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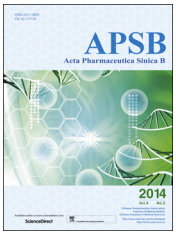

ORIGINAL ARTICLE

Preclinical investigation of the pharmacokinetics, metabolism, and protein and red blood cell binding of DRDE-07: a prophylactic agent against sulphur mustard

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Protein binding;
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Abstract DRDE-07, a newly synthesized amifostine analog currently under clinical investigation in a phase I trial, is a potent antidote against sulfur mustard toxicity. The purpose of this research was to evaluate the pharmacokinetic profile of DRDE-07 in female Swiss Albino mice after a single oral dose of 400 or 600 mg/kg. The physicochemical properties of DRDE-07, including solubility, pK_a, Log *P*, plasma protein binding and plasma/blood partitioning, were determined to support the pharmacokinetic characterization. DRDE-07 concentration was determined by an HPLC-UV method. The profile of plasma concentration *versus* time was analyzed using a non-compartmental model. Plasma protein binding was assessed using ultrafiltration. DRDE-07 appeared rapidly in plasma after oral administration with peak plasma levels (C_{max}) observed in less than 15 min. There was a rapid decline in the plasma levels followed by a smaller second peak about 90 min after dosing. The plasma protein binding of DRDE-07 was found to be less than 25% at all concentrations studied. Plasma clearance of DRDE-07 is expected to be ~1.5 fold higher than the blood clearance of DRDE-07. The probable metabolite of DRDE-07 was identified as phenyl-*S*-ethyl amine.

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1. Introduction

Nerve agents and blistering agents continue to be a threat as chemical warfare agents in spite of controls imposed by the Chemical Weapon Convention (CWC). The CWC prohibits the production, storage, transport and use of chemicals on enemy forces[1]. One of such chemical is sulfur mustard (SM), commonly known as mustard gas and included in the Schedule I of the CWC. Reports are available of its use in several instances before the CWC came into force[2–4]. In spite of the CWC, the threat exists that SM will be used clandestinely during war or by terrorist organizations because of its simple method of preparation. Despite 90 years of research, there is still no antidote for mustard. This fact is especially crucial when we consider that probably at least a dozen countries have SM in their arsenals today. Development of an effective prophylactic or therapeutic antidote is an immediate requirement for personnel and particularly for OPCW (Organization for the Prohibition of Chemical Weapons) officials engaged in the destruction of SM.

Taking into consideration the increase in terrorist activities, drug development against SM is needed not only for army personnel but also for civilians. A large number of chemicals and drugs including sulfur compounds have been tested against SM or nitrogen mustard toxicity in various protocols but have been found to have little or no protective effect against their systemic toxicity[5,6]. There still no effective treatment for SM toxicity and it poses a challenge even today[7,8].

DRDE-07, an analog of amifostine, was found to be the most potent agent against sulfur mustard toxicity. It has been proven that orally administration of DRDE-07 is more efficacious than amifostine against percutaneously administered SM. DNA damage, the decrease in body weight, and the depletion of GSH induced by SM were significantly protected by DRDE-07. Both *in vitro* and *in vivo* data indicate promising roles of DRDE-07 as a prophylactic agent against SM poisoning[9–11]. The physicochemical properties of a drug determine its pharmacokinetic fate in the body. Therefore, physicochemical data can be used to explain the ADME profile obtained in animal studies. Since DRDE-07 is a new compound, preclinical characterization of DRDE-07 is essential for further studies of this agent. Presently, there is no detailed pre-clinical pharmacokinetic data of DRDE-07 in mice. Hence, the purpose of this study was to determine the pharmacokinetic profile of DRDE-07 and to establish the solubility, pK_a, log *P*, plasma protein binding and red blood cell (RBC)/plasma partitioning of DRDE-07, and to identify the possible metabolites of DRDE-07 in mice.

2. Materials and methods

2.1. Chemicals

DRDE-07 (Fig. 1) was developed in the synthetic chemistry laboratory of the Defense Research and Development Establishment, Gwalior, with its chemical structure confirmed by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry. The HPLC purity of DRDE-07 was greater than 99.5%. Heptane sulfonic acid (HSA), tetra methyl ammonium chloride (TMAC), hydrochloric acid and *n*-octanol were purchased from Aldrich (USA), while acetone, ethyl acetate orthophophoric acid, sodium chloride, dibasic and monobasic sodium hydrogen phosphate and dibasic and monobasic potassium hydrogen phosphate were purchased from Merck (Germany). HPLC grade acetonitrile and methanol were obtained from J.T. Baker (Philipsburg, NJ). Millipore water was prepared *via* a Millipore purification system (Milford, MA). All other chemicals and reagents were of analytical/regent grade. Blood was collected from healthy Swiss Albino mice in heparinized tubes and was centrifuged to separate plasma so as to generate drug-free mice plasma.

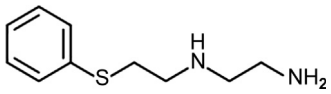

Figure 1 Structure of DRDE-07 [(2-aminoethyl)(2-(phenylsulfanyl) ethylamine].

