

Determination of Oral Bioavailability of Fusaric Acid in Male Sprague-Dawley Rats

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Published online: 6 June 2014

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Abstract Head and neck squamous cell cancer accounts for 3 % of new cancer cases and 2 % of cancer mortality annually in the United States. Current treatment options for most head and neck cancers continue to be surgical excision with or without radiation, radiation alone, or chemotherapy with radiation depending on location, stage of disease, and patient preference. Fusaric acid (FA) is a novel compound from a novel class of nicotinic acid derivatives that have activity against head and neck squamous cell carcinoma (HNSCC). Although its exact mechanism is still unknown, FA is thought to be active by increasing damage to DNA and preventing its synthesis and repair. The novel mechanism of FA provides an alternative to present therapies, as a single agent whether given parenterally or orally. It has synergy with conventional agents taxol, carboplatin, and erlotinib. In order to determine if FA has reasonable oral bioavailability, we have determined the pharmacokinetics of FA in male Sprague Dawley rats following administration by gavage and by intravenous injection. The bioavailability of FA was sufficient (58 %) to suggest that FA may be viable as an orally administered medication. Despite the encouraging bioavailability of FA, the intravenous (IV) pharmacokinetics suggested non-linear behavior within the IV dose range of 10, 25, and 75 mg/kg. These results demonstrate that further

pharmacokinetic and toxicity studies in larger animals such as dogs and non-human primates are warranted.

1 Introduction

Head and neck squamous cell cancer accounts for 3 % of new cancer cases and 2 % of cancer mortality annually in the United States [1]. Globally, head and neck squamous cell carcinoma (HNSCC) affects over 500,000 patients each year, making it the sixth in incidence and the seventh in mortality in the world [2].

Current treatment options for most head and neck cancers continue to be surgical excision with or without radiation, radiation alone, or chemotherapy with radiation depending on location, stage of disease, and patient preference. While advances have been made in the delivery of treatment, little change has been seen in the overall survival of head and neck cancer patients for decades [2]. Currently, no effective single agent chemotherapy treatment regimen is available for head and neck cancer. Additionally, oral chemotherapy is currently limited in its use, usually as second- or third-line therapy or in a clinical trial.

Fusaric acid (FA) is a novel compound from a novel class of nicotinic acid derivatives, which have activity against HNSCC. FA is produced by *Fusarium* species as a mycotoxin [3]. Mycotoxins are highly toxic compounds produced by fungi usually for the purposes of self-defense or to dissolve cell membranes as part of their fungal pathogenicity. Also known as 5-butylpicolinic acid, FA has been reported to have a number of pharmacologic effects in mammals including cardiovascular [4] and potential adverse neurological effects [5]. The therapeutic effects were observed at doses in the range of 10–30 mg/kg, while

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adverse effects were observed at a significantly higher dose of 100 mg/kg [5].

Although its exact mechanism is still unknown, FA is thought to be active by increasing damage to DNA and preventing its synthesis and repair [6]. This may be in part from chelation of divalent cations from catalytic DNA-associated metalloproteins. Chelation as a mechanism has been observed as the effect of other compounds upon cancer. Sorenson and Wanglia [7] reported tetrathiomolybdate chelates copper from proangiogenic molecules, thereby causing a reversible growth arrest in squamous cell carcinoma (SCC) in vitro and caused by decreased vascular proliferation within the tumor bed. Conversely, chelation has also been shown to activate proangiogenic genes including vascular endothelial growth factor (VEGF) in other models [8]. We have also observed significant cytokine changes induced by FA and this may also explain the cytostatic or cytotoxic effects of FA [9].

FA has demonstrated anti-tumorigenic activity in non-epidermoid carcinomas such as adenocarcinoma and hepatocellular carcinoma [6, 10]. Our data demonstrate a suppressive effect of FA upon two HNSCC (epidermoid) lines (Hep-2 and UMSSC-1) in vitro [11]. Additionally, in a docetaxel-resistant head and neck cancer cell line, FA demonstrates a concentration-driven suppression of cell growth [9].

The novel mechanism of FA provides an alternative to present therapies [9] as a single agent whether given parenterally or orally. It has synergy with conventional agents taxol, carboplatin, and erlotinib. It has shown effect upon resistant cell lines in culture and in laboratory animals, which may offer the possibility of its use in the setting of treatment failure. Preliminary data show no evidence of toxicity at therapeutic doses. The efficacy and potency of orally administered FA suggests that it would be practical as an ambulatory oral therapy [12, 13].

