Chapter - 2
Analytical method development

2. Introduction

The analytical methods exhibit a pivotal role in formulation development. It is employed to estimate entrapment efficiency, assay, in-vitro drug release, ex-vivo drug release, and in-vivo studies. There is a need for a sensitive, accurate, reliable, and reproducible method to evaluate the designed formulation for quality, safety, and performance of the formulation. The suitability of the method for the intended use and compliance with standards (accuracy, precision, sensitivity, and specificity) was emphasized by the ICH, US Food and Drug Administration (USFDA) [1,2].

2.1. Analytical method development of Apremilast

The analytical methods for estimating Apremilast and its impurities are reported by high-pressure liquid chromatography (HPLC). Few studies have been reported for the estimation of Apremilast using HPLC. However, the reported HPLC methods for determination of Apremilast were restricted to bulk and oral tablet preparation. The reported method demonstrated the sensitivity to the micro gram level. Additionally, there was no such method which can determine Apremilast in topical gel or complex nanocarriers delivery systems and skin tissue. We developed a sensitive, specific, and stability-indicating HPLC method to estimate the Apremilast based on reported studies. To evaluate the method selectivity and its application for stability testing, force degradation studies were performed by subjecting Apremilast to acid, base, oxidation, and thermal degradation. Further, the method was validated for the assessment of Apremilast in developed nanoformulations and skin samples (biological samples) for the application in dermatokinetics studies of designed formulation.

2.2. Materials, reagents, and chemicals

Acetonitrile, methanol (HPLC grade), orthophosphoric acid 85% emplurar, potassium dihydrogen phosphate, hydrochloric acid, and ammonium acetate were procured from Merck,

Mumbai, India. Sodium hydroxide and Hydrochloric acid were procured from Central Drug House (P) Ltd. New Delhi. Milli-Q water was obtained from an in-house Milli-Q water purification system (Millipore Bedford, Bedford, MA, USA), which was used in aqueous buffer preparation.

2.3. Stock, standard preparation and buffer preparation

The primary stock solution of Apremilast (1000 µg/mL) was prepared by dissolving the required weighed amount in acetonitrile. The secondary stock (100 µg/mL) was prepared from the primary stock solution using the mobile phase. Further standard samples were prepared from the secondary stock solution. The 10 mM phosphate buffer was prepared by dissolving the potassium dihydrogen phosphate (1360.0 mg) in 1000 mL milli-Q water. The required pH of the solution was adjusted using orthophosphoric acid. The 10 mM acetate buffer was prepared by dissolving the ammonium acetate (770.8 mg) in 1000 mL milli-Q water. The prepared buffer solutions were filtered through a 0.22 µm membrane filter and sonicated to remove dissolved gases before the experiment.

2.4. Chromatographic conditions

The analytical method was developed and validated using a high-pressure liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with a binary pump (LC10AT), autosampler (SIL-HT), column oven (CTO-10ASP), and Photo Diode Array detector (SPD-M20A). The elution of Apremilast was performed using Agilent, eclipse XDB-C18 analytical column (150 ×4.6 mm, 5µm). The acetonitrile and 10 mM potassium phosphate (pH 3.5) buffer pH adjusted with orthophosphoric acid were used for method development. Before initiation of the sample analysis, the system was equilibrated for 40 to 60 min. The LC solution software version 1.22 SP1 was used to control hardware, data acquisition and interpretation of data.

2.5. Method validation

The proposed method was validated as per the regulatory guidelines for analytical method validation [1,2].

2.5.1. System suitability

System suitability was performed to check the system performance for the intended application of the analytical system to obtain the expected outcomes. The test was used to determine column efficiency, retention time, reproducibility, tailing factor, and peak area. The system suitability was evaluated by injecting six replicates of 1000 ng/mL of drug concentration [3,4].

2.5.2. Linearity and range

The linearity of the Apremilast was performed using six standard solutions (100, 500, 1000, 2000, 5000, 10000 ng/mL) in the range of 100-10000 ng/mL. The linear regression was implemented on the obtained data from the least-squares of regression. Peak areas of the analyte were plotted on Y-axis versus concentrations on X-axis [3].

2.5.3. Limit of detection and limit of quantification

The lowest concentration of the analyte detected and discriminated from the noise level is defined as the limit of detection (LOD). The lowest concentration of analyte, which can be quantified with accuracy and precision as per the accepted range, is defined as a limit of quantification (LOQ). LOD and LOQ represent the sensitivity of the analytical method. The signal-to-noise ratio (S/N) method was employed for the determination of LOD and LOQ. The desired S/N ratio for LOD is 3:1 and LOQ is 10:1; the LOQ is considered the first point in the linearity range [5,6].

