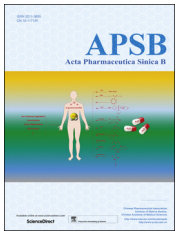
Acta Pharmaceutica Sinica B 2015;5(5):467–472

HOSTED BY

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Validated LC–MS/MS method for determination of YH-8, a novel PKnB inhibitor, in rat plasma and its application to pharmacokinetic study

Qianqian Zhai[a,b], Jing Pang[a], Guoqing Li[a], Congran Li[a], Xinyi Yang[a], Liyan Yu[a], Yucheng Wang[a], Jian Li[b], Xuefu You[a,*]

[a]*Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China*
[b]*Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China*

Received 1 March 2015; received in revised form 31 March 2015; accepted 15 April 2015

KEY WORDS

YH-8;
LC–MS/MS;
Pharmacokinetics;
Rat plasma;
Method validation

Abstract (*E*)-Methyl-4-aryl-4-oxabut-2-enoate (YH-8) is a novel PKnB protein kinase inhibitor with good anti-tuberculosis activity. To evaluate its pharmacokinetics in rats, a sensitive and selective high performance liquid chromatography–tandem mass spectrometric (LC–MS/MS) method has been developed and validated for the quantification of YH-8 in rat plasma for the first time. Samples were pre-treated using a liquid–liquid extraction with ethyl acetate and the chromatographic separation was performed on a C18 column by gradient elution with methanol–water as the mobile phase. YH-8 was detected using a tandem mass spectrometer in positive selected reaction monitoring (SRM) mode. Method validation revealed good linearity over the range of 1–500 ng/mL for YH-8 with a lower limit of quantification (LLOQ) of 1 ng/mL. Intra- and inter-day precision of YH-8 assay in rat plasma samples were 2.0%–6.8%, with accuracy of the method being 100.69%–106.18%. Stability test showed that when spiked into rat plasma, YH-8 was stable for 12 h at room temperature, for up to 15 days at −70 °C, and after three freeze-thaw cycles. Extracted samples were found to be stable over 12 h in an auto-sampler. The method was successfully applied to the pharmacokinetic study of YH-8 in rats after oral administration at 100 mg/kg and 200 mg/kg.

© 2015 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

*Corresponding author. Tel.: +86 10 67061033; fax: +86 10 63017302.
E-mail address: xuefuyou84@163.com (Xuefu You).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2015.04.001

2211-3835 © 2015 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

PKnB is one of the most important serine/threonine protein kinases for *Mycobacterium tuberculosis* (TB) and its structure consists of extracellular domain, intracellular domain and transmembrane helixes[1]. The intracellular domain of PknB has the main activity of the holoenzyme, and is able to autophosphorylate and combine with ATP and its analogues[2–5]. PKnB plays an important role in the growth of TB, and is necessary for survival of TB[6–8]. Change of expression index or phosphorylation of PKnB can lead to alteration of growth rate and morphology of TB, due to the defects in cell wall synthesis and cell division[8–11]. Because of the significant differences between PKnB and the human protein kinases, it is widely accepted as the drug target for anti-TB. To date, several PKnB inhibitors have been reported, and some of them have shown certain degree of anti-TB capability. Most of these compounds are aminopyrimidines, aminoguanidines and anthraquinones[12–17].

YH-8, namely (*E*)-methyl-4-aryl-4-oxabut-2-enoate (Fig. 1), is a representative of the small molecule class of unsaturated crotonic acid derivatives. Previous studies have shown that YH-8 could inhibit the activity of PKnB[18], and the anti-TB activity of YH-8 is significantly higher than the reported PknB inhibitors of aminopyrimidines, aminoguanidines and anthraquinones classes, making it a promising anti-TB candidate for further research[12–18]. Preclinical pharmacokinetic characteristics can help to predict drug's behavior in human body and thus concurring to its safe and efficient clinical application. However, no information about the pharmacokinetics of YH-8 has been reported so far. Hence, it is necessary to develop a robust assay for the pharmacokinetic study of YH-8.

