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LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRYMETHOD FOR ESTIMATION OF ROFLUMILAST IN HUMAN PLASMA

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ABSTRACT

Roflumilast was estimated by LCMS/MS in presence roflumilast N-oxide. LLE method was used for sample preparation. A gradient (solution A: 2 mM ammonium acetate and solution B:acetonitrile) was used to elute the sample from a C_8 column. Flow rate was maintained as 1.0 – 1.5 mL/min. Roflumilast D4 was used as an internal standard. The analysis was performed on LCMS/MS API 4000 using positive atmospheric pressure ionization mode. MRM transitions were monitored for roflumilast as m/z 402.9→ 187.0 (fragment 1) and m/z 402.9→ 241.1 (fragment 2). A linear response was observed over the concentration ranges 75.70pg/mL to 16149.46pg/mL. The intra-day and inter-day precisions were within 14.0%. The assay accuracy was 81.0 –105.0%. Mean recovery was 62.80% (3.30%). The limit of detection was 18.94pg/mL. This LC–MS/MS method for determination of roflumilast in human plasma is relatively simple, fast, sensitive and specific. This method is also cost effective and can be successfully used in a bioequivalence study.

Keywords: Roflumilast, COPD, LLE, LCMS/MS, validation.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is associated with an abnormal inflammatory response leading to chronic airflow limitation which is not fully reversible. It is generally but not exclusively associated with tobacco smoking which is however, considered the most important risk factor for COPD worldwide. It is among the world's most prevalent diseases and the fourth leading cause of death in Europe[1–5].

Roflumilast [3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy) benzamide] is an anti-inflammatory medicine used for the treatment of chronic obstructive pulmonary disease. It is a selective, long acting phosphodiesterase-4 (PDE4) inhibitor. It inhibits the breakdown of cAMP leading to an increase in the intracellular concentration of cAMP. This, in turn, activates protein kinase A (PKA), which catalyzes the phosphorylation of proteins, leading subsequently to a reduction of inflammation. Roflumilast N-oxide, the main metabolite of roflumilast in humans and inmost animal species, is pharmacodynamically active and adds considerably to the overall biological activity of the parent compound. Thisis formed by phase I metabolism of roflumilast with CYP3A4 and CYP1A2 as major enzymes and a minor contribution of CYP2C19 and CYP1A1[6 - 8].

Few spectrophotometric/chromatographic methods for determination of roflumilast in serum/plasma are available [9-12]. Knebel et al. developed a sensitive method for quantification of roflumilast in human plasma using LCMS/MS. Its lower limit of quantification was 0.1 ng/mL and the calibration graph was linear till 50 ng/mL. However, in order to increase the sample-throughput and to reduce the run time per sample a cumbersome column-switching technique was implemented using a dual analytical column chromatography approach [9].

A spectrophotometric method for estimation of roflumilast in human plasma is reported by Raveendra et al. [10]. Though the method is simple but its quantification limit is only 2.37µg/ml. The linearity range achieved by

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Belal et al. [11] in their HPLC method was 2.5–200 µg /mL. Although LCMS/MS method developed by Cui et al.[12] has the quantification limit of 0.02 ng/mL for roflumilast, it uses SPE method for sample preparation which is not cost-effective. Hence, we developed a new, sensitive and relatively simple LC–MS/MS method for estimation of roflumilast in human plasma. The limit of quantification of roflumilast in human plasma achieved is75.70 pg/mL.LLE method is utilized for sample preparation. This method is validated as per FDA regulations [13] and can be used for pharmacokinetic study.

MATERIALS AND METHODS

Roflumilast (purity: 99.91 %), Roflumilast D4 (purity: 99.92 %) used as an internal standard, and the metabolite, Roflumilast N-Oxide, (purity: 98.30 %) were purchased from Vivan Life sciences, India. Methanol (HPLC-grade), acetonitrile and ammonium acetate of highest purity grade were purchased locally. Milli Q purified water (Millipore, Milford, MA) was used throughout the study. Plasma lots used for the experiments were collected in-house.

Preparation of analyte, metabolite and internal standard solutions

Stock solution of roflumilast (200µg/mL) was prepared in acetonitrile. Its potency and actual amount weighed were used to correct the concentration. The stock solution of roflumilast was then diluted together with diluent (acetonitrile:Milli-Q-water: 80:20 v/v) to concentration ranges of 3.75ng/ml to 812.00ng/mL.

