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Electrospray ionization LC-MS/MS validated method for the determination of the active metabolite (R-138727) of prasugrel in human plasma and its application to a bioequivalence study

Ojikumar Lukram,* Mukund Zarapkar, Chandan Kumar Jha, Shivaji Parmar, Keshav S. Tomar and Amit Hande

A rapid and sensitive liquid chromatography tandem mass spectrometry method has been developed and validated for the determination of the active metabolite (R-138727) of prasugrel in human plasma. Because R-138727 contains a thiol group, it requires stabilization by derivatizing with N-ethyl maleimide. Commercially available trandolapril was used as the internal standard (IS). The derivatives of R-138727 and IS were extracted from human plasma using a liquid-liquid extraction technique. Chromatography was performed on a Hypurity C18, 5 μ (50 mm × 4.6 mm, i.d.) column, with the mobile phase consisting of acetonitrile and 10 mM ammonium formate (pH 3.0, 50 : 50 V/V), followed by detection using mass spectrometry. No significant endogenous peaks corresponding to R-138727 or IS were detected in the blank human plasma samples and no significant matrix effect was observed for R-138727 and IS in the human plasma samples. The mean recovery for R-138727 ranged from 90.1 to 104.1%, with the lower limit of quantification set at 1 ng/ml. Linearity was established for concentrations in the range of 1.0–500.12 ng/ml, with a coefficient of determination (r^2) of 0.9958. The derivatized R-138727 was stable in human plasma for 3 months at −20 °C. This method increased the sensitivity and selectivity, resulting in high-throughput analysis of R-138727 using trandolapril as the IS in pharmacokinetic and bioequivalence studies, with a chromatographic run time of 3.7 min.

Keywords: prasugrel; R-138727; LC-MS/MS; human plasma; trandolapril

Introduction

Prasugrel, 5-[(1*RS*)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate hydrochloride, corresponding to the molecular formula $C_{20}H_{20}FNO_3S{\bullet}HCl$ and molecular mass 409.90, is a novel member of the thienopyridine class of adenosine diphosphate (ADP) receptor inhibitors. Like other thienopyridines, prasugrel is a prodrug that is inactive *in vitro*. Prasugrel is rapidly metabolized by esterases to a thiolactone, R-95 913, which is then oxidatively metabolized by several cytochrome P450 enzymes to R-138 727. This active metabolite, R-138 727, binds specifically and irreversibly to the platelet P2Y12 purinergic receptor thus inhibiting ADP-mediated platelet activation and aggregation.[1,2,3,4] Prasugrel (trade name: Efient®) inhibits ADP-induced platelet aggregation more rapidly than any other member of the thienopyridine class of compounds.[2,5]

Pharmacokinetic and bioequivalence studies require a simple, sensitive, reliable, and economical bioanalytical method with a small sample volume and lower turnaround times. To the best of our knowledge, only a few methods have been reported in the literature for elucidating the plasma levels of R-138727 using the derivatizing method of sample preparation using liquid chromatography tandem mass spectrometry (LC-MS/MS).[6] These methods were particularly used for the estimation and interaction of the active and inactive metabolites of prasugrel in a clinical pharmacokinetic study. The chiral separation and determination of the four stereoisomers to the total R-138727 present in human plasma has also been reported using chiral LC-MS/MS.[7] Determination of R-138727, an inactive metabolite in urine and faeces, has also has been successfully accomplished by the LC-MS/MS method.[8] Several LC-MS/MS methods have been developed for the determination of thiol-containing compounds using different derivatizing agents.[9–13] An examination of the literature reveals that stabilization of thiol-containing compounds with a suitable derivatizing agent in biological matrix is the first thing to be performed before subjecting it to analysis. Prior selection of a suitable derivatizing agent for stabilizing thiol-containing compounds in biological matrix is one of the most challenging areas in bioanalysis.

Although the reported assays are sufficiently sensitive, the limitation with them is that they use complicated chromatographic conditions and sample clean-up procedures, and the derivatizing

* *Correspondence to: Ojikumar Lukram, LifeSan Clinical Research, Division of Centaur Pharmaceuticals Pvt. Ltd., Centaur House, Near Hotel Grand Hyatt, Vakola, Santacruz (E), Mumbai, India 400055. E-mail: ojitkr@yahoo.co.in*

LifeSan Clinical Research, Division of Centaur Pharmaceuticals Pvt. Ltd., Centaur House, Near Hotel Grand Hyatt, Vakola, Santacruz (E), Mumbai, India 400055

agent used in the methods is expensive. Moreover, the assay methods seemed to be unreliable with regard to selectivity and high throughput, especially in bioequivalence studies. Similarly, there is no reported literature for the determination of R-138727 in human plasma by LC-MS/MS using the liquid–liquid sample extraction procedure. Determination of drug in the biological matrix by LC-MS/MS becomes a need for bioanalysis in order to increase the sensitivity and selectivity.[14–21] The chromatography separation technique, when coupled with tandem mass spectrometry, enables improving the sensitivity and selectivity, with a significantly lower turnaround time. Therefore, it was the need of the hour to develop a simple, specific, rapid, economic, and sensitive analytical method for the quantification of R-138727 in human plasma.

