



Original Article

Assay development for determination of DZ2002, a new reversible SAHH inhibitor, and its acid metabolite DZA in blood and application to rat pharmacokinetic study

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ABSTRACT

Methyl (S)-4-(6-amino-9H-purin-9-yl)-2-hydroxybutanoate (DZ2002) is a potent reversible inhibitor of S-adenosyl-L-homocysteine hydrolase (SAHH). Due to its ester structure, DZ2002 is rapidly hydrolyzed in rat blood to 4-(6-amino-9H-purin-9-yl)-2-hydroxybutyric acid (DZA) during and after blood sampling from rats; this hampers accurate determination of the circulating DZ2002 and its acid metabolite DZA in rats. To this end, a method for determining the blood concentrations of DZ2002 and DZA in rats was developed by using methanol to immediately deactivate blood carboxylesterases during sampling. The newly developed bioanalytical assay possessed favorable accuracy and precision with lower limit of quantification of 31 nM for DZ2002 and DZA. This validated assay was applied to a rat pharmacokinetic study of DZ2002. After oral administration, DZ2002 was found to be extensively converted into DZA. The level of systemic exposure to DZ2002 was significantly lower than that of DZA. The apparent oral bioavailability of DZ2002 was 90%–159%. The mean terminal half-lives of DZ2002 and DZA were 0.3–0.9 and 1.3–5.1 h, respectively. The sample preparation method illustrated here may be adopted for determination of other circulating ester drugs and their acid metabolites in rodents.

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1. Introduction

Carboxylesterases can cleave ester-linked drugs and prodrugs into alcohol and carboxylic acid [1]. Methyl (S)-4-(6-amino-9H-purin-9-yl)-2-hydroxybutanoate (DZ2002), a new reversible inhibitor of S-adenosyl-L-homocysteine hydrolase (SAHH), has immunomodulatory properties, including reducing pro-inflammatory cytokine production from macrophage [2], inhibiting antigen-induced specific immune responses [3], suppressing T cell activation [4], regulating Toll-like receptor-triggered antigen-presenting cell functions [5], and suppressing TNF- α /IFN- γ -induced inflammatory response in keratinocytes and T cell-derived IL-17 [6]. DZ2002 has an ester functional group and is converted into its

acid metabolite 4-(6-amino-9H-purin-9-yl)-2-hydroxybutyric acid (DZA) by carboxylesterases in many tissues, including the liver, intestine and blood. Significant interspecies differences have been reported in catalytic activity of blood carboxylesterases, with such activity, for instance, being high in rats but low in dogs [7,8]. DZ2002 was found to be rapidly hydrolyzed in blood to its acid metabolite DZA during and after blood sampling from rats. This hampers accurate determination of circulating DZ2002 and DZA in rats, which are also used in nonclinical safety assessment of DZ2002. Previously, carboxylesterase inhibitors, such as bis(4-nitrophenyl)phosphate, were tested and used to solve such analytical issue, resulting in the development of an accurate assay for determining simmitemcan, an ester anticancer prodrug, and its bioactive acid metabolite chimmitemcan in rat plasma [9]. Although various carboxylesterase inhibitors, including bis(4-nitrophenyl)phosphate, had also been tested for DZ2002, none of them could completely terminate the hydrolysis of DZ2002 into DZA in rat blood samples. To this end, a novel sample preparation method for immediate deactivation of carboxylesterases during blood sampling was developed; the method could be useful for

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measurement of ester-linked drugs in rodent blood. This investigation was designed to develop the first bioanalytical assay for measuring concentrations of DZ2002 and its metabolite DZA in rat blood. After validation, the newly developed assay was applied to a rat pharmacokinetic study of DZ2002. This assay was developed to support preclinical pharmacokinetic evaluation of DZ2002, which is under phase I clinical trial as a potential therapy of systemic lupus erythematosus.

2. Experimental

2.1. Reference standards and materials

Methyl (S)-4-(6-amino-9H-purin-9-yl)-2-hydroxybutanoate (DZ2002; purity > 99%), 4-(6-amino-9H-purin-9-yl)-2-hydroxybutyric acid (DZA; > 99%), and 3-(6-amino-9H-purin-9-yl)-2,2-diethylpropanoic acid (internal standard; > 99%) were obtained from Shanghai Institute of Materia Medica (Shanghai, China). The chemical structures of these three compounds are shown in Fig. 1. The internal standard was dissolved in methanol (500 mL) to prepare its stock solution (50 μ M), which was then diluted in methanol to yield internal standard solution at 500 nM. Meanwhile, DZ2002 and DZA were dissolved in the internal standard solution (50 mL) to prepare an analyte stock solution containing both compounds (each at 800 μ M), as well as the internal standard at 500 nM. This stock solution was then serially diluted with the internal standard solution to generate calibration solutions at 2500, 833, 278, 92.6, 30.9, 15.4, and 7.72 nM for each of DZ2002 and DZA; the concentration of the internal standard was 500 nM in all these solutions. For assay validation, quality control solutions containing DZ2002 and DZA were prepared from an independent weighing of the compounds. DZ2002, DZA, and the internal standard solids were weighed on a Mettler-Toledo XP56 micro balance (Greifensee, Switzerland) that was connected with a Mettler-Toledo P25 printer.

HPLC-grade methanol, ammonium formate, dimethyl sulfoxide, and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade deionized water was prepared using a Millipore Milli-Q Integral 3 cabinet water purifying system (Milford, MA, USA). All other chemicals and reagents were obtained from Sino-pharm Chemical Reagent (Shanghai, China). Protocol for collection of blank rat blood was reviewed and approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica (Shanghai, China).

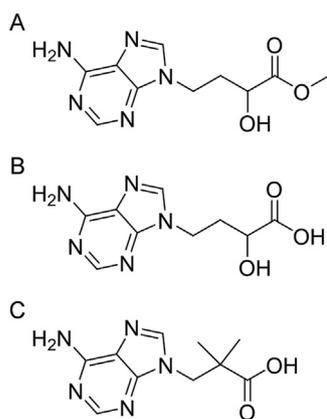


Fig. 1. Chemical structures of (A) methyl (S)-4-(6-amino-9H-purin-9-yl)-2-hydroxybutanoate (DZ2002; $C_{10}H_{13}N_5O_3$; MW, 251), (B) 4-(6-amino-9H-purin-9-yl)-2-hydroxybutyric acid (DZA; $C_9H_{21}N_5O_3$; MW, 237), and (C) 3-(6-amino-9H-purin-9-yl)-2,2-diethylpropanoic acid (internal standard; $C_{10}H_{13}N_5O_2$; MW, 235).

