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Pharmacokinetic Study on the Main Active Components of Total Coumarins of *Cnidii monnieri* in Rats

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***SUMMARY*. Cnidii Monnieri [*Cnidium monnieri* (L.) Cusson] is one of the most common used herbal plants in the treatment of numerous ailments including reproductive diseases for hundred of years. Total coumarins of Cnidii Monnieri (TCCM) is a key ingredient. Osthole (Ost) and Imperatorin (Imp) are two main effective ingredients enriched in TCCM. The purpose of this study was to investigate the pharmacokinetics of the main active components of TCCM in rats and the interaction of multi-component in TCCM. The pharmacokinetics study on Ost and Imp in rat plasma was achieved by using an optimized HPLC technique. After intravenous injection and oral administration among TCCM, Ost and Imp separately, plasma concentration of Ost and Imp of male Sprague-Dawley rats were determined at different times (1, 3, 5, 10, 20, 40,60, 120, and 240 min). When only Ost was given, the elimination of Ost was very fast, t1/2 was only 38 min. When intravenous injection of TCCM was done, the elimination of Ost slowed down apparently, $t_{1/2}$ was prolonged to 109.849 ± 9.833 min. Similar behavior was observed with Ost, $t_{1/2}$ of Imp through oral administration was longer than intravenous injection of TCCM, which means that the elimination of Imp in TCCM is slower than using Imp separately. Ost and Imp could be absorbed quickly in rats, and arrived peak concentration in 1 h in plasma after oral administration. The bioavailability of Ost and Imp in rats was 45.491 and 52.244%, respectively, which showed that both components were well absorbed. These results indicated that the absorption of Ost and Imp were rapidly in rats, and the bioavailabilities of both two components were relatively high. The pharmacokinetic profiles of Ost and Imp were different from each other in two administration routes, and so does the using of TCCM while them separately. Other components in TCCM can slow down the elimination of Ost and Imp significantly in rats.**

INTRODUCTION

Cnidii is the dried and ripe fruit of an annual herb plant, *Cnidium monnieri* (L.) Cusson, which could be used widely in clinical, for external as well as oral use for killing parasites to relieve itching and warming the kidney. There are many domestic and international researches about total coumarins of *Cnidii monnieri* (TCCM), such as immunization, nerve endocrine, cardiovascular, and reproduction which are used as anti-inflammatory, anti-allergic, central inhibition, anti-arrhythmia, and anti-tumor [1-5]. Other researches showed that active constituents of fructus cnidii are TCCM which involves Osthole, Imperatorin, and isoborneol [6-9].

Unlike the pharmacodynamics of TCCM, there is little literature available about the pharmacokinetics of TCCM. Currently, only Zhang & Xu [10] and An *et al.* [11] have studied the pharmacokinetics of Osthole of TCCM in rats and rabbits, but these studies were totally about one component of TCCM while the interaction of multi-component of TCCM had not yet been reported anywhere in the world.

As the two highest active components, osthole and imperatorin were often used as the control indicators of quality evaluation of cnidium monnieri medicinal materials and chinese patent drug (containing fructus cnidii proprietary) that comes from different places [12]. Thus

KEY WORDS: Cnidii Monnieri total coumarins, HPLC, Imperatorin, Osthole, Pharmacokinetics.

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we choosed the two major active components of TCCM, Osthole (Ost) and Imperatorin (Imp), as quantitative indicators to investigate the pharmacokinetic progress of the two major active components of TCCM in rats, which were given through intravenous injection and oral administration routes [13,15] aiming at the experimental evidences to verify the materials of pharmacodynamics in TCCM and studying further about the interaction of multi-component in TCCM through comparing the pharmacokinetic progress of TCCM with Ost and Imp, which were given separately.

MATERIALS AND METHODS

Reagents

The Chinese herb Fructus Cnidii was obtained and its components osthole and imperatorin standard were provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), mifepristone standard (the internal standard, IS) was prepared by Beigium company. Methanol (Merk, Germany) and water were used for the HPLC analysis. All other chemicals were of HPLC grade and provided by Shandong Yuwang Chemical Factory (Shandong, China).

Animals

Healthy, male Sprague Dawley (SD) rats, weighing 300 ± 20 g (Certificate No. JXA0319602), were obtained from the Experimental Animals Centers of Medical College of Nanchang University (Nanchang, China).Animals were kept in a standard room and allowed free access to food and water. Food was withdrawn 12 h prior to experimentation. The animal facilities and protocols were approved by the Institutional Animal Care and Use Com-mittee, Nanchang University.All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animal (The National Academies Press, revised edition 2010).

