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Comparative Pharmacokinetics of Five Rhubarb Anthraquinones in Normal and Thrombotic Focal Cerebral Ischemia-Induced Rats

Su-xiang Feng,[1,2] Jian-sheng Li,[3]* Ling-bo Qu,[1,4]* Yan-mei Shi[1] and Di Zhao[2]

[1]College of Chemistry and Molecular Engineering, Zhengzhou University, Zhengzhou 450001, China
[2]College of Pharmacy, Henan University of Traditional Chinese Medicine, Zhengzhou 450008, China
[3]Institute of Geriatrics, Henan University of Traditional Chinese Medicine, Zhengzhou 450008, China
[4]College of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou 450001, China

A comparative oral pharmacokinetic study of five anthraquinones (aloe-emodin, emodin, rhein, chrysophanol and physcion) from the extract of Rheum palmatum L. was performed in normal and thrombotic focal cerebral ischemia (TFCI)-induced rats. The plasma samples were clarified through solid phase extraction prior to simultaneous determination of the anthraquinones with a validated high-performance liquid chromatography -fluorescence system. The results indicated that the C_{max}, $t_{1/2}$ and AUC_{0-t}, of aloe-emodin, rhein, emodin and chrysophanol in TFCI-induced rats were nearly double, whereas the CL values were remarkably decreased ($p < 0.05$) over those of the normal rats. The plasma drug concentration–time data of five anthraquinones to rats fitted a two-compartment open model. The five anthraquinones in rat plasma were absorbed quickly and eliminated slowly in both groups. The obtained results could be helpful for evaluating the impact of the efficacy and safety of the drug in clinical applications.

Keywords: rhubarb extract; anthraquinones; pharmacokinetics comparison; normal rats; thrombotic focal cerebral ischemia-induced rats.

INTRODUCTION

Rhubarb (Rhei rhizoma) is one of the important herbal drugs used in many traditional Chinese medicines for treating obstipation, gastrointestinal indigestion, diarrhea and jaundices in China and other Asian countries for thousands of years (Tang and Eisenbrand, 1992; Newall *et al*., 1996). The main active components in rhubarb are aloe-emodin, rhein, emodin, chrysophanol, physcion (shown in Fig. 1) and their glucosides. A number of studies showed that rhubarb can improve the nervous function of acute ischemic stroke (Liu *et al*., 2008) and alleviate cerebral edema (Tang *et al*., 2006). Zhang *et al*. found physcion could decrease the expressing of interleukin-1 beta and intercellular adhesion molecule-1 to protect brain tissue from cerebral ischemia-reperfusion injury (Zhang *et al*., 2005). Chrysophanol could improve the impairments of memory with cerebral ischemia reperfusion and increase the brain index (Li *et al*., 2010a). Lately, a great amount of works has been done on rhubarb anthraquinones (Li *et al*., 2010b; Li *et al*., 2011; Liu *et al*., 2010). They found that rhubarb anthraquinones possessed of protective effects against focal cerebral ischemia by decreasing plasminogen and fibrinogen, prolonging blood coagulation or resisting the aggregation and adhesion of platelet. Therefore, the potency of five anthraquinones makes it a promising candidate for development as a novel anti-cerebral ischemia drug.

* Correspondence to: Jian-sheng Li, Institute of Geriatrics, Henan University of Traditional Chinese Medicine, Zhengzhou, Henan Province 450008, China; Ling-bo Qu, College of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou, Henan Province 450001, China.
E-mail: li_js8@163.com; qulingbo@zzu.edu.cn

Several pharmacokinetic studies on anthraquinones have been performed on healthy volunteers (Lee *et al*., 2003; Zhu *et al*., 2005) and normal animals (Gong *et al*., 2011; Yan *et al*., 2009; Yan and Ma, 2007), but very little on the diseased conditions. It has been reported that the elimination rate of puerarin, a principal bioactive component of *Pueraria lobata* (*Willd*.) *Ohwi*, was significantly slower in the cerebral ischemia reperfusion rat than in the normal rat (Yan *et al*., 2005). C_{max}, AUC_{0-t}, Vd and CL of hydroxysafflower yellow A, an active marker component of Carthamus tinctorius L., showed obvious differences between the normal and acute blood stasis rats (Tian *et al*., 2010). The purpose of this study was to investigate the pharmacokinetics in rats and compare the blood levels of the anthraquinones between normal and thrombotic focal cerebral ischemia (TFCI)-induced rats after oral administration of rhubarb extract. The results may provide useful information for clinical applications.