This paper describes the development and validation of the bioanalytical method that can be used for the determination of R-138727 in human plasma using trandolapril as an internal standard (IS) employing the LC-MS/MS method. The method used a Quattro Premier API triple quadrupole mass spectrometer (Waters® Corporation, Milford, MA, USA) equipped with an AcQuity UPLC system obtained from Waters® Corporation as the solvent delivery system.

Experimental method

Chemicals and reagents

The chemicals and reagents used were acetonitrile (ACN), methanol and methyl-tert butyl ether, which were of high-performance liquid chromatography (HPLC) grade from JT Baker (Mallinckrodt Baker, Deutschland, Germany). A MilliQ water purification system (Millipore Co., Bedford, MA, USA) was used to obtain purified water for the HPLC analysis. R-138727 and trandolapril (purity >98%) were obtained from MSN Laboratories (Hyderabad, India) and USP (Bangalore, India), respectively. Formic acid, ammonium chloride, and ammonia (30%) were obtained from Merck (Merck KGaA, Darmstadt, Germany). Ammonium formate of MS grade, N-ethyl maleimide (NEM) derivatizing and 2-bromo-3′-methoxyacetophenone (MPB) agent were procured from Fluka (Sigma-Aldrich Chemie GmbH, Switzerland). Ethylene diamine tetra acetic acid (EDTA) plasma of healthy volunteers was obtained from Yash Pathological Laboratories (Mumbai, India).

Clinical protocol

The Regulatory Authority of India (Central Drug Standard Control Organization) and the Independent Ethics Committee on human studies to be conducted at the clinical facility of LifeSan Clinical Research approved the present pharmacokinetic study in compliance with the current regulations for conducting bioequivalence studies in India. Six healthy Indian male volunteers aged 18–25 years and weighing 53–73 kg were enrolled in the study. All of them completed the study successfully. The study was performed at the clinical facility of LifeSan Clinical Research, India. Based on medical history, clinical examinations and laboratory tests, which included haematology, blood biochemistry, and urine analysis, the subjects were enrolled in the crossover, comparative pharmacokinetic study. No subject had a history or evidence of hepatic, renal, gastrointestinal or haematological deviations or any acute or chronic diseases or drug allergy. Subjects were instructed to abstain from taking any medication or xanthine-containing foods for at least two weeks prior to and during the study period. No milk or dairy products were allowed during the study. Informed consent was obtained from all subjects after the nature and purpose of the study had been explained. After an overnight fast of more than 10 h, subjects were given a single dose of one tablet of prasugrel 10 mg (test or reference as per randomization) with 240 ml of water. Subjects were called at each dosing station at the scheduled time. After administration of the study drug, the subjects fasted for an additional 4 h, followed by consumption of a standardized meal. Drinking water was restricted 1 h before and 2 h after dosing. During the 24-h period, after drug administration, strenuous physical or mental activity was not permitted. Lunch, evening snacks, and dinner were served at 4, 8, and 12 h, respectively, after administration of the study drug. A series of blood samples (5 ml each) were collected using an indwelling catheter or by direct venepuncture in pre-labelled K_2EDTA vaccutainers at the following time points: 0.0 h (pre-dose) and 0.17, 0.34, 0.50, 0.67, 0.83, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 12.00, 16.00, 24.00, 30.00, 36.00, and 48.00 h after administration of the study drug. Immediately after blood draw, a volume of 25 µl of NEM (500 mM in ACN) was added to the collected blood samples in order to ensure stability of the metabolite, R-138727. All the collected samples were centrifuged in a refrigerated centrifuge at $10^{\circ} \pm 2\,^{\circ}C$, at 3500 rpm, for 10 min to separate plasma. The separated plasma was divided and transferred into two aliquots and then frozen at $-20^{\circ} \pm 5\,^{\circ}C$.

Liquid chromatographic and mass spectrometric conditions

A chromatographic analysis was performed on the AcQuity UPLC system (Waters® Corporation, Milford, MA, USA) at ambient temperatures, with the mobile phase consisting of ACN, 10 mM ammonium formate and formic acid (50:50:0.2, V/V/V). A Hypurity C18, 5 µ (50 mm × 4.6 mm, i.d.) column obtained from Thermo Hypersil (West Palm Beach FL, USA) was used for the chromatographic separation at a flow rate of 0.250 ml/min. The samples were placed in a UPLC auto sampler to enable control of the temperature at $5^{\circ} \pm 2\,^{\circ}C$. A weak needle wash (ACN 80:20) was used to flush the needle and the auto sampler sample loop to prevent a carry over. The mobile phase was delivered by the UPLC solvent delivery system and the sample was injected by a UPLC auto sampler (Waters® Corporation, Milford, MA, USA).

Drug monitoring and quantification were performed using a Quattro Premier API triple quadrupole mass spectrometer equipped with an electro spray ionization (ESI) source and run by MassLynx™ 4.1 software (Waters® Corporation, Milford, MA, USA). The mass spectrometer was operated in the positive ion mode with Multiple Reaction Monitoring. The mass spectra of derivatized R-138727 and IS are shown in Figures 1 and 1A. The optimum parameters were optimized for the determination of R-138727 and the conditions are presented in Table 1.

