline separation of the components, carried out number of experiments by varying different components like percentage of organic phase in the mobile phase, total p^H of the selected mobile phase and flow rate by changing one at a time and keeping all other parameters constant. The optimum conditions obtained were included in the procedure proposed.

1. Detection Characteristics

To test whether Ametoctradin had been linearly eluted from the column, different amounts of Ametoctradin were taken and analyzed by the above mentioned procedures. The peak area ratios of component areas were calculated and the values are graphically represented in Fig1.1, the linear fit of the system was illustrated graphically. Least square regression analysis for the method was carried out for the slope, Intercepts and correlation coefficient. The results are presented in Table -1.

2. . Performance Calculations

To ascertain the system suitability for the proposed method, a number of statistical values have been calculated with the observed readings and the results are recorded in Table-1.

3. Method validations

The UV absorption maximum for Ametoctradin was fixed at 310 nm respectively. As the final detection was made by the UV - absorption spectrum, each method was validated by linear fit curve.

4. Precision

The precision of the method was ascertained separately from the peak area ratios obtained by actual determination of a fixed amount of sample. The percent of Relative Standard deviation calculated for Ametoctradinand are presented in Tables-7,8,9,10,11&12. The precision of the assays was also determined in terms of intra and inter-day variation in the peak areas for a set of sample solution was calculated in terms of coefficient of variation (CV)

5. Interference Studies

The effect of wide range of excipients and other additives usually present in the formulations of Ametoctradinin the determinations under optimum conditions were investigated. The common excipients such as colloidal Silicon dioxide, ethyl cellulose, hydroxyl propyl methyl cellulose, magnesium state, microcrystalline cellulose provide have been added to the sample solutions and injected. They have not disturbed the elution or quantification of Drug. In fact many have no absorption at this $\lambda_{max.}$

6. Analysis of Formulation

To find out the stability of the proposed methods for the assay of formulations containing Ametoctradin was analyzed by the proposed and reference methods. The proposed method does not differ significantly in precision from reference method. The results are recorded in Table-3.

7. 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 Ametoctradin peak is passing.

Hence, the method is very precise, selective and specific to the estimation of Assay of Ametoctradinin SG 700 g/l by HPLC and the same method is stability indicating, as the degraded products are well separated from Ametoctradinand as well from each adjacent peaks.

8. Ruggedness and Robustness

Ruggedness of the proposed method was determined by carrying out the analysis by two different analysts using similar operational i.e. Robustness with Change in Column Lot, Change in Flow rate, Change in wavelength and Change in p^H of the Mobile phase . The results were indicated by % CV in Tables – 13,14,15,16,17,18,19,20. Robustness of the method was determined by carrying out the analysis at two different wavelengths i.e. at 308 nm and 312 nm and the results were indicated by % CV in Table -18.

9. Solution Stability

The stability of the solutions under study was established by keeping the solution at room temperature for 48 Hours. The results indicate no significant change in assay values indicating stability of Drug in the solvent used during analysis. The results are recorded in Table -23.

CONCLUSION

The method was found to be accurate and precise, as indicated by recovery studies close to 100 and % RSD is not more than 2. The summery of validation parameters of proposed HPLC method is given.

The simple, accurate and precise HPLC method for the determination of Ametoctradinas bulk and form has been developed. The method may be recommended for routine and quality control analysis of the investigated drug in bulk and formulations. The analytical solution is found to be stable up to 48 Hrs 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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