YH-8 is difficult to be detected in plasma in virtue of its low concentration. LC–UV has poor sensitivity and specificity for YH-8 in plasma samples, so it cannot meet the requirements of the measurement. LC–MS/MS has demonstrated to be the most valuable tool for pharmacokinetic study because of its higher sensitivity and specificity compared with other analytical tools, such as HPLC–UV[19–23]. Therefore, the present study was to establish and validate a sensitive, accurate and reliable LC-MS/MS assay for routine analysis of YH-8 in rat plasma and to characterize the pharmacokinetic profile of YH-8 in rats. Furthermore, to the best of our knowledge, no any biological analytical method for determination of YH-8 to characterize the pharmacokinetic property has been reported so far.

2. Experimental

2.1. Chemicals and reagents

YH-8 (purity > 98%) was synthesized and purified by Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences (Beijing, China). HPLC-grade methanol was purchased from Fisher Scientific (New Jersey, USA). Ultrapure water was obtained from a Millipore system (Bedford, USA). All other reagents were of analytical grade. Nitrogen gas was provided by the Gas Company of Beijing (China).

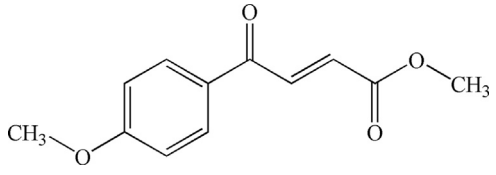


Figure 1 The chemical structure of YH-8.

2.2. Instrumentation and conditions

A Finnigan Surveyor Plus HPLC system (Thermo Finnigan, San Jose, USA), equipped with a quaternary pump, a vacuum degasser, an auto-sampler and a column heater-cooler, was connected by an electrospray ionization (ESI) interface to a Thermo Finnigan TSQ Quantum mass spectrometer (Thermo Finnigan, San Jose, USA). Samples were separated on a Waters XTerra® MS C18 column (50 mm × 2.1 mm, 3.5 μm, Waters XTerra®, Ireland) with the column temperature set at 35 °C. The mobile phase consisting of ultrapure water (A) and methanol (B) was delivered for separation of analytes using a gradient elution program at a flow rate of 0.3 mL/min. The gradient elution program was conducted as follows: 0–5.0 min, 10% B; 5.0–6.0 min, 10%–50% B; 6.0–14.0 min, 50% B; 14.0–14.01 min, 50%–10% B; 14.01–16.0 min, 10% B. The auto-sampler was maintained at 4 °C and the injection volume was 5 μL. Solvent eluted from chromatography column during the first four minutes was switched to waste before it entered the ion source. The MS was set in positive selected reaction monitoring mode with the transition of *m/z* 221 → 161 for YH-8. The MS operating conditions were optimized and set as follows: sheath gas pressure, 30 arbitrary units; auxiliary gas pressure, 10 arbitrary units; capillary temperature, 350 °C; spray voltage, 3800 V and source collision-induces dissociation (CID), 15 V. The collision gas was argon and the collision energy was 17 eV. Data were processed using Xcalibur software (Thermo Finnigan, San Jose, USA).

2.3. Animals

Twelve Sprague–Dawley (SD) rats, weighing 180–220 g (Certificate No. SCXK (Army) 2007-004), were supplied by Academy of Military Medical Sciences (Beijing, China). The rats were housed under controlled environmental conditions (temperature 23 ± 3 °C; humidity, 55%–75%) with a commercial food diet and water freely available. All animals were acclimated in the laboratory for at least seven days prior to the experiments and fasted for 12 h but allowed water *ad libitum* before experiments. Animal experiments were carried out under the permission and supervision of the Ethics Committee of Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

2.4. Preparation of calibration standards and quality control samples

Stock solution of YH-8 at 1.0 mg/mL was prepared by dissolving appropriate weight of the compound in methanol. Aliquots of YH-8 stock solutions were further diluted with methanol to yield the working standard solutions of 0.5, 5 and 50 μg/mL, respectively. All of the solutions were stored at 4 °C. Calibration standards were prepared by spiking blank rat plasma with working standard solutions of YH-8, and the final concentrations were 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL. Quality control (QC) samples were prepared similarly, with final concentrations of 2.5, 40 and 400 ng/mL, respectively. These samples were stored under the same conditions as the experimental samples.