Preparation of the stock and working standard solutions

The stock solution of R-138727 (10 mg/ml) was prepared by dissolving it with 0.5 ml of ACN and further dissolving with 0.1M ammonium chloride solution (pH 7) for preparing the calibration standards (CS) and quality control (QC) standards. The solution was derivatized by mixing with 0.5 ml of NEM. The stock solution was kept at room temperature for 15 min and then further diluted with ACN to get the concentration of 1000 µg/ml, and stored at

2°–8 °C for further preparation of working solution. The working stock solution of R-138727-NEM was diluted with ACN to get concentrations of 5.006, 21.255, 15.006, 10.009, 5.005, 2.502, 1.251, and 0.100 µg/ml for the CS and 18.759, 12.512, 3.754, 0.151, and 50.15 µg/ml for the QCs. The stock solution of trandolapril (1 mg/ml) was also prepared in ACN and this was further diluted with water : ACN (50 : 50) to acquire a concentration equivalent to 12.5 ng/ml. A working solution of the IS was freshly prepared every day before spiking to the plasma samples. All the stock and working stock solutions were stored at 2°–8 °C when not in use.

Preparation of calibration spiked plasma standards and quality control samples

The CSs were prepared by bulk spiking of the screened pooled plasma with the corresponding working standard solutions to give concentrations of 1.0, 2.0, 25.2, 50.5, 100.9, 200.18, 300.12, 425.10, and 500.12 ng/ml of R-138727-NEM. The QC samples were also prepared by bulk spiking of the screened pooled plasma with the corresponding working solutions to give concentrations of 1.00, 3.00, 75.07, 250.25, and 375.18 ng/ml as the lower limit-quality control (LLQC), low-quality control (LQC), medium1-quality control (M1QC), medium2-quality control (M2QC), and high-quality control (HQC) of R-138727-NEM.

Sample preparation

Sample preparation was carried out using a liquid–liquid extraction (LLE) technique. The frozen plasma sample was removed from the freezer and was allowed to thaw at room temperature. The thawed samples were vortexed using a mutipulse vortexer to ensure complete mixing. Five hundred microlitres of plasma were aliquoted into pre-labelled 10-ml stoppered test tubes filled with 500 µL of 4% formic acid. In this tube, freshly prepared 25 µl of the IS, equivalent to 12.5 ng/ml, was added and the same was vortexed to ensure complete mixing. After this, methyl tert.-butyl ether (4.0 ml) was added to each acidified plasma solution and the samples were then vortexed for 4 min at 80 rpm on a mutipulse vortexer followed by centrifugation at 5000 rpm at 10 °C for 5 min. The organic layer was then transferred to pre-labelled evaporating tubes and methyl tert.-butyl ether was dried under the stream of nitrogen gas for 10 min in a Turbovap (Caterpillar, Mumbai, India) at 10 °C.

Each of the dried extracts was reconstituted by dissolving with 200 µl of the mobile phase and the samples were transferred to maximum recovery vials for analysis.

Data treatment

The linearity of our method for the determination of R-138427 in human plasma was tested for the concentration range

