Original research paper

# Quantitative analysis and pharmacokinetic study of a novel diarylurea EGFR inhibitor (ZCJ14) in rat plasma using a validated LC-MS/MS method

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1-(4-(Pyrrolidin-1-yl-methyl)phenyl)-3-(4-((3-(trifluoromethyl) phenyl)amino)quinazolin-6-yl)urea (ZCJ14), a novel epidermal growth factor receptor (EGFR) inhibitor, with diarylurea moiety, displays anticancer effect. In the present study, an LC-MS/MS method was established to determine the concentration of ZCJ14 in rat plasma. Furthermore, the method was applied to investigate the pharmacokinetic characteristics of ZCJ14. Chromatographic separation of ZCJ14 and internal standard (IS) [1-phenyl-3-(4-((3-(trifluoromethyl)phenyl)amino)quinazolin-6-yl)urea] was accomplished by gradient elution using the Kromasil $C_{18}$ column. The selected reaction monitoring transitions were performed at $m/z$ 507.24→436.18 and 424.13→330.96 for ZCJ14 and IS, resp. The established method was linear over the concentration range of 10–1000 ng $mL^{-1}$. The intra- and inter-day precisions were < 11.0 % (except for *LLOQ* which was up to 14.3 %) and the respective accuracies were within the range of 87.5–99.0 %. The extraction recovery and matrix effect were within the range of 88.4–104.5 % and 87.3–109.9 %, resp. ZCJ14 was stable under all storage conditions. The validated method was successfully applied to the pharmacokinetic study of ZCJ14 in rats, and the pharmacokinetic parameters have been determined. The oral bioavailability of ZCJ14 was found to be 46.1 %. Overall, this accurate and reliable quantification method might be useful for other diarylurea moiety-containing drugs.

The epidermal growth factor receptor (EGFR) belongs to the family of receptor tyrosine kinases (RTKs), which plays a vital role in the regulation of tumor growth, differentiation, survival and invasiveness (1). Among the recognized RTKs, EGFR has been extensively studied and clinically validated as an important target for chemical therapies (2, 3). Various small molecules were synthesized and evaluated as potential inhibitors of EGFR. Among them, gefitinib, erlotinib and lapatinib were launched successfully to treat cancer

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in clinical studies (4, 5). The common structure shared by gefitinib, erlotinib and lapatinib is 4-anilinoquinazoline, whose structure-activity relationship was elucidated (6). However, in most of the patients that initially showed a dramatic response to these EGFR inhibitors, there was a progression of disease due to the acquisition of a second mutation in the EGFR-TK gene (7, 8). Thereupon, third-generation of EGFR inhibitors (osimertinib and WZ4002) was developed to overcome the second mutation, *viz.*, T790M mutation in the gatekeeper of the kinase domain (9–11). What is more, a great number of compounds with various structures have been developed as EGFR multi-target inhibitors to enhance the anticancer effects (12–18).

In our previous work, we integrated tertiary amine group, urea moiety and 4-anilinoquinazoline scaffold into one molecule to look for novel EGFR-TK inhibitors and anticancer agents (19). The results of biological activity evaluation *in vitro* indicated that the cell-based activity of the compound 1-(4-(pyrrolidin-1-yl-methyl)phenyl)-3-(4-((3-(trifluoromethyl)phenyl)amino)quinazolin-6-yl)urea (ZCJ14) increased by 7-fold compared to gefitinib against the lung adenocarcinoma epithelial cell line (A549). Further studies showed that compound ZCJ14 can potently suppress the tumor growth *in vivo*. Thus, ZCJ14 might serve as a potential EGFR inhibitor and anticancer candidate.

To carry on the pharmacokinetic study of ZCJ14, it is necessary to develop a new method that could determine ZCJ14 in biological samples. Different analytical methods for EGFR inhibitors have been searched for, developed and validated in diverse biological samples (20–23). Compared with HPLC and other methods, due to high sensitivity and specificity, LC-MS/MS method is more favorable for the pharmacokinetic study of a novel compound. Thus, the purpose of the present work is to establish a reliable method to extract and determine ZCJ14 from biological samples by using the LC-MS/MS method. To prove its feasibility, we verified indicators such as selectivity, precision, accuracy, matrix effect, recovery, stability and dilution effect of ZCJ14. Subsequently, the method was applied to the pharmacokinetics and bioavailability study of ZCJ14 *in vivo*. The data thus obtained could serve for further preclinical investigation and direct the clinical application of ZCJ14.