2.5. Sample preparation

Before analysis, plasma samples were thawed at room temperature together with calibration standards and QC samples. A 100 μL volume of the plasma sample was transferred into a 1.5 mL centrifuge tube. The sample was extracted with 400 μL ethyl acetate by vortex for 1 min. The sample was then centrifuged at 8000 rpm for 15 min and the supernatant was transferred to a new tube. The residue was extracted one more time with 400 μL ethyl acetate, and the supernatant was combined with the first round supernatant. These sample extracts were evaporated to dryness at room temperature under a stream of nitrogen, reconstituted with 200 μL of mobile phase, vortexed for 1 min, filtered through a 0.22 μm pore size filter (Millipore), and then 5 μL of the solution was injected into the LC–MS/MS system.

2.6. Method validation

The proposed assay method was validated for selectivity, linearity, accuracy, precision, recovery, stability and matrix effect according to the standard guidelines[24,25].

2.6.1. Selectivity

The selectivity of the method was evaluated by analyzing six different sources of blank rat plasma, blank plasma spiked with YH-8 at lower limit of quantification (LLOQ) and a rat plasma sample collected after oral administration of YH-8. No significant interfering peaks should be present at the retention time of YH-8.

2.6.2. Linearity

Linearity of the proposed method was assessed by spiking YH-8 in drug-free rat plasma covering the range of 1–500 ng/mL. Calibration curves were constructed by weighted ($1/x^2$) least squares linear regression of the peak areas (y) of YH-8 *versus* the concentration (x) in ng/mL. The LLOQ was established as the lowest concentration of YH-8 in the calibration curve at which precision was within 20% of the relative standard deviation (RSD) and accuracy was between 80% and 120% of the theoretical value.

2.6.3. Precision and accuracy

Intra-day and inter-day accuracy and precision were assessed by analyzing five replicates of QC standards at LLOQ and three concentrations (2.5, 40 and 400 ng/mL) within the same day or on three different days. The accuracy (%) was calculated as (mean measured concentration/nominal concentration) × 100, and the precision was expressed by the RSD (%) at each concentration level. The accuracies should be within ±15%, and the precisions should not exceed 15% at all QC levels except the LLOQ which should not exceed 20%.

2.6.4. Extraction recovery and matrix effect

Extraction and matrix effect were evaluated by employing Matuszewski method[26]. The recovery of YH-8 was determined by comparing the responses of the analytes from QC samples (2.5, 40 and 400 ng/mL) with analytes spiked in post-extracted blank rat plasma at equivalent concentrations. The matrix effect was measured by comparing the responses obtained from post-extraction blank rat plasma spiked samples with that of mobile phase spiked with corresponding concentrations of YH-8. A recovery of ≥70% and a matrix effect between 80% and 120% with a variation below 15% are considered ideal.

2.6.5. Stability

The stabilities of YH-8 in rat plasma were evaluated by analyzing five replicates of plasma samples at concentrations of 2.5, 40 and 400 ng/mL after being exposed to different conditions, and comparing the results with those obtained from freshly prepared plasma samples. The long-term stability of YH-8 in rat plasma was assessed by carrying out the experiment after 15 days of storage at −70 °C. Freeze and thaw stability was determined by thawing at room temperature and then refreezing at −70 °C for 24 h for three cycles. The short-term room temperature stability was examined by keeping the QC plasma samples at room temperature for 12 h. The post-preparative stability was evaluated by analyzing processed QC samples stored in autosampler at 4 °C for 12 h. For all experiments, mean concentrations should be within ±15% of the nominal values.

