

# LC-MS/MS Determination and Pharmacokinetics Study of Apremilast after Oral Administration in Rabbits

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## ABSTRACT

**Objectives:** A selective and reproducible method has been optimised for evaluation of Apremilast in rabbit biological matrices by UPLC-ESI-MS/MS. **Methods:** The analytical technique was implemented to quantify the apremilast in rabbit plasma samples with Apremilast-D5 as deuterated internal standard. The chromatographic separation tuned with 10mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20:40:40%, v/v/v) using the CORTECS C<sub>18</sub>, 2.7  $\mu$ .m, 4.6 m.m X 150 m.m analytical column with analysis time four minutes. The flow of mobile phase through column is 0.5 m.L/min. The mass spectrometric ions of Apremilast and Apremilast-D5 obtained were m/z 461.5→257.1 and 466.5→257.1. **Results:** The curve indicates correlation coefficient ( $r^2$ ) was superior than 0.998 with linear range of 0.03-48.0 n.g/m.L. The developed method was tuned to apply efficaciously for analyzing the pharmacokinetic parameters of Apremilast in rabbit plasma samples. **Conclusion:** An accurate and reproducible novel method was fabricated for estimation of Apremilast in rabbit biological matrices by UPLC-ESI-MS/MS will be used for regular analysis and appropriate for therapeutic drug monitoring.

**Key words:** Apremilast, Rabbit plasma, UPLC-ESI-MS/MS, Bio-analysis, Pharmacokinetic, Therapeutic drug monitoring.

## INTRODUCTION

Apremilast (AM) is an micro-molecule inhibitor of phosphodiesterase four (P.D.E.4) precise for cyclic adenosine monophosphate (c.A.M.P) P.D.E.4 inhibition consequences in improved intra-cellular c.A.M.P ranges. It's unique system by using which apremilast exert healing movement in psoriatic arthritis suffering patients.<sup>1</sup>

Apremilast prohibits phosphodiesterase 4 (P.D.E.4). It is synthetically N-[2-[(1S)-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl]-2-three-dihydro-1-three-dioxo-1H-isoindol-four-yl]-acetamide. Molecular form is C<sub>22</sub>H<sub>24</sub>N<sub>2</sub>O<sub>7</sub>S with mass of 460.5.<sup>2-4</sup>

A through literature survey reveals that various analytical and bioanalytical methods

were published to describe the quantification of Apremilast in active pharmaceutical ingredient and pharmaceutical dosage forms to study the purity, degradation products by UV-Spectrophotometric method,<sup>5-6</sup> FTIR,<sup>7</sup> RP-HPLC,<sup>8-21</sup> Pharmacokinetic studies in beagle dog plasma,<sup>22</sup> human plasma and cerebro spinal fluid,<sup>23-27</sup> rat plasma<sup>28-30</sup> by LC-MS/MS. The published methods by LC-MS/MS was calibrated 0.1–100 ng/m.L of apremilast in biological samples. Literature survey reveals, the reported LC-MS/MS methods with pit falls of sensitivity and reproducibility. To our experience, there could be no revealed reports within literature that demonstrates the quantification of apremilast in rabbit biological matrices

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with addition of Apremilast-D<sub>5</sub> utilised as deuterated Internal standard (I.S). To enhance the accurateness and robustness of method Apremilast-D<sub>5</sub> utilized as internal standard (I.S).

Hence, to meet the requirement of Biopharmaceutical studies with wider range of dosage forms, it essential to develop greater responsive and reproducible pre-clinical pharmacokinetic experiments to determine Apremilast in rabbit plasma matrices using Apremilast-D<sub>5</sub> as internal-standard (I.S) by UPLC–MS–M. as per F.D.A guidelines.<sup>31–36</sup>

The purpose of this technique is to prove designed analytical procedure for evaluation of Apremilast in plasma matrices as according to the F.D.A guidelines through UPLC–MS–MS. The fabricated method was implemented effectively to a pharmacokinetic experiment of Apremilast in rabbit's plasma samples under fasting conditions.