The concentration of LOD and LOQ were determined by Equation 2.1

$$\frac{\text{Concentration of Standard}}{\frac{S}{N}\text{Value of Standard}} \times \text{Desired}\,\frac{S}{N}\text{value} \qquad \text{(Eq. 2.1)}$$

2.5.4. Accuracy and precision

The developed method was validated for accuracy and precision for lower quality control (LQC), middle-quality control (MQC), and higher quality control (HQC) samples. The intraday accuracy and precision were ascertained for six replicate samples of the same concentration on the same day. The inter-day accuracy and precision were also ascertained for quality control samples for three days in six replicates [7,8].

2.5.5. Carryover effect

The reappearance of the analyte with respect to the previous run in the next run due to the overload of the sample is termed the carryover effect. The carryover effect of the Apremilast was determined by injecting three continuous higher concentration samples of the linearity curve followed by the blank sample. As per guidelines, the carryover acceptance criteria should not exceed 20% of the LOQ area [9].

2.5.6. Selectivity and stress degradation studies

The developed method was evaluated for the selectivity and stress degradation studies to check the interference of the formulation excipients and degradants of the drug. The Apremilast was subjected to acid, base, oxidation, and thermal degradation. In brief, acid degradation was performed using 0.5 M hydrochloric acid, base degradation using 0.5 M sodium hydroxide solution, and oxidative degradation using 3% hydrogen peroxide. Apremilast's 200 µg/mL solution was prepared using the solutions mentioned above and placed in a water bath and subjected to heat at 80 ºC for 4 h. Thermal degradation studies were performed by preparing the 200 µg/mL of Apremilast diluted with milli-Q water. The prepared solution was subjected to heat at 80 ºC for 4 h. After completion of the study, the samples were cooled to room temperature. The acid samples were neutralized with dilute sodium hydroxide solution. The base samples were neutralized with dilute hydrochloric acid. The samples were diluted and

filtered through 0.22 µm filter and subjected to analysis. The Apremilast spectra obtained in degradation studies were evaluated for peak purity. It was compared with the spectral peak purity of the standard Apremilast peak chromatogram of LC solution software version 1.22 SP1 [3,4,10].

2.5.7. Estimation of the drug recovery in the skin samples

The designed nanoformulation embedded gel was used for topical application in the psoriatic skin condition. The permeation and skin retention of encapsulated Apremilast need to be quantified in ex-vivo and in-vivo studies. Hence, the method applicability was determined in the presence of skin tissue and in-vitro sample matrix (tape strip).

In brief, the known amount of the drug was spiked to skin tissue (1 cm^2), and the skin was subjected to homogenization in acetonitrile (5 mL). Similarly, the tape strips utilized for drug retention study up to 15 strips were chopped, and a known amount of drug was spiked. The drug solution, along with tape strips, was sonicated for 8 h. The homogenized samples, sonicated samples were centrifuged, and the supernatant solution was collected. The collected samples were filtered through 0.22 µm analyzed for drug content using the developed method. The amount of Apremilast recovered was calculated [5].

2.6. Results and discussion

2.6.1. Development and optimization of the method using design of experiments

The method development was performed by screening various mobile phase ratios. The λ_{max} 229 nm was used for the detection of the Apremilast during the method development and validation. Initially, different proportions of methanol: water and acetonitrile: water were used to separate Apremilast to get the desired peak characteristics. Peak splitting and broad peaks were observed in the case of methanol, whereas sharp peak was found in acetonitrile. Further, acetonitrile was combined with different proportions of aqueous buffers (10 mM acetate buffer

and 10 mM phosphate buffer) and evaluated. Based on the peak properties such as peak area, tailing factor, and retention time, phosphate buffer was found to be more suitable than acetate buffer. All the above screening was performed with an organic to aqueous mobile phase ratio of 50:50. Acetonitrile and phosphate buffer were selected for further evaluation.

The mobile phase with 50% acetonitrile and 50% phosphate buffer pH 3.5 was found to be desirable with retention time and tailing factor from the data obtained from various trials. The combination of acetonitrile and phosphate buffer pH 3.5 in a 50:50 ratio was selected as optimized mobile phase for chromatographic evaluation of Apremilast. The flow rate of 0.8, 1.0, and 1.2 mL/min were screened to differentiate the peak from the solvent front. The flow rate 0.8 mL/min was found to be suitable with acceptable peak properties. The retention time and tailing factor were found to be 3.98 min and 1.19 respectively using optimized mobile phase with 0.8 mL/min flow rate which indicated acceptable chromatogram properties.

2.6.2. Validation

2.6.2.1. System suitability

The six replicates of the Apremilast solution (1000 ng/mL) were injected to evaluate the system suitability. The peak properties were found to be within the acceptable range. The % RSD for the peak area (0.056) and retention time (0.030) were less than 2, and the tailing factor was less than 1.5.

