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Quantification of *trans*-2,6-difluoro-4'-N,N-dimethylaminostilbene in rat plasma: Application to a pharmacokinetic study

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Abstract

trans-2,6-Difluoro-4'-N,N-dimethylaminostilbene (DFS), a synthetic stilbene, displayed potent preclinical anti-cancer activities exceeding that observed for naturally occurring resveratrol. In this study, a simple and sensitive HPLC method was developed and validated to quantify DFS in rat plasma. The lower limit of quantification (LLOQ) was 5 ng/ml. The intra- and inter-day variation in terms of relative standard deviation (RSD) was all less than 10%. The bias rate ranged from -11.5% to 6.2% while the absolute recovery ranged from 94.1 ± 2.3 to $97.3 \pm 4.4\%$. The pharmacokinetic profiles of DFS were examined in Sprague-Dawley rats after intravenous administration (2 mg/kg). DFS displayed moderate clearance ($Cl = 61.5 \pm 17.7$ ml/min/kg) and a relatively prolonged terminal elimination half-life ($t_{1/2\lambda_z}$) of 351 ± 180 min. Aqueous solubility played a crucial role in the oral absorption of DFS. When DFS was given as a suspension (6 mg/kg), the absolute oral bioavailability (F) was almost negligible. However, when DFS was given in a solution prepared with hydroxypropyl- β -cyclodextrin (6 mg/kg), the F was $12.4 \pm 10.7\%$. Dose-escalation to 15 mg/kg resulted in much higher systemic exposure ($F = 40.2 \pm 10.0\%$). As DFS is orally available after formulation with hydroxypropyl- β -cyclodextrin and pharmacologically active systemic concentrations could be achieved after a single oral dose, the use of DFS as a cancer hemopreventive/chemotherapeutic agent is possible.

Keywords

trans-2,6-Difluoro-4'-N,N-dimethylaminostilbene; HPLC; Pharmacokinetics; Absolute oral bioavailability; Dose-escalation

1. Introduction

Naturally occurring resveratrol (*trans*-3,5,4'-trihydroxystilbene, **1**, Fig. 1) is a phytoalexin that possesses numerous effects including anti-aging, anti-diabetic, anti-inflammation, anti-obesity, anti-oxidation, cancer chemoprevention, cardio- and neuro-protection [1]. Due to its inferior pharmacokinetics, such as its short half-life, extensive metabolism *via* glucuronidation or sulfation and poor oral bioavailability [1], resveratrol is not a suitable candidate for further drug development. Therefore, the development of analogs with

superior potency, improved pharmacokinetics and most importantly, selective clinical applications, is of interest.

Fluorinated N,N-dialkylaminostilbenes are innovative compounds that display selectivity as anti-cancer agents [2]. Among the recently reported fluorinated N,N-dialkylaminostilbenes, *trans*-2,6-difluoro-4'-N,N-dimethylaminostilbene (DFS, **2**, Fig. 1) displayed the most potent anti-neoplastic activities [2]. It inhibited Wnt/ β -catenin, a signaling pathway that plays an important role in tumorigenesis, at a sub-micromolar level [2]. Subsequently, the proliferation of colorectal cancer cells was suppressed in a mouse xenograft model [2]. Thus, DFS appears to be a promising drug candidate for further pharmaceutical development.

In this study, a simple and sensitive HPLC–UV method was developed and validated for the quantification of DFS in rat plasma. The pharmacokinetic profiles of DFS were subsequently examined in Sprague-Dawley rats after single intravenous and oral administration. To the authors' knowledge, this is the first report of the pharmacokinetics of DFS. Findings from the present study are necessary in evaluating the potential clinical application of DFS.

2. Materials and methods

2.1. Special precautions

All laboratory procedures involving the manipulation of DFS and resveratrol trimethyl ether (RTE) were executed in a dimly lit environment in order to prevent photo-isomerization of stilbenes.

2.2. Chemicals and reagents

trans-2,6-Difluoro-4'-N,N-dimethylaminostilbene (DFS, **2**, Fig. 1) was prepared in a previous study (purity > 97%) [2]. RTE (*trans*-3,5,4'-trimethoxystilbene, **3**, Fig. 1) (purity 97%) and the sodium salt of carboxymethylcellulose (CMC) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma–Aldrich (St. Louis, MO 63178, USA), respectively. 2-Hydroxypropyl- β -cyclodextrin (HP- β -CD, degree of substitution: about 0.6) was a generous gift from Roquette (Lestrem, France).