2.2. Collection and preparation of blood samples

Before sampling, 400 μ L of methanol containing the internal standard at 500 nM was added into 1.5 mL heparinized polypropylene microcentrifuge tubes, which were then weighed. Rat blood samples (50–200 μ L) were directly collected into the tubes. After vortex-mixing, the tubes were weighed again and centrifuged for 10 min at 21,100g. The resulting supernatants (3 μ L) were applied to LC-MS/MS analysis.

2.3. LC-MS/MS conditions

Analyses were performed on a TSQ Vantage mass spectrometer (Thermo Fisher, San Jose, CA, USA), interfaced via a HESI source with an Agilent 1290 infinity LC system (Waldbronn, Germany). The chromatographic separation was achieved on a Phenomenex Synergi Fusion-RP column (50 mm \times 2.0 mm i.d., 4 μ m; Torrance, CA, USA). The mobile phases, which consisted of solvent A (water/methanol, 99:1 (v/v), containing 3.2 mM ammonium formate and 1 mM formic acid) and solvent B (water/methanol, 1:99 (v/v), containing 3.2 mM ammonium formate and 1 mM formic acid), were delivered at 0.3 mL/min. A 6 min gradient elution method was used as follows: 0–3 min, at 1%–100% solvent B; 3–4 min, at 100% solvent B; 4–4.1 min, at 100%–1% solvent B; and 4.1–6 min, at 1% solvent B.

The mass spectrometer was operated in the positive ionization mode with precursor-product ion pairs for selected reaction monitoring (SRM) of DZ2002, DZA, and the internal standard at m/z 252.1 \rightarrow 136.1, 238.1 \rightarrow 136.1, and 236.1 \rightarrow 119.0, respectively. Both the source-dependent and compound-dependent instrument parameters were optimized for these compounds to maximize generation of the protonated molecules and to efficiently produce the characteristic fragment ions.

2.4. Construction of calibration curves

To construct matrix-matched calibration curves of DZ2002 and DZA, freshly collected rat blood samples (100 μ L; heparinized) were precipitated with 400 μ L of methanol containing DZ2002 (7.72–2500 nM), DZA (7.72–2500 nM), and the internal standard (500 nM) (the calibration solutions). The calibration curves were constructed by using weighted (1/X) linear regression of the peak area ratio of the analytes to the internal standard (Y) against the amounts of respective analytes (X: 1000, 333, 111, 37.0, 12.3, 6.17, and 3.09 pmol), which corresponded to blood concentration of 10000, 3333, 1111, 370, 123, 62, and 31 nM, respectively, for the sample volume of 100 μ L.

2.5. Estimation of stability of DZ2002 in rat blood and deactivation of blood carboxylesterases by methanol

DZ2002 was dissolved in water to prepare a 100 μ M solution, which was added into freshly collected rat blood to obtain concentration of 1000 nM. The blood sample was incubated at 23 or 37 $^{\circ}$ C for 0, 2, 5, 10, 15, and 30 min in triplicate. The samples (200 μ L) were treated with methanol (400 μ L) at the set points of incubation time; after centrifugation, the supernatants were analyzed by LC-MS/MS to determine the concentrations of DZ2002 and DZA over time. In addition, methanol (400 μ L) was added into freshly collected rat blood (200 μ L). After vortex-mixing, DZ2002 was added into the blood mixture. After incubation in triplicate at 23 $^{\circ}$ C for 0, 2, 5, 10, 15, and 30 min, the sample was centrifuged, and the supernatants were analyzed by LC-MS/MS to detect the occurrence of DZA.

2.6. Assay validation

The newly developed assay was validated, based on the European Medicines Agency Guideline on Bioanalytical Method Validation (2012), to demonstrate its reliability for the intended use. Assay selectivity was assessed by monitoring DZ2002, DZA, and the internal standard, at their respective precursor-product ion pairs, using blank blood samples obtained from three male and three female rats. Assay carry-over potential was assessed by injecting blank rat blood samples prepared with the quality control standard solution for the upper limit of quantification followed by injecting blank rat blood samples prepared with methanol.

Extraction efficiency (a measure of influence of spiking analytes and the internal standard into methanol on preparation of blood sample) was assessed by comparing LC-MS/MS responses of DZA and the internal standard (the measured peak areas) between two different sample sets. DZ2002 was not tested because it is unstable to carboxylesterases in rat blood. For sample set 1, DZA and the internal standard were spiked into freshly collected blank rat blood in sextuplicate to yield three levels of blood samples (100 μ L), i.e., one level using 8 pmol of DZA and 50 pmol of internal standard, another level using 80 pmol of DZA and 50 pmol of the internal standard, and the third level using 800 pmol of DZA and 50 pmol of the internal standard. These blood samples were then mixed with 400 μ L of methanol and centrifuged; the supernatants were analyzed by LC-MS/MS. For set 2, same amounts of DZA and the internal standard were spiked, in sextuplicate, into 400 μ L of methanol, which was then mixed with 100 μ L of freshly collected rat blood. After centrifugation, the supernatants were analyzed by LC-MS/MS. Dividing compounds' responses from set 2 by the respective mean responses from set 1 yielded the extraction efficiency.

