

Original Article

Pharmacokinetic study of ACT-132577 in rat plasma by ultra performance liquid chromatography-tandem mass spectrometry

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Abstract: It was reported that macitentan was metabolized predominantly by cytochrome P450 3A4, and ACT-132577, its pharmacologically active metabolite, is fivefold less potent at blocking ET receptors than macitentan. In this work, a sensitive and selective ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for determination of ACT-132577 in rat plasma was developed and validated. After addition of diazepam as an internal standard (IS), protein precipitation by acetonitrile was used to prepare samples. Chromatographic separation was achieved on a UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) with 0.2% formic acid and methanol as the mobile phase with gradient elution. An electrospray ionization source was applied and operated in positive ion mode; multiple reactions monitoring (MRM) mode was used for quantification using target fragment ions m/z 546.9→200.6 for ACT-132577, and m/z 285.1→193.1 for IS. Calibration plots were linear throughout the range 10-4000 ng/mL for ACT-132577 in rat plasma. Mean recovery of ACT-132577 in rat plasma ranged from 82.6% to 90.6%, matrix effect of ACT-132577 in rat plasma ranged from 101.4% to 115.2%. RSD of intra-day and inter-day precision were both less than 11%. The accuracy of the method ranged from 96.1% to 103.5%. The method was successfully applied to pharmacokinetic study of ACT-132577 after oral and intravenous administration of macitentan.

Keywords: ACT-132577, UPLC-MS/MS, pharmacokinetics, rat

Introduction

Pulmonary arterial hypertension (PAH) is a chronic, progressive and fatal disease, characterized by increasing pulmonary vascular resistance leading to right ventricular failure and premature death. PAH has been shown to be associated with increased endothelin (ET) and ET-1 receptor upregulation, suggesting that the ET system is a therapeutic target for PAH [1, 2].

Macitentan is a novel dual orally active endothelin-1 receptor antagonist (ERA) to be used in patients with PAH. In preclinical and clinical studies, when compared with other ERAs, it exhibited sustained receptor binding and enhanced tissue penetration [3-5]. Macitentan reaches a maximum plasma concentration

(C_{max}) after approximately 8 hours (t_{max}), with an elimination half-life (t_{1/2}) of 17.5 hours [6]. It was reported that macitentan was metabolized predominantly by cytochrome P450 3A4, and ACT-132577 (**Figure 1A**), its pharmacologically active metabolite, is fivefold less potent at blocking ET receptors than macitentan [4]. However, due to its long half-life of about 48 hours, this metabolite is prone to accumulate upon repeated dosing and, therefore, significantly contributes to the overall effect [6, 7].

Up to now, several high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) methods have been reported to determine ACT-132577 [6-8]. Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) is a faster and more effi-