MATERIALS AND METHODS

Materials and reagents. The reference standards of aloe-emodin, rhein, emodin, chrysophanol, physcion and 1, 8-dihydroxyanthraquinone (internal standard (IS)) were purchased from the National Institutes for Food and Drug Control (Beijing, China). The purity of all the compounds was more than 98% on high-performance liquid chromatography (HPLC). Solid phase extraction (SPE) cartridges (C_{18}, 3 mL, packed with 200 mg of 50 μm particle sizes) were purchased from Bonna-Agela Technologies (Tianjin, China). HPLC grade methanol

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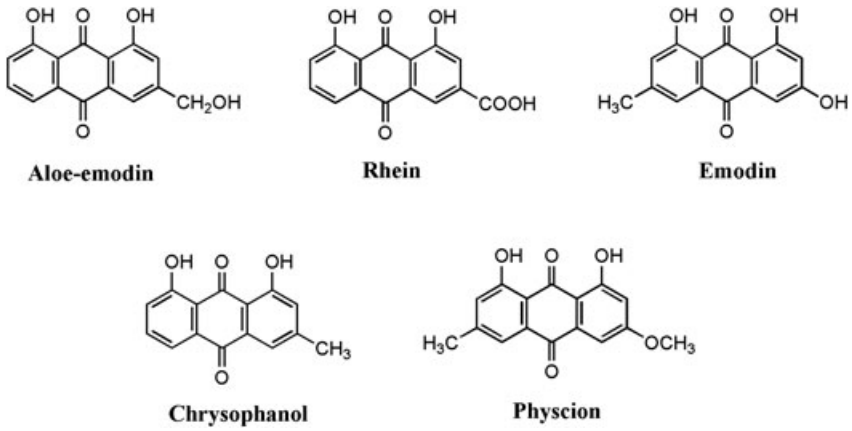

Figure 1. The chemical structure of five anthraquinones.

was purchased from Dikma Technology Inc. (Beijing, China). Purified water was obtained from a Millipore Milli-Q system (Bedford, MA, USA). The other chemicals were all of analytical grade. *Rheum palmatum L.*, collected from Sichuan, was purchased from Henan Medical Limited Company (Zhengzhou, China) and authenticated by Dr. Sui-qing Chen (College of Pharmacy, Henan University of Traditional Chinese Medicine). Voucher specimens (No. Rhu.20110301) were deposited in the desiccators of our laboratory at Henan University of Traditional Chinese Medicine (Zhengzhou, China).

Chromatographic condition. HPLC system (Waters, USA) was equipped with a 2695-separation module composed of an auto-sampler, a 2475-fluorescence detector and an Empower-2 chromatography analytical workstation. The chromatographic separation was performed on a Venusil XBP C_{18} (L) column (4.6×250 mm, 5 μm, Bonna-Agela Technologies, Tianjin, China) protected by a Waters security guard C_{18} column (4.6×20 mm, 5 μm). The mobile phase consisted of a mixture of methanol-0.1% phosphoric acid water (75:25, v/v) at a flow rate of 1.0 mL/min. The analysis was performed at 30 °C. The column eluent was monitored with a fluorescence detector at wavelengths of 435 nm and 515 nm for excitation and emission, respectively.

Preparation of rhubarb extract. Rhubarb extract was prepared in our laboratory. Briefly, the powder of *Rheum palmatum L.* was extracted twice using 75% ethanol, and then filtered. The combined filtrate was evaporated and refluxed with 8% hydrochloric acid for 0.75 h, then concentrated to dryness. The extraction was loaded to column chromatography on polyamide and eluted with 40% and 95% ethanol in turn. Afterward, the fraction eluted with 95% ethanol was collected and evaporated by rotary evaporation below 45 °C. Finally, the residue was evaporated to dryness at 40 °C. The quantitative analysis of anthraquinones was determined by internal standardization under the same chromatography conditions above. The content of aloe-emodin, rhein, emodin, chrysophanol and physcion in the rhubarb extract was 87, 115, 231, 263 and 176 mg/g, respectively.

