Ritesh P. Bhole et al /J. Pharm. Sci. & Res. Vol. 11(5), 2019, 1861-1869

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

A Stability Indicating HPTLC Method for Apremilast and Identification of degradation products using MS/MS.

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Abstract

Aim: Apremilast is used for treatment of certain types of psoriasis, psoriatic arthritisand plaque psoriasis is a type of skin condition. An extensive literature search revealed that there exist very few methods on RP-HPLC reported and HPTLC method was not reported .This study deals with simple, precise, accurate and economical stability indicating HPTLC method development with validation as per ICH guidelines with application of developed method on in Apremilast pharmaceutical formulations and Identified and Characterization of degradation products using MS/MS.

Method: Precoated Silica gels plates were used as stationary phase. Toluene: Ethyl Acetate (4:6; v/v) was delivered best separation at 236 nm (Rf 0.55±0.02) by densitometry analysis. Degradation analysis was performed as per ICH guidelines (Q2R1). Isolation of degradation product by HPTLC method and identify by MS/MS method.

Results: The linearity was 100-600 ng with R^2 of 0.997 while, % RSD was in range. LOD and LOQ of Apremilast were found 0.77ng/spot and 2.35ng/spot respectively. The recovery of Apremilast was found to be 99.70±0.23%. The % assay of active substance was found in a range 80.62 to 100. HPTLC and MS/MS method revealed possible degradation mechanism of 11 degradant products.

Conclusion: The Proposed developed and validated HPTLC method was found to be more sensitive, simple, precise, accurate, cost effective and robust. This method could be applied for analysis of bulk drug and tablet formulation, degradation study. This degradation pathway of drug will help to identify the degradation products of Apremilast.

Keywords: HPTLC, Aprimilast, MS-MS studies, Degredation Mechanism

1. INTRODUCTION

Apremilast (APL) is a chemically N-{2-[(1S)-1-(3-Ethoxy-4-methoxyphenyl)-2- (methylsulfonyl) ethyl]-1, 3-dioxo-2, 3-dihydro-1H-isoindol-4-yl} acetamide Fig. (1). It may be also helpful for other immune system related inflammatory diseases. APL is a small molecule inhibitor of phosphodiesterase type 4 inhibitor (PDE4)and tumour necrosis factor α (TNF α). APL is used to block the action of PDE4 on cyclic adenosine monophosphate (cAMP). In inflammatory cells, PDE4 is a key enzyme is liable for this reaction [1-3].

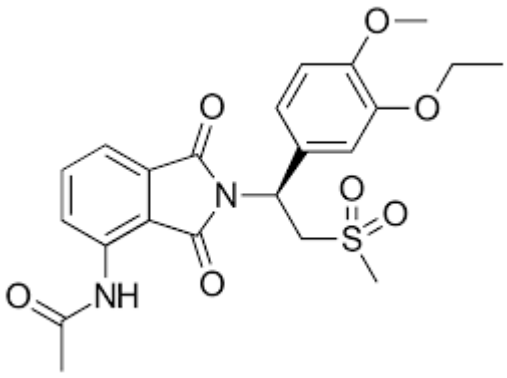

Fig (1) Structure of Aprimilast

APL (Otezla®**)** is a USFDA-approved drug, for the treatment of different types of psoriasis and psoriatic arthritis. It was developed by Celgene Corporation Company [4-6].The anticipated method was optimized with validation as per the ICH Q2 R1 and stability indicating study performs as per the ICH Q1A R2 and ICH Q1B guidelines [7-9]. Literature survey shown that very less methods are available for the analysis of APL in bulk and in pharmaceutical formulations. The estimation of APL in rat plasma by UPLC-MS-MS and its use to a pharmacokinetic study [10]. Development and validation of stability indicating RP-HPLC, UV spectrophotometric method for the estimation of APLwas also reported [11-12]. Development and validation of stability indicating fast RP-LC method was performed for estimation of process and degradation interrelated impurities of APL, an anti-inflammatory drugin the said research work the eight process interrelated impurities (Imp-1 to Imp-8) have been identified by develop RP-LC method and were characterized using MS, FT-IR also NMR spectroscopic techniques [13]. Identification and characterization of process-related substances alongwith its degradation products in APL process optimization and degradation pathway elucidation by LC-MS/MS analysis are found to be 12 impurities. [14].the identification, characterization and HPLC quantification for impurities of APL by and they found that the impurities in the mentioned research work as(Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-F) are generated in the process from the intermidate-1 to APL. Imp-A, Imp-B, Imp-C and Imp-D are identified by LC-MS and Imp-E, Imp-F are found by Column Chromatography and preparative Liquid Chromatography [15].The oral dose formulation containing APL 10mg, 20mg, 30mg is available in the market with a brand name Otezla. Till now no method was reported with HPTLC stability indicating method for estimation of APL in pharmaceutical formulation. So it is beneficial to develop a novel stability indicating method for APL. The present work focused on development of simple, economic, accurate, precise, rapid, and selective High-Performance Thin-Layer Chromatographic method for determination of APL in bulk

and pharmaceutical formulation. The proposed method was optimized and validated as per the (ICH) Q2 R1 guidelines.