Potential applications of FA might include use as a second-line drug for patients who have failed first-line therapy, inhibition of growth of known metastatic carcinoma (chronic therapy), prophylactic therapy against recurrent or second primary disease given to high-risk patients (patients with the previous diagnosis of HNSCC), or as a first-line agent given in combination with another chemotherapy using an alternative mechanism of action.

We have accumulated substantial animal evidence to pursue phase I trials of FA in humans. These data suggest that an oral dose of 25 mg/kg per day is efficacious toward HNSCC in mice [11–13]. Prior to phase I clinical trials, the oral bioavailability of FA in an animal model must be evaluated to guide a phase I experimental design. In the study described here, the oral bioavailability was determined from the ratio of the area under the serum concentration–time curve following oral administration (AUC_{PO})

to the area under the serum concentration–time curve following intravenous administration (AUC_{IV}). The bioavailability was calculated from each animal since each received an IV dose and an oral (PO) dose. Additional studies will determine the linearity of the pharmacokinetic parameters at the different IV doses of FA.

2 Materials and Methods

2.1 Chemicals and Supplies

FA from *Gibberella fujikuroi* was purchased from Sigma-Aldrich (St. Louis, MO). The liquid chromatography-mass spectrometry (LC-MS) internal standard, citrulline ($5\text{-}^{13}\text{C}$, 99 %; 4,4,5,5-D₄, 95 %), was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). FA was prepared for dosing by dissolving an appropriate amount of compound in preservative-free sterile saline (University hospital supply). Formic acid and trifluoroacetic acid were LC-MS grade and purchased from Thermo Fisher Scientific (Pittsburgh, PA). Water, acetonitrile, and methanol were Optima LC-MS grade and obtained from Thermo Fisher. Control plasma was obtained from Innovative Research (Novi, MI).

2.2 Pharmacokinetic Studies

The pharmacokinetics of FA administered orally and intravenously were characterized. Sprague-Dawley rats surgically implanted with catheters in the left and right jugular veins (JV) were used for all studies. All surgical procedures were performed by the vendor (Charles River Laboratories) prior to shipment. Animals were placed in separate cages and allowed to free feed for 3 days.

On the first experimental day, a 250- μL blood sample was removed from the right JV catheter (JVC) as control. Each animal was administered 25 mg/kg IV FA in saline vehicle through the left JVC in a volume of 1 mL/kg. Blood samples (200 μL) were removed from the right JVC at 5, 10, 30, 50, 60 minutes, and 2, 4, 6, and 8 hours following drug administration. Prior to the removal of each experimental blood sample, the catheter was cleared of vehicle by removing approximately 150 μL . This dead volume was replaced after collecting the experimental sample. Saline solution (100 μL) was used to flush the catheter after each draw. Animals were fasted beginning at approximately 5 pm on the day prior to oral administration of FA. On the following day, the animal was administered 25 mg/kg PO FA in saline vehicle by gavage (4 mm tip stainless steel blunt needle). Experimental samples were collected as before at 5, 10, 30, 50, 60 minutes, and 2, 4, 6, and 8 hours. All animal experiments were approved and

performed in compliance with the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee guidelines.

Blood samples were allowed to clot for at least 20 minutes and then centrifuged ($12,000\times g$) for 10 minutes. The serum from each sample was promptly removed and stored at $-20\text{ }^{\circ}\text{C}$ until analysis by liquid chromatography with mass spectrometric detection. AUC values and elimination half-life values were determined using an Excel-based non-compartmental analysis program (PK Solutions 2.0, Summit Research Services, Montrose, CO).

24-hour urine samples were collected by placing rats in metabolism cages (Nalgene Model 655-0100, Rochester, NY) following administration of either 10 mg/kg ($n = 3$) or 25 mg/kg ($n = 7$) FA (IV). Animals were allowed free access to food and water while housed in metabolism cages.

2.3 Sample Preparation and LC-MS

Protein precipitation of serum samples (10 μL) and serum standards (10 μL) was performed in 96-well Strata Impact 2 ml filtration plates (Phenomenex, Torrance, CA). To each well was added 490 μL acetonitrile:water:formic acid (85:14.8:0.2 v/v) containing citrulline+5 stable isotope as internal standard (IS). This was followed by the addition of 10 μL of serum. After mixing gently, the plate was covered, allowed to stand for 5 minutes, and the filtrate was collected under vacuum. The 96-well collection plate was loaded into the Acquity (Waters, Corp., Milford, MA) sample manager and the sample (3 μL) was injected onto the analytical column.