Effects of blood matrix on the determination of DZ2002, DZA, and the internal standard were assessed, using the method described by Matuszewski et al. [10], i.e., by comparing LC-MS/MS responses of DZ2002, DZA, and the internal standard between two different sample sets and by examining the internal standard-normalized LC-MS/MS response. For sample set 1, two quality control methanol solutions (400 μ L; one containing DZ2002 at 20 nM, DZA at 20 nM, and the internal standard at 500 nM; the other containing DZ2002 at 2000 nM, DZA at 2000 nM, and the internal standard at 500 nM) were mixed with water (100 μ L) in quintuplicate before LC-MS/MS analysis. For set 2, the samples were prepared in the same way as those in set 1, except for heparinized rat blood (100 μ L; freshly collected from three male and three female rats) substituting water (100 μ L), and analyzed by LC-MS/MS. Dividing compounds' responses from set 2 by the respective mean responses from set 1 yielded the absolute matrix effects (ionization suppression or enhancement) for DZ2002, DZA, and the internal standard. The internal standard-normalized matrix effects for DZ2002 and DZA were also estimated by dividing the absolute matrix effects of individual blood samples on analyte determination by those of respective blood samples on internal standard determination.

Stability of DZ2002 and DZA was evaluated under conditions mimicking situations likely to be encountered during sample storage and preparation and other analytical steps. DZ2002 and DZA were each tested at nominal blood concentrations of 80 and 8000 nM under conditions of short-term storage (4 h at room temperature), three cycles of freeze and thaw ($-70^{\circ}\text{C} \leftrightarrow 23^{\circ}\text{C}$), and long-term storage (60 days at -70°C). Stability of these compounds in the supernatant (prepared by centrifuging rat blood pre-treated with four volumes of methanol) was also evaluated up to 24 h at 10°C . Stability of DZ2002 and DZA in stock solutions at -70°C for 6 months was also evaluated.

Because rat blood samples collected ranged from 50 μ L to 200 μ L and were all treated with a fixed volume (400 μ L) of

methanol containing the internal standard at 500 nM, assay precision and accuracy were assessed by determination of DZ2002 and DZA using three different volumes of heparinized blank rat blood, i.e., 50, 100, and 200 μ L. Because amounts of DZ2002 and DZA spiked into methanol (for preparing blood samples) were 3.1, 8, 80, and 800 pmol for each compound, four nominal blood concentrations of these two compounds were 16,000, 1600, 160, and 62 nM for the 50 μ L blood set; 8000, 800, 80, and 31 nM for the 100 μ L blood set; and 4000, 400, 40, and 15.5 nM for the 200 μ L blood set. Together with the DZ2002 and DZA, the internal standard was pre-spiked into methanol at 500 nM; the amount of internal standard was identical for all the blood samples no matter how large volume of blood (50–200 μ L) was. The relative standard deviation of measured concentration was used to show assay precision, while the deviation of the mean measured concentration away from the corresponding nominal concentration was used to show assay accuracy. Within-run precision and accuracy were based on sextuplicate determinations of blood concentrations of DZ2002 and DZA during a single analytical run, while between-run precision and accuracy were based on sextuplicate determinations of the compounds' blood concentrations on three analytical runs over two consecutive days.

The dilution integrity was assessed, in triplicate, with respect to precision and accuracy. Heparinized blank rat blood samples (100 μ L) were used to obtain nominal concentration of 80,000 nM for DZ2002 and for DZA. The concentration was prepared using the quality control solutions (400 μ L; containing DZ2002 at 20,000 nM, DZA at 20,000 nM, and the internal standard at 500 nM). After centrifugation, the supernatant (10 μ L) was diluted with 90 μ L of blank supernatant (prepared by centrifuging rat blood pre-treated with four volumes of methanol containing the internal standard at 500 nM) and the resulting solution was analyzed by LC-MS/MS.

2.7. Rat pharmacokinetic studies

All animal care and experimental procedures complied with the *Guidance for Ethical Treatment of Laboratory Animals* issued by the Ministry of Science and Technology of China in 2006. Rat study was implemented according to protocols that were reviewed and approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica (Shanghai, China). Sprague-Dawley rats (230–280 g) were obtained from Sino-British SIPPR/BK Laboratory Animal Co., Ltd. (Shanghai, China). Rats were maintained under specific-pathogen-free conditions in a unidirectional airflow room at $20\text{--}24^{\circ}\text{C}$ and relative humidity of 30%–70% with a 12 h light/dark cycle. Rats were given filtered tap water and commercial rat chow ad libitum and allowed to acclimate to the facilities and environment for 3 days before use. A total of 24 rats were used in the experiment described here.

Rats were randomly divided into four groups (three male and three female rats in each group) and received a single intravenous bolus dose of DZ2002 (dissolved in normal saline) at 5 mg/kg from the tail vein or a single oral dose of DZ2002 at 5, 10, and 25 mg/kg via gavage. Serial blood samples (around 100 μ L and not exceeding the range 50–200 μ L; 0, 5, 15, 30 min and 1, 2, 4, 6, 8, 11, and 24 h) were directly collected into pre-weighed 1.5 mL heparinized polypropylene microcentrifuge tubes containing 400 μ L of methanol that had been fortified with the internal standard at 500 nM. After sample preparation (see Section 2.2), LC-MS/MS-based analyses were performed to determine concentrations of circulating DZ2002 and DZA in the rats.

2.8. Data processing

Blood pharmacokinetic parameters were estimated by non-compartmental analysis using Thermo Kinetica software package

(version 5.0; InnaPhase, Philadelphia, PA, USA). The oral bioavailability (F) of DZ2002 after orally dosing DZ2002 was calculated using the following equations:

$$F_1 = \left(\text{AUC}_{\text{po(DZ2002)}} \times \text{Dose}_{\text{iv}} \right) / \left(\text{AUC}_{\text{iv(DZ2002)}} \times \text{Dose}_{\text{po}} \right) \quad (1)$$

$$F_2 = \left[\left(\text{AUC}_{\text{po(DZ2002)}} + \text{AUC}_{\text{po(DZA)}} \right) \times \text{Dose}_{\text{iv}} \right] / \left[\left(\text{AUC}_{\text{iv(DZ2002)}} + \text{AUC}_{\text{iv(DZA)}} \right) \times \text{Dose}_{\text{po}} \right] \quad (2)$$

where AUC is area under concentration-time curve from zero to infinity and Dose is the dose of DZ2002 via oral (po) or intravenous (iv) administration route. All data obtained from this study were assumed to describe a normal standard distribution and are expressed as the mean \pm standard deviation (SD), along with relative standard deviation (RSD). Statistical analysis was performed using IBM SPSS Statistics Software (version 19.0; Somers, NY, USA). A value of $P \leq 0.05$ was considered to be the minimum level of statistical significance.

