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Determination of Apremilast In Human Plasma by Using LC–ESI–MS/MS

Velamakanni Satish Ramanatham [1, 2]*, Vinayender Adireddy [2] and Venkateswarlu Padala [3]

1. University College of Chemistry, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad–500 085, India.
2. AnaCipher Clinical Research, Ramanthapur, Hyderabad–500 013, India.
3. Vimta Labs Limited, Cherlapally, Hyderabad–500 051, India.

ABSTRACT

A simple, rapid and sensitive liquid chromatography / electro spray ionization tandem mass spectrometry (LC–ESI–MS/MS) assay method has been proposed for the determination of apremilast in human plasma samples using apremilast d_5 as internal standard (IS). Analyte and the IS were extracted from the 200 µL of human plasma *via* simple solid–phase extraction. The Chromatographic separation was obtained on a C_{18} column operating at a flow rate of 1.0 mL/min by using a mobile phase comprising of mixture of 5mm ammonium acetate in 0.2% formic acid buffer (15:85, v/v) and acetonitrile. A linear ($r^2 \geq 0.99$) of the calibration curve was obtained over the concentration range of 2.03–808 ng/mL. As per FDA guidelines method validation was performed and the results met the acceptance criteria. The intra–day and inter–day precision (%CV) and accuracy results in five validation batches across five concentration levels were well within the acceptance limits. To analyze the more number of samples in short time, thus increasing the productivity a short run time of 2.25 min for each sample was applied and this was made possible. The method was successfully applied to a pharmacokinetic study in humans.

Keywords: Apremilast, human plasma, solid–phase extraction, LC–ESI-MS/MS, Pharmacokinetics.

*Corresponding Author Email: velamakanni4273@gmail.com
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INTRODUCTION

Apremilast is a selective, medicine used to treat psoriasis arthiritis, plaque psoriasis and rhematoid arthiritis[1-4]. Abnormal red patches on the skin which are for a long period auto immune diseases or disorders is psoriasis which are scaly and itchy in nature. As apremilast is given to people suffering from plaque psoriasis and psoriasis arthiritis, tumor necrosis factor alpha (TNFα) is inhibited continuously from the rhematoid syrovial cells and also the enzyme phosphodiesterase 4 (PDE4) by the drug apremilast[5]. Apremilast falls in the category of small molecules where the enzyme cyclic adenosine monophosphate breaks which inhibits phosphodiesterase 4 (PDE4)[6]. The anti inflammatory interleukin 10, interleukin 17, interleukin 23 and the tumor necrosis factor alpha (TNFα) all of whose increase and decrease levels are being regulated by the most predominent enzyme phosphodiesterase 4 (PDE4) [5]. Apremilast is given orally once daily (QD), to start with an initial dose of 10 mg with up–titration to 20 mg or 30 mg QD, depending on the rate and magnitude of the increase observed in serum sodium concentrations. Single and multiple oral doses of apremilast exhibited dose–dependent aquaretic effects[7]. The absolute bioavailability of apremilast is reported to be 73% Apremilast is well tolerated at all doses and can be administered with or without food[5].

As per literature, very few of the methods and studies have been conducted which have investigated apremilast pharmacokinetics in all the healthy subjects. In this one method employed protein precipitation (PP) and others liquid-liquid extraction (LLE) [1,6,8-10]. By employing PP and LLE there are chances of ion suppression and endogenous compounds such as lipids, fatty acids, phospholipids[11-13] cannot be removed. In the method specified by Liu *et al.*, methotrexate and apremilast are given as coadministration drugs to subjects with rheumatoid arthiritis and psoriatic arthiritis where LLE was employed. LLE requires larger volume of solvents, gives low recoveries, less reproducibility and also matrix effect cannot be removed or reduced [14,15] also LLE is a time taking process involving evaporation by maintaining temperature there by loss of stability may also occur if drug is thermally instable. The method reported by Iqbal *et al.*, was done in rat plasma using UPLC/MS-MS following LLE[8] where LOQ is 3.04 ng/mL. Similarly other method prescribed by Hoffmann M *et al.*, also employed LLE but the run time is too high of 80 minutes. A study was conducted in rat plasma by employing UPLC/MS-MS using PP with diazepam as internal standard[9]. With clopidogrel as internal standard using LLE[10] a study performed on beagle dogs with an LOQ of 2 ng/mL. But the purpose was to develop a method with no matrix effect or minimum matrix effect which can be achieved using deuterated or stable labeled isotope as IS. All

these drawbacks are overcome by the present work which we have developed and validated for the quantification of apremilast in human plasma with a LLOQ of 2.00 ng/mL using a high throughput LC/MS-MS. Presently LC-MS/MS is a unique analytical tool for the quantification of drugs and metabolites both nanogram to picogram level in biological matrices[16]. Solid phase extraction technique (SPE) was employed to get clear extracts for the sample preparation and also in order to avoid the variability in recoveries of analyte and internal standard (IS) and to potential matrix effect related problems apremilast d_5 was used as IS. We achieved a higher sensitivity using low plasma volume (200µL) compared to other earlier reports. Thus, the proposed method was applied under fasting or fed conditions thereby clinical pharmacokinetic study in healthy male subjects was successfully studied following the oral administration of apremilast. The authenticity in the measurement of study data is demonstrated through incurred sample reanalysis (ISR).