2.MATERIALS AND METHODS

2.1 Materials

Pure drug of APL are received as a gift sample from Megafine Pharma (P) Ltd., Mumbai, India. Otezla (10 mg of Apremilast), is purchased from local market. All solvents used in the chromatography such as methanol, toluene, and ethyl acetate were of AR grade and precoated Aluminium TLC plates with silica gel 60 F_{254} (0.2 mm thick) were purchased from E. Merck Ltd., Mumbai India.

2.2 Methods

2.2.1 Equipments

HPTLC System : Camag TLC system (Muttenz, Switzerland)
Software: WinCAT's version (1.4.3.6336)
Syringe for Sample Application : 100 µl syringe (Hamilton, Bonaduz, Switzerland).
TLC Plate:Aluminium plates precoated with silica gel 60 F_{254} plates (E.Merck, Germany)
Sample Applicator: Camag Linomat-V
Gas for Sample Application: Nitrogen
Development Chamber: Camag twin trough chamber (10 X 10 cm)
TLC Scanner: Camag TLC scanner III
Ultrasonicator:Wensar (WUC-2L)
LC-MS : Shimadzu 8040 with Mass range: 2 to 2000 m/z. Ionisation sources: Electro spray Ionisation (ESI) and Atmospheric Pressure Chemical Ionization (APCI).

2.2.2 Chromatographic condition

Stationary Phase : AluminiumTLC plate precoated with silica gel 60 F_{254} (10 x 10 cm)
Mobile Phase of APL: Toluene: Ethyl Acetate (4:6v/v)
Saturation Time of Mobile Phase : 15 min
Development Time (Run) : 10 min
Development Distance on plate : 8 cm
Densitometry of scanning mode : Absorbance–Reflectance.
Scanning Wavelength of APL : 236 nm.

2.3 Selection of detection wavelength

After development of chromatographic condition the HPTLC plate were scanned in Camag TLC scanner III using UV lamp at the wavelength of 200-700 nm. It was observed that the APL shown strong absorbance at 236.0 nm wavelength was selected for the HPTLC method validation shown in fig. (2).

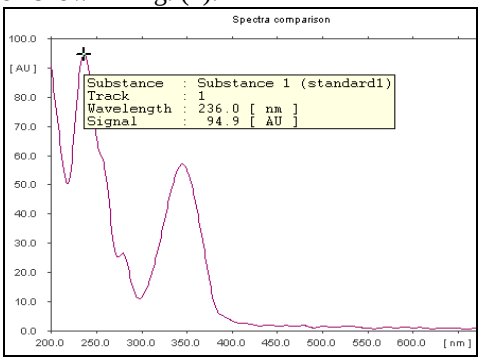

Fig. (2) Spectra of Apremilast (236nm)

2.4 Preparation of Stock Solution

Precisely weighed quantity of APL 10 mg was transferred in 10 ml volumetric flask, dissolved in 5 ml of methanol and ultrasonicated for 10 min. The volume was made up with methanol. The solution of APL was filtered with a whatman filter paper no 42.(Concentration 1000 µg/ml of APL).

2.5 Preparation of Sample Solution

From the stock solution, 1.0 ml is diluted with methanol to make final volume, 10.0 ml. (Concentration 100 µg/ml of APL).

2.6 Validation of HPTLC Method

The proposed HPTLC method was developed and validated as per the International Conference on Harmonization ICH Q2 (R1) guidelines.

2.6.1 Accuracy

The accurately weigh amount of a sample equivalent to 10 mg APL(Label clime of Otezla tablet) was transfer individually in to a nine, 10.0 ml volumetric flasks, added 8.0, 10.0 and 12.0 mg of APL to the sample for 80%, 100%, and 120% level of recovery, respectively. All dilution were made with methanol in triplicate. Accuracy was determined as per the ICH Q2 (R1) guidelines and expressed as % recovery. Accuracy is sometime termed as trueness or recovery study.

2.6.2 Precision

Precision can be considered as a three levels intermediate precision, repeatability, and reproducibility. To establish the reproducibility and repeatability (intra-assay precision) of the method precision study was performed. Similarly the sample solution was prepared and analysed as described under analysis of marketed formulation. The intermediate precision was established by intra-day and inter-day analysis and repeated three times- same day and three consecutive days. The results of precision were expressed in terms of SD and RSD.

The RSD used for intra-day (inside or within the day) and inter-day precision was found less than 2 indicating the repeatability and reproducibility of the method.

Robustness is a measure of the performance of a method when small, deliberate
changes are made to the specified method parameters

2.6.3 Robustness

The robustness of the proposed method was studied to measure the performance of a method when small, deliberate changes to the specified method were made. The changes in the parameters such as a change in mobile phase composition (± 0.1ml), volume of mobile phase (± 1ml), duration for chamber saturation (± 5min), spotting to development and development to scanning (5 min, 20 min, 30 min, and 1hr) were done. The effect variations in the process parameters was studied on RF values and Peak area of the sample. Individual sample was examined in triplicate (n= 3) and the peak areas obtained were utilized to calculate % RSD.