A stock solution (200µg/ml) of roflumilast-N-Oxide in acetonitrile was prepared. This solution was then diluted to final concentration of 500 ng/ml by using acetonitrile:Milli-Q-water: 80:20, v/v. Similarly, stock solution of roflumilast D4 (200µg/mL) prepared in acetonitrile was diluted to 100ng/mL using diluent (acetonitrile:Milli-Q-water: 50:50 v/v). The concentrations of the stock solutions of both roflumilast-N-Oxide and roflumilast D4 were corrected as mentioned above.

Preparation of calibration standards

To prepare calibration curve standards, 20 µL of the diluted samples of roflumilast was added to 980 µL of K_2EDTA pooled plasma to obtain a concentration range about 75.70 pg/mL to 16149.46 pg/mL. All these bulk spiked samples were stored at about -70°C in aliquot of 600 µL.

Preparation of Quality Control Samples

Stock solution of roflumilast was diluted with 80% acetonitrile in water to obtain the concentration ranges of 3.79ng/mL to 630.00ng/mL.20 µL of each diluted solution was added into the mixture of 960 µL of K_2EDTA plasma and 20µL of roflumilast-N-Oxide (approximately10 ng/mL) to obtain final concentration range of75.85pg/mL to 12600.00 pg/mL for roflumilast.

Sample preparation

50 µl of internal standard mixture (roflumilast D4) was added to all RIA vials except blank. 500 µl of sample was then added to each labeled RIA vials.200 µl of 100 mMdi-sodium hydrogen phosphate dihydrate was added to respective RIA vials and mixed by vortex.2 mL of extraction solvent (ethyl acetate: n-hexane, 25:75, v/v) was then added to all vials, capped them and then placed onvibramax at 2500 RPM for 10 mins. They were centrifuged at 4000 RPM for 5 mins at about 4°C. In a fresh RIA vial, 1.6 mL of supernatant was transferred and dried at 40°C in nitrogen evaporator. 0.3 mL of Reconstitution Solvent (2mM ammonium acetate: acetonitrile: 40:60 v/v) was added, transferred into a labeled HPLC vial after vortex and then placed in the autosampler.

Chromatography

25 µL of sample was injected on a reversed phase column (Zorbax SB C8, 4.6 x 100mm, 3.5µm). The column oven was set at 40^0C. The sample was analyzed on API 4000 Mass spectrometer (Applied Biosystems, USA) attached to Waters UPLC by using below mentioned gradient flow (Table 1) with a splitter. The run time was 5.0 minutes. The column was then equilibrated to the initial condition before injecting the next sample.

Mass Spectrometry

Atmospheric pressure ionization (API) interface operated in positive ionization mode was used for the multiple reaction monitoring (MRM). The operational conditions were optimized by infusing diluted stock solution of analyte and internal standard (Table 2).

. Unit resolution was set for quadrupoles Q1 and Q3. For roflumilast, MRM transitions were monitored as m/z 402.9→ 187.0 (Fragment 1) and m/z 402.9 → 241.1 (Fragment 2).

For roflumilast D4 and roflumilast N-oxide, MRM transitions were 407.0 → 245.2 and 419.1 → 187.1, respectively.

Linear regression analysis using the analyst software 1.6.2 was used to calculate sample concentration. Quantification of Roflumilast is done by the summation of Fragment1 and Fragment 2 peak areas. Data was processed by peak area ratio. The concentration of unknown was calculated from the equation (Y= mX+ c) using regression analysis of spiked plasma calibration standards with reciprocal of the square of the drug concentration ($1/X^2$).

RESULTS AND DISCUSSION

Method Development

Sensitive and selective LC–MS/MS assays for

determination of very low concentration levels of pharmaceutical compounds present in biological samples requires specific and effective sample clean-up procedures. Three commonly used samples clean–up methods are protein precipitation (PPT), liquid–liquid extraction (LLE) and solid-phase extraction (SPE).The simplest among them is protein precipitation method using organic solvent but chances of matrix effect prevails. SPE technique for sample extraction is usually good; however, it requires an extra cost. Roflumilast being a water insoluble compound can easily be extracted by LLE method. This technique was found to be robust, provided clean samples and gave good and reproducible recoveries of both analyte and IS. The extraction recovery of analyte was determined by comparing peak areas from plasma samples (n = 6) spiked before extraction with those from aqueous samples. The mean recoveries across QC levels (with precision) were 62.8% (3.30%) for roflumilast and 64.12% for roflumilast D4 (IS).