Pharmacokinetic study of ACT-132577 in rat

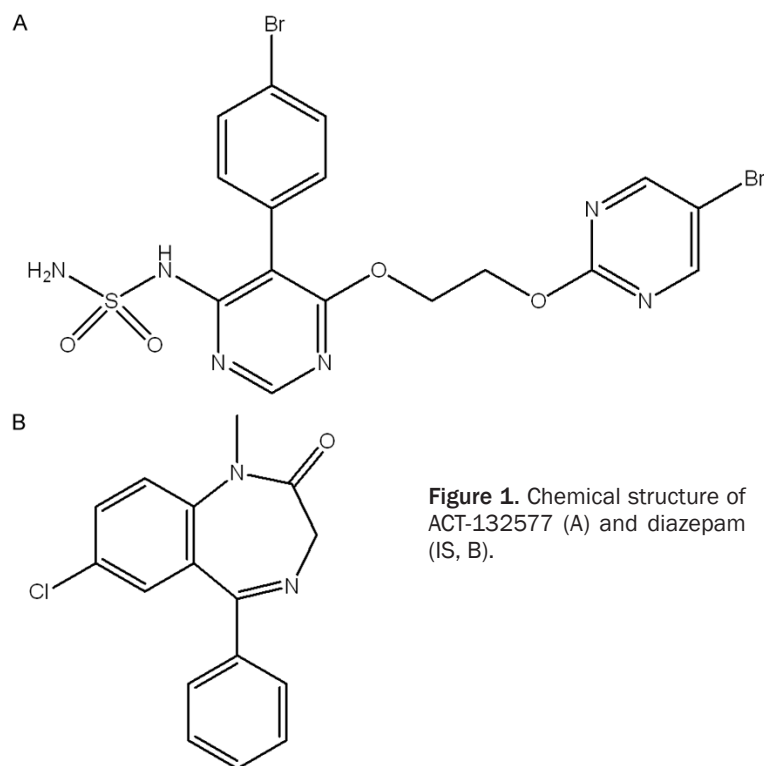


Figure 1. Chemical structure of ACT-132577 (A) and diazepam (IS, B).

cient analytical tool compared with HPLC-MS/MS [9, 10]. In present work, a fast, sensitive and reliable UPLC-MS/MS method was developed and validated for determination of ACT-132577 in plasma for the first time, and the method was successfully applied to a pharmacokinetic study in rats following oral and intravenous administration of macitentan.

Experimental

Chemicals and reagents

ACT-132577 (purity > 98%) and diazepam (IS, purity > 98%) were purchased from the Beijing Sunflower and Technology Development CO. LTD. (Beijing, China). LC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany). Ultra-pure water was prepared by Millipore Milli-Q purification system (Bedford, MA, USA). Rat blank plasma samples were supplied from drug-free rats.

Instrumentation and conditions

A UPLC-MS/MS system with ACQUITY I-Class UPLC and a XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA), equipped with an electrospray ionization inter-

face, was used to analyze the compounds. The UPLC system was comprised of a Binary Solvent Manager and a Sample Manager with Flow-Through Needle. Masslynx 4.1 software (Waters Corp., Milford, MA, USA) was used for data acquisition and instrument control.

ACT-132577 and diazepam (IS) were separated on an UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) maintained at 40°C. The initial mobile phase consisted of 0.2% formic acid and methanol with gradient elution at a flow rate of 0.4 mL/min and an injection volume of 2 μL. Elution was in a linear gradient, where the methanol increased from 40% to 90% between 0 and 2.0 min, maintained at 90% for 0.5 min,

then decreased to 40% within 0.1 min, then maintained at 40% for 0.4 min. The total run time of the analytes was 3 min. After each injection, the sample manager underwent a needle wash process, including both a strong wash (methanol-water, 50/50, v/v) and a weak wash (methanol-water, 10/90, v/v).

Nitrogen was used as the desolvation gas (1000 L/h) and cone gas (50 L/h). Ion monitoring conditions were defined as capillary voltage of 2.5 kV, source temperature of 150°C, and desolvation temperature of 500°C. MRM modes of m/z 546.9→200.6 for ACT-132577, and m/z 285.1→193.1 for IS were utilized to conduct quantitative analysis.

Calibration standards and quality control samples

The stock solutions of ACT-132577 (1.0 mg/mL) and diazepam (IS) (100 μg/mL) were prepared in methanol-water (50:50, v/v). The 0.25 μg/mL working standard solution of the IS was prepared from the IS stock solution by dilution with methanol; working solutions for calibration and controls were prepared from stock solutions in the same manner. All of the solutions