EXPERIMENTAL

*Reagents and solvents*

ZCJ14 (purity 96.9 %) and internal standard [IS, 1-phenyl-3-(4-((3-(trifluoromethyl)phenyl) amino)quinazolin-6-yl)urea, purity 98.0 %] were synthesized in our laboratory (struc-
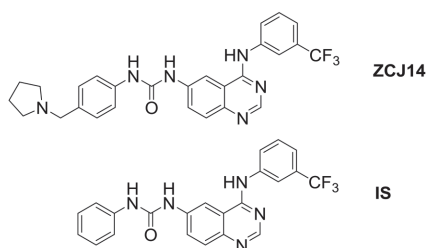


Fig. 1. Chemical structures of 1-(4-(pyrrolidin-1-yl-methyl)phenyl)-3-(4-((3-(trifluoromethyl)phenyl) amino)quinazolin-6-yl)urea (ZCJ14) and 1-phenyl-3-(4-((3-(trifluoromethyl)phenyl) amino)quinazolin-6-yl)urea (IS).

ture identification of ZCJ14 and IS are given in the supplementary material). The structures of both compounds are shown in Fig. 1. Methanol and acetonitrile (HPLC grade) were purchased from Merck (Germany). Formic acid (HPLC grade) was purchased from Kemiou Chemicals (China). The tri-distilled water was obtained by the Millipore water purification machine (Millipore, USA).

## Animals

Blank rat plasma was collected from Sprague-Dawley rats. Thirty male Sprague-Dawley rats (body mass: 250 ± 10 g) were obtained from the Laboratory Animal Center of Xi'an Jiaotong University. They fasted overnight before the experiment and had free access to water. The experimental procedures were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University (Shaanxi, China).

## Instrumentation

TSQ Vantage triple quadrupole LC-MS/MS system (Thermo Fisher Scientific, USA) was used. The chromatographic analysis was conducted by a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific) stocked with an Ultimate 3000 Pump and CTC Pal auto-sampler (CTC Analytics AG, Switzerland). Chromatographic separation of ZCJ14 and IS were accomplished by a Kromasil $C_{18}$ column (AkzoNobel, 150 × 4.6 mm, 5.0 μm).

Centrifugation was performed on a cryogenic high-speed centrifuge (Allegra X-22R, Beckman, USA). Vortex generator (Vortex-Genie2, Scientific Industries, USA) was used in sample preparation. The Xcalibur software (version 3.0.63) was used for instrument control and data processing.

## Optimization of LC-MS/MS conditions

Various experimental conditions were optimized to obtain symmetric and high-resolution peak shapes for the analyte. Mobile phases, such as acetonitrile/water, methanol/water and acetonitrile/methanol/water in different ratios, were evaluated to optimize the chromatographic conditions. It was discovered that the ionization and separation effects of methanol were more successful than those of acetonitrile. Formic acid was essential for the mobile phase to form a symmetric peak shape. Thus, as a result, two mobile phase eluents in a gradient mode were used for chromatographic analysis, including a mixture of 0.1 % formic acid (A) and methanol (B). The flow rate was 0.5 mL min$^{-1}$. The gradient elution was programmed as Table I. Ten (10 μL) of the samples were injected into the system through an auto-sampler, with the column temperature set to room temperature, and the tray temperature in the auto-sampler maintained at 4 °C.

*Table I. Gradient elution program*

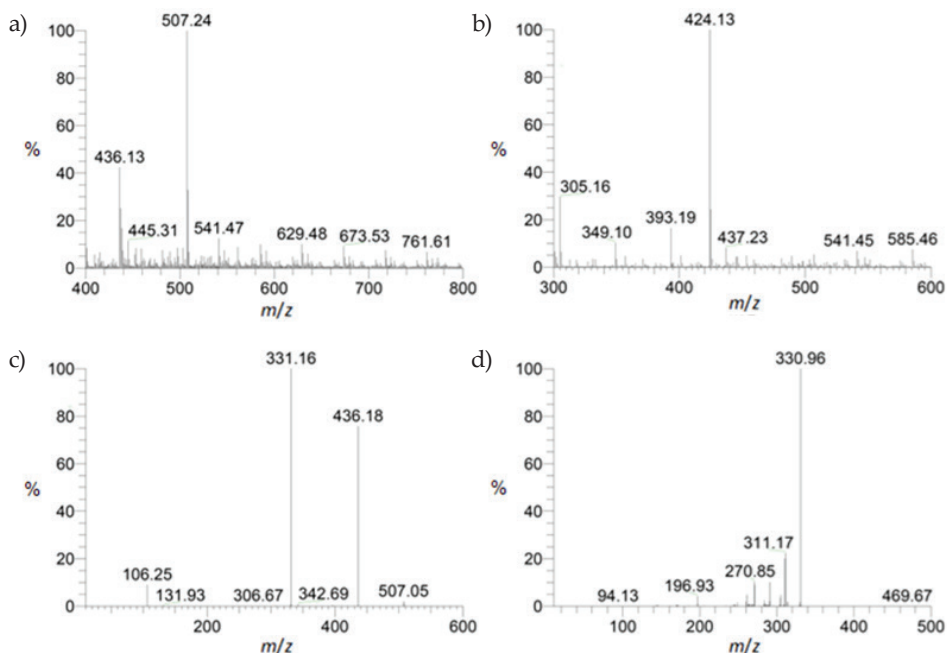| Time (min) | | 0 | 1 | 2 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|
| Mobile phase | A | 50 | 50 | 2 | 2 | 50 | 50 |
| | B | 50 | 50 | 98 | 98 | 50 | 50 |

Fig. 2. a) Precursor ion (*m/z* 507.24) of ZCJ14, b) precursor ion (*m/z* 424.13) of IS, c) product ions (*m/z* 436.18, 331.16, 106.25) of ZCJ14, d) product ions (*m/z* 330.96, 311.17, 270.85) of IS.