2.7. Pharmacokinetic study

The 12 SD rats were acclimatized to the facilities for 7 days and then were randomly allocated into two groups (three males and three females for each group). The rats were fasted with free access to water for 12 h prior to oral administration of YH-8 at doses of 100 and 200 mg/kg, respectively, and fasted for another 4 h after administration. YH-8 suspensions were freshly prepared in 1% (*w/v*) sodium carboxymethyl cellulose (CMC-Na). Blood samples of 0.3 mL were taken *via* the post-orbital venous plexus vein at 0.167, 0.333, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 48 and 60 h after oral administration. The blood sample was transferred into a heparinized eppendorf tube, mixed gently, and then centrifuged (5000 rpm, 15 min) to obtain 100 μL of plasma, which was kept at −70 °C until analysis. Pharmacokinetic parameters of YH-8 were calculated by WinNonlin® (Version 6.1, Pharsight Corp., Mountain View, USA) using non-compartmental model. Data were expressed as mean ± SD.

3. Results and discussions

3.1. Optimization of LC–MS/MS conditions

To optimize MS/MS parameters, 1 μg/mL YH-8 solution was directly infused into the mass spectrometer in both positive and negative mode. The observed mass spectral response and signal stability of YH-8 was higher in positive mode. The MS spectrum was recorded. YH-8 showed the $[M+H]^+$ ion at m/z 221. The $[M+H]^+$ ion was used as the precursor to select product ion formed by CID. The strongest fragment for YH-8 was the ion at m/z 161. The transition m/z 221 → 161 was then used to optimize the CID and other MS parameters for YH-8. Various mobile phases and a number of commercially available HPLC columns were evaluated for the chromatographic behavior and the ionization response of YH-8. The best response was obtained from methanol and water. The gradient elution mode and a Waters XTerra MS C18 column (50 mm × 2.1 mm, 3.5 μm) were applied in chromatographic separation, and they provided satisfactory chromatographic results with minimal matrix effects. No significant signal suppression or enhancement was found using the current conditions. Liquid–liquid extraction and protein precipitation were compared as methods of sample preparation, and the former was chosen since it produced a cleaner background. Ethyl acetate, diethyl ether and trichloromethane were investigated as extraction solvents, and ethyl acetate

was selected since it gave stable recovery and is more environmental friendly.

3.2. Selectivity

The typical chromatograms of blank rat plasma, the LLOQ sample and *in vivo* plasma sample after oral administration of YH-8 are presented in Fig. 2. Compared to spiked plasma samples, all blank plasma samples from six individual rats were found to be free from interference of endogenous substances at the retention time of YH-8 in the SRM scan mode. The representative chromatogram of real plasma samples showed similar chromatographic behavior to spiked plasma samples. Obviously, no significant endogenous interference at the retention time of YH-8, indicating that the assay was selective.

3.3. Linearity and LLOQ

The calibration curves were established by plotting the peak area *versus* nominal concentration using least squares linear regression analysis with a weighting factor of $1/x^2$. The calibration curve for YH-8 exhibited good linearity over the concentration range of 1–500 ng/mL, with correlation coefficient (R^2) > 0.99 (Fig. 3). The typical standard curve was described by the equation $y = 3679.19 + 3158.49x$, where y is the peak area and x is the concentration of YH-8. According to FDA guidelines, the deviation for the calculated concentrations from the nominal concentrations should be within 15% with an exception of 20% for the LLOQ. The accuracy and precision of calculated value at each nominal concentration in this study was within the acceptable range. Moreover, the LLOQ of YH-8 was 1.0 ng/mL with precision within 20% and accuracy between 80% and 120%.

3.4. Precision and accuracy

The precision and accuracy of the method were assessed using QC samples (2.5, 40 and 400 ng/mL). Intra-day and inter-day precisions were lower than 5.3% and lower than 6.8% respectively for the different QC levels studied. The accuracy of the method was found to be 100.69%–106.18% for the three QC levels (Table 1). The accuracies and precisions for the tested levels were all within the defined acceptance criteria, demonstrating that the method was accurate and precise.