2.6.4 Limit of detection (LOD) and Limit of quantification(LOQ)

The sensitivity of a proposed methods was estimated in terms of LOD and LOQ. The SD of the responses and slopes of calibration curves (n = 3) as described by the ICH

Q2 (R1) guidelines were used for the determination of LOD and LOQ during HPTLC method validation. The LOD and LOQ are calculated using equations
LOD = 3.3.SD/S and LOQ = 10·SD/S
Where, SD of peak areas of the drug (n=3) taken as a measure of noise and S is slope calibration curve.

2.6.5 Forced degradation studies

To develop the stability–indicating HPTLC method, APL is stressed under various conditions as per ICH Q1A (R2) and photo stability as per Q1B guidelines. The API concentration was (1 mg/ml). For forced degradation, six samples were prepared by transferring 10 mg of APL in 10ml volumetric flasks and added 3 ml of 0.1M HCl, 0.1M NaOH, 3% H_2O_2 and H_2O in each flask. Similarly thermal and photolytic study was performed as per the protocol given in ICH guideline.
Above studies were performed at 80 °C for 2 hr. Thermal degradation study was done by sealing the sample in 10 ml volumetric flask and placed in a hot air oven at 60 °C for 1 hr. and photolytic study was performed by exposed the sample UV light at 257nm for 3 days. (72 hr)

2.6.6 Isolation and identification of DPs by using HPTLC and MS/MS

The forced degradation was doneby exposing sample to the stress conditions depicted in 2.6.5. After Fixed time interval the samples solutions are applied on the spots marked on the line in equal distances on TLC plate with sample volume of about 5µl/band. The development of TLC plate was done under optimized chromatographic conditions for APL.
After development of the plate, the plates were kept under the UV cabinet. The RF value value of the DPs were used for separation. The plated were cut with respected RF values and soaked overnight in methanol. The samples was filtered off through whatman filter paper no. 42 before MS/MS analysis. The MS-MS spectra was used to characterize the DPs and their degradation path.

3. Result And Discussion

3.1 Selection and optimization of mobile phase composition

To get good resolution and reproducible peaks, different mobile phase compositions were tried. After different trials the suitable mobile phase was found to Toluene: Ethyl Acetate (4:6v/v). The TLC plate was scanned at 236 nm wavelength. A sharp and well resolved peak was obtained for APL at RF of 0.55(±0.02. The HPTLC densitogram of APL shown in Fig. (3).

3.2 Method Validation

The meyhod validation was done using Q2(R1) , ICH guideline

3.2.1 Linearity and Range

The linearity was found in the range of **100-600ng/band.** The linearity of APL at 236.0 nm was shown in Fig. (4) And its 3D spectra shown in Fig. (5). The linearity equation for APL is **y=11.175x+44.906** with coefficient of correlation (R^2), **0.9978** Fig. (6),

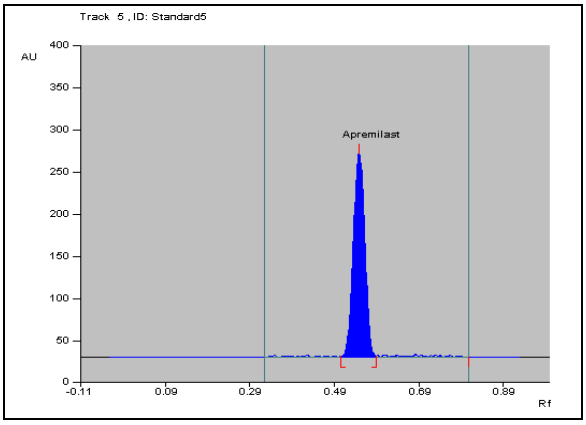

Fig. (3) Densitogram of Apremilast (Rf- 0.55)

