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ANALYTICAL QUALITY BY DESIGN APPROACH BASED STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF IMEGLIMIN HCL AND VILDAGLIPTIN IN A SYNTHETIC MIXTURE

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ABSTRACT

The concept of Quality by design (QbD) has recently gained importance in the area of analytical method development and involves understanding of the critical factors and their interaction effects by a desired set of experiments. So, the present work describes the development of Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method by AQbD approach using Design of Experiments and subsequent validation for analysis of Imeglimin HCL and Vildagliptin in synthetic mixture. An efficient experimental design based on systematic scouting of all three key components of the RP-HPLC method (Proportion of methanol, flow rate, pH of mobile phase) is presented. The significance and interaction effects of these parameters on the response variables (Retention time, tailing factor and resolution) were evaluated through statistical analysis tools like Analysis of Variance (ANOVA) and plots which revealed the final chromatographic conditions of the method. The developed method was validated and forced degradation studies were also carried out in order to determine the stability-indicating nature of the method. The chromatographic separation was achieved on HIBAR C18, 250*4.6 mm, 5µm column using Acetonitrile: Water (60:40) as

mobile phase and detection was carried out using photo diode array detector at 219 nm. The developed method is linear over a range of 50 to 250 µg/ml for Imeglimin HCL and 2.5 to 12.5 µg/ml for Vildagliptin, correlation coefficient R2 value as 0.9999 and 0.9963 for Imeglimin HCL and Vildagliptin respectively. The %RSD for precision and accuracy of the method was found to be less than 2%. Forced Degradation studies revealed that the method was found to be stability-indicating. The results showed that the proposed method is suitable for the precise and accurate determination of Imeglimin HCL and Vildagliptin in synthetic mixture.

Keywords: Imeglimin HCL, Vildagliptin, RP-HPLC Method, Analytical Quality By Design, Stress testing

INTRODUCTION

Hyperglycemia is a hallmark of diabetes mellitus, a collection of physiological dysfunctions caused by either insufficient insulin secretion, excessive glucagon secretion, or insulin resistance. The autoimmune condition known as type 1 diabetes (T1D) causes the beta-cells in the pancreas to be destroyed. The more prevalent kind of diabetes (T2D) is mainly characterized by increasingly poor glucose management brought on by a confluence of insulin resistance and malfunctioning pancreatic beta cells **[1]**. Imeglimin HCL (IML) is an investigational first-in-class novel oral agent for the treatment of type 2 diabetes worked by amplification of glucose-stimulated insulin secretion and preservation of β-cell mass and enhanced insulin action, including the potential for inhibition of hepatic glucose output and improvement in insulin signalling in both liver and skeletal muscle **[2]**. Vildagliptin (VIL) is a strong and specific inhibitor of dipeptidyl peptidase-4 (DPP-4), the enzyme that breaks down the incretin hormones glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). This activity improves insulin secretion by raising levels of active incretins and improving the responsiveness of pancreatic islet α- and β-cells to glucose A methodical approach to method development, AQbD regulates every phase of the life cycle of the analytical technique. The analytical target profile (ATP) is defined, critical method parameters or factors are identified, and critical method attributes (CMAs) or responses are chosen. Screening and response-surface experimental designs allow the recognition of significant factors and their optimization by statistical analysis **[3]**. It is widely acknowledged that the development of robust analytical processes and analytical testing depend heavily on analytical quality by design, or AQbD. Since its inception, the method has proven valuable in producing high-performing pharmaceutical treatments. In addition to its

advantages in product development, AQbD has several uses in analytical development. Within analytical science, liquid chromatography technologies are increasingly being used for regular pharmaceutical examination and drug product quality assessment **[4]**. There are no analytical methods for estimating both medications. There is a plan to construct an analytical technique utilizing the analytical quality by design approach, conduct a force degradation studies, and validate the developed method.

MATERIAL AND METHOD

Instruments- The analysis of drugs was carried out by Agilent Infinity 1200 HPLC equipped with open lab software, Quaternary Gradient pump and PDA detector. Wavelength selection was done using Shimadzu 1900i having UV Probe 2.42 Software.

Chemical and Reagent- Standard Imeglimin HCL and vildagliptin obtained as a gift sample from Exemed Pharmaceutical, Vapi and Emcure Pharmaceutical, Mehsana respectively. HPLC grade methanol, water, acetonitrile was purchased from Merck Life Science Pvt. Ltd.