Instrument and equipment

HPLC (LC-10AD), UV detector (Shimadzu, Japan); HW-2000 workstation (Dalian Scientific Instrument Co., Ltd): Diamonsil C18 analytical column, 5 μm, 4.6 mm × 150 mm (Dima Co. Ltd., Orlando, FL); high-speed refrigerated centrifuge GS-15r (USs Beckman Co.); electronic analytical balance BP61s (Sartorius Co., Germany); Coolwork ultrapure water machine (Taiwan Cool Work Co.); KA - 1000 type bench centrifuge (Shanghai Anting Scientific Instruments Plant).

Chromatographic conditions

The mobile phase consisted of a mixture of methanol-water (70:30, v/v). The analysis was carried out at a flow rate of 1.0 mL/min with the detection wavelength set at 320 nm. The analyte was determined at room temperature (25 °C) on an analytical column (Diamonsil C18, 5 μm, 4.6 mm × 150 mm).

Preparation of TCCM

TCCM ultrasonic extraction was done with 95% ethanol [13-14], 10 g fructus cnidii powder was treated by this method and the ultimate residue was dissolved by 100 mL 10% ethanol, the solution was filtered by 0.22 μm filtration membrane and stored at 4 °C. Extraction rate: Imp 0.31 mg/g, Ost 2.05 mg/g) could be gain by HPLC method.

Pharmacokinetic of TCCM through intravenous injection and oral administration

Ten mL/kg TCCM solution was given through intravenous injection at vena caudalis (Ost was 2050 μg/kg and Imp was 310 μg/kg). Blood sample (0.2 mL) was taken from retrobulbar venous plexus of rats and put into heparinized EP tube before and after the administration of 1, 3, 5, 10, 20, 40, 60, 120, and 240min. Blood samples were centrifugated at 3000 rpm for 10 min, then the upper plasma was withdrawn and stored at -20 °C until to be used.

The dispose of oral administration (Ost was 2050 μg/kg and Imp was 310 μg/kg) was the same that used in intravenous injection.

Pharmacokinetic of Ost and Imp through intravenous injection

The rats were divided into two groups randomly, one group received iv 2050 μg/kg Ost through vena caudalis, the other received iv 310 μg/kg Imp through the same route. Blood samples (0.2 mL) were taken from retrobulbar venous plexus of rats and placed into heparinized EP tubes before and after administration at 1, 3, 5, 10, 20, 40, 60, 120, and 240 min. Blood samples were centrifuged by 3000 rpm for 10 min, then the upper plasma was withdrawn and stored at -20°C until to be used.

Pretreatment of biological samples

Ninety μL of plasma sample was mixed with 10 μL IS (400 ng/L of Mfs, mifepristone standard), 300 μL of acetonitrile and 500 μL ethyl acetate. After vortering for 2 min and standing 10 min, the mixture was centrifuged at 15000

rpm for 10 min. The supernatant layer was transferred into another tube and evaporated to dryness at 40 °C under nitrogen . The residue was reconstituted with 60 µL of absolute alcohol and 10 µL was injected into HPLC system for analysis.

HPLC assay validation

The specificity was evaluated by comparing chromatograms of blank solution, blank solution spiked with Ost, Imp, and Mfs (blank plasma, blank plasma spiked with Ost, Imp, and Mfs, and plasma samples obtained from rats which were injected intravenously by TCCM.

As presented in Fig. 1, no significant endogenous peak interfering with Ost, Imp, and Mfs was obtained in blank plasma. The chromatograms were free of interfering peaks at the retention times of Imp (8.4 min), Ost (12.5 min), and Mfs (16.2 min).

Preparation of standard curve

The evaluation of the assay was performed with a seven-point calibration curve over the concentration range 10.94 to 700 µg/L (10.94, 21.88, 43.75, 87.50, 175.00, 350.00, and 700.00 ng/mL) of Ost and over the concentration range 15.63 to 1000 µg/L (15.63, 31.25, 62.50, 125.00, 250.00, 500.00, and 1000.00 µg/L) of Imp. Internal standard substance, Mfs (final concentration for 400 ng/mL), 20 µL one time. The regression equation of five standard curves was: Ost: $Y = 0.004589x + 0.1001$, $R^2 = 0.999163$, *Imp:* $Y = 0.003197x + 0.03572$, $R^2 = 0.999942$. Each calibration curve was constructed with six different concentrations by plotting the peak areas ratios of Ost or Imp to the internal standard (Fig. 2).