2.6.2.2. Linearity, range, limit of detection and limit of quantification

Linearity of the Apremilast was performed for six concentrations (100, 500, 1000, 2000, 5000, 10000 ng/mL) and at six replicates. The linearity concentrations and their respective peak area are represented in **Table 2.1**. The linearity was found to be reproducible with $r^2 \geq 0.999$. The regression equation for the developed method was (Peak area) Y = 103.71 X (concentration)+456.4. The statistical analysis of the obtained linearity values revealed no

significant difference for both the analytes ($p < 0.05$). The regression of the linearity curve revealed $F_{calculated} > F_{critical}$ value according to ANOVA. The limit of detection and low limit of quantification was found to be 30 ng/mL and 100 ng/mL, respectively. The LOD and LOQ values were obtained at λ_{max} 229 nm with 20 µL injection volume.

Table 2.1. Table representing linearity curve data (n=6).

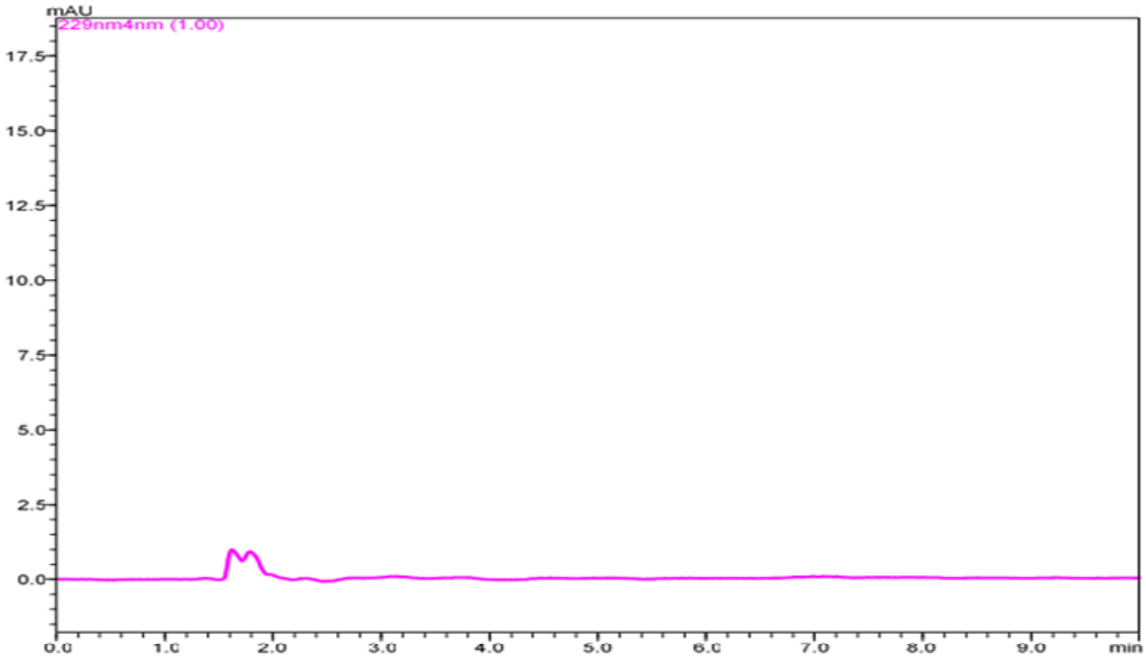
Concentration (ng/mL)	Area ± SD	% RSD
100	10775 ± 237.96	0.00023
500	50552 ± 313.11	0.00006
1000	102226 ± 6368.58	0.00060
2000	205102 ± 3422.92	0.00017
5000	530336 ± 1679.18	0.00003
10000	1032672 ± 1256.09	0.00001