Pharmacokinetic study of ACT-132577 in rat

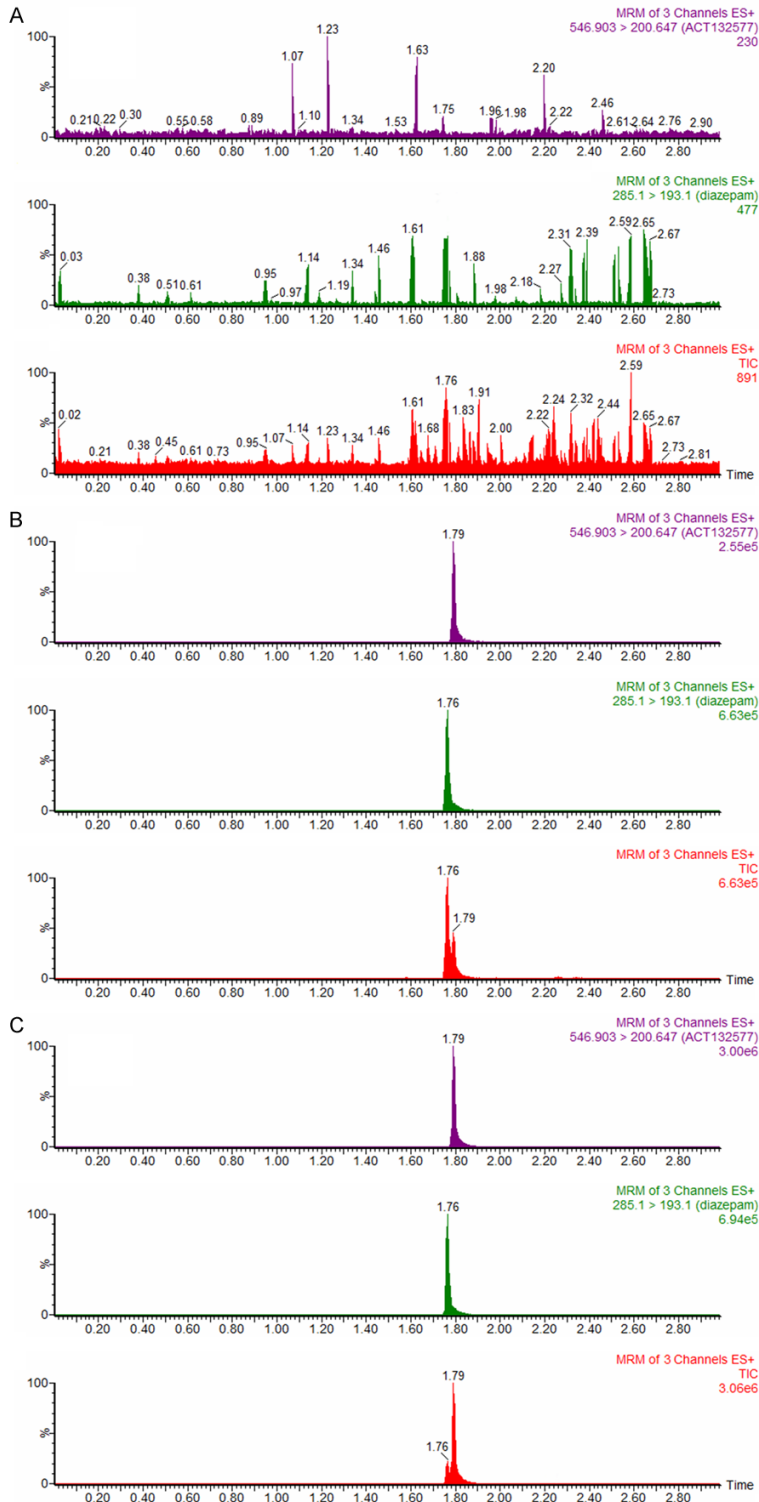


Figure 2. Representative UPLC-MS/MS chromatograms of ACT-132577 and diazepam (IS). (A) blank plasma, (B) blank plasma spiked with ACT-132577 (50 ng/mL) and IS (50 ng/mL), (C) a rat plasma sample 24 h after intravenous administration of single dosage 5 mg/kg macitentan.

were stored at 4°C and were brought to room temperature before use.

set of calibration standards and six replicates of QC plasma samples.

ACT-132577 calibration standards were prepared by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were offset to range between 10-4000 ng/mL for ACT-132577 in rat plasma (10, 20, 50, 100, 200, 500, 1000, 2000 and 4000 ng/mL). Quality-control (QC) samples were prepared in the same manner as the calibration standards, in three different plasma concentrations (20, 1800, and 3600 ng/mL). The analytical standards and QC samples were stored at -20°C.

Sample preparation

Before analysis, the plasma sample was thawed to room temperature. An aliquot of 10 µL of the IS working solution (0.25 µg/mL) was added to 50 µL of the collected plasma sample in a 1.5 mL centrifuge tube, followed by the addition of 150 µL of acetonitrile. The tubes were vortex mixed for 1.0 min. After centrifugation at 14900 × g for 10 min, the supernatant (2 µL) was injected into the UPLC-MS/MS system for analysis.