Precision and accuracy

Three levels of QC samples (21.88, 87.50, and 350.00 µg/L for Ost and 31.25, 125.00, and 500.00 µg/L for Imp) were used for the precision assay. The results are shown in Tables 1 and 2.

Extraction recovery

The extraction recoveries of Ost and Imp were determined at three different concentration levels by comparing the peak areas of the analytes obtained from five replicates of QC plasma samples with those from the standard. The ex-

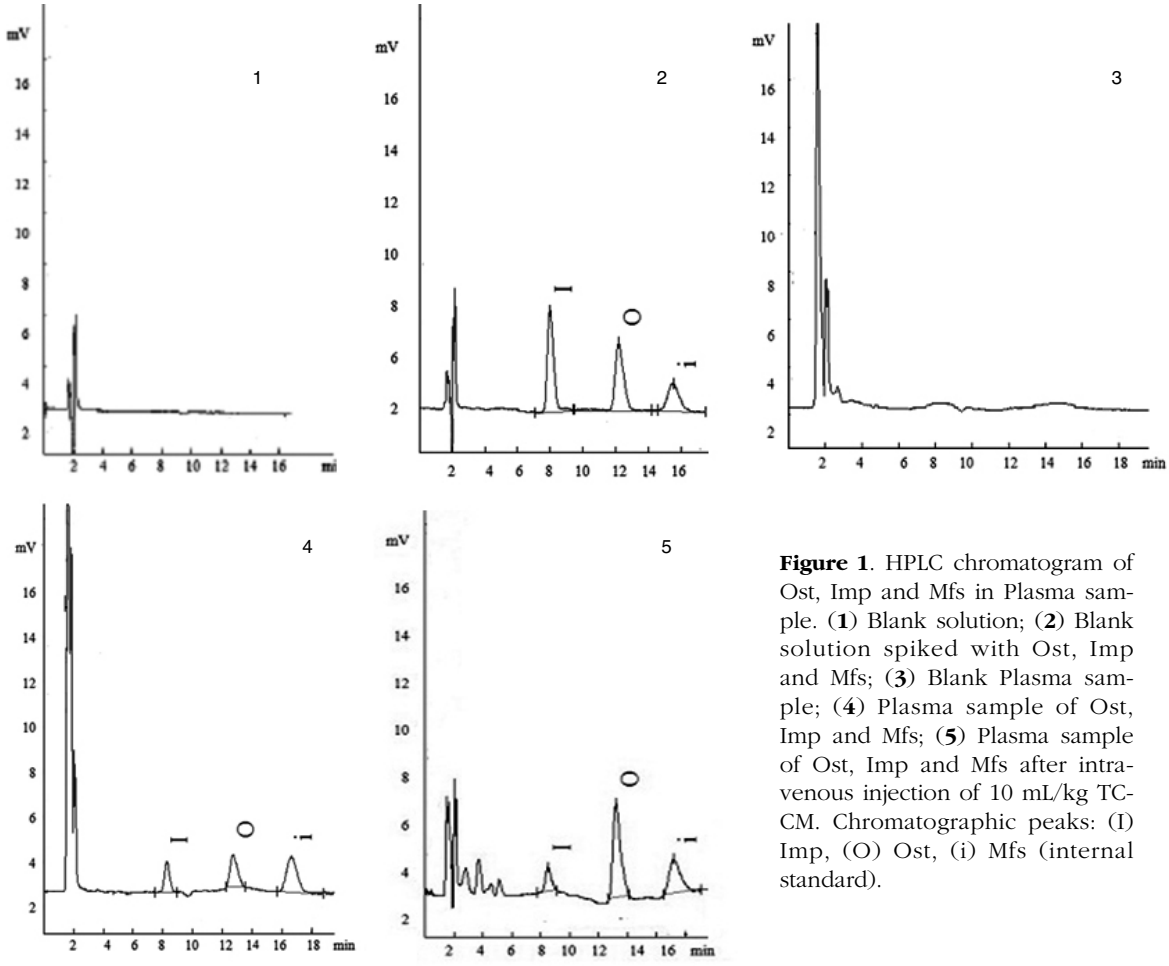

Figure 1. HPLC chromatogram of Ost, Imp and Mfs in Plasma sample. (**1**) Blank solution; (**2**) Blank solution spiked with Ost, Imp and Mfs; (**3**) Blank Plasma sample; (**4**) Plasma sample of Ost, Imp and Mfs; (**5**) Plasma sample of Ost, Imp and Mfs after intravenous injection of 10 mL/kg TCCM. Chromatographic peaks: (I) Imp, (O) Ost, (i) Mfs (internal standard).