2.2. Bioanalysis

The DRDE-07 concentrations in mouse plasma and urine were determined using an HPLC-UV method described in our previous papers[12,13]. The least squares linear regression coefficient of determination (R^2) was greater than 0.9998 and 0.9986 (minimum value) in all analytical tests for plasma and urine analysis, respectively. Details of accuracy, precision, recovery and stability of DRDE-07 are shown in Table 1[12,13]. Reverse-phased HPLC-UV methods were used for the determination of DRDE-07 in samples. The mobile phase consisting of (A) double-distilled H_2O containing 10 mmol/L sodium dihydrogen phosphate, 0.5 mmol/L HSA and 1 mmol/L of TMAC (pH 3.5) and (B) acetonitrile was freshly made daily and degassed before use. The mobile phase was delivered isocratically in a ratio of 78% (A) and 22% (B) through a Waters X-Tera, MS C18 column coupled with a Waters X-Tera, MS C18 guard column at 30 °C and a flow rate of 1.0 mL/min. All samples and standard solutions were detected at 249 nm where the maximum absorbance of the substance investigated was observed.

2.3. Solubility

The aqueous solubility of DRDE-07 in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) was measured using a shaking flask method[14]. DRDE-07 powder was weighed (50 mg) in glass vial and SIF (pH 6.8) or SGF (pH 1.2) was added to produce saturated solutions. Samples were prepared in triplicate. Samples were placed for 24 h in a shaking water bath at 37 °C. The samples were collected and filtered through 0.22 μm filter. The concentration of DRDE-07 in the diluted supernatant (SIF or SGF) was determined using a Waters HPLC system.

2.4. Drug lipophilicity (Log P)

The octanol:water partition coefficient, a measure of drug lipophilicity, was measured by established methods[15–17]. Phosphate buffer (0.1 mol/L) was prepared from deionized water and dibasic sodium phosphate. The pH was adjusted to 7.4 using 85% phosphoric acid. This aqueous buffer solution was fortified with DRDE-07 at 10, 25 and 50 μg/mL and added to an equal volume of *n*-octanol in a screw top tube. The tube was gently rocked for 1 h at room temperature to equally disperse the drug into each phase of the tube and then centrifuged for 10 min at 2000 rpm. Before and after incubation and shaking, the drug concentration in the aqueous layer was analyzed by HPLC. The partition coefficient

Table 1 Accuracy, precision, recovery and stability of DRDE-07 in mice plasma and urine[a].

Data item	Spiked concentration in plasma (μg/mL)			Spiked concentration in urine (μg/mL)		
	1	10	100	1	10	100
Accuracy and precision						
Intra-day precision ($n=5$)						
Measured concentration (μg/mL)	1.000 ± 0.02	9.98 ± 0.17	95.56 ± 4.23	0.84 ± 0.04	9.55 ± 0.21	95.12 ± 1.17
Accuracy (%)	100.07	99.78	95.56	84.26	95.46	95.12
RSD (%)	2.15	1.75	4.38	4.16	2.24	1.23
Difference (%)	0.07	−0.22	−3.25	−15.74	−4.54	−4.88
Inter-day precision ($n=5$)						
Measured concentration (μg/mL)	1.01 ± 0.02	9.57 ± 0.11	98.67 ± 3.16	0.86 ± 0.03	9.62 ± 0.09	94.45 ± 0.66
Accuracy (%)	101.02	95.69	98.67	85.48	96.16	94.45
RSD (%)	2.09	1.07	3.21	3.49	0.96	0.70
Difference (%)	1.02	−4.31	−1.33	−14.52	−3.84	−5.55
Recovery (%)	96.87 ± 1.89	96.43 ± 1.41	88.26 ± 1.49	40.66 ± 0.83	65.05 ± 0.83	61.84 ± 1.11
Stability of samples ($n=3$)						
Freeze-thaw for three cycles (%)	93.16 ± 1.21	ND	95.12 ± 2.62	90.66 ± 2.07	ND	94.78 ± 2.27
Short-term (room temperature, 24 h)(%)	95.91 ± 3.76	ND	94.76 ± 2.73	93.79 ± 2.66	ND	96.09 ± 2.91
Long-term (−20 °C, 30 days) (%)	92.14 ± 3.16	ND	96.03 ± 0.56	94.12 ± 1.77	ND	93.72 ± 1.50

[a]SD: standard deviation; RSD: relative standard deviation; ND: not determined.

(PC) was calculated using Eq. (1). Log *P* was calculated by taking logarithm of partition coefficient (PC).

$$\mathrm{PC} = \frac{\mathrm{Concentration}_{\mathrm{Pre-incubation}} - \mathrm{Concentration}_{\mathrm{Post-incubation}}}{\mathrm{Concentration}_{\mathrm{Post-incubation}}} \quad (1)$$