2.3. Liquid chromatography

A Shimadzu (Kyoto, Japan) 2010A Liquid Chromatography was used throughout the study. This integrated HPLC apparatus consists of a quaternary gradient low-pressure mixing pump, an online degasser, an auto-sampler, a column oven, a dual-wavelength UV–vis detector and a system controller. It was controlled by a personal computer using Shimadzu Class-VP Version 6.12 SP1 software (Shimadzu, Kyoto, Japan). Chromatographic data analysis was also performed with the same software.

The chromatographic conditions were optimized by modifying the recent methods for quantification of various stilbenes in rat plasma [3–9]. The chromatographic separation was obtained by a reversed phase HPLC column (Agilent Zorbax Eclipse Plus C18: 250 $\times$ 4.6 mm i.d., 5 μ m), which was protected by a guard column (Agilent Zorbax Eclipse Plus C18: 12.5 mm $\times$ 4.6 mm i.d., 5 μ m) through a 12.5-min gradient delivery of a mixture of acetonitrile and water at a flow rate of 1.5 ml/min at 50 $^{\circ}$ C. The gradient schedule was: (a) 0–4.5 min, acetonitrile: 65%; (b) 4.5–5.5 min, acetonitrile: 65 $\rightarrow$ 90%; (c) 5.5–9 min, acetonitrile: 90%; (d) 9–12.5 min, acetonitrile: 65%. Ultraviolet (UV) absorbance at 356 (maximal UV absorption wavelength of DFS) was recorded while the absorbance at 320 nm was used as reference.

2.4. Sample preparation

DFS stock solutions were prepared with dimethyl sulfoxide (DMSO) and stored at room temperature (25 °C). The calibration standards (5, 50, 100, 250, 500, 1000 and 1500 ng/ml) and quality control (QC) samples (14, 140 and 1400 ng/ml) were freshly prepared daily by diluting the stock solutions with blank rat plasma. A working solution of RTE (internal standard) was prepared in acetonitrile at a concentration of 750 ng/ml. During the sample cleanup procedure, three volumes of RTE-acetonitrile working solution were added to one volume of rat plasma. The samples were vortexed vigorously followed by centrifugation at $10000 \times g$ for 5 min at 4 °C. Finally, the supernatant was carefully transferred into a glass insert pre-installed in a 1.5 ml amber auto-sampler vial and 100 μ l of the analyte solution was injected into the HPLC system.

2.5. Assay validation

This analytical method was validated by assessing its specificity, sensitivity, linearity (R^2), precision (intra- and inter-day variation), accuracy (bias rate) and the stability profiles of DFS. The specificity was examined by comparing the chromatograms of 6 individual blank rat plasma samples and the corresponding plasma samples spiked with DFS and RTE. The specificity of the assay was further confirmed with the actual pharmacokinetic samples by chromatographic comparison between pre-dosing and post-dosing plasma samples. The assay sensitivity was represented by the lower limit of quantification (LLOQ), which was defined as the concentration producing a signal to noise ratio equal to 5 [10]. The ratio between the peak area of DFS and RTE (internal standard) recorded at λ equal to 356 nm was defined as the analytical response. Linear regression was carried out with GraphPad Prism Version 5.04 (La Jolla, CA 92037, USA), where x was the concentration of DFS, y was the analytical response, and $1/x^2$ was used as a weighting factor [5,9]. The calibration standards (5, 50, 100, 250, 500, 1000 and 1500 ng/ml) were used to assess the linearity. The calibration was carried out over 5 consecutive days. For intra-day studies, 5 sample replicates were analyzed. For inter-day studies, duplicate samples were measured. The intra- and inter-day variation in terms of relative standard derivation (RSD) of analytical response at each concentration were calculated and used as precision indicators. The QC samples (14, 140 and 1400 ng/ml) were prepared and analyzed in the same way. The precision (RSD), accuracy (bias) and absolute recovery (%) were measured with the QC samples [3,5]. The stability of DFS under various conditions was assessed with the QC samples.