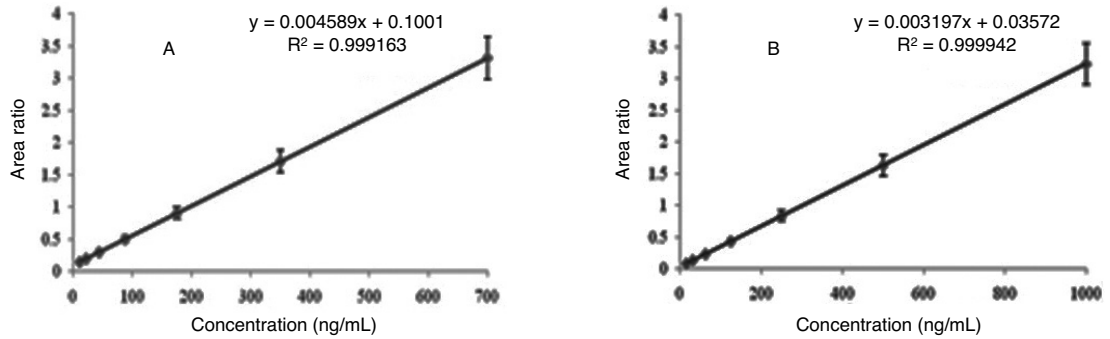

Figure 2. Standard curves of Ost, Imp in plasma sample determined by HPLC (n = 5, Mean ± SD). **A**) Standard curves of Ost in plasma sample. **B**) Standard curves of Imp in plasma sample

Concentration (ng/mL)	Intra-day			Inter-day		
	$\bar{x}$ ± SD (ng/mL)	RSD (%)	Accuracy (%)	Concentration (ng/mL)	RSD (%)	Accuracy (%)
21.88	21.35 ± 0.21	0.98	97.58	21.51 ± 0.26	1.21	98.31
87.50	87.19 ± 1.36	1.56	99.65	87.30 ± 1.53	1.75	99.77
350.00	356.49 ± 11.77	3.30	101.85	349.76 ± 10.65	3.04	99.93

Table 1. Precision and accuracy of the assay of osthole ($\bar{x}$ ± SD, n = 5).

Concentration (ng/mL)	Intra-day			Inter-day		
	$\bar{x}$ ± SD (ng/mL)	RSD (%)	Accuracy (%)	Concentration (ng/mL)	RSD (%)	Accuracy (%)
31.25	31.11 ± 0.36	1.16	99.55	31.05 ± 0.41	1.32	99.36
125.00	125.14 ± 2.24	1.79	100.11	125.60 ± 1.89	1.50	100.48
500.00	479.69 ± 13.38	2.79	95.94	475.51 ± 15.71	3.30	95.10

Table 2. Precision and accuracy of the assay of imperatorin ($\bar{x}$ ± SD, n = 5).

traction recoveries were calculated by the peak area ratios of Ost and Imp in plasma samples and the same concentration of Ost and Imp standards. The results of Ost and Imp were 98.81, 97.88, and 96.97%, RSD 0.62, 1.23, and 1.75%, and 93.04, 93.26, and 98.37%, RSD2.28, 1.11, and 1.14%, respectively.

Stability

The stability of analytes in the plasma was assessed using three concentrations (high, medium and low) of spiked samples under two conditions(25 °C, 24 h; -20 °C, 7d). The concentration of Ost and Imp in plasma under two conditions deviated to less than 10% from those in freshly spiked plasma, which demonstrated a good stability of Ost and Imp in the overall steps of the determination.

Data calculation and statistic method

All the results are expressed as Mean ± SD. Plasma concentration versus time profiles of Ost and Imp were analyzed by non-compartmental models using DAS2.0. The pharmacokinetic parameters, including half-life of elimination ($t_{1/2}$), apparent volume distribution (V_d), plasma clearance (CL), mean residence time ($MRT_{0-\infty}$), time to reach C_{max} (T_{max}), the area under concentration-time curve ($AUC_{0-\infty}$) were obtained from analysis of experimental data. A p value less than 0.05 was considered to be significantly different using paired student's *t*-test.