Animals. Healthy male Sprague–Dawley (SD) rats weighing 250–280g were purchased from Henan Laboratory Animal Centra (Zhengzhou, China) with the license number SCXK2010-0002, which were housed in an air-conditioned room at a temperature of 22 ± 2°C, a relative humidity of 50 ± 10%. They were fed standard laboratory chow and had access to water *ad libitum* for at least one week. Prior to administration, all the rats were fasted with free access to water for 12 h. Animal experiments were approved by the Ethics Committee for the Use of Experimental Animals in Zhengzhou University

Preparation of plasma sample. SPE cartridges were equilibrated with 2 mL methanol and 2 mL purified water. A 200 μL plasma sample was piped into a test tube and 50 μL 1, 8-dihydroxyanthraquinone (1.6 μg/mL) as IS was added. Then, 2 mL methanol was introduced to the mixture. The tube was shaken quickly for 5 min and then centrifuged at 12,000 rpm for 15 min at 4 °C (Refrigerated Centrifuge 3-18k, Sigma, German). The supernatant was collected carefully and diluted with 5 mL 0.1% phosphoric acid. Then, the mixture was loaded into the cartridge. After washing the SPE column with 2 mL purified water, the analytes were eluted with 2 mL methanol. Afterward, the eluate was evaporated to dryness under a stream of nitrogen at room temperature. Finally, the residue was dissolved in 100 μL methanol, and 20 μL of the mixture was injected into HPLC system for analysis.

Preparation of calibration standards and quality control samples. Stock solutions of aloe-emodin, rhein, emodin, chrysophanol and physcion were prepared by dissolving the accurately weighed reference substance in methanol, respectively, and an appropriate amount of 1, 8-dihydroxyanthraquinone as IS was dissolved in methanol to a final concentration of 1.6 μg/mL.

The working solutions of calibration curve were prepared by mixing and diluting the above stock solutions with methanol. Seven calibration samples were prepared by spiking the standard mixture into 200 μL blank plasma in tubes containing 50 μL IS. The calibration curves of aloe-emodin, rhein, emodin, chrysophanol and physcion were linear over the range of 22.8–2224, 40.0–19520, 30.2–7360, 21.0–2048 and 24.2–2368 ng/mL, respectively.

The quality control (QC) samples were prepared from blank plasma at high, medium and low concentrations for five anthraquinones. All plasma samples were processed according to the procedures described above.

Method validation. The linearity of calibration curve was determined by plotting the peak ratio of each anthraquinone over the IS against the respective concentration of aloe-emodin, rhein, emodin, chrysophanol and physcion with the lower limit of quantification (LLOQ) established as the lowest concentration point. The limit of detection (LLOD) was determined at a signal-to-noise ratio of 3.

The accuracy of the established method was assessed by exterminating QC samples at low, medium and high concentrations and the intra-day accuracy, by assaying the six replicates at each concentration level on the same day while inter-day accuracy was assayed by analyzing the six duplicates during five consecutive days.

The extraction recoveries of anthraquinones in rat plasma were determined by comparing the peak area ratios obtained with the ratios of standard samples at the same three concentrations as QC samples. The recovery of IS was determined in the same way.

The stability of anthraquinones in rat plasma was evaluated under a variety of storage and handling conditions using the low, medium and high QC samples in six replicates. Freeze–thaw stability was tested on three freeze (−20 °C)/thaw (room temperature) cycles consecutively. Storage stability was assessed by analyzing samples that were kept at −20 °C for 30 days.