Characterization of API- Characterization of API was done using IR spectroscopy. Melting point of both drugs were done using differential scanning calorimetry. Solubility study was also performed to check solubility of API

Preparation of standard solution of IML- Accurately weighed 1 gm IML was dissolved in 100 ml methyl alcohol (10000 µg.ml -1). 1.0 ml from above Solution was withdraw volume was make up to 10 ml with methyl alcohol (1000 µg.ml-1 Master stock solution). Withdrawal of 1.0 ml from Master Stock Solution was done and made up to 10 ml with methyl alcohol (100 µg.ml-1)

Preparation of standard solution of VIL- Accurately weighed 50 mg of VIL was dissolved in 100 ml methyl alcohol (500 µg.ml -1). Withdrawal of 1.0 ml from above solution and volume was made up to 10 ml with methyl alcohol (50 µg.ml-1 Master stock solution). Withdrawal of 1.0 ml from master stock solution and volume was made up to 10 ml with methyl alcohol (5 µg.ml-1).

Preparation of standard solution of mixture- Accurately weighed IML + VIL (1 gm + 50 mg) dissolved in 100 mL methyl alcohol (10000+500 µg.ml-1). Withdrawal of 1.0 ml from above Solution and volume was make up to 10 ml with methyl alcohol (IML+VIL = 1000+50 µg.ml-1 Stock solution). 1.0 ml from Stock Solution was withdraw and volume was made up to 10 mL with methyl alcohol (IML+VIL = 100+5 µg.ml-1)

Software aided method development-A new RP-HPLC method was develop to estimate IML and VIL using AQbD approach. Method optimization was performed with full factorial design

comprising critical method attribute (CPMs) which include three significant factors (Proportion of methanol, flow rate, pH of mobile phase), encompassing three levels. Twenty-seven experimental runs were performed. The proportion of methanol was tested at 60,70,80 ml, the flow rate was 0.8,1,1.2 ml/min, the pH was 4,4.5,5. The response considered were retention time (R_t), tailing factor (T_f), resolution which were designated as critical analytical attribute (CAA). The data were analysed and the model was validated using Design-Expert software. The contour plot revealed a virtuous correlation with experimental data executed for design space navigation. The 3D surface plot was scrutinized to assess the indicative critical factors impact upon the observed responses or CAAs found within the predicted range. The predicted values from the experimental responses were found to be satisfactory and it was confirmed that it was acquired within the design space of the optimized results.

Optimization of chromatographic method using analytical QbD approach – Initially trial and error practice were executed to obtain rigorous data for the selected chromatographic method's performance and its finding of vital independent variables with its considerable effect upon the dependent variables**.** The statistical design intensifies ANOVA principles for establishing the optimized experimental condition of the method. AQbD based RP-HPLC method was developed which is also stability indicating. This was further subsequently validated as per international conference on Harmonization (ICH) **[5]**.

Optimizes chromatographic Condition- The isocratic flow rate was maintained at 0.9 ml/min. Analysis was carried out by HIBAR C18, 250*4.6 mm, 5µm. Methanol: water (70:30) was used as a mobile phase. Injection volume was 20 µL. Eluted sample was monitored at 219 nm.

Forced Degradation Studies Forced degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions. It is required to demonstrate specificity of stability indicating methods and also provides an insight into degradation pathways and degradation products of the drug substance and helps in elucidation of the structure of the degradation products **[6]**. New chemical entities and drug products must undergo forced degradation studies which would be helpful in developing and demonstrating the specificity of such stability indicating methods. At every stage of drug development practical recommendations are provided which will help to avoid failures **[7]**. To prove the stability indicating nature of the method, the stock solution of the IML and VIL was stressed under different conditions as

follows to promote degradation. The standard solution and the stressed solutions were prepared as follows

Preparation of standard solution- About 1 gm of IML and 50 mg of VIL weighed and transferred into a 100 ml volumetric flask. The volume was made up to mark with methanol. Withdraw 1.0 ml from above solution and make up to 10 ml with methyl alcohol.

Acid hydrolysis- From the standard solution,1 ml was transfer it to a 10 ml volumetric flask and add 5 ml of 0.5N HCl. The flask was heated at 70 Degrees on hot plate for 1 hr. After the heating cool down the solution and neutralize the contents with 0.5N NaOH. The volume was made up to mark with methanol.

Base hydrolysis- From the standard solution,1 ml was transfer it to a 10 ml volumetric flask and add 5 ml of 0.5N NaOH. The flask was heated at 70 Degrees on hot plate for 1 hr. After the heating cool down the solution and neutralize the contents with 0.5N HCL. The volume was made up to mark with methanol.

Oxidative Stress- From the standard solution,1 ml was transfer it to a 10 ml volumetric flask and add 5 ml of 3% hydrogen peroxide. The flask was heated at 70 Degrees on hot plate for 1 hr. After the heating cool down the solution. The volume was made up to mark with methanol.

Thermal Stress- About 1 gm of IML and 50 mg of VIL was weighed and transferred into a petri plate and keep it under hot air oven at 70 degrees for 3 hours. After exposure the plate was removed and rinse the contents with methanol in to a 100 ml volumetric flask. The volume was made up to mark with methanol. Further 1 ml from the above solution was taken in a 10 ml volumetric flask and dilute the contents with methanol.1 ml from the above solution was pipette out in a 10 ml volumetric flask and dilute volume was made up with methanol.