2.5. *Plasma protein binding (PPB)*

The plasma protein binding of DRDE-07 was estimated using the ultra-filtration method. Briefly, freshly collected female mouse plasma was spiked with DRDE-07 to obtain the plasma concentrations of 12.5, 25 and 50 μg/mL. The spiked plasma was allowed to equilibrate at 37 °C for 60 min before the start of the study. The separation of bound and unbound forms of DRDE-07 and preparation of protein-free filtrate were achieved by filtration through Amicon Ultra-4 centrifugal filters (molecular weight cut-off: 3 kDa) from Millipore (Bedford, MA). Samples at each concentration (1 mL, $n=3$) were placed in Centrifree devices and centrifuged at 3600 rpm for 15 min at 37 °C to collect approximate 200 μL of the original volume of plasma as ultra-filtrate. The concentrations of *in-vitro* plasma samples and their respective ultra-filtrates were analyzed by an HPLC method[12]. Eq. (2) was used to calculate the percent-bound drug from plasma ($\mathrm{Conc}_{\mathrm{plasma}}$) and ultrafiltrate ($\mathrm{Conc}_{\mathrm{ultrafiltrate}}$) concentrations. Before conducting the plasma-protein binding experiments, the non-specific binding ability of DRDE-07 to the ultrafiltration device membrane was investigated. Two aqueous concentrations of drug, 10 and 50 μg/mL, were prepared from a stock solution (1 mg/mL). These samples (1 mL each) were then placed in ultrafiltration devices and centrifuged at 3600 rpm for 15 min. The concentrations of DRDE-07 in the ultrafiltrate and the remaining aqueous solution (did not undergo ultrafiltration and represent total concentration) were then analyzed by HPLC. Eq. (3) was used to determine the unbound percentage, and thus the percentage of drug bound to the ultrafiltration device membrane was obtained.

$$\mathrm{Bound}\ (\%) = \frac{\mathrm{Concentration}_{\mathrm{Ultrafiltrate}}}{\mathrm{Concentration}_{\mathrm{Plasma}}} \times 100 \quad (2)$$

$$\mathrm{Unbound}\ (\%) = \frac{\mathrm{Peak\ area}_{\mathrm{UF}}}{\mathrm{Peak\ area}_{\mathrm{Total}}} \times 100 \quad (3)$$

2.6. *Blood/plasma partitioning*

The extent of blood cell partitioning of DRDE-07 was determined in mice. DRDE-07 (1 mg/mL) in saline was added to mouse blood that contained sodium heparin at a ratio of 1:50 to give a final concentration of 100 μg/mL. Samples were incubated at 37 °C for 30 min. Plasma samples were obtained by centrifuging aliquots of heparinized blood samples. The blood or plasma samples (100 μL) were then processed and analyzed according to a validated HPLC method[12]. All experiments were performed in triplicate. The blood-to-plasma partition ratio (C_B/C_P) of DRDE-07 was calculated from the drug concentration in blood and plasma. Blood cell distribution was calculated as C_B, C_P and hematocrit (HCT) using[18]

$$\mathrm{Blood\ cell\ distribution}\ (\%) = \frac{100 \times [C_B - C_P(1-\mathrm{HCT})]}{C_B} \quad (4)$$

For determination of HCT, heparinised whole blood (1.0 mL) was taken in an hematocrit tube and centrifuged at 2000*g* for 30 min. The HCT for whole mouse blood was calculated by using[19]

$$\mathrm{HCT} = \frac{\mathrm{Volume\ of\ packed\ cell}}{\mathrm{Total\ blood\ volume}} \quad (5)$$

2.7. *Prediction of pK_a*

The acid dissociation constant of DRDE-07 was determined by the method described by Albert and Serjeant[20]. The method is based

on direct determination of the ratio of molecular species (protonated) to dissociated (deprotonated species) in a series of non-absorbing buffer solutions. For this purpose, the absorption spectra of the molecular species were obtained first in a buffer solution at the pH in which the compound of interest would be present wholly in either form. 30 µL of DRDE-07 stock solution (1 mg/mL) was diluted to 3 mL in a cuvette containing either 0.1 mol/L hydrochloric acid or 0.1 mol/L sodium hydroxide solution and the absorption spectrum of DRDE-07 in acid or alkali were determined over the wavelength 200–400 nm with a reference to blank solution at 25 ± 1 °C. The spectra obtained in acid or alkali were of protonated (D_m) and deprotonated (D_i) molecules. Nine different pH values, ranging from 5.2 to 6.8, were selected to determine the pK_a of DRDE-07. For this, appropriate buffer consisting of phosphate (pH 5.2–6.8) was used to determine the dissociation constants of DRDE-07. 30 µL of DRDE-07 stock solution (1 mg/mL) was diluted to 3 mL in each buffer and the optical densities were determined at analytical wavelengths using buffer blank at 25 ± 1 °C. A set of 9 values of pK_a were obtained using

$$pK_a = pH + \log \frac{D_i - D}{D - D_m} \quad (6)$$

where D_m and D_i correspond to the optical density of protonated and deprotonated forms of DRDE-07, and D is the optical density in the buffer. The average of the 9 measurements was considered the pK_a of the DRDE-07. A Shimadzu UV-1800 UV–visible double-beam spectrophotometer with quartz cells of 10 mm was used for spectrometric analysis. pH values of buffers were determined using pH/ion selective meter (Model 1112000, Thermo Scientific; USA). The pH meter was calibrated at 25 °C using a two-point calibration method with commercially available standard buffer solutions pH 4.0 and 7.0.

