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Validated LC–MS/MS method for the determination of sarpogrelate in human plasma: Application to a pharmacokinetic and bioequivalence study in Chinese volunteers

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ABSTRACT

A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was established for the determination of sarpogrelate in human plasma. One-step protein precipitation with acetonitrile was used to extract the analytes from the plasma. Sarpogrelate and tramadol (internal standard, I.S.) were separated on a Venusil MP-C_{18} column within 1.7 min, using acetonitrile:ammonium acetate (10 mM, pH 6.8) (55:45, v/v) as mobile phase at a flow rate of 1.2 mL/min with an approximately 1:1 split entering the mass spectrometer. Detection was performed on electrospray positive ionization mass spectrometry by multiple reaction monitoring of the transitions of sarpogrelate at m/z 430.3 → 135.3 and of I.S. at m/z 264.1 → 58.0. The assay was validated over the concentration range of 1–1000 ng/mL with a lower limit of quantitation (LLOQ) of 1 ng/mL using 50 μL of plasma. The intra- and inter-day precision (relative standard deviation, R.S.D.) were ≤6.4% and ≤5.4%, respectively, with accuracy (relative error, R.E.) in the range 0.5–3.6%. The method was successfully applied to a pharmacokinetic and bioequivalence study enrolling 22 Chinese volunteers administered sarpogrelate tablets.

1. Introduction

Recent report indicates that atherothrombotic vascular diseases are major causes of morbidity and mortality throughout the world [1]. Thrombus propagation on disrupted plaque induces acute coronary events or serious complication after coronary intervention. The 5-HT_{2A} receptor appears to play a crucial role in thrombus formation in diseased arteries via platelet aggregation and vasoconstriction. Sarpogrelate, a specific 5-HT_{2A} receptor inhibitor, is now commercially available as an anti-platelet aggregation agent for the prevention of arterial thrombosis. By virtue of its anti-platelet action, it is used for the treatment of peripheral vascular disease [2], for this agent inhibiting 5-HT mediated changes in intracellular Ca^{2+}, which induced proliferation of vascular smooth muscle cells [3–5]. Additionally, sarpogrelate has beneficial effects in coronary artery disease [6], restenosis after coronary stenting [7], gastroesophageal reflux [8], and diabetes mellitus [9]. The wide use, low toxicity and excellent pharmacological properties of sarpogrelate attracted much attention from pharmaceutical industry to perform pharmacokinetics and bioequivalence studies, however, up to now limited information is available on its pharmacokinetic behavior [10,11]. To current knowledge, sarpogrelate disappears from the plasma more rapidly after oral administration, of which a small proportion is converted to its intermediate metabolite (M-1) in plasma. In view of the relatively low plasma concentration (<1/10 of sarpogrelate) of M-1 [12] and limited systemic knowledge with respect to its pharmacological properties, sarpogrelate remains to be target-component monitored in the pharmacokinetic or bioequivalence study. Recently, an LC–MS/MS method [11] to determine sarpogrelate in human plasma was reported. However, this method suffered from several disadvantages, including relatively inadequate sensitivity with LLOQ (5 ng/mL) using large sample volume of 0.5 mL, a tedious and time-consuming sample preparation consisting of protein precipitation and solid-phase extraction. In addition, the lack of matrix effect evaluation, application to real clinical studies, as well as freeze/thaw and long-term sample stability study that are considerably important in sample storage [13], may invalidate the analytical results [14] and preclude its further utilization in clinical trials. Given the limitations of existing analytical methods, the requirement for an alternative, simplified, more sensitive assay that could facilitate clinical research regarding sarpogrelate formulations is clear.

The present paper reported an original LC–MS/MS method for the quantification of sarpogrelate in human plasma, requiring only a small sample volume (50 μL) and employing a simple sam-

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ple treatment using one-step precipitation. This method exhibited excellent performance in terms of recovery and matrix effect, and high sensitivity (LLOQ of 1 ng/mL). Eventually, the suitability of this LC–MS/MS method for routine bioanalysis is proved by its application to pharmacokinetic samples from clinical bioequivalence trials of two branded sarpogrelate tablets.