A gradient method is developed for the quantification of roflumilast in K_2EDTA human plasma in presence of its metabolite, roflumilast-N-Oxide.The total run time including recalibration of the column was 5.5 min. The retention times for roflumilast and roflumilast D4 were 2.90min and 2.87min, respectively.

Method Validation

Specificity, linearity, intra- and inter-day precision & accuracy, and stability were validated in this method as per FDA Guidelines[13].

Specificity and Selectivity

Eight individual human plasma lots including one haemolysed and one lipemic lot spiked with LLOQ and intended concentrations of internal standard were processed for determination of specificity and selectivity. No interference were observed at the retention times of analyte and internal standard when peak responses in blank lots (Figure 1) were compared against the response of spiked LLOQ containing IS mixtures (Figure 2) indicating the specificity of the method. The signal to noise ratio was found to be more than 18.08. The signal to noise ratio> 5 is acceptable for selectivity.

Linearity and Sensitivity

Roflumilast concentrations ranging from75.70 pg/mL to 16149.46 pg/mL were used to prepare an eight-point calibration curve. The peak-area ratio (y) of analytes to internal standards was plotted against the nominal concentration ratio (x) of analyte to internal standard to determine the linearity of each calibration curve. Excellent linearity was achieved with correlation coefficients greater than 0.99 for all validation batches (**Figure 3**).

The accuracy of each calibration point was obtained after back calculation of concentrations of calibration standards. The ranges of the calibration points' accuracy were98.05 – 103.67%.

The sensitivity of this method was assessed by injecting six processed LLOQ samples along with a 'Precision and Accuracy' batch. Precision and accuracy for roflumilast at the LLOQs were 9.0% and 98.9% respectively. The LLOQ of the method is 75.70 pg/mL which is lower than the reported one [9]. Limit of detection was 18.94 pg/mL. This method is therefore quite sensitive even for a pharmacokinetic study. Even at 18.94 pg/mL, signal to noise ratio was > 12.93 (4 times more than the required one). This indicates that the method can be used to quantify even lower concentration of roflumilast or the volume of plasma can be further decreased. Thus the application of this method even widens further to the pediatric patients where sample volume is always a challenge.

Precision and accuracy

Precision and accuracy were determined by injecting a set of calibration curve samples and quality control samples. The correlation coefficient of calibration curve was more than 0.99(r) as required by FDA guidelines. The accuracy and precision were 98.58 – 103.67% and < 6.5 which were within acceptable limits.

Six replicate analyses of QC samples (n=6) at four different concentrations– Lower Limit Of Quantification Quality Control (LOQQC), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC), were used to determine precision and accuracy for intra- and inter-day batches for all analytes. The respective concentrations for roflumilast were 76.18 pg/mL, 198.88 pg/mL, 4971.92 pg/mLand12429.80 pg/mL for LOQQC, LQC, MQC and HQC. Results of precision and accuracy of quality control samples were presented in Table 3.

The intra-day and inter-day precision were within 14.0%. The assay accuracy was 96.62 – 99.08% of the nominal values. Assay accuracy was calculated by using the formula [(mean observed concentration) / (spiked concentration)] x 100%. Relative standard deviation (RSD) was used for evaluation of precision.

Matrix effect

Eight blank matrix lots including one heamolysed and one lipemic lot from different sources were processed. 500 μL of blank plasma from each lot was processed as mentioned in sample preparation. Post extracted samples (presence of matrix) were prepared by adding aqueous solution of roflumilast (analyte), either at LQC or HQC level, and known concentration of internal standard (roflumilast D4)to each of the processed plasma.

Similarly, the aqueous solution of roflumilast either at LQC or HQC level containing same concentration of IS as above was prepared with their constitution solvent and was considered as aqueous samples (absence of

matrix). Six replicates of each aqueous sample along with post extracted samples of LQC or HQC were injected.

Mean analyte and IS area responses of the aqueous sample were compared with respective analyte and IS area responses of each post extracted sample. Matrix effect was calculated using the formula: Matrix effect (%) = A_2/ A_1 x 100 (%), Where A_1= response of aqueous concentrations and A_2 is response of post-extracted concentrations.