2.8. *Pharmacokinetic study*

Swiss Albino female mice were used in this study because DRDE-07 offered better protection against SM in female mice[11]. All animals weighed 25–30 g were obtained from the laboratory division of the Defense Research and Development Establishment, Gwalior, and were used for *in vivo* pharmacokinetic experiments. All protocols were approved by the Animal Ethics Committee of DRDE, Gwalior, India. The animals were individually housed in a temperature-controlled room with 12 h light/dark cycle for at least 48 h before the experiment. A total of 44 mice were used for each independent experiment (per dose). DRDE-07 was dissolved in sterile saline (0.9% NaCl) and was administered orally at two doses level 400 and 600 mg/kg, to the 2 h fasted mice using a mice feeding needle and syringe. Blood samples were collected at 5, 15, 30, 60, 90, 120, 240, 360, 480, 720, and 1440 min *via* retroorbital sinus puncture from the standard heparinized micro-hematocrit capillary tubes into heparinized 0.5-mL centrifuge tubes, (typically 300 µL is collected from each animal, 4 animals per each time point). The care was taken to minimize the trauma due to pain during blood sampling by the use of appropriate diethyl ether anesthesia. Blood samples were promptly centrifuged at 4000 rpm for 10 min. Plasma samples were and stored at −20 °C and analyzed using a validated HPLC method[12]. The pharmacokinetic characterization of DRDE-07 was analyzed using PK Solver, Version 2.0[21] and non-compartmental modeling (NCA) was carried out according to conventional pharmacokinetic principles.

2.9. *Metabolite identification*

The HPLC chromatogram of plasma obtained after p.o. dosing of DRDE-07 showed an extra peak along with the DRDE-07 peak, which was not present in the HPLC chromatogram of blank plasma (Fig. 2). This extra peak may be because of the presence of one of the metabolites (M1) of the DRDE-07. To identify this metabolite the HPLC fractions with the metabolite peak were collected and analyzed by mass spectrometry.

2.9.1. *Fraction collection*

Liquid chromatographic separation was performed Waters HPLC system equipped with Waters 2996 photodiode array detector (PDA). The mobile phase[12] used for quantifying the DRDE-07 in plasma and urine contain phosphates and organic acid which are not suitable for mass analysis, and therefore a different mobile phase was used to collect the metabolite fractions. The mobile phase consisted of (A) DD-H_2O containing 0.2% *v/v* trifluroacetic acid and (B) acetonitrile, and was delivered isocratically in a ratio of 75% (A) and 22% (B) through a Waters X-Tera, C18 column at 30 °C and a flow rate of 1.0 mL/min. This mobile phase was not suitable to quantify the DRDE-07 in plasma because overlapping peaks of DRDE-07 and blank plasma protein were observed at the same retention time. Several metabolite fractions of DRDE-07 (M1) were collected manually using the above-described isocratic program. The fractions of pure metabolite (M1) were combined and evaporated to dryness using a Heto Vacuum Centrifuge, Maxi-Dry Plus type.

2.9.2. *Mass analysis*

Chromatographic separation of the metabolite fractions (M1) was carried out with Thermo Electron HPLC with surveyor LC pump (San Jose, CA, USA) equipped with Thermo Electron Hypercarb, 100 mm × 2.1 mm microbore column. The following solvent composition was made for sample introduction: solvent A (20 mmol/L ammonium hydroxide in water) and solvent B (methanol). The pH of solvent A was adjusted to 8 by adding 25% solution of ammonia hydroxide. The same amount of ammonium hydroxide was added to solvent B. Chromatographic separations were performed using 90% B and 10% A at a flow rate of 200 µL/min. Column temperature was kept at 30 °C. Samples were injected through a Rheodyne injector (Model 7010) fitted with 2 µL loop. The LC system was coupled on-line to a LCQ Advantage ion trap spectrometer (Thermo Electron Corporation) equipped with an orthogonal electrospray interface. The system was operated in a positive ion mode. Helium was continually flowing into the collision cell at a pressure of 0.1 Pa during the electrospray ionization tandem-mass spectrometry (ESI-MS^n)

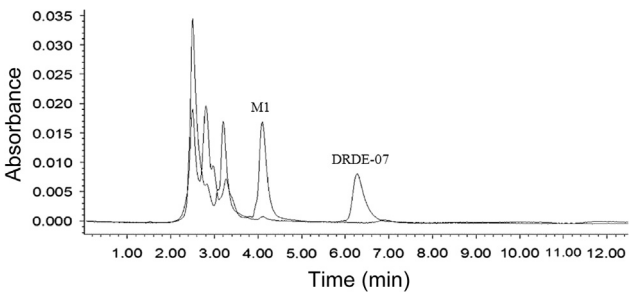

Figure 2 HPLC chromatograms of blank plasma and plasma after oral dosing of DRDE-07 (400 mg/kg).

operation. The ESI-MS^n data were acquired over the mass range of *m/z* 50–300 amu. MS^n spectra were obtained at the collision energy of 30 eV.

3. Results

3.1. Aqueous solubility in simulated intestinal/gastric fluid

Good linearity between the peak areas to the aqueous DRDE-07 concentrations ranging from 1.0 to 100 μg/mL was obtained. The linear regression correlation coefficient (R^2) was 0.9997. The aqueous solubility, as determined by the mean value of DRDE-07 concentrations at equilibrium, was found to be 0.985 ± 0.04 g/mL (n = 3) and 0.747 ± 0.03 g/mL (n = 3) in SIF and SGF, respectively.