2. Experimental

2.1. Chemicals and reagents

Sarpogrelate hydrochloride (purity >99.0%) and tramadol hydrochloride (purity >99.5%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). The reference formulation was sarpogrelate tablet (each tablet containing 100 mg sarpogrelate) provided by Mitsubishi Pharmaceutical Company (Tokyo, Japan). Acetonitrile (HPLC-grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical grade and used without further purification. Distilled water prepared from demineralized water was used throughout the study. Heparinized blank (drug-free) human plasma was obtained from Changchun Blood Donor Service (Changchun, PR China) and kept at −20 °C until used.

2.2. Instrumentation

The LC–MS/MS system consisted of an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), which equipped with electrospray ionization (ESI) source for ion production. High purity nitrogen used for mass spectrometer was supplied by Changchun Institute of Optics, Fine Mechanics and Physics, Chinese Academy of Sciences. Data acquisition and integration were controlled by Analyst Software (Version 1.3, Applied Biosystem).

2.3. LC–MS/MS conditions

The chromatographic separation was performed on a Venusil MP-C_{18} column (100 mm × 4.6 mm I.D., 5 μm, Agela Technologies Inc., USA) maintained at 35 °C using a mobile phase of acetonitrile:ammonium acetate (10 mM, pH 6.8) (55:45, v/v) at a flow rate of 1.2 mL/min. The column effluent was split so that approximately 0.6 mL/min entered the mass spectrometer and the injection volume via autosampler (set as 18 °C) was 10 μL. The ionspray voltage (IS) was set at 5000 V for positive ionization and the heater gas temperature was 500 °C. Nitrogen was used as nebulizing gas (55 p.s.i), auxiliary gas (50 p.s.i) and curtain gas (15 p.s.i). The multiple reaction monitoring (MRM) experiments were conducted by monitoring the precursor ion to product ion transitions in the positive ion mode for sarpogrelate from *m*/*z* 430.3 (Q1) to *m*/*z* 135.3 (Q3) with Declustering Potential (DP) of 39 V and collision energy (CE) of 40 eV, and for I.S. from *m*/*z* 264.1 (Q1) to *m*/*z* 58.0 (Q3) with DP of 50 V and CE of 26 eV. The pause time was set at 10 ms and the dwell time at 200 ms.

2.4. Preparation of standard and quality control (QC) samples

A stock solution (1 mg/mL) of sarpogrelate in methanol was diluted with acetonitrile:water (80:20, v/v) to produce standard solutions with concentrations of 0.05, 0.15, 0.5, 1.5, 5, 15 and 50 μg/mL. Quality control (QC) solutions were prepared independently at concentrations of 0.15, 1.5, 15 and 40 μg/mL in the same way. 50 μL aliquot of each standard solution was mixed with 2450 μL of blank plasma to produce calibration standards of sarpogrelate with concentrations of 1, 3, 10, 30, 100, 300 and 1000 ng/mL. QC samples (3, 30, 300 and 800 ng/mL) were generated from QC solutions with the same procedure. A stock solution (1 mg/mL) of I.S. in methanol was diluted with acetonitrile:water (80:20, v/v) to give a final concentration of 10 ng/mL. All the solutions and QC samples were stored at −20 °C when not in use.

Table 1
The list of drugs that may be potentially coadministered with sarpogrelate and tramadol during clinical application.

Aspirin	Propranolol
Nimodipine	Captopril
Felodipine	Enalapril
Amlodipine	Losartan
Azelnidipine	Valsartan
Morphine	Metoprolol
5-Fluorouracil	Hydrochlorothiazide
Felbinac	Lovastatin
Ibuprofen	Simvastatin
Paracetamol	Glibenclamide
Pseudoephedrine	Glimepiride
Chlorphenamine	Gliclazide
Codeine	Glipizide
Nalmefene	Metformin
Probenecid	Repaglinide
Verapamil	Tolbutamide
Omeprazole	Domperidone

2.5. Sample preparation

The frozen samples were thawed at ambient temperature. A 50 μL aliquot of human plasma (or standard or QC samples) was placed into 1.5 mL Eppendorf tube. After adding 100 μL of I.S., the tube was briefly vortexed and 300 μL of acetonitrile was added to precipitate proteins. The mixture was vortex-mixed for 1 min and centrifuged at 12 000 × *g* for 10 min. The upper clear solution layer was collected, of which 10 μL aliquot was injected into the LC–MS/MS system for analysis.

