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## Short Communication

# UPLC–MS/MS for the determination of azilsartan in beagle dog plasma and its application in a pharmacokinetics study

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## ABSTRACT

The purpose of the study is to develop an ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) to determinate the concentration of azilsartan in the dog plasma. After precipitated by methanol, the plasma sample containing azilsartan and diazepam (internal standard, IS) was determined by UPLC–MS/MS. The mobile phase consisted of acetonitrile–water was pumped at a flow rate of 0.3 ml/min in gradient elution. Kinetex 2.6  $\mu$  XB-C18 column (50  $\times$  2.1 mm, 100  $\text{\AA}$ ; Phenomenex, USA) were used for LC separations. The column temperature was 30  $^{\circ}\text{C}$  and the injection volume was 5  $\mu\text{l}$ . The electrospray ionization (ESI) and multiple reaction monitoring (MRM) were applied at the transitions of  $m/z$  457  $\rightarrow$  279 (azilsartan) and  $m/z$  285  $\rightarrow$  193 (diazepam), respectively. The developed method was identified a good linearity over a concentration range of 2.5–5000 ng/ml. The lower limit of quantitation (LLOQ) was 2.5 ng/ml. The intra-day and inter-day precision (relative standard deviation, RSD%) were less than 10% and accuracy (relative error, RE %) was less than 5% at three quality control levels. The extraction recovery of azilsartan at three quality control levels were  $82.41 \pm 0.68\%$ ,  $98.66 \pm 11.00\%$ ,  $102.43 \pm 0.82\%$ . And the recovery for IS (100 ng/ml) was  $91.75 \pm 0.54\%$ . A validated UPLC–MS/MS method was firstly developed for the quantification of azilsartan in dog plasma and it was applied to the pharmacokinetics study.

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## 1. Introduction

Azilsartan, a new generation of angiotensin II receptor blocker (ARB), was successfully developed by Takeda in 2012 and the trade name is Azilva<sup>®</sup>. It is the main active metabolite of azilsartan medoxomil [1] which was approved as an anti-hypertension drug by US Food and Drug Administration (FDA) in 2011. Azilsartan selectively binds to the angiotensin II AT<sub>1</sub> receptor to inhibit the actions of angiotensin II and results in lowering blood pressure (BP) [2]. According to the drug label and literatures, azilsartan is principally absorbed in the upper parts of small intestine, and the best absorption sites are duodenum and jejunum [3–5]. The volume of distribution of azilsartan is approximately 16 L and it is highly bound to human plasma proteins [6]. Azilsartan is metabolized to two main metabolites by cytochrome P450 2C9 (CYP2C9). And the major pathways for the elimination of azilsartan are feces and urine [5,7]. The recommended dose and maximum dose in adults are 20 mg and 40 mg taken orally per day. Compared with olmesartan and valsartan at maximal approved doses, azilsartan has superior efficacy at its maximal dose [8] and no significant increases in adverse events (cardiovascular, cerebrovascular and renal events) are observed [9,10]. Azilsartan shows sustaining reduction in BP even after 24 h administrated to rat and dog [11]. What's more, azilsartan is well tolerated in patients with hypertension [12].

At present, most literatures about azilsartan or azilsartan medoxomil focus on its antihypertensive efficacy and safety [13–16]. For future pharmacokinetics study, the method to quantify the concentration of azilsartan in plasma is needed. However, there is no reported UPLC–MS/MS method for determination of azilsartan in plasma.

In this study, methanol was added to the plasma sample to precipitate the protein, and the analyte and endogenous components were separated by the gradient elution. The UPLC–MS/MS method with simple procedure, high-sensitivity (LLOQ = 2.5 ng/ml) and short analytical time (3.5 min per sample) was first developed to assay the azilsartan in beagle dog plasma and was successfully applied to the pharmacokinetic study.

