DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF APREMILAST IN HUMAN PLASMA (K_3EDTA) BY USING LC-MS/MS

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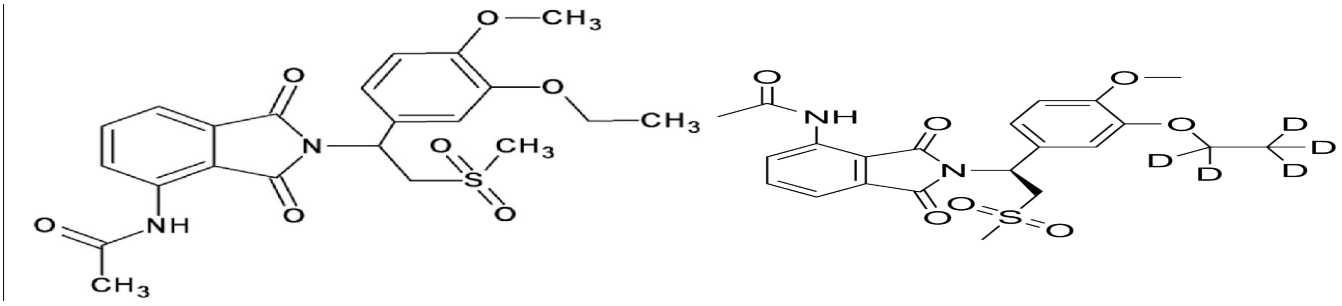
Abstract— A sensitive and reliable liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the quantification of Apremilast, a novel therapeutic agent, in human plasma samples. The method utilizes multiple reaction monitoring (MRM) mode with specific transitions for Apremilast and its stable isotope-labeled internal standard (Apremilast-D5). Chromatographic separation was achieved on a Unisol C18, 4.6x100mm, 5µm column using a mobile phase consisting of acetonitrile and water with 0.2%formic acid(90:10v/v). Liquid-liquid extraction (LLE) was optimized for efficient extraction of Apremilast from plasma matrices, ensuring minimal matrix interference and high recovery. Method validation was performed according to International Council for Harmonisation (ICH) guidelines, demonstrating satisfactory results for specificity, linearity, accuracy, precision, sensitivity, dilution integrity, and stability. The method exhibited a linear calibration range of 1.0–1000.0 ng/mL, with excellent accuracy (% accuracy ranged from 95.82% to 105.85%) and precision (%CV ≤ 15%) across quality control samples. Furthermore, stability testing confirmed the stability of Apremilast in various conditions, including refrigerated storage, autosampler conditions, and freeze-thaw cycles. The developed LC-MS/MS method offers a robust and sensitive approach for the quantitative determination of Apremilast in plasma samples, suitable for pharmacokinetic studies and therapeutic drug monitoring.

Index Terms—Apremilast, LC-MS/MS, Human Plasma(K_3EDTA).

INTRODUCTION

Apremilast, a novel small-molecule inhibitor of phosphodiesterase 4 (PDE4), has emerged as a promising therapeutic agent for the treatment of various inflammatory diseases, including psoriasis, psoriatic arthritis, and Behçet's disease. Its mechanism of action involves modulating inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-17 (IL-17), and interleukin-23 (IL-23), thereby exerting anti-inflammatory and immunomodulatory effects. [1] Given its potential clinical benefits, accurate and precise quantification of apremilast in biological samples is essential for pharmacokinetic studies, therapeutic drug monitoring, and dose optimization. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a preferred analytical technique for the quantification of small molecules due to its high sensitivity, selectivity, and specificity.

[2] In this context, we aimed to develop and validate a robust LC-MS/MS method for the quantification of apremilast in human plasma. The method development involved optimizing chromatographic conditions, selecting suitable extraction techniques, and validating the method according to regulatory guidelines. The validated method provides a reliable and sensitive analytical tool for determining apremilast concentrations in clinical samples, facilitating pharmacokinetic studies and personalized therapeutic strategies for patients with inflammatory diseases. [2]



A **B**

Figure 1: Chemical Structures of A) Apremilast B) Apremilast D5

MATERIALS AND METHODS

Chemicals

Apremilast, Apremilast D5(Figure 1), Methanol, Acetonitrile, HPLC Grade Water, Formic acid, Ethyl Acetate, Human Plasma

Instrumentation

HPLC- Shimadzu SIL-40C XR, Mass Spectrometer- Sciex Triple Quad 4500, Microbalance- Mettler Toledo XPR26, Refrigerated Centrifuge- Thermo Scientific Sorvall ST4R plus, Multitube Vortexer- MR Scientifics Vibex, Nitrogen Evaporator- PCI EV-144 plus, Micropipettes- Brand Transferpette® α; Thermo Scientific Finnpipette® F2, Multipette- Brand Handy step® S, Hand Vortexer- D Lab MX-S.

Preparations of Solutions and Standards

Preparation of Apremilast (Analyte) standard stock solution: 2.000 mg of Apremilast has weighed accurately and transferred to 2 ml volumetric flask, then 0.400 ml added DMSO to dissolve and made up to mark with methanol to get the 1 mg/ml solution of Apremilast.

Preparation of Apremilast D5 (Internal Standard or ISTD) stock solution: 1.000 mg of Apremilast D5 has weighed accurately and transferred to 10 ml volumetric flask, then added methanol to dissolve and made up to mark with methanol to get the 0.1 mg/ml solution of Apremilast D5.

Preparation of Internal standard working solution (ISTD WS): Transfer about 0.500 mL of Internal standard Stock Solution into a 500 mL volumetric flask, make up the volume with Methanol:water(50:50V/V) up to the mark to get the 500 ng/ml solution of ISTDWS.

Preparation of Mobile Phase Buffer / Extraction Buffer: 0.2% Formic acid in Water.

Preparation of Mobile Phase: Acetonitrile : Mobile Phase Buffer (90:10 V/V).

Preparation of Calibration Curve Standards and Quality Control Standards:

CC & QC spiking solutions are prepared as per the dilutions given in the Table 1 to 4 by using Methanol:Water(50:50V/V).

Table 1: Preparation of Calibration Curve spiking solutions:

Solution	Analyte Con.	Volume Taken (mL)	Volume of Plasma (mL)	Total Volume (mL)	Spiking Con.	Spiking Solution
Apremilast Stock	1000000.000	0.251	4.749	5.000	50200.000	SS STD10
SS STD10	50200.000	4.000	1.000	5.000	40160.000	SS STD9
SS STD9	40160.000	3.125	1.875	5.000	25100.000	SS STD8
SS STD8	25100.000	2.500	2.500	5.000	12550.000	SS STD7
SS STD7	12550.000	2.000	3.000	5.000	5020.000	SS STD6
SS STD6	5020.000	2.500	2.500	5.000	2510.000	SS STD5
SS STD5	2510.000	2.000	3.000	5.000	1004.000	SS STD4
SS STD4	1004.000	2.500	2.500	5.000	502.000	SS STD3
SS STD3	502.000	1.013	3.987	5.000	101.705	SS STD2
SS STD2	101.705	2.500	2.500	5.000	50.853	SS STD1

Table 2: Calibration Curve Spiking in Biological matrix:

Spiking Solution	Spiking Con.	Spiking Solution Vol (mL)	Volume of Diluent (mL)	Final Matrix Volume (mL)	Analyte Final Con.(ng/mL)	CC
SS STD10	50200.000	0.02	0.980	1	1004.000	STD10
SS STD9	40160.000	0.02	0.980	1	803.200	STD9
SS STD8	25100.000	0.02	0.980	1	502.000	STD8
SS STD7	12550.000	0.02	0.980	1	251.000	STD7