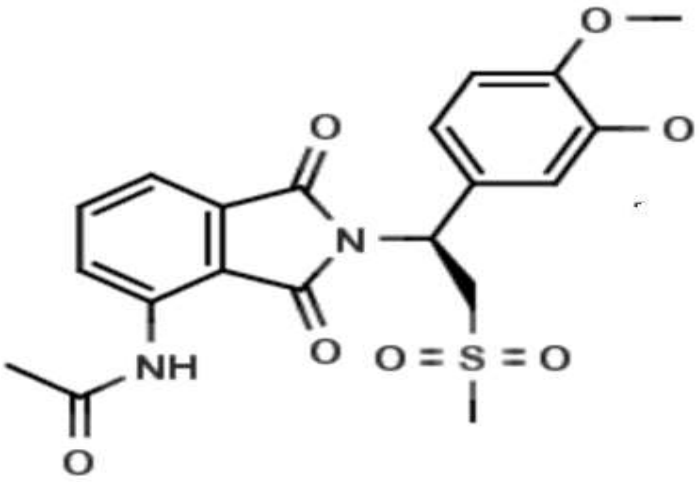

Figure 1: Chemical structures of apremilast

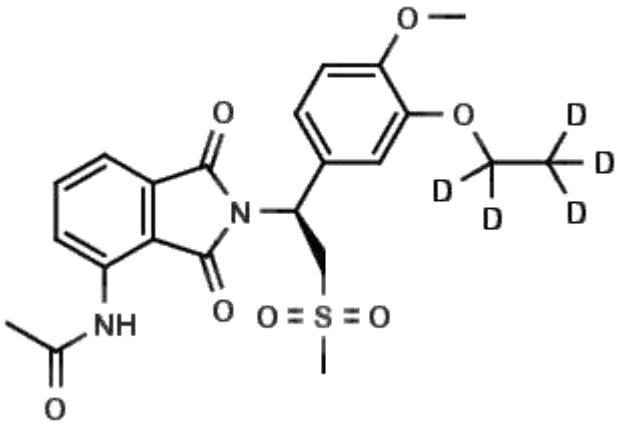

Figure 2: Chemical structures of apremilast-d_5

MATERIALS AND METHOD

Standards and chemicals

Reference sample of apremilast (97.78%) and also apremilast d_5 (97.55%) were procured from Clearsynth Labs Limited, Mumbai, India (Fig. 1 and Fig. 2). Milli Q water purification system procured from Millipore (Bangalore, India) was used as a source of water which is used for the LC–MS/MS analysis. Acetonitrile was purchased from J.T. Baker (Phillipsburg, USA) of the HPLC grade and from Merck Ltd (Mumbai, India) analytical grade ammonium acetate was purchased. From Deccan's Pathological Lab's (Hyderabad, India) the control K_2–human plasma sample was procured.

LC–MS/MS instrument and conditions

For the study a Zodiac C_{18} column (100 mm × 4.6 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA) in an HPLC system (Shimadzu, Kyoto, Japan) consisting of a solvent degasser (DGU–$20A_3$), an auto sampler (SIL–HTc) and binary LC–20AD prominence pump was used. An aliquot of 15 µL of the processed samples kept at ambient temperature were injected into the column. To separate the analyte from the endogenous compounds the flow rate was optimized at 1.0 mL/min and passed into the electrospray ionization chamber of the mass spectrometer. An isocratic mobile phase consisting of a mixture of acetonitrile and 5mM ammonium acetate in 0.2 % formic acid (85:15, v/v) was used for the purpose. MS–MS detection in positive ion mode for the analyte and the IS using an MDS Sciex API–4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ the quantification was achieved. The interface was set at 550 °C and ion spray voltage set at 5500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 40, 40, 10, and 10 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 87, 15, 10, 6 V for apremilast and 86, 15, 10, 6 V for the IS. By carefully observing the transition pairs of *m/z* 461.1 precursor ion to the *m/z* 257.1 for apremilast and *m/z* 466.1 precursor ion to the *m/z* 262.1 product ion for the IS, the detection of the ions was carried out in the multiple–reaction monitoring mode (MRM) and unit resolution for both the Quadrupoles Q1 and Q3 were set. The analysis data obtained were processed by Analyst Software™ (version 1.4.2).

Preparation of plasma standards and quality controls

The standard stock solution of apremilast and IS (1 mg/mL) were prepared in methanol and further was diluted in diluent (60:40, v/v; diluent). The IS working solution (5000 ng/mL) was also

prepared in diluent. Stock solutions of apremilast and IS were found to be stable for 8 days at 2–8 °C.

The apremilast standard calibration concentrations of 2.03, 4.05, 8.10, 32.4, 81.0, 162, 324, 485, 647 and 808 ng/mL were prepared in K_2 EDTA human plasma. Similarly, quality control (QC) samples were also prepared at each concentration levels as a bulk based on an individual weights of standard drug, at concentrations levels of 2.05 (LLOQ), 6.02 (low), 120 (middle 1), 401 (middle 2) and 603 ng/mL (high). The aliquots of the calibration and control bulk samples were obtained by distributing into micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at –70 ± 10 °C until analyses.