Average (n=8) matrix factors were 98.4% with a CV of 4.4% at LQC level and 96.4% with a CV of 1.6% at HQC level which is within the accepted limit (% CV ≤15) (**Table 4**).

Dilution integrity

Dilution integrity was evaluated after spiking interference free human plasma with 2 times of HQC concentration of roflumilast (i.e. 2 x 12429.80 pg/mL = 24859.60pg/mL). These spiked plasma were diluted either 2 fold (2T) or 4 fold (4T) with interference -free human plasma. These samples (Six replicates of each dilution) were processed and then analyzed against a set of freshly spiked calibration standards. The mean accuracy and precision were 96.37% and 3.40% for 2T and 99.81% and 1.92% for 4T (data not shown).

Carry –over Effect

The cleaning ability of wash solvent (Acetonitrile: Milli-Q-water: 50:50v/v) used for rinsing the injection needle and port was evaluated to avoid any carry – over of injected sample in subsequent runs. The order of placing samples was: LLOQ of individual analyte, blank plasma, upper limit of quantitation (ULOQ) of individual analyte and blank plasma. There were no carry over observed during the experiment (data not shown).

Stability

Both aqueous and matrix based samples were subjected for stability evaluations. Short-term and long-term stabilities for aqueous solutions were determined as follows:

a) Stability in aqueous solution:

i)Short – Term stock solution stability (STSS):

Stock solutions of both analyte and IS were prepared separately and kept at 25^{0}C for about 25 h and named as stability stock. MQC concentration of analyte was prepared from the stability stock solution and stored at 25^{0}C for about 25 h and marked as stability working solution. Just before injection, fresh stock analyte solution and stock IS were diluted to MQC concentration of analyte and intended concentration of IS. MQC sample (both stock and working solutions) and diluted IS solutions were injected in six replicates and the results were compared with those obtained from the freshly prepared MQC solution. No significant differences were noticed indicating that analyte were stable at 25^{0}C (Table 5). For IS, stability was >97% after 25h (data not shown).Criteria accepted for the ratio of mean response for stability samples is 90-110%.

ii) Long term stock solution stability (LTSS)

Aqueous MQC sample of analyte and solution of internal standard with known concentration were prepared by dilution from respective stock solutions and stored at 2-8 ^{0}C for 19 days. Mean area response of stored stock solution was then compared against freshly prepared MQC stock solution. Similarly, mean area response for internal standard was also compared. Mean percent stabilities for roflumilast was 98.5and 99.8 for roflumilast D4 (data not shown) were well within accepted limit (90 – 110%). This indicated that both analyte and internal standard solutions were stable for 19 days at 2-8 ^{0}C (Table 5).

b) Stability in human plasma

i) Bench-top stability

Six aliquots of each analyte in K_2EDTA containing human plasma (at LQC and HQC concentrations) from the -70^{0}Cwere allowed to thaw unassisted at room temperature (25^{0}C) for 6.5 h. A set of freshly prepared calibration standards were processed with these LQC and HQC samples. The stabilities for LQC and HQC samples were 93.7% and 98.5% respectively.

ii) Freeze thaw stability

After 4 freeze thaw cycles, the stability of roflumilast were101.6% for LQC and 100.1% for HQC.

iii) In-injector stability

The stability for LQC and HQC samples kept in auto-sampler at 10^{0}C for about 25 h were 97.3% and 98.9% respectively. IS stability was found to be 100.35%.

iv)Wet extract stability

The stability of roflumilast after 4 h at 25°C was 97.7% for LQC and 98.8% for HQC. As per FDA, accepted range for all the stability studies mentioned above is that the mean concentration for stability samples should be 85-115% of the mean concentration of freshly prepared samples. Thus all the analytes were stable during the analysis process.

Results of stability studies in human plasma were presented in Table 6.

c) Stability in human blood

Analyte and metabolite solutions were prepared at MQC level by dilution of respective stock solution. Similarly, analyte and metabolite solutions (at MQC level) in whole blood were prepared by adding the required amount of individual stock solutions directly to the blood. All solutions were kept on ice bath for 4hrs before processing as mentioned under sample preparation. Internal standard was added just before processing.