Validation of optimized condition [8-11]: Method validation was performed to substantiate that the analytical procedure employed for a definite experiment is appropriate for use. The outcomes of method validation parameters can be highly requisite to judge the reliability, quality, and steadiness of analytical results. Validation of a method for parameters specificity, linearity, accuracy, LOD, LOQ and precision was done according to recommendations of ICH guidelines

System Suitability: System suitability testing is an integral part of any analytical procedure. System suitability testing was carried out by injecting 6 replicates of 10µg/ml standard IML and VIL solution. In this test, system suitability parameters like retention time, number of theoretical plates and tailing factor, resolution were evaluated.

Specificity: Specificity of the method was determined by recording the chromatogram of standard stock solution of IML and VIL (100+5 µg/ml) and blank chromatogram (only diluent). Specificity signifies the identification of analyte, interference from other peaks and peak purity.

Linearity and Range: The linearity of the method was evaluated in the range of 50 - 250 % and 2.5 -12.5 % of the working concentration level of IML and VIL respectively. Then each level was injected six times into HPLC, chromatograms were recorded and peak area was recorded for all the peaks. The calibration graphs were plotted as peak area of the analyte against the concentration of the drug in µg/ml.

Accuracy: The accuracy of the developed method was studied to make sure the agreement between the true and reference values in three significant levels of 50%, 100%, and 150%. The percentage recoveries of three various concentrations were observed.

Precision: Precision of the system suitability test for six replicate standard injections was calculated, within the acceptance criteria, that is, NMT 2%, and both intraday and interday precisions through three replicate injections of standards were calculated. Data of % RSD were reported.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solution of IML and VIL using the developed HPLC method. The detection limit was derived as a signal-to-noise (S/N) ratio of 3:1, whereas the quantification limit was indicated as a S/N ratio of 10:1, as an effect of the response by the detector.

Assay: To perform assay accurately weighed amount of 104 mg placebo, IML (1 gm) and VIL (50 mg) was taken into the volumetric flask (100 ml) and volume of the flask was raised to 100 ml with methanol to give stock solution. 1.0 ml from above solution was pipette out and volume was made up to 10 ml with methyl alcohol. From the above solution 1.0 ml was pipette out in 10 mL volumetric flask; make up the volume with methanol

RESULT AND DISCUSSION

Development and optimization of new RP-HPLC method for IML and VIL using AQbD approach:

- A Quality by Design with Design of Experiments approach to the development of an analytical method mainly involves two phases. **1: Screening Phase-** The first phase of the method development involves the screening of the major effectors. The high-risk parameters are selected using Ishikawa fish bone diagram and Risk estimation matrix.

(Fig:3) This screening phase was carried out using Design Expert 10 software. Full factorial design was chosen to optimize the CMPs. The responses obtained after carrying out the 27 experimental trial runs under full factorial design were fed back to DoE software. The values of responses (retention time, tailing factor and resolution) are tabulated in **Table 1**. **2: Statistical Analysis and Final Optimisation-** Statistical Analysis was used to identify the significant influential chromatographic factors like Proportion of methanol, flow rate, pH of mobile phase (CMP) and their interaction impact on the three responses i.e. Retention, Tailing factor and Resolution. The analysis of 3D-response surface plots and Graph plots were used to estimate as to which method parameter gave the most acceptable responses. Statistical analysis tool like ANOVA was evaluated for each individual response to determine the most influential chromatographic parameter. The design matrix of the design and experimental runs are shown in **Table 2**. The statistically constructed model was suitably validated by interaction studies using the effect of various factors upon the found responses. The 3D surface plot and contour plot for various response for IML and VIL are shown in figure 4-13 elucidates the parameters intended for numerical optimization for desirability and optimized data for factors indorsed by design. Finally, the optimized apparent chromatographic conditions can be well predicted from the arithmetical model, and it has strongly been recommended for the developed analytical method. Chromatographic Condition after optimization by Design Expert Software is described in **Table 3**. In all parameters values of "Prob > F" less than 0.0500 indicate model terms are significant.

Forced Degradation Studies: Forced degradation studies was done on standard IML and VIL indicated that IML was degraded 17.59%, 14.85%, 14.98%, 7.67% while VIL was degraded up to 15.55%, 12.33%, 16.11%, 8.42% when it exposed to acid hydrolysis **(Fig:14)**, base hydrolysis **(Fig:15)**, oxidative degradation **(Fig:16)** and thermal degradation **(Fig:17)** respectively **(Table 4)**. This shows that both drugs susceptible to acid hydrolysis, base hydrolysis and oxidation. While, the drug was found to be stable after thermal degradation and photolytic degradation. The results of forced degradation studies reveal

that all the degradation products were fully resolved and do not interfere with the analyte peak which indicates specificity of the method. Thus, the method can be employed for monitoring the stability of both drug in bulk drug. Moreover, these studies also determine the physical and chemical stability of drug substance and drug product which may be further useful to determine the storage conditions for the drug product.

