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Pharmacokinetic and Metabolism Studies of CT-011 by HPLC-UV and LC-MS/MS

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Abstract Z)-N-tert-butyl-1-(5-((((E)-3-(3,4-dihydroxyphenyl)acryloyl)oxy)methyl)-3,6-dimethylpyrazin-2-yl) methani mine oxide (CT-011), a novel derivative of tetramethylpyrazine (TMP) was designed and synthesized in our laboratory, can effectively scavenge free radicals and protect nerve cells. A developed and validated high performance liquid chromatography (HPLC) was used to evaluate the pharmacokinetics of CT-011, the limit of quantitywas0.02 μg/mL. Within the intravenous dose of CT-011 range from 10 to 90 mg/kg in rats, the plasma concentration decreased rapidly, and the half-life of terminal elimination ranges from 22.833 to 42.257 min. The concentration of CT-011 in lung tissue was significantly higher than that in other tissues, suggesting that lung tissue may be its main target organ. The main metabolites of CT-011 in rats were caffeic acid and 2-N-tert-butyl nitrone-5-hydroxymethyl-3,6-dimethylpyrazine (5-OH-TBN) *in vivo* and *in vitro*. The results provide basic information for the pharmacodynamic study and further clinical study of CT-011.

Keywords Pharmacokinetic, Metabolism, Quantitative determination, Plasma concentration, Tissues distribution

1. Introduction

Ligustrazine, chemically known as 2,3,5,6-tetramethylpyrazine (TMP, Figure 1), is an alkaloid monomer extracted from Ligusticum chuanxiong. It is one of the effective components of Ligusticum chuanxiong. Studies have shown that Ligustrazine can play a variety of pharmacological activities. Ligustrazine can maintain mitochondrial structure and functional integrity, scavenge oxygen free radicals, and thus protect against oxidative damage [1]. Ligustrazine inhibits platelet aggregation, inhibits platelet activation during cardiopulmonary bypass, and depolymerizes platelets that have formed, thereby exerting antithrombotic effects [2,3]. In addition, ligustrazine has also been shown to improve the ability of mice to learn and memory [4], improve microcirculation [5], reduce lipid peroxidation [6], regulate the expression of apoptosis inhibitor Bcl-2 and apoptosis promoting factor p53 [7], thereby exerting neuroprotective effects.

Caffeic acid (CA, Figure 1), chemically known as 3-(3,4-dihydroxyphenyl)-2-acrylic acid, is widely found in various plants. Recent studies have shown that caffeic acid has various pharmacological activities, such as antioxidation [8], antibacterial and antiviral effects [9,10], and neuroprotective [11], elevated white blood cells, platelets and anti-platelet aggregation [12]. Thus, it can be applied to cardiovascular and cerebrovascular systems, blood systems, immune systems, tumors and other diseases [13].

Nitrone compound has strong free radical scavenging ability. Numerous studies have shown that nitrone compound can treat neuronal dysfunction caused by neurodegenerative diseases such as stroke, Parkinson's disease and Alzheimer's disease [14-16]. Because of the various pharmacological activities of TMP, CA and nitrone compound, The CT-011 (Figure 1) in our laboratory was designed and synthesizedwith tetramethylpyrazine as the backbone, combined with caffeic acid and nitrone. Our previous studies have shown that CT-011 has a variety of

pharmacological effects. For example, CT-011 can activate the PI3K-Akt-GS3β signaling pathway to protect glutamate-induced neuronal damage, as well as directly eliminate and inhibit excessive production of free radicals in nerve cells, which suggesting that CT-011 may have good pharmacological activity in vivo and may become a potential new drug. We developed and validated an HPLC-UV method for the determination of CT-011 in rats, and used LC-MS/MS method to speculate the metabolites of CT-011. The results provide support for the pharmacodynamic study of CT-011 and contribute to the pharmacokinetic study of compounds with similar structure to CT-011.

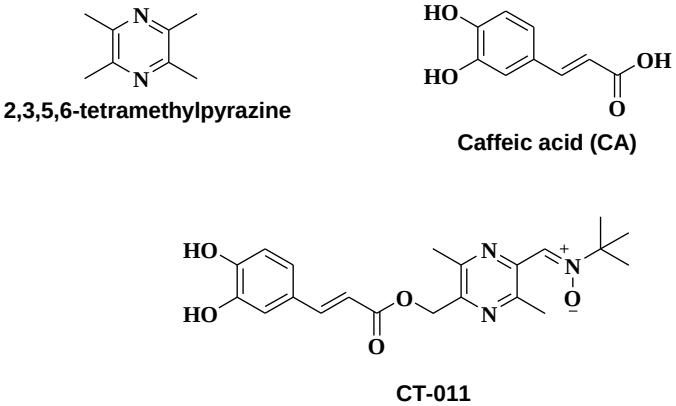

Figure 1: Structures of TMP, CA and CT-011

2. Materials and Methods

2.1. Chemicals Materials

CT-011, TN-2 (internal standard, IS) and 5-OH-TBN were synthesized in our laboratory with a purity greater than 98%. Caffeic acid was purchased from aladdin industrial corporation (Shanghai, China). Methanol and acetonitrile (HPLC grade) was purchased from fan-hong company (Guangzhou, China). Ultra-pure water was obtained using a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

2.2. HPLC and LC-MS Conditions

Liquid chromatography was performed on Agilent 1260 Infinity LC system with Agilent Eclipse C18 column (250mm×4.6mm, 5μm) and was separated at 30℃. The mobile phase is methanol and water, using a gradient elution, 0-5 min, methanol is increased from 30% to 70%, after running for 5 minutes in 70% methanol, 10-11.5 min, methanol is reduced from 70