SS STD6	5020.000	0.02	0.980	1	100.400	STD6
SS STD5	2510.000	0.02	0.980	1	50.200	STD5
SS STD4	1004.000	0.02	0.980	1	20.080	STD4
SS STD3	502.000	0.02	0.980	1	10.040	STD3
SS STD2	101.705	0.02	0.980	1	2.034	STD2
SS STD1	50.853	0.02	0.980	1	1.017	STD1

Table 3: Quality Control/System /Suitability /Stabilization spiking solutions:

Solution	Analyte Con.	Volume Taken (mL)	Volume of Plasma (mL)	Total Volume (mL)	Spiking Con.	Spiking Solution
Apremilast Stock	1000000.000	0.189	4.811	5.000	37800.000	SS HQC
SS HQC	37800.000	3.000	2.000	5.000	22680.000	SS MQC1
SS MQC1	22680.000	0.665	4.335	5.000	3016.440	SS MQC2
SS MQC2	3016.440	0.215	4.785	5.000	129.707	SS LQC
SS LQC	129.707	1.986	3.014	5.000	51.520	SS LLOQ
Apremilast Stock	1000000.000	0.256	1.744	2.000	128000.000	SS DQC
Apremilast Stock	1000000.000	0.153	4.847	5.000	30600.000	SS SYS
SS SYS	30600.000	0.013	4.987	5.000	79.560	SS STAB

Table 4: Quality Control/Stabilization Spiking in Biological matrix:

Spiking Solution	Spiking Con.	Spiking Solution Vol (mL)	Volume of Diluent (mL)	Final Matrix Volume (mL)	Analyte Final Con.(ng/mL)	QC
SS HQC	37800.000	0.02	0.980	1	756.000	HQC
SS MQC1	22680.000	0.02	0.980	1	453.600	MQC1
SS MQC2	3016.440	0.02	0.980	1	60.329	MQC2
SS LQC	129.707	0.02	0.980	1	2.594	LQC
SS LLOQ	51.520	0.02	0.980	1	1.030	LLOQ
SS DQC	128000.000	0.02	0.980	1	2560.000	DQC
SS STAB	79.560	0.02	0.980	1	1.591	STAB

Instrument Parameters

Optimization of Mass-spectroscopic conditions:

Apremilast and Apremilast D5 of 100.00 ng/mL were prepared in methanol and infused with a stream rate of 15µL/min into positive particle mode to ramp or tune of mass spectrometer conditions. After ramping or tuning of mass conditions, m/z (amu) 461.200/257.100 and 466.200/262.100 ions were produced for Apremilast and Apremilast D5. Declustering potential (DP) was 80.0, Entrance potential (EP) was 10.0, Collision energy (CE) was 18.0, Collision cell exit potential (CXP) was 10.0, Dwell time (msec) was 200.0. The mass spectra's were represented in Figure 2.

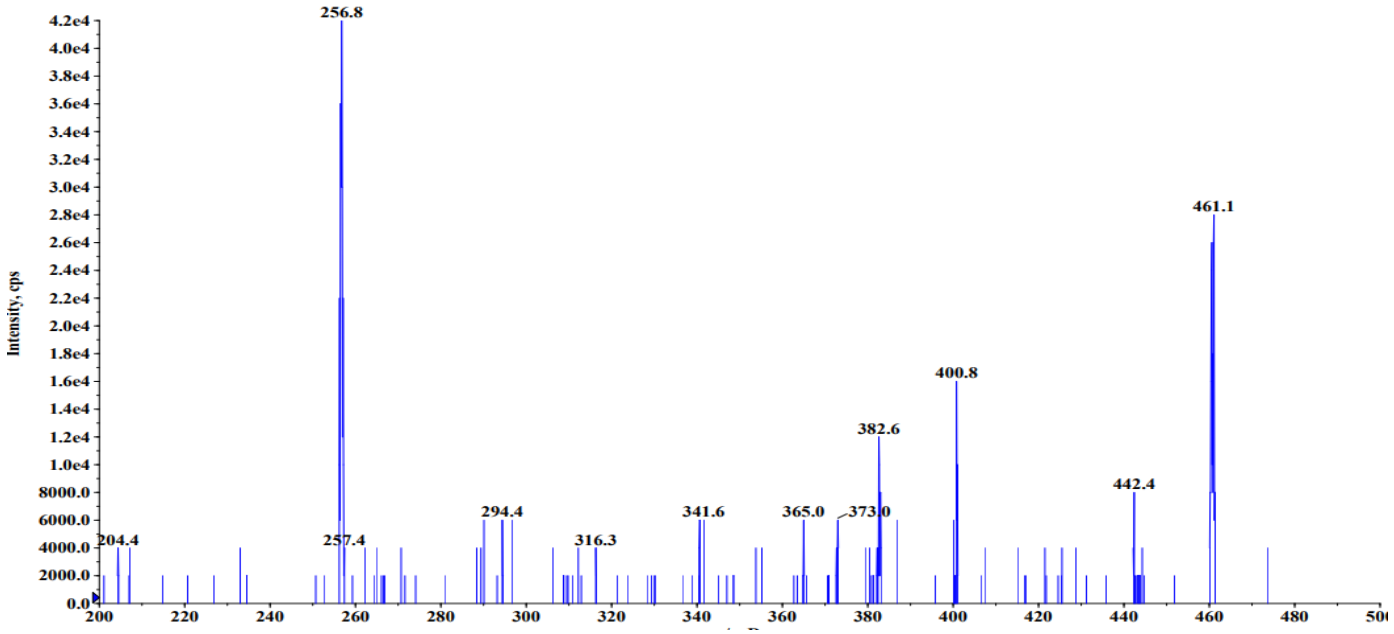


Figure 2: Mass Spectra of Apremilast

Optimization of Chromatographic conditions:

After a series of trials, the chromatographic conditions was optimized with 0.2% Formic acid (Mobile Phase Buffer): Acetonitrile, (10:90%, v/v) by utilizing the Unisol C18, 4.6x100mm, 5µm Column gave the best peak shape. The Apremilast and Apremilast D5 Peak were eluted at 1.24 min ± 0.5 min and 1.24 min ± 0.5 min. The total chromatographic duration was 2.00 min with flow of 1.000 mL/min and Column oven temperature and Auto sampler temperature were set at 40°C and 5°C and Injection Volume was 15.00 µL**.**

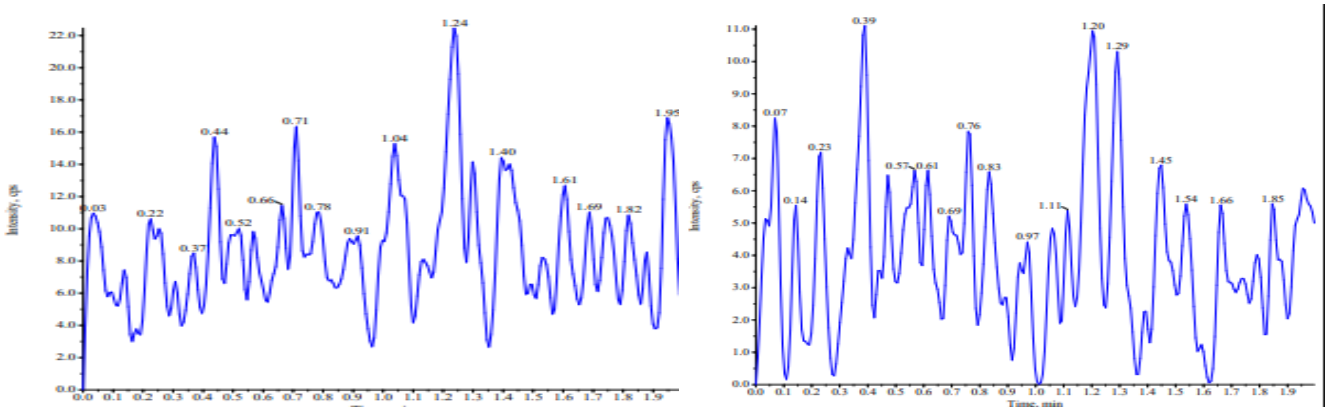


Figure 3: Blank plasma chromatogram of interference free Apremilast and free Apremilast D5.