Validation of the optimized method: Once the chromatographic conditions were set, method validation was done on IML and VIL for: System suitability, Specificity, Limit of Detection, Limit of Detection, Linearity, Range, Accuracy and Precision.

Evaluation of System Suitability: The standard solution (IML:100 µg/ml, VIL: 5 µg/ml) was injected six times. The %RSD obtained from six replicate injections was found to be less than 2.0%. Tailing factor was less than 2.0. Theoretical plates were also found to be above 2000. Thus, all the parameters evaluated for system suitability were found to be within the acceptance criteria and the system was suitable for analysis of IML and VIL.

LOD and LOQ: The LOD and LOQ was obtained by successively decreasing the concentration of both drugs. As long as a signal to noise ratio of not less than 3:1 and 10:1 is maintained respectively. The LOD of IML was found to be 1.05 µg/ml and 0.04 µg/ml for VIL. The LOQ for IML and VIL was found to be 3.20 µg/ml and 0.13 µg/ml respectively. These values indicate that the method developed is sensitive.

Specificity: Blank (Diluent) and Standard solution (IML:100 µg/ml, VIL: 5 µg/ml) were injected. The method was quite selective for IML and VIL as there was no other interfering peak around the retention time of drugs **(Fig.18-20)**.

Linearity and Range: Linearity was evaluated in the range of 50 to 250 µg/ml for IML and 2.5 to 12.5 µg/ml for VIL of the working concentration level. The Linearity was confirmed in the range. The co-efficient of co-relation (R2) was found to be 0.999 and 0.9963, Regression Equation was y = 515.21x - 244.17 and y = 3657.6x - 270.65 as evident from the below calibration curve for IML and VIL respectively. Thus, the data shows that the response is found to be linear **(Fig:21)**. This clearly indicates that an excellent correlation existed between the peak area and concentration of the analyte. The linearity curve for IML is shown in **Fig:22** and for VIL is shown in **Fig: 23**.

Precision and Accuracy: Precision is reported in terms of Relative Standard deviation (RSD) over the range of quantitation for a single experiment in which standards are assayed in replicate (Intraday) and for a series of experiments in which standards are assayed in over several experiments (Interday). The data for

intraday and inter day is shown in **Table 5 and 6** respectively. Accuracy was calculated for the same solutions which were injected for Intraday Precision. The results for Accuracy are shown in **Table 7**. The precision of the method for the standard solution of IML and VIL shows that the Relative Standard Deviation (RSD) for both intraday and inter day falls within the limits i.e. within 2%. Moreover, the accuracy data shows that the % mean recovery of drugs at each level is within the acceptance criteria of 98.0% - 102.0%.

Assay: Assay was performed on placebo. Percentage recovery for both drugs is shown in **Table 8**.

CONCLUSION: The RP-HPLC method developed for Imeglimin HCL and Vildagliptin by AQbD approach is linear, accurate, precise, reproducible and specific as evident from the validation results. The developed method is also stability indicating and can be conveniently used for quality control to determine the assay in regular product development, production and stability samples.

Table 1: ANNOVA by selecting 3^3 designs

Factor	Name	Units	Minimum	Maximum	Coded	Values	Mean
A	Proportion of ACN	ml	60	80	-1.000=60	1.000=80	70
B	Flow Rate	ml/min	0.8	1.2	-1.000=0.8	1.000=1.2	1
C	pH of mobile phase	-	4	5	-1.000=4	1.000=5	4.5

Table 2: Experimental run by selecting full factorial 3^3 designs

Run	Factor 1 Proportion of ACN	Factor 2 Flow rate	Factor 3 pH of mobile phase	Rt of IML	Rt of VIL	Tf of IML	Tf of VIL	Resolution
1	80	1.2	4	4.877	9.586	0.951	1.391	5.782
2	80	1.2	4.5	4.905	9.614	1.002	1.423	5.814
3	80	1.2	5	4.928	9.659	1.041	1.466	5.856
4	80	1	4	4.956	9.702	1.071	1.489	5.899
5	80	1	4.5	4.992	9.761	1.099	1.503	5.922
6	80	1	5	5.062	9.834	1.121	1.538	5.948
7	80	0.8	4	5.124	9.869	1.164	1.571	5.988
8	80	0.8	4.5	5.158	9.893	1.184	1.599	6.064
9	80	0.8	5	5.192	9.931	1.202	1.643	6.091
10	70	1.2	4	4.811	9.532	0.983	1.486	5.701
11	70	1.2	4.5	4.857	9.581	1.021	1.524	5.748
12	70	1.2	5	4.905	9.634	1.066	1.573	5.781
13	70	1	4	4.912	9.677	1.087	1.594	5.803
14	70	1	4.5	4.981	9.754	1.127	1.656	5.869
15	70	1	5	5.045	9.812	1.158	1.698	5.901
16	70	0.8	4	5.098	9.841	1.171	1.712	5.924
17	70	0.8	4.5	5.119	9.872	1.196	1.743	5.963
18	70	0.8	5	5.156	9.899	1.251	1.781	5.989
19	60	1.2	4	4.786	9.477	1.156	1.541	5.614
20	60	1.2	4.5	4.802	9.499	1.193	1.592	5.668