The high-performance liquid chromatography (HPLC) system was a Waters Acquity series (Waters) equipped with a sample manager, binary pump, in-line degasser, and a column thermostat. The mass spectrometer was a Quattro Premier equipped with an electrospray ionization probe (Waters). Analytical separation was optimally achieved on a Phenomenex 1.7 μm KinetexDiol analytical column [50 \times 2.1 mm (i.d.)].

FA was separated using a linear binary gradient in hydrophilic interaction liquid chromatography (HILIC) mode (Mobile phase A: acetonitrile containing 0.1 % formic acid, 0.2 % acetic acid and 0.005 % trifluoroacetic acid; Mobile phase B: water containing 0.1 % formic acid, 0.2 % acetic acid and 0.005 % trifluoroacetic acid). Initially the flow rate was 0.4 mL/min. The gradient was increased from 10 to 80 % B in the first 2.3 minutes and held at 80 % B for 0.2 minutes while the flow rate was increased to 0.6 mL/min. The gradient was returned to 10 % B over 1 minute. The total run time was 5.0 minutes. Detection of $5\text{-}^{13}\text{C}$, $4,4,5,5\text{-}^2\text{H}$ -citrulline (citrulline+5) and FA was achieved following electrospray ionization

interfaced to a Quattro Premier triple quadrupole mass spectrometer (Waters). Positive ions for FA and citrulline+5 were generated using a cone voltage of 22 and 18 V, respectively. Product ions were generated using argon collision-induced dissociation at collision energy of 10 eV while maintaining a collision cell pressure of 2.8×10^{-3} torr. Detection was achieved in the multiple-reaction-monitoring (MRM) mode using the precursor \rightarrow product ions, m/z 180.2 \rightarrow 162 and 181 \rightarrow 164, for FA and citrulline+5, respectively. Citrulline+5 (5 μM) served as the internal standard. Matrix ion effects were evaluated using the post-column infusion technique, which has been described elsewhere [14]. Separate citrulline+5 (10 μM) and FA (10 μM) solutions were prepared in acetonitrile containing 20 % water. These were infused in separate experiments at a rate of 10 $\mu\text{L}/\text{min}$ and mixed with column eluent during an injection of extracted serum.

Analytical recovery and inter-day precision were evaluated using quality control standards prepared from a separated stock solution of FA. Quality control samples were prepared in FA-free mouse serum (Innovative Research, Novi, MI) at 10, 100, and 3,000 μM FA. Quality control standards were taken through seven freeze-thaw cycles by removing standards from $-20\text{ }^{\circ}\text{C}$, removing a 10 μL aliquot for analysis, thawing at room temperature, and returning the standards to $-20\text{ }^{\circ}\text{C}$. At least 1 day elapsed between freeze-thaw cycles with a seven-cycle freeze-thaw study taking place over a period of 30 days.

3 Results

3.1 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

A representative chromatogram of an extracted serum FA standard (30 μM) containing internal standard is shown in Fig. 1. The lower limit of quantitation (LLOQ) was 10 μM (from 10 μL sample) and the upper limit of quantitation was 3,000 μM . The LLOQ was defined as the lowest calibration standard that resulted in an analytical recovery of 80–120 % and a reproducibility of $\pm 20\%$. The analytical recovery for FA was $116 \pm 14\%$ at 10 μM . Inter-day precision and accuracy results are shown in Table 1. Quality control standards were quantified on seven non-consecutive days covering a period of 30 days. Matrix ion suppression was not observed for either the internal standard or FA. Increases in the MRM trace (1.80–2.3 min) for FA (m/z 180.2 \rightarrow 162) while infusing 10 μM FA during an injection of extracted serum were associated with changes in the gradient and not unique to the extracted serum. The MRM trace for citrulline+5 was stable through the chromatographic run.