## 2. Materials and methods

### 2.1. Materials

Azilsartan (99.8%) was kindly provided by Weigao Pharm. Co. Ltd. (Shandong, China). Diazepam (internal standard, IS > 99.0% purity) was purchased from Baoyuan Pharm. Co. Ltd. (Shanxi, China). HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade Formic acid was purchased from Dikma (Richmond Hill, NY, USA). Double distilled water was used throughout the study. The azilsartan tablets (Azilva<sup>®</sup>) were purchased from Takeda Pharm. Co. Ltd. (Tokyo, Japan). The azilsartan test tablets were prepared in the laboratory.

### 2.2. Instrumentation

The UPLC–MS/MS system consisted of a Waters ACQUITY UPLC system and a Tandem Quadrupole (TQ) Detector with an electrospray ionization (ESI) interface (Waters Corp., USA). All data were processed by MassLynx 4.1 software with the QuanLynx program (Waters Corp., USA).

### 2.3. UPLC/MS/MS conditions

The analytical column used was Kinetex 2.6  $\mu$  XB-C18 column (50  $\times$  2.1 mm, 100  $\text{\AA}$ ; Phenomenex, USA). The column was maintained at 30 °C. The mobile phase was composed of solvent A (acetonitrile with 0.2% Formic acid) and solvent B (0.2% Formic acid). The gradient program started with 40% solvent A for 1.0 min. The percentage of solvent A was increased linearly to 80% from 1.0 min to 2.5 min and was decreased linearly to 40% from 2.51 min to 3.0 min. Then the percentage of solvent A maintained 40% from 3.0 min to 3.5 min and the gradient program was completed. The flow was always at 0.3 ml/min in the gradient program.

For MS detection, the electrospray ionization source was operated in a positive mode (ESI+). Cone voltage and collision energy were 20 V, 14 V for azilsartan and 30 V, 25 V for diazepam (internal standard, IS), respectively. The temperature and gas flow of de-solvent were 350 °C and 650 L/h. To analyze the drugs in plasma, multiple reaction monitoring (MRM) was used at the transitions of  $m/z$  457  $\rightarrow$  279 for azilsartan and  $m/z$  285  $\rightarrow$  193 for diazepam, respectively, with a scan time of 0.2 s.

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

The stock solutions of azilsartan and IS were prepared in methanol at 100  $\mu$ g/ml and 1 mg/ml, respectively. Then they were diluted to 10  $\mu$ g/ml for azilsartan and 100 ng/ml for IS with methanol-water (80:20, v/v) to obtain standard solutions. The standard solutions of azilsartan were used for preparing the calibration curve and QC samples. The calibration curve was prepared by spiking 200  $\mu$ l of blank plasma with 200  $\mu$ l of appropriate azilsartan standard solution and 200  $\mu$ l IS standard solution. Then 400  $\mu$ l of methanol was added to the sample. Finally, the calibration curve was prepared at the concentration of 2.5, 5, 10, 20, 50, 200, 500, 1000, 4000 and 5000 ng/ml. And the QC samples for azilsartan at the concentration of 5, 200, 4000 ng/ml was prepared as the same process.

### 2.5. Plasma sample preparation

200  $\mu$ l of IS standard solution (100 ng/ml) and 200  $\mu$ l of methanol-water (80:20, v/v) were added to 200  $\mu$ l plasma. The mixture was vortex-mixed for 3 min. 400  $\mu$ l of methanol was added to the sample to precipitate the protein and then the mixture was vortex-mixed for 3 min again. After that, the sample was centrifuged at 13,000 rpm for 10 min. At the end, 5  $\mu$ l supernatant was injected into the UPLC–MS/MS system.

## 2.6. Method validation

### 2.6.1. Selectivity

The selectivity of the method was to distinguish and quantify the analyte in the plasma samples. To confirm the selectivity of the method, the chromatograms of six different batches of blank plasma and the corresponding spiked samples were compared.