3.5. Matrix effect and recovery

The mean matrix effects at the three QC concentrations (2.5, 40 and 400 ng/mL) were 89.21%, 89.91% and 86.42%, with the variation coefficient of 2.81%–6.32%. The data convincingly suggested the absence of ion-suppression or ion-enhancement for YH-8. The mean extraction recoveries of YH-8 from spiked rat plasma under the liquid extraction conditions were 85.61%, 88.34% and 87.58% at concentrations of 2.5, 40 and 400 ng/mL, respectively, with the variation coefficient of 4.61%–7.13%. The recoveries of all three QC levels are consistent, precise and reproducible, indicating the suitability of liquid extraction procedures for separation of YH-8 from rat plasma samples[27].

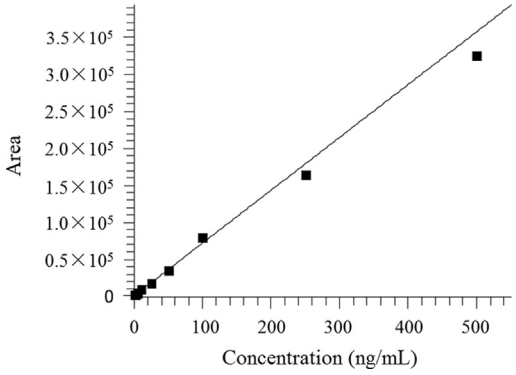


Figure 3 The calibration curve for YH-8 over the concentration range of 1–500 ng/mL in rat plasma.

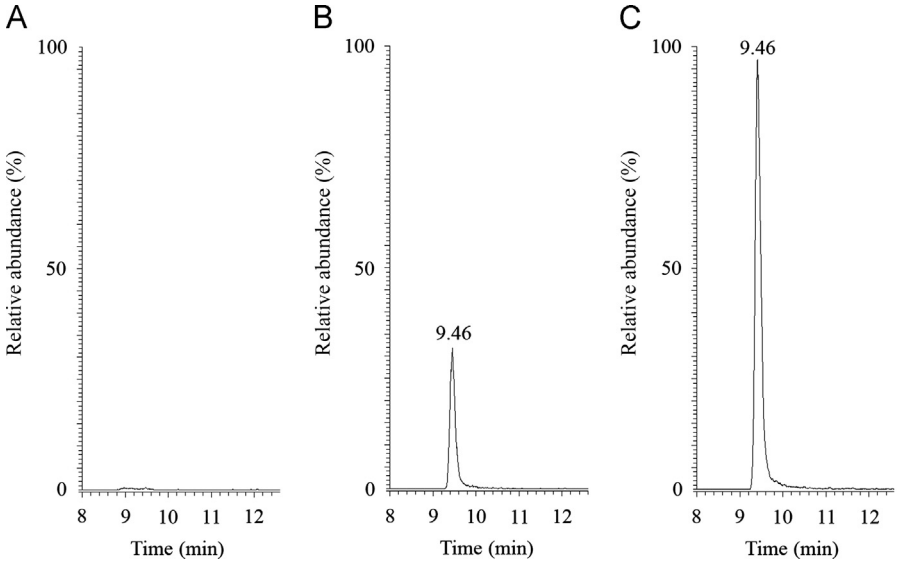


Figure 2 Chromatograms of YH-8 in selected reaction monitoring mode in matrix. (A) Blank rat plasma; (B) blank plasma spiked with YH-8 at LLOQ; (C) a plasma sample collected at 45 min after oral administration of YH-8 at 100 mg/kg to the rat.

Table 1 Precision and accuracy for the determination of YH-8 in rat plasma.

Nominal concentration (ng/mL)	Intra-day accuracy (%, $n=5$)	Inter-day accuracy (%, $n=15$)	Intra-day precision (RSD%, $n=5$)	Inter-day precision (RSD%, $n=15$)
1	100.92	103.71	6.5	8.2
2.5	101.28	103.68	5.3	5.8
40	100.69	104.86	3.5	6.8
400	106.18	101.80	2.0	4.4

Table 2 Stability of YH-8 in rat plasma ($n=5$; mean ± SD).