3.2. Lipophilicity

The average octanol:water partition coefficient at concentrations of 10, 25 and 50 μg/mL was 0.25 ± 0.01. This is equivalent to a logarithm of the partition coefficient (log *P*) of −0.6 (Table 2).

3.3. Plasma protein binding

In the assay of plasma filtrate, the peak-area of DRDE-07 showed a linear relationship with the nominal concentrations of DRDE-07 in the tested range of 2.5–100 μg/mL. The linear regression correlation coefficient (R^2) was 0.9974. There was no binding of DRDE-07 to the ultrafiltration device membrane; therefore the method is considered suitable for use in the protein binding studies. These results (Table 3) show DRDE-07 binding to mouse plasma proteins (21.82%–24.54%). The fraction of plasma protein binding of DRDE-07 was found to be less than 25% at all concentrations studied.

3.4. Blood/plasma partitioning

The extent of blood partitioning of DRDE-07 was measured in mouse blood at a concentration of 100 μg/mL at 37 °C. The blood to plasma partition ratio (C_B/C_P) of DRDE-07 was found to be 1.52 ± 0.09. The HCT value was found to be 0.448 in mice and the percent distribution of DRDE-07 in blood cells was found to be 18.74 ± 2.31, suggesting that DRDE-07 distributed primarily into the blood compartment (Table 4). The plasma clearance is more widely used in pharmacokinetics than the blood clearance owing to sample collection and analysis are easier in plasma than in blood. However, the clearance estimates of an organ (*e.g.*, liver) are generally described by blood clearance. The relationships between blood clearance (CL_b) and plasma clearance (CL_p) can be described by[22]

$$CL_b = \frac{dA/dt}{C_B(t)} \text{ and } CL_p = \frac{dA/dt}{C_P(t)} \quad (7)$$

Therefore, the blood *versus* plasma concentration ratio can be used to represent the ratio of plasma clearance *versus* blood clearance as

$$\frac{C_B}{C_p} = \frac{CL_P}{CL_B} \quad (8)$$

DRDE-07 was found to be distributed mostly into the blood than rather the plasma. Hence, the plasma clearance of DRDE-07 is expected to be about 1.5-fold higher than the blood clearance of DRDE-07.

3.5. pK_a

DRDE-07, a nitrogen-containing drug, is generally regarded as a basic drug. In this study, the pK_a value of DRDE-07 was predicted by a spectrophotometric method. Although a conventional titration method can be used for experimental measurement of the pK_a. Table 5 contains the pH and absorbance data. The average pK_a value of DRDE-07 was found to be 5.95 ± 0.28.

3.6. Pharmacokinetic study

The concentration–time profiles of DRDE-07 after oral (400 and 600 mg/kg) administration are shown in Fig. 3. Following oral administration (400 and 600 mg/kg) the compound exhibited two peak plasma concentrations. $C_{max,1}$ (9.88 ± 4.31 and 22.39 ± 4.52, respectively) was observed at 15 min, while $C_{max,2}$ (4.39 ± 0.87 and 10.77 ± 4.10, respectively) was observed at 90 min but was lower than $C_{max,1}$. Pharmacokinetic parameters obtained from non-compartmental analysis of oral plasma levels of DRDE-07 in mice are summarized in Table 6. The systemic plasma clearance (CL/*F*) was 320.10 and 192.21 mL/min/kg after 400 and 600 mg/kg oral doses respectively. The volume of distribution (V_z/F) was found as 213.73 and 102.50 L/kg after 400 and 600 mg/kg oral doses, respectively, which is greater than the total body water.

Table 3 Protein binding data analysis for mice plasma[a].

Nominal concentration (μg/mL)	Unbound concentration (μg/mL)	Bound (%)
12.5	9.77 ± 0.46	21.82 ± 4.66
25	18.86 ± 0.09	24.54 ± 0.37
50	39.03 ± 0.85	21.94 ± 1.69

[a]Data based on an average of 3 replicate trials, presented as average ± SD.

Table 2 Calculation of Log *P*.

Nominal concentration (μg/mL)	Pre-incubation concentration (μg/mL)	Post-incubation concentration (μg/mL)	PC	Log *P*
10	10.22 ± 0.40	8.30 ± 0.09	0.23 ± 0.04	−0.60 ± 0.08
25	23.25 ± 0.43	18.88 ± 0.07	0.24 ± 0.02	−0.60 ± 0.03
50	50.86 ± 1.27	39.41 ± 1.39	0.26 ± 0.03	−0.59 ± 0.05
Average			0.25 ± 0.01	−0.60 ± 0.01

Data based on an average of 3 replicate trials, presented as average ± SD.

Table 4 Blood/plasma partitioning data analysis for mice[a].

Nominal concentration (μg/mL)	Drug concentration in plasma (μg/mL)	Drug concentration in blood (μg/mL)	C_B/C_P	Blood cell distribution (%)
100	36.27±2.51	54.89±0.88	1.52±0.09	18.74±2.31

[a]Data based on an average of 3 replicate trials, presented as average±SD.