21	60	1.2	5	4.859	9.529	1.222	1.611	5.693
22	60	1	4	4.888	9.556	1.248	1.658	5.738
23	60	1	4.5	4.934	9.582	1.283	1.696	5.782
24	60	1	5	4.982	9.614	1.308	1.722	5.811
25	60	0.8	4	5.014	9.666	1.336	1.751	5.841
26	60	0.8	4.5	5.066	9.683	1.374	1.788	5.883
27	60	0.8	5	5.099	9.701	1.398	1.801	5.903

Table 3: Chromatographic Condition after optimization by Design Expert Software

Proportion of ACN	ACN (63.2 v/v) - Optimized
pH	4.4 - Optimized
Selection of Stationary phase	HIBAR C_{18}, 250*4.6 mm, 5µm
Flow rate	0.9 ml/minutes - Optimized
Injection volume	20 µl
Analytical wavelength	219 nm

Table 4: Result of forced degradation studies

Degradation method	Optimized condition	% Degradation	
		IML	VIL
Acid hydrolysis	0.5 N HCl and refluxed for 60 min. at 70°C	17.59 %	15.55 %
Base hydrolysis	0.5 N NaOH and refluxed for 30 min. at 70°C	14.85 %	12.33 %
Oxidative degradation	3% H_2O_2 at room temperature for 1 hr.	14.98 %	16.11 %
Thermal degradation	90°C for 4 hrs.	7.67 %	8.42 %

Table 5: Intraday precision of IML and VIL

IML			VIL		
Conc. (µg/ml)	Area Mean ± SD (n=3)	%RSD	Conc. (µg/ml)	Area Mean ± SD (n=3)	%RSD
50	25721.67 ± 311.01	1.21	2.5	9178.33 ± 165.84	1.81
150	76597.00 ± 713.75	0.93	7.5	165.84 ± 393.42	1.52
250	128807.00 ± 757.76	0.59	12.5	46895.00 ± 614.02	1.31

Table 6: Inter day precision of IML and VIL

IML			VIL		
Conc. (µg/ml)	Area Mean ± SD (n=3)	%RSD	Conc. (µg/ml)	Area Mean ± SD (n=3)	%RSD
50	25758.33 ± 333.80	1.30	2.5	9166.00 ± 176.31	1.92
150	76683.33 ± 801.05	1.04	7.5	25907.00 ± 418.03	1.61
250	128767.67 ± 899.71	1.70	12.5	46945.33 ± 666.85	1.42

Table 7: Recovery data for IML and VIL

IML (n=3)			VIL (n=3)		
Amount of drug added (µg/ml)	Amount of drug recovered (µg/ml) ± SD	% Recovery	Amount of drug added (µg/ml)	Amount of drug recovered (µg/ml) ± SD	% Recovery
50	49.82 ±1.00	99.65	2.5	2.48±0.03	99.33
100	99.57±1.48	99.57	5	4.97±0.08	99.47
150	149.33±1.53	99.56	7.5	7.50±0.10	100.00

Table 8: Assay for IML and VIL

IML			VIL		
Amount taken	Amount found ± SD	% Assay	Amount taken	Amount found ± SD	% Assay
100 µg/ml	98.56	98.56	5 µg/ml	4.93	98.60
	99.71	99.71		4.99	99.80
	101.38	101.38		5.07	101.40
Mean	99.88	99.88	Mean	5.00	99.93
SD	1.42	1.42	SD	0.07	1.40
% RSD	1.42	1.42	% RSD	1.41	1.41

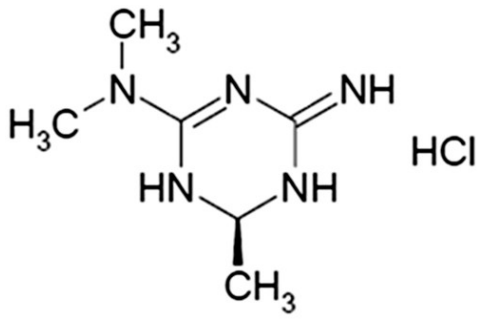

Fig- 1 Chemical Structure of IML

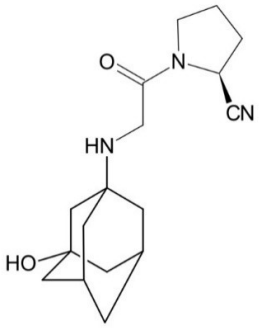

Fig- 2 Chemical Structure of VIL[11]