Sample processing

Before starting of the analysis calibration standards, quality control samples and all frozen subject samples, were thawed and allowed to equilibrate at room temperature. Prior to spiking the samples were vortexed to mix for 15 s. An aliquot of 200 µL of human plasma sample was mixed with 20 µL of the internal standard working solution (5000 ng/mL of apremilast d_5). To this, 300 µL of Milli Q/HPLC grade water was added and vortexed. The sample mixture will be loaded onto Starata^{TM}-X, 33µm, polymeric sorbent cartridges (30mg/1mL) that were pre-conditioned with 1.0 mL of HPLC grade methanol followed by 1.0 mL Milli Q/HPLC grade water. After applying the maximum pressure the extraction cartridge will be washed with 3mL of Milli Q/HPLC grade water (each time 1mL). Analytes and IS are eluted with 1.0 mL of mobile phase. Aliquot of 15µL of the extract was injected into the LC– MS/MS system.

Bioanalytical method validation

A thorough and complete method validation of apremilast in human plasma was carried out as per US FDA guidelines[17]. The method was validated for sensitivity, selectivity, specificity, carryover test, matrix effect, recovery, linearity, precision, accuracy, dilution integrity and stability.

Pharmacokinetic study design

A single dose pharmacokinetic study was performed in healthy South Indian male subjects (*n*=6). The Ethics Committee (Samkshema Independent Ethics Committee, Hyderabad, India) approved the protocol and the volunteers provided with written informed consent. Under the fasting condition blood samples were collected following oral administration of apremilast (30 mg tablet) from healthy subjects at pre–dose and 0.0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 3.0, 3.50, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, 24.0, 36.0 and 48 h, in K_2–EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The plasma was collected by centrifuging the tubes at 3200 rpm

for 10 min and the collected plasma samples were stored at –70 ± 10 °C till their use. As described earlier the plasma samples were spiked with the IS and processed as per the extraction procedure. Using WinNonlin Version 5.2 by non–compartmental model, the main pharmacokinetic parameters of apremilast were calculated. An incurred sample re–analysis was also conducted by selecting the 6 subject samples (2 samples from each subject) near C_{max} and the elimination phase. The percent change in the value should not be more than ±20% [18, 19].

RESULTS AND DISCUSSION

Mass spectrometry

A standard analyte concentration of 100 ng/mL was infused at a flow rate of 10µL/min in positive and negative ionization modes into the mass spectrometer. By using electrospray as the ionization source carefully mass parameters were optimized in the multiple reaction monitoring mode (MRM) during method development. However, the response observed was much better in positive ionization mode for the analyte and IS compared to the negative mode. Protonated form of analyte and IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from m/z 461.1 to 257.1 for apremilast and from m/z 466.1 to 262.1 for the IS. The dwell time for each transition was 200 ms(Fig. 3 and Fig. 4). The very powerful and important technique for pharmacokinetic studies is LC–MRM because it provides sensitivity requirements for analytical methods[20]. Thus for the assay development the present MRM was chosen.

Method development

LC-MS/MS based methods are preferable because of highly selective and sensitive and widely opted in bioanalytical laboratories [21, 22]. The main objective of the present work was to develop and fully validate an LC–MS/MS method for the determination of apremilast in human plasma with high sensitivity to measure the concentration of apremilast for the pharmacokinetic/bioequivalence studies. The method development requires selection of a chromatography column, organic solvent, mobile phase and to fix a flow rate along with the injection volume. These parameters should be carefully monitored to produce the required resolution from endogenous components which in turn affect sensitivity and reproducibility of the analytical method by ion suppression. Once organic solvent, mobile phase pH, Chromatographic column all are set then column temperature, flow rate, buffer type and concentration can be manipulated for attaining better response.