Table 5 Calculation of pK_a from absorbance data at various pH.

pH	Absorbance (d)	pK_a=pH+log[(D_i-D)/($D-D_m$)]
5.2	0.22725	5.511648
5.4	0.22935	5.633931
5.6	0.23210	5.737252
5.8	0.23368	5.883337
6.0	0.23628	5.995804
6.2	0.23903	6.10309
6.4	0.24045	6.254476
6.6	0.24620	6.244145
6.8	0.26318	6.223706
pK_a		5.95±0.28

D_m=0.21028 (in 0.1 mol/L HCl); D_i=0.26203 (in 0.1 mol/L NaOH).

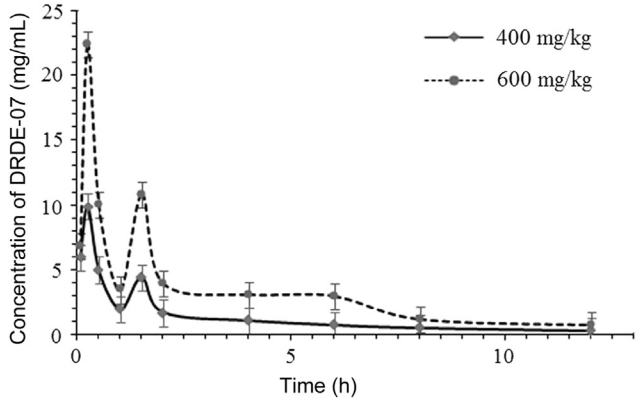

Figure 3 Mean concentration–time curve in mice after a single p.o. doses of DRDE-07 (400 and 600 mg/kg). Each point represents the plasma concentration (mean±SD) for 4 animals.

The estimated terminal half-life was 7.7 and 6.16 h and MRT was 8.48 and 8.99 h after 400 and 600 mg/kg oral doses respectively.

3.7. *Metabolite identification*

The metabolite identification was conducted by comparing the LC/MS chromatogram of the metabolite fraction with that of the expected metabolite *i.e.* phenyl-*S*-ethyl amine and standard DRDE-07 samples (Figs. 4, 5 and 6). The metabolite, M1, was a result of cleavage of the ethylene amine moiety, most probably in the form of aziridine from DRDE-07 because the observed $(M+H)^+$ ion (154 Da) was 43 Da lower than DRDE-07 (197 Da). The LC/MS spectrum of the DRDE-07 metabolite resembled the ESI-MS/MS spectrum of phenyl-*S*-ethyl amine indicating that the probable metabolite of DRDE-07 may be phenyl-*S*-ethyl amine.

Table 6 Pharmacokinetic parameters of DRDE-07 in mice calculated non-compartmental analysis after oral administration.

Parameter	400 mg/kg	600 mg/kg
$C_{max,1}$ (μg/mL)	9.88±4.31	22.39±4.52
$C_{max,2}$ (μg/mL)	4.39±0.87	10.77±4.10
$T_{max,1}$ (min)	15	15
$T_{max,2}$ (min)	90	90
λ_z (min^{-1})	0.0015	0.0019
$t_{1/2}$ (h)	7.7	6.16
T_{lag} (min)	0	0
C_{last}/C_{max}	0.02	0.03
AUC_{0-t} (μg min/mL)	1113.66	2762.99
$AUC_{0-\infty}$ (μg min/mL)	1249.61	3121.52
$AUC_{0-t/0-\infty}$	0.891	0.885
$AUMC_{0-\infty}$ (μg min^2/mL)	635,805.41	1,682,825.74
$MRT_{0-\infty}$ (h)	8.48	8.99
V_z/F (L/kg)	213.73	102.49
CL/F (mL/min/kg)	320.099	192.214

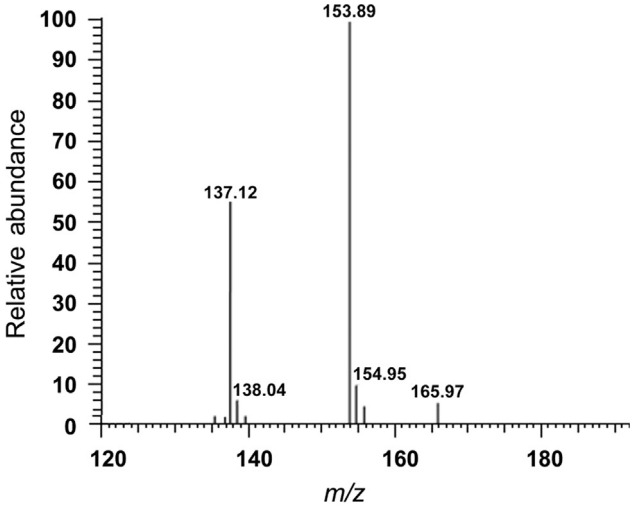

Figure 4 LC–MS spectrum of DRDE-07.

The proposed MS/MS fragments of DRDE-07 and its metabolite are shown in Table 7 and Fig. 7.