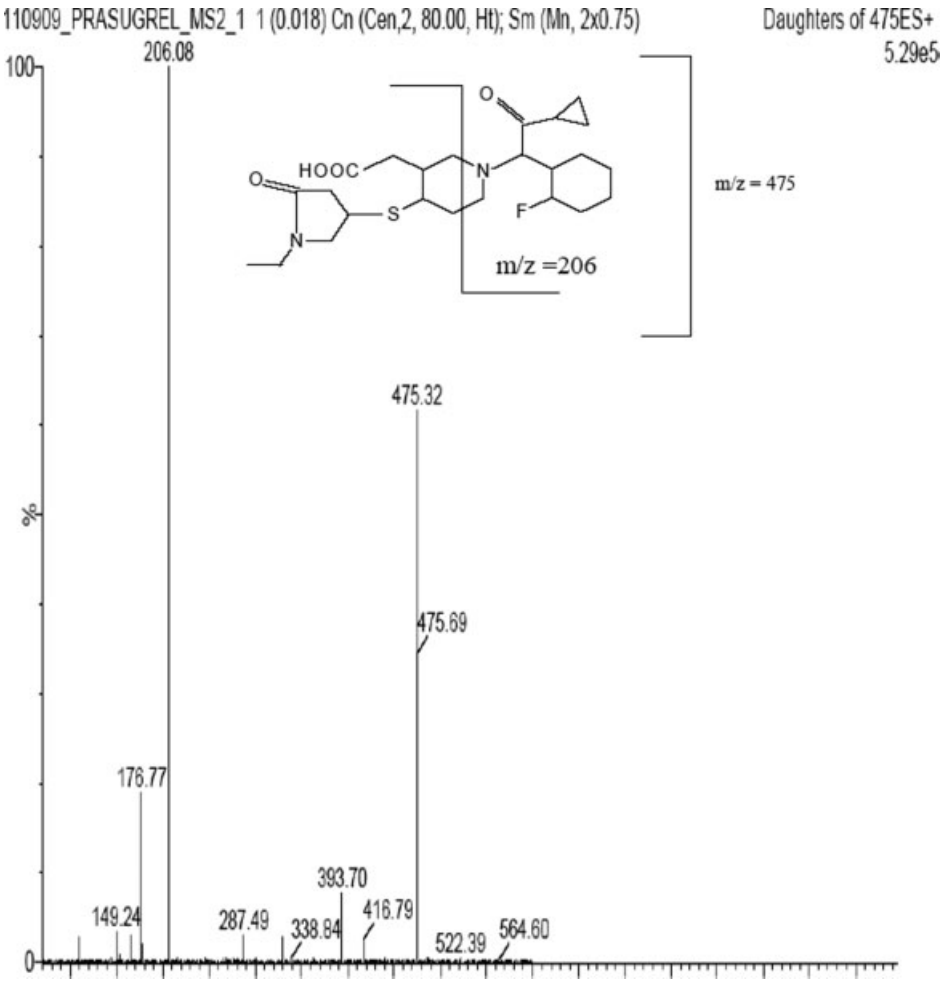

Figure 1. Full scan product ion spectra of R-138727-NEM.

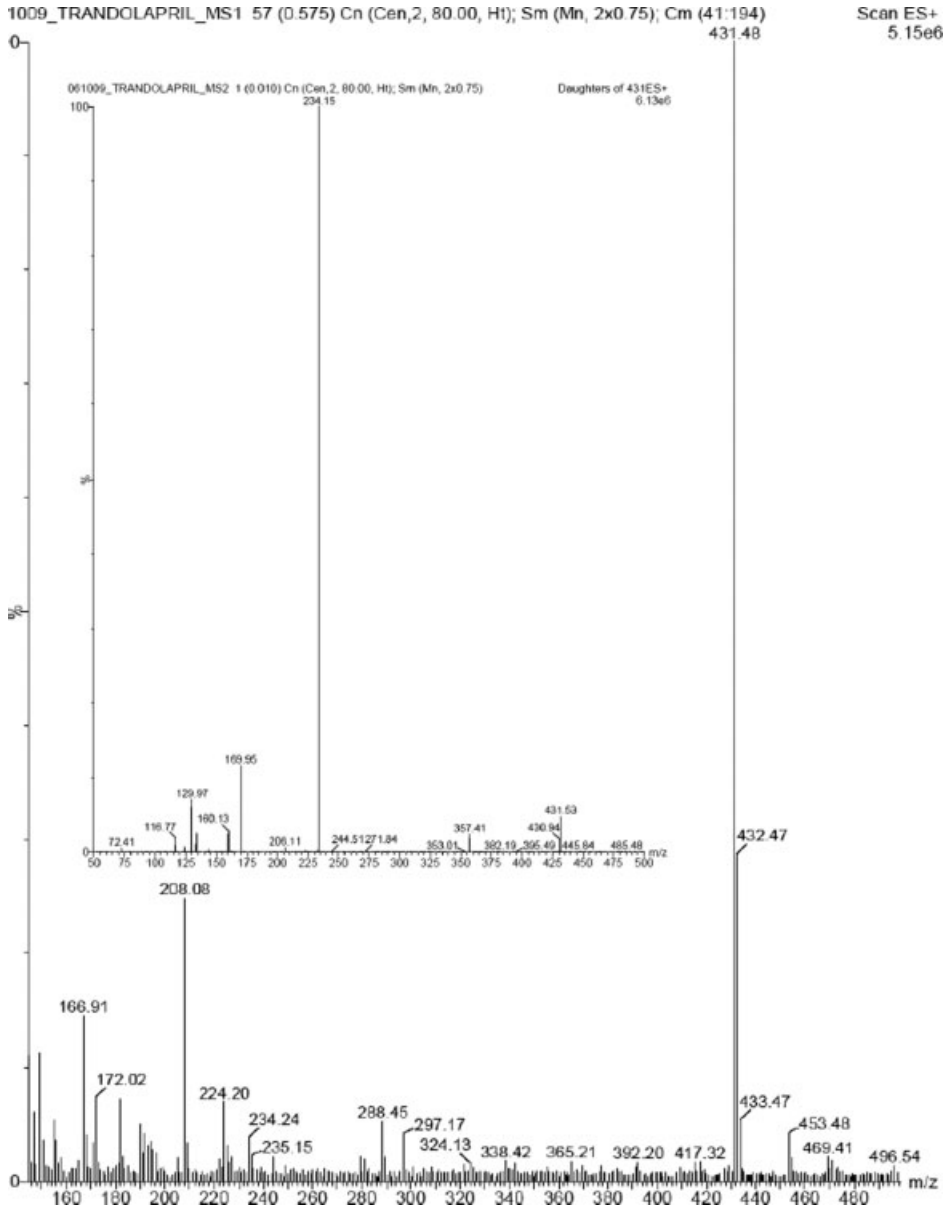

Figure 1A. Full scan parent ion and product ion spectra of trandolapril.

Table 1. Mass spectrometry parameter for R-138727-NEM and trandolapril (IS)

Parameters	Values	
	R-138727-NEM	Trandolapril
Source temperature, °C	120	120
Desolvation temperature, °C	450	450
Desolvation gas, L/h	1000	1000
Cone gas flow, L/h	20	20
Capillary voltage, kV	1.09	1.09
Extractor voltage,	5	5
Cone voltage, V	20	30
Collision energy, eV	21	15
Polarity	Positive	Positive
Ion transition, m/z	475.42/206.04	431.24/234.12

1–500.12 ng/ml. The standard curves were calculated from the peak area ratio (p.a.r.) of R-138727-NEM/IS using the linear regression equation, $y = ax + b$, with $1/x^2$ as the weighing factor. R-138727-NEM concentrations (ng/ml) for QCs in a batch were calculated by interpolating the p.a.r. from the corresponding standard curves.