The mass spectrometer was operated in the electron spray ion positive mode. The acquisition was conducted at multiple reaction monitoring (MRM) modes. ZCJ14 and IS gave abundant singly charged protonated precursor ions [M+H]$^+$ at *m/z* 507.24 and 424.13 in Q1 scan mode, resp. (see Fig. 2). ZCJ14 and IS gave in abundance product ions at *m/z* 436.18 and 330.96 in the product ion scan, resp. (see Fig. 2). Therefore, the selected reaction monitoring transitions for ZCJ14 and IS were performed at *m/z* 507.24→436.18 (collision energy: 15.3 V) and 424.13→330.96 (collision energy: 26.1 V), resp. The radiofrequency (RF) lenses for ZCJ14 and IS were 158.3 V and 182.3 V, resp. Other mass spectrometer conditions were as follows: sheath gas pressure of 241.3 kPa, aux gas pressure of 55.2 kPa, positive ion spray voltage 3500 V, negative ion spray voltage 2500 V, ion transfer tube temperature 300 °C and vaporizer temperature 400 °C.

*Preparation of stock standards and quality control (QC) samples*

ZCJ14 and IS were dissolved in methanol to a concentration of 1.0 mg mL$^{-1}$ to prepare the stock solutions. Stock solutions were then diluted with methanol to prepare the working standards of ZCJ14 ranging from 200 to 20,000 ng mL$^{-1}$ and an IS solution of 200 ng mL$^{-1}$. Calibration standards in plasma were prepared as follows: 5 µL of each working standard was spiked with 95 µL blank rat plasma to reach the concentration of 10, 25, 50, 100, 250, 500, 1000 ng mL$^{-1}$. QC plasma samples were prepared at 20, 100, and 800 ng mL$^{-1}$ in the same manner.

## Sample preparation

The plasma samples were prepared *ut infra*: 100 µL of each plasma sample was spiked with 5 µL IS solution (200 ng mL⁻¹), followed by the addition of 500 µL acetonitrile. After a 20-s vigorous vortex mixing, the mixture was centrifuged for 15 min at 13000 rpm; the supernatant liquid was then filtered through Millipore filter and 10 µL of the filtrate was injected into the LC-MS/MS system.

## Method validation

During the course of method validation, selectivity, calibration curve, the lower limit of quantification (*LLOQ*), accuracy, precision, recovery, matrix effect, dilution effect and stability were evaluated (24).

*Selectivity.* – The selectivity of the method was validated by evaluating the interference of endogenous substances at the retention time ($t_R$) of ZCJ14 and IS. The chromatograms of six different blank rat plasma samples were compared with blank rat plasma spiked with ZCJ14 or IS, and rat plasma sample after intravenous administration or intragastrical administration of ZCJ14. The method is considered selective if the peak response of the endogenous substances was not exceeding 20.0 % of the mean peak response of ZCJ14 at *LLOQ* and not exceeding 5.0 % of the mean peak response of IS.

*Calibration curve and limits of quantification.* – Each calibration curve should consist of seven concentration levels (10, 25, 50, 100, 250, 500 and 1000 ng mL⁻¹). The ratio of peak area of ZCJ14 to IS was plotted against the corresponding standard nominal concentration of ZCJ14 to acquire the calibration curve. All the curves were fitted to the data by linear least square regression analysis ($1/x^2$ weighting) to determine the intercept, the slope and the correlation coefficient. The lower limit of quantification (*LLOQ*) was defined as the lowest concentration on the calibration curve which should have a signal/noise (*S/N*) ratio greater than 10. The calibration curve was acceptable if at least 75.0 % of the back-calculated concentrations of the standards were within ± 15.0 % of their nominal values, or within ± 20.0 % for the *LLOQ* (24). The *LLOQ* was estimated as 10 ng mL⁻¹ by measuring five replicates of the samples spiked with blank plasma, where the accuracy and precision (*n* = 5) should have been within ± 20.0 %. The upper limit of quantification (*ULOQ*) was evaluated by analyzing plasma samples with concentration levels over the highest calibration standard (1000 ng mL⁻¹). These plasma samples were analyzed after a tenfold dilution with blank rat plasma. The *ULOQ* was set at 1000 ng mL⁻¹ with precision and accuracy within ± 15.0 %.