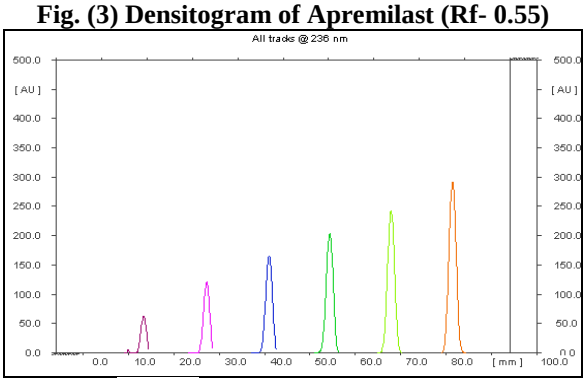

Fig. (4)Linearity of Apremilast

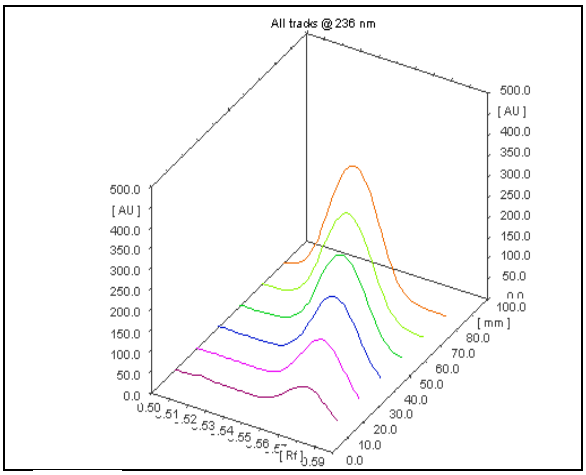

Fig. (5) Linearity of Apremilast in 3D Spectra

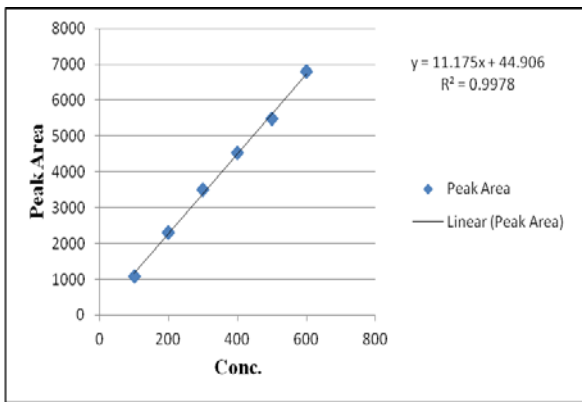

Fig. (6) Calibration curve of Apremilast

3.2.2Accuracy (Recovery studies)

To examine the accuracy of proposed method, recovery studies were performed by standard addition method and the results are expressed as percent recovery. The mean percentage recovery for each compound was calculated at each concentration level and reported as standard deviation. The percentage recovery at a three levels 80%, 100% and 120% for the APL was satisfactory. Accuracy data was shown in Table 1.

Table 1.Result of Accuracy (Recovery study) for APL

Sr. No.	Level of recovery	Weight of tablet powder taken (mg)	Amount of drug added (mg)	Amount of drug recovered (mg)	% Recovery	% RSD
1	80%	114.83	8.1	7.94	99.44	0.74
2	100%	114.83	10	9.94	99.86	0.67
3	120%	114.8	12.03	11.97	99.81	0.64

Above Data obtained from three replicate (n=3) from each concentration

3.2.3Precision

Repeatability, Intra-day and inter-day precisions were observed using six repetitive measurements in fix concentration level (1000 ug/ml). The application volume of APL solution was **0.5ul** fixed for the HPTLC method validation. The precision of developed method was expressed in terms of SD and % RSD of the peak area. A result for the precision is depicted in Table 2.

Table 2. Result of Precision Study of APL

Drug Conc. (ng/band)	Intra-day Precision		Inter-day Precision	
	% Amount of drug found	%RSD	% Amount of drug found	%RSD
500	99.15	0. 30	100.1	0.39
500	100.0	0.28	98.96	0.60
500	98.94	0.52	99.92	0.34

3.2.4 Robustness

As there was no significant change in the RF value of APL (standard RF values 0.55 ± 0.02 and standard peak area is 5476.7).Each sample was studied in triplicate (n= 3) and the obtained peak areas are utilized to calculate % RSD, which was found to be less than 2 suggesting method is robust. The result of robustness is depicted in Table 3.

3.2.5 LOD & LOQ

The sensitivity of proposed methods was estimated in terms of a Limit of Detection (LOD) and Limit of Quantification (LOQ). The LOD and LOQ were found to be **0.7782** and **2.3583**ng/band, respectively.

Table 3. Result of Robustness Study of APL

Factor	Level	Peak area	Rf
Mobile phase composition (± 0.1 ml)			
3.9:6.1	– 0.1	5434.12	0.52
4 : 6	0	5476.90	0.55
4.1:5.9	+ 0.1	5497.38	0.57
	R.S.D.	**0.30**	
Duration for chamber saturation (± 5 min)			
10 min	- 5	5215.41	0.53
15min	0	5476.40	0.55
20 min	+ 5	5492.72	0.56
	R.S.D.	**1.06**	
Spotting to development			
5 min	-	5496.13	0.58
15 min	-	5476.20	0.55
30 min	-	5570.45	0.53
1 hr	-	5642.83	0.52
	R.S.D.	**0.76**	
Development to scanning			
5 min	-	5462.70	0.56
15 min	-	5476.62	0.55
30 min	-	5492.80	0.54
1 hr	-	5689.31	0.52
	R.S.D.	**1.01**	
Volume of mobile phase (± 1 ml)			
9.0	– 1	5395.3	0.53
10.0	0	5476.70	0.55
11.0	+ 1	5645.20	0.56
	R.S.D.	**1.19**	

Table 4. Result of degradation studies

Sr. No.	Stress Condition	Temperature and Time	% assay of active substance	Rf of degraded product
1	**Acid** (0.1N HCl)	80^0C for 2hr	80.62	0.12, 0.19, 0.24, 0.61
2	**Alkali** (0.1N NaOH)	80^0C for 2hr	83.59	0.12, 0.19, 0.24, 0.61
3	**Neutral** (H_2O)	80^0C for 2hr	90.44	0.12,0.26,0.30, 0.81,0.88
4	**Oxide** (3% H_2O_2)	80^0C for 2hr	94.85	0.10, 0.29, 0.49, 0.51, 0.75
5	**Thermal** (Heat)	60^0C for 1hr	93.87	0.32
6	**Photolytic**	72 hrs	100	-

3.2.6 Forced degradation study

The degradation was found maximum in Acidic condition (19.38%), minimum in oxidative condition (5.15%) and stable in photolytic condition. The percent assay of active substance and their RF values of degradation products are given in Table 4. Densitogram of acid, alkaline, neutral,oxide, thermal and photolytic treated APL are shown in Figures (7), (8), (9), (10),(11) and (12) respectively.