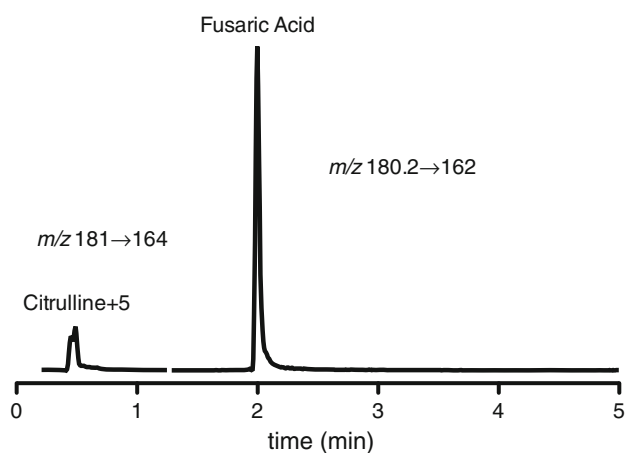


Fig. 1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) chromatogram of extracted serum sample spiked with 30 μ M fusaric acid and 30 μ M citrulline+5 (internal standard)

Table 1 Inter-day precision and accuracy at low, intermediate, and high concentrations

Nominal concentration	Predicted concentration ^a	SD	CV	Accuracy
10	10.1	3.6	36	101
100	109.4	20.2	18	109
3000	2799.9	421.2	15	93

SD Standard deviation ($n = 7$). CV Coefficient of variation = $100 \times (\text{SD}/\text{Predicted Conc.})$

^a Values are the mean

The chromatographic peak shape for the internal standard ($k = 0.75$) was not optimized and peak splitting was observed. Since peak splitting was not observed for FA ($k = 6.3$), and the precision for the integrated peak area for the internal standard was not compromised due to volume overload, it was reasoned that the poor peak shape associated with the internal standard was not detrimental to the quantitation of FA.

3.2 Bioavailability

FA was administered to nine animals for bioavailability studies, with each animal receiving an IV and PO dose at 25 mg/kg. Toxicity (i.e., chromodacyrea) was not observed in any of these animals. After completion of a study, each animal was sacrificed in a CO₂ chamber. Complete studies (8 h) were completed on only five animals. The initial study was designed anticipating an elimination half-life of about 30 minutes. During the course of these preliminary studies, the analytical sensitivity for the quantitation of FA was improved and blood samples were collected for 8 hours instead of 2 hours as originally planned. The results reported here are from rats in which blood samples

were collected for 8 hours. Rat 3 was delivered with a non-patent catheter and could not be used for these studies. In all animals, the FA serum concentration fell below the lower limit of quantitation (i.e., 10 μ M) within 4 hours of FA administration.

Serum concentration-time profiles following IV and PO administration of 25 mg/kg FA are shown in Fig. 2 and the corresponding pharmacokinetic parameters derived from these data are provided in Table 2. The average oral bioavailability for FA was quite favorable at 58 %. Curiously, there was a significant difference in the elimination half-life when comparing IV- (33 ± 6 min) with PO- (24 ± 4 min) administered FA ($p = 0.01$). For well behaved compounds, the elimination half-life should be independent of the route of administration, but it is possible that an insufficient number of blood samples were collected beyond the adsorption/distribution phase of FA disposition. This would effectively shorten the elimination half-life obtained following administration by gavage. Another explanation for the apparent effect of route of administration on elimination half-life is that either the volume of distribution or the clearance is affected on the route of administration.

Careful consideration of the apparent volume of distribution and clearance suggests that volume of distribution is affected to a greater extent by oral administration than for clearance. The apparent volume of distribution for FA was significantly higher ($p = 0.002$) following IV administration (251 ± 28 ml) versus oral administration (182 ± 27 ml). Clearance values for the two routes of administration were not significantly different ($p = 0.8$). Very little of the FA administered by IV was excreted unchanged in the urine. Following IV administration of 10 or 25 mg/kg, 1.7 and 2.0 % FA was excreted in urine (24 h).

3.3 IV Dose Effects

FA was well tolerated in rats at IV doses of 10, 25, and 75 mg/kg with no adverse effects observed. The pharmacokinetics were not well behaved and the results, which are summarized in Tables 2 and 3, suggest non-linear pharmacokinetic behavior for FA over the dose range studied. While there was larger than expected variation in the clearance at 10 mg/kg (47 ± 34 mL/h), there was no significant difference in the clearance at any of the doses studied. The clearance at 25 and 75 mg/kg was 81 ± 14 and 40 ± 5 mL/h, respectively. Though statistical differences in clearance at these doses were not observed, the data are strongly suggestive of non-linear pharmacokinetics. The effects of dose on maximum concentration (C_{max}) and time to C_{max} (T_{max}) are clearly important since these parameters are directly related to the rate and extent of