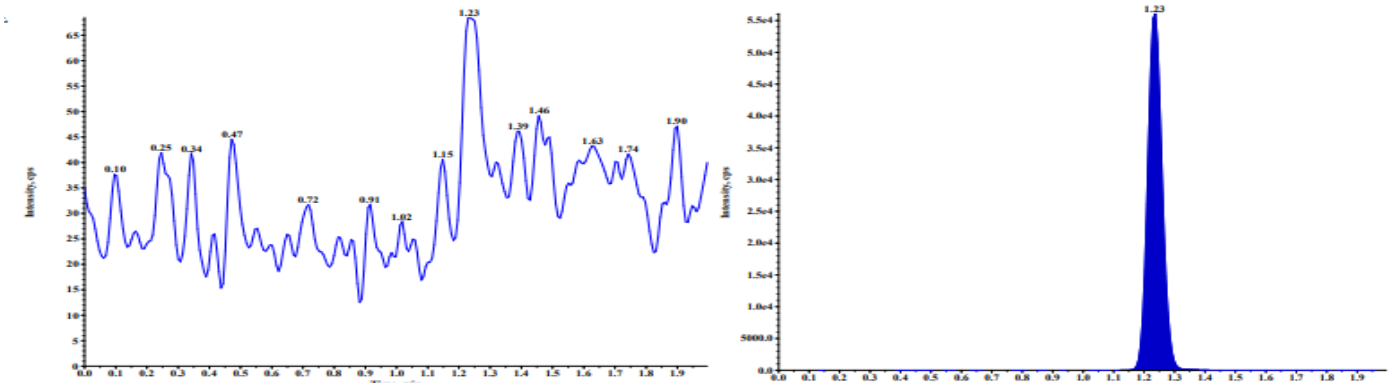


Figure 4: Blank plasma chromatogram of interference free Apremilast and Apremilast D5.

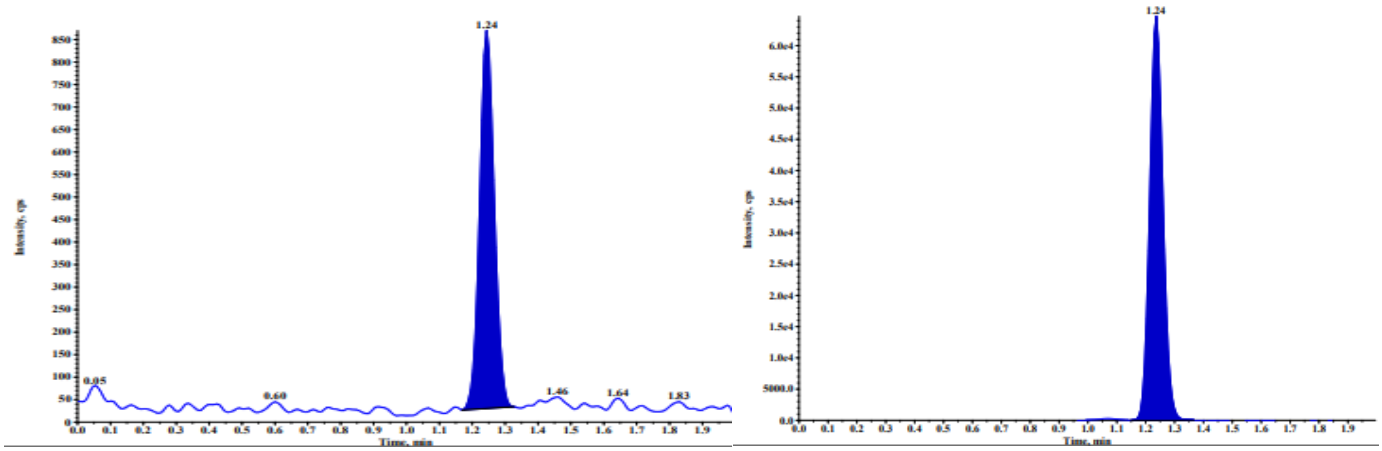


Figure 5: Chromatogram of LLOQ sample contains Apremilast and Apremilast D5.

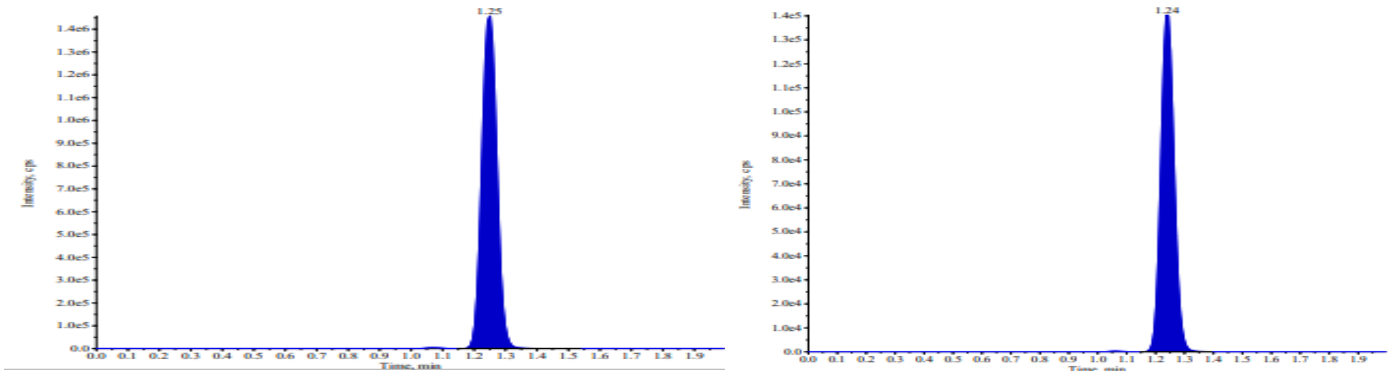


Figure 6: Chromatogram of ULOQ sample contains Apremilast and Apremilast D5.

Optimization Extraction technique:

Various extraction techniques were optimized to extract Apremilast and Apremilast D5 from human biological matrices. Ultimately, Liquid -Liquid Extraction (LLE) was appropriate as a result of larger free matrix interference and good recovery.

Sample extraction procedure (Sample Preparation):

Step 1: Retrieve the required of number samples from the deep freezer as per the request.

Step 2: Arrange the samples as per sequence and thaw at ICE Bath.

Step 3: Add 0.050 mL of Internal standard working solution (ISTD WS) into pre-labeled ria vials except standard Blank and add 50.000 µL of diluent in Standard Blank to compensate with ISTD WS.

Step 4: Aliquot 0.300 mL of plasma samples into the above pre-labeled ria vials and vortex to mix.

Step 5: Add 0.100 mL Mobile phase buffer/extraction buffer and vortex for few seconds.

Note: From Step 2 to Step 5 were Performed in Ice Bath.

Step 6: Add 2.000 mL of tBME (Tertiary Butyl Methyl Ether) and samples are placed in Multitube vortexer at 2500 rpm for about 03minutes.
Step 7: Centrifuge the samples at 4000 rpm, at 05±01°C for about 05 minutes.

Step 8: Separate the supernatant by flash freezing/calibrated pipette in to pre-labeled ria vials.

Step 9: Evaporate the samples in Nitrogen Evaporator at 40±05°C and apply nitrogen gas Pressure and increase the pressure gradually till complete dryness of tubes.

Step10: Add 0.250 mL of Reconstitution solution into all dried vials and vortex.