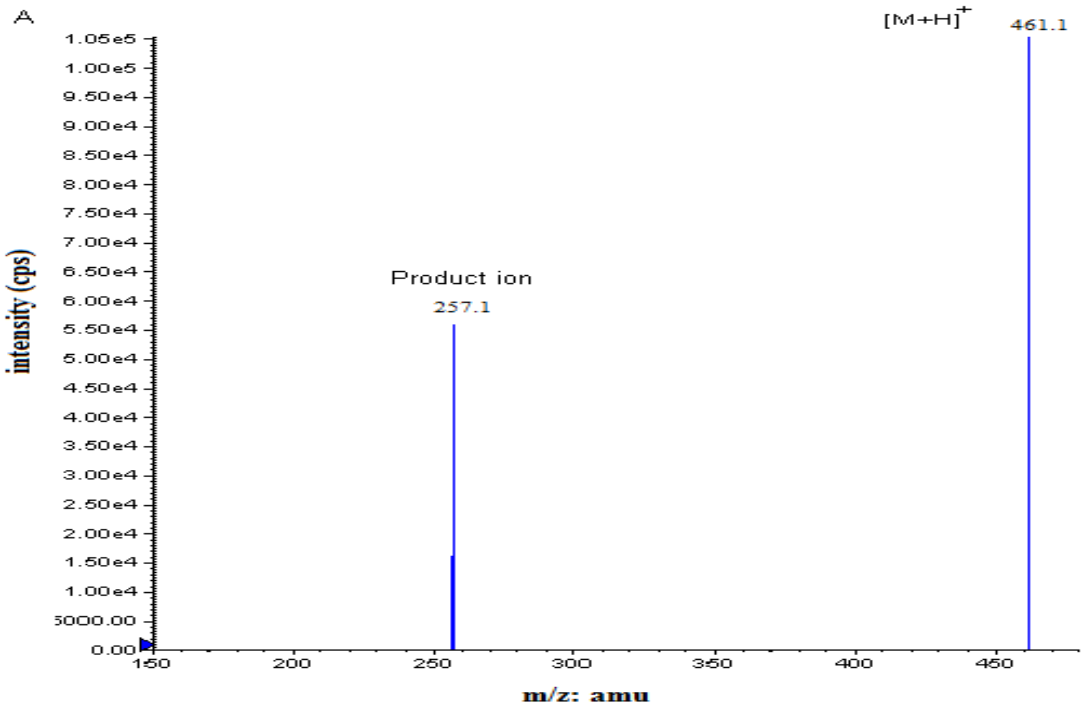

Figure 3: Product ion mass spectra of $[M+H]^+$ of apremilast

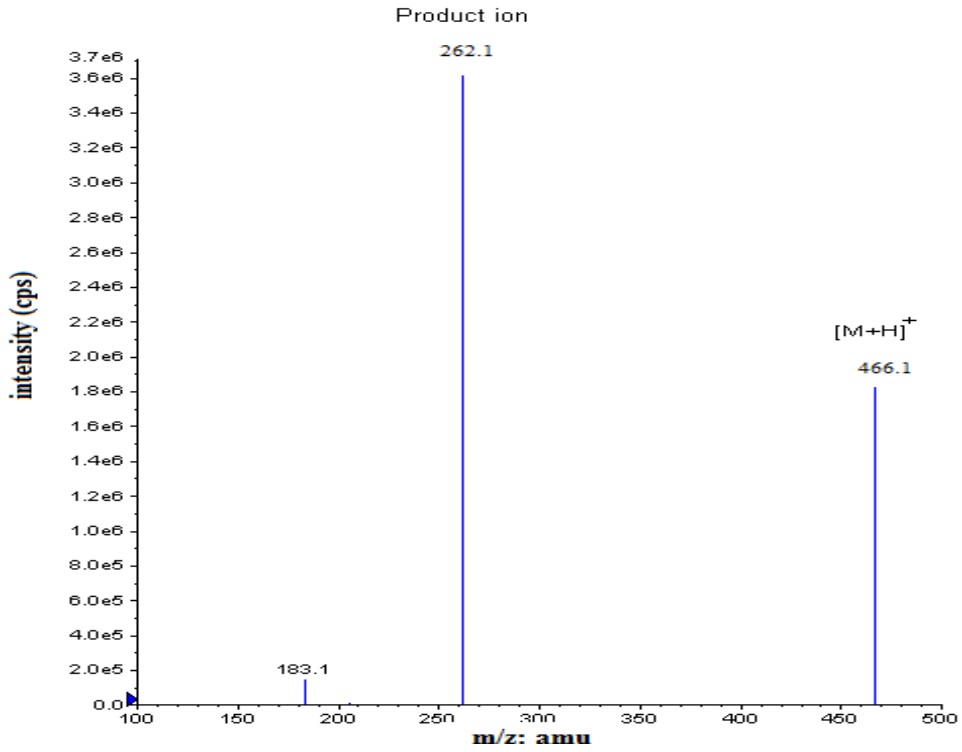

Figure 4: Product ion mass spectra of $[M+H]^+$ of apremilast- d_5

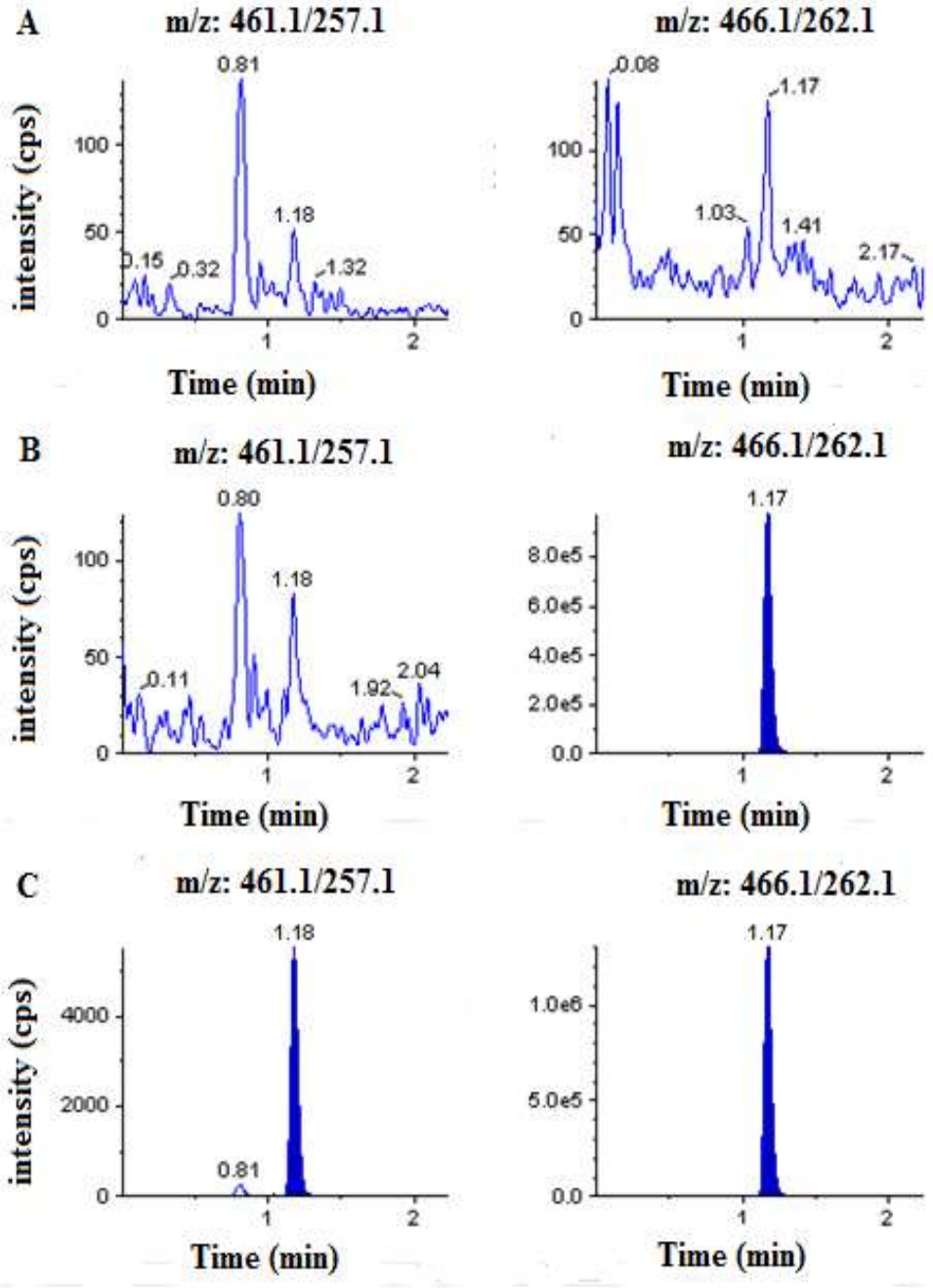

Figure 5: Typical MRM chromatograms of apremilast (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

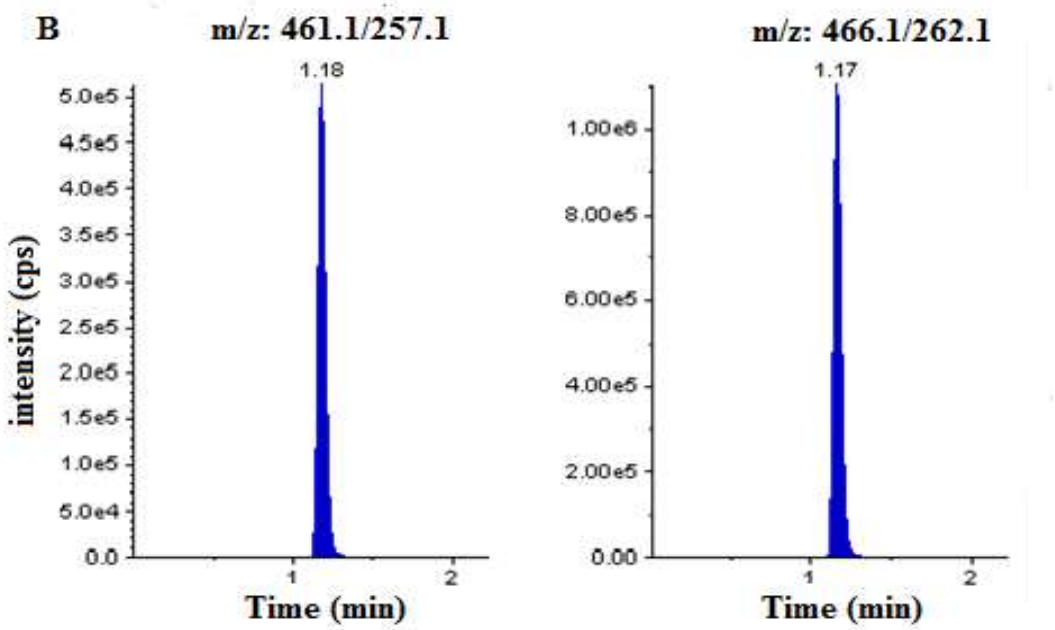

Figure 7: MRM chromatograms resulting from the analysis of 3.50 h of subject plasma sample, after the administration of a 30 mg oral single dose of apremilast tablet. The sample concentration was determined to be 287 ng/mL.