## MATERIALS AND METHODS

### Materials

#### Chemical Resources

Apremilast (ALSACHIM), Apremilast-D<sub>5</sub> (ALSACHIM) (Figure 1), high purity Methanol, Acetonitrile, Water, Ammonium acetate, Acetic acid, rabbit biological matrices.

#### Instrument Resources

QSight® Triple Quad UPLC-ESI-MS/MS system (Perkin Elmer) Combined with QSight LX50 UHPLC, data acquisition with Simplicity™ 3Q software.

### Methods

#### Preparation of solutions and standards

Apremilast (1.0 mg/mL) and Apremilast-D<sub>5</sub> (1.0 mg/mL) standard solutions were prepared by precisely weighing around 10 mg and diluted to 10 mL with Methanol.

#### Preparation of 10mM Ammonium acetate Buffer (pH: 4.0)

Precisely weighed, 0.770g of ammonium acetate and diluted to 100.0mL with ultra unadulterated water and pH was set to adjusted to pH: 4.0.

#### Preparation of mobile-phase

10mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20:40:40%, v/v/v).

#### Preparation of 10% of acetone in Acetonitrile (Extraction solvent)

Transferred 10.0mL of acetone diluted to 100.0mL acetonitrile.

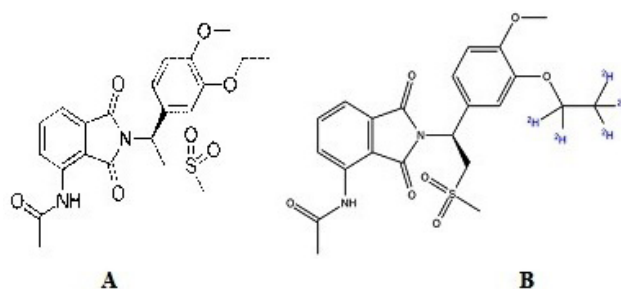


Figure 1: Chemical Structures of A) Apremilast B) Apremilast-D<sub>5</sub>.

### Analytical and Quality control standards

Stock of apremilast (1.0 mg/mL) was used to 0.03, 0.09, 0.15, 0.3, 0.6, 1.5, 3.0, 12.0, 24.0, 36.0, 48.0 ng/mL for calibration standards and 0.030 ng/mL (L.L.O.Q), 0.095 ng/mL (L.Q.C), 25.0 ng/mL (M.Q.C) and 40.0 ng/mL (H.Q.C) for quality control (Q.C) standards prepare analyte free screened rabbit plasma.

### Strategy of method optimisation

For optimisation of method strategy, a logical investigation of the impact of different components were attempted by changing one parameter at once and keeping every single other condition steady. Method strategy comprises of choosing the suitable mass parameters and chromatographic conditions, Internal standard, extraction process.

#### Optimisation of internal standard

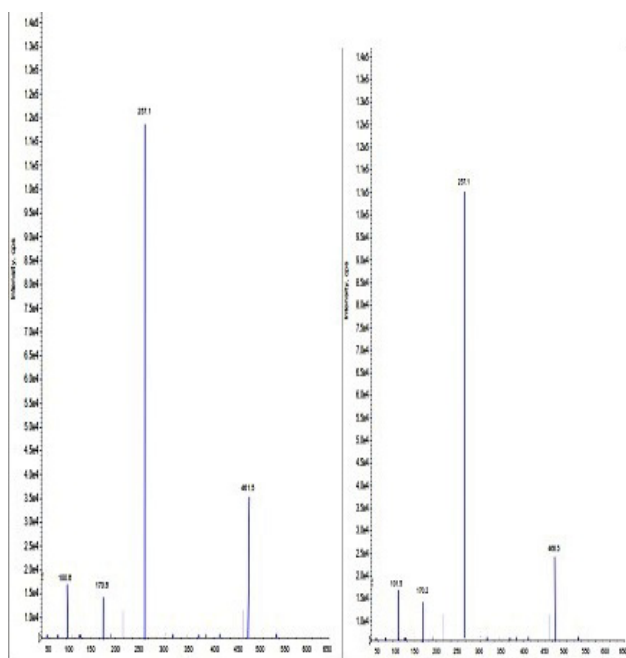
For determination of internal-standard; clopidogrel, prasugrel, ticagrelor were attempted. At last Apremilast-D<sub>5</sub> (AMIS) was chosen as internal-standard because of its similarity separation conditions with analyte.