3. Results and discussion

3.1. Deactivation of blood carboxylesterases and assay reliability

As shown in Fig. 2, DZ2002 was quickly transformed into its acid metabolite DZA in rat blood. Efficient deactivation of ester hydrolysis was a prerequisite for accurate measurement of circulating ester-linked drugs and their acid metabolite in rats.

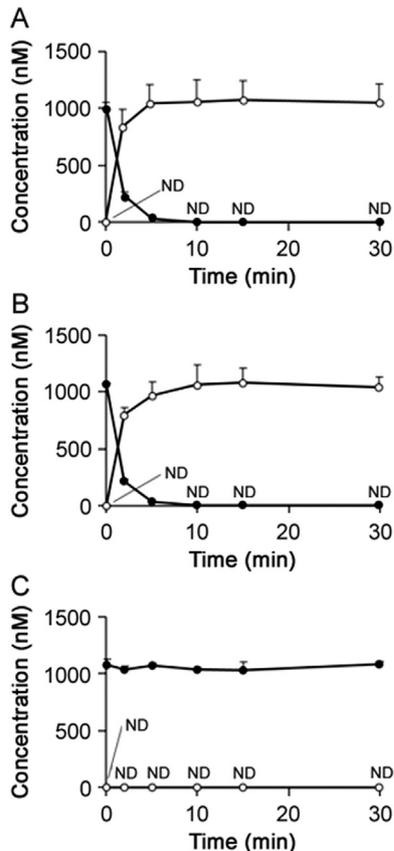


Fig. 2. Stability of DZ2002 (solid circle) in (A) rat blood at 37 °C and (B) 23 °C and in (C) methanol-pretreated rat blood at 23 °C, as well as formation of DZA (open circle) from DZ2002. ND: not detected.

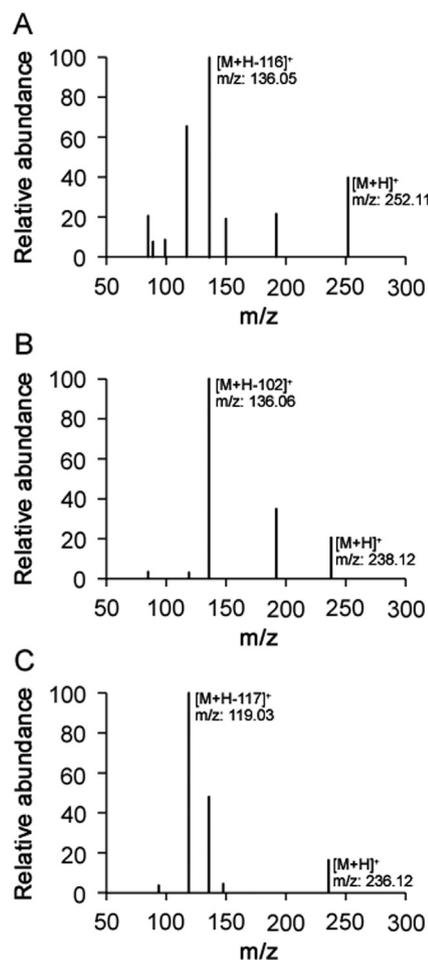


Fig. 3. MS/MS spectra of (A) DZ2002, (B) DZA, and (C) internal standard.

Previously, various carboxylesterase inhibitors, such as benzil, bis(4-nitrophenyl)phosphate, and thenoyltrifluoroacetone, were tested with respect to their inhibitory capability and a sensitive and reliable assay was successfully developed for determination of the anticancer prodrug simmitemcan and its active metabolite chimimitecan in rat plasma [9]. The assay was based on immediate deactivation of blood carboxylesterases using bis(4-nitrophenyl) phosphate. However, such use of carboxylesterase inhibitors may be limited by their potential for hemolysis of the blood samples and additional matrix effects on electrospray ionization of compounds. Instead of carboxylesterase inhibitors, methanol was used, in this investigation, to immediately deactivate rat blood carboxylesterases at blood sampling for determination of circulating DZ2002 and its acid metabolite DZA in rats. Using 400 μL of methanol to treat 50–200 μL of freshly collected rat blood samples completely deactivated the blood carboxylesterases, as indicated by DZA being undetected in the methanol-treated blood samples that were spiked with DZ2002 (Fig. 2). Although the principle of methanol-induced deactivation of carboxylesterases appears simple, development of a reliable assay for measurement of an ester-linked drug in rodent blood samples requires an assay sample preparation step that can integrate smoothly with the animal study by immediately deactivating blood carboxylesterases at sample collection, without impairing the follow-up LC-MS/MS-based quantification of the analytes. These requirements result in this sample preparation being different from conventional sample preparations for compounds stable in rodent plasma. The differences include using a volume range of blood samples (50–200 μL ; rather than a fixed volume), weighing the tubes before and after

blood sampling, calibration, and adding the internal standard in a different way. Because deactivating the carboxylesterases is the top priority at blood sampling for accurate and precise quantification of DZ2002 and DZA, adding internal standard into the rat blood samples could not be performed after blood sampling. For the quantification, the internal standard was introduced at sample collection by adding it into methanol that was used to prepare samples and it could monitor the fluctuations related to DZ2002 and DZA in the follow-up steps of quantification.

The newly developed assay for determination of DZ2002 and DZA in rat blood was fully validated before use. DZ2002, DZA, and the internal standard formed predominantly the protonated molecules ($[M + H]^+$) in the electrospray ionization source; their MS/MS spectra are shown in Fig. 3. Chromatographic separation was optimized to avoid ion crosstalk or cross-interference among the analytes and the internal standard (Fig. 4). None of the analytes (DZ2002 and DZA) or the internal standard was detected in blood samples collected from three male and three female rats before dosing DZ2002. The assay had limited carry-over (checked by injecting calibration standard of upper limit of quantification at 10,000 nM), as indicated by the response for the subsequent blank sample being less than 20% of the lower limit of quantification for DZ2002 and DZA, and being less than 5% of the response for the internal standard.