4. Discussion

Preclinical pharmacokinetic studies aid in the determination and optimization of various therapy-related parameters, including the dosing, frequency of administration, formulation, and potential sites of toxicity of the agent. Such information is essential to the

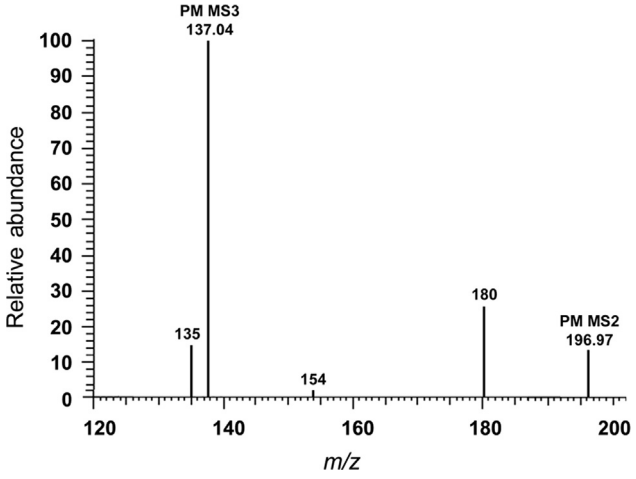

Figure 5 LC–MS spectrum of metabolite (M1).

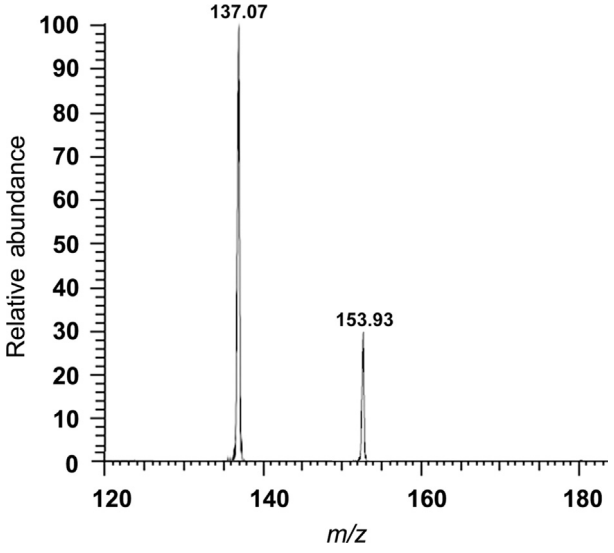

Figure 6 ESI-MS/MS spectrum of pheny-*S*-ethylamine (M1).

Table 7 LC/MS/MS analysis of metabolites of DRDE-07.

Sample	Molecular weight	$[M+H]^+$	MS/MS fragment
DRDE-07	196	197	137, 180
Metabolite (M1)	153	154	137
Phenyl-*S*-ethyl amine	153	154	137

development of a compound toward phase I clinical trials. The good SIG and SGF solubility of DRDE07 contributes to the favorable oral absorption and will also make this drug amenable to compounding in various aqueous formulations. It also presented a relatively low octanol:water partition coefficient (−0.6) which indicates its passage through the epithelium *via* paracellular channels. DRDE-07 was found to be distributed mostly into the blood than rather the plasma. Hence, the plasma clearance of DRDE-07 is expected to be about 1.5 fold higher than the blood clearance of DRDE-07. At oral doses, the kinetics of DRDE-07

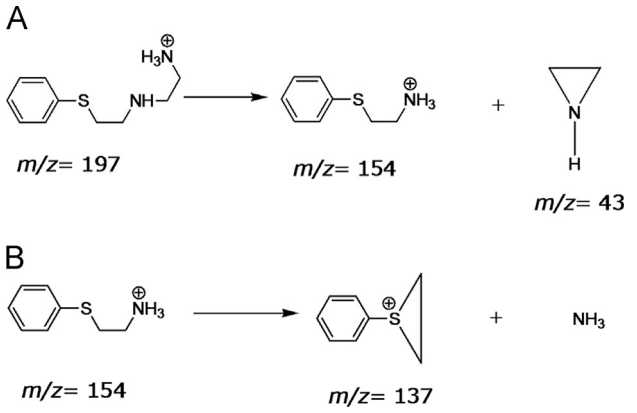

Figure 7 Proposed fragmentations of (A) DRDE-07 and (B) metabolite, M1.

appears to show no dose-proportionality. Nevertheless, C_{max} and AUC did not increase proportionally with the dose, indicating that DRDE-07 follows non-linear kinetics in mice. As C_{max} is dependent on the absorption of the compound, thus no co-relation was found between the ratio of the dose administered and the C_{max}. DRDE-07 showed low binding to proteins in mice plasma (<25%), which may result in a much higher clearance that favors the extensive first-pass effect, resulting in low bioavailability. Low plasma-protein binding also leads to less DRDE-07 being present in the central blood compartment and better extracellular penetration, thus contributing a higher volume of distribution. DRDE-07 appeared rapidly in plasma after oral administration with peak plasma levels (C_{max}) observed in less than 15 min after administration; afterwards there was a rapid decline in the plasma levels followed by a smaller second peak about 90 min after the dose administration. The possibility of enterohepatic recirculation of the compound or erratic absorption was indicated by the presence of the second peak in the profile after oral administration. The volume of distribution (V_z/F) was found to be much larger than the blood volume of mice, indicating rapid uptake of the compound by quickly perfused organs *viz*, liver and kidney. A double-peak phenomenon was observed in four out of total four animals receiving the 200 and 600 mg/kg oral dose, which implies that the DRDE-07 and/or its conjugated metabolites may be excreted into the bile, undergo hydrolysis in the gut (if a conjugated metabolite exists), and be reabsorbed into systemic circulation[23]. The LC/MS spectrum of the DRDE-07 metabolite resembled the ESI-MS/MS spectrum of phenyl-*S*-ethyl amine, indicating that the probable metabolite of DRDE-07 may be phenyl-*S*-ethyl amine. The results obtained from this study should prove useful for further research of DRDE-07.