#### Optimisation of Mass-spectroscopic conditions

Apremilast (AM) and Apremilast-D<sub>5</sub> (AMIS) of 100.00 µg/mL were prepared in methanol and infused with a stream rate of 5µL/min into positive particle mode to ramp of mass spectrometer conditions. After ramping of mass conditions, m/z (amu) 461.5 / 257.1 and 466.5 / 257.1 ions were produced for AM and AMIS. The mass spectras were represented in Figure 2.

#### Optimisation of Chromatographic conditions

After series of trials, the chromatographic conditions was accomplished with 10m.M Ammonium Acetate Buffer with pH of 4.0. along with Methanol: Acetonitrile, (20:40:40%, v/v/v) by utilizing the stationary phase CORTECS C<sub>18</sub>, 2.7 µ.m, 4.6 mm X 150 mm gave the best peak shape. The Apremilast and Apremilast-D<sub>5</sub> were eluted at 1.94 min±0.05 and 1.94 min±0.05 min. The total chromatographic duration was 4.0 min with



**Figure 2: Parent and product ion mass spectra (Q1) and (Q3) of Apremilast and Apremilast-D5.**

flow of 0.5 mL/min and column chamber was set at 40°C.

### Optimisation Extraction technique

Different extraction techniques were optimised to extract of Apremilast and Apremilast-D<sub>5</sub> from rabbit biological matrix. Eventually protein precipitation (PPT) was appropriate as a result of larger free matrix interference and recovery.

### Sample extraction and Cleanup procedure (Sample Preparation)

To each tube 100 µL of I.S (200.00 pg/mL) was mixed with the 100 µL plasma sample, 2.5ml 10% of acetone in acetonitrile, mixture was blended for ten minutes and matrix was isolated by centrifuged at 5000 rpm for 20 min at 25°C.

The organic layer was separated and dried at 40°C utilizing turbo evaporator. To the evaporated dried residue was dissolved in 200µL of chromatographic solution and infused in to UPLC-ESI-MS/MS.

### Validation of Bioanalytical Technique

The optimised technique method was calibrated with linear concentration range of 0.030–48.0 n.g/m.L.

### Selectivity and Specificity

Ten set of plasma samples were examined, out of that six were free from plasma interference. The biological

matrix interference free plasma samples used for evaluating the selectivity and specificity experiment.

### Lower level of Quantification

Six Lower level of quantification standards were used to quantify S/N ratio of LOQ and LOD of analyte.

### Linearity

The developed method was calibrated with standards of 0.03, 0.09, 0.15, 0.3, 0.6, 1.5, 3.0, 12.0, 24.0, 36.0, 48.0 n.g/mL in five replicates.

### Precision and Accuracy

Quality control standards of 0.030 n.g/mL (LLOQ), 0.095 ng/mL (LQC), 25.0 n.g/mL (MQC) and 40.0 ng/mL (HQC) standards in six replicates on a similar day (Intra-day) and five completely different days (Inter-days).

### Matrix Effect

Extracted blank matrix samples were reconstituted with the aqueous standard of 25.00 ng/mL and compared with spiked plasma standard of the similar standard.

### Recovery (Extraction efficiency)

Method extraction potency determined by comparison by comparing the plasma extracted standards with non-plasma extracted standards using 0.095 ng/mL (LQC), 25.0 ng/mL (MQC) and 40.0 ng/mL (HQC) concentrations.

### Assessment of stability of analyte

#### Stability of analyte at laboratory room temperature

Drug spiked lower and higher quality control standards stored at room temperature with duration of 48 hr. After completion of storage period, stability samples cross examined with freshly extracted lower and higher quality control standards.