Likewise, three replicates at LLOQ concentration of analyte were also processed. All samples were then analyzed in LCMS as mentioned earlier. % stability of roflumilast and roflumilast-N-Oxide were found to be 102.27% and 106.62% for 4 hrs in whole blood respectively. Whole blood stability was found to be within acceptance limits i.e. the mean area ratio of the stored MQC samples should be within ±15% of freshly processed MQC samples (data not shown).

Back Conversion test

Solutions (MQC level) of analyte (roflumilast) and its metabolite (roflumilast N-oxide) were prepared in aqueous solution and also in human plasma. After 24 hrs of storage at 2-8°C the samples were processed and analyzed in LCMS. There was no back conversion observed for both roflumilast and roflumilast N-Oxide i.e. the average area response of metabolite/analyte in individual spiked analyte/metabolite sample should be less than or equal to average area response of LLOQ area of that metabolite/analyte (data not shown).

Extended precision and accuracy run

One set of CC and 40 sets of LQC and HQC as a batch (total 90 samples) were processed and then analyzed. Results of precision and accuracy were presented in Table 7.

The precisions were 6.2% for LQC and 3.1% for HQC. The accuracies were 102.3% for LQC and 97.1% for HQC.

Fig 1. Chromatogram of extracted blank

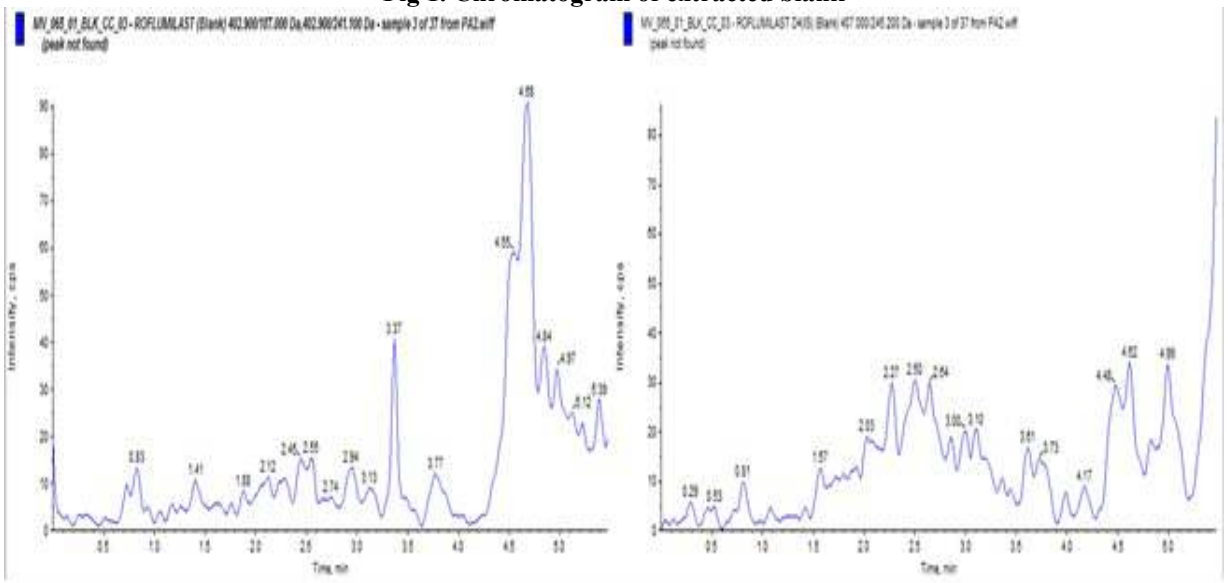

Fig 2. Chromatogram of an LLOQ calibration curve standard with IS

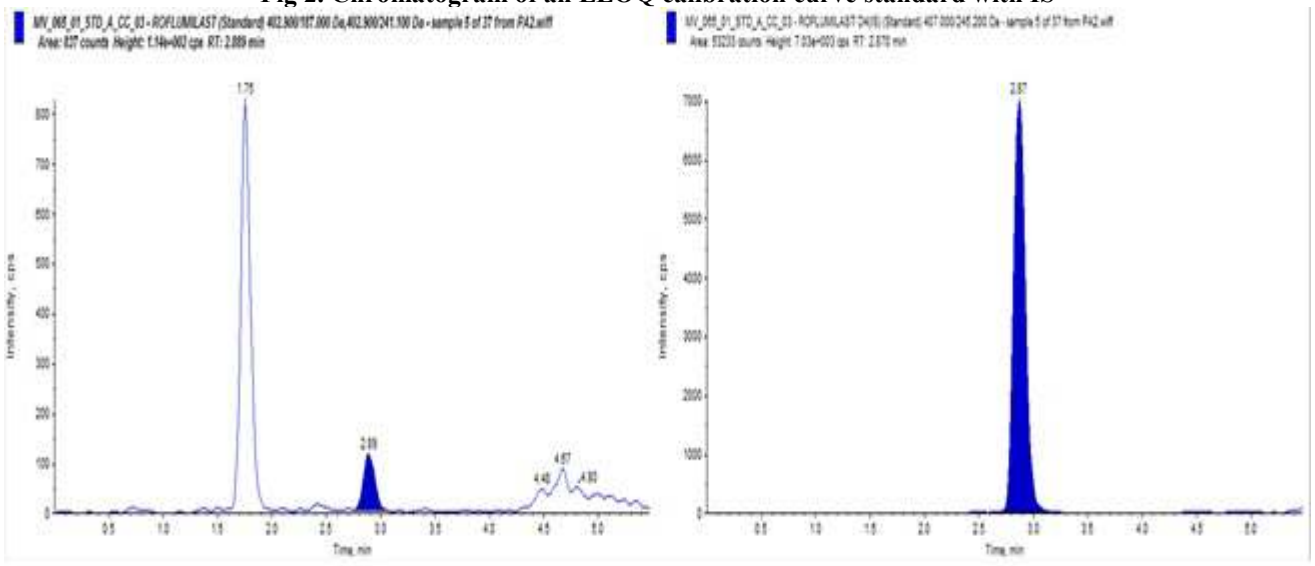

Fig 3. Calibration curve for Roflumilast

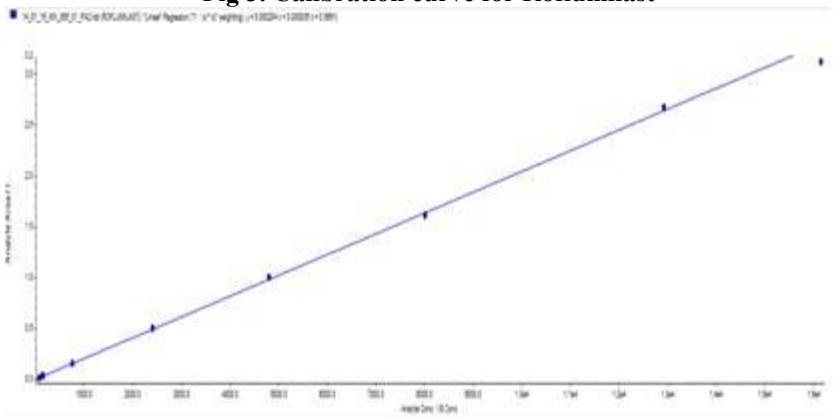

Table 1. Gradient elution program

Time (min)	Flow Rate (mL/min)	Pump A (%) (2 mM Ammonium acetate)	Pump B (%) (Acetonitrile)
Initial	1.000	40	60
3.75	1.000	40	60
3.76	1.500	10	90
5.00	1.500	10	90
5.01	1.000	40	60
5.50	1.000	40	60

Table 2. MS parameters optimized for analysis

Analyte/IS	Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Energy (CE) (V)	Collision Cell Exit Potential (CXP) (V)	Collision activated dissociation (CAD) (psi)	Dwell Time (ms)	Ion source voltage (V)	Curtain gas flow (CUR) (psi)
Roflumilast								
Fragment 1	90	10	36	12	8	300	5500	30
Fragment 2	90	10	26	12	8	300	5500	30
Roflumilast N-Oxide	90	10	36	12	8	300	5500	30
Roflumilast D4	90	10	26	12	8	300	5500	30

Table 3. Intra-day and inter-day accuracy and precision for the determination of roflumilast in human plasma