2.6.2.3. Accuracy and precision

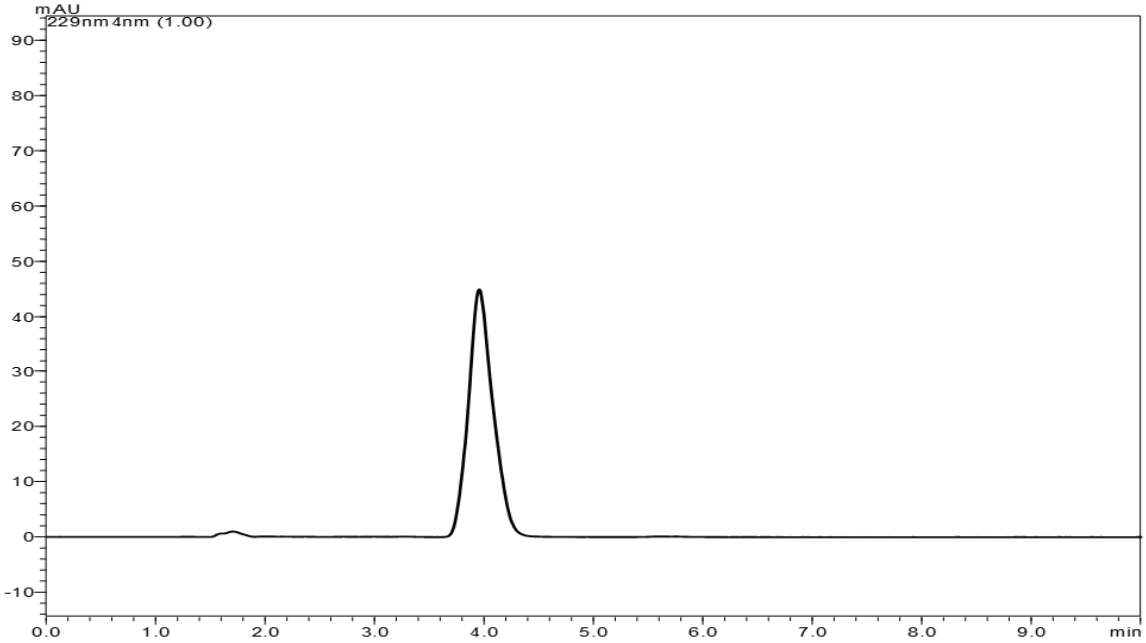
The accuracy and precision of three different quality control samples i.e. LQC (250 ng/mL), MQC (4000 ng/mL), and HQC (8000 ng/mL)) were analyzed intraday and interday in six replicates. The chromatograms are represented in **Figure 2.1 (A-D)**. The results obtained from accuracy and precision studies are depicted in **Table 2.2**.

Table 2.2. Accuracy and precision data of intraday and interday analysis of quality control samples (n=6).

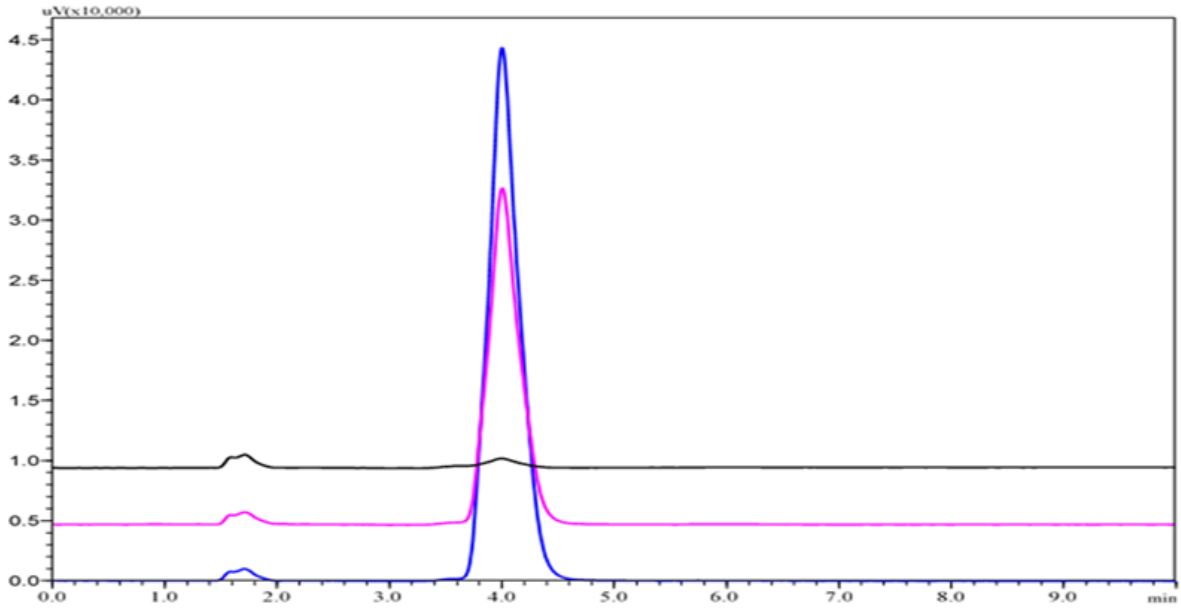
Levels	Intraday			Interday		
	Measured concentration (ng/mL)	% RSD	Accuracy (% bias)	Measured concentration (ng/mL)	% RSD	Accuracy (% bias)
LQC	251.75 ± 0.09	0.04	-0.70	251.79 ± 0.06	0.02	-0.71
MQC	4068.58 ± 1.28	0.03	-1.71	4068.39 ± 1.32	0.03	-1.70
HQC	8092.41 ± 1.72	0.02	-1.15	8092.73 ± 0.10	0.01	-1.16