#### Stability of analyte in freeze and thaw cycles

Drug spiked lower and higher quality control standards were exposed to three freeze-thaw cycles of twenty-four, thirty six and forty eight hoursh (-70°C to room temperature). Finally, stability samples compared with freshly extracted lower and higher quality control standards.

#### Processed plasma samples stability (stability in UPLC Auto-sampler)

Extracted lower and higher quality control standards were stored in HPLC auto-sampler tray at 2-8°C with duration of 36 hr. Finally, stability samples compared with freshly extracted lower and higher quality control standards.

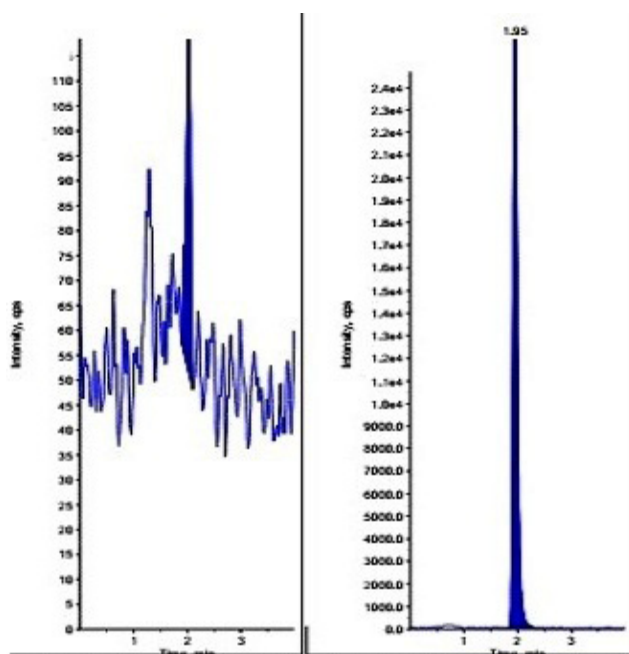


Figure 3: Blank plasma chromatogram of interference free Apremilast and Apremilast-D5.

### Stability of plasma samples at -70°C up to 120 Days

Upon completion of storage period (-70°C up to 120 days), stability samples of lower and higher quality control standards crossed verified with freshly prepared lower and higher quality control standards.

### Pharmacokinetic Application

#### Selection of animals

The present examination was connected to bioavailability test sample. The investigation was affirmed by the animal ethics (IAEC) and performed according to the CPCSEA guidelines. Six New Zealand albino male rabbits (1.9 kg of body mass) were stored in separate cages up to 14 days.

#### Study design

Test formulation of Apremilast with 1.5 m.g/1.9 K.g of body mass (Equivalent dose of 10 mg Apremilast tablet) was administered through oral cavity under fasting condition. Blood samples were collected from rabbit ear vein with volume of 0.2 ml to 0.4 ml at 0.0, 0.1, 0.2, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2, 4, 6, 8, 12 and 24 hr. Each sample was separated by centrifugation and stored at -70°C. The diagrammatical pharmacokinetic profile of Apremilast was depicted in Figure 6.

#### Pharmacokinetic Analysis

Pharmacokinetic parameters like were evaluate by a non-compartmental statistic model using Win Non-Lin