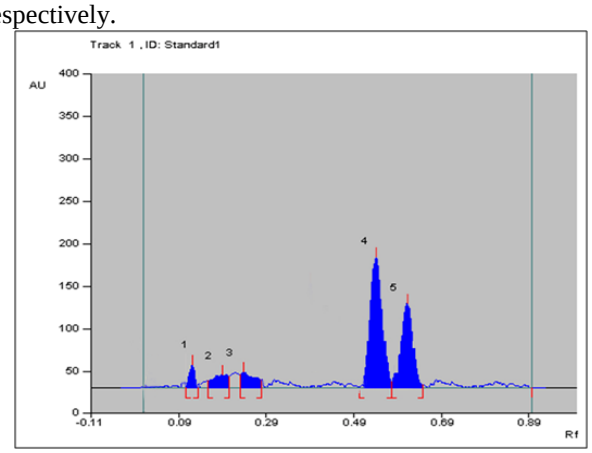

Fig. (7) Densitogram of Acid (0.1 N HCl) treated APL

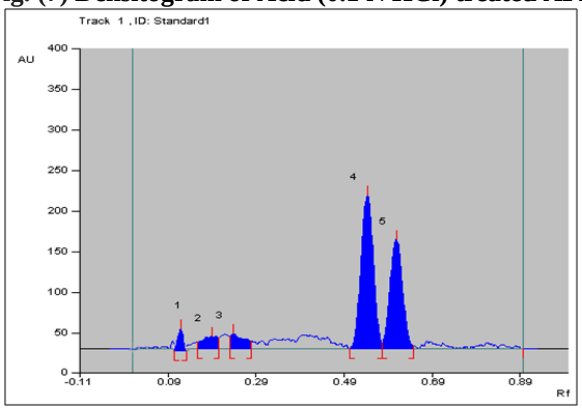

Fig. (8) Densitogram of Alkali (0.1N NaOH) treated APL

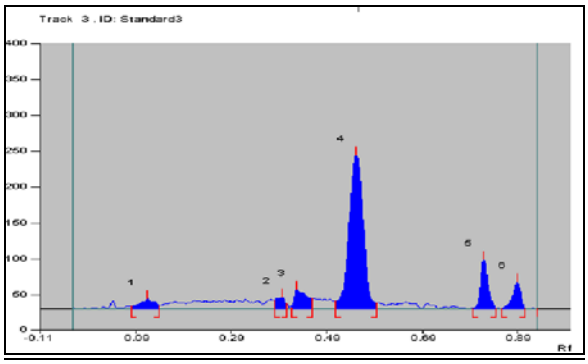

Fig. (9) Densitogram of Neutral (H_2O) hydrolysis treated APL

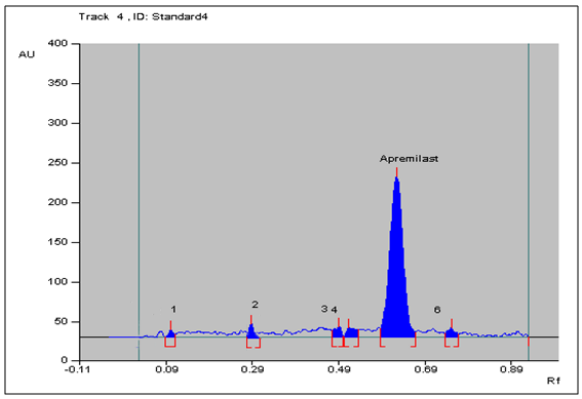

Fig. (10) Densitogram of Oxide (3% H_2O_2) treated APL

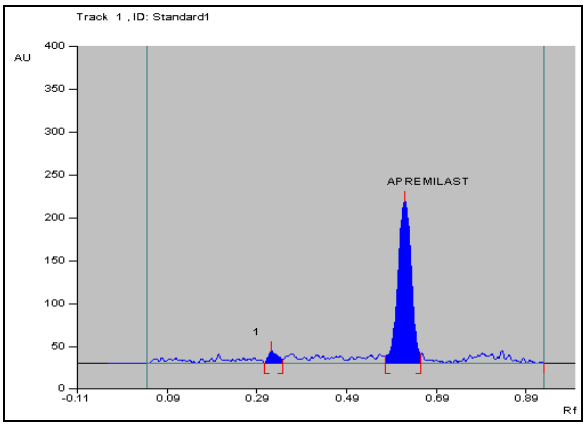

Fig. (11) Densitogram of Thermal (Dry heat) treated APL

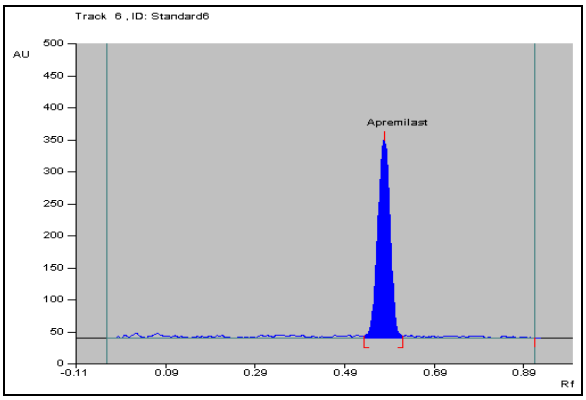

Fig. (12) Densitogram of sample exposed to UV radiations

3.3Isolation and identification of degrade product by HPTLC and MS/MS (Tandem mass spectroscopy) method.