The measured p.a.r. of the QC samples was converted into concentrations using the following equation:

$$\text{R-138727-NEM concentration } (x) = [\text{p.a.r. (R-138727-NEM/IS)} - b]/a \quad (1)$$

where **a** is slope of the corresponding standard curve and **b** is intercept of the corresponding standard curve. The concentrations were reported in nanogram per millilitre plasma for R-138727-NEM.

Validation procedure

The selectivity, sensitivity, linearity, precision, accuracy, recovery and stability, matrix effect, and dilution integrity of the method were validated according to the US Food and Drug Administration guidance for the validation of bioanalytical methods[22] and European Medicines Agency (EMA).[26–27]

Validation of selectivity was performed by analyzing plasma samples from different sources (or donors), including haemolized and lipemic plasma, to test for interference at the retention times of both R-138727-NEM and IS. The sensitivity was determined analysing five replicates of blank human plasma and plasma spiked with the analyte at the lowest level of the calibration curve. Replicate ($n = 4$) analyses of the QC samples at the LOQ extracted from the sample batch were performed to determine the inter-run and intra-run accuracies. The inter-run precision and accuracy of the CSs was assessed using the four calibration curves used for assay validation.

Accuracy, defined as the percent relative error (%RE), was calculated using the formula:

$$\%RE = (E\text{-}T)(100/T) \quad (2)$$

where **E** is experimentally determined concentration and **T** is theoretical concentration.

Assay precision is calculated using the formula:

$$\%RSD = (SD/M)(100) \quad (3)$$

where **M** is mean of the experimentally determined concentrations and **SD** is standard deviation of M.

The extraction efficiencies of R-138727-NEM and IS were determined by comparing the peak area of the extracted analytes with the peak area of the non-extracted standards. The extraction efficiency value can be calculated as follows:

$$\text{Extraction efficiency } (\%) = C/D \times 100 \quad (4)$$

where **C** is peak area of the extracted standard and **D** is peak area of the non-extracted standard. Dilution integrity with acceptable precision and accuracy was performed to extend the upper concentration limits. Five replicates each at concentrations two-times the highest concentration were prepared and diluted to two-fold and four-fold with blank plasma. These were then subject to processing.

Stability of the processed sample was evaluated by comparing the extracted plasma samples that were injected immediately (time 0) with the samples that were re-injected after keeping in the auto sampler at 5 °C for 24.0 h. Stability of the spiked human plasma, stored at room temperature (bench-top stability), was evaluated for 6 h, and this was compared with the stability of the freshly prepared extracted samples. Freeze–thaw stability was evaluated by comparing the stability of the samples that had been frozen and thawed three times with the stability of freshly spiked QC samples. Long-term stability of the spiked human plasma stored at −20 °C was evaluated by analyzing the MQC and HQC samples that were stored at −20 °C for 60 days together with the freshly spiked CS and QC samples. All stability evaluations were based on back-calculated concentrations. The analytes were considered stable if the deviation of the mean test responses was within 15% of the freshly prepared or comparison samples.

Results and discussion

Method development

The main objective of our method was to stabilize R-138727 (active metabolite of prasugrel) using NEM as the derivatizing agent and to complete the analysis within a short period of time. Several derivatizing agents were studied by Farid *et al.*[3] and Srinivas *et al.*[9] In the present study, the derivatizing agents NEM and MPB were investigated using the commercially available IS, trandolapril. Maximum recovery and highly reproducible results were found when NEM was used as the derivatizing agent than when using MPB. The chromatographic conditions, especially the composition of the mobile phase, were optimized using different organic modifiers, such as ammonium acetate, ammonium formate, and formic acid alone or in combination in different concentrations. It was found that 10 mM aqueous ammonium formate in combination with ACN and formic acid (50 : 50:0.2, V/V/V) gave the best results. The percentage of formic acid was investigated to get good ionization and fragmentation in the mass spectrometer. The sensitivity of R-138727-NEM was investigated by replacing the 0.010″ i.d. tubing that is commonly used with the smaller i.d. (<0.007″) tubing length to a minimum. The sensitivity increases with smaller i.d. but there was slight increase in the back pressure of the column; however, this remained constant throughout the study. Clean sample extraction techniques are essential for minimizing the ion suppression and matrix effect in the LC-MS/MS analyses. LLE was used for the sample preparation in this method. Different commonly used extraction solvents, such as methyl tert.-butyl ether, ethyl acetate, diethyl ether, and dichloromethane alone or in combination in different proportions, were investigated. It was found that methyl tert.-butyl ether showed good recovery with a clean background noise in blank human plasma.

Previous reported literature[3,9,8,23] has reported that R-138727 degrades in whole blood within 10–15 min after collection and when kept in an ice bath. The stability of R-138727 after reacting with NEM in whole blood and in plasma after separation from blood cells was studied using two different concentrations (3.0 and 375.18 ng/ml) at two different temperatures (room temperature and ice bath). The derivatized R-138727 was found to be stable in whole blood for 1 h when keep in the ice bath and for 6 h in plasma after separation from the blood cells.