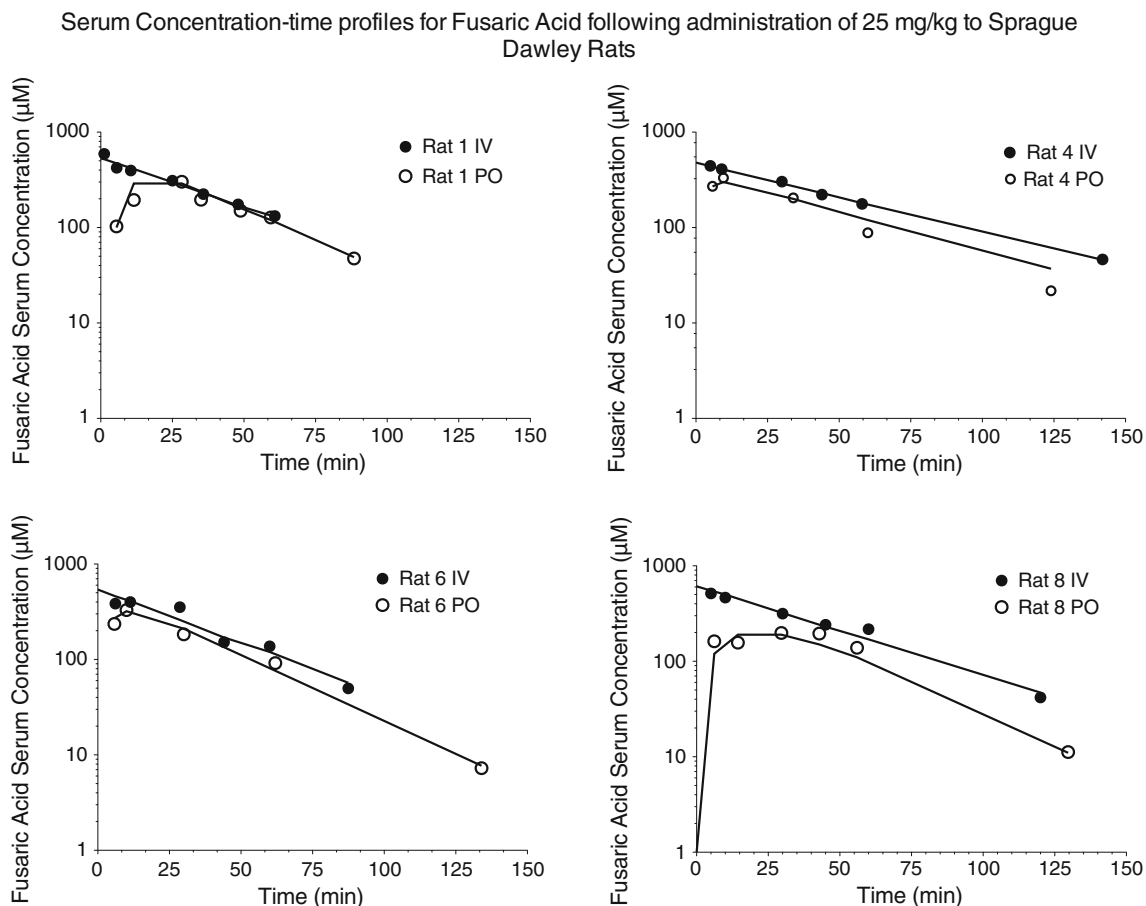


Fig. 2 Serum concentration-time profile for fusaric acid following administration of 25 mg/kg fusaric acid. Fusaric acid was administered by either the intravenous (IV) (closed circles) or oral (PO) (open circles) route. A 1-week wash-out period was allowed between IV

and PO administrations. Fusaric acid concentrations were determined by hydrophilic interaction liquid chromatography (HILIC)-tandem mass spectrometry (MS/MS) following protein precipitation and filtration of serum samples (10 µl)

absorption. Since these dose effects were not determined here, these studies should be undertaken in the future.

4 Discussion

Few descriptions of the pharmacokinetics of FA can be found in the literature. Matsuzaki et al. reported the disposition of FA following an oral dose of 20 mg/kg in the rat [15]. In this study, the acyl carbon was labeled with the radioisotope and total radioactivity in various tissues was determined. Peak radioactivity was achieved in 30 minutes with a calculated FA concentration of $42 \pm 7.4 \mu\text{g/mL}$. These results are in good agreement with the results reported here and shown in Table 2. A concentration of $290 \mu\text{M}$ is equivalent to $52 \pm 11 \mu\text{g/mL}$ FA. A simple unpaired t-test indicates that there is no significant difference in the C_{max} reported herein and that reported by Matsuzaki et al. [15] ($p = 0.24$, alpha 0.05, 95 % clearance). In the present study, little of the FA was excreted unchanged