2.6. Pharmacokinetic study

The animal handling protocol had been reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS). This animal model has been used extensively to assess the pharmacokinetics of various stilbenes [3–9,11,12]. Twenty-three Sprague-Dawley rats (male, 320 ± 19 g, bred by the Center for Animal Resources, NUS) were divided into four groups. Group 1 ($n = 5$) received a single bolus intravenous administration of DFS solution prepared with 0.01 M HCl-0.3 M HP- β -CD (2 mg/kg), serial blood samples were collected before dosing and at 5, 15, 30, 60, 90, 120, 180, 300, 480 and 720 min post dosing. Group 2 ($n = 6$) received a single oral administration of DFS in 0.3% CMC suspension (6 mg/kg) through gavage and serial blood samples were collected before dosing and at 30, 60, 90, 120, 150, 180, 240 and 300 min post administration. Group 3 ($n = 6$) received a single oral administration of DFS solution prepared with 0.01 M HCl-0.3 M HP- β -CD (6 mg/kg) through gavage and serial blood samples were collected before dosing and at 15, 30, 45, 60, 90, 120, 180, 300, 480 and 720 min post dosing. Group 4 ($n = 6$) received a single oral administration of DFS at 15 mg/kg using the same formulation, dosing route and blood sampling schedule as in Group 3. During each sampling, 120 μ l blood was collected. The harvested plasma was kept at -80 °C until HPLC assay, which was performed within 7 days after the pharmacokinetic study.

Pharmacokinetic analysis was performed by WinNonlin standard version 1.0 (Scientific Consulting Inc., Apex, NC 27502, USA) as described before [4,5]. As the intravenous plasma pharmacokinetic profile of DFS displayed a typical bi-exponential decline, its plasma concentration–time data were fitted to the classical two-compartment first-order open model ($C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t}$) using a nonlinear least squares method with a weighting factor of $1/y^2$ [4,5]. The plasma exposure (area under the plasma concentration–time curve ($AUC_{0 \rightarrow last}$)), clearance ($Cl_{0 \rightarrow last}$), terminal elimination half-life ($t_{1/2 \lambda_z}$) and mean transition time ($MTT_{0 \rightarrow last}$) was calculated through non-compartmental analysis [4,5].

2.7. Statistics

All statistical calculations were performed with GraphPad Prism Version 5.04 or GraphPad InStat version 3.10 (GraphPad Software, Inc., La Jolla, CA, USA). A contingency table was compared by a two-tailed Chi-square test. As the data for the observed time to maximal concentration (t_{max}) in a pharmacokinetic study are non-continuous and restricted by the sampling schedule, the Mann–Whitney test was used to compare the t_{max} data between Groups 3 and 4. For the other pharmacokinetic parameters in Groups 3 and 4, the normality of the distribution of data was assessed with the Kolmogorov–Smirnov test. When the distribution was Gaussian, a two-tailed unpaired *t*-test was applied; otherwise, the Mann–Whitney test was used. A *p* value less than 0.05 was adopted to indicate statistical significant difference.

3. Results and discussion

3.1. Assay validation

As *trans*-stilbene has been used extensively as internal standard to quantify various pharmacologically active stilbenes in rat plasma [3–7,9,12,13], we tried it as internal standard at the beginning. However, since *trans*-stilbene co-eluted with one of the unidentified metabolites, we had to reselect the internal standard. After a rapid screening on the stilbene compounds that we have, RTE was chosen as internal standard. The specificity of this HPLC method for the quantification of DFS in rat plasma was demonstrated. Under our chromatographic conditions, DFS and RTE eluted at ~8.7 and ~7.0 min, respectively (Fig. 2A). No notable interference peak was observed in the chromatograms acquired from either blank plasma samples ($n = 6$) or pre-dosing plasma samples ($n = 23$) (a typical chromatogram of a pre-dosing sample is shown in Fig. 2B). Moreover, DFS and RTE were well separated from various metabolites in the chromatograms acquired from post-dosing samples (Fig. 2C and D).

The LLOQ of DFS was found to be 5 ng/ml. The calibration curves were linear (R^2 at least >0.9986). This HPLC assay was quite precise as the intra-day or inter-day RSDs were all less than 10% (calibration data are not shown, QC data are shown in Table 1). The accuracy of the assay was also confirmed with the QC samples and bias rate ranged from $-11.5 \pm 6.2\%$ (Table 1). Similarly, the absolute recovery rates were $97.3 \pm 4.4\%$, $93.3 \pm 3.0\%$ and $94.1 \pm 2.3\%$ at 14, 400, and 1400 ng/ml, respectively. The stability profiles of DFS were assessed and the results are shown in Table 2.