Method validation

Specificity and selectivity

No significant interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. This was evaluated by injecting the extracted blank plasma samples of specified anticoagulant and then comparing the interferences with the response of the extracted LLOQ samples processed with the IS. In addition to the specified normal K_2EDTA plasma lots, selectivity evaluation was also carried out haemolized and lipemic plasma lots of the same anticoagulant to prove that there is no effect in the extraction efficiency of the analyte when the properties of the plasma condition change. Figure 2 shows the representative chromatogram of the blank plasma.

Linearity

Linearity of the standard curves was established by calculating the weighted linear regression from the p.a.r. of R-138727-NEM/IS. The

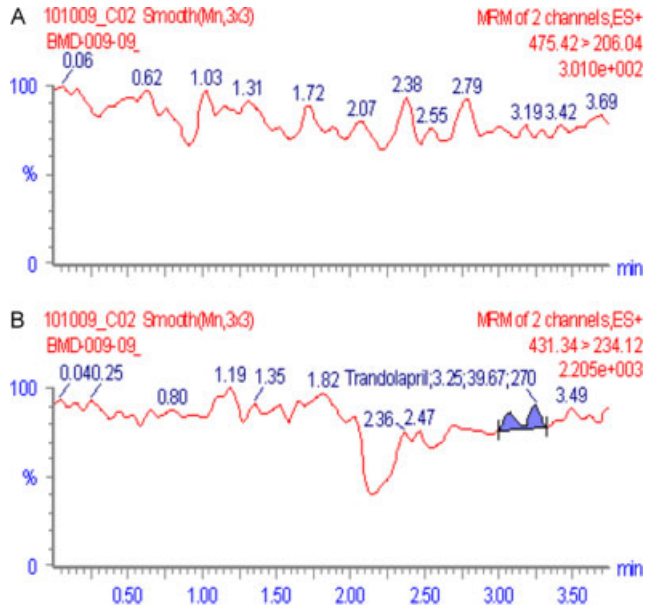

Figure 2. Ion chromatogram of R-138727-NEM (A) and IS (B) from extracted blank plasma.

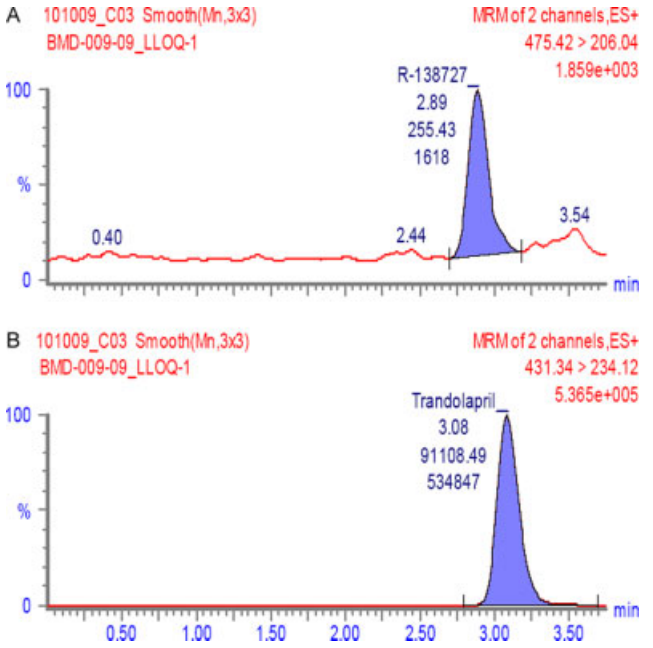

Figure 3. Ion chromatogram of R-138727-NEM (A) and IS (B) from extracted LLOQ (1 ng/ml) plasma standard.

Table 2. Back-calculated concentrations of calibration curve standards for R-138727-NEM in human plasma (n = 3)

Standard ID	Nominal concentration (ng/mL)	Concentration found (ng/mL)	Precision (CV %)	Accuracy (RE %)
CS 1	1.00	1.047 ± 0.0231	2.21	4.70
CS 2	2.00	1.813 ± 0.1021	5.63	−9.35
CS 3	25.02	23.013 ± 1.3031	5.66	−8.02
CS 4	50.05	50.823 ± 0.9372	1.84	1.54
CS 5	100.09	106.393 ± 1.6481	1.55	6.30
CS 6	200.18	208.667 ± 6.7683	3.24	4.24
CS 7	300.12	298.337 ± 3.6329	1.22	−0.59
CS 8	425.10	433.527 ± 3.5458	0.82	1.98
CS 9	500.12	494.967 ± 6.3499	1.28	−1.03
r^2	0.9958667			
Slope	0.0032271			
Intercept	0.00065065			

obtained values of the slope (a) and the intercept (b) were used in the equation y = ax + b to calculate the concentration of the QCs (x) from the measured p.a.r. (y).