For accurate measurement of DZ2002 and DZA in rat blood samples, methanol was used to deactivate the blood carboxylesterases and to remove blood protein from the samples. To this end, rat blood samples after dosing DZ2002 were directly collected into microcentrifuge tubes containing methanol that had been fortified with the internal standard, while the calibration

standards were prepared by spiking DZ2002, DZA, and internal standard into methanol that was used to precipitate blank rat blood. As shown in Table 1, LC-MS/MS responses of DZA and internal standard in sample set 2 (spiking these compounds into methanol that was used to treat blank rat blood samples) were quite close to the respective compounds' responses in sample set 1 (spiking them into blank rat blood samples that were treated with methanol). This suggested limited influence of spiking DZA and the internal standard into methanol on sample preparation. Similar scenario is expected to take place for DZ2002. Matrix effect on electrospray ionization of DZ2002, DZA, and internal standard was assessed and the results are shown in Table 2. Regarding absolute matrix effects, the responses of DZ2002, DZA, and the internal standard in blood matrix components-containing solutions (set 2) were 79.2%–83.1%, 79.7%–82.7%, and 92.4% of the respective compounds' responses in blood matrix components-free solutions (set 1), indicating that the blood matrix components caused 17%–21% and 8% ionization suppression for the analytes and the internal standard, respectively. Meanwhile, the relative standard deviations for set 2 (8.2%–10.0% for DZ2002, 10.0%–13.7% for DZA, and 2.6% for the internal standard) were slightly greater than those for set 1 (0.1%–7.4%, 3.1%–4.2%, and 1.7%, respectively), indicating the presence of a small relative matrix effect. The use of internal standard did not significantly change the relative matrix effects.

As an integral part of the method validation, the stabilities of DZ2002, DZA, and the internal standard were evaluated under conditions mimicking those experienced by test blood samples. The results summarized in Table 3 indicated that the analytes and the internal standard were all acceptably stable under the tested conditions.

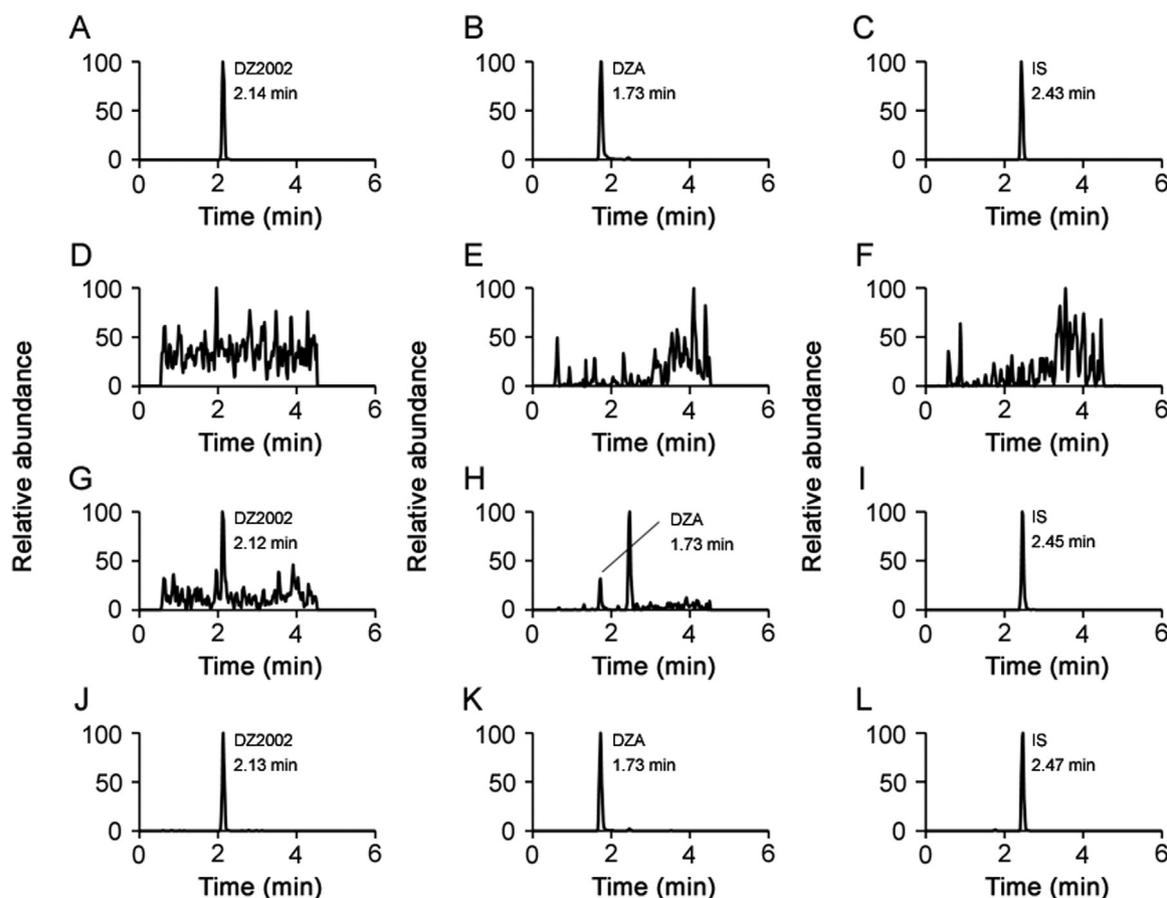


Fig. 4. Chromatograms of DZ2002, DZA, and the internal standard (IS), detected by tandem mass spectrometry, in (A–C) a solution containing the three compounds, in (D–F) a typical blank rat blood sample, in (G–I) the same blank blood sample spiked with DZ2002 (at its lower limit of quantification), DZA (at its lower limit of quantification), and the internal standard (at 500 nM), and in (J–L) an 500 nM internal standard-spiked blood sample collected at 0.5 h after an oral dose of DZ2002 at 5 mg/kg.

Table 1
Extraction of DZA and the internal standard.