In summary, a simple and sensitive HPLC method was developed and validated for the quantification of DFS in rat plasma. This HPLC method appeared to be appropriate for the pre-clinical pharmacokinetic investigation of DFS in rats.

3.2. Application to a pharmacokinetic study

As DFS is water insoluble, 0.01 M HCl-0.3 M HP- β -CD was used to solubilize DFS. The intravenous pharmacokinetic profile of DFS was examined after a bolus injection of DFS at 2 mg/kg (Group 1). The plasma DFS *versus* time profile is shown in Fig. 3A. Upon intravenous dosing, plasma DFS levels declined bi-exponentially (*i.e.*, a distribution phase followed by a terminal elimination phase). Therefore, the classical two-compartment first-order open model was applied to describe the intravenous pharmacokinetic profile of DFS. The major pharmacokinetic parameters of DFS are listed in Table 3. DFS exhibited a moderate apparent volume of distribution in the central compartment (V_c) of 2.14 ± 0.75 L kg^{-1} , a moderate clearance (Cl) of 61.5 ± 17.7 ml/min/kg, and a mean residence time (MTT) of 60.9 ± 26.1 min, with a relatively prolonged terminal elimination half-life ($t_{1/2\lambda_z}$) of 351 ± 180 min. Clearly, the elimination rate of DFS appears to be quite favorable for its development as a potential therapeutic agent. Three metabolites (peak 3–5) were observed in the plasma samples collected at 15 min after intravenous injection (2 mg/kg) (Fig. 2C). Peak 5 may be the *cis*-isomer of DFS. Peaks 3 and 4 appeared to be more polar than DFS as they eluted earlier than DFS under reversed-phase HPLC. The signal of peak 3 was consistently higher than peak 4.

In recent studies, the aqueous solubility has been found to be a barrier to the oral absorption of various stilbenes such as resveratrol, its methylated analog (RTE), and *trans*-2,4,3',4',5'-pentamethoxystilbene [4,5,11]. To elucidate the influence of aqueous solubility on the oral bioavailability of DFS, the oral pharmacokinetic profiles of DFS were compared using two formulations, namely DFS suspended in 0.3% CMC and DFS solubilized in an aqueous solution containing 0.01 M HCl-0.3 M HP- β -CD. When a suspension was given (Group 2, 6 mg/kg), the oral bioavailability of DFS was almost nil. Among 54 post-dosing plasma samples in Group 2, DFS was quantifiable in only 5 samples from two rats. When a solution was given (Group 3, 6 mg/kg), DFS was absorbed rapidly with a peak plasma level observed at 30 min post-gavage (Fig. 3B). Among 60 post-dosing plasma samples in Group 3, DFS was quantified in 46 samples (Group 2 *versus* Group 3, two-tailed Chi-square test: $p < 0.0001$). However, the maximal plasma concentration ($C_{\max}$) was fairly low ($C_{\max} = 88.5 \pm 37.8$ ng/ml). After achieving $C_{\max}$, the DFS plasma concentrations dropped rapidly and declined to unquantifiable level 3–8 h after oral administration. At the dose of 6 mg/kg, the plasma exposure of DFS was limited, with an absolute oral bioavailability (F) of only $12.4 \pm 10.7\%$. Clearly, aqueous solubility was a barrier to the oral bioavailability of DFS as evident by the improved oral absorption when DFS was solubilized with 0.01 M HCl-0.3 M HP- β -CD.

The oral pharmacokinetic profile of DFS was also assessed at a higher dose (Group 4, 15 mg/kg) with the solution formulation. Interestingly, non-linear pharmacokinetics was observed (Table 3). At the higher dose, the dose-normalized $C_{\max}$ ($C_{\max}/\text{dose}$) was significantly higher (two-tailed unpaired t -test: $p < 0.05$) while the MTT and $t_{\max}$ were longer (Mann–Whitney test: $p < 0.01$). Most importantly, dose-escalation from 6 mg/kg to 15 mg/kg led to a greater than 2.5-fold enhancement in F ($12.4 \pm 10.7\%$ versus $40.2 \pm 10.0\%$, two-tailed unpaired t -test: $p < 0.001$). The elimination appeared to be slower at higher dose as the plasma DFS levels declined more gradually after achieving $C_{\max}$ and DFS still remained at measureable levels 12 h post-oral administration. Clearly, the 15 mg/kg dose of DFS produced greater plasma exposure (Fig. 3B) than the lower dose.