Pharmacokinetic study. Rats were randomly divided into two groups, normal and TFCI-induced model group. TFCI-induced rats were duplicated through the occlusion of middle cerebral artery (MCA) by using the embolus of rat autologous blood blot inserted with nylon thread (Zhang *et al*., 1997; Li *et al*., 2006). Unilateral stroke was induced in male rats by embolic occlusion of the right MCA. Briefly, animals were anesthetized with 10% chloral hydrate. Under sterile conditions, the right carotid artery comprising the external and internal carotid arteries was exposed and isolated. A silicon-coated nylon thread (30 mm long, 0.26–0.30 mm in diameter) was carefully inserted into the right middle carotid artery via external carotid artery, and the nylon thread was pulled out to an appropriate length to allow blood to coagulate. Rectal temperature was monitored and maintained at 37 °C to 38 °C throughout the experiment. After occlusion of the blood flow for 60 min, the nylon thread was withdrawn from the external carotid artery to allow reperfusion for 24 h. During the 24 h period, the rats were put in the cages separately and fed with water and food.

Each rat was orally administered at a single intragastric gavage dose of 300 mg/kg rhubarb extract. The dose of 300 mg/kg is ten times to the effective dose of rats (the dose recommended for human is 6 mg/kg, amounting to 300–360 mg/d to a 60 kg person). The blood samples (0.5 mL) were collected into heparinized tubes from the tail vein before and after oral administration at time points of 0, 0.083, 0.17, 0.333, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h, then centrifuged them immediately for 20 min at 3000 rpm to separate plasma. Each plasma sample was stored in 0.5 mL polypropylene tubes at −70 °C prior to analysis.

The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were noted directly from the observed concentrations versus time data. The pharmacokinetic parameters such as $t_{1/2}$ (half-life), CL (total body clearance) and AUC_{0-t} (area under curve) were calculated by drug and statistics software, version 2.0 (Shanghai, China).

Statistical analysis. The statistical analysis was performed with SPSS for Windows Version 16.0 (SPSS Inc., Chicago, IL, USA), and statistical analyses were made using the two-tailed Student's *t*-test.

RESULTS AND DISCUSSION

Assay validation

The typical chromatography of five anthraquinones and IS are presented in Fig. 2, which indicates that five analytes and IS are not detected in plasma blank (A) and could be well separated and detected in plasma spiked (B), plasma in normal rats (C) and TFCI-induced rats (D) after oral administration. In addition, there also appeared several unknown peaks, and they do not interfere with the separation and analysis of the five analytes and IS, which may be caused by the unknown compounds existing in the plasma sample matrix. The regression equations, correlation coefficient, linearity range were listed in Table 1. The calibration curves showed good linearity over the concentration range with a correlation coefficient (r) greater than 0.9972. The LLOD were estimated to be 7.6, 13.3, 10.1, 7.0 and 8.1 ng/mL for aloe-emodin, rhein, emodin, chrysophanol and physcion, respectively. The LLOQ of aloe-emodin, rhein, emodin, chrysophanol and physcion were established as 22.8, 40.0, 30.2, 21.0 and 24.2 ng/mL in rat plasma, respectively.

The mean extraction recovery rates of aloe-emodin, rhein, emodin, chrysophanol and physcion in rat plasma were 94.4 – 97.9%, 96.4 – 110.4%, 94.2 – 98.8%, 94.6 – 97.3% and 96.0 – 98.6% with RSD less than 7.4%. The average recovery of the IS was $96.2 \pm 3.7\%$. The mean RSD values in the intra- and inter-day precisions ranged 3.3 – 9.1% and 1.8 – 9.1%, respectively. The results indicated that an acceptable precision and accuracy of the present method was obtained to determination of five anthraquinones in rat plasma.

Stability of the anthraquinones in rat plasma was evaluated by analyzing the low, medium and high QC samples. The RSD values were 3.4 – 8.6% for five analytes after three freeze/thaw cycles and 3.9 – 9.5% for 30 days stored at −20 °C. These results indicated that the analytes were all stable under the above storage and process conditions.