### 2.6.2. Calibration curve and lower limit of quantitation

The calibration curve was determined by plotting the peak area ratio (A, area of analyte/area of IS) versus the concentration using weighted ( $1/C^2$ ) least square linear regression. The lower limit of quantitation (LLOQ), 10 times greater than the ratio of signal-to-noise, was determined.

### 2.6.3. Precision and accuracy

The precision and accuracy expressed as relative standard deviation (RSD) and relative error (RE), respectively, were calculated by analyzing the QC samples at low (5 ng/ml), medium (200 ng/ml) and high (4000 ng/ml) concentration on three validation days.

### 2.6.4. Extraction recovery and matrix effect

The extraction recovery of azilsartan was assessed by comparing the peak areas of the extracted QC samples with those of the samples which mixed corresponding standard solutions with the extracted blank plasma. The matrix effect of azilsartan was determined by comparing the peak areas of the analyte at low, medium and high concentration in the extracted blank plasma with those of the corresponding

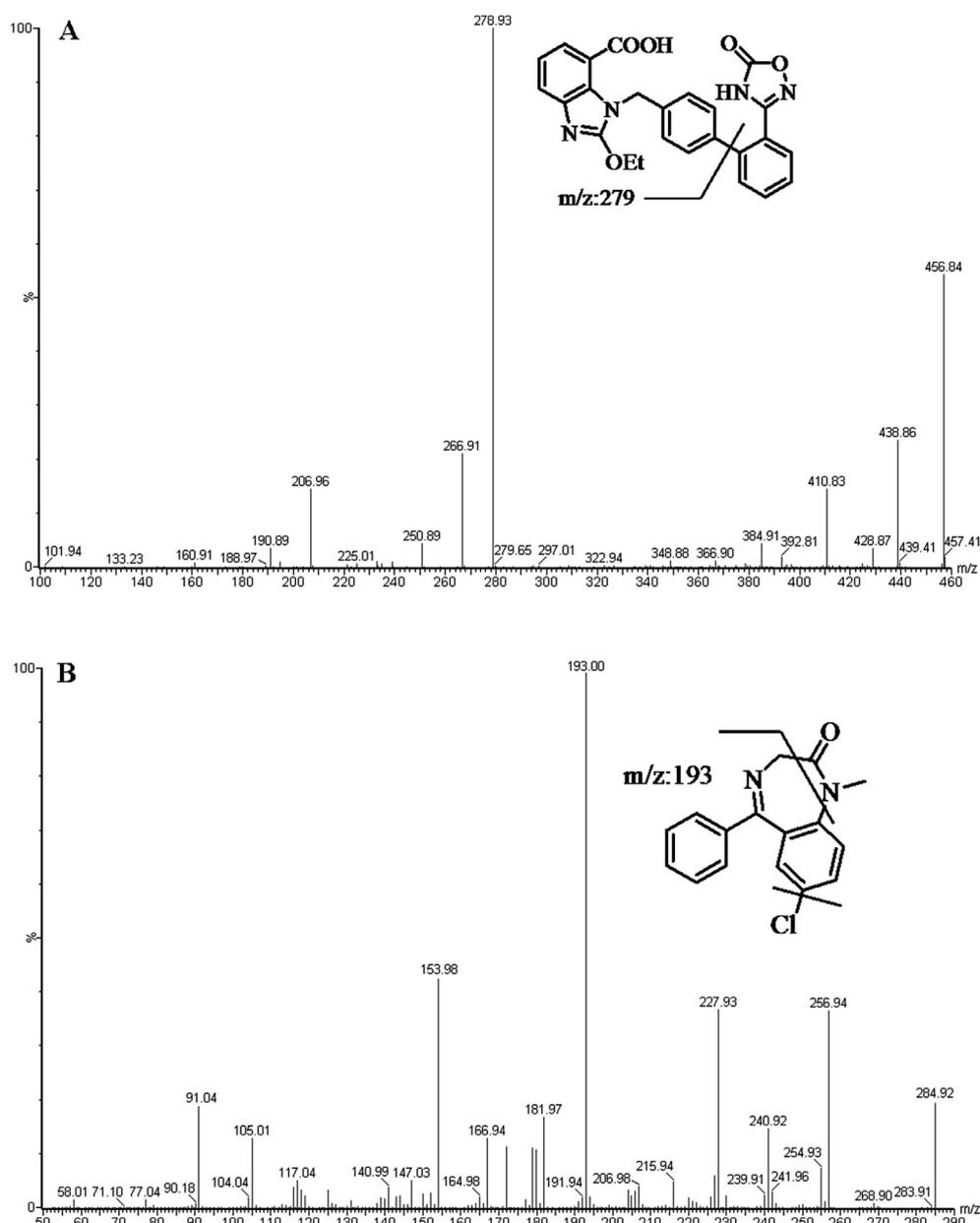


Fig. 1 – Mass spectra of azilsartan (A) and IS (B) in full scan positive ionization mode.