Test compound (pmol)	LC-MS/MS response (peak area, $\times 10^4$) (Mean \pm SD (%RSD))		Extraction efficiency (%) (set 2/set 1)
	Sample set 1	Sample set 2	
Level 1 ($n = 6$)			
8 (DZA)	0.27 \pm 0.00 (1.2)	0.26 \pm 0.01 (3.8)	95.8
50 (internal standard)	17.7 \pm 0.4 (2.4)	17.8 \pm 0.6 (3.6)	101
Level 2 ($n = 6$)			
80 (DZA)	2.65 \pm 0.03 (1.0)	2.63 \pm 0.06 (2.4)	99.4
50 (internal standard)	14.7 \pm 0.5 (3.2)	15.7 \pm 0.4 (2.4)	107
Level 3 ($n = 6$)			
800 (DZA)	22.0 \pm 0.6 (2.6)	22.0 \pm 0.5 (2.2)	100
50 (internal standard)	15.7 \pm 0.27 (1.7)	15.9 \pm 0.18 (1.1)	101

For sample set 1, DZA and the internal standard were spiked into freshly collected blank rat blood to yield three levels of blood samples (100 μ L), i.e., one level using 8 pmol of DZA and 50 pmol of the internal standard, another level using 80 pmol of DZA and 50 pmol of the internal standard, and the third level using 800 pmol of DZA and 50 pmol of the internal standard. These blood samples were then mixed with 400 μ L of methanol and centrifuged; the supernatants were analyzed by LC-MS/MS. For sample set 2, same amounts of DZA and the internal standard were spiked into 400 μ L of methanol, which was used to mix with 100 μ L of freshly collected rat blood. After centrifugation, the supernatants were analyzed by LC-MS/MS.

Table 2
Matrix effects on measurement of DZ2002, DZA, and the internal standard. Blank blood samples were collected from six rats.

Test concentration (nM)	LC-MS/MS response (peak area, $\times 10^4$)		Absolute matrix effect (%)	IS-normalized matrix effect (%)
	Sample set 1 Mean \pm SD (%RSD)	Sample set 2 Mean \pm SD (%RSD)		
DZ2002				
80	0.52 \pm 0.04 (7.4)	0.44 \pm 0.04 (8.2)	83.1	88.5 \pm 6.8 (7.7)
8000	50.2 \pm 0.5 (0.1)	39.8 \pm 4.0 (10.0)	79.2	87.4 \pm 10.2 (11.7)
DZA				
80	0.26 \pm 0.01 (4.2)	0.23 \pm 0.03 (13.7)	82.7	87.8 \pm 10.0 (11.4)
8000	26.8 \pm 0.8 (3.1)	21.3 \pm 2.1 (10.0)	79.7	87.9 \pm 10.4 (11.8)
Internal standard				
500	17.7 \pm 0.3 (1.7)	16.3 \pm 0.4 (2.6)	92.4	–

For sample set 1, two quality control methanol solutions (400 μ L; one containing DZ2002 at 20 nM, DZA at 20 nM, and the internal standard at 500 nM; the other containing DZ2002 at 2000 nM, DZA at 2000 nM, and the internal standard at 500 nM) were mixed with water (100 μ L) before LC-MS/MS analysis.

For sample set 2, the samples were prepared in the same way as those in sample set 1, except for heparinized rat blood (100 μ L; freshly collected from three male and three female rats) substituting water (100 μ L), and analyzed by LC-MS/MS.

Within-run accuracy and precision data for the newly developed assay are summarized in Table 4. The assay showed good accuracy and precision, which were not influenced by different volumes of rat blood samples collected. The assay's lower limit of quantification (LLOQ) was 3.09 pmol for DZ2002 and DZA in blood (50–200 μ L), while the upper limit of quantification (ULOQ) was 1000 pmol for the two compounds in blood (50–200 μ L). Because

some of the early time-point blood samples in the high oral dose group (25 mg/kg) had DZ2002 and DZA concentrations above the upper limit of the standard curve (10,000 nM), the impact of sample dilution on the accuracy and precision of the assay was also evaluated. As a result, the accuracy (89.0% for DZ2002 and 108% for DZA) and precision (2.56% and 3.51%, respectively) demonstrated that the assay was reliable even for rat blood samples

Table 3
Stability of DZ2002, DZA, and the internal standard.

Condition	Response (peak area; Mean \pm SD, $\times 10^4$) (%Control)				
	DZ2002		DZA		Internal standard (500 nM)
	80 nM	8000 nM	80 nM	8000 nM	
In rat blood at 23 °C for 4 h	0.46 \pm 0.03 (93.6)	46.3 \pm 0.4 (102)	0.24 \pm 0.01 (95.2)	23.4 \pm 0.4 (97.5)	16.3 \pm 0.5 (108)
After three cycles of freeze and thaw of –70 °C \leftrightarrow 23 °C	0.53 \pm 0.05 (108)	46.4 \pm 0.4 (102)	0.25 \pm 0.00 (98.1)	21.5 \pm 0.1 (89.5)	15.0 \pm 0.1 (98.4)
In rat blood at –70 °C for 60 days	0.46 \pm 0.02 (94.6)	42.1 \pm 0.0 (92.4)	0.27 \pm 0.02 (108)	26.8 \pm 2.5 (112)	15.7 \pm 1.1 (104)
In supernatant of methanol- treated rat blood at 10 °C for 24 h	0.53 \pm 0.05 (108)	49.4 \pm 0.8 (108)	0.26 \pm 0.02 (106)	26.3 \pm 0.2 (109)	16.1 \pm 0.2 (106)
In stock solutions of DZ2002 and DZA at –70 °C for 6 months ^a	38.1 \pm 0.6 (101)		13.1 \pm 0.5 (103)		38.6 \pm 0.5 (97.9)

^a The peak areas ($\times 10^4$) of DZ2002 and DZA at diluted concentration of 1000 nM from the stock solutions were compared to those of control samples, which were freshly prepared at the same concentration from freshly made stock solutions.

Table 4

Within-run and between-run accuracy and precision for assay for measurement of DZ2002 and DZA when 50, 100, and 200 μL of rat blood samples were applied to sample preparation with 400 μL of methanol.