2.6. Method validation procedure

2.6.1. Specificity and selectivity

Assay specificity and selectivity were defined by evidence of non-interference at retention times and ion channels identical to that of the analytes of interest in the blank samples and in the presence of concomitant medications. To investigate whether endogenous matrix constituents interfered with the assay, blank plasma samples from six different healthy subjects were analyzed. Cross-reactivity of 34 drugs (Table 1) that may be concomitantly administered with sarpogrelate and tramadol was also evaluated.

2.6.2. Precision and accuracy

Validation runs were conducted on 5 consecutive days. Each validation run consisted of three sets of calibration standards and six replicates of QC plasma samples at four concentrations (3, 30, 300 and 800 ng/mL), which were used to evaluate the precision and accuracy of the developed method. The intra- and inter-day precision (R.S.D.) were required to be below 15%, and the accuracy (R.E.) to be within ±15% [13].

2.6.3. Linearity and lower limit of quantitation and detection

Linearity in the range of 1–1000 ng/mL was assessed by weighted ($1/x^2$) least squares linear regression of calibration curves prepared and assayed in triplicate, which were constructed using the analyte/I.S. peak area ratio versus the analyte concentration. Lower limit of quantification (LLOQ) was defined as the lowest concentration of analyte that could be quantitatively determined with precision of ≤20% and accuracy within ±20% [13].

2.6.4. Recovery and matrix effect

The extraction recoveries of analyte were evaluated by a comparison of the peak area of analytes in extracted QC samples (3, 30, 300 and 800 ng/mL) in six replicates with those of blank plasma spiked after extraction at the corresponding concentrations. Matrix effects were determined by comparing the peak areas of blank plasma spiked after precipitation at four QC concentration levels (3, 30, 300 and 800 ng/mL) in six replicates with the mean peak areas of the standard solutions, which were prepared in the same way as QC samples except for the substitution of water for human plasma. Recovery and matrix effect of I.S. at 10 ng/mL were also investigated in the similar way.

2.6.5. Stability

The stability of sarpogrelate was evaluated under all the storage conditions at four QC concentration levels (3, 30, 300 and 800 ng/mL) in three replicates. The freeze/thaw stability test was performed after three complete freeze/thaw cycles (−20 to 25 °C). The long-term stability was assessed after storage of QC samples at −20 °C for 30 days. For the short-term stability, QC samples were kept at ambient temperature (25 °C) for 6 h and then analyzed. Stability of processed samples was assessed by re-injection of extracted QC samples after conservation 4 h in the autosampler (set at 18 °C). The stock solutions of sarpogrelate and internal standard stored at −20 °C were compared to a freshly prepared stock solution during 1 month.

2.7. Application of the method

A randomized, single-dose, two-period, two-sequence, and crossover design was used for the bioequivalence assessment of two branded sarpogrelate tablets, approved by the Ethic Committee of Affiliated Hospital, Academy of Military Medical Science of the CPLA. Twenty-two male volunteers (age 23.0 ± 2.7 years; body weight 64.1 ± 12.2 kg) were judged to be in good health through medical history, physical examination and routine laboratory tests. After a 12 h fast, subjects received a single oral dose of 100 mg sarpogrelate tablet (reference or test). Blood samples were collected into heparinized Eppendorf tube at pre-dose, and 0.08, 0.17, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 3.5 and 4 h after dosing. Plasma samples were separated by centrifugation at 12 000 × *g* for 10 min and stored at −20 °C until analysis. Plasma concentration–time data of sarpogrelate was analyzed by non-compartmental method using BAPP 3.0 software (programmed by China Pharmaceutical Uni-