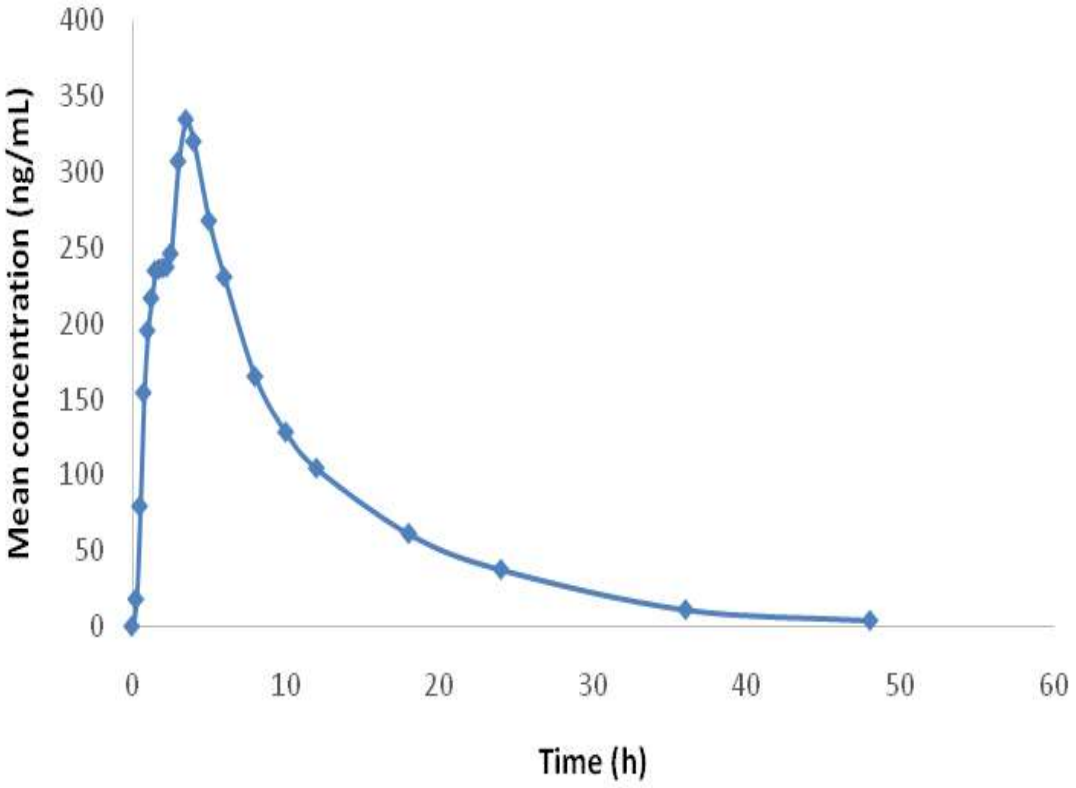

Figure 8: Mean plasma concentration–time profile of apremilast in human plasma following oral administration of apremilast (30mg tablet) to healthy volunteers (*n*=6).

A trial was done for the separation using organic solvents such as methanol and acetonitrile in different volume ratio with buffers like ammonium formate, ammonium acetate (2–10 mM) as well as acid additives like acetic acid and formic acid (0.1–1.0%) by changing the strength on different columns like C_{18} and C_8 of different makes (Hypurity advance, Grace, Kromasil, Zorbax, Inertsil, Hypersil etc). It was observed that acetonitrile and 5mM ammonium acetate in 0.2% formic acid (85:15, v/v) as the mobile phase was most appropriate to give best peak shape, efficiency and sensitivity. Zodiac C_{18} (100 x 4.6 mm, 5µm) column gave good response even at lowest concentration (2.03 ng/mL) level for the analyte along with good peak shapes. Also for the achievement of the chromatographic peak shapes, the effect of flow rate was also optimized from 0.4 to 1.0 mL/min, which was also responsible for acceptable results and finally flow rate was set at 1.0 mL/min. Finally chromatographic run time of 2.25 min was achieved with the retention time of analyte and the IS being very low enough (1.17 and 1.17 min).

The earlier reported procedures have employed PP in rats plasma[9], LLE in rats plasma[8], LLE in human plasma[1,6,10] to extract apremilast. Based on these, different sample pre–treatment methods were investigated. Initially, PP was carried out using acetonitrile and methanol (9:1) solvents in combination under normal conditions as reported previously[10]. However, the response was inconsistent especially at the LLOQ level in these two solvents in combination and also individually. These two solvents caused considerable ion suppression. Hence, LLE was carried out using solvents like dichloromethane, cyclo hexane, ethyl acetate, diethyl ether, hexane and methyl tert–butyl ether (MTBE), alone and in combination under acidic conditions. LLE with various organic solvents and their mixtures resulted in the poor chromatography and non-reproducible recoveries of the analytes and also intereferences in blank plasma. Thus, finally SPE was carried out using Strata^{TM}-X, 33µm, polymeric sorbent cartridges (30mg/1mL) in the presence of ammonium acetate in acidic condition. Thus, SPE is helpful in avoiding the introduction of non–volatile materials onto the column and MS system spectroscopically and producing clean sample. Always clean samples are essential for minimizing ion suppression and matrix effect on LC–MS/MS. Consistent and quantitative recovery (~70%) was obtained with Strata^{TM}-X, 33µm, polymeric sorbent cartridges (30mg/1mL) cartridges at all QC levels for all the analytes. Thus, the simple SPE technique was employed for the sample preparation in this work and provided high recoveries of the drugs. The mean recoveries for analytes and the IS were good and reproducible. There was no significant improvement in the recovery of the analyte by addition of acidic or basic additives to the plasma samples in different volume ratios. When a significant matrix effect is possible, by using a stable isotope–labeled drugs as internal standards matrix effect can be nullified

or can be reduced to a certain extent [19, 20]. Thus, the most stable and suitable IS apremilast d_5 was opted for the purpose.