Nominal concentration (ng/mL)	Recovered (%)			
	Short-term (r.t., 12 h)	Long-term (−70 °C, 15 d)	Freeze-thaw (3 cycles)	Post-preparative (4 °C, 12 h)
2.5	100.85 ± 3.47	97.17 ± 8.44	92.90 ± 3.87	99.17 ± 4.48
40	101.35 ± 1.91	97.30 ± 4.02	95.03 ± 2.82	98.02 ± 5.12
400	101.15 ± 6.32	97.48 ± 9.13	93.27 ± 8.90	98.38 ± 7.23

3.6. *Stability*

The results for stability study of YH-8 (long-term storage, room temperature storage, freeze-thaw cycles and post-preparation storage) are shown in Table 2. For all experiments, mean concentrations were within ±15% of the nominal values. The data demonstrated that YH-8 was stable in rat plasma stored at −70 °C for 15 days, at room temperature for 12 h and after three freeze-thaw cycles. It was also stable in the ready-to-inject sample placed in autosampler at 4 °C for 12 h. The stability was thus satisfactory for a routine pharmacokinetic study.

3.7. *Pharmacokinetic application*

The validated assay method was successfully applied to the pharmacokinetic study of YH-8 in rats following oral administration at 100 and 200 mg/kg. The bioanalytical method was sensitive enough to quantify YH-8 concentration in plasma up to 60 h. Pharmacokinetic data of YH-8 were processed with Phoenix WinNonlin version 6.1 software simulating through non-compartmental analysis. The mean plasma concentration profile is shown in Fig. 4 and mean pharmacokinetic parameters are shown in Table 3. After oral administration at 100 and 200 mg/kg, YH-8 rapidly reached peak level at about 0.6 h and eliminated with elimination half-life of about 7.2 h without significant difference between the two doses. Both C_{max} and AUC of YH-8 increased in a dose-dependent manner. The apparent volumes of distribution (Vz/F) values were 1028.07 ± 155.71 and 996.43 ± 163.73 L/kg for 100 and 200 mg/kg groups, indicating extensive distribution of YH-8 into different organs and tissues. As YH-8 is a lipophilic drug, and its concentration in the liver is much higher than that in the kidney after oral administration (unpublished data), the clearance (CL/F) values obtained suggested that YH-8 was rapidly cleared *via* hepatic clearance. The pharmacokinetic profile of YH-8 in rats was characterized for the first time.

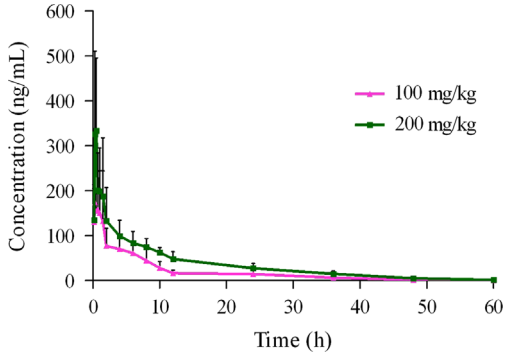


Figure 4 Mean plasma concentration-time profile of YH-8 in rats after oral administration at 100 and 200 mg/kg. Each point and bar represents the mean ± SD ($n=6$).

Table 3 Mean plasma concentration-time profile of YH-8 in rats after oral administration at 100 and 200 mg/kg. Each point and bar represents the mean ± SD ($n=6$).[a]

Pharmacokinetic parameters	100 mg/kg	200 mg/kg
$T_{1/2}$ (h)	7.17 ± 0.56	7.21 ± 0.57
T_{max} (h)	0.63 ± 0.49	0.58 ± 0.46
C_{max} (ng/mL)	289.72 ± 37.54	457.08 ± 109.77
AUC_{0-t} (ng · h/mL)	1137.41 ± 167.62	2101.66 ± 270.92
$AUC_{0-\infty}$ (ng · h/mL)	1148.46 ± 167.47	2119.45 ± 269.42
Vz/F (L/kg)	1028.07 ± 155.71	996.43 ± 163.73
CL/F (L · h/kg)	98.11 ± 16.03	95.66 ± 12.30
MRT (h)	11.73 ± 2.74	13.11 ± 3.18