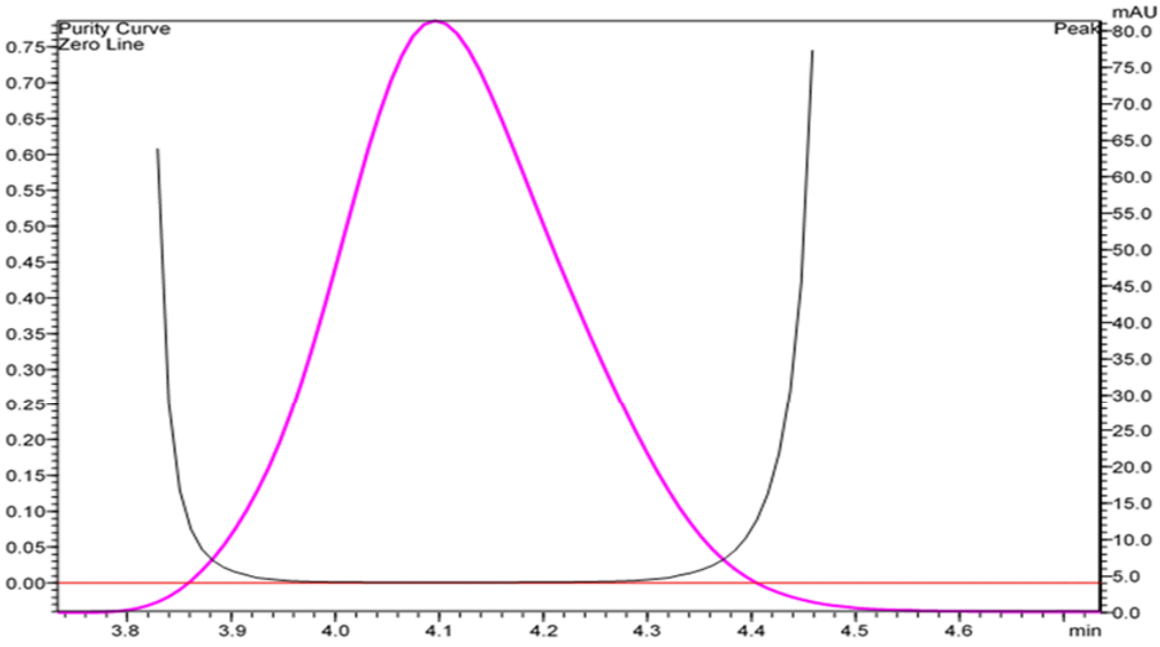
2.1 (A) Blank



2.1 (B) Sample



2.1 (C) LQC, MQC, HQC overlay



2.1 (D) Peak purity

Figure 2.1. Chromatographs obtained for Apremilast method validation:

2.1. A. Chromatogram depicting the blank sample; **2.1. B.** Chromatogram depicting the sample; **2.1. C.** Chromatogram depicting the overlay of lower quality control (LQC) (250 ng/mL), middle quality control (MQC) (4000 ng/mL), and higher quality control (HQC) (8000 ng/mL) sample; **2.1. D.** Chromatogram depicting the peak purity of sample.

The intraday accuracy (% bias) results for LQC, MQC, and HQC were found to be -0.70%, -1.71%, and -1.15%, respectively. Whereas interday accuracy (% bias) results for LQC, MQC and HQC were -0.71%, -1.70%, and -1.16%, respectively. The (% RSD for intraday and interday precision) results were less than 0.05% for all the concentration levels. The accuracy and precision limits were within acceptable limits, i.e., % RSD less than ± 2%. The results indicated that the developed method was accurate and precise for estimating Apremilast in analytical samples.

2.6.2.4. Carryover effect

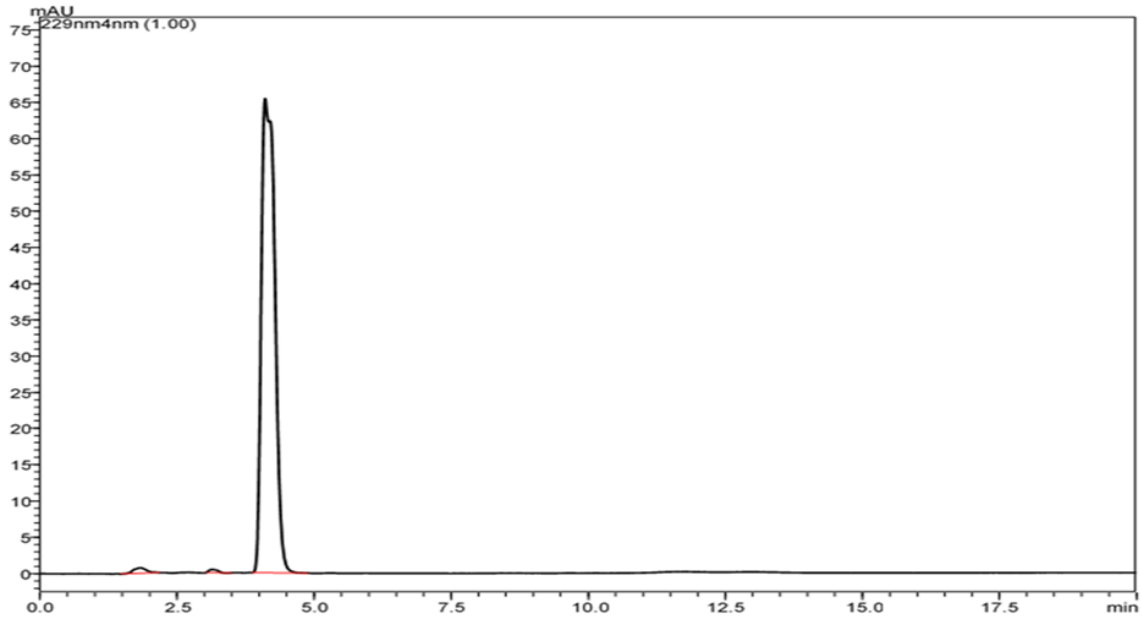
After continuous three injections of a high concentration sample of the linearity curve (10000 ng/mL), a blank sample was injected. The absence of analyte peak in the blank sample indicated the developed method has zero carryover effect. The results suggested that the method can be utilized for a continuous run for a more significant number of samples for routine analysis.