Carryover effect

Carryover was done to show that it does not affect the accuracy and precision of the specific method followed. After injection of the highest concentration of analyte (ULQ; upper limit of quantitation) no significant carryover was observed in blank sample which indicates no carry–over of the analyte in subsequent samples (data not shown).

Selectivity and chromatography

The selectivity of the method was performed by examining the blank plasma. There was no significant interference observed in the blank plasma from the endogenous substances in drug–free human plasma at the retention time of the analyte and the IS and this was achieved by analyzing blank human plasma extract (Fig. 5A) and an extract spiked only with the IS (Fig. 5B). Likewise, Fig. 5B shows the absence of direct interference from the IS to the MRM channel of the analyte. Fig. 5C shows a representative ion–chromatogram for the LLOQ (STD-A) sample (2.03 ng/mL). Likewise, no significant interference was observed by using the most important commonly used medications such as acetaminophen, nicotine, pantoprazole, ibuprofen, caffeine, diphenhydramine, pseudoephedrine and dicyclomine (data not shown). Here in this, after the administration of a 30 mg oral single dose of apremilast, a representative chromatograms resulting from the analysis of subject blank plasma sample and 3.50 h subject plasma sample are shown in Fig. 6 and Fig. 7.

Matrix effect

Matrix effect assessment was done with the aim to check the effect of different lots of plasma on the back calculated value of QC's nominal concentration. The results found were well within the acceptable limits as shown in Table 1. No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high quality control concentrations. Also, the extraction method was rugged enough and gave accurate and consistent results when applied to real subject samples.

Table 1: Matrix effect of apremilast in human plasma (*n* = 6)

Plasma lot	LQC (6.02 ng/mL)		HQC (603 ng/mL)	
	Concentration found (mean ± SD; ng/mL)	% Accuracy	Concentration found (mean ± SD; ng/mL)	% Accuracy
Lot 1	6.09 ± 0.08	101	597 ± 13.2	99.0
Lot 2	6.08 ± 0.17	101	602 ± 8.40	100
Lot 3	5.90 ± 0.09	98.0	598 ± 8.65	99.2
Lot 4	6.04 ± 0.06	100	612 ± 10.1	102
Lot 5	6.21 ± 0.18	103	613 ± 12.4	102

Lot 6	5.91 ± 0.10	98.3	595 ± 13.3	98.7

SD- Standard Deviation

Linearity, precision and accuracy

The ten–point calibration curve was found to be linear over the concentration range of 2.03–808 ng/mL for the apremilast. After evaluation and computing and also comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration are the best fit for the concentration–detector response relationship. The generated results shows that during the validation the mean correlation coefficient of the weighted calibration curves were was ≥ 0.99.

The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Table 2. All the results of intra–day and inter day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle and high quality control level, whereas within 20% at LLOQ QCs level. Also the intra–day and inter–day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle, and high quality control level, whereas within 100± 20% at LLOQ QCs level. The results revealed good precision and accuracy.

Table 2: Precision and accuracy data for apremilast

Quality control	Run	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
Intra–day variations (Six replicates at each concentration)				
LLOQ	1	2.16± 0.12	5.38	106
	2	2.16± 0.13	5.99	105
	3	2.15± 0.14	6.38	105
	4	2.21± 0.11	5.00	108
	5	2.24± 0.21	9.32	109
LQC	1	5.72± 0.19	3.31	95.0
	2	5,68±0.25	4.45	94.5
	3	5.90± 0.22	3.67	98.1
	4	5.79± 0.15	2.60	96.3
	5	6.56± 0.84	12.81	109
MQC1	1	129± 3.91	3.03	107
	2	126± 3.68	2.92	104
	3	131± 4.18	3.20	109
	4	130± 2.40	1.84	108
	5	125± 8.51	6.80	104
MQC2	1	423 ± 16.98	4.01	105
	2	432 ± 18.09	4.18	108
	3	430 ± 14.57	3.39	107
	4	428± 15.48	3.62	107

	5	427± 30.33	7.11	106
HQC	1	630± 26.01	4.13	104
	2	662± 23.75	3.59	110
	3	635± 29.80	4.70	105
	4	634± 26.64	4.20	105
	5	618± 40.63	6.58	102
Inter–day variations (Thirty replicates at each concentration)				
LLOQ		2.18 ± 0.14	6.35	107
LQC		5.93 ± 0.51	8.56	98.6
MQC1		128 ± 5.20	4.06	107
MQC2		428± 18.8	4.39	107
HQC		636 ± 31.5	4.96	105

Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 2.05, 6.02, 120, 401 and 603 ng/mL, respectively.

Recovery

For recovery determination, six replicates at low, medium–2 and high quality control concentration for apremilast were prepared. The mean overall recovery of apremilast was 100.5±4.58% with the precision range of 0.87–11.55% and the recovery of IS was 96% with the precision range of 2.09–7.52%.

Stability studies and dilution integrity

In the different stability experiments carried out viz. bench top stability (8 h), autosampler stability (52 h), wet extract stability (48 h), repeated freeze–thaw cycles (4 cycles), reinjection stability (43 h) and long term stability at –70 °C for 34 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 3). Thus, the results were found to be within the acceptable limits during the entire validation.