RESULTS AND DISCUSSION

Pharmacokinetic results and discussion of Ost

From this, we can see Ost pharmacokinetic process after intravenous injection and oral administration TCCM presents significant difference. The $MRT_{(0-t)}$, $MRT_{(0-\infty)}$ and $t_{1/2}$ of Ost through oral are longer than intravenous injection and so as the Apparent volume of distribution-V_d. This may be because of two different

Parameter	Intravenous injection	Oral	P
$t_{1/2}$ (min)	109.849 ± 9.833	213.332 ± 34.506	0.000
V_d (L/kg)	6.124 ± 1.014	66.207 ± 17.531	0.000
CL (L/min.kg)	0.039 ± 0.005	0.212 ± 0.025	0.000
$MRT_{(0-t)}$ (min)	79.828 ± 2.665	237.002 ± 12.978	0.000
$MRT_{(0-\infty)}$ (min)	147.562 ± 14.592	317.835 ± 46.643	0.000
Zeta	0.006 ± 0.001	0.003 ± 0.001	0.000

Table 3. Comparison of main pharmacokinetic parameters of Ost in rats after intravenous injection of 10 mL/kg TCCM and oral adminstration of 20 mL/kg TCCM, respectively ($\bar{x}$ ± SD, n = 10).

Parameter	TCCM		Ost
	Intravenous injection ($\bar{x}$ ± SD)	Oral ($\bar{x}$ ± SD)	Intravenous injection ($\bar{x}$ ± SD)
$t_{1/2}$ (min)	109.849 ± 9.833	213.332 ± 34.506	38.198 ± 3.386*#†
V_d (L/kg)	6.124 ± 1.014	66.207 ± 17.531	4.467 ± 0.452*†
C (L/min·kg)	0.039 ± 0.005	0.212 ± 0.025	0.081 ± 0.010*#†
$MRT_{(0-t)}$ (min)	79.828 ± 2.665	237.002 ± 12.978	36.531 ± 2.500*#†
$MRT_{(0-\infty)}$ (min)	147.562 ± 14.592	317.835 ± 46.643	51.622 ± 5.585*#†
Zeta	0.006 ± 0.001	0.003 ± 0.001	0.018 ± 0.002*#†

Table 4. Comparison of main pharmacokinetic parameters of Ost in rats after intravenous injection of 10 mL/kg TCCM, 2050 μg/kg Ost and Oral 20 mL/kg TCCM respectively ($\bar{x}$ ± SD, n = 10). *P < 0.05 in group 12, #P < 0.05 in group 13, †P < 0.05 in group 23.

routes of administration lead to different components of TCCM in blood, which shows different pharmacokinetic interactions, such as some components of TCCM, which can quick biotransformation and excretion, may could not be absorbed from gastrointestinal. So the elimination of Ost through intravenous injection was faster than oral.

From Fig. 3, it can be seen that the pharmacokinetic progress of Ost presents significant difference between multi-component of TCCM and single Ost. When we gave Ost only, the elimination of Ost was very fast, $t_{1/2}$ was only 38 min, $MRT_{(0-t)}$ is 36.531, ± 2.500 min, and $MRT_{(0-\infty)}$ is 51.622 ± 5.585 min. When intravenous injection of TCCM, the elimination of Ost slowed down apparently, $t_{1/2}$ prolonged to 109.849 ± 9.833min, $MRT_{(0-t)}$ is 79.828 ± 2.665 min, and $MRT_{(0-\infty)}$ is 147.562±14.592 min. This showed that other components of TCCM restrained the elimination of Ost in rats.

Most components in TCCM have similar structure, and all of them are coumarins, which may have similar pharmacokinetic process. For example, they may share the same transporter or substrate of metabolic enzymes, and have similar metabolic process as well as excretion

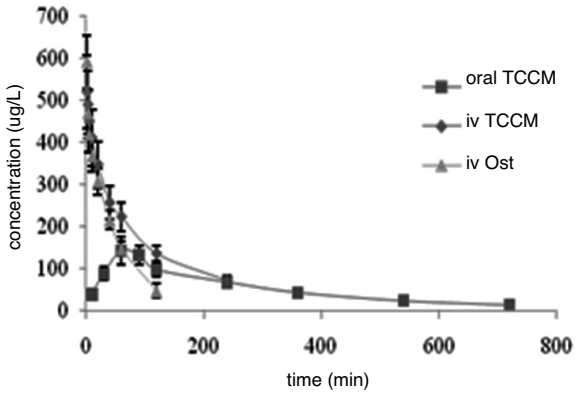

Figure 3. Plasma concentration time curve of Ost in rats after after intravenous injection adminstration of 10 mL/kg TCCM, 2.05 mg/kg Ost, and Oral 20 mL/kg TCCM, respectively.

pathway. In that way, it may be different from using Ost only about the pharmacokinetic process, due to the interaction of components in TCCM. Liver is the main transformation organ of Ost in rats. Our study gave a further confirmation that CYP3A is the main metabolic enzyme of Ost in the liver of rats. So other components in TCCM may have an influence on the metabolic process in rats through inhibit CYP3A.