Method validation

Rigorous tests for selectivity, linearity, accuracy, precision, recovery, and stability, according to the guidelines set by the United States Food and Drug Administration (FDA) [11] and European Medicines Agency (EMA) [12], were conducted in order to thoroughly validate the proposed bio-analytical method. Validation runs were conducted on three consecutive days. Each validation run consisted of one

Pharmacokinetic study of ACT-132577 in rat

Table 1. Precision, accuracy, and recovery for ACT-132577 of QC samples in rat plasma (n=6)

Concentration (ng/mL)	RSD (%)		Accuracy (%)		Recovery (%)
	Intra-day	Inter-day	Intra-day	Inter-day	
20	8.8	10.2	102.8	103.5	90.6
1800	7.9	5.9	96.1	97.1	82.6
3600	5.0	10.1	103.5	101.8	88.7

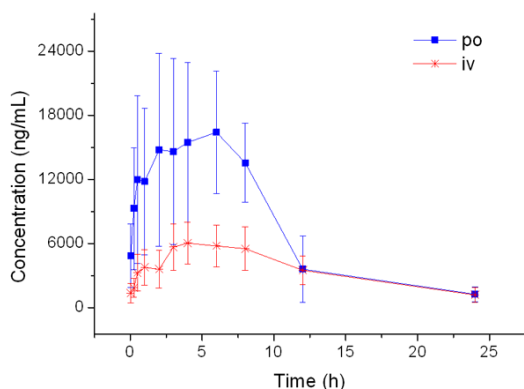


Figure 3. Mean ACT-132577 plasma concentration time profile after oral (po, 15 mg/kg) and intravenous (iv, 5 mg/kg) administration of macitentan in rats.

The selectivity of the method was evaluated by analyzing blank rat plasma, blank plasma-spiked ACT-132577 and IS, and a rat plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of ACT-132577-to-IS were plotted against analyte concentrations. Resultant standard curves were well fitted to the equations by linear regression, with a weighting factor of the reciprocal of the concentration ($1/x$) in the concentration range of 10-4000 ng/mL. The lower Limit of quantitation (LLOQ) was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank rat plasma was extracted and spiked with the analyte at 20, 1800, and 3600 ng/mL concentrations. The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, this peak area ratio is defined as the matrix effect. The matrix effect of the IS was evaluated at a concentration of 50 ng/mL in a similar manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (20, 1800, and 3600 ng/mL) over three days of validation testing. The precision is expressed as RSD.

The recovery of ACT-132577 was evaluated by comparing the peak area of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts (n=6). The recovery of the IS was determined in the same way.

Carry-over was assessed following injection of a blank plasma sample immediately after 3 repeats of the upper limit of quantification (ULOQ), after which the response was checked for accuracy [13].

Stability of ACT-132577 in rat plasma were evaluated by analyzing three replicates of plasma samples at concentrations of 20 or 3600 ng/mL which were all exposed to different conditions. These results were compared with the freshly-prepared plasma samples. Short-term stability was determined after the exposure of the spiked samples to room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 h. Freeze/thaw stability was evaluated after three complete freeze/thaw cycles (-20 to 25°C) on consecutive days. Long-term stability was assessed after storage of the standard spiked plasma samples at -20°C for 20 days. The stability of the IS (50 ng/mL) was evaluated similarly [14, 15].

Pharmacokinetic study

All twelve Male Sprague-Dawley rats (200-220 g) were obtained from the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China). The ethical number of the experiment animals was wyd2013-0071. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.2 mL) were collected from the caudal vein into heparinized 1.5 mL tapered plastic centrifuge tubes at 0.0333, 0.15, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 h after oral (15 mg/kg, n=6) and intravenous (5 mg/kg, n=6) administration of

Pharmacokinetic study of ACT-132577 in rat

Table 2. Primary ACT-132577 pharmacokinetic parameters after oral and intravenous administration of macitentan in rats (n=6)