Sample ID	LOQQC (Nominal Conc. 76.180 pg/ml)			LQC (Nominal Conc 198.880 pg/ml)			MQC (Nominal Conc 4971.920 pg/ml)			HQC (Nominal Conc 12429.800 pg/ml)		
	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Calculated Conc (pg/ml)	Mean accuracy (%)	% CV
PA - 1	76.2	100.0	14.0	202.5	101.8	5.3	4869.1	97.9	2.3	12294.8	98.9	2.3
PA – 2	71.6	94.0	6.4	190.4	95.7	8.4	4819.3	96.9	3.2	12294.9	98.9	1.8
PA – 3	78.1	102.5	8.6	189.2	95.1	10.1	4772.6	96.0	2.5	12124.7	97.5	2.5
PA – 4	62.0	81.4	5.7	196.5	98.8	3.1	5030.2	101.2	3.0	12576.5	101.2	3.5
PA – 5	80.1	105.1	12.1	197.2	99.2	5.4	4811.0	96.8	3.1	12284.9	98.8	2.5
Inter-day	73.6	96.6	12.9	195.1	98.1	6.7	4860.4	97.8	3.2	12315.2	99.1	2.6

Table 4. Matrix effect for roflumilast in human plasma

Matrix ID	LQC			HQC		
	Absence of matrix	Presence of matrix	Matrix Factor	Absence of matrix	Presence of matrix	Matrix Factor
PL-696	2893	2567	91.0	178297	169086	95.7
PL-697	2849	2907	103.1	175366	170229	96.3
PL-699	2891	2921	103.6	179262	167358	94.7
PL-700	2812	2637	93.5	176437	169379	95.8
PL-701	2711	2796	99.2	174391	172464	97.6
PL-702	2762	2780	98.6	176829	175939	99.5
LPL-643		2829	100.3		167740	94.9
HPL-566		2763	98.0		170865	96.7
Average	2819.7	2775.0	98.4	176763.7	170382.5	96.4
SD	72.8	122.2	4.3	1804.8	2784.7	1.6
%CV	2.6	4.4	4.4	1.0	1.6	1.6

Table 5. Short and long –term stability of roflumilast aqueous solution

Short-term stability at 25^{0}C for 26h						Long-term stability at 2-8^{0}C for 19 days		
Stock solution			Working solution			Stock solution		
Average area		% Stability	Average area		% Stability	Average area		% Stability
Stock solution	Fresh solution		Working solution	Fresh working solution		Stock solution	Fresh solution	
85747.3	85452.8	99.7	85651.2	85452.8	99.6	68612.5	70459.2	98.5

Table 6. Stability studies of roflumilast in plasma

Parameters	Bench-top stability for 6h		Freeze-thaw stability after 4 cycles		In-injector stability for 25h		Wet extract stability for 4h	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Nominal (pg/mL)	198.9	12429.8	198.9	12429.8	198.9	12429.8	198.9	12429.8
Mean calculated conc. (pg/ml)	182.1	12220.0	197.5	12422.7	189.0	12269.2	194.3	12257.4
SD	9.6	358.8	10.4	555.5	7.2	427.6	16.0	388.6
%CV	5.3	2.9	5.3	4.5	3.8	3.5	8.2	3.2
% Stability	93.7	98.5	101.6	100.1	97.3	98.9	97.7	98.8

Table 7. Extended precision and accuracy of roflumilast

LQC				HQC			
Nominal conc. (pg/mL)	Mean calculated conc. (pg/mL)	Accuracy (%)	% CV	Nominal conc. (pg/mL)	Mean calculated conc. (pg/mL)	Accuracy (%)	% CV
198.9	203.4	102.3	6.2	12429.8	12065.9	97.1	3.1

SUMMARY & CONCLUSION

This LC–MS/MS method for determination of roflumilast in human plasma is relatively simple, fast, sensitive and specific. This method utilizes liquid-liquid extraction technique for this relatively non-polar molecule. This offers consistent and reproducible recoveries with insignificant interference and matrix effect. On the top of it, this method is also user friendly and cost-effective compared to the reported LCMS/MS method which involves column switching. FDA guideline [13] mentions that internal standard should preferably be identical to the analyte and hence this method was developed and validated using deuterated roflumilast. By using 500 µL plasma samples, the lower limits of quantification were achieved. It demonstrates that the method is reproducible, sensitive and suitable for high-throughput sample analysis. Moreover, as the sensitivity of this method is quite high this can be used even for analysis of pediatric samples

where sample volume is always a challenge. This estimation method of roflumilast is developed in presence of roflumilast N-oxide and hence can be used for estimation of both analyte and its metabolite, if required. This method has the potential to be useful for bioequivalence studies and routine therapeutic drug monitoring.

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CONFLICT OF INTEREST

None.

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