Pharmacokinetics study

The validated method was applied to a pharmacokinetic study in normal and TFCI-induced rats. The mean

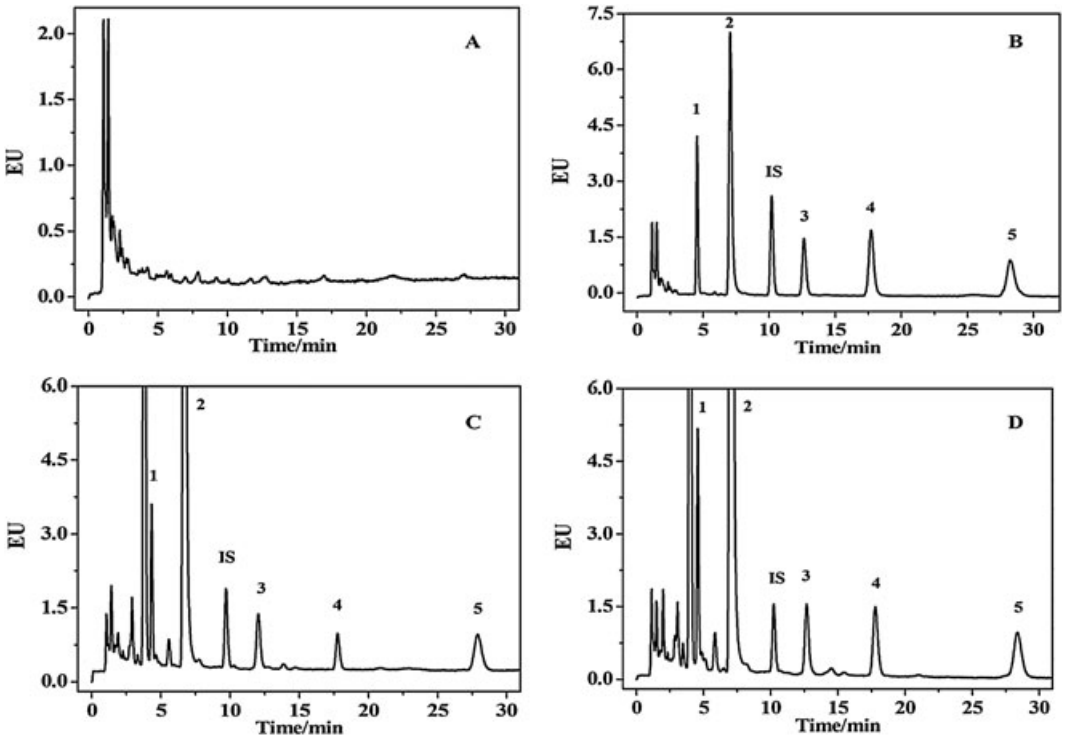

Figure 2. HPLC chromatograms of rat plasma samples: (A) blank plasma; (B) plasma spiked with aloe-emodin (1), rhein (2), internal standards (I.S.), emodin (3), chrysophanol (4) and physcion (5); (C) plasma sample at collected 0.5 h in normal rats after oral administration of rhubarb extract;(D) plasma sample at collected 0.75 h in TFCI-induced rats after oral administration of rhubarb extract.

Table 1. Regression equation, correlation coefficient (*r*) and linearity range of anthraquinones in rat plasma samples

Analytes	Regression equation	Correlation coefficient (r)	Linearity range (ng/mL)
Aloe-emodin	$y = 0.2186 + 0.0039x$	0.9987	22.8–2224
Rhein	$y = 0.1406 + 0.0015x$	0.9997	40.0–19,520
Emodin	$y = 0.4618 + 0.0039x$	0.9972	30.2–7360
Chrysophanol	$y = 0.2275 + 0.0019x$	0.9991	21.0–2048
Physcion	$y = 0.2913 + 0.0057x$	0.9995	24.2–2368