Step 11: Transfer appropriate volume into pre-labeled auto sampler vials.

Note: For the Matrix Factor and Post Extracted Recovery, Blank samples will be processed and reconstituted with Spiking solutions of HQC, MQC1 & LQC after completion of evaporation.

Aqueous solution preparation Procedure:

Step 1: Take 0.970 ml of Mobile phase into prelabelled vials.

Step 2: Add 0.250 ml of ISTD working solution and vortex to mix.

Step 3: Add 0.030 mL of Respective Spiking Solution and vortex to mix.

Aqueous DQC solution preparation Procedure (1/5 Dilution):

Step 1: Take 4.970 ml of Reconstitution solution into prelabelled vials.

Step 2: Add 1.250 ml of ISTD working solution and vortex to mix.

Step 3: Add 0.030 ml of Respective Spiking Solution and vortex to mix.

Validation Procedure:

A validation according to the ICH M10, FDA, WHO and ANVISA guidelines was performed for the assay of Apremilast in Human Plasma. [3] [4] [5]

System Suitability

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture SYS concentration of the calibration curve for analyte and 500 ng/ml for ISTD WS. System suitability was performed at the start of the method validation and after every three days or while changing the mobile phase solution.

Autosampler Carry Over

Carryover is the appearance of an analyte and internal standard signal in blank sample peaks after the analysis of samples with a high analyte concentration.

Selectivity

The selectivity was established by screening the standards blanks of different lots of Human Plasma. Eleven different lots of plasma were screened for the Experiment. All Eleven lots were found to be free of Significant interferences at the Retention time of all analytes in standard blank samples was ≤ 20.00% of the area of the drug in the Extracted LLOQ (Lower Limit of Quantification) Samples; area of peak at the Retention time of IS in the standard blank samples was ≤ 5.00% of the area of the IS in the Extracted LLOQ Sample as per acceptance limit.

Sensitivity
The sensitivity was evaluated by analyzing by taking 6 LLOQ of 3 Accepted Precision and Accuracy Batch's used to quantified S/N ratio of analyte.

Calibration Curve/Linearity
The linearity of the method was determined by using a regression analysis of standard plots associated with a Ten-point standard curve. Calibration curve analyzed during the course of validation were found to be linear for the standard concentration ranging from 1-1000 ng/ml range.

Precision
The precision of the method was evaluated by the % CV at different concentration levels corresponding to LLOQ, LQC, MQC, HQC and DQC during the course of validation.

Within-batch precision
The % CV of back calculated concentrations for all quality control samples at to LLOQ, LQC, MQC, HQC and DQC concentration levels with Six replicates were spiked combined with plasma sample and were being analyzed.

Between-batch precision
The % CV of back calculated concentrations for all quality control samples at to LLOQ, LQC, MQC, HQC and DQC concentration levels from three different batches of Six replicates at each QC levels were spiked combined with plasma sample and were being analyzed.

Accuracy
The accuracy of the method was evaluated by the % nominal concentration at different concentration levels corresponding to to LLOQ, LQC, MQC, HQC and DQC during the course of validation.

Within-batch accuracy
The percentage nominal of back calculated concentrations for all quality control samples of to LLOQ, LQC, MQC, HQC and DQC concentration levels with six replicates were spiked combined with plasma sample and were being analyzed.

Between-batch accuracy
The percentage nominal of back calculated concentrations for all quality control samples at to LLOQ, LQC, MQC, HQC and DQC concentration levels from three different batches of Six replicates at each QC levels were spiked combined with plasma sample and were being analyzed.

Recovery
The percentage mean recoveries were determined by measuring the responses of the quality control samples spiked into plasma against respective aqueous quality control samples at LQC, MQC and HQC levels.

Dilution Integrity
Dilution Integrity was performed by diluting the DQC Sample by 5 Times. DQC1/5 samples was processed along with the Precision and Accuracy batch.

Best Fit Analysis / Weighting Factor
Three calibration curves were analysed by least-squares linear regression analysis with weighing factors of $1/x$ and $1/x^2$.

Matrix effect

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. The matrix effect between different independent lots were evaluated.

STABILITIES

Long Term Stock/Working Solutions Stability of Analytes and Internal Standard

Long term stock/working solution stability for the Apremilast and IS at concentration 500 ng/ml were determined by using stock and working solution, after storage of primary stock solution over a period of 08 days 20 hrs at 2-8°C. Stability was assessed by comparing against the freshly prepared stock. The % mean stability was calculated.

Bench Top Stability

Bench top stability of the spiked quality control samples was determined for a period of 22 hrs 22 mins. stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards.

Auto Sampler Stability

Auto sampler stability of the processed quality control samples was determined for a period of 04 days 22 hrs 38 mins by storing them in auto sampler maintained at 5°C. Stability was assessed by comparing processed sample against the freshly spiked calibration standards

Post Extract Stability at Room Temperature

Post Extract stability of the processed quality control samples was determined for a period of 22 hrs 15 mins. stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards.

Freeze Thaw Stability

Freeze thaw stability of the spiked quality control samples was determined after three freeze thaw cycles stored at -70 °C. Stability was assessed by comparing them against the freshly spiked calibration standards.

Long Term Stability in Matrix

Long term stability of the spiked quality control samples was determined after stored at -70 °C for 38 days. Stability was assessed by comparing them against the freshly spiked calibration standards.

RESULTS AND DISCUSSION

Method Development

After several Trials the LC Condations were Optimised and given in Below table

LC Conditions:

S. No.	Parameter	Conditions
1.	Flow rate	1.000 mL/min
2.	Injection volume	15.00 µL
3.	Auto sampler temperature	5°C
4.	Column oven temperature	40°C
5.	Run time	2.00 mins
6.	Column specifications	Unisol C18, 4.6x100mm, 5µm
7.	Retention Time	Apremilast: 1.24 min ± 0.5 min
		Apremilast D5: 1.24 min ± 0.5 min
8.	Pump mode	Isocratic
9.	Split Ratio	75% split in to drain

Tuning of Apremilast and Apremilast D5 were optimized and given in below table.

MS/MS Conditions:

S. No.	Compound Parameters	Apremilast	Apremilast D5
1.	Multiple reaction monitoring (MRM) (Q1/Q3) (m/z)	461.200/257.100	466.200/262.100

2.	Declustering potential (DP)	80.00
3.	Entrance potential (EP)	10.00
4.	Collision energy (CE)	18.00
5.	Collision cell exit potential (CXP)	10.00
6.	Dwell time (msec)	200.0

By Checking or optimization of source parameters, the final source parameters are given in below table.

Source Dependent Parameters:

S. No.	Source Parameters	
1.	Ion Source	Turbo Spray
2.	Polarity	Positive
3.	Curtain gas (CUR)	38.00
4.	Collision associated dissociation (CAD)	8.00
5.	Ion spray voltage (IS)	5500.00
6.	Heater temperature (TEM)	550.00
7.	Nebulizer gas (GS1)	45.00
8.	Heater gas (GS2)	45.00

Extraction techniques were optimized to extract Apremilast and Apremilast D5 from human biological matrices. Ultimately, Liquid -Liquid Extraction (LLE) was appropriate as a result of larger free matrix interference and good recovery.

After optimization of the above conditions, the method was validated according to the ICH guidelines.

METHOD VALIDATION

System Suitability

The %CV of the retention times was found to be ≤ 0.00 for all analytes and IS. The %CV of the peak area was found to be ≤ 2.8 for analyte and IS. Acceptance limit for retention time (Rt) deviation and area deviation 5% and 5%CV respectively were passed. The results are summarized in Below Table.