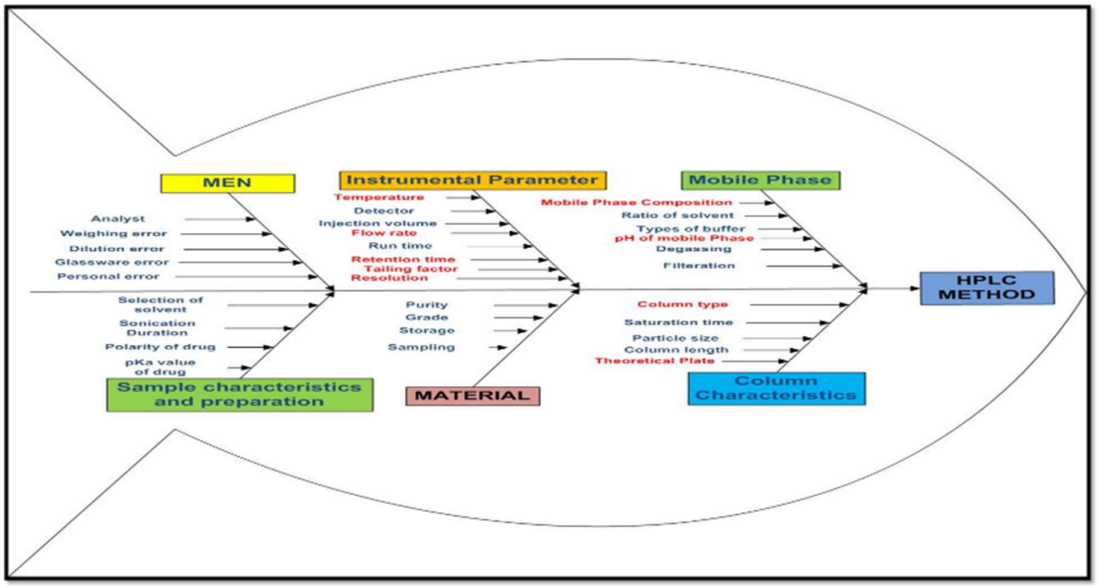

Fig: 3 Ishikawa fish- bone diagram

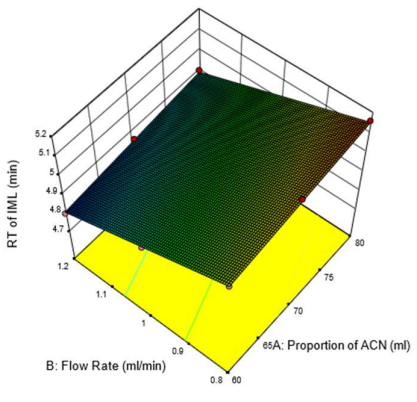

Fig- 4 3D Surface Plot of RT of IML

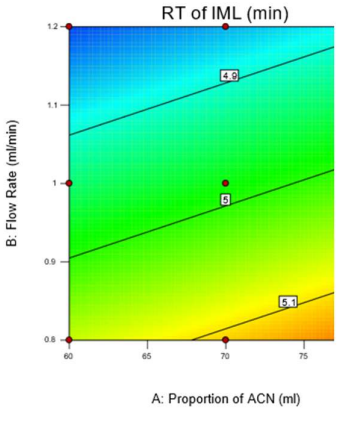

Fig-5 Contour Plot for RT of IML

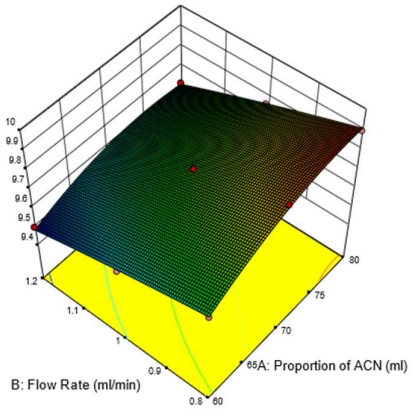

Fig-6 3D Surface Plot of RT of VIL

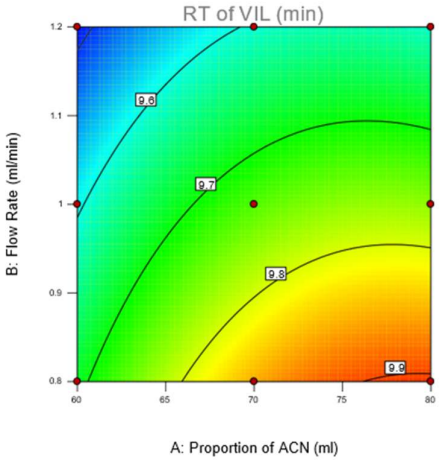

Fig-7 Contour Plot for RT of VIL

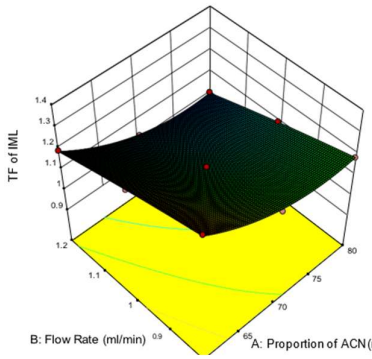

Fig-8 3D Surface Plot of TF of IML

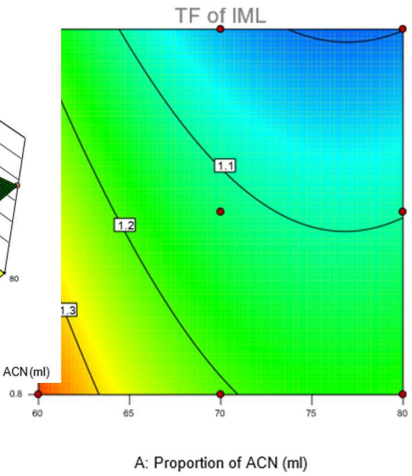

Fig-9 Contour Plot for TF of IML

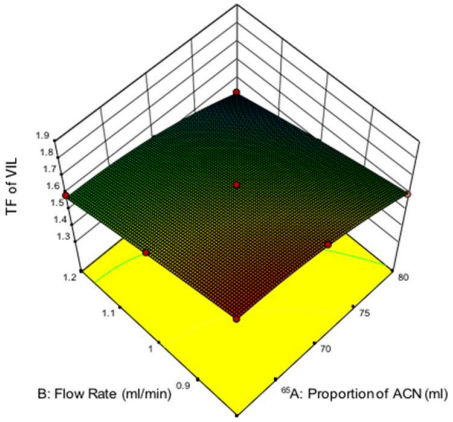