*Accuracy and precision.* – Accuracy and precision of the established method were measured at four QC levels (10, 20, 100 and 800 ng mL⁻¹) in five replicates on the same day (intra-day) and five consecutive days (inter-day). Calibration curves were prepared daily and were used to determine the actual concentration at each QC level. The intra- and inter-day accuracy of the analytical method was expressed as a percentage, which was calculated by the ratio of the measured concentration to the nominal value. The precision of the analytical method was described by the proximity of repeated measurements of the analyte (expressed as RSD, %). The acceptance standard should be within 85.0–115.0 % of nominal concentration and 15.0 % RSD for accuracy and precision, resp., whereas for *LLOQ* QC (10 ng mL⁻¹), the accuracy and precision should be within ± 20.0 %.

*Extraction recovery and matrix effect.* – The extraction efficiency is evaluated by comparison of peak areas of plasma spiked with ZCJ14 carried by the sample preparation and processing procedures of the method *vs.* post-extraction blank plasma spiked with ZCJ14. The extraction recovery of ZCJ14 was measured at three levels of QC samples (20, 100, and 800 ng mL$^{-1}$) with five replicates.

The study of matrix effect (matrix factor) was carried out by comparing peak areas of the post-extraction blank matrix spiked with ZCJ14 *vs.* pure solution of ZCJ14. The measurement was performed at three QC levels of ZCJ14 (20, 100 and 800 ng mL$^{-1}$) in five replicates. The extraction recovery and matrix effect for IS were determined at the working concentration (200 ng mL$^{-1}$) in the same manner. The extraction recovery and matrix effect are acceptable if the inaccuracy and RSD are within ± 15.0 %.

*Dilution effect.* – The dilution effect experiment was performed to analyze real samples with the concentration of analyte beyond the upper limit of the calibration curve. Such plasma samples were diluted by ten folds with blank rat plasma and the dilution effect was measured by comparing the calculated concentrations of diluted plasma samples with the nominal values (expressed as a percentage). The acceptance standard should be within 85.0–115.0 % of nominal concentration (one-tenth) for the dilution effect.

*Stability.* – Stability investigations were conducted by assaying the plasma samples at three levels of QC samples (20, 100 and 800 ng mL$^{-1}$, each *n* = 5) under the following circumstances: (*i*) short-term stability was determined after storing at room temperature (25 °C) for 24 h, (*ii*) long-term stability was determined after storing at –20 °C for 15 days, (*iii*) three cycles of freeze-thaw (–20 to 25 °C) was conducted to determine the freeze-thaw stability. In each cycle, the samples were frozen for at least 24 h and thawed at room temperature. The analytes are assumed stable if the mean value do not exceed ± 15.0 % around the nominal concentration.

*Pharmacokinetic study and incurred sample reanalysis*

The ZCJ14 powder was dissolved in dimethyl sulfoxide (10.0 %, *V/V*), diluted with PEG 400 (70.0 %, *V/V*) and water (20.0 %, *V/V*) in attempt to prepare a solution with a concentration of 10 mg mL$^{-1}$ for oral application. Likewise, the ZCJ14 powder was dissolved in 1.0 % acetic acid aqueous solution (20.0 %, *V/V*), diluted with PEG 400 (10.0 %, *V/V*) and 5.0 % glucose injection (70.0 %, *V/V*) to obtain a solution of 5 mg mL$^{-1}$ for the *i.v.* administration.

Pharmacokinetic and bioavailability studies were carried out using twelve Sprague-Dawley rats. They were randomly divided into two groups (*n* = 6). The first group received intravenous injection through a jugular vein (25 mg kg$^{-1}$ bm) and the second group received an oral dose (50 mg kg$^{-1}$ bm ) *via* oral gavage. The dosage of this arose from the previous study (19). A maximum of 200 µL blood samples was collected from the rat femoral artery into heparin containing polypropylene tubes before dosing and at 0.167, 0.5, 1, 1.5, 2, 4, 6, 10, 20 h after the *i.v.* administration. For peroral administration, blood sampling was performed in the same way, before dosing and at 0.167, 0.5, 1, 2, 4, 12, 16, 18, 20, 22, 24, 26, 28, 36, 44 h after administration. Plasma was harvested after centrifuging the blood samples at 3000 rpm for 10 min at each time point. Plasma was then stored at –20 °C before analysis.

Pharmacokinetic parameters of ZCJ14 were calculated by PKSolver 1.0 (25) using non-compartmental methods (26). The maximum peak plasma concentration ($C_{max}$) and the time to reach $C_{max}$ ($T_{max}$) were determined based on the observed concentration-time curves. The area under the plasma concentration-time curve ($AUC_{0-inf\_obs}$) following administration *per os* was obtained by PKSolver 1.0. The *AUC* following the *i.v.* administration was calculated using the modified method, which was based on the trapezoidal rule and pseudo-equilibrium (27). The absolute oral bioavailability (*F*) was calculated using the following Equation *1*:

$$F(\%) = \frac{AUC(i.g.) / \text{dose}(i.g.)}{AUC(i.v.) / \text{dose}(i.v.)} \tag{1}$$

Incurred sample reanalysis (ISR) was conducted using 24 blood samples from twelve Sprague-Dawley rats mentioned above: two samples from each subject, one near $C_{max}$ and the other at the elimination phase. When the reanalyzed results are compared with the original data, the percentage difference in the value should be within ± 20.0 %.