Injection Number	Retention Time (min)		Area / Area Ratio
	Apremilast	Apremilast D5	
1	1.25	1.24	4.5115
2	1.25	1.24	4.5875
3	1.25	1.24	4.5834
4	1.25	1.24	4.7598
5	1.25	1.24	4.4882
6	1.25	1.24	4.7995
N	**6**	**6**	**6**
Average	**1.25**	**1.24**	**4.622**
Standard Deviation	**0.0000**	**0.0000**	**0.1291**
% CV	**0.0**	**0.0**	**2.8**
Acceptance Criteria	Retention Time: %CV ≤5.0 & Area / Intensity Ratio: %CV ≤5.0		

Autosampler Carry Over

No carry over was observed at Apremilast and Apremilast D5 peak area. Auto sampler carry over was observed with in the acceptance criteria. The results are summarized in Below Table.

Sample ID	Peak Area		% Carryover	
	Apremilast	Apremilast D5	Apremilast	Apremilast D5
Unextracted samples				
RS	0	0	**N/A**	
AQ ULOQ	4648359	434555		
RS	0	0	**0.00**	**0.00**
RS	0	0	**N/A**	
AQ LLOQ	6890	531923		
Extracted samples				
STD Blank	0	0	**N/A**	
ULOQ	4680240	384850		
STD Blank	211	1960	**13.37**	**0.42**
STD Blank	784	0	**N/A**	
LLOQ	5866	466236		
Acceptance Criteria: 1) The carryover response in subsequent injection of RS after AQ ULOQ should not be more than 20% of AQ LLOQ response for analyte and should not be more than 5% of AQ LLOQ response for IS. 2) The carryover response in subsequent injection of STD Blk after ULOQ should not be more than 20% of LLOQ response for analyte and should not be more than 5% of LLOQ response for IS.				

Selectivity

Selectivity All Eleven lots were found to be free of Significant interferences at the Retention time of all analytes in standard blank samples was ≤ 20.00% of the area of the drug in the Extracted LLOQ (Lower Limit of Quantification) Samples; area of peak at the Retention time of IS in the standard blank samples was ≤ 5.00% of the area of the IS in the Extracted LLOQ Sample as per acceptance limit. In optimization trials we choose such method where plasma lots were found to be free of significant interferences at the Retention time of all analytes in standard blank samples. The results are summarized in Below Table.

Matrix ID	Apremilast			Apremilast D5		
	Response in Blank	Response in LLOQ	% Interference	Response in Blank	Response in LLOQ	% Interference
Plasma lot-01	0	4272	0.00	0	338927	0.00
Plasma lot-02	0	4455	0.00	0	365689	0.00
Plasma lot-03	0	4334	0.00	0	381646	0.00
Plasma lot-04	0	4227	0.00	0	342890	0.00
Plasma lot-05	0	4065	0.00	0	351971	0.00
Plasma lot-06	0	3721	0.00	0	285755	0.00
Plasma lot-07	0	4171	0.00	0	342783	0.00

Plasma lot-08	0	4015	0.00	0	356217	0.00
Plasma lot-09	0	4473	0.00	0	371875	0.00
Plasma lot-10	0	4405	0.00	0	357467	0.00
Plasma lot-11	0	4148	0.00	0	354829	0.00
Acceptance Criteria	Peak area at RT of analyte in blank sample should not be more than 20% (i.e. ≤ 20.0%) of the analyte peak area observed in respective LLOQ sample.					
	Peak area at RT of ISTD in blank sample should not be more than 5% (i.e. ≤ 5.0%) of the ISTD peak area observed in respective LLOQ sample.					

Sensitivity:

LLOQ Samples were taken from the 3 Qualified or Accepted Precision and Accuracy batches used to quantified S/N ratio of analyte. S/N ratio were met the acceptance criteria. The results are summarized in Below Table.

Nominal Conc. (ng/mL)	**1.03**	
Nominal Conc. Lower Range (ng/mL)	**0.824**	
Nominal Conc. Upper Range (ng/mL)	**1.236**	
LLOQ ID	**Back Calculated Conc. (ng/mL)**	**S/N Ratio**
FS-01 LLOQ QC-001	1.09	511
FS-01 LLOQ QC-002	1.044	557
FS-01 LLOQ QC-003	1.121	433
FS-01 LLOQ QC-004	1.198	271
FS-01 LLOQ QC-005	1.118	251
FS-01 LLOQ QC-006	1.101	291
Mean	**1.112**	**385.7**
SD	**0.05048**	**NA**
% CV	**4.54**	
% Accuracy	107.96	
FS-01 LLOQ QC-001	1.167	574
FS-01 LLOQ QC-002	1.166	476
FS-01 LLOQ QC-003	1.089	284
FS-01 LLOQ QC-004	1.117	246
FS-01 LLOQ QC-005	1.153	574
FS-01 LLOQ QC-006	1.046	373
Mean	**1.123**	**421.2**
SD	**0.04859**	**NA**
% CV	**4.33**	
% Accuracy	**109.03**	
FS-02 LLOQ QC-001	1.034	147
FS-02 LLOQ QC-002	1.186	285
FS-02 LLOQ QC-003	1.07	342
FS-02 LLOQ QC-004	1.027	161

FS-02 LLOQ QC-005	1.215	153
FS-02 LLOQ QC-006	1.175	239
Mean	**1.1178**	**221.2**
SD	**0.08357**	**NA**
% CV	**7.48**	
% Accuracy	**108.52**	
Inter run Mean	**1.118**	**342.7**
Inter run SD	**0.05933**	**NA**
Inter run Precision (%)	**5.31**	
Inter run accuracy (%)	**108.5**	

Intra batch min-max range			
%Accuracy Range	**107.96**	**109.03**	**N/A**
%CV Range	**4.33**	**7.48**	
S/N ratio Range	**147**	**574**	

Acceptance Criteria: 1. The Inter and Intra run %CV for LLOQ samples must be ≤ 20% and the Inter and Intra run accuracy must be within 80%-120% of nominal LLOQ concentration.
2. The signal-to-noise (S/N) ratio for all six LLOQ samples shall be ≤ 5.

Calibration Curve/Linearity

Representative calibration curve is shown in figures which are obtained during the precision and accuracy batch. The average correlation coefficient (R^2) was ≥ 0.99 during the course of validation. Data of calculated calibration standard concentration are shown in below Table.

CC ID	Nominal Con. (ng/mL)	Back Calculated Con. (ng/mL)	% Accuracy
STD1	1.017	0.993	97.6
STD2	2.033	2.114	103.98
STD3	10.042	10.629	105.85
STD4	20.083	19.722	98.2
STD5	50.208	48.733	97.06
STD6	100.417	97.982	97.58
STD7	251.042	249.519	99.39
STD8	502.084	481.115	95.82
STD9	803.335	847.437	105.49
STD10	1004.168	994.375	99.02
HQC	756.528	750.0667	99.15
MQC1	452.403	451.4528	99.79
MQC2	60.396	60.8938	100.82
LQC	2.597	2.6083	100.44
LLOQ	1.03	1.112	107.96
DQC	2558.439	2481.829	97.01

Precision

Within batch precision

The %CV of back calculated concentrations for all quality control samples of LLOQ, LQC, MQC, HQC and DQC concentration levels with Six replicates were within 2.83% to 5.31%. Acceptances criteria are that at least 67% of QC samples must be within 15% except LLOQ where limit is within 20%.

Between batch precision

The %CV of back calculated concentrations for all quality control samples at LLOQ, LQC, MQC, HQC and DQC concentration levels from three different batches of six replicate at each QC levels were found within 4.00% to 7.48%. Acceptances criteria are that at least 67% of QC samples must be within 15% except LLOQ where limit is within 20%. The results are summarized in Below Table.

Accuracy

Within batch accuracy

The percentage nominal of back calculated concentrations for all quality control samples of LLOQ, LQC, MQC and HQC concentration levels with six replicates were within 96.03%-108.50%. Acceptance criteria are that at least 67% of QC samples must be within 85-115%.