Volume of rat blood ^a (μL)	Compound	Amount in blood (pmol)	Nominal concentration (nM)	Within-run ($n = 6$)		Between-run ($n = 3$)	
				Mean \pm SD (%RSD)	Accuracy (%)	Mean \pm SD (%RSD)	Accuracy (%)
50	DZ2002	3.1	62	72 \pm 8 (11.1)	116	75 \pm 9 (12.0)	121
		8	160	176 \pm 9 (8.8)	110	182 \pm 10 (5.6)	114
		80	1600	1645 \pm 75 (4.6)	103	1662 \pm 72 (4.3)	104
		800	16,000	13,669 \pm 275 (2.0)	85	14,030 \pm 636 (4.5)	88
	DZA	3.1	62	59 \pm 9 (15.3)	95	62 \pm 11 (17.7)	100
		8	160	153 \pm 7 (4.4)	95	156 \pm 17 (10.7)	97
		80	1600	1521 \pm 55 (3.6)	95	1527 \pm 56 (3.7)	95
		800	16,000	15,456 \pm 644 (4.2)	97	15,376 \pm 598 (3.9)	96
100	DZ2002	3.1	31	34.0 \pm 3.0 (8.8)	110	32.9 \pm 4.0 (12.1)	106
		8	80	83.3 \pm 7.2 (8.7)	104	85.1 \pm 6.1 (7.2)	106
		80	800	811 \pm 17 (2.0)	101	812 \pm 33 (4.1)	102
		800	8000	7150 \pm 275 (3.9)	89	7164 \pm 487 (6.8)	90
	DZA	3.1	31	35.2 \pm 7.0 (19.8)	114	33.5 \pm 6.5 (19.3)	108
		8	80	85.6 \pm 13.2 (15.4)	107	83.4 \pm 9.5 (11.4)	104
		80	800	781 \pm 24 (3.0)	98	792 \pm 36 (4.6)	99
		800	8000	7437 \pm 285 (3.8)	93	7608 \pm 308 (4.1)	95
200	DZ2002	3.1	15.5	15.7 \pm 1.9 (12.2)	101	16.6 \pm 2.6 (15.6)	107
		8	40	43.2 \pm 1.2 (2.7)	108	41.5 \pm 5.2 (12.4)	104
		80	400	408 \pm 29 (7.1)	102	414 \pm 25 (5.9)	104
		800	4000	3832 \pm 37 (1.0)	96	3835 \pm 165 (4.3)	96
	DZA	3.1	15.5	18 \pm 2 (11.1)	113	19 \pm 3 (15.8)	119
		8	40	45 \pm 6 (13.3)	113	47 \pm 5 (10.6)	118
		80	400	439 \pm 26 (6.0)	110	438 \pm 22 (5.1)	110
		800	4000	4301 \pm 85 (2.0)	108	4269 \pm 131 (3.1)	107

^a All of these rat blood samples were treated with 400 μL of methanol.

having very high analyte concentrations ($>$ ULOQ). Collectively, the newly developed assay showed good reliability and reproducibility for the intended use.

3.2. Pharmacokinetics of circulating DZ2002 and DZA in rats

The newly developed assay was applied to measuring DZ2002 and its acid metabolite DZA circulating in rats that received oral and intravenous DZ2002. Fig. 5 shows blood concentrations of DZ2002 and DZA over time after dosing; Table 5 summarizes the pharmacokinetic parameters of DZ2002 and DZA. After intravenous administration of DZ2002 at 5 mg/kg, DZA exhibited a higher level of systemic exposure than that of DZ2002. The elimination half-life of DZ2002 was significantly shorter than that of DZA. The systemic

clearance of DZ2002 ($CL_{\text{tot,b}}$) exceeded the rat hepatic blood flow rate (3.31 L/h/kg [11]) by around 13 times and even the rat cardiac output (17.8 L/h/kg [11]) by around 2 times. This suggests that DZ2002 was subjected to extensive extrahepatic elimination; its hydrolysis into DZA by blood carboxylesterases contributed, at least in part, to the high systemic clearance of DZ2002 in rats. After oral administration of DZ2002 at 5 mg/kg, its AUC was significantly higher than the AUC of DZ2002 after intravenous administration of DZ2002 at 5 mg/kg, while the AUC of DZA from the oral DZ2002 was lower than that from the intravenous DZ2002. This is probably due to the metabolic capability of rat blood carboxylesterases being more potent than those of carboxylesterases in other tissues, such as the liver [8]. Oral administration results in DZ2002 passing through the intestine and the liver during its presystemic absorption and disposition, while

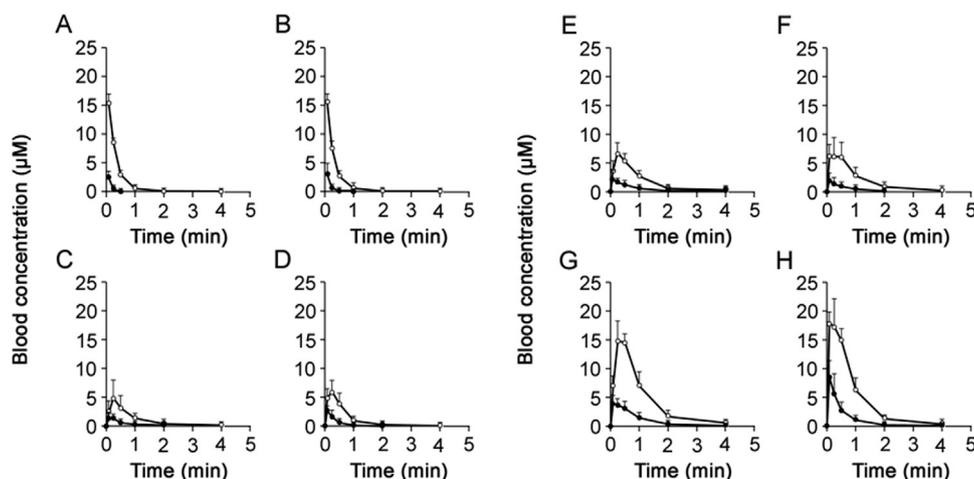


Fig. 5. Blood concentrations of DZ2002 (solid circles) and DZA (open circles) over time after a bolus intravenous dose of DZ2002 at (A and B) 5 mg/kg or a single oral dose of DZ2002 at (C and D) 5, (E and F) 10, or (G and H) 25 mg/kg in (A, C, E, and G) male and (B, D, F, and H) female rats. The blood pharmacokinetic parameters are summarized in Table 5.