In all eleven degradation products was identify based on MS/MS spectra. The eleven degrading product found were DP-1, DP-2, DP-3, DP-4, DP-5, DP-6, DP-7, DP-8, DP-9, DP-10, and DP-11 respectively. The correlation of the degradation product in Apremilast and its stress condition shown in Fig. (13) and Fig. (14).

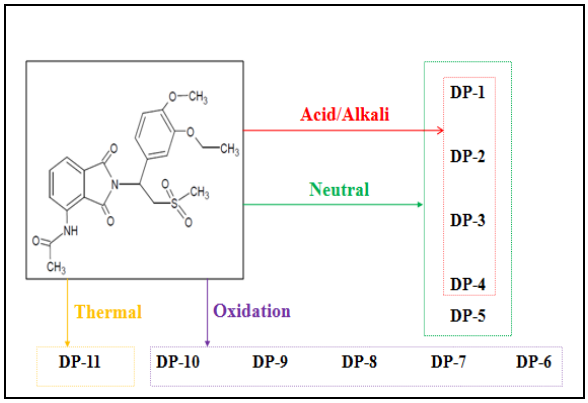

Fig. (13) Correlation of the degradation product in Apremilast and its stress condition

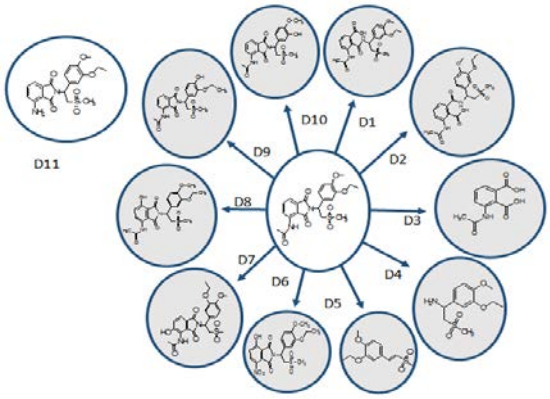

Fig. (14) Degradation product of Apremilast

The drug degraded into DP-1, DP-2, DP-3, and DP-4, under acid and alkali condition respectively. In neutral stress condition, generation of degradation product DP-1 to DP-4 may follow similar type of degradation pathway as proposed in acid and alkali stress condition. Moreover, DP-4 was shown further degradation by loss of NH_2 group to generate DP-5.In oxidative and thermal stress condition, generation of degradation product DP-6, DP-7, DP-8, DP-9, DP-10, and DP-11 respectively. The result of the HPTLC and MS/MS was given below,

The degradation of APL in acid and alkali and neutral stress condition follow similar type of degradation pathway.

DP-1 (m/z 478.48)the parent ion of m/z 478.48 is observed in mass spectra. The DP-1 was formed by forced hydrolytic condition it was observed that, isoindole ring of APL in the said stress condition may be opened and addition of OH group generated DP-1.

DP-2 (m/z 478.48)the same was characterised by mass spectroscopy further degradation product of this product DP-1 shows rearrangement of OH and NH functional group and form DP-2. Mass spectra of DP-1 and DP-2 shown in Fig. (15)

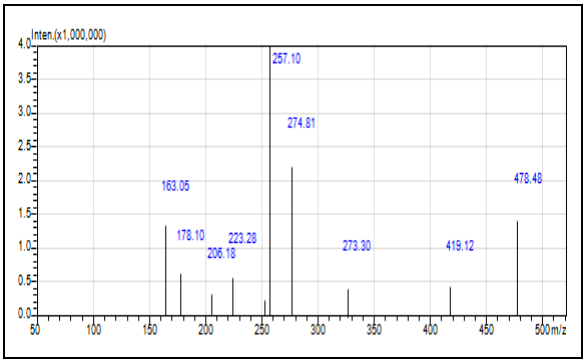

Fig. (15) Mass spectra of DP-1 and DP-2

DP-3&DP-4 (m/z223.23 &m/z 273.30)the breakdown of DP-2 to form DP-3 and DP-4. Mass spectra of DP-3 and DP-4 shown in Fig. (16) and (17) respectively.

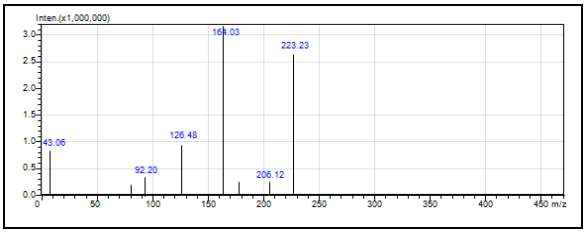

Fig. (16) Mass Spectra of DP-3

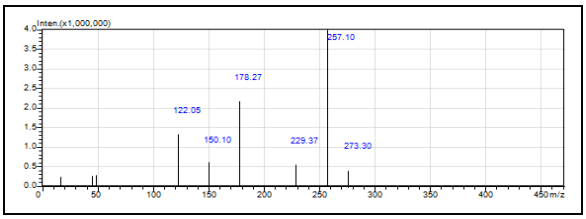

Fig. (17) Mass Spectra of DP-4

DP-5 (m/z 256.095) the parent ion of m/z 256.095 the DP-5 was formed by neutral stress condition in DP-4 loss of NH2 group to form DP-5. Mass spectra of DP-5 shown in Fig. (18)