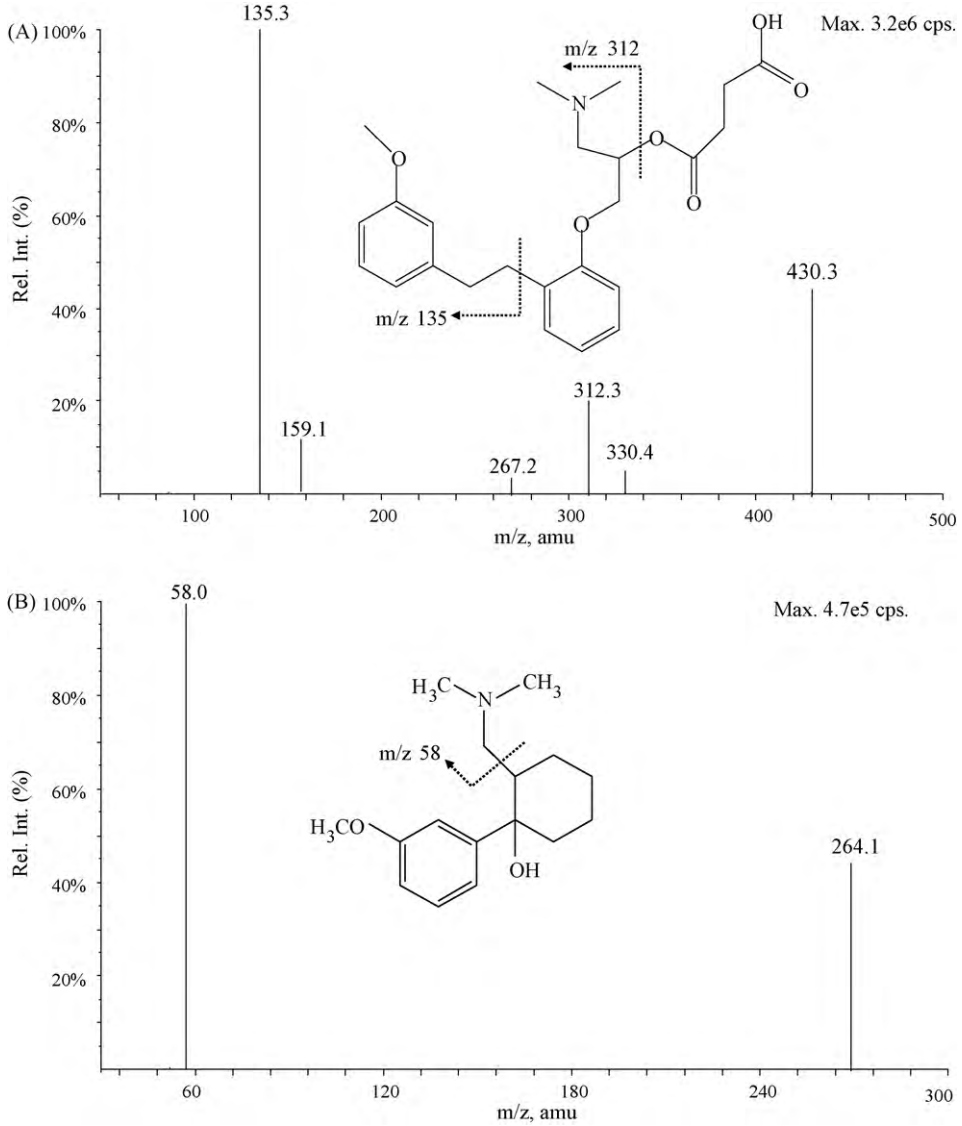

Fig. 1. Full-scan product ion mass spectra of $[M+H]^+$ ions for (A) sarpogrelate and (B) I.S.