Table 5
Pharmacokinetic parameters of DZ2002 and DZA after intravenous and oral administration of DZ2002 in rats.

Administration route and dose of DZ2002	Pharmacokinetic parameter ^a	Male rat		Female rat	
		DZ2002	DZA	DZ2002	DZA
Intravenous bolus at 5 mg/kg	$C_{5\text{ min}}$ (nM)	2463 ± 597	15,340 ± 1211	3027 ± 1453	15,548 ± 983
	AUC_{0-t} (nM h)	495 ± 142	4936 ± 226	660 ± 376	4713 ± 340
	$AUC_{0-\infty}$ (nM h)	504 ± 146	5025 ± 212	680 ± 396	4796 ± 300
	$t_{1/2}$ (h)	0.08 ± 0.01	1.61 ± 1.73	0.15 ± 0.11	1.22 ± 0.44
	MRT (h)	0.11 ± 0.02	0.56 ± 0.28	0.15 ± 0.06	0.51 ± 0.03
	V_{SS} (L/kg)	4.50 ± 1.22	–	4.59 ± 0.96	–
	$CL_{\text{tot,b}}$ (L/h/kg)	42.4 ± 14.8	–	35.4 ± 15.7	–
	C_{max} (nM)	1379 ± 457	5226 ± 2588	2718 ± 418	6493 ± 1040
Oral at 5 mg/kg	T_{max} (h)	0.19 ± 0.1	0.33 ± 0.14	0.08 ± 0	0.19 ± 0.1
	AUC_{0-t} (nM h)	729 ± 52	3772 ± 608	963 ± 182	4267 ± 669
	$AUC_{0-\infty}$ (nM h)	790 ± 92	3895 ± 501	985 ± 184	4355 ± 701
	$t_{1/2}$ (h)	0.45 ± 0.23	1.33 ± 0.64	0.31 ± 0.12	2.06 ± 1.14
	MRT (h)	0.67 ± 0.32	1.24 ± 0.68	0.43 ± 0.11	1.01 ± 0.09
	F_1 (%)	157 ± 18	–	145 ± 27	–
	F_2 (%)	83 ± 10	–	98 ± 16	–
	C_{max} (nM)	2360 ± 1100	7025 ± 1048	2131 ± 742	7229 ± 1978
Oral at 10 mg/kg	T_{max} (h)	0.14 ± 0.1	0.33 ± 0.14	0.14 ± 0.1	0.28 ± 0.21
	AUC_{0-t} (nM h)	1405 ± 171	6707 ± 267	1184 ± 366	7599 ± 1026
	$AUC_{0-\infty}$ (nM h)	1472 ± 199	6811 ± 223	1226 ± 348	7808 ± 846
	$t_{1/2}$ (h)	0.68 ± 0.49	1.46 ± 0.23	0.54 ± 0.18	3.88 ± 3.75
	MRT (h)	0.83 ± 0.24	1.31 ± 0.19	0.74 ± 0.16	1.63 ± 0.74
	F_1 (%)	146 ± 20	–	90 ± 27	–
	F_2 (%)	75 ± 1	–	83 ± 11	–
	C_{max} (nM)	4023 ± 898	16,039 ± 1630	8487 ± 2490	19,848 ± 535
Oral at 25 mg/kg	T_{max} (h)	0.14 ± 0.10	0.42 ± 0.14	0.08 ± 0.00	0.19 ± 0.10
	AUC_{0-t} (nM h)	3850 ± 1153	18,075 ± 1745	3946 ± 1434	17,606 ± 1114
	$AUC_{0-\infty}$ (nM h)	4001 ± 1210	18,352 ± 1935	4020 ± 1399	18,398 ± 1984
	$t_{1/2}$ (h)	0.93 ± 0.27	2.78 ± 1.38	0.71 ± 0.42	5.09 ± 3.64
	MRT (h)	1.20 ± 0.37	1.60 ± 0.31	0.67 ± 0.23	2.95 ± 2.77
	F_1 (%)	159 ± 48	–	118 ± 41	–
	F_2 (%)	81 ± 11	–	82 ± 11	–

^a AUC, area under concentration-time curve; $C_{5\text{ min}}$, concentration at 5 min after a bolus intravenous dose of DZ2002; C_{max} , maximum concentration after an oral administration of DZ2002; $CL_{\text{tot,b}}$, total blood clearance (systemic clearance); F_1 and F_2 oral bioavailability (the equations for estimation of F_1 and F_2 being shown in the subsection 'Data Processing'); MRT, mean residence time; $t_{1/2}$, elimination half-life; T_{max} , time taken to achieve the maximum concentration; V_{SS} , distribution volume at steady state.

intravenous administration delivers the compound directly into the blood stream. We found that enzymatic capability of rat intestinal and hepatic carboxylesterases for DZ2002 hydrolysis was lower than that of rat blood carboxylesterases (details pending publication elsewhere). Regarding oral bioavailability of DZ2002, F_1 ranged from 90% to 159%, while F_2 ranged from 75% to 98%. The levels of systemic exposure to DZ2002 and DZA increased as the oral dose of DZ2002 increased from 5 to 25 mg/kg, but not in a proportional manner [12].

4. Conclusions

Carboxylesterase-mediated hydrolysis during and after blood sampling represents an analytical issue that hampers accurate determination of circulating ester drugs and their acid metabolites in rodents that are commonly used in preclinical drug evaluations. DZ2002 is such an investigational ester drug that has been approved recently for phase I clinical evaluation by the China Food and Drug Administration. Here, a validated assay, based on immediate deactivation of blood carboxylesterases with methanol during sampling, was developed for accurate and sensitive determination of DZ2002 and its acid metabolite DZA in rat blood. This methanol treatment of sample is reported here as an efficient and simple way to solve the analytical issue for DZ2002; it also avoids additional matrix effects on electrospray ionization-based measurement of the analytes due to using carboxylesterase inhibitors for sample preparation. Based on this newly developed bioanalytical assay, rat pharmacokinetics of circulating DZ2002 and its acid metabolite DZA was accurately characterized. The assay has also been successfully applied to a related rat

toxicokinetic study that supports nonclinical safety assessment of DZ2002 (the details pending publication elsewhere).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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