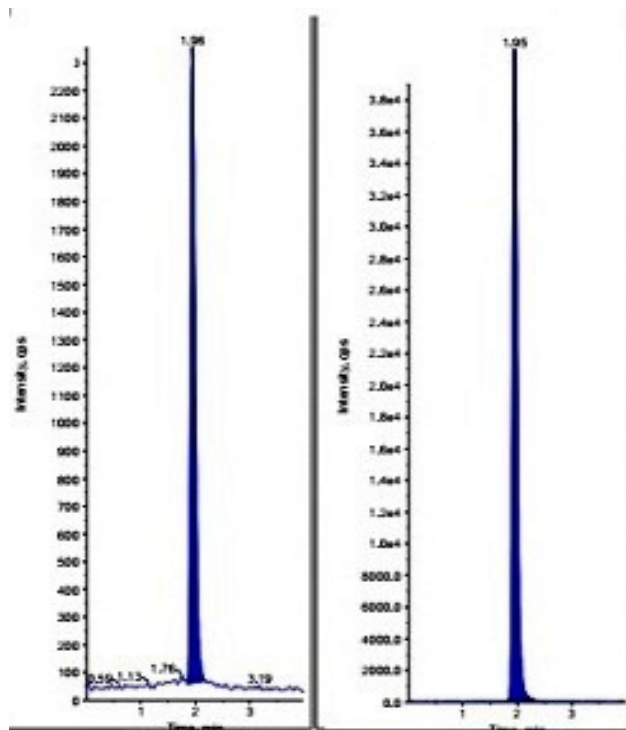


Figure 4: Chromatogram of LLOQ sample contains Apremilast and Apremilast-D5.

5.1 software version (Pharsight, USA). The results were tabulated in Table 4.

## RESULTS AND DISCUSSION

### Method development

High intense mass ions like  $m/z$  461.5, 466.5, 257.1 and 257.1 were identified by mass spectroscopy for Apremilast and Apremilast-D5 molecules.

To optimize shape and reducing tailing effect of peak, Ammonium Acetate buffer pH of 4.0 with Methanol: Acetonitrile, (20:40:40%, v/v/v) was used as mobile phase using CORTECS  $C_{18}$ , 2.7  $\mu$ m, 4.6 mm X 150 mm stationary phase at temperature 40°C. The total analysis run time was optimized 4.0 min with sample acquiring volume 10  $\mu$ L.

Before bioanalysis, endogenous samples were separated by plasma precipitation technique using 10% acetone in acetonitrile. The developed extraction method improved recovery, reproducibility and found less matrix interference.

### Method validation

No response was identified at peak elution times of apremilast and Apremilast-D5 in blank samples. The lower level concentration is 30.0 p.g/m.L (Figure 3 and 4). Linearity was quantified by peak area ratio methodology (Apremilast peak area / Apremilast-D5 peak area Vs



concentration (n.g/m.L)) using linear regression analysis at range of 0.03 to 48.00 n.g/m.L. The %CV was lower than 15% and mean % accuracy was varies from 97.78 to 102.11% (Table 1 and Figure 5). Intra and inter batch %accuracy for apremilast varies from 99.14-102.93 and 98.07-100.07. %CV is 0.04- 2.27 and 3.75-4.17 (Table 2).

The mean %recovery for lower, medium and high Q.Cs samples of apremilast were 101.27%, 97.14%, 99.91%, respectively. The %recovery and %CV of apremilast across QC levels is 99.44% and 2.57%. For the Apremilast-D5, the mean % recovery and %CV is 99.25% and 0.99%.

No significant matrix effect found in rabbit plasma samples for Apremilast and Apremilast-D5. The %CV was found to be 7.97.

Stability of apremilast and apremilast-D5 stock solutions was examined and found that % change was less than 5%. From stability data, it indicates that stock solutions were stable at 2-8°C up to 90 hr.

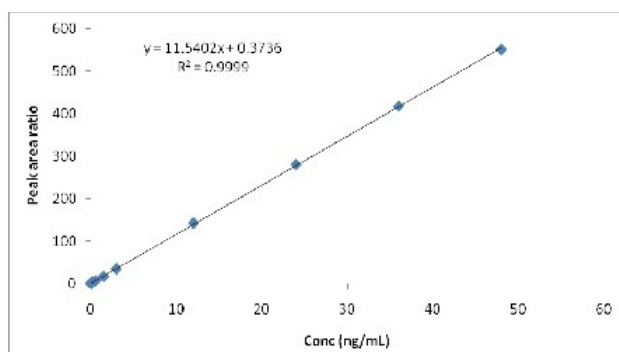


Figure 5: Calibration curve of Apremilast.