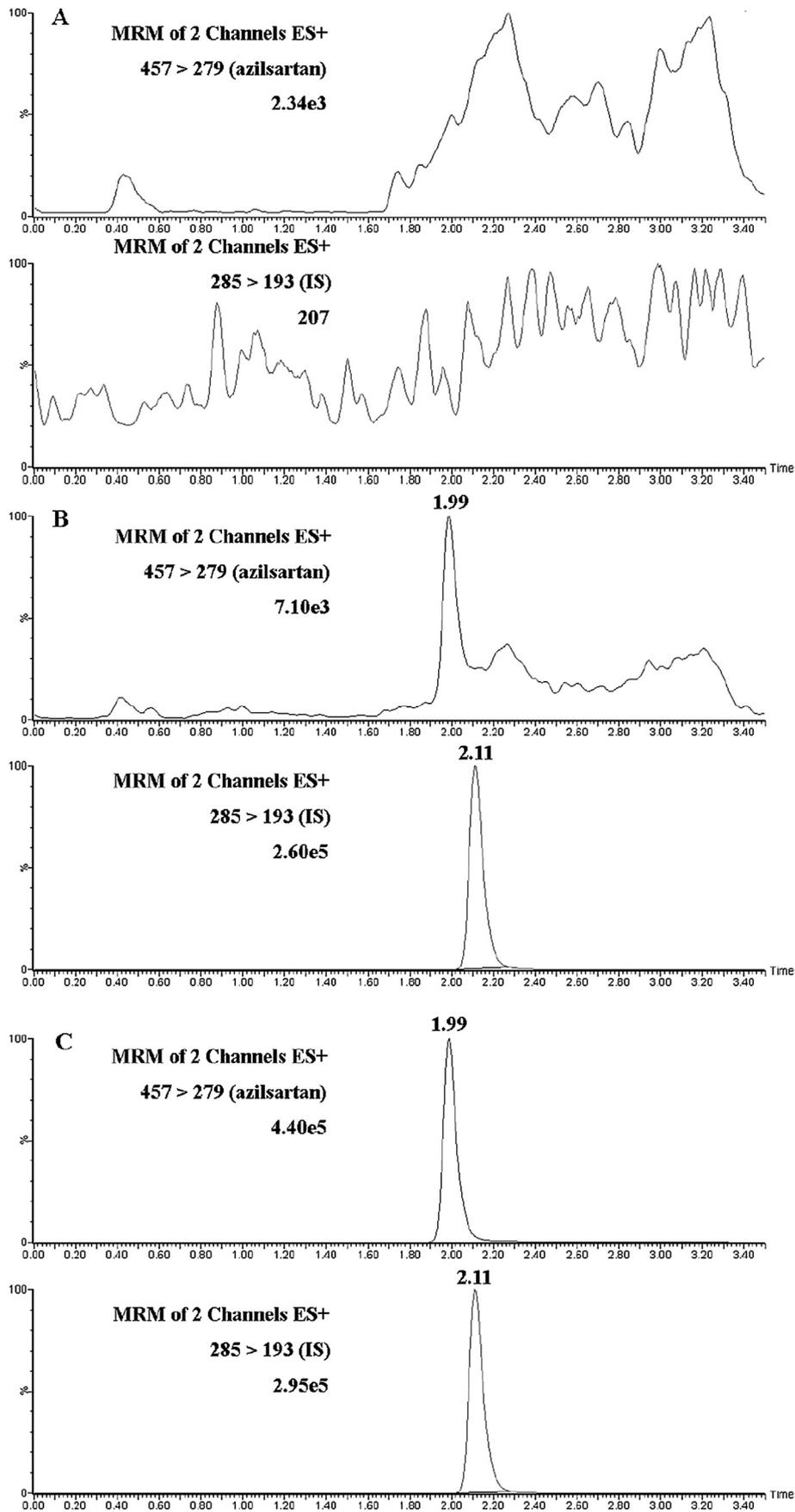


Fig. 2 – Representative MRM chromatograms of azilsartan and diazepam (IS) in dog plasma samples: (A) blank plasma, (B) blank plasma spiked with azilsartan (2.5 ng/ml) and diazepam (100 ng/ml), (C) plasma from a dog after single oral administration of 20 mg azilsartan.

standard solutions. The recovery and matrix effect of diazepam (IS) were also determined.

### 2.6.5. Stability

The stability test was regarded as an important part of the method validation. The stability of azilsartan in the plasma samples was evaluated under various conditions, including at ambient temperature (25 °C) for 12 h, post-processed for 12 h, freeze–thaw cycles for 3 times and long-term stability (–20 °C for 20 days). All stability tests were analyzed by QC samples ( $n = 3$ ) at low, medium and high concentration.

### 2.7. Pharmacokinetics application

The above-mentioned validated method was successfully used to analyze plasma samples for a pharmacokinetic study of azilsartan. The animal protocols in the study were approved by the Shenyang Pharmaceutical University Animal Care and Use Committee.

Six healthy beagle dogs (weight 10–12 kg) were segmented into two groups randomly, and study in a crossover experimental design. The washout period is a week. Dogs were fasted for a night before the experiment, but have free access to water. Each group was administered orally with commercial tablets (Azilva<sup>®</sup>, Reference) and self-made tablets (Test), respectively. The dosage of administration is 20 mg.

Blood samples (2 ml) were collected in heparinized tubes from forearm vein of the dog before drug administration and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 14 and 24 h post-dosing. Then the plasma was separated by centrifugation at 3000 rpm for 10 min and kept in the refrigerator at –20 °C until analysis.

The main pharmacokinetic parameters were calculated by the DAS 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

## 3. Results and discussion

### 3.1. Optimization of LC-MS/MS condition

Both azilsartan and diazepam (IS) are polar compounds. The electrospray ionization source was set to a positive mode (ESI+) in order to offer higher signal intensity for them. The cone voltage and collision energy of azilsartan and IS were developed for the best response of the parent and daughter ions. The protonated molecular ions  $[M + H]^+$  with greatest response in the daughter-ion mass spectrum were  $m/z$  279 for azilsartan and  $m/z$  193 for IS as shown in Fig. 1. Various mobile phases were attempted in this study. For the sake of obtaining

good peak shapes with short retention time, the mobile phase consisted of 0.2% formic acid and acetonitrile with 0.2% formic acid. The gradient elution in this method was developed to ensure that the assay was not interfered by endogenous substances.