($\leq 2 \%$) in the urine. Matsuzaki et al. reported 87 % of the total radioactivity administered was recovered in urine (24 h). This apparent difference can be explained in light of the fact that Matsuzaki et al. used FA labeled at the acyl carbon. Previous studies have shown that this acyl carbon is retained in FA metabolites [16], so it is not surprising that 87 % of the radioactivity was excreted in the earlier study since much of this radioactivity would be associated with metabolites. Umezawa has also shown that $< 5 \%$ FA is excreted unchanged in the urine [16].

Linear pharmacokinetics were not observed for the IV doses administered in this study. Non-linear pharmacokinetic parameters suggest that metabolic enzymes, transporters, and protein-FA interactions are saturated at the concentrations produced within the dose range of 10–75 mg/kg. These are the first and only studies of this type conducted in any species. Earlier reports on the acute toxicity observed mild gastrointestinal hemorrhage and erosion in Wistar male rats following administration of 32 mg/kg FA by gavage [17]. This dose is very close to the

Table 2 Pharmacokinetic parameters for fusaric acid (FA) following administration of a 25-mg/kg dose

Rat ^a	$t_{1/2}$ (min) ^b		Vd (ml/kg) ^c		CL (ml/min/kg)		T_{max} (min)	C_{max} (μ M)	AUC_{iv} (mol-min/L)	AUC_{po} (mol-min/L)	(F %)
	IV	PO	IV	PO	IV	PO					
1	32.1	21.2	262	180	6.09	5.42	28.3	302	22986	14972	65.1
4	32.4	22.6	282	221	4.65	4.83	9.6	332	30136	16806	55.8
6	26.8	21.8	245	168	6.34	5.35	10	329	22098	15179	68.7
8	42	28.5	215	161	4.63	5.63	29.6	198	30412	15158	49.8
Average	33 \pm 6	24 \pm 3	251 \pm 28	182 \pm 27	5.4 \pm 0.9	5.3 \pm 0.3	19 \pm 11	290 \pm 63	26408 \pm 4480	15529 \pm 857	60 \pm 9

AUC_{iv} area under the serum concentration–time curve following intravenous administration, AUC_{po} area under the serum concentration–time curve following oral administration, CL clearance, C_{max} maximum concentration, IV intravenous, PO oral, $T_{1/2}$ half-life, T_{max} time to maximum concentration, Vd volume of distribution

^a Catheters were not patent in Rats 2 and 3. A complete oral gavage was not administered to Rats 5 and 9. Rats 5 and 9 were injured by gavage needle. IV pharmacokinetic parameters for Rat 7 were deemed outliers by the Grubbs Test

^b Elimination half-life following IV and PO administration were statistically different ($p = 0.01$) according to a paired, two-tailed t test ($\alpha = 0.05$, $n = 4$)

^c Apparent volume of distribution was significantly effected by the route of administration ($p = 0.002$)

Table 3 Effects of dose on IV pharmacokinetic parameters of fusaric acid in Sprague Dawley rats

PK parameter	Dose		
	10 mg/kg	25 mg/kg	75 mg/kg
$t_{1/2}$ (min)	40.3 \pm 19.2	32.7 \pm 6.6	41.4 \pm 2.8
AUC_{∞} (mol-min/L)	26723 \pm 17931	26408 \pm 4480	157283 \pm 19338
Vd (ml)	135.7 \pm 30.8	251 \pm 28	161.5 \pm 25.0
CL (min/ml-kg)	3.07 \pm 2.4	5.4 \pm 0.9	2.70 \pm 0.3

AUC_{∞} area under the serum concentration–time curve from zero to infinity, CL clearance, PK pharmacokinetic, $T_{1/2}$ half-life, Vd volume of distribution

25 mg/kg dose administered in the present study and therefore some of the same gastrointestinal effects might be expected here as well. Since necropsies were not performed in the current study, the degree of intestinal damage was not assessed.

The bioavailability of FA (58 %), while not optimal, demonstrates that further pharmacokinetic and toxicity studies in larger animals such as dogs and non-human primates are warranted. The effects of dose on the IV pharmacokinetic parameters raise some questions on the ability to safely scale the dosage from rat to human use. Repeating these studies in higher order animal species, such as non-human primates, should in part answer questions of dose scalability of FA use in humans.

Conflict of interest None.

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