P.a.r. for the nine-point calibration curve was found to be linear over the concentration range of 1.00–500.12 ng/ml. The values for r^2 (overall >0.99) indicate linearity over the whole calibration range. The range of accuracy and precision of the back-calculated concentration of the standard curve points for R-138727-NEM was from 90.65 to 106.30% and 0.82 to 5.66%, respectively. Table 2 summarizes the calibration curve results. The chromatogram of the extracted 1 ng/ml R-138727-NEM and IS are shown in Figure 3.

Accuracy and precision

The determination of within-batch precision and accuracy were evaluated by analysing six sets of QC samples in a single analytical batch whereas the between-batch precision and accuracy were evaluated by analysing six sets of QCs in three different batches. The nominal concentration of the QCs was calculated from freshly prepared CSs and the results of the between-batch precision were within 20% for the LLOQ QC and within 15% for the other QCs, while the accuracy was 100 ± 20% or better for the LLOQ QC and 100 ± 15% or better for the other QCs. The data of the within- and between-run are summarized in Table 3.

Table 3. The data of accuracy and precision for the determination of R-138727-NEM in human plasma

Within and between run accuracy and precision of R-138727 NEM		Precision (CV %)	Accuracy (RE %)
Batch I	LLOQ QC	6.78	−8.30
n = 6	LQC	4.39	−6.83
	M1QC	6.82	2.77
	MQC	3.19	−0.09
	HQC	3.55	−8.21
Batch II	LLOQ QC	6.49	−3.80
n = 6	LQC	5.84	−0.60
	M1QC	2.69	3.70
	MQC	2.87	6.13
	HQC	3.17	−2.26
Batch III	LLOQ QC	3.95	−7.60
n = 6	LQC	6.74	−4.80
	M1QC	5.63	4.08
	MQC	5.35	4.69
	HQC	4.92	−2.71
Between run	LLOQ QC	6.04	−6.50
n = 18	LQC	6.04	−4.03
	M1QC	5.04	3.52
	MQC	4.57	3.57
	HQC	4.73	−4.39

Table 4. Extraction recovery comparison of R-138727 using N-ethyl maleimide (NEM) vs 2-bromo-3′-methoxyacetophenone (MPB) derivatizing agent

	R-138727 -NEM			R-138727 -MPB	
	Non-extracted	Extracted	%Recovery	Extracted	%Recovery
	LQC	LQC		LQC	
Mean (N = 6)	1049.3	1008.2		1053.2	
SD±	26.46	29.5	96.08	76.4	100.37
%CV	2.52	3.16		9.513	
	MQC	MQC		MQC	
Mean (N = 6)	24 875	25 896		18593.3	
SD±	1259.14	1094.86	104.10	1266.5	74.75
%CV	5.06	4.5		7.058	
	M1QC	M1QC		M1QC	
Mean (N = 6)	82977.7	79904.2		75570.8	
SD±	963.69	2809.61	96.30	9783.6	91.07
%CV	1.16	3.54		13.864	
	HQC	HQC		HQC	
Mean (N = 6)	131449.7	118480.7		123769.3	
SD±	1154.82	2062.73	90.13	8797.3	94.16
%CV	0.88	1.76		7.599	
	Global Statistics				
	Mean		96.65		90.09
	SD±		5.729		10.933
	%CV		5.9		12.1

Recovery

The absolute recovery was performed by comparing the extracted QCs (LOQ, M1QC, MQC, and HQC) samples using NEM and MPB derivatizing agents[3] with the non-extracted equivalent standards. The recovery obtained at each level was within 90.1–104.1% for NEM, with mean precision less than 5.9 when compared with MPB. The recovery of the IS was 95.50%. The results are summarized in Table 4.

Dilution integrity and matrix effect

Dilution integrity for R-138727-NEM was evaluated by preparing QC samples with a concentration 1.6-times the concentration of the highest standard. Dilution QCs with a concentration of 800.38 ng/ml R-138727-NEM were prepared in plasma. These were diluted to two-fold and four-fold of the original concentration using screened pooled plasma and analysed against a freshly prepared calibration curve. The accuracy of the samples for the two-fold and four-fold dilutions of R-138727-NEM was 94.42 and 100.79%, respectively, which was within the acceptance criteria of 85–115% of nominal. Precision of R-138727-NEM for the dilutions two-fold and four-fold was 1.72 and 4.33%, respectively, which was within the acceptance criteria of ≤15%, proving the ability of the method to dilute the specimen samples in a linear fashion when the concentration of the unknown samples falls outside the calibration range. The results are depicted in Table 5.

Table 5. Dilution QC data of R-138727-NEM at 1/2 and 1/4 dilutions

	Nominal concentration (ng/mL):	
Sr. No.	Dilution factor 2 1/2	Dilution factor 4 1/4
1	744.88	793.35
2	757.46	789.19
3	752.59	801.65
4	763.13	779.45
5	776.04	875.98
6	740.16	800.38
Mean	755.710	806.660
SD±	12.9634	34.9071
%CV	1.72	4.33
% Nominal	94.42	100.79

Matrix effect was determined by the IS-normalization matrix factor[25] at the three concentration levels of LQC, MQC, and HQC using six different plasma lots that passed the selectivity criteria. Samples were processed in triplicate at each level to ensure that the concentration was independent of variability in matrix due to its physiological nature.