Table 3: Stability data for apremilast in plasma (*n*=6)

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Process[a]	6.02	6.34 ± 0.11	1.77	105
	603	627± 5.40	0.86	104
Wet extract[b]	6.02	5.91± 0.49	8.29	98.3
	603	633± 5.88	0.93	105
Bench top[c]	6.02	6.30± 0.20	3.12	105
	603	632± 10.2	1.61	105
FT[d]	6.02	6.27± 0.21	3.41	104
	603	636 ± 7.94	1.25	105
Reinjection[e]	6.02	6.08 ± 0.22	3.68	106

	603	628± 18.7	2.97	99.8
Long–term[f]	6.02	5.96 ± 0.25	4.20	98.9
	603	614± 23.6	3.85	102

[a] after 52 h in autosampler at 10°C; [b] after 47 h at room temperature; [c] after 8 h at room temperature; [d] after 4 freeze and thaw cycles; [e] after 43 h of Reinjection;
[f] at –70°C for 34 days

Stock solutions of apremilast and IS were found to be stable for 8 days at 2–8 °C. The percentage stability (with the precision range) of apremilast and IS was 101% (0.96–1.02%) and 99% (0.58–0.86%), respectively.

The upper concentration limit of apremilast can be extended to 2622 ng/mL for by 1/2 and 1/4 dilutions with screened human blank plasma. The mean back–calculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal value and the coefficients of variations (%CV) were in the range of 3.35- 4.61%.

Pharmacokinetic study results

Thus under the fasting condition, the validated method was successfully applied for a pharmacokinetic study of apremilast in 6 healthy adult male subjects who received 30 mg, respectively. With the reported lowest LLOQ (2.03 ng/mL) apremilast was quantifiable beyond 48 h of post–dosing. The mean plasma concentration–time profile of apremilast was presented in Fig. 8 and the corresponding pharmacokinetic parameters were listed in Table 4. The pharmacokinetic parameters were calculated based on the plasma concentration of apremilast by a non-compartmental model. The rate constant kernel (kel) is the slope of the linear regression of the log transformed concentration values versus time data at the terminal phase and the value of kernel obtained is 0.12 and the elimination half life obtained is 6.48 h. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the trapezoidal rule and the value obtained is 3531. The maximum plasma concentration (C_{max}) obtained is 356 ng/mL. The sum of the ratio of last measurable concentration to the elimination rate constant plus AUC_{0-t} gives the area under the curve extrapolated to infinity ($AUC_{0-\infty}$) and the value achieved is 3575. When the *C*max is achieved, the rate of absorption equals to the rate of elimination which is t_{max} and the value obtained is 3.33 h and the time taken to lose half of its pharmacological activity, physiological activity is $t_{1/2}$ and the value that is obtained is 6.48 h.

Table 4: Pharmacokinetic parameters of apremilast after single oral administration of 30 mg to healthy male subjects (n=6, Mean ± SD)

PK parameter	Mean ± SD

	30 mg
t_{max} (h)	3.33±0.52
C_{max} (ng/mL)	356±37.0
AUC_{0-t} (ng h/mL)	3531±823
AUC_{0-inf} (ng h/mL)	3575±857
$t_{1/2}$ (h)	6.48±2.12
Kel (h^{-1})	0.12±0.05

Incurred sample reanalysis

As the FDA has prescribed the importance of incurred sample reanalysis evaluation at the Crystal City III meeting [17,21], by using dosed subject samples it is compulsory to prove assay reproducibility. Incurred sample reanalysis was evaluated by picking up two plasma samples from each subject and re–assayed in a separate batch run. By applying the current method, it showed the good reproducibility and this was confirmed because the differences in concentrations between the ISR and the initial values for all the tested samples were less than 20% (Table 5).

Table 5: Incurred samples re–analysis data of apremilast

Apremilast dose of 30 mg			
Sample	**Initial conc. (pg/mL)**	**Re–assay conc. (pg/mL)**	**Difference (%)**
1	344.65	340.35	-1.25
2	11.56	11.01	-4.86
3	360.01	364.25	1.17
4	11.43	11.89	3.92
5	287.41	279.41	-2.82
6	22.25	22.03	-0.98
7	358.82	361.50	0.74
8	13.42	13.93	3.76
9	420.65	422.22	0.37
10	15.45	15.34	-0.68
11	487.32	478.98	-1.73
12	17.53	17.19	-1.94
Expressed as [(initial conc.−re–assay conc.)/average]×100%			

CONCLUSION

Thus according to commonly acceptable FDA guidelines the mentioned paper indicates the successful development and validation of a simple, sensitive and rapid LC–MS/MS method for the determination of apremilast in human plasma samples. The method presented has the highest sensitivity (2.03 ng/mL) and employs low plasma volume (200 µL) for analysis compared to other procedures. Moreover, the total analysis time (extraction and chromatography) is the shortest. Thus, a higher output can be achieved by this method and relatively more number of samples can

be analyzed in less time. The simple SPE procedure minimizes the chances of errors, saves considerable time and simplifies the sample preparation procedure. The method showed suitability for pharmacokinetic studies in humans. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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