### 3.2. Method validation

#### 3.2.1. Specificity

The specificity of the analytical method was evaluated by comparing the representative MRM chromatograms of (A) blank plasma, (B) blank plasma spiked with azilsartan (at the concentration of LLOQ) and IS (100 ng/ml), (C) plasma from a dog after single oral administration of 20 mg azilsartan in Fig. 2. And no endogenous interference for azilsartan or IS was observed. The analytical time was 3.50 min for each sample. The retention times of azilsartan and IS were 1.99 min and 2.11 min, respectively.

#### 3.2.2. Linearity and sensitivity

The calibration curves over the concentration range of 2.5–5000 ng/ml showed a good linearity. The typical regression equation was shown as the following equation:  $A = 0.130049C + 0.110229$  (correlation coefficient,  $r = 0.9971$ ), where A stands for the ratio of the peak area of analyte to the peak area of IS and C stands for the concentration of azilsartan in the plasma. All correlation coefficients exceeded 0.9950 on three validation days.

LLOQ was determined as 2.5 ng/ml and the precision (RSD %) and accuracy (RE %) values were 6.75% and –10.80%, respectively, within the acceptable range of  $\pm 20\%$ .

#### 3.2.3. Precision and accuracy

The intra- and inter-day precision and accuracy for the determination of azilsartan were listed in Table 1. From the results, it indicated that the intra-day precision and accuracy were all less than 6% while the inter-day precision and accuracy were all less than 9%. All the results were within the acceptable range of  $\pm 15\%$ .

#### 3.2.4. Extraction recovery and matrix effect

The extraction recoveries for azilsartan at low (5 ng/ml), medium (200 ng/ml) and high (4000 ng/ml) concentrations were  $82.41 \pm 0.68\%$ ,  $98.66 \pm 11.00\%$ ,  $102.43 \pm 0.82\%$ . The recovery for IS (100 ng/ml) was  $91.75 \pm 0.54\%$  ( $n = 6$ ).

The matrix effects for azilsartan at three concentration levels (5, 200 and 4000 ng/ml) were  $105.43 \pm 8.16$ ,  $99.41 \pm 3.43$ , and  $104.37 \pm 1.99\%$ , respectively. The matrix effects of IS was  $93.66 \pm 3.76\%$ . All the ratios of matrix effects for azilsartan and IS were within the acceptable range of  $\pm 15\%$ . The results

**Table 1 – Precision and accuracy for the determination of azilsartan in dog plasma samples by UPLC–MS/MS (intra-day,  $n = 6$ ; inter-day,  $n = 18$ ; mean  $\pm$  SD).**

| Added (ng/ml) | Intra-day          |         |        | Inter-day            |         |        |
|---------------|--------------------|---------|--------|----------------------|---------|--------|
|               | Found (ng/ml)      | RSD (%) | RE (%) | Found (ng/ml)        | RSD (%) | RE (%) |
| 5             | 4.75 $\pm$ 0.19    | 3.9     | –5.0   | 4.94 $\pm$ 0.44      | 8.9     | –1.2   |
| 200           | 191.05 $\pm$ 6.93  | 3.6     | –4.5   | 203.37 $\pm$ 13.03   | 6.4     | 1.7    |
| 4000          | 4144.72 $\pm$ 7.60 | 1.7     | 3.6    | 3994.24 $\pm$ 119.04 | 3.0     | –0.1   |