2.6.2.5. Selectivity and force degradation studies

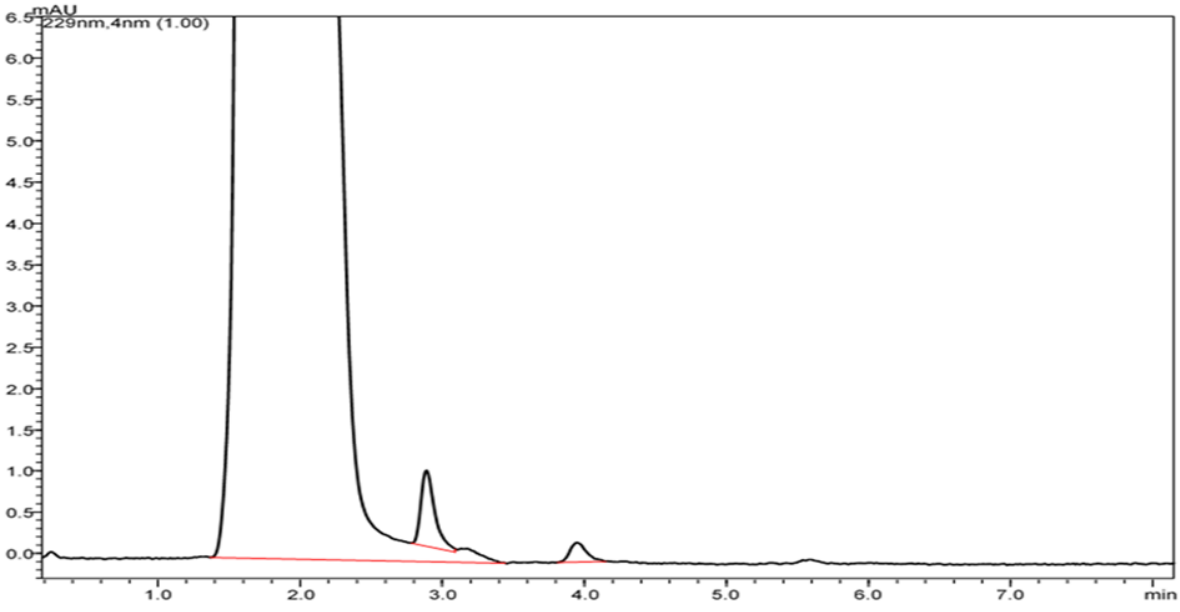
In selectivity studies with formulation excipients, there was no appearance of the extra peaks and the interference of excipients (glyceryl monostearate, glyceryl di behenate, glyceryl palmitostearate, Labrafil M 2125, Labrfac cc, and oleic acid), indicating the selectivity of the developed method. No extra peak of excipient observed at analyte retention time indicated that the method could selectively measure the Apremilast.