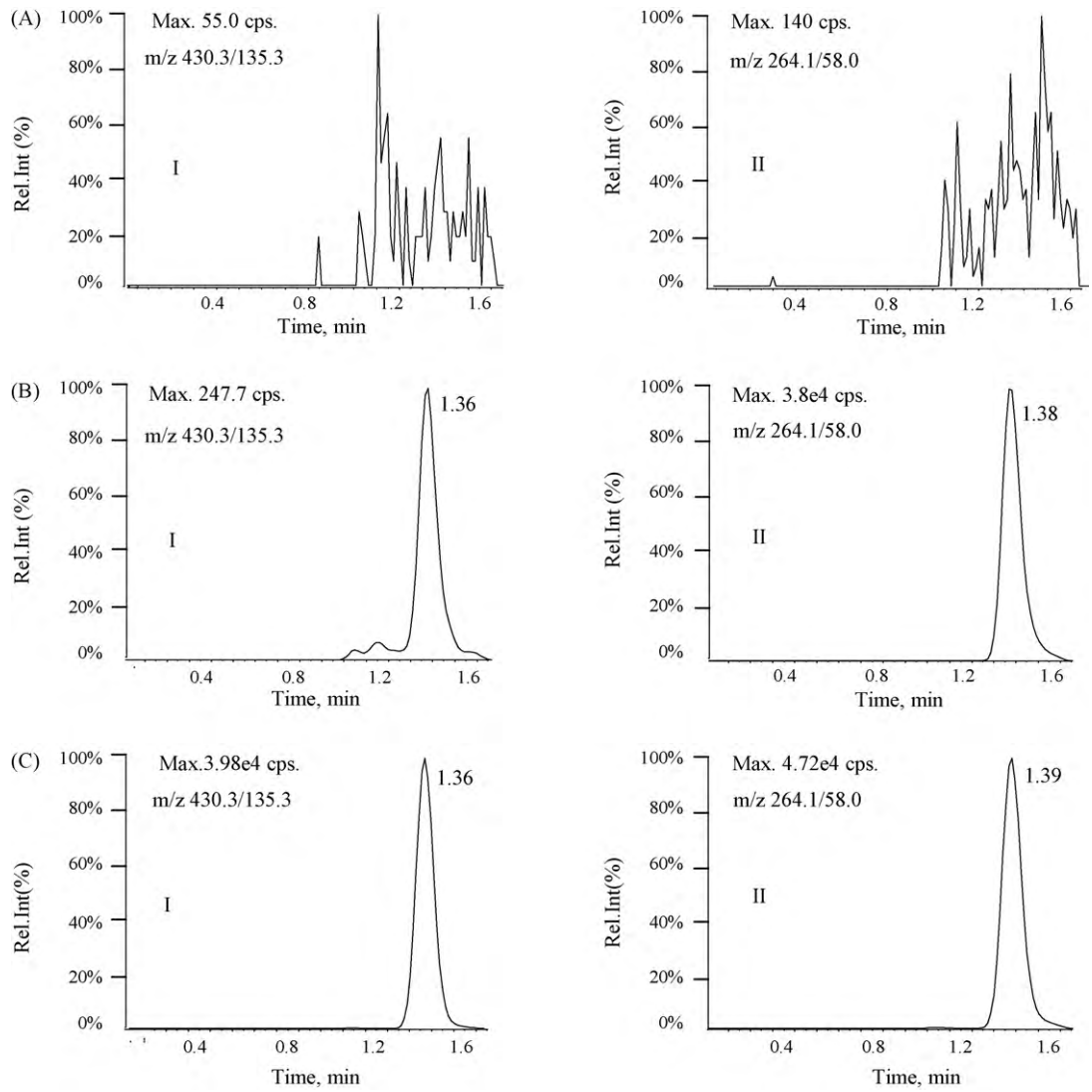

Fig. 2. Typical MRM chromatograms for (I) sarpogrelate and (II) I.S. in human plasma: (A) blank plasma; (B) blank plasma spiked with 1 ng/mL (LLOQ) sarpogrelate; and (C) a plasma sample (136 ng/mL) collected 1 h after a single sarpogrelate 100 mg tablet to a healthy volunteer.

versity). The bioequivalence of drugs was determined on basis of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}, by analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratios test/reference (logarithmically transformed data).

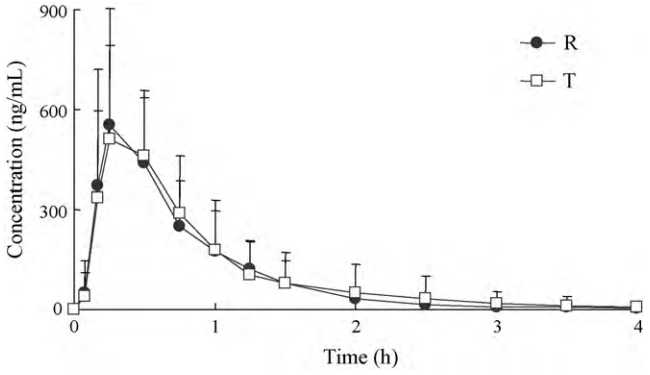

Fig. 3. Mean plasma concentration–time profiles of the reference (R) and test (T) formulations of sarpogrelate tablets in 22 healthy volunteers after a single oral dose of 100 mg (data are mean ± S.D., $n = 22$).