## RESULTS AND DISCUSSION

### Method validation

*Specificity and selectivity.* – Representative chromatograms of blank rat plasma, blank rat plasma spiked with IS and ZCJ14 (*LLOQ*, 10 ng mL$^{-1}$), a plasma sample harvested at 0.5 h after the *i.v.* administration of 25 mg kg$^{-1}$ ZCJ14 to rats are shown in Fig. 3. The chromatograms revealed clearly that the interference peak from the endogenous matrix was not observed during the retention period. Chromatographic parameters investigated by the characterization of QC samples are shown in Table II. The results indicated the suitability of the method.
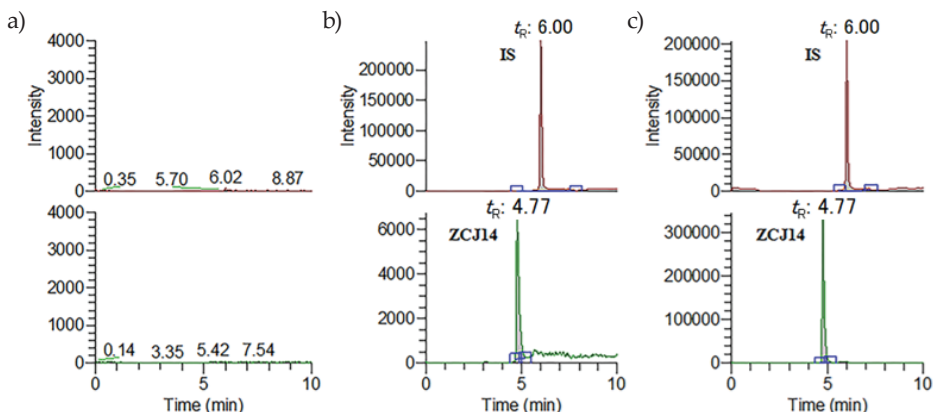


Fig. 3. Representative selected monitoring LC-MS/MS chromatograms [intensity (cps) *versus* time (min)] including: a) blank rat plasma, b) blank rat plasma spiked with 10 ng mL$^{-1}$ (*LLOQ*) ZCJ14 and IS, c) rat plasma sample collected 0.5 h after *i.v.* administration of ZCJ14.

*Table II. Chromatographic parameters*

| Compound | $t_R$ (min) | Instrument precision (RSD, %) | Capacity factor ($k'$) | Resolution ($R_s$) | Tailing factor ($T_f$) | Plate count ($N$) |
|---|---|---|---|---|---|---|
| ZCJ14 | 4.77 | 0.26 | 6.22 | – | 1.28 | 5919 |
| IS | 6.00 | 0.39 | 8.15 | 4.11 | 1.17 | 14994 |

*Calibration curve,* ULOQ *and* LLOQ. – Within the concentration range of 10–1000 ng mL$^{-1}$, calibration curves of rat plasma spiked with known amounts of ZCJ14 were linear with the coefficient of determination ≥ 0.99. All the back-calculated concentrations of the standards were within ± 12.5 % of their nominal values. The representative linear regression equation acquired by least squared regression was $y = 0.00367x − 0.00294437$ ($R^2 = 0.9904$), where $y$ is the ZCJ14/IS peak area ratio and $x$ is the concentration of ZCJ14.

The *LLOQ* for ZCJ14 was 10 ng mL$^{-1}$ (*S/N* > 10) and the precision and accuracy at *LLOQ* were within ± 12.1 %. The *ULOQ* for ZCJ14 was 1000 ng mL$^{-1}$ with the precision and accuracy within ± 8.6 %.

*Precision and accuracy.* – The precision and accuracy of ZCJ14 in rat plasma gained from analyzing QC samples at *LLOQ*, low, medium, and high levels (10, 20, 100 and 800 ng mL$^{-1}$) were satisfactory under the present method validation conditions. The intra- and inter-day precision of ZCJ14, as presented in Table III, were within the acceptance limits (RSD ≤ 14.3 %). Assay accuracy was found to be within the range of 87.5–99.0 % (*n* = 5).

The results indicated that the bioanalytical method developed in this study was precise and accurate enough for assaying ZCJ14 in rat plasma within the established concentration range.