In the base degradation, the impurity generation was high, followed by oxidation. The degradation in the case of acid and thermal was low. The results are depicted in **Table 2.3.** The base degradation revealed extra peaks at 2.11, 2.68, and 3.11 min. The degradation in base hydrolysis was found to be 90 to 92%. The oxidation study revealed a degradation peak at 1.89 min. At retention time 2.63 and 3.12 min, the extra degradation peaks were observed with lesser intensity and within the detectable range. The acid degradation was minimal; the variation in

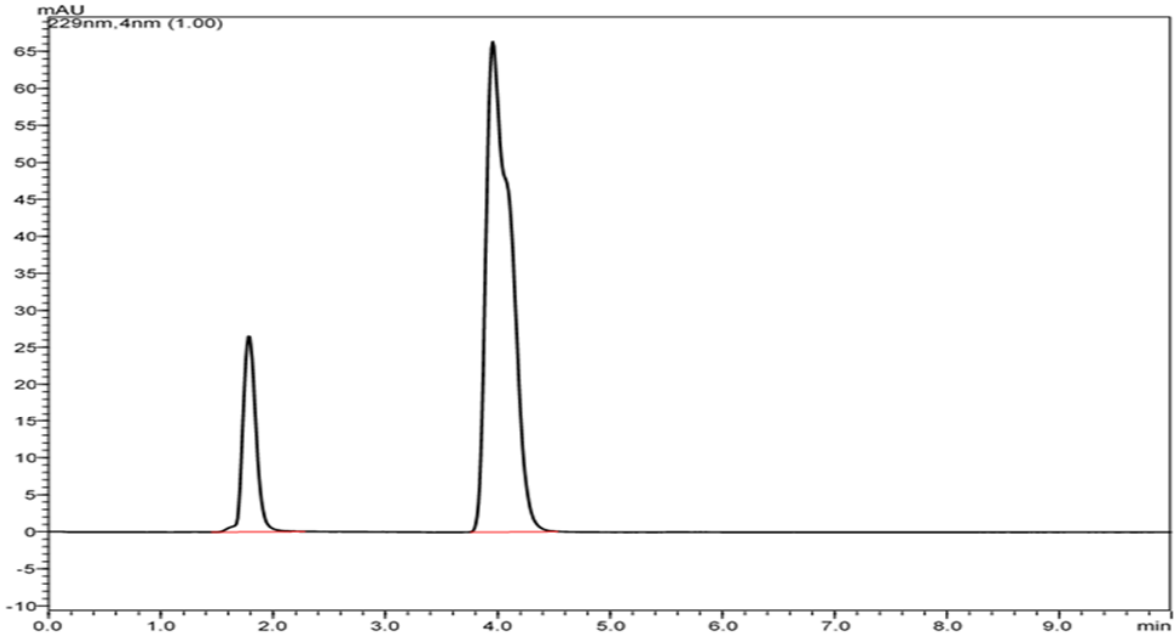
the peak percent was less than 6%, with an impurity peak at 3.21 min. The force degradation study chromatograms are represented in **Figure 2.2 (A-D).**



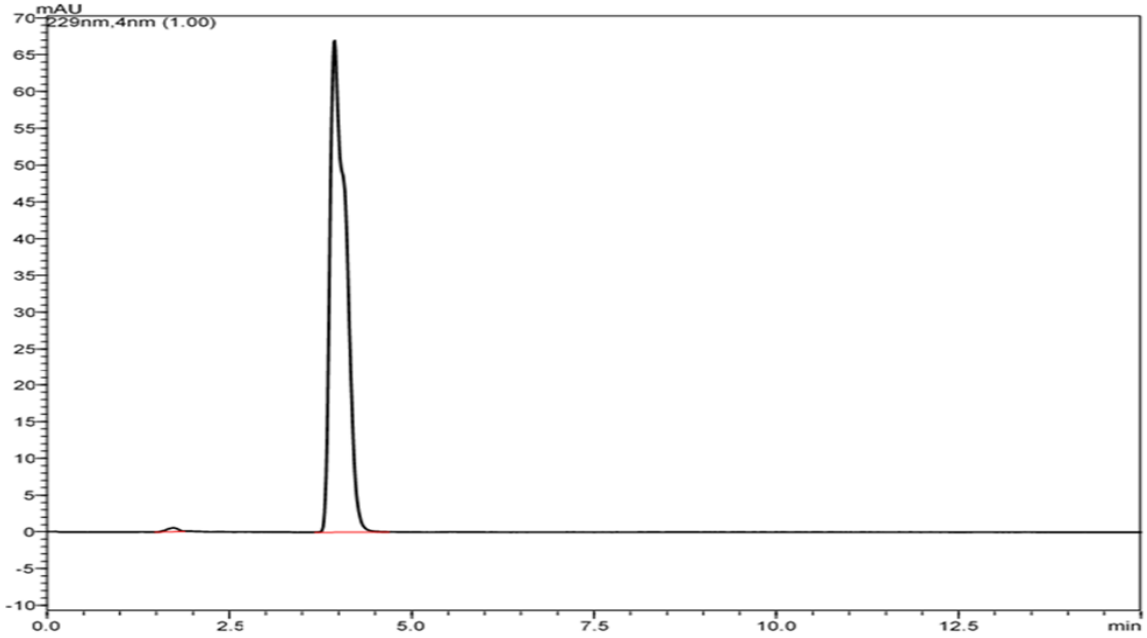
2.2 (A) Acid degradation



2.2 (B) Base degradation



2.2 (C) Oxidative degradation



2.2 (D) Thermal degradation

Figure 2.2. Chromatographs obtained for force degradation study of Apremilast;
2.2. A. Chromatogram depicting the acid degradation; **2.2. B.** Chromatogram depicting the base degradation; **2.2. C.** Chromatogram depicting the oxidative degradation; **2.2. D.** Chromatogram depicting the thermal degradation.