The percentage coefficient of variance of the LQC, MQC, M1QC, and HQC was 3.99%, 4.70%, 4.75%, and 3.20%, respectively, which was within the acceptance range of ≤15%.

Table 6. Stability data

Stability	Storage condition	Level	Mean comparison sample concentration found (ng/ml)	%CV	Mean stability sample concentration found (ng/ml)	%CV	Mean % changes
Bench top stability	Room temp (12h)	LQC	2.942	4.09	2.953	4.49	0.37
		HQC	378.085	5.233	385.012	1.2	1.82
Auto sampler stability	10 °C (48 h)	LQC	2.942	4.09	3.057	2.55	3.91
		HQC	378.085	5.233	385.402	0.68	1.93
Dry extract stability at	−20 °C	LQC	2.942	4.09	2.958	4.94	0.54
		HQC	378.085	5.233	382.485	1.8	1.16
Long-term stability	−20 °C (90 Days)	LQC	2.942	4.09	2.847	2.43	−3.23
		HQC	378.085	5.233	370.097	1.21	−2.11
Freeze and thaw	−20 °C (3 Cycle)	LQC	2.868	3.48	2.928	5.7	2.09
		HQC	356.463	2.18	359.222	3.13	0.77

Stock solution stability		Mean comparison peak area	%CV	Mean stability peak area	%CV	Mean % changes
Room temp (12h)	Analyte	67 598	1.91	69 408	3.3	2.68
	IS	70 185	0.93	65 672	1.29	−6.43
−20 °C (11 days)	Analyte	83 371	1.67	88 309	2.26	5.92
	IS	86 430	0.79	92 038	0.55	6.49

Table 7. Pharmacokinetic parameters

Treatment	Statistics	C_{max} (ng/ml)	AUC_{0-t} (ng/mL h)	AUC_{0-inf} (ng/mL h)	T_{max} (h)	T_{half} (h)
Treat(T)	Mean	118.52	228.86	239.94	0.71	5.97
	SD	43.555	36.642	40.103	0.1938	4.039
Referance®	Mean	163.56	278.89	290.22	0.608	5.6625
	SD	61.858	67.075	65.939	0.2153	1.82591
90% CI		52.25–100.45	71.41–97.36	71.74–97.56		
Intra subject CV %		34.61	16.06	15.92		

Stability

The stability experiment was performed to evaluate the stability of R-138727-NEM human plasma under different temperatures, timing conditions, and handling. To determine the stability, the screened pooled plasma was spiked with the LQC and HQC and was evaluated as short term at room temperature for 6 h, process stability at auto sampler for 48 h, dry-extract stability at −20 °C, long-term stability when stored at −20 °C for 90 days, and freeze–thaw stability. The stability samples were analysed and compared with freshly prepared QCs. The results obtained from the experiment were well within the acceptance criteria (≤15%). The results are summarized in Table 6. The stability of R-138727-NEM and IS in the stock solution was examined at room temperature for a period of 12.0 h and the long-term stability was examined when stored at 2°–8 °C for a period of 11 days, and the results were found to be within the acceptance limit (90–110%).

Application

This method was successfully applied to determine the plasma concentration of R-138727 following oral administration of a single 10-mg prasugrel tablet in six healthy subjects as part of a pilot bioequivalence study. Pharmacokinetic parameters were calculated from all the subjects who successfully completed periods I and II of the study. Some of the main pharmacokinetic parameters are given in Table 7. The mean plasma concentration versus time profile is shown in Figure 4.

The maximum mean concentrations of the test and the reference products were 118.52 ± 43.555 and 163.56 ± 61.858 ng/ml. The mean area under the plasma concentration–time curve was 228.86 ± 36.642 and 278.89 ± 67.075 ng.h/ml. The high intra-subject coefficient of variance in C_{max} recommended a larger sample size for the estimation of bioequivalence.

Conclusion

For the first time, a highly sensitive and selective method for the determination of the active metabolite of prasugrel R-138727 in plasma was developed using LC–MS/MS with the turbo-ESI method. The validation was performed in compliance with the EMA[26–27] and FDA[22] guidelines. All validation parameters met the acceptance criteria, which proved that the method for the determination of R-138727 after derivatizing with NEM in human plasma led to reliable results. This validated assay method was used in a pharmacokinetic study for the first time in which six Indian healthy male volunteers were given a dose of 10 mg of prasugrel.

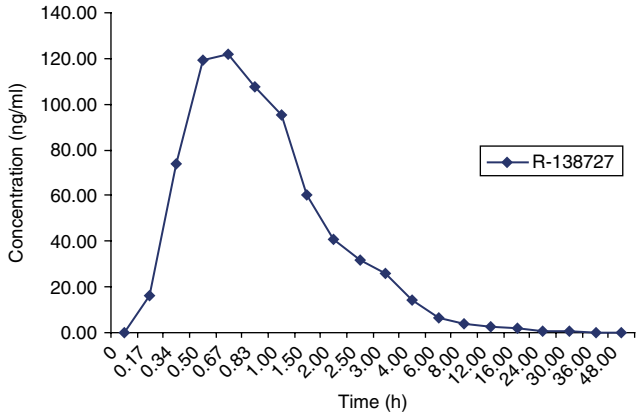

Figure 4. The mean plasma concentration versus time profile of R-138727.

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