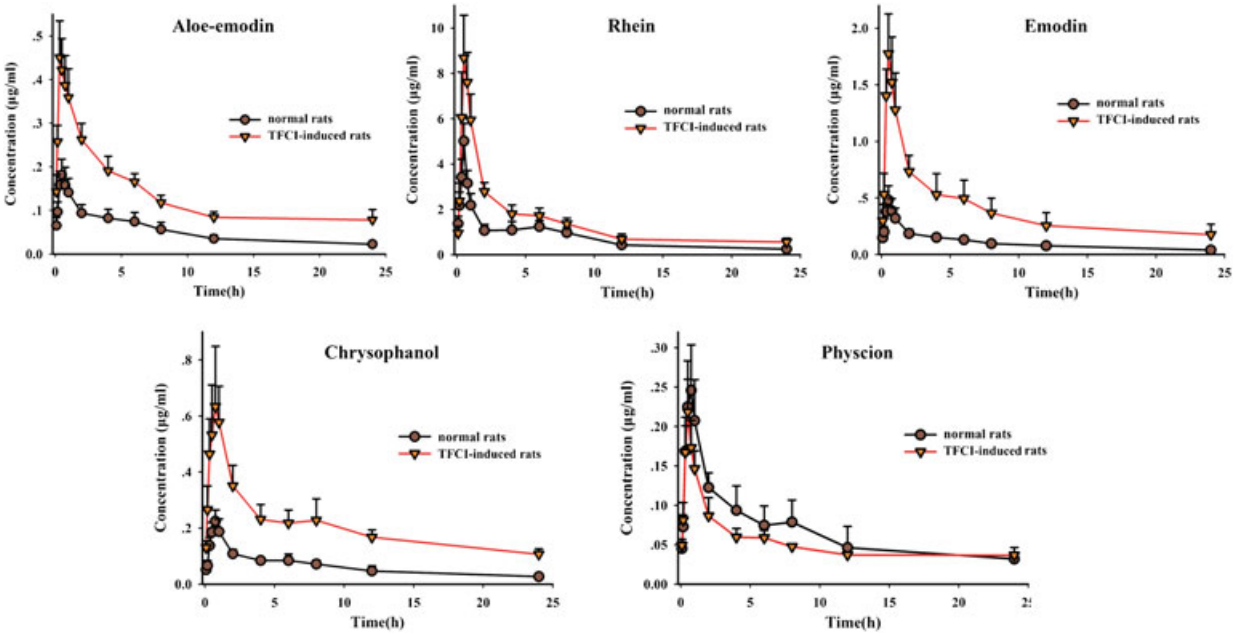

Figure 3. Mean concentration–time curves of five anthraquinones in normal rats and TFCI-induced rats (A) aloe-emodin; (B) rhein; (C) emodin; (D) chrysophanol; (E) physcion.

Table 2. Pharmacokinetic parameters of five anthraquinones in normal rats and TFCI-induced rats after oral administration of rhubarb extract (mean ± SD, $n = 6$).

Parameters	Aloe-emodin		Rhein		Emodin		Chrysophanol		Physcion	
	normal rats	TFCI-induced rats	normal rats	TFCI-induced rats	normal rats	TFCI-induced rats	normal rats	TFCI-induced rats	normal rats	TFCI-induced rats
$AUC_{(0\text{-}t)}$ (μg h/mL)	1.24 ± 0.09	3.10 ± 0.06*	17.79 ± 0.21	32.72 ± 0.12*	2.46 ± 0.26	9.18 ± 0.31*	1.49 ± 0.23	4.83 ± 0.19*	1.57 ± 0.35	1.24 ± 0.09
$AUC_{(0\text{-}\infty)}$(μg h/mL)	1.59 ± 0.16	4.67 ± 0.39*	20.30 ± 0.30	45.44 ± 0.33*	2.95 ± 0.32	11.16 ± 0.38*	1.92 ± 0.34	8.06 ± 0.31*	2.19 ± 0.41	3.29 ± 0.60
C_{max} (μg/mL)	0.18 ± 0.11	0.46 ± 0.18*	5.01 ± 0.16	8.91 ± 0.19*	0.48 ± 0.25	1.80 ± 0.21*	0.22 ± 0.18	0.65 ± 0.30*	0.26 ± 0.22	0.21 ± 0.18
T_{max} (h)	0.47 ± 0.15	0.36 ± 0.19*	0.50 ± 0.00	0.54 ± 0.18	0.47 ± 0.15	0.54 ± 0.19	0.75 ± 0.00	0.83 ± 0.15	0.70 ± 0.14	0.50 ± 0.00*
$t_{1/2}$ (h)	10.54 ± 0.21	14.63 ± 0.70	6.80 ± 0.41	14.23 ± 0.77*	9.05 ± 0.26	9.61 ± 0.46	10.86 ± 0.48	20.99 ± 0.74*	12.91 ± 0.19	39.12 ± 0.86*
$MRT_{(0\text{-}t)}$(h)	8.03 ± 0.05	8.64 ± 0.12	7.12 ± 0.11	7.03 ± 0.11	7.65 ± 0.08	7.66 ± 0.13	8.14 ± 0.12	8.97 ± 0.06	8.10 ± 0.09	9.41 ± 0.07*
$MRT_{(0\text{-}\infty)}$(h)	14.76 ± 0.20	21.01 ± 0.68	10.14 ± 0.33	18.15 ± 0.70*	12.33 ± 0.25	12.83 ± 0. 41	15.08 ± 0.45	28.55 ± 0.75*	17.51 ± 0.19	55.13 ± 0.87*
CL(L/h/kg)	16.78 ± 0.17	6.15 ± 0.29*	1.83 ± 0.30	0.82 ± 0.26*	25.27 ± 0.26	7.24 ± 0.47*	44.74 ± 0.30	10.57 ± 0.28*	27.35 ± 0.36	22.14 ± 0.58
Vd(L/kg)	248.5 ± 0.10	108.1 ± 0.36*	16.38 ± 0.16	14.10 ± 0.48	319.0 ± 0.27	93.28 ± 0.55*	649.2 ± 0.28	275.3 ± 0.44*	489.9 ± 0.30	762.1 ± 0.40*