Parameters	Unit	Mean	SD	Mean	SD
		Oral, 15 mg/kg		Intravenous, 5 mg/kg	
AUC _(0-t)	ng/mL*h	271898.5	90431.9	85919.3	28881.5
AUC _(0-∞)	ng/mL*h	343000.3	116453.6	99860.0	37412.2
t _{1/2}	h	7.4	2.9	7.6	2.0
CL	L/h/kg	0.05	0.02	0.06	0.03
V	L/kg	0.5	0.2	0.6	0.2
C _{max}	ng/mL	17208.9	7281.3	6570.9	2073.3

macitentan, respectively. The caudal vein of rat was cleaned by 75% alcohol, after that the end of caudal vein was cut by scissors. A 1.5 mL tapered plastic centrifuge tube was used to collect the blood which dropped from the end of caudal vein by squeezing and massaging gently. The samples were immediately centrifuged at 3000 × g for 10 min. The plasma as-obtained (50 µL) was stored at -20°C until UPLC-MS/MS analysis. Plasma ACT-132577 concentration versus time data for each rat was analyzed by DAS (Drug and Statistics) software (Version 2.0, Wenzhou Medical University, China).

Results and discussion

Method development

The mobile phase played a critical role in achieving good chromatographic behavior and appropriate ionization [16-21]. Methanol was selected for the organic phase, as it provides sharper peak shape and better sensitivity compared to acetonitrile. Methanol and water (containing 0.2% formic acid) were chosen as the mobile phase because the combination provides proper retention time and peak shape. The total run time for each injection was 3 min. Ultra performance liquid chromatography system using a gradient elution method could elute more residual impurities from column for each sample.

Efficient removal of proteins and other potential interference in the bio-samples prior to LC-MS analysis was a crucial step in the development of this method [22-27]. Then the simple protein precipitation was employed in our work, acetonitrile was chosen as the protein precipitation solvent because it exhibited acceptable recovery (between 82.6% and

90.6%) and matrix effect (between 101.4% and 115.2%).

Selectivity and matrix effect

Figure 2 shows typical chromatograms of a blank plasma sample, a blank plasma sample spiked with ACT-132577 and IS, and a plasma sample. There were no interfering endogenous substances observed at the retention time of the ACT-132577 and IS.

The matrix effect for ACT-132577 at concentrations of 20, 1800, and 3600 ng/mL were measured to be 101.4%, 113.2% and 115.2% (n=6). The matrix effect for IS (50 ng/mL) was 97.2% (n=6). As a result, matrix effect from plasma was considered negligible in this method.

Calibration curve and sensitivity

Linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 10-4000 ng/mL for ACT-132577 in rat plasma. The equation utilized to express the calibration curve was: $y=0.000289854*x+0.000559052$, $r=0.9953$, where y represents the ratios of ACT-132577 peak area to that of IS, and x represents the plasma concentration. The LLOQ for the determination of ACT-132577 in plasma was 10 ng/mL. The precision and accuracy at LLOQ were 13.8% and 92.6%.

Precision, accuracy and recovery

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three days of validation tests. Intra-day precision was 9% or less, and inter-day precision was 11% or less at each QC level. The accuracy of the method ranged from 96.1% and 103.5% at each QC level. Mean recovery of ACT-132577 were higher than 82.6%. The recovery of the IS (50 ng/mL) was 87.4%. Assay performance data was presented below in **Table 1**.

Carry-over

None of the analytes showed any significant peak (≥20% of the LLOQ and 5% of the IS) in blank samples injected after the ULOQ sam-

ples. Adding 0.4 extra minutes to the end of the gradient elution effectively washed the system between samples, thereby eliminating carry-over [13].

Stability

Results from the auto-sampler showed that the ACT-132577 was stable under room temperature, freeze-thaw, and long-term (20 days) conditions, confirmed because the bias in concentrations were within 90% and 115% of their nominal values. To this effect, the established method was suitable for pharmacokinetic study.

Application

The method was applied to a pharmacokinetic study in rats. The mean ACT-132577 plasma concentration-time curve after oral (15 mg/kg) and intravenous (5 mg/kg) administration of macitentan was shown in **Figure 3**. Primary pharmacokinetic parameters, based on non-compartment model analysis, were summarized in **Table 2**.

Conclusion

The developed and validated UPLC-MS/MS method, utilizing only 50 µL of plasma with an LLOQ of 10 ng/mL, was successfully applied to a pharmacokinetic study of ACT-132577 after both oral and intravenous administration of macitentan.

Disclosure of conflict of interest

None.

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Pharmacokinetic study of ACT-132577 in rat

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