[a] C_{max}: the maximum concentration; T_{max}: the time to maximum concentration; AUC_{0-t}: area under curve from time zero to the last sampling time; $AUC_{0-\infty}$: area under curve from time zero to infinity; $T_{1/2}$: the plasma elimination half-life; MRT: mean residence time; CL: Clearance; Vz: volume of distribution; F: bioavailability.

4. Conclusions

In this study, a reliable, sensitive and specific LC–MS/MS method for analysis of YH-8 in rat plasma was successfully developed and validated, and used in pharmacokinetic study of YH-8 in rats. The peaks obtained using the LC–MS/MS approach enabled the detection of the analyte at very low concentration with high resolution. A sample preparation method employing the liquid–liquid extraction of YH-8 was also successfully developed. To our

knowledge, this is the first report of an LC–MS/MS method on the determination of YH-8 in rats with a low detection limit. The pharmacokinetic parameters of YH-8 in the present work enriched our understanding of the pharmacologic features of YH-8, and provided helpful information for its further development.

Acknowledgments

This work was supported by the National Mega-Project for Innovative Drugs (Nos. 2012ZX09301002 and 2014ZX09507-009), and the National Natural Science Foundation of China, China (Nos. 81321004 and 81361138020).

References

1. Barthe P, Mukamolova GV, Roumestand C, Cohen-Gonsaud M. The structure of PknB extracellular PASTA domain from *Mycobacterium tuberculosis* suggests a ligand-dependent kinase activation. *Structure* 2010;**18**:606–15.
2. Young TA, Delagoutte B, Endrizzi JA, Falick AM, Alber T. Structure of *Mycobacterium tuberculosis* PknB supports a universal activation mechanism for Ser/Thr protein kinases. *Nat Struct Mol Biol* 2003;**10**:168–74.
3. Av-Gay Y, Jamil S, Drews SJ. Expression and characterization of the *Mycobacterium tuberculosis* serine/threonine protein kinase PknB. *Infect Immun* 1999;**67**:5676–82.
4. Fernandez P, Saint-Joanis B, Barilone N, Jackson M, Gicquel B, Cole ST, et al. The Ser/Thr protein kinase PknB is essential for sustaining mycobacterial growth. *J Bacteriol* 2006;**188**:7778–84.
5. Mieczkowski C, Iavarone AT, Alber T. Auto-activation mechanism of the *Mycobacterium tuberculosis* PknB receptor Ser/Thr kinase. *EMBO J* 2008;**27**:3186–97.
6. Sharma K, Gupta M, Krupa A, Srinivasan N, EmbR Singh Y. a regulatory protein with ATPase activity, is a substrate of multiple serine/threonine kinases and phosphatase in *Mycobacterium tuberculosis*. *FEBS J* 2006;**273**:2711–21.
7. Dasgupta A, Datta P, Kundu M, Basu J. The serine/threonine kinase PknB of *Mycobacterium tuberculosis* phosphorylates PBPA, a penicillin-binding protein required for cell division. *Microbiology* 2006;**152**:493–504.
8. Kang CM, Abbott DW, Park ST, Dascher CC, Cantley LC, Husson RN. The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. *Gene Dev* 2005;**19**:1692–704.
9. Ortiz-Lombardía M, Pompeo F, Boitel B, Alzari PM. Crystal structure of the catalytic domain of the PknB serine/threonine kinase from *Mycobacterium tuberculosis*. *J Biol Chem* 2003;**278**:13094–100.
10. Narayan A, Sachdeva P, Sharma K, Saini AK, Tyagi AK, Singh Y. Serine threonine protein kinases of mycobacterial genus: phylogeny to function. *Physiol Genomics* 2007;**29**:66–75.
