DMD Fast Forward. Published on September 2, 2016 as DOI: 10.1124/dmd.116.071647
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Short Communication

Pharmacokinetics and Disposition of Circulating Iridoids and Organic Acids in Rats Intravenously Receiving ReDuNing Injection

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DMD Fast Forward. Published on September 2, 2016 as DOI: 10.1124/dmd.116.071647
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Running Title: Rat Pharmacokinetic Studies of ReDuNing Injection

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Number of Text Pages: 8

Number of Tables: 1

Number of Figures: 3

Number of References: 13

Number of Words in Abstract Section: 248

Number of Words in Introduction Section: 328

Number of Words in Results and Discussion Section: 1266

ABBREVIATIONS: $AUC_{0-\infty}$, area under plasma concentration-time curve to infinity; COMT, catechol-*O*-methyltransferase; CR, carbonyl reductase; $Cum.A_{e\text{-}B,0\text{-}24h}$, cumulative amount excreted into bile collected 0–24 h after dosing started; $Cum.A_{e\text{-}U,0\text{-}24h}$, cumulative amount excreted into urine collected 0–24 h after dosing started; $f_{e\text{-}B}$, fraction of dose excreted into bile; $f_{e\text{-}U}$, fraction of dose excreted into urine; GSH, glutathione; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; SAM, *S*-adenosylmethionine; SULT, sulfotransferase; $t_{1/2}$, elimination half-life; UDPGA, uridine 5'-diphospho-glucuronic acid; UGT, uridine 5'-diphospho-glucuronosyltransferase.

ABSTRACT

ReDuNing injection, prepared from a combination of *Gardenia Jasminoides* fruits, *Lonicera japonica* flower buds, and *Artemisia annua* aerial part, is extensively used for treatment of viral upper respiratory tract infection in China. Iridoids, organic acids, and flavonoids are probably important for the herbal injection because of their reported pharmacological properties. This study was designed to characterize pharmacokinetics and disposition of major circulating herbal compounds in rats intravenously receiving the injection. ReDuNing injection was found to contain 19 iridoids (content levels, 0.01–27.93 mM), 16 organic acids (0.04–19.06 mM), and 11 flavonoids (<0.08 mM). After dosing the injection, the iridoids geniposide, secologanic acid, secoxyloganin, genipin-1-*β*-gentiobioside, geniposidic acid, sweroside, and shanzhiside and the organic acids chlorogenic acid, quinic acid, cryptochlorogenic acid, and neochlorogenic acid were found to be the major circulating compounds with mean elimination half-lives of 0.2–0.9 hour, whereas other plasma compounds were at low exposure levels. These major circulating compounds exhibited small apparent volumes of distribution (0.03–0.34 l/kg). Most of the iridoids were eliminated predominantly via renal excretion of the unchanged compounds, whereas the organic acids were eliminated via methylation and sulfation and excreted into urine as the unchanged and metabolized compounds. The methylated metabolites also underwent subsequent conjugations before hepatobiliary and renal excretion. In vitro data suggested that the preceding metabolism of the organic acids in rats also occurred in humans. The current pharmacokinetic research could serve as a crucial step in identifying the chemical basis responsible for the therapeutic action of ReDuNing injection.

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Introduction

ReDuNing is an herbal injection, which was approved in 2005 by the China Food and Drug Administration (China FDA) for treatment of viral upper respiratory tract infection associated with high fever, chills, headache, myalgia, and cough with phlegm. Each milliliter of the injection is prepared from a combination of 0.60 g of *Gardenia jasminoides* fruits (Zhizi), 0.75 g of *Lonicera japonica* flower buds (Jinyinhua), and 1.25 g of *Artemisia annua* aerial part (Qinghao), yielding an herb-to-injection ratio of 2.6:1. The injection is available as a sterile and nonpyrogenic dosage form for intravenous administration at a dose of 20 ml once daily for three days. ReDuNing therapy appears to have low incidence of side effects (Xu et al., 2009). Recently, ReDuNing injection is also used as an add-on therapy in conventional treatment of hand, foot, and mouth disease in children infected with coxsackievirus type A16 or enterovirus type 71 (Li et al., 2014). Despite its extensive use in clinics, little is known about the chemical basis responsible for the therapeutic action of ReDuNing injection.

Pharmacologically active constituents existing in adequate abundance in an herbal medicine and exhibiting favorable pharmacokinetic profiles are most likely to form the chemical basis responsible for the medicine's therapeutic action. ReDuNing injection has been reported to contain iridoids, organic acids, and flavonoids (Li et al., 2015a). These compounds are important because of their reported antiviral, anti-inflammatory, and antioxidative properties as pure isolates (Shang et al., 2011; Liu et al., 2013). Although bioanalytical assays were developed for measurement of concentrations of some iridoids and organic acids in human and rat plasma after dosing ReDuNing injection (Ni et al., 2015; Wang et al., 2015), pharmacokinetic information about ReDuNing injection is still limited. This hinders understanding the

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chemical basis responsible for the therapeutic action of ReDuNing injection. The current study was designed to assess systemic exposure to ReDuNing compounds in rats intravenously receiving the herbal injection and to investigate pharmacokinetics and elimination of the major circulating herbal compounds.

Materials and Methods

A detailed description of materials and methods is provided in Supplemental Materials and Methods, which are available online.

ReDuNing Injection and Its Component Herbs. Samples of six lots of ReDuNing injection (China FDA drug ratification number, GuoYaoZhunZi-Z20050217) and samples of its component herbs *G. Jasminoides* fruits, *L. japonicae* flower buds, and *A. annua* aerial part were obtained from Jiangsu Kanion Pharmaceutical Corporation (Lianyungang, Jiangsu Province, China).

Chemicals and Reagents. Reference standards of iridoids, organic acids, and flavonoids (purity, ≥98% by HPLC) were obtained from Tauto Biotech (Shanghai, China), Shanghai Nature Standard R&D and Biotech (Shanghai, China), BioBioPha (Kunming, Yunnan Province, China) , and Sigma-Aldrich (St. Louis, MO).

Rat Studies. All animal care and experimental procedures were in compliance with the Guidance for Ethical Treatment of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and approved by an Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica (Shanghai, China). Male Sprague Dawley rats (200–230 g) were obtained from the Sino-British SIPPR/BK Laboratory Animal (Shanghai, China). Three rat studies were performed by giving a single 30-minute intravenous infusion of ReDuNing injection at 2 ml/kg via the tail veins. The rat dose was derived from the label daily dose of ReDuNing injection (20 ml/person) according to dose normalization by body surface area

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(Reagan-Shaw et al., 2008). In the first rat study, blood samples (~80 μl) were collected from four rats, into heparinized tubes, before and 0.083, 0.25, 0.5, 0.58, 0.75, 1, 1.5, 2.5, 4.5, 6.5, 8.5, and 24 hours after starting infusion; the plasma fractions were prepared by centrifuging the blood samples. In the second rat study, urine samples were collected, from four rats, before and 0–4, 4–8, and 8–24 hours after starting infusion. In the third rat study, bile samples were collected, from four rats, before and 0–2, 2–4, 4–6, 6–8, and 8–24 hours after starting infusion.

In Vitro Metabolism Studies. To characterize metabolism of herbal compounds in rats and to facilitate prediction of the metabolism in humans, metabolic capacities of cytochrome P450 (P450), carbonyl reductase (CR), catechol-*O*-methyltransferase (COMT), UDP-glucuronosyltransferase (UGT), and sulfotransferase (SULT) for ReDuNing compounds were assessed using rat liver microsomes and cytosol, as well as human liver microsomes and cytosol. The preceding metabolic reactions were repeated by adding glutathione (GSH) into the incubations. Also, interplays of COMT with UGT and with SULT were also evaluated using methods by Li et al. (2015b).

Determination of Plasma Protein Binding. Fractions of unbound compounds to rat plasma proteins (f_u) were assessed by an ultrafiltration method (Guo et al., 2006).

Liquid Chromatography/Mass Spectrometry-Based Assays. A Waters Synapt G2 high definition time-of-flight mass spectrometer (Manchester, UK), interfaced via a Zspray/LockSpray ESI source with a Waters Acquity UPLC separation module (Milford, MA), was used for analysis of unchanged and metabolized herbal compounds in ReDuNing injection, the component herbs, rat biosamples, and in vitro metabolism study samples.

Data Processing. Pharmacokinetic parameters were estimated by noncompartmental analysis using Kinetica software (version 5.0; Thermo Scientific,

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Philadelphia, PA). All data are expressed as the mean ± standard deviation.

Results and Discussion

Analysis of the chemical composition of ReDuNing injection was the first step in pharmacokinetic research on the injection. The aims of the analysis were to understand which, and how much, herbal compounds were introduced into the bloodstream by dosing the injection and to understand the injection's lot-to-lot variability. A total of 46 compounds (19 iridoids, 16 organic acids, and 11 flavonoids) were detected in ReDuNing injection (Fig. 1). The compounds could be graded according to their dose levels from ReDuNing injection at the label daily dose 20 ml/person. The iridoids geniposide (**6**) and secologanic acid (**4**) and the organic acids chlorogenic acid (**30**), quinic acid (**25**), cryptochlorogenic acid (**31**), and neochlorogenic acid (**29**) exhibited dose levels of >100 μmol/person (135–592 μmol/person); the injection exhibited small lot-to-lot variability (3.1–7.9%) for these compounds, except for **4** being 26.4% (Supplemental Table 1). The sum of the dose levels of **6** and **4** was 78.2% of the total dose of iridoids present in ReDuNing injection, whereas the sum of the dose levels of **30**, **25**, **31**, and **29** was 89.6% of the total dose of organic acids in the injection. The dose levels of secoxyloganin (**12**), genipin-1-β-gentiobioside (**16**), geniposidic acid (**3**), sweroside (**2**), shanzhiside (**9**), isochlorogenic acid C (**35**), caffeic acid (**24**), isochlorogenic acid B (**33**), and isochlorogenic acid A (**34**) were in the range 10–100 μmol/person (13–69 μmol/person); the lot-to-lot variability for these compounds was 6.0–16.1%. The remaining ReDuNing compounds (including all the detected flavonoids) were in the range <10 μmol/person (0.0007–8 μmol/person); the lot-to-lot variability for these minor compounds was 6.3–61.7%. Most of the ReDuNing iridoids originated from the component herbs Zhizi and Jinyinhua. However, **6** originated predominantly from

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Zhizi, whereas **4** originated predominantly from Jinyinhua. Most of the ReDuNing organic acids originated from Zhizi, Jinyinhua, and Qinghao. However, **30** and **25** originated predominantly from Jinyinhua. Despite being minor constituents present in the component herbs, **31** and **29** were also major ReDuNing organic acids, which, in part, were transformed from **30** during the preparation of the injection. Dawidowicz et al. (2014) reported heating-induced conversion of chlorogenic acid into cryptochlorogenic acid and neochlorogenic acid in water.

To understand systemic exposure to ReDuNing compounds, rats received a 30-minute intravenous infusion of the herbal injection. As shown in Fig. 2, a total of 39 unchanged herbal compounds (19 iridoids, 16 organic acids, and 4 flavonoids) were detected in plasma samples of rats after starting infusion. The iridoids geniposide (**6**), secologanic acid (**4**), secoxyloganin (**12**), and geniposidic acid (**3**) and the organic acids chlorogenic acid (**30**), quinic acid (**25**), cryptochlorogenic acid (**31**), and neochlorogenic acid (**29**) exhibited high levels of systemic exposure; whereas the iridoids sweroside (**2**), genipin-1-β-gentiobioside (**16**), and shanzhiside (**9**) were at lower levels. Chemical structures of these major circulating ReDuNing compounds are shown in Fig. 2. The plasma flavonoids detected were at quite low levels. A total of 40 unchanged ReDuNing compounds were detected in rat urine samples, whereas only 24 such compounds were detected, all at low levels, in bile samples (Fig. 2). Renal excretion of unchanged compound was found to be the main elimination pathway for the major circulating iridoids **6**, **12**, **16**, **2**, and **9** with fractions of doses excreted into urine ($f_{e\text{-}U}$) of 64.0–87.5%, whereas the $f_{e\text{-}U}$ of the iridoid **3** was 334.8%. As shown in Supplemental Table 2B, an in vitro metabolism study using rat liver microsomes suggested that P450-mediated and NADPH-dependent oxidative ester cleavage of **6** probably took place in rats to yield **3**. Unlike for these iridoids, renal

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excretion was a minor elimination pathway for the iridoid **4** ($f_{e\text{-}U}$, 7.3%); so was biliary excretion (fraction of dose excreted into bile, $f_{e\text{-}B}$; 0.1%). A reduced metabolite of **4** (**M4$_R$**) was detected in rat plasma, urine, and bile samples (Supplemental Fig. 1 and Supplemental Table 2A); the metabolism was probably mediated by carbonyl reductase (CR) in rat liver cytosol fortified with NADPH (Supplemental Table 2B) and could be inhibited by quercetin. The organic acid **25** was also primarily eliminated unchanged via renal excretion ($f_{e\text{-}U}$, 89.7%). The $f_{e\text{-}U}$ values of the other organic acids **30**, **31**, and **29** (31.9–44.2%) were lower than that of **25**; several metabolites of these compounds were detected in rats (Supplemental Fig. 1 and Supplemental Table 2A). Because **30**, **31**, and **29** were isomers with the same molecular mass 354 daltons, characterization of the parent compound and metabolite relationship was achieved by in vitro metabolism study using purified compounds as substrates (Supplemental Table 2B). As a result, the detected plasma metabolites were methylated metabolites of **30** (**M30$_{M\text{-}1}$** and **M30$_{M\text{-}2}$**), **31** (**M31$_{M\text{-}1}$** and **M31$_{M\text{-}2}$**), and **29** (**M29$_{M\text{-}1}$** and **M29$_{M\text{-}2}$**) and their sulfated metabolites (**M30$_{S\text{-}2}$**, **M31$_S$**, and **M29$_S$**); these metabolites were excreted into urine and, all to less extent, into bile. In addition, the methylated metabolites were subsequently glucuronized to yield **M30$_{M\text{-}G}$**, **M31$_{M\text{-}G}$**, and **M29$_{M\text{-}G}$** (excreted into urine and bile) or conjugated with GSH to yield **M30$_{M\text{-}Gsh\text{-}1}$**, **M30$_{M\text{-}Gsh\text{-}2}$**, **M31$_{M\text{-}Gsh}$**, and **M29$_{M\text{-}Gsh}$** (all excreted only into bile). Figure 3 shows proposed metabolic pathways of **6**, **4**, **30**, **31**, and **29** in rats intravenously receiving ReDuNing injection. In vitro metabolism studies using human liver microsomes and cytosol suggested that the preceding metabolism of **6**, **4**, **30**, **31**, and **29** in rats probably also occurred in humans (Supplemental Table 2B).

Table 1 summarizes the pharmacokinetic parameters of major circulating herbal compounds in rats receiving ReDuNing injection; their plasma concentrations rose as

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the 30-minute infusion was continued with the maximum plasma concentrations (C_{max}) being measured just prior to completion of the infusions (Supplemental Fig. 2). The ReDuNing compounds exhibited short elimination half-lives ($t_{1/2}$), i.e. 0.3–0.9 hour for the iridoids geniposide (**6**), secologanic acid (**4**), secoxyloganin (**12**), genipin-1-β-gentiobioside (**16**), geniposidic acid (**3**), sweroside (**2**), and shanzhiside (**9**) and 0.2–0.5 hour for the organic acids chlorogenic acid (**30**), quinic acid (**25**), cryptochlorogenic acid (**31**), and neochlorogenic acid (**29**). The mean total plasma clearance ($CL_{tot,p}$) values of the iridoids and organic acids were 3–22% and 7–21% of rat cardiac plasma output (7.3 l/h/kg; Toutain et al., 2004), respectively, indicating the clearance of these compounds was not high. The mean distribution volumes at steady state (V_{SS}) of iridoids (0.2–0.4 l/kg) and organic acids (0.3–0.4 l/kg) were close to the rat extracellular volume (0.3 l/kg; Davies and Morris, 1993). The iridoids and organic acids were poorly bound to rat plasma protein with fractions of compounds unbound to rat plasma proteins (f_u) of 75.2–99.0% and 48.8–65.8%, respectively.

Chinese herbal medicines are often combinations of multiple herbs and have very complex chemical composition. Pharmacokinetic research, serving as compound sieve (Liu, et al. 2009), is a crucial step in identifying the chemical basis responsible for the therapeutic actions of herbal medicines. In the current study, 11 unchanged herbal compounds (seven iridoids and four organic acids) showed considerably high levels of systemic exposure with short $t_{1/2}$ (<1 hour) in rats intravenously receiving ReDuNing injection. Also, several methylated and sulfated metabolites of the organic acids exhibited high levels of systemic exposure. Further investigation of these major circulating herbal compounds, including assessing their pharmacodynamic activities related to the injection's therapeutic action, exploring the translation of the compounds' individual activities into the injection's overall action, and evaluating

their pharmacokinetic compatibility (i.e., exhibiting limited pharmacokinetic interaction problems that can counteract the compounds' synergistic or additive pharmacodynamic effects), will finally lead to understanding the chemical basis responsible for the therapeutic action of ReDuNing injection. The current pharmacokinetic study in rats also facilitates designing a future clinical pharmacokinetic study of ReDuNing injection and future systematic evaluation of potential pharmacokinetic herb-drug interactions related to the injection.

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Authorship Contributions

Participated in research design: C. Li, Cheng.

Conducted experiments: Cheng, Du, Yu, Xu, Wang, L. Li, Olaleye, Yang, Chen, Zhong, Liu, J. Li.

Contributed new reagents: Xiao, Wang.

Performed data analysis: C. Li, Cheng.

Wrote or contributed to the writing of the manuscript: C. Li, Cheng.

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Footnotes

This work was supported by the National Science Fund of China for Distinguished Young Scholars [Grant 30925044], the National Science and Technology Major Project of China “Key New Drug Creation and Manufacturing Program” [Grants 2009ZX09304-002], and the National Basic Research Program of China [Grant 2012CB518403].

This work was previously presented as a poster presentation at the following workshop: Cheng et al. (2016) Pharmacokinetic study of ReDuNing injection in rats. *The 13th Asia Pacific Federation of Pharmacologist Meeting “New Paradigms in Pharmacology for Global Health”*, 2016 February 1–3; Bangkok, Thailand.

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Legends for Figures

Fig. 1. Iridoids (both compound names and ID numbers, black), organic acids (purple), and flavonoids (brown) present in ReDuNing injection. (**A**) Stacked chromatograms of herbal compounds from a typical sample of ReDuNing injection by mass spectrometry-based monitoring of their ionized molecules; (**B**), (**C**), and (**D**) content levels (mg/g) of compounds in the component herbs Zhizi (*G. Jasminoides* fruits), Jinyinhua (*L. japonicae* flower buds), and Qinghao (*A. annua* aerial part), respectively; (**E**) content levels (mM) of the compounds in samples of six lots of ReDuNing injection; (**F**) compound dose levels (μmol/person) of the compounds from ReDuNing injection (lot number, 141106) at the label daily dose 20 ml/person; (**G**) percentage doses of iridoids in the total dose of iridoids and percentage doses of organic acids in the total dose of organic acids in ReDuNing injection (141106). See Supplemental Table 1 for the compounds' names and the associated detection and characterization information.

Fig. 2. Systemic exposure to and excretion of unchanged iridoids (compound ID numbers, black), organic acids (purple), and flavonoids (brown) in rats receiving a 30-minute intravenous infusion of ReDuNing injection (lot number, 141106) at 2 ml/kg and chemical structures of the major circulating herbal compounds. ReDuNing compounds (shown as compound ID) in (**A**–**C**) are ranked according to their content levels in ReDuNing injection (Fig. 1E). See Supplemental Table 1 for the compounds' names. The symbol "×" (in red) denotes that the compound was not detected.

Fig. 3. Proposed metabolic pathways for the iridoids (in black) geniposide (**6**) and secologanic acid (**4**) and the organic acids (in purple) chlorogenic acid (**30**), cryptochlorogenic acid (**31**), and neochlorogenic acid (**29**) in rats intravenously receiving ReDuNing injection. The metabolite ID is used to indicate the compound being a metabolite, showing its parent compounds, type of metabolism, and metabolite isomer. For instance, **M30** in $\mathbf{M30_{M\text{-}1}}$ denotes that the compound is a metabolite of chlorogenic acid (**30**). The subscript letter **M** denotes "methylation" and the subscript number **1** denotes the first eluted metabolite isomer. The subscript letters **R**, **G**, **S**, and **Gsh** in other metabolite IDs denote "reduction", "glucuronidation", "sulfation", and "glutathionylation", respectively. Glc, β-D-glucopyranosyl; Glu, glucuronosyl; Gsh, glutathione.

TABLE 1

Pharmacokinetics of major circulating iridoids and organic acids in rats receiving a 30-minute intravenous infusion of ReDuNing injection

C_{max}, maximum plasma concentration; $AUC_{0\text{-}24h}$, area under the plasma concentration-time curve from 0 to 24 hours after starting infusion; $t_{1/2}$, elimination half-life; MRT, mean residence time; $CL_{tot,p}$, total plasma clearance; V_{SS}, apparent volume of distribution at steady state; $f_{e\text{-}U}$, fraction of dose excreted into urine; CL_R, renal clearance; and f_u, fraction of compound unbound to rat plasma proteins. **6**, geniposide; **4**, secologanic acid; **12**, secoxyloganin; **16**, genipin-1-*β*-gentiobioside; **3**, geniposidic acid; **2**, sweroside; **9**, shanzhiside; **30**, chlorogenic acid; **25**, quinic acid; **31**; cryptochlorogenic acid; and **29**, neochlorogenic acid.

PK parameter	Iridoid							Organic acid			
	6	**4**	**12**	**16**	**3**	**2**	**9**	**30**	**25**	**31**	**29**
C_{max} (μM)	71.7 ± 6.4	30.8 ± 4.0	19.1 ± 1.1	6.7 ± 0.9	11.3 ± 2.4	6.3 ± 0.4	4.6 ± 0.2	55.7 ± 4.2	51.9 ± 5.3	24.5 ± 2.1	19.0 ± 2.2
$AUC_{0\text{-}24h}$ (μM·hour)	36.4 ± 3.4	16.2 ± 3.0	12.1 ± 1.8	4.4 ± 0.6	16.1 ± 4.2	4.0 ± 0.6	2.9 ± 0.6	28.1 ± 3.0	35.3 ± 7.0	12.3 ± 1.5	8.8 ± 1.1
$t_{1/2}$ (hour)	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.9 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
MRT (hour)	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	1.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.0
$CL_{tot,\ p}$ (l/hour/kg)	1.6 ± 0.2	1.4 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.2 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	1.3 ± 0.1	0.5 ± 0.1	1.3 ± 0.2	1.6 ± 0.2
V_{SS} (l/kg)	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
$f_{e\text{-}U}$ (%)	74.3 ± 15.4	7.3 ± 3.2	87.5 ± 20.8	71.3 ± 18.4	334.8 ± 90.3	80.3 ± 24.6	64.0 ± 28.5	35.2 ± 11.8	89.7 ± 15.0	31.9 ± 14.9	44.2 ± 11.2
$f_{e\text{-}B}$ (%)	0.3 ± 0.1	0.1 ± 0.0	1.0 ± 0.2	1.4 ± 0.3	38.4 ± 24.7	1.3 ± 0.2	0.3 ± 0.1	0.0 ± 0.0	0.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
f_u (%)	92.3	91.2	75.2	80.4	88.0	95.7	99.0	56.9	65.8	48.8	65.3

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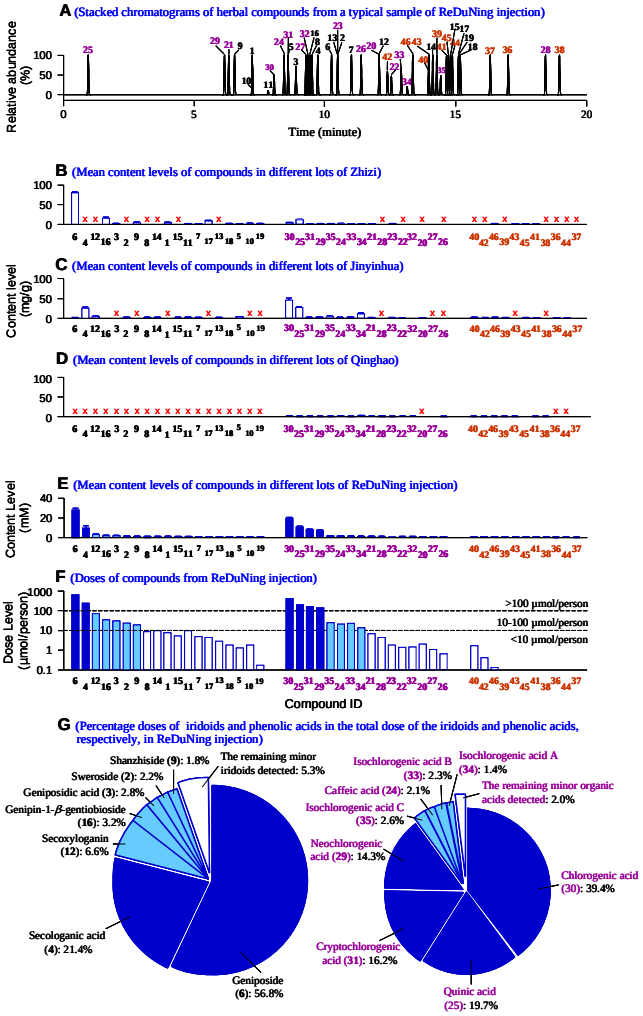
DMD #71647 - Figure 1



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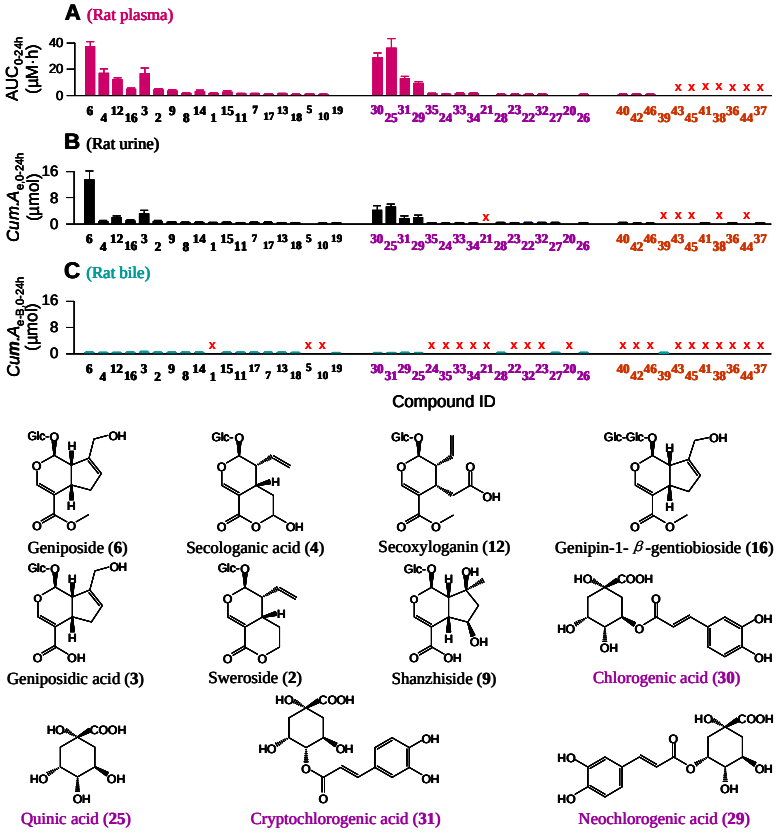

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DMD #71647 - Figure 3