*Matrix effect and extraction recovery.* – Table IV summarizes the matrix effect and extraction recovery of ZCJ14 and IS. The matrix effect for ZCJ14 was 93.6 ± 4.9, 109.9 ± 7.2 and 87.3 ± 5.3 % at concentration levels of 20, 100 and 800 ng mL$^{-1}$, resp. The matrix effect for IS (200 ng

*Table III. Inter- and intra-day precision and accuracy of ZCJ14 in rat plasma*

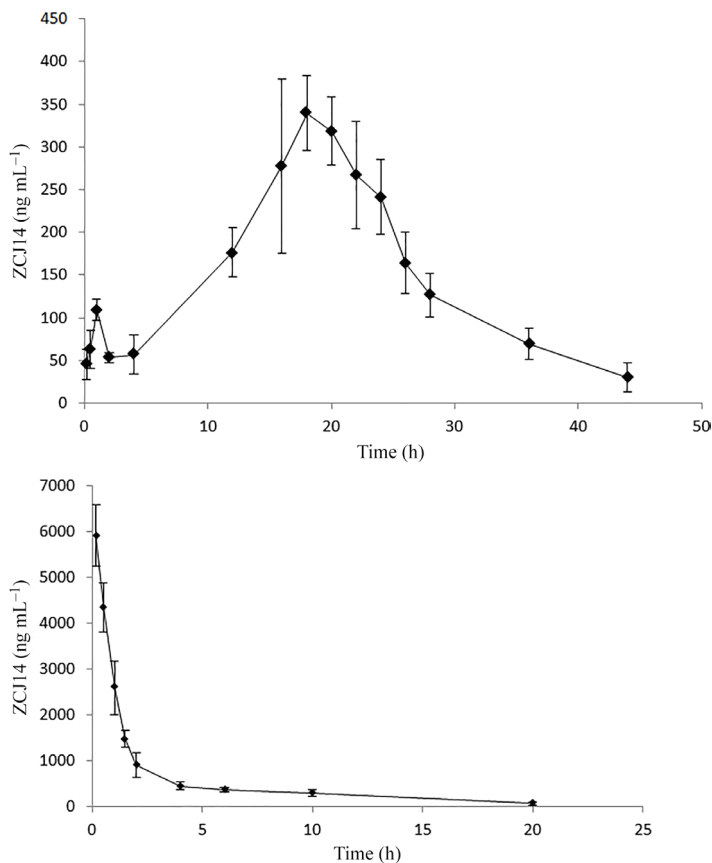| ZCJ14 nominal conc. (ng mL$^{-1}$) | Inter-day | | | Intra-day | | |
|---|---|---|---|---|---|---|
| | Measured concentration (ng mL$^{-1}$)[a] | Precison (RSD, %) | Model accuracy (%) | Measured concentration (ng mL$^{-1}$)[a] | Precison (RSD, %) | Model accuracy (%) |
| 10.0 | 9.9 ± 1.2 | 12.1 | 99.0 | 9.8 ± 1.4 | 14.3 | 98.0 |
| 20.0 | 17.5 ± 0.8 | 4.6 | 87.5 | 18.7 ± 2.0 | 10.7 | 93.5 |
| 100.0 | 94.5 ± 7.3 | 7.7 | 94.5 | 95.8 ± 5.8 | 6.1 | 95.8 |
| 800.0 | 743.5 ± 81.6 | 11.0 | 92.9 | 725.2 ± 49.4 | 6.8 | 90.7 |

[a] Mean ± SD, *n* = 5.

Fig. 4. Plasma concentration-time profiles of ZCJ14 in rats following a single peroral dose of: a) 50 mg kg$^{-1}$ and b) *i.v.* dose of 25 mg kg$^{-1}$ of ZCJ14. Data are expressed as mean ± SD (*n* = 6).

mL$^{-1}$) was 87.8 ± 5.2 %. The results showed that under the working conditions the matrix effects on ZCJ14 and IS were acceptable. At the same concentrations, the extraction recovery for ZCJ14 was 92.1 ± 12.7, 101.0 ± 2.9 and 88.4 ± 6.2 %, resp., whereas for IS it was 104.5 ± 9.7 %.

These data demonstrated that the extraction of the analyte from rat plasma could offer acceptable recovery with suitable precision and accuracy.

*Dilution effect.* – The dilution effect for ZCJ14 was measured by analyzing plasma samples at three concentrations (200, 1000 and 8000 ng mL$^{-1}$). Each sample was diluted 10 times with blank rat plasma before analyzing and the accuracy was 87.2, 86.9 and 105.4 %, resp. The results confirmed that the plasma samples could be diluted with blank rat plasma and analyzed with reasonable accuracy.