The degradation was less than 2% on exposure to high temperature. The force degradation studies demonstrated developed method was selective and stability-indicating. The results revealed the detection and differentiation of impurity peaks from the Apremilast retention time. The peak purity of the Apremilast was found to be within the threshold limit (threshold ~0.999), which indicated that no degradant peak eluting at the retention time of Apremilast. The developed stability-indicating analytical method can quantify Apremilast in pre-formulation samples, compatibility samples, and stability samples [11,12].

Table 2.3. Forced degradation study representing impurity peaks retention time and percent degradation(n=4).

Force degradation performed	**Impurity peak observed at retention time (min)**	**% Degradation of the Apremilast**
Thermal	-	0.86733 ± 0.186
Acid	3.21	5.76066 ± 0.139
Base	2.11, 2.68,3.11	91.18644 ± 0.476
Oxidation	1.89, 2.63, 3.12	15.51918 ± 0.502

2.6.2.6. Estimation of the Apremilast recovery in the skin samples

The collected samples were analysed for the % recovery of the Apremilast from both skin tissue and tape strip. The average percent recovery for the six replicates in tape strips and skin tissue was 98.922 ± 0.555% and 98.129 ± 0.233%, respectively. The chromatogram of the Apremilast in the presence of skin tissue is illustrated in **Figure 2.3.**

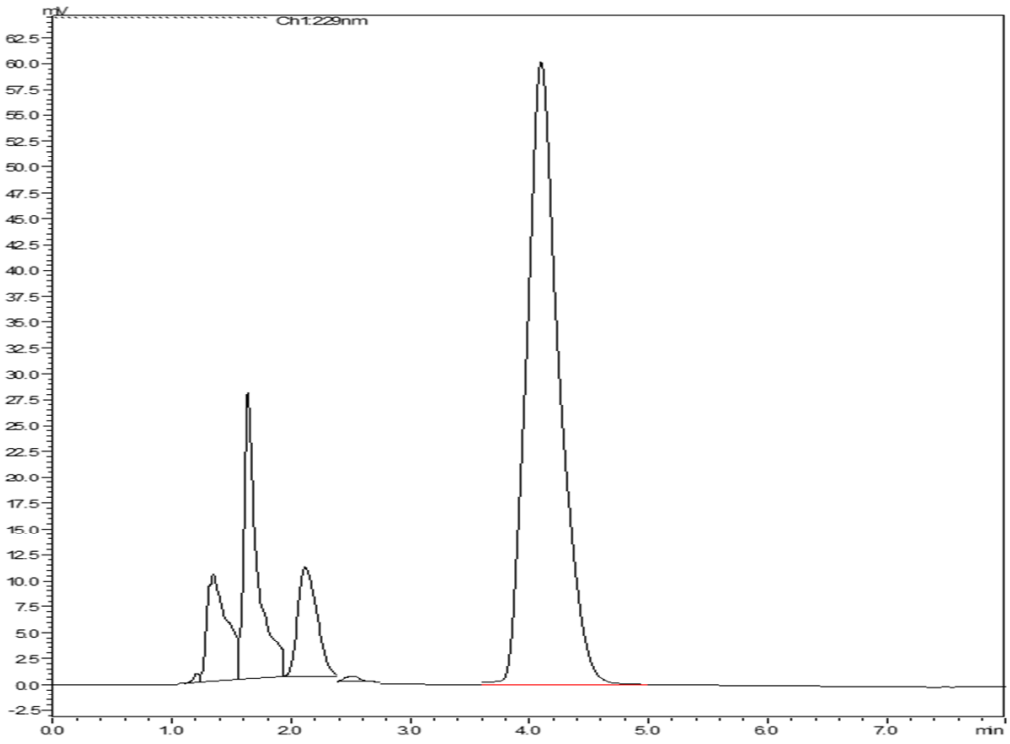


Figure 2.3. Chromatogram depicting the Apremilast in presence of skin tissue

2.7. Conclusion

An isocratic HPLC method development and validation was performed for the estimation of Apremilast. The developed method was precise, accurate, and sensitive to determine Apremilast in lipid-based nanoformulation for topical application. The developed method is sensitive enough to detect 100 ng/mL samples and linearity was 100 ng/mL to 10000 ng/mL. The developed method was found to be stability-indicating, with no interference of excipients used for topical lipid-based formulation. The method can be used to estimate the drug retention in the skin and applicable for dermatokinetics studies.

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