*$P < 0.05$, versus normal rats.

plasma concentration–time curves ($n = 6$) of five anthraquinones are illustrated in Fig. 3, and the main pharmacokinetic parameters of the five compounds in rats are shown in Table 2. The plasma drug concentration–time data of aloe-emodin, rhein, emodin, chrysophanol and physcion after a single oral administration of rhubarb extract to rats fitted a two-compartment open model. Comparison of the pharmacokinetic data of normal and TFCI-induced rats showed that there were significant differences ($P < 0.05$) in the main pharmacokinetic parameters such as $t_{1/2}$, $AUC_{0\text{-}t}$, C_{max} and CL.

In summary, the results of present study indicated disease condition influenced the potential pharmacokinetic of rhubarb anthraquinones. Under the pathologic condition, rhubarb anthraquinones had a better absorption effect in TFCI-induced rats with higher concentrations and larger AUC_{0-t} than normal rats, and it proved the rationality of using it in cerebrovascular disease, which would improve the therapeutic efficacy. The C_{max} and AUC_{0-t} of aloe-emodin, rhein, emodin and chrysophanol in TFCI-induced rats were nearly double than normal rats after a single oral dose of rhubarb extract, while the CL remarkably decreased ($P < 0.05$) compared with normal rats. The time courses showed that five anthraquinones were absorbed rapidly in rat plasma in 5 min after oral administration. However, the elimination of anthraquinones was slow both in normal and TFCI-induced rats. The $t_{1/2}$ of the five anthraquinones was long and still detectable at 48 h for aloe-emodin and rhein, 32 h for emodin, 60 h for chrysophanol and physcion. Similar research was wide spread in other drugs or other disease models (Deng *et al.*, 2008; Lu *et al.*, 2002).

CONCLUSIONS

A study was conducted to evaluate and compare the pharmacokinetics differences of rhubarb anthraquinones in normal and TFCI-induced rats. The results showed that the main pharmacokinetic parameters of five anthraquinones such as the C_{max}, $t_{1/2}$, $AUC_{0\text{-}t}$, and CL were significantly different between normal rats and TFCI-induced rats. It indicated disease influenced the potential pharmacokinetic of rhubarb anthraquinones. In the pathologic condition, a better absorption of the anthraquinones was found in TFCI-induced rats than normal rats, and it proves the rationality of using it in cerebrovascular disease, which would improve the therapeutic efficacy. The five anthraquinones in rat plasma were absorbed quickly and eliminated slowly in both groups after oral administration of rhubarb extract. The obtained results could be helpful for evaluating the impact of the efficacy and safety of the drug in clinical applications.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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