References

1. Krutzsch W, Trapp R. In: *A commentary on the chemical weapons convention*. London: Martinus Nijhoff Publishers; 1994.
2. Smith WJ, Dunn MA. Medical defense against blistering chemical warfare agents. *Arch Dermatol* 1991;**127**:1207–13.
3. Eisenmenger W, Drasch G, von Clarmann M, Kretschmer E, Roider G. Clinical and morphological findings on mustard gas [bis(2-chloroethyl) sulphide] poisoning. *J Forensic Sci* 1991;**36**:1688–98.
4. Momeni AZ, Enshaeih S, Meghdadi M, Amindjavaheri M. Skin manifestations of mustard gas. A clinical study of 535 patients exposed to mustard gas. *Arch Dermatol* 1992;**128**:775–80.

5. Papirmeister B, Feister AJ, Robinson SI, Ford RD. In: *Medical defense against mustard gas: toxic mechanisms and pharmacological implications*. Boca Raton: CRC Press; 1991.
6. Sugendran K, Kumar P, Vijayaraghavan R. Treatment for sulphur mustard poisoning—a review. *Def Sci J* 1998;**48**:155–62.
7. Dabrowska MI, Becks LL, Lelli JL Jr, Levee MG, Hinshaw DB. Sulfur mustard induces apoptosis and necrosis in endothelial cells. *Toxicol Appl Pharmacol* 1996;**141**:568–83.
8. Reddy PMK, Dubey DK, Kumar P, Vijayaraghavan R. Evaluation of CC-2 as a decontaminant at various time intervals against topically applied sulphur mustard in mice. *Indian J Pharmacol* 1996;**28**:227–31.
9. Bhattacharya R, Rao PVL, Pant SC, Kumar P, Tulsawani RK, Pathak U, et al. Protective effects of amifostine and its analogues on sulfur mustard toxicity *in vitro* and *in vivo*. *Toxicol Appl Pharmacol* 2001;**176**:24–33.
10. Vijayaraghavan R, Kumar P, Joshi U, Raza SK, Rao PVL, Malhotra RC, et al. Prophylactic efficacy of amifostine and its analogues against sulphur mustard toxicity. *Toxicology* 2001;**163**:83–91.
11. Kumar P, Vijayaraghavan R, Kulkarni AS, Pathak U, Raza SK, Jaiswal DK. *In vivo* protection by amifostine and DRDE-07 against mustard toxicity. *Hum Exp Toxicol* 2002;**21**:371–6.
12. Verma P, Gautam A, Vijayaraghvan R. HPLC determination of DRDE-07 in mice plasma: probable antidote for sulphur mustard toxicity. *J Liq Chromatogr Relat Technol* 2013;**36**:2785–95.
13. Verma P, Gautam A, Vijayaraghvan R. Quantitative estimation of DRDE-07 in mice urine using ion-paired reversed-phase high-performance liquid chromatography. *Anal Methods* 2012;**4**:1019–23.
14. Stephen J, Stephen T. (Binary Systems, Part 1). *Solubility of inorganic and organic compounds.* Vol. 1. New York: The Macmillian Co.; 1963.
15. Purcell WP, Bass GE, Clayton JM. In: *Strategy of drug design: a guide to biological activity*. New York: Wiley; 1973.
16. Ashby J, Piddock LJV, Wise R. An investigation of the hydrophobicity of the quinolones. *J Antimicrob Chemother* 1985;**16**:805–8.
17. Asuquo AE, Piddock LJV. Accumulation and killing kinetics of fifteen quinolones for *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 1993;**31**:865–80.
18. Kamath AV, Wang J, Lee FY, Marathe PH. Preclinical pharmacokinetics and *in vitro* metabolism of dasatinib (BMS-354825): a potent oral multi-targeted kinase inhibitor against SRC and BCR-ABL. *Cancer Chemother Pharmacol* 2008;**61**:365–76.
19. Mehrotra N, Lal J, Puri SK, Madhusudanan KP, Gupta RC. *In vitro* and *in vivo* pharmacokinetic studies of bulaquine (analogue of primaquine), a novel antirelapse antimalarial, in rat, rabbit and monkey—highlighting species similarities and differences. *Biopharm Drug Dispos* 2007;**28**:209–27.
20. Albert A, Serjeant EP. In: *The determination of ionization constants: a laboratory manual*. London: Chapman and Hall; 1971.
21. Zhang Y, Huo M, Zhou J, Xie S. PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Comput Methods Programs Biomed* 2010;**99**:306–14.
22. Kwon Y. *Blood vs. plasma clearance*. *Handbook of essential pharmacokinetics, pharmacodynamics and drug metabolism for industrial scientists*. New York: Kluwer Academic Publishers; 2002.
23. Peris-Ribera JE, Molina FT, Carbonell MCG, Aristorena JC, Granero L. General treatment of the enterohepatic recirculation of drugs and its influence on the area under the plasma level curves, bioavailability, and clearance. *Pharm Res* 1992;**9**:1306–13.