3. Results and discussion

3.1. Method development

Mass spectrometric detection was carried out on an API 4000 triple quadrupole instrument equipped with an ESI source operated in the positive ion mode. During optimization of the mass spectrometric parameters, strong and stable signals of sarpogrelate and I.S. can be observed in the form of their $[M+H]^+$ molecular ions with mass to charge ratios of m/z 430.3 and m/z 264.1, respectively. Different collision energy (CE) values for sarpogrelate were tested, and two major fragment ions of $[M+H]^+$ were obtained. At higher CE (40 eV), a major fragment ion at m/z 135.3 was formed, while at lower CE (25 eV), a major fragment ion was at m/z 312.3. Comparing the two transitions, the former gave a considerably better response and a higher signal. Fragmentation of I.S. produced the most abundant product ion at m/z 58.0. Therefore, the ion transitions m/z 430.3 → 135.3 and 264.1 → 58.0 (Fig. 1) were selected for MRM of the sarpogrelate and the I.S., respectively.

The separation of sarpogrelate was investigated on several different reversed phase columns (Zorbax extend-C_{18}, Hypersil-C_{18} and Venusil MP-C_{18}) to obtain a suitable retention time and symmetrical peak shape. The mobile phase optimization was

Table 2
Precision and accuracy for the determination of sarpogrelate in human plasma (data are based an assay of six replicates on 5 different days).

Spiked conc. (ng/mL)	Calculated conc. (ng/mL) (mean ± S.D.)	Intra-day, R.S.D. (%)	Inter-day, R.S.D. (%)	Accuracy, R.E. (%)
1 (LLOQ)	1.04 ± 0.05	6.2	5.1	3.6
3	3.01 ± 0.10	3.4	3.2	0.5
30	30.3 ± 1.2	1.4	4.3	1.0
300	304 ± 14	6.4	4.2	1.3
800	824 ± 43	4.4	5.4	3.1

Table 3
Stability of sarpogrelate in human plasma under various storage conditions ($n = 3$).

Storage conditions	Added conc. (ng/mL)	Calculated conc. (ng/mL) (mean ± S.D.)	R.E. (%)
In human plasma at room temperature for 6 h	3	3.08 ± 0.17	2.7
	30	28.5 ± 0.1	−5.0
	300	302 ± 3	0.7
	800	819 ± 32	2.4
In processed samples at 18 °C for 4 h	3	3.12 ± 0.19	4.0
	30	27.8 ± 0.7	−7.3
	300	301 ± 4	0.3
	800	816 ± 50	2.0
In human plasma after three freeze/thaw cycles	3	3.03 ± 0.22	1.0
	30	30.1 ± 0.7	0.3
	300	291 ± 6	−3.0
	800	771 ± 17	−3.6
In human plasma for 30 days at −20 °C	3	2.78 ± 0.10	−7.3
	30	30.7 ± 3.1	2.3
	300	326 ± 4	8.7
	800	791 ± 44	−1.1

Table 4
Pharmacokinetic parameters of reference and test sarpogrelate tablets (100 mg) based on its plasma concentrations (data are mean ± S.D., $n = 22$).

Parameters	Reference (mean ± S.D.)	Test (mean ± S.D.)	90% confidence interval
AUC_{0-t} (ng h/mL)	436.2 ± 141.0	459.1 ± 220.5	90.6–114.2%
$AUC_{0-\infty}$ (ng h/mL)	440.4 ± 142.6	465.6 ± 228.3	90.7–114.6%
C_{max} (ng/mL)	669.7 ± 320.3	650.1 ± 237.7	85.6–116.7%
T_{max} (h)	0.4 ± 0.2	0.4 ± 0.2	–
$t_{1/2}$ (h)	0.8 ± 0.1	0.8 ± 0.1	–

accomplished by comparing various solvent systems composed of mixtures of methanol, acetonitrile, water and ammonium acetate. Symmetrical peaks and satisfactory sensitivity were achieved, when water/acetonitrile mixture was used as the mobile phase instead of water/methanol, but retention time of the analyte was poorly reproducible on repeated injections. Thus, this mobile phase was deemed unacceptable for practical, routine used. Ammonium acetate (10 mM)/acetonitrile was preferable in consideration of peak shapes and reproducibility compared with water/acetonitrile. Lower proportion of the organic modifier of 25–35% had a long retention time and low ionization efficiency, while high proportion of organic modifier of 75–85% led to retention time falling into the column dead time, which caused severe matrix effects by co-eluting endogenous compounds. Therefore, the best combinations of peak shape and retention time were achieved using Venusil MP-C_{18} column (4.6 mm × 100 mm I.D., 5 μm) with a mobile phase of acetonitrile: ammonium acetate (55:45, v/v).