Fig-10 3D Surface Plot of TF of VIL

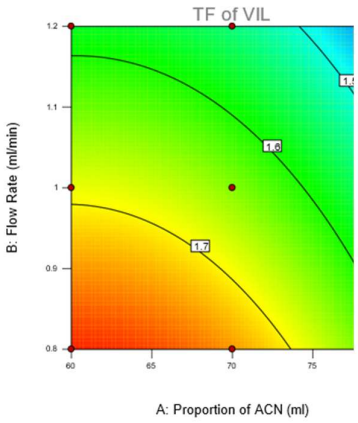

Fig-11 Contour Plot for TF of VIL

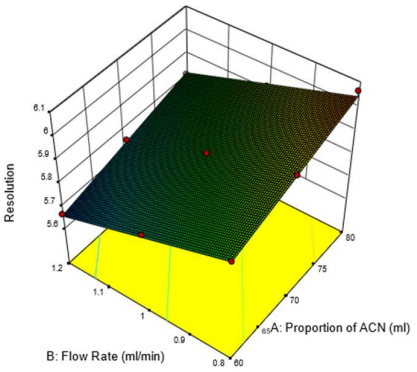

Fig-12 3D Surface Plot of Resolution

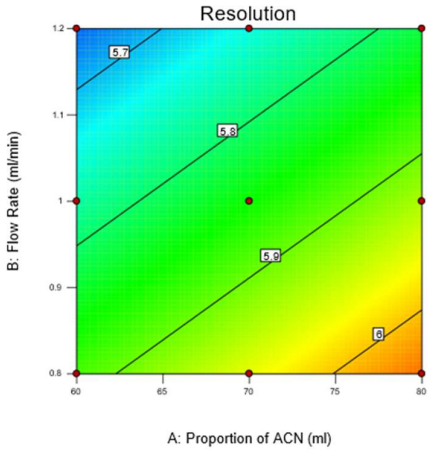

Fig:13 Contour Plot for resolution

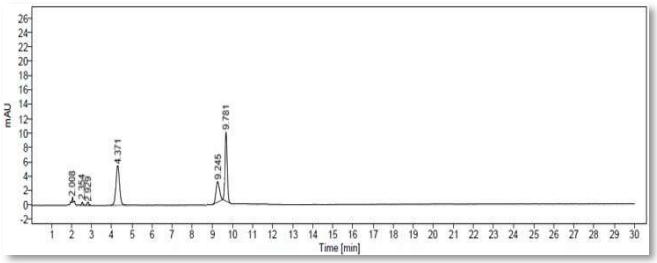

Fig:14 Chromatogram of Acid Hydrolysis

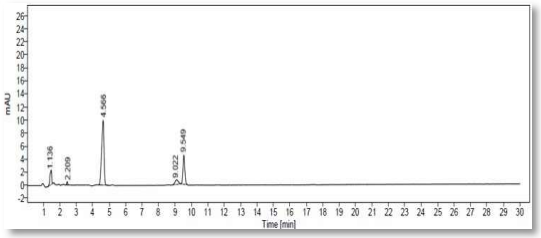

Fig:15 Chromatogram of Base hydrolysis

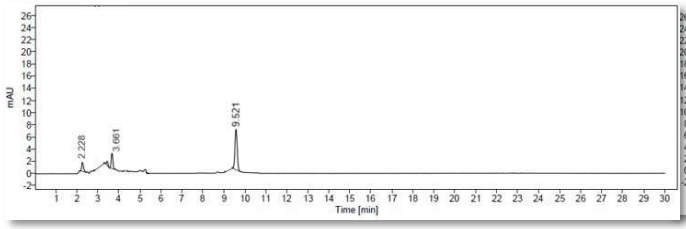

Fig:16 Chromatogram of oxidative degradation

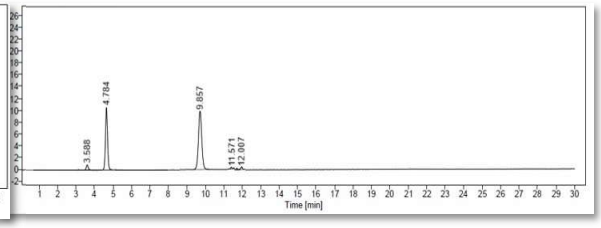

Fig:17 Chromatogram of thermal degradation