Between batch accuracy

The percentage nominal of back calculated concentrations for all quality control samples of LLOQ, LQC, MQC and HQC concentration levels with six replicates of three different batches were within 96.03%-108.50%. Acceptances criteria are that at least 67% of QC samples must be within 85-115%. The results are summarized in Below Table.

QC Level	HQC	M1QC	M2QC	LQC	LLOQ QC	DIQC
Nominal Con. (ng/mL)	**756.528**	**452.403**	**60.396**	**2.597**	**1.03**	**2558.439**
Lower Limit (ng/mL)	**643.049**	**384.543**	**51.337**	**2.207**	**0.824**	**2174.673**
Upper Limit (ng/mL)	**870.007**	**520.263**	**69.455**	**2.987**	**1.236**	**2942.205**
QC ID	**Back Calculated Con. (ng/mL)**					
1	758.059	455.522	59.902	2.688	1.09	2485.8
2	779.681	449.298	66.791	2.691	1.044	2457.881
3	727.268	449.406	60.74	2.604	1.121	2488.99
4	739.356	467.937	58.277	2.423	1.198	2499.194
5	733.216	444.961	58.965	2.627	1.118	2499.562
6	762.82	441.593	60.688	2.617	1.101	2459.547
Mean	**750.0667**	**451.4528**	**60.8938**	**2.6083**	**1.112**	**2481.829**
SD	**20.10958**	**9.34322**	**3.04613**	**0.09795**	**0.05048**	**18.72456**
% CV	**2.68**	**2.07**	**5.00**	**3.76**	**4.54**	**0.75**
% Accuracy	**99.15**	**99.79**	**100.82**	**100.44**	**107.96**	**97.01**
1	768.262	464.282	61.686	2.821	1.167	2484.813
2	766.224	453.243	61.342	2.394	1.166	2494.104
3	748.049	447.238	57.309	2.794	1.089	2394.074
4	723.212	485.36	63.525	2.724	1.117	2396.221
5	723.557	442.551	59.246	2.594	1.153	2424.171
6	770.478	431.658	61.907	2.683	1.046	2328.862
Mean	**749.9637**	**454.0553**	**60.8358**	**2.6683**	**1.123**	**2420.374**
SD	**22.07576**	**18.79198**	**2.20563**	**0.15697**	**0.04859**	**62.05227**
% CV	**2.94**	**4.14**	**3.63**	**5.88**	**4.33**	**2.56**
% Accuracy	**99.13**	**100.37**	**100.73**	**102.75**	**109.03**	**94.6**
1	803.529	481.803	63.104	2.698	1.034	2437.086
2	798.204	468.633	62.289	2.659	1.186	2651.799
3	764.161	447.321	59.293	2.576	1.07	2503.632
4	785.308	437.311	60.478	2.721	1.027	2382.773

5	753.758	447.929	62.962	2.751	1.215	2423.273
6	709.322	451.559	58.412	2.645	1.175	2409.737
Mean	**769.047**	**455.7593**	**61.0897**	**2.675**	**1.1178**	**2468.05**
SD	**35.00932**	**16.33712**	**1.98865**	**0.06225**	**0.08357**	**98.65708**
% CV	**4.55**	**3.58**	**3.26**	**2.33**	**7.48**	**4**
% Accuracy	**101.65**	**100.74**	**101.15**	**103**	**108.52**	**96.47**
Inter run (Global) Precision and Accuracy Range						
Mean	756.3591	453.7558	60.9398	2.6506	1.1176	2456.7511
SD	26.6081	14.5382	2.3099	0.1103	0.0593	69.5154
% CV	3.52	3.2	3.79	4.16	5.31	2.83
% Accuracy	99.98	100.3	100.9	102.06	108.5	96.03
Intra run Precision and Accuracy Range						
	HQC	MQC	LQC	LLOQ QC	DIQC	MQC2
%CV Min	2.68	2.07	2.33	4.33	0.75	3.26
%CV Max	4.55	4.14	5.88	7.48	4	5
%Nom Min	99.13	99.79	100.44	107.96	94.6	100.73
%Nom Max	101.65	100.74	103	109.03	97.01	101.15

Acceptance Limits: 1) Intra-run Precision: The %CV for HQC, MQC and LQC samples should be ≤15 % and for LLOQQC should be ≤ 20%.

2) Intra-run accuracy: The % Accuracy should be within 85%-115% for HQC, MQC and LQC and 80%-120% for LLOQ QC of the respective Nominal concentrations.

Note: The Intra-run precision and accuracy must be reported as a range of minimum and maximum %CV observed at each level among the accepted precision and accuracy runs.

3) Inter-run Precision: The %CV for HQC, MQC and LQC samples from at least 3 Precision and Accuracy runs (including ruggedness P&A) analyzed on at least three different days should be ≤ 15 % and for LLOQQC should be ≤ 20%.

4) Inter-run Accuracy: The % Accuracy for HQC, MQC and LQC samples from at least 3 Precision and Accuracy runs (including ruggedness P&A) analyzed on at least three different days should be 85-115% and for LLOQQC should be 80-120% of the respective Nominal concentrations.

At least two consecutive Precision and Accuracy runs must meet the above acceptance criteria.

Recovery

The % mean recovery of both analyte and IS acceptable limit was % CV of 15. The results are summarized in Below Table's.

RECOVERY OF APREMILAST						
	Apremilast					
	LQC		**MQC**		**HQC**	
	Extracted	**Post Extracted**	**Extracted**	**Post Extracted**	**Extracted**	**Post Extracted**
Average/Mean	4635	5634	841917	952140	1303163	1510918
Standard Deviation	144	448	49986	26638	67494	42328
CV (Precision%)	3.10	7.95	5.94	2.80	5.18	2.8
%Recovery	**82.27**		**88.42**		**86.25**	
Overall Recovery	**85.65**					
Standard Deviation	**3.12**					
Global CV (Precision%)	**3.64**					

Acceptance Criteria	The recovery %CV should not be more than 15.0% at each QC level for analyte Globally.

RECOVERY OFAPREMILAST D5						
	Apremilast D5					
	LQC		**MQC**		**HQC**	
	Extracted	**Post Extracted**	**Extracted**	**Post Extracted**	**Extracted**	**Post Extracted**
Average/Mean	243278	285737	236555	280835	236765	275060
Standard Deviation	12503	15565	13211	10627	10395	9776
CV (Precision%)	5.14	5.45	5.58	3.78	4.39	3.55
%Recovery	**85.14**		**84.23**		**86.08**	
Overall Recovery	**85.15**					
Standard Deviation	**0.92**					
Global CV (Precision%)	**1.08**					
Acceptance Criteria	The recovery %CV should not be more than 15.0% at each QC level for ISTD and Globally.					

Dilution Integrity

Dilution Integrity was performed by diluting the DQC Sample by 5 Times. DQC1/5 samples was processed along with the Precision and Accuracy batch, met the acceptance criteria.

Weighting Factor/Best Fit Analysis

Linear regression with $1/x^2$ weighting was selected as weighting Factor. The results are summarized in Below Table.