11. Villarino A, Duran R, Wehenkel A, Fernandez P, England P, Brodin P, et al. Proteomic identification of *M. tuberculosis* protein kinase substrates: PknB recruits GarA, a FHA domain–containing protein, through activation loop–mediated interactions. *J Mol Biol* 2005;**350**:953–63.
12. Chapman TM, Bouloc N, Buxton RS, Chugh J, Lougheed KEA, Osborne SA, et al. Substituted aminopyrimidine protein kinase B (PknB) inhibitors show activity against *Mycobacterium tuberculosis*. *Bioorg Med Chem Lett* 2012;**22**:3349–53.
13. Lougheed KEA, Osborne SA, Saxty B, Whalley D, Chapman T, Bouloc N, et al. Effective inhibitors of the essential kinase PknB and their potential as anti-mycobacterial agents. *Tuberculosis* 2011;**91**:277–86.
14. Seal A, Yogeeswari P, Sriram D. Consortium O, Wild DJ. Enhanced ranking of PknB inhibitors using data fusion methods. *J Cheminform* 2013;**5**:2.
15. Székely R, Wáczek F, Szabadkai I, Németh G, Hegymegi-Barakonyi B, Erős D, et al. A novel drug discovery concept for tuberculosis: inhibition of bacterial and host cell signalling. *Immunol Lett* 2008;**116**:225–31.
16. Hegymegi-Barakonyi B, Szekely R, Varga Z, Kiss R, Borbely G, Nemeth G, et al. Signalling inhibitors against *Mycobacterium tuberculosis*—early days of a new therapeutic concept in tuberculosis. *Curr Med Chem* 2008;**15**:2760–70.
17. Wehenkel A, Fernandez P, Bellinzoni M, Catherinot V, Barilone N, Labesse G, et al. The structure of PknB in complex with mitoxantrone, an ATP-competitive inhibitor, suggests a mode of protein kinase regulation in mycobacteria. *FEBS Lett* 2006;**580**:3018–22.
18. Xing Y, Huang B, Xu J, Zhao LL, Si SY, Wang YC, et al. The establishment and application of a high throughput screening assay for inhibitors of *Mycobacterium tuberculosis* protein kinase B. *Microbiol China* 2014;**41**:646–53.
19. Liu QW, Wang JS, Yang L, Jia YW, Kong LY. A rapid and sensitive LC–MS/MS assay for the determination of berbamine in rat plasma with application to preclinical pharmacokinetic study. *J Chromatogr B* 2013;**929**:70–5.
20. Song M, Lee D, Lee T, Lee S. Determination of leelamine in mouse plasma by LC–MS/MS and its pharmacokinetics. *J Chromatogr B* 2013;**931**:170–3.
21. Liu AC, Zhao LX, Xing J, Gao J, Lou HX. LC–MS/MS method for the determination of a new puerarin derivative and its application in pharmacokinetic studies in rats. *Chin J Nat Med* 2013;**11**:566–71.
22. Qi Q, Hao K, Li FY, Cao LJ, Wang GJ, Hao HP. The identification and pharmacokinetic studies of metabolites of salvianolic acid B after intravenous administration in rats. *Chin J Nat Med* 2013;**11**:560–5.
23. Li GL, Han WL, Jiang W, Zhang DM, Ye WC, Chen XJ, et al. Quantitative determination of arenobufagin in rat plasma by ultra fast liquid chromatography—tandem mass spectrometry and its application in a pharmacokinetic study. *J Chromatogr B* 2013;**939**:86–91.
24. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Veterinary Medicine. *Guidance for Industry: bioanalytical Method validation*. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf.
25. Science and Medicinal Health, European Medicines Agency (EMEA). *Guideline on bioanalytical method validation*. EMEA/CHMP/EWP/192217/2009, 21 July 2011.
26. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Anal Chem* 2003;**75**:3019–30.
27. Bansal S, DeStefano A. Key elements of bioanalytical method validation for small molecules. *AAPS J* 2007;**9**:E109–14.