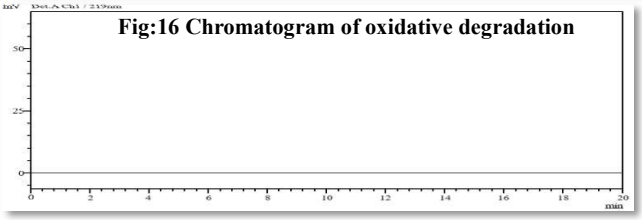

Fig-18 Specificity chromatogram of diluent

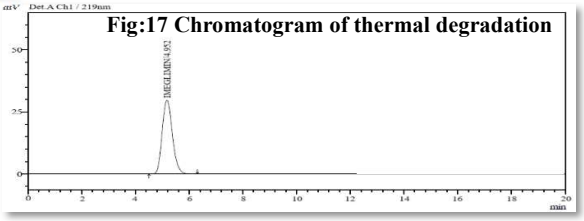

Fig-19 Specificity chromatogram of standard IML (100 µg/ml)

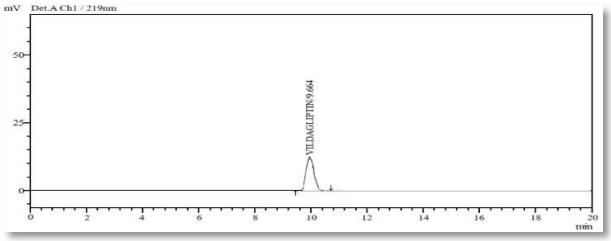

Fig:20 Specificity chromatogram of standard VIL (5 µg/ml)

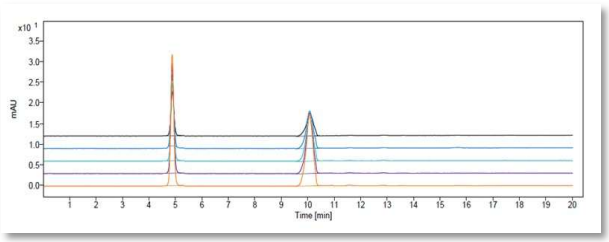

Fig: 21 Overlay linearity graph of IML and VIL

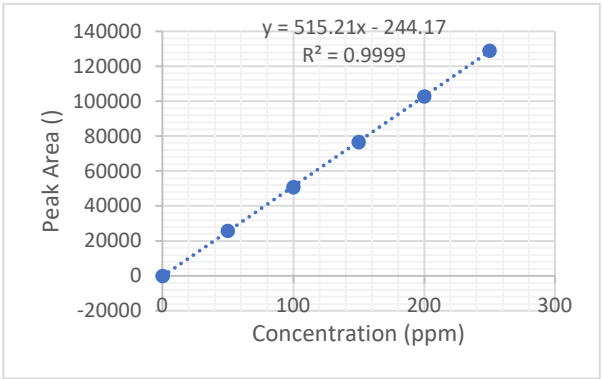

Fig:22 Calibration curve of IML

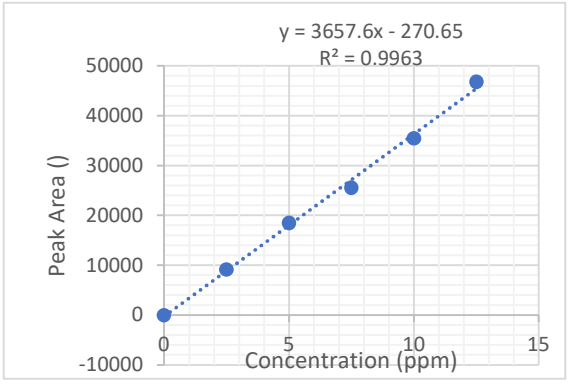

Fig:23 Calibration curve of VIL

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