Table 1: Calibration curve details of Apremilast			
Spiked plasma Concentration (ng/ml)	Concentration measured (ng/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
0.03	0.03± 0.002	7.83	98.00
0.09	0.09± 0.002	2.57	99.56
0.15	0.15± 0.003	2.17	97.78
0.3	0.30± 0.04	1.06	99.67
0.6	0.60±0.003	0.50	99.60
1.5	1.48±0.031	2.07	98.56
3.0	3.03±0.11	3.47	101.11
12.0	12.02±0.13	1.09	100.19
24.0	24.15±0.40	1.66	100.61
36.0	36.76±0.42	1.15	102.11
48.0	48.12±0.16	0.33	100.25

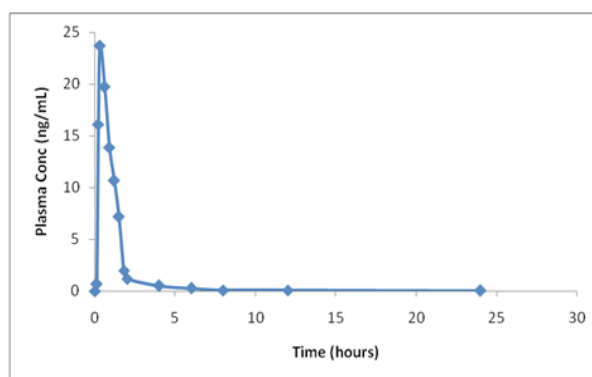


Figure 6: Mean plasma concentrations vs. time graph of Apremilast after oral administration of 1.5 mg/1.9 kg in male rabbits.

Table 2: Precision and accuracy (Analysis with spiked samples at three different concentrations) of Apremilast						
Spiked Plasma Concentration (ng/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Concentration measured (n=6;ng/ml;mean±S.D)	%CV	%Accuracy	Concentration measured (n=6;ng/ml;mean±S.D)	%CV	%Accuracy
0.095	0.009±0.001	1.80	99.14	0.09±0.003	3.75	98.07
25.0	25.17±0.01	0.04	100.69	25.02±0.95	3.78	100.07
40.0	41.17±0.93	2.27	102.93	39.92±1.67	4.17	98.81

Table 3: Stability studies of Apremilast in rabbit plasma spiked samples								
Spiked Plasma concentration (ng/ml)	Room temperature Stability		Processed sample Stability		Long term stability		Freeze and thaw stability	
	48h		36h		120 days		Cycle (48h)	
	Concentration measured (n=6;ng/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;ng/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;ng/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;ng/ml; mean±S.D)	%CV (n=6)
0.095	0.09±0.001	1.80	0.10±0.01	5.76	0.09±0.003	3.01	0.10±0.003	3.36
40.00	41.57±0.79	1.91	40.42±0.11	0.28	40.79±1.50	3.68	41.56±1.91	4.60

**Table 4: Mean pharmacokinetic parameters of Apremilast in rabbit plasma after oral administration of 1.5 mg/ 1.9 kg in male rabbits.**

Pharmacokinetic parameters	Calculated Value
AUC <sub>0-t</sub> (ng x h/mL)	26.31
C <sub>max</sub> (ng/mL)	23.73
AUC <sub>0-∞</sub> (ng x h/mL)	27.36
T <sub>max</sub> (h)	0.30
Kel (h <sup>-1</sup> )	1.04
t <sub>1/2</sub> (h)	1.5

AUC<sub>0-∞</sub>: area under the curve extrapolated to infinity;  
 AUC<sub>0-t</sub>: area under the curve up to the last sampling;  
 C<sub>max</sub>: the maximum plasma concentration;  
 T<sub>max</sub>: the time to reach peak concentration; and  
 Kel: the apparent elimination rate constant.

Stability of plasma samples for apremilast was proved at L.Q.C and H.Q.C levels. The results discovered that apremilast was stable in rabbit biological matrices at room temperature, refrigerated conditions up to 48 and 36 hr, respectively. Further it was identified that, Freeze-thaw cycles (-70°C) does not affect the stability of plasma samples. After long storage of plasma samples at -70°C, it was examined that apremilast was stable up to 120 days. The stability results were depicted in Table 3.