**Table 2 – Stability of azilsartan in beagle dog plasma under various conditions (mean  $\pm$  SD,  $n = 3$ ).**

|   | Add<br>(ng/ml) | Found<br>(ng/ml)    | RSD<br>(%) | RE%<br>(%) |
|---|----------------|---------------------|------------|------------|
| Ambient (25 °C) for 12 h                    | 5              | 4.67 $\pm$ 0.40     | 8.7        | -6.7       |
|   | 200            | 205.07 $\pm$ 5.06   | 2.5        | 2.5        |
|   | 4000           | 3619.10 $\pm$ 21.80 | 0.6        | -9.5       |
| Post preparation for 12 h                   | 5              | 4.43 $\pm$ 0.51     | 11.6       | -11.3      |
|   | 200            | 199.80 $\pm$ 5.39   | 2.7        | -0.1       |
|   | 4000           | 3620.73 $\pm$ 40.43 | 1.1        | -9.5       |
| Three freeze–thaw cycles                    | 5              | 4.47 $\pm$ 0.38     | 8.5        | -10.7      |
|   | 200            | 203.70 $\pm$ 5.14   | 2.5        | 1.8        |
|   | 4000           | 3646.47 $\pm$ 44.62 | 1.2        | -8.8       |
| Long-term stability<br>(-20 °C for 20 days) | 5              | 4.53 $\pm$ 0.35     | 7.7        | 9.3        |
|   | 200            | 203.77 $\pm$ 1.40   | 0.7        | 1.9        |
|   | 4000           | 3618.20 $\pm$ 40.85 | 1.1        | 9.4        |

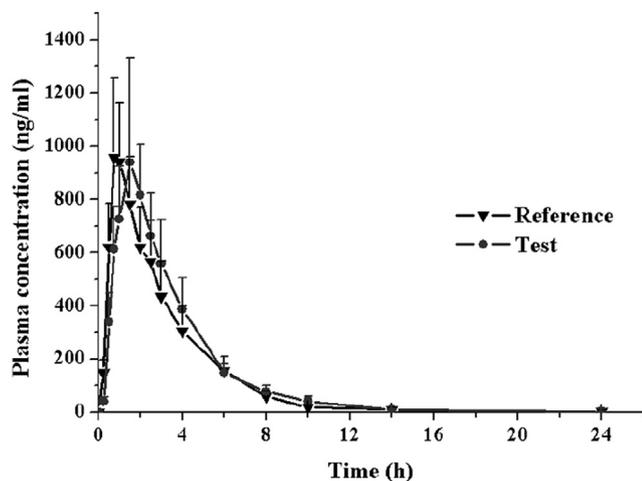
showed that no significant matrix effects for azilsartan and IS were discovered in this study.

### 3.2.5. Stability

The stability test was performed by analyzing replicates ( $n = 3$ ) of QC samples at low, medium and high concentrations under different conditions. All the data of the stability test were shown in Table 2. It suggested that the azilsartan showed a good stability in the dog plasma during the analytical procedure.

### 3.3. Pharmacokinetic studies

The validated UPLC–MS/MS method was first used for the study of pharmacokinetic behaviors of azilsartan in beagle dogs. The mean plasma concentration–time curves for azilsartan marketed tablets (Reference) and self-made tablets (Test) after oral administration to six healthy beagle dogs are shown in Fig. 3. The  $T_{max}$  and  $C_{max}$  were obtained directly from the curves. It was clearly indicated that  $C_{max}$  were 955.33  $\pm$  272.39 ng/ml for Reference and 939.68  $\pm$  335.41 ng/ml for Test, respectively, while



**Fig. 3 – Mean plasma concentration–time curves for azilsartan marketed tablets (Reference), self-made tablets (Test) after oral administration to six healthy beagle dogs (data were mean  $\pm$  SE,  $n = 6$ ).**

the  $T_{max}$  were 0.75  $\pm$  0.09 h and 1.50  $\pm$  0.28 h.  $AUC_{0-24h}$  values for Reference and the Test were found to be 3387.36  $\pm$  866.96 ng/ml·h and 3121.05  $\pm$  770.54 ng/ml·h, respectively. The data were shown as mean  $\pm$  standard error ( $n = 6$ ). The relative bioavailability of Test compared with Reference was 108.53%.

## 4. Conclusion

In this study, a rapid, selective and sensitive UPLC–MS/MS method was developed for the determination of azilsartan in beagle dog plasma. The precision, extraction recovery, matrix effect and stability of this method have been validated. It has been successfully used for the pharmacokinetic study of azilsartan in beagle dog plasma for the first time.

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