Parameter	Intravenous injection ($\bar{x}$ ± SD)	Oral ($\bar{x}$ ± SD)	P
$t_{1/2}$ (min)	37.509 ± 1.479	205.203 ± 39.892	0.000
V_d (L/kg)	1.463 ± 0.226	22.322 ± 5.040	0.000
CL (L/min.kg)	0.027 ± 0.004	0.075 ± 0.005	0.000
$MRT_{(0-t)}$ (min)	36.255 ± 1.020	157.198 ± 3.421	0.000
$MRT_{(0-\infty)}$ (min)	50.641 ± 2.369	316.453 ± 55.298	0.000
Zeta	0.018 ± 0.001	0.004 ± 0.001	0.000

Table 5. Comparison of main pharmacokinetic parameters of Imp in rats after intravenous injection of 10 mL/kg TCCM and oral adminstration of 20 mL/kg TCCM respectively ($\bar{x}$ ± SD, n = 10).

Parameter	TCCM		Imp
	Intravenous injection ($\bar{x}$ ± SD)	Oral ($\bar{x}$ ± SD)	Intravenous injection ($\bar{x}$ ± SD)
$t_{1/2}$ (min)	37.509 ± 1.479	205.203 ± 39.892	33.112 ± 5.019*†
V_d (L/kg)	1.463 ± 0.226	22.322 ± 5.040	1.886 ± 0.348*†
CL (L/min.kg)	0.027 ± 0.004	0.075 ± 0.005	0.040 ± 0.005*#†
$MRT_{(0-t)}$ (min)	36.255 ± 1.020	157.198 ± .421	34.461 ± 1.195*†
$MRT_{(0-\infty)}$ (min)	50.641 ± 2.369	316.453 ± 55.298	44.592 ± 5.155*†
Zeta	0.018 ± 0.001	0.004 ± 0.001	0.021 ± 0.003*#†

Table 6. Comparison of pharmacokinetic parameters of Imp in rats after intravenous injection of 10 mL/kg TCCM, 0.31 mg/kg Imp and Oral 20 mL/kg TCCM respectively ($\bar{x}$ ± SD, n=10). *P < 0.05 in group 12, #P < 0.05 in group 13, †P < 0.05 in group 23.

But, compared with the pharmacokinetic of Ost through oral administration TCCM, $t_{1/2}$ of Ost is shorter through intravenous injection which showed that it is very complicated about the interaction of components in TCCM. Some of them may inhibit metabolic and excretion process, but others may have opposite impacts. In that way, there may be different interactions due to different components *in vivo*.

Pharmacokinetics results and discussion of Imp

From Table 5 it can be seen that two routes of administration present significant difference. As the same with Ost, $MRT_{(0-t)}$, $MRT_{(0-\infty)}$ and $t_{1/2z}$ of Imp is longer than intravenous injection and so is V_d. This may be the reason that Imp and Ost have similar structure, so they have similar pharmacokinetic process.

From Table 6 and Fig. 4 it can be seen that it showed different pharmacokinetic processes between two routes of administration and intravenous injection of Imp. Similarly with Ost, $MRT_{(0-t)}$, $MRT_{(0-\infty)}$ and $t_{1/2z}$ of Ost through oral are longer than intravenous injection which means the elimination Imp in TCCM is slower than using Imp separately, and this may be the reason that most of compounds in TCCM have similar structure.

CONCLUSION

This research studied the differences between Ost and Imp through lots of ways, such as different routes of administration and administration as multi-component or single component. This verified components in TCCM had influence on pharmacokinetics, especially the elimination of Ost and Imp in rats. From the results we can see that the interaction of components in TCCM is very complicated, and other components in TCCM inhibited the elimination of Ost as well as Imp. Due to the expensive use of TCCM in clinical, this result could help increase its curative effect. For further developing clinical pharmacology research and blood concentration-efficacy combining research, this study also will have great significance.

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