Weighting Factor: 1/X									
Batch IDs	**Batch-01**			**Batch-02**			**Batch-03**		
Non-Zero Calibration Standards	**% Accuracy**	**Absolute Difference From 100 %**	**Difference2**	**% Accuracy**	**Absolute Difference From 100 %**	**Difference2**	**% Accuracy**	**Absolute Difference From 100 %**	**Difference2**
STD1	99.1	0.9	0.81	99.8	0.2	0.04	108.56	8.56	73.2736
STD2	104.57	4.57	20.8849	94.7	5.3	28.09	94.23	5.77	33.2929
STD3	105.71	5.71	32.6041	95.41	4.59	21.0681	89.91	10.09	101.8081
STD4	98	2	4	99.32	0.68	0.4624	100.24	0.24	0.0576
STD5	96.8	3.2	10.24	105.33	5.33	28.4089	104.25	4.25	18.0625
STD6	97.3	2.7	7.29	106.4	6.4	40.96	105.9	5.9	34.81
STD7	99.1	0.9	0.81	101.02	1.02	1.0404	96.45	3.55	12.6025
STD8	95.54	4.46	19.8916	100.07	0.07	0.0049	101.32	1.32	1.7424
STD9	105.17	5.17	26.7289	95.37	4.63	21.4369	98.11	1.89	3.5721
STD10	98.73	1.27	1.6129	102.57	2.57	6.6049	101.04	1.04	1.0816
	SUM(A)	30.88	NA	SUM(A)	30.79	NA	SUM(A)	42.61	NA

	SUM of Difference2		124.872	SUM of Difference2		148.1165	SUM of Difference2		280.3033
	Square Root (B)		11.1746	Square Root (B)		12.17031	Square Root (B)		16.74226
	Sum of % RE (A+B)		**42.055**	**Sum of % RE (A+B)**		**42.9603**	**Sum of % RE (A+B)**		**59.3523**
Weighting Factor: 1/X2									
Batch ID	**Batch-01**			**Batch-02**			**Batch-03**		
Non-Zero Calibration Standards	**% Accuracy**	**Absolute Difference From 100 %**	**Difference2**	**% Accuracy**	**Absolute Difference From 100 %**	**Difference2**	**% Accuracy**	**Absolute Difference From 100 %**	**Difference2**
STD1	97.6	2.4	5.76	102.48	2.48	6.1504	104.52	4.52	20.4304
STD2	103.98	3.98	15.8404	95.8	4.2	17.64	92.54	7.46	55.6516
STD3	105.85	5.85	34.2225	95.22	4.78	22.8484	90.17	9.83	96.6289
STD4	98.2	1.8	3.24	98.94	1.06	1.1236	100.82	0.82	0.6724
STD5	97.06	2.94	8.6436	104.82	4.82	23.2324	105.02	5.02	25.2004
STD6	97.58	2.42	5.8564	105.85	5.85	34.2225	106.73	6.73	45.2929
STD7	99.39	0.61	0.3721	100.48	0.48	0.2304	97.24	2.76	7.6176
STD8	95.82	4.18	17.4724	99.53	0.47	0.2209	102.15	2.15	4.6225
STD9	105.49	5.49	30.1401	94.86	5.14	26.4196	98.93	1.07	1.1449
STD10	99.02	0.98	0.9604	102.02	2.02	4.0804	101.88	1.88	3.5344
	SUM(A)	30.65	NA	SUM(A)	31.3	NA	SUM(A)	42.24	NA
	SUM of Difference2		122.508	SUM of Difference2		136.1686	SUM of Difference2		260.796
	Square Root (B)		11.0683	Square Root (B)		11.66913	Square Root (B)		16.14918
	Sum of % RE (A+B)		**41.718**	**Sum of % RE (A+B)**		**42.9691**	**Sum of % RE (A+B)**		**58.3892**

Batch No.	**Weighting Factor: 1/X**	**Weighting Factor: 1/X2**
Batch-01	42.05463198	41.71832869
Batch-02	42.96031224	42.96913022
Batch-03	59.3522609	58.38917955
SUM	**144.3672051**	**143.0766385**

Matrix effect

The matrix effect between different independent lots were be evaluated. No significant matrix effect found in Human plasma samples for Apremilast and Apremilast D5. The % mean Accuracy and % CV of each independent lots were met the acceptance Criteria. The results are summarized in Below Table.

Analyte Name	**Apremilast**		**ISTD Name**	**Apremilast D5**
Nominal Conc. (ng/mL) at LQC Level	**2.597**	**Nominal Conc. (ng/mL) at HQC Level**	**756.528**	
Matrix ID	**LQC**		**HQC**	

	Calculated Conc. (ng/mL)	Mean	SD	%CV	% Accuracy	Calculated Conc. (ng/mL)	Mean	SD	%CV	% Accuracy
Plasma Lot-01	2.332	2.38	0.0746	3.14	91.64	687.3	711.324	21.1	2.97	94.02
	2.342					719.9				
	2.466					726.8				
Plasma Lot-02	2.302	2.318	0.1467	6.33	89.26	721.4	711.058	9.277	1.3	93.99
	2.18					703.4				
	2.472					708.4				
Plasma Lot-03	2.406	2.322	0.0872	3.75	89.41	717	707.104	8.553	1.21	93.47
	2.328					702.8				
	2.232					701.6				
Plasma Lot-04	2.501	2.42433	0.0755	3.12	93.35	692.6	700.855	24.95	3.56	92.64
	2.35					728.9				
	2.422					681.1				
Plasma Lot-05	2.311	2.331	0.1163	4.99	89.76	681.2	697.083	17.9	2.57	92.14
	2.456					693.6				
	2.226					716.5				
Plasma Lot-06	2.372	2.438	0.0584	2.4	93.88	700.6	728.596	37.29	5.12	96.31
	2.459					714.3				
	2.483					770.9				
Plasma Lot-01 Hemolyzed	2.186	2.29367	0.1342	5.85	88.32	724.3	708.873	35.23	4.97	93.7
	2.251					668.6				
	2.444					733.7				
Plasma Lot-01 Hemolyzed	2.518	2.43833	0.1252	5.14	93.89	705.1	716.996	24.32	3.39	94.77
	2.294					745				
	2.503					700.9				
Plasma Lot-01 Lipemic	2.562	2.48933	0.0637	2.56	95.85	712.5	677.694	36.63	5.4	89.58
	2.443					639.5				
	2.463					681.1				
Plasma Lot-02 Lipemic	2.473	2.48367	0.0129	0.52	95.64	675.3	709.313	31.33	4.42	93.76
	2.48					715.6				
	2.498					737				
Acceptance Criteria	Mean % Accuracy of QC Sample should be within 85.0% to 115.0% and %CV should be ≤ 15% at each QC level and in each lot.									
	At least 80% of matrices should be within above acceptance criteria and both Hemolytic, Lipemic plasma lot should meet the above acceptance criteria.									

STABILITIES

Aqueous Solution Stabilities

Long Term Stock Solution/working Stability (At Refrigerated Temperature, 2-8°C)

Long term stock/working solution stability for the Analyte and IS at concentration 500 ng/ml were determined by using stock and working solution of Analyte and IS respectively, after storage of primary stock and Working solution over a period of 08 days 20 hrs at 2-8°C. Stability was assessed by comparing against the freshly prepared stock. which is within the acceptance limit of 90.00 – 110.00%. The results are summarized in Below Table..

Long Term Stock Solution Stability (At Refrigerated Temperature)

Analyte Name	Apremilast		ISTD Name	Apremilast D5		
	Analyte Peak Area				ISTD Peak Area	
	At LLOQ level		At ULOQ level		At ULOQ level	
	Fresh Samples	Stability Samples	Fresh Samples	Stability Samples	Fresh Samples	Stability Samples
Average	3106.5	3278	2370487	2440729	215472	221566.8
Standard Deviation	158.872	265.4626	87814.71356	107727	7287.639	11321.69
%CV	**5.11**	**8.10**	**3.70**	**4.41**	**3.38**	**5.11**
Nominal Conc. (ng/mL)	1.027	1.018	1027.15	1018.192	507.144	506.746
%Stability	NA	**106.45**	NA	**103.87**	NA	**102.91**
Acceptance Criteria	The %Stability should be within 90.0-110.0%.					