*Stability.* – As shown in Table V, room temperature storage of QC samples for up to 24 h has little effect on the quantification of ZCJ14 (accuracy between 93.0 and 112.4 %, RSD

*Table IV. Matrix effect and recovery of ZCJ14 and IS in rat plasma*

| Compound | Conc. (ng mL$^{-1}$) | Matrix effect (%)[a] | RSD (%) | Recovery (%)[a] | RSD (%) |
|---|---|---|---|---|---|
| ZCJ14 | 20 | 93.6 ± 4.9 | 5.2 | 92.1 ± 11.7 | 12.7 |
| | 100 | 109.9 ± 7.2 | 6.6 | 101.0 ± 2.9 | 2.9 |
| | 800 | 87.3 ± 5.3 | 6.1 | 88.4 ± 6.2 | 7.0 |
| IS | 200 | 87.8 ± 5.2 | 5.9 | 104.5 ± 9.7 | 9.3 |

[a] Mean ± SD, $n = 5$.

*Table V. Stability of ZCJ14 in rat plasma*

| Conditions | Nominal concentration (ng mL$^{-1}$)[a] | Found concentration (ng mL$^{-1}$)[a] | Accuracy (%) | RSD (%) |
|---|---|---|---|---|
| 24 h, room temperature | 20.0 | 18.6 ± 2.2 | 93.0 | 11.9 |
| | 100.0 | 106.2 ± 9.1 | 106.2 | 8.6 |
| | 800.0 | 899.5 ± 126.2 | 112.4 | 14.0 |
| 15 days, –20 °C | 20.0 | 19.9 ± 2.7 | 99.5 | 13.8 |
| | 100.0 | 112.9 ± 8.9 | 112.9 | 7.8 |
| | 800.0 | 814.6 ± 55.1 | 101.8 | 6.8 |
| Three freeze-thaw cycles | 20.0 | 19.6 ± 2.7 | 98.0 | 13.9 |
| | 100.0 | 109.8 ± 4.0 | 109.8 | 3.7 |
| | 800.0 | 806.3 ± 106.4 | 100.8 | 13.2 |

[a] Mean ± SD, $n = 5$.

*Table VI. Mean pharmacokinetic parameters of ZCJ14 in rats following per os or intravenous administration*

| Parameter | Type of administration | |
|---|---|---|
| | *p.o.* (dose: 50 mg kg$^{-1}$)[a] | *i.v.* (dose: 25 mg kg$^{-1}$)[a] |
| $AUC_{0\text{-inf\_obs}}$ (ng h mL$^{-1}$) | 6731.7 ± 694.8 | 7294.3 ± 1499.6 |
| $MRT_{0\text{-inf\_obs}}$ (h) | 22.2 ± 2.1 | 5.3 ± 1.5 |
| $t_{1/2}$ (h) | 7.7 ± 2.9 | 5.9 ± 1.4 |
| $T_{max}$ (h) | 18.0 ± 1.3 | – |
| $C_{max}$ (ng mL$^{-1}$) | 350.4 ± 63.7 | – |
| $Cl$ (mL h$^{-1}$ g$^{-1}$) | – | 2.0 ± 0.2 |
| $Vz$ (mL g$^{-1}$) | – | 17.2 ± 3.3 |
| $F$ (%) | 46.1 | – |

[a] Mean ± SD, $n = 6$.

within 14.0 %). ZCJ14 was stable at –20 °C for 15 days with accuracy values 99.5–112.9 % and RSD < 13.8 %. The changes in ZCJ14 concentration after three freeze-thaw cycles indicated no stability problems (accuracy between 98.0 and 109.8 %, RSD < 13.9 %).

## Pharmacokinetic study and incurred sample re-analysis

The newly-established LC-MS/MS method has been successfully applied in this research and achieved satisfying results for the quantification of ZCJ14 in rat plasma after *i.v.* or *per os* administration of 25 or 50 mg kg⁻¹ ZCJ14, resp. As some of the plasma samples exceeded the standard curve range, proper dilution of these samples was carried out with blank rat plasma using the verified dilution factors and re-analyzed. The mean plasma concentration-time profiles are shown in Fig. 4. The pharmacokinetic parameters calculated by non-compartmental analysis are summarized in Table VI.

In the plasma concentration-time profiles of ZCJ14 after administration *per os*, two peak concentrations were observed after 1 h and 18 h and the concentration of the first peak was lower than the latter one. Therefore, we put forward two possible reasons for this double-peak phenomenon. Firstly, the occurrence of the first peak concentration may be related to the solubility of ZCJ14. Compound ZCJ14 is lipophilic. After peroral administration, only one portion of ZCJ14 was absorbed into the bloodstream. Thus the absorption of ZCJ14 was slow. With absorbing the ZCJ14, blood concentration increased slowly, which led to the formation of a second peak after a long period of time. Secondly, gastric motility may be affected by ZCJ14. This may explain the formation of double peaks in the