The methodology was utilized in the quantification of apremilast in plasma samples for calculating the pharmacological parameters with dose of 1.5 mg/1.9 kg of body mass (equivalent to ten mg of tablet) through oral route in six rabbits. The pharmacokinetic profile of apremilast was depicted in Figure 6 and calculated concentrations were between linearity ranges (Table 4).

## CONCLUSION

The methodology demonstrates great performance in terms of linearity, accuracy, precision, recovery, robust, stability in plasma matrices. The developed technique was applied to study the pharmacokinetic effect of apremilast in rabbit plasma. This method is sensitive enough for quantitative detection of the analyte in biological samples by UPLC-MS/MS.

This technique contains a short analysis run time and usage of deuterated internal standard is an advantage comparing to reported methods. The projected technique will be used for routine analysis and appropriate for therapeutic drug observation (pharmacokinetic or bioequivalence studies) of pharmaceutical tablets containing Apremilast.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**AM:** Apremilast; **AMIS:** Apremilast-D<sub>5</sub>; **IS:** Internal standard; **RP-HPLC:** Reverse phase High performance Liquid chromatography; **UPLC-ESI-MS/MS:** Ultra performance liquid chromatography-Electro spray Ionisation coupled with tandem mass spectrometry; **ng/mL:** nano gram per milli liter; **mg:** Milli gram; **µg:** Micro gram; **h:** Hours; **min:** Minutes; **mM:** Milli molar; **mL:** Milli liter; **C<sub>18</sub>:** Octa decyl silane; **mm:** Milli meter; **Kg:** Kilogram; **g:** Grams; **µm:** Micro meter; **LLOQ:** Lower limit of quality control; **LQC:** Lower quality control; **MQC:** Medium quality control; **H.Q.C:** High quality control; **LC-MS:** Liquid chromatography coupled with tandem mass spectrometry; **D<sub>5</sub>:** Five deuterated carbon atoms; **amu:** Atomic mass units.

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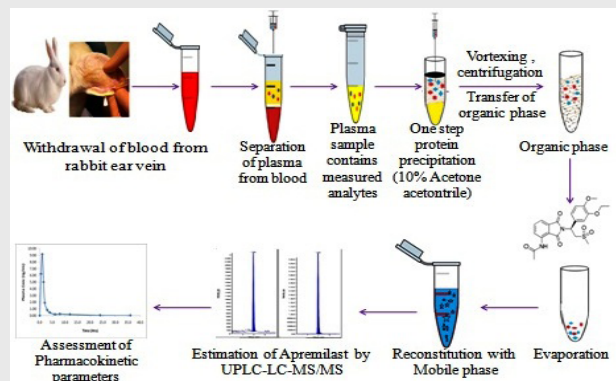
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## SUMMARY

- From reported methods, there are not many published by LC-MS and HPLC for quantification of apremilast in Pharmaceuticals compounds and Biological samples. There is no methodology for quantification of Apremilast in biological matrices by UPLC-LC-MS/MS.
- The assay of this methodology is additionally extremely specific due to the inherent selectivity, sensitivity of UPLC and has major advantages like novel, sensitive, economical, rapid, precise, free matrix effect, use of small sample volume and Apremilast-D<sub>5</sub> was utilized as an internal standard.
- The optimised technique was validated as per the FDA guidelines over a range of 0.03–48.0 n.g/m.L using protein precipitation for recovery of drug and internal standard. In this article an UPLC has been introduced for the rapid and reliable measurement of Apremilast concentrations in rabbit plasma samples.
- The mobile phase consists of 10mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20:40:40%, v/v/v) using the CORTECS C<sub>18</sub>, 2.7 μ.m, 4.6 m.m X 150 m.m analytical column. Each sample requires 4 min of analysis time and was eluted at 1.94 min±0.05 and 1.94 min±0.05 min respectively.
- This methodology was successfully utilized, for estimation of rabbit plasma samples following administrat- ing the apremilast (1.5 mg) in male healthy rabbits under fasting condition through oral route.

### PICTORIAL ABSTRACT



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