The non-linear oral pharmacokinetics of DFS can be rationalized by saturable first-pass metabolism. Various metabolites were observed in plasma samples collected after oral administration (15 mg/kg) (Fig. 3D). Although these metabolites have not yet been identified, their chromatographic signals were fairly intense. Interestingly, less metabolite was observed after intravenous administration (Fig. 3C). For example, the plasma

concentration of DFS collected at 15 min after intravenous administration (2 mg/kg) was comparable to the $C_{\max}$ level ($t_{\max}$: ~60 min) after oral administration (15 mg/kg), while the signal intensity of metabolite peak 3 in Group 1 was much lower. Moreover, metabolite peaks 6–8 were observed in Fig. 3D but not in Fig. 3C although they were observed at low intensity levels in the chromatograms of the first post-dosing sample (5 min) of Group 1. Clearly, DFS undergoes partial first-pass metabolism after oral administration. At a higher oral dose (*e.g.*, 15 mg/kg), the first-pass metabolism may be saturated or partially saturated, leading to a slower elimination, more abundant plasma exposure, and much higher F than that seen at the lower dose of 6 mg/kg.

Besides DFS, the pharmacokinetic profiles of various other stilbenes including resveratrol [11], pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene) [8], RTE [4], *cis*-RTE [3], oxyresveratrol tetramethyl ether (OTE, *trans*-2,4,3',5'-tetramethoxystilbene) [3], piceatannol (PTE, *trans*-3,4,3',5'-tetramethoxystilbene) [7], DMU-212 (*trans*-3,4,5,4'-tetramethoxystilbene) [9], *trans*-3,5,3',4',5'-pentamethoxystilbene [6], *trans*-2,4,3',4',5'-pentamethoxystilbene [5] have been recently assessed with the same animal model, allowing a pharmacokinetic comparison. Obviously, the pharmacokinetic profiles of DFS were more favorable than those of resveratrol as DFS had a much slower elimination rate and longer elimination half-life than resveratrol. The major intravenous pharmacokinetic parameters of DFS, namely V_c , $t_{1/2\lambda_z}$, MTT and Cl were comparable to those of the other methoxylated stilbenes [3–9,12]. However, the oral pharmacokinetics of DFS was more favorable than OTE [3], DMU-212[9], *trans*-2,4,3',4',5'-pentamethoxystilbene [5] and *cis*-RTE [13] as DFS possessed more abundant plasma exposure and quite high oral bioavailability when given in solution form at a relatively higher dose.

DFS displayed potent anti-cancer activities and effectively inhibited the proliferation of colorectal cancer cells at 0.3 μ M [2]. Such concentrations could be obtained and maintained for ~6 h in plasma after a single oral dose of DFS at 15 mg/kg. Therefore, the pharmacokinetic properties of DFS will not hinder its application as an orally active chemopreventive agent. Moreover, as DFS is susceptible to first-pass metabolism, its intestinal/hepatic levels should be even higher than those reported here. Clearly, DFS has properties that make it a promising candidate for the prevention of colorectal cancer.

4. Conclusions

In summary, a simple and sensitive HPLC method has been developed and validated to quantify DFS in rat plasma. This HPLC method has been used to examine the pharmacokinetic profiles of DFS in Sprague-Dawley rats. Similar to many other stilbenes, aqueous solubility has been identified as a barrier to the oral absorption of DFS, but this barrier was overcome by enhancing DFS water-solubility using an cyclodextrin complexing agent such as HP- β -CD. Dose-escalation led to much higher oral bioavailability than seen at low doses. As DFS was orally available and pharmacologically active plasma concentrations could be achieved after a single oral dose, future investigations on DFS as cancer chemopreventive/chemotherapeutic agent are warranted.