3.2. Selection of I.S.

An ideal I.S. used to guarantee high accuracy of LC–MS/MS assay should track the analyte during the extraction with almost the same recovery of analyte. It was also eluted close to the analyte on the column and compensated for potential inconsistent response for matrix effects. Due to the lack of stable isotopes for sarpogrelate in commerce, tramadol was adopted for its satisfactory positive-ion electrospray ionization, similar extraction efficiency and retention behavior to the analyte.

3.3. Method validation

The chromatograms were visually inspected for interfering chromatographic peaks from endogenous substances and concomitant medications. No visible interfering peak was observed in the retention time window of the analyte and I.S. (Fig. 2), and they were separated from the biological background with retention times of 1.36 and 1.38 min, respectively. The total analysis time for each run was 1.7 min. An excellent chromatographic specificity was also observed with no interference resulting from the 34 potentially concomitant drugs tested.

The calibration curves showed a good linearity in the concentration range of 1–1000 ng/mL with correlation coefficient ($r > 0.9975$) in triplicate on five separate occasions and the LLOQ for sarpogrelate was proved to be 1 ng/mL with acceptable precision and accuracy in Table 2.

The reproducibility of the method was defined by examining both intra- and inter-day accuracy and precision. Table 2 presents the results of the QC samples obtained during the method validation on 5 different days. The intra- and inter-day precision were ≤6.4% and ≤5.4%, respectively, with accuracy in the range 0.5–3.6%. The results above demonstrate that all these values are within the acceptable range and the method is accurate and precise.

The matrix effects ranged 95.9–101.9% for sarpogrelate and 90.7% for I.S., respectively, indicating no significant interfering resulted from co-eluting endogenous substances with the ionization of sarpogrelate or I.S. under the given conditions. The extraction recoveries of sarpogrelate from human plasma were 96.7 ± 2.5%, 99.3 ± 3.5%, 99.9 ± 1.9% and 97.0 ± 2.6% for the QC samples at four different concentrations, respectively, whereas 97.6 ± 5.1% for I.S. at 10 ng/mL. The results were shown to be consistent, precise and reproducible.

Stability results in Table 3 demonstrate that sarpogrelate is stable with decomposition <15% under the indicated conditions for the pharmacokinetic study. The stock solutions of sarpogrelate and internal standard stored for 1 month at −20 °C were comparable to the freshly prepared ones.

3.4. Clinical pharmacokinetics and bioequivalence study

The plasma concentration–time profiles after a single oral dose (100 mg sarpogrelate) of two tablet formulations are shown in Fig. 3. It is well absorbed after oral administration with a T_{max} of 0.4 h, which disappeared from the plasma rapidly with a $t_{1/2}$ of 0.8 h. The mean estimated pharmacokinetic parameters derived from the plasma concentration profiles of sarpogrelate are shown in Table 4. No significant differences were observed between the two formulations in relation to T_{max}, $t_{1/2}$, C_{max}, AUC_{0-t} and $AUC_{0-\infty}$, although these parameters showed great interindividual variation among subjects. The 90% confidence intervals (CIs) of the individual ratios (test formulation/reference formulation) of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ (after log-transformed) were within the bioequivalence acceptance range 80–125% adopted by US-FDA [15], indicating that the two tablet formulations were found to be bioequivalent.

4. Conclusion

A rapid, selective, sensitive and robust LC–MS/MS method was developed and validated for the determination of sarpogrelate in human plasma. The main advantages of this method were only a small volume of sample required (50 μL), simple and rapid sample treatment, excellent recovery and minor matrix effect. The method was shown to be adequate and reliable for application to a bioequivalence and pharmacokinetics study of two branded sarpogrelate formulations that depict pharmacokinetic behavior of sarpogrelate in Chinese volunteers.

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