Long Term Spiking/working Solution Stability (At Refrigerated Temperature)						
Analyte Name	**Apremilast**		**ISTD Name**	**Apremilast D5**		
	Analyte Peak Area				**ISTD Peak Area**	
	At LLOQ level		**At ULOQ level**		**At ULOQ level**	
	Fresh Samples	**Stability Samples**	**Fresh Samples**	**Stability Samples**	**Fresh Samples**	**Stability Samples**
Average	3106.5	3332.5	2370487	2377867	215472	216535.2
Standard Deviation	158.872	488.9355	87814.71356	74317.92	7287.639	10384.48
%CV	5.11	14.67	3.70	3.13	3.38	4.80
Nominal Conc. (ng/mL)	1.027	1.017	1027.15	1004.616	507.144	500.554
%Stability	NA	**108.33**	NA	**102.56**	NA	**101.82**
Acceptance Criteria	The %Stability should be within 90.0-110.0%.					

Extracted Stabilities

Auto-sampler Stability

Auto sampler stability of the processed quality control samples was determined for a period of 04 days 22 hrs 38 mins by storing them in auto sampler maintained at 5°C. Stability was assessed by comparing processed sample against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 99.4% & 98.7%. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should not fail. The results are summarized in Below Table.

Auto sampler Stability				
	LQC		HQC	
	Fresh Samples	Stability samples	Fresh Samples	Stability samples
Average	2.6025	2.586833	706.9911667	697.8585
Standard Deviation	0.051675	0.185023	30.39608892	18.83368
%CV	**1.99**	**7.15**	**4.30**	**2.70**
Nominal Conc. (ng/mL)	2.597	2.597	756.528	756.528

%Accuracy	**100.21**	**99.61**	**93.45**	**92.24**
% Stability (comparision with Fresh Samples)	NA	**99.40**	NA	**98.71**
Acceptance Criteria	%CV should not be more than 15.0% at each QC level. The mean % accuracy should be within 85.0% to 115.0% at each QC level.			
	%Stability should be within 85.0-115.0%.			

Post Extract Stability at Room Temperature

Post Extract Stability of the processed quality control samples was determined for a period of 22 hrs 15 mins. stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 96.1% & 102.8%. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should not fail. The results are summarized in Below Table.

Post Extract Stability at Room Temperature				
	LQC		**HQC**	
	Fresh Samples	**Stability samples**	**Fresh Samples**	**Stability samples**
Average	2.6025	2.499833	706.9911667	727.099
Standard Deviation	0.051675	0.169179	30.39608892	19.79977
%CV	**1.99**	**6.77**	**4.30**	**2.72**
Nominal Conc. (ng/mL)	2.597	2.597	756.528	756.528
%Accuracy	**100.21**	**96.26**	**93.45**	**96.11**
% Stability (comparision with Fresh Samples)		**96.06**	NA	**102.84**
Acceptance Criteria	%CV should not be more than 15.0% at each QC level. The mean % accuracy should be within 85.0% to 115.0% at each QC level.			
	%Stability should be within 85.0-115.0%.			

Bench Top Stability

Bench top stability of the spiked quality control samples was determined for a period of 22 hrs 22 mins. stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 97.3% & 103.8%. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should not fail. The results are summarized in Below Table.

Bench Top Stability				
	LQC		**HQC**	
	Fresh Samples	**Stability samples**	**Fresh Samples**	**Stability samples**
Average	2.6025	2.533167	706.9911667	734.087
Standard Deviation	0.051675	0.130293	30.39608892	14.51765
%CV	**1.99**	**5.14**	**4.30**	**1.98**
Nominal Conc. (ng/mL)	2.597	2.597	756.528	756.528
%Accuracy	**100.21**	**97.54**	**93.45**	**97.03**
% Stability (comparision with Fresh Samples)	NA	**97.34**	NA	**103.83**

Acceptance Criteria	%CV should not be more than 15.0% at each QC level. The mean % accuracy should be within 85.0% to 115.0% at each QC level.
	%Stability should be within 85.0-115.0%.

Freeze Thaw Stability at -70±15°C

Freeze thaw stability of the spiked quality control samples was determined after Five freeze thaw cycles stored at -80 °C. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 98.4% & 103.5%. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should not fail. The results are summarized in Below Table.

Freeze Thaw Stability at -70±15°C				
	LQC		**HQC**	
	Fresh Samples	**Stability samples**	**Fresh Samples**	**Stability samples**
Average	2.6025	2.561333	706.9911667	735.1907
Standard Deviation	0.051675	0.151678	30.39608892	18.80737
%CV	**1.99**	**5.92**	**4.30**	**2.56**
Nominal Conc. (ng/mL)	2.597	2.597	756.528	760.25
%Accuracy	**100.21**	**98.63**	**93.45**	**96.70**
% Stability (comparision with Fresh Samples)		**98.42**	NA	**103.48**
Acceptance Criteria	%CV should not be more than 15.0% at each QC level. The mean % accuracy should be within 85.0% to 115.0% at each QC level.			
	%Stability should be within 85.0-115.0%.			

Long Term Stability in Matrix at -70±15°C

Long Term Stability Long term stability of the spiked quality control samples was determined after stored at -80 °C for 14 days. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 97.1% & 103.1%. This is within the acceptance limit. Acceptance Criteria is at least 67% QC samples should pass acceptance limit of 85-115% and more than 50% at each QC level should not fail. The results are summarized in Below Table.

Long Term Stability in Matrix at -70±15°C				
	LQC		**HQC**	
	Fresh Samples	**Stability samples**	**Fresh Samples**	**Stability samples**
Average/Mean	2.6025	2.527	706.9911667	732.1672
Standard Deviation	0.051675	0.138749	30.39608892	21.7852
%CV	**1.99**	**5.49**	**4.30**	**2.98**
Nominal Conc. (ng/mL)	2.597	2.597	756.528	760.25
%Accuracy	**100.21**	**97.30**	**93.45**	**96.31**
% Stability (comparision with Fresh Samples)		**97.10**	NA	**103.05**
Acceptance Criteria	%CV should not be more than 15.0% at each QC level. The mean % accuracy should be within 85.0% to 115.0% at each QC level.			
	%Stability should be within 85.0-115.0%.			

Conclusion

Based on the comprehensive method development, validation, and stability testing conducted for the analysis of Apremilast and Apremilast D5 in human biological matrices, the following conclusions can be drawn:

- Method Suitability: The developed method utilizing liquid-liquid extraction and optimized chromatographic conditions demonstrates suitability for the accurate and precise quantification of Apremilast and Apremilast D5 in human plasma samples.
- Validation Compliance: The method validation results comply with ICH guidelines, demonstrating the method's accuracy, precision, linearity, sensitivity, dilution integrity, and absence of matrix effects. The method is thus deemed suitable for routine analysis.
- Stability Assessment: Stability testing indicates that both stock solutions and extracted samples of Apremilast and Apremilast D5 remain stable under various conditions, including refrigerated, room temperature, and freeze-thaw cycles. Long-term stability in matrix storage at -70°C for up to 14 days further confirms the method's robustness.
- Reliability and Reproducibility: The method exhibits consistent and reproducible results across different validation parameters and stability conditions. The absence of carryover, interference, and significant matrix effects further enhances the reliability of the analytical data.
- Applicability: The validated method can be applied effectively in pharmacokinetic studies, bioequivalence assessments, and therapeutic drug monitoring of Apremilast, ensuring accurate measurement of drug concentrations in clinical samples.
- Compliance: Overall, the method meets the stringent requirements of regulatory authorities, ensuring compliance with quality standards and guidelines for bioanalytical method validation.

In conclusion, the developed and validated method provides a robust, reliable, and sensitive approach for the quantification of Apremilast and Apremilast D5 in human plasma samples, offering a valuable tool for pharmacokinetic studies and clinical drug monitoring in various therapeutic settings.

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