*Table VII. Incurred sample re-analysis data of ZCJ14*

| | Per os (dose: 50 mg kg⁻¹ bm) | | | | | | Intravenous (dose: 25 mg kg⁻¹ bm) | | | | |
| Subject no. | Sampling point (h) | Initial conc. (ng mL⁻¹) | Reanalyzed conc. (ng mL⁻¹) | Mean | Difference (%)ᵃ | Subject no. | Sampling point (h) | Initial conc. (ng mL⁻¹) | Reanalyzed conc. (ng mL⁻¹) | Mean | Difference (%)ᵃ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 18.0 | 328.3 | 320.7 | 324.5 | 2.3 | 7 | 0.2 | 5045.7 | 5187.3 | 5116.5 | -2.8 |
| 1 | 44.0 | 56.7 | 51.9 | 54.3 | 8.8 | 7 | 10.0 | 282.9 | 292.1 | 287.5 | -3.2 |
| 2 | 18.0 | 300.2 | 319.7 | 310.0 | -6.3 | 8 | 0.2 | 6060.2 | 6005.5 | 6032.8 | 0.9 |
| 2 | 36.0 | 60.7 | 59.8 | 60.2 | 1.5 | 8 | 10.0 | 375.1 | 351.7 | 363.4 | 6.4 |
| 3 | 20.0 | 291.6 | 278.6 | 285.1 | 4.6 | 9 | 0.2 | 5563.0 | 5479.7 | 5521.4 | 1.5 |
| 3 | 36.0 | 45.6 | 41.7 | 43.6 | 8.9 | 9 | 10.0 | 389.3 | 390.0 | 389.6 | -0.2 |
| 4 | 18.0 | 339.1 | 328.4 | 333.8 | 3.2 | 10 | 0.2 | 6115.7 | 5914.4 | 6015.0 | 3.3 |
| 4 | 36.0 | 57.8 | 52.4 | 55.1 | 9.8 | 10 | 10.0 | 276.1 | 299.6 | 287.8 | -8.2 |
| 5 | 18.0 | 379.6 | 401.2 | 390.4 | -5.5 | 11 | 0.2 | 5705.6 | 5863.9 | 5784.8 | -2.7 |
| 5 | 44.0 | 22.1 | 24.1 | 23.1 | -8.6 | 11 | 10.0 | 307.7 | 290.3 | 299.0 | 5.8 |
| 6 | 16.0 | 463.8 | 456.6 | 460.2 | 1.6 | 12 | 0.2 | 7013.6 | 6583.1 | 6798.4 | 6.3 |
| 6 | 36.0 | 88.2 | 80.5 | 84.4 | 9.1 | 12 | 6.0 | 290.5 | 306.2 | 298.4 | -5.3 |

ᵃRepresented as [(initial conc. − reanalyzed conc.)/mean] × 100.

plasma (28). In fact, the irritation of the stomach after administration of gefitinib or other EGFR-TK inhibitors has been regarded as one of the major side-effects of EGFR-TK inhibitors (29). Thus, as an EGFR-TK inhibitor, ZCJ14 may also have this common problem which may cause an adverse impact of drug absorption and therefore lead to the double peaks phenomenon.

Despite the first peak, the plasma concentration of ZCJ14 increased to $C_{max}$ of 350.4 ± 63.7 ng mL$^{-1}$ within 18.0 ± 1.3 h ($T_{max}$) following peroral administration. Compared with the published pharmacokinetic parameters of gefitinib in rats (30), in which $T_{max}$ was 2 h and $C_{max}$ was 836 ng mL$^{-1}$ at the oral dose 20 mg kg$^{-1}$, the $T_{max}$ of ZCJ14 was longer and the $C_{max}$ of ZCJ14 was lower. The elimination half-lives ($t_{1/2}$) of ZCJ14 were 7.7 ± 2.9 h and 5.9 ± 1.4 h after *per os* or *i.v.* administration, resp. The plasma clearance (*Cl*) of ZCJ14 is 2.0 mL h$^{-1}$ g$^{-1}$, which is higher than the *Cl* of gefitinib (1.5 mL h$^{-1}$ g$^{-1}$) in rats. The oral bioavailability (*F*) of ZCJ14 in rats is 46.1 %, which is lower than that of gefitinib (59.3 %). The present pharmacokinetic data can serve as a basis for preclinical study and may help the research and development of appropriate dosage forms for clinical application of ZCJ14 under the condition of rational administration.

During the course of incurred sample reanalysis, the difference between reanalyzed results and initial data of the same subject samples were within ± 9.8 % (Table VII), which is within the acceptance criterion (± 20.0 %).

CONCLUSIONS

In this study, a fast, sensitive and specific LC-MS/MS method was established and validated. Also, the quantification with a complete and detailed elaboration of a novel diarylurea EGFR tyrosine kinase inhibitor (ZCJ14) in rat plasma was conducted for the first time. The newly developed method was successfully applied to the pharmacokinetics and bioavailability studies of ZCJ14 after *p.o.* or *i.v.* administration to SD rats. The results suggest that this method is rapid, simple, sensitive and accurate, and may serve as a valuable tool in further clinical research. The present study can shed some light on the preclinical research of this novel anticancer agent and may help predict the pharmacokinetic parameters in the human body. Studies regarding tissue distribution, metabolism and excretion are required to be done in the future.

Supplementary material available upon request.

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