The formation of DP-1, DP-2, DP-3, DP-4 and DP-6 after stress condition (Acid, Alkaline, and Neutral) degradation study pathway is shown in Fig. (19)

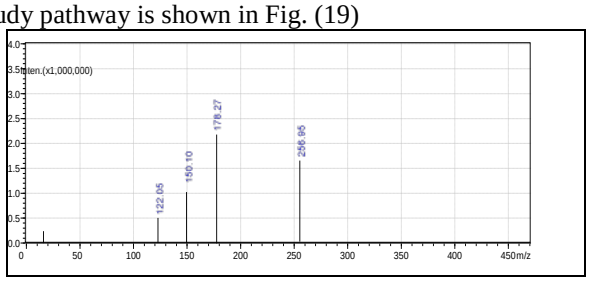

Fig. (18) Mass Spectra of DP-5

The degradation of APL in oxidative stress condition to form five degradation products DP-5, DP-7, DP-8, DP-9 and DP-10.

DP-6(m/z 448.05) the parent ion of m/z 448.05 is observed in mass spectra Fig. (20) The DP-5 was formed by oxidative stress condition it was observed that, loss of carbonyl radical and addition of OH group in isoindole ring of APL and to form DP-5.

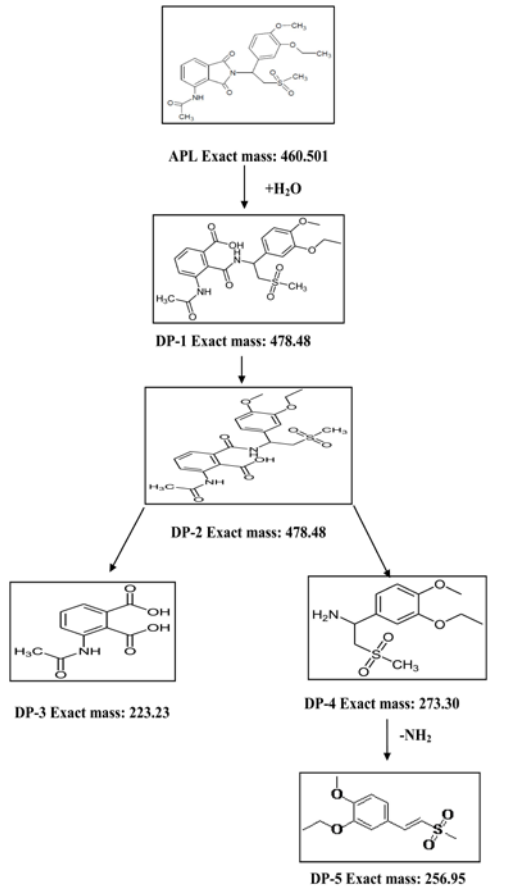

Fig. 19 Formation of DP-1, DP-2, DP-3, DP-4 and DP-5 after stress condition (Acid, Alkaline, and Neutral) degradation study pathway.

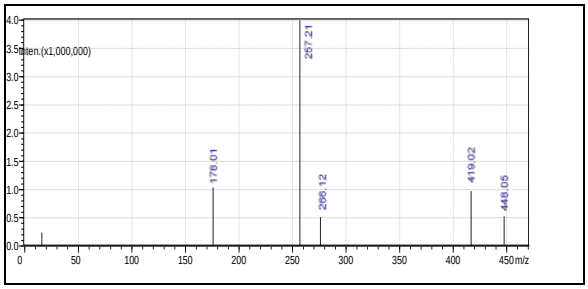

Fig. (20) Mass Spectra of DP-6

DP-7 & DP-8 (m/z 447.35) the parent ion of m/z 448.05 is observed in mass spectra Fig. (21) The DP-7 & DP-8 was formed by oxidative stress condition it was noted that, addition of OH group respectively and rearrangement of OH group in DP-8.

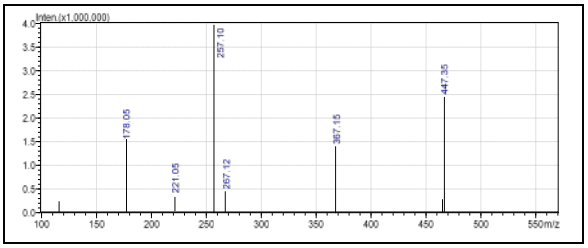

Fig. (21) Mass Spectra of DP-7& DP-8

DP-9 (m/z 446.62) the parent ion of m/z 446.62 is observed in mass spectra Fig. (22) The DP-9 was formed by loss of methyl radical in phenyl ring of APL to form DP-9.

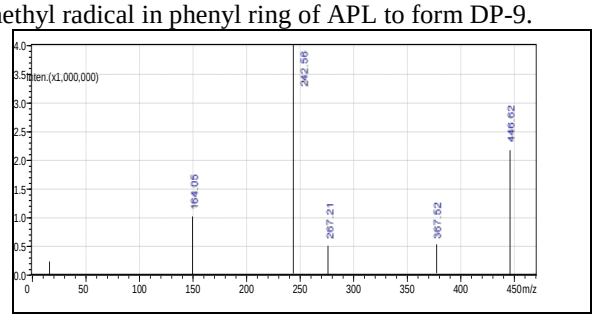

Fig. (22) Mass Spectra of DP-9

DP-10 (m/z 432.27) the parent ion of m/z 432.27 is observed in mass spectra. Fig. (23) The DP-10 was formed by loss of ethyl radical in phenyl ring of APL to form DP-10.

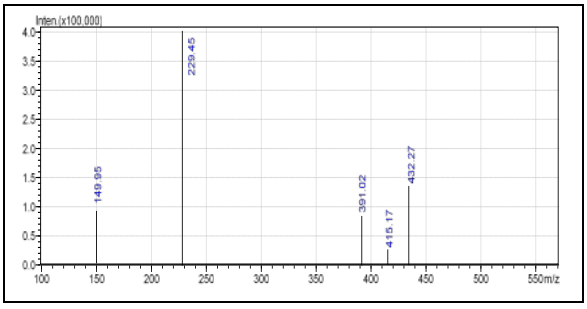

Fig. (23) Mass Spectra of DP-10

The formation of DP-5, DP-7, DP-8, DP-9 and DP-10 after stress condition (Oxidative) degradation study pathway is shown in Fig. (24).

Figure-24 **Formation of DP-6,DP-7, DP-8, DP-9 and DP-10 after stress condition (Oxidative) degradation study pathway**

The degradation of APL in thermal stress condition to form DP-11.

DP-11(m/z 419.24) the parent ion of m/z 419.24 is observed in mass spectra. Fig. (25) the DP-11 was formed by thermal stress condition.Itmay be because ofloss for the carbonyl radical group in isoindole ring of APL and to form DP-11.

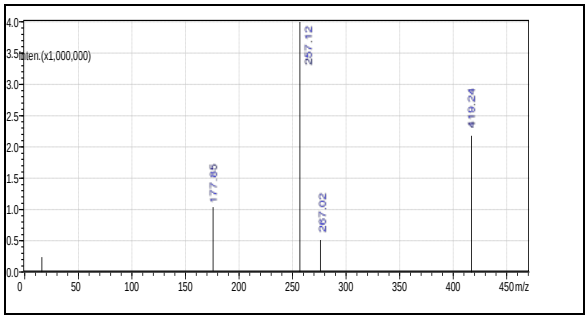

Fig. (25) Mass Spectra of DP-11

The formation of DP-11 after stress condition (Thermal) degradation study pathway is shown in Fig. (26)

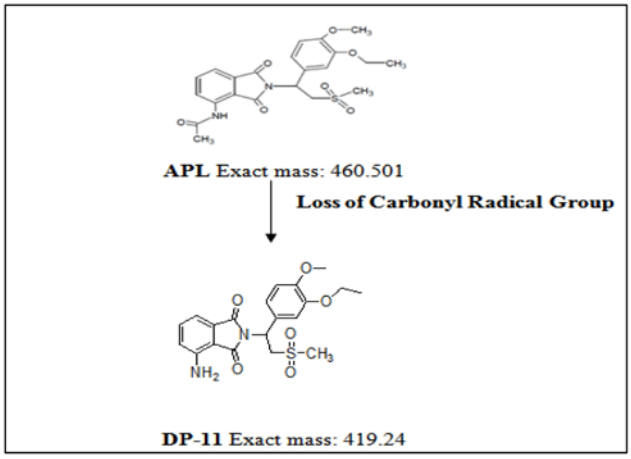

Fig. 26 Formation of DP-11 after stress condition (Thermal) degradation study pathway.

CONCLUSION

The Proposed HPTLC method was found to be a more sensitive, precise, economic, less time consuming and cost effective. The developed HPTLC method was validated as per ICH Q2 (R1) guidelines for the estimation of APL, which provided useful information about degradation behaviour of APL using different stress conditions. Also, the method was found to be simple, accurate, and reproducible.

In stability testing, of Apremilast were found susceptible to acid and alkaline degradation.The stability indicating study was performed as per the ICH Q1A (R2) guidelines. The stability indicating study of APL shows degradation in stress conditions generation of eleven degradation products. These degradation products were characterized and identified by MS/MS methods. Mass analysis showed four DPs in acid and alkali condition while in neutral degradationsame DPs were found , further DP-4 after losing of NH_2 group formed DP-6. In oxidative and thermal degradation five and one degradantes were found respectively. This methos may be useful for the routine analysis of APL in Pharmaceutical tablet dosage form.

List of Abbreviations:APL: Apremilast, HPTLC: High Performance Thin–Layer Chromatography, ICH: International Conference on Harmonization, API: Active Pharmaceutical Ingredient, AR: Analytical Reagent, RF: Retardation Factor, SD: Standard Deviation, RSD: Relative Standard Deviation, LOD: Limit of Detection, LOQ: Limit of Quantification, DP- Degradation Products.

ACKNOWLEDGMENT

The author is thankful to the Dr. P.D Patil, Chairman and Dr. S. S. Chitlange, Principal, Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune for providing excellent infrastructures and facilities for undertaking this research work.

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