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(1)

(2)

(3)

Fig. 1.
Chemical structures of resveratrol (1), *trans*-2,6-difluoro-4'-N,N-dimethylaminostilbene (DFS) (2), and resveratrol trimethyl ether (RTE) (3).

Fig. 2.

Typical chromatograms (UV absorbance, $\lambda = 356$ nm) of (A) a blank plasma sample spiked with DFS (50 ng/ml) and RTE (internal standard) (2.25 μ g/ml), (B) a pre-dosing plasma sample, (C) a plasma sample taken from a rat at 15 min after receiving an intravenous dose of DFS (2 mg/kg) (without internal standard), and (D) a plasma sample taken from a rat at 60 min after receiving an oral dose of DFS (15 mg/kg) (with internal standard). Peak 1, DFS; peak 2, internal standard; peaks 3–8, unidentified metabolites.

Fig. 3.
Pharmacokinetic profiles of DFS in Sprague-Dawley rats. Symbols represent mean values and error bars represent SD.

Table 1

Analytical accuracy and precision of DFS in rat plasma.^a

Amount spiked (ng/ml)	Intra-day			Inter-day		
	Amount measured (ng/ml)	RSD (%)	Bias rate range (%)	Amount measured (ng/ml)	RSD (%)	Bias rate range (%)
14.0	13.7 ± 0.7	4.8	-10.0 to 1.6	13.5 ± 0.7	5.2	-10.0 to 6.2
140.0	136.8 ± 4.5	3.3	-5.8 to 2.4	140.7 ± 5.8	4.1	-7.0 to 6.0
1400.0	1410.1 ± 34.8	2.5	-2.0 to 4.1	1383.2 ± 67.5	4.9	-11.5 to 4.1

^aResults were presented as mean ± SD (n = 5).

Table 2Stability profiles of DFS.^a

Stability (% remained)	Spiked concentration (ng/ml)		
	14.0	140.0	1400.0
Stock solution stored at 24 °C for 7 days	101.1 ± 7.7	96.3 ± 3.9	101.5 ± 2.1
Plasma samples stored at 4 °C for 24 h	86.9 ± 3.2	89.9 ± 1.9	90.5 ± 2.3
Post-preparative samples stored at 24 °C for 24 h	92.1 ± 4.4	92.7 ± 4.3	93.5 ± 3.3
Plasma samples after three freeze–thaw cycles	96.2 ± 4.7	97.6 ± 4.8	94.2 ± 1.3
Plasma samples stored at –80 °C for 14 days	93.1 ± 7.0	91.5 ± 2.1	90.5 ± 0.9

^aResults were presented as mean ± SD (*n* = 5).

Table 3Pharmacokinetic parameters of DFS.^a

Parameters	Intravenous (<i>n</i> = 5)	Oral (<i>n</i> = 6)	
Formulation	HP-β-CD	HP-β-CD	HP-β-CD
Dose (mg/kg)	2	6	15
<i>A</i> (ng/ml)	970 ± 351	–	–
<i>B</i> (ng/ml)	63.4 ± 18.9	–	–
α ($10^{-2} \times \text{min}^{-1}$)	9.50 ± 2.65	–	–
β ($10^{-3} \times \text{min}^{-1}$)	3.72 ± 1.03	–	–
<i>V_c</i> (L/kg)	2.14 ± 0.75	–	–
<i>AUC</i> _{0→last} ($10^4 \times \text{min ng/ml}$)	2.90 ± 1.06	1.08 ± 0.93	8.73 ± 2.16
<i>Cl</i> (ml/min/kg)	61.5 ± 17.7	–	–
<i>t</i> _{1/2λ_Z} (min)	351 ± 180	–	–
<i>MTT</i> _{0→last} (min)	115 ± 39	97.8 ± 55.7	218.9 ± 34.8 ^{**}
<i>C</i> _{max} (ng/ml)	–	88.5 ± 37.8	361.3 ± 115.9
<i>C</i> _{max} /dose (mg/ml)	–	14.7 ± 6.3	24.1 ± 7.7 [*]
<i>t</i> _{max} (min)	–	30	45 or 60 ^{**}
<i>F</i> (%)		12.4 ± 10.7	40.2 ± 10.0 ^{***}

^aResults were presented as mean ± SD.^{*}*p* < 0.05.^{**}*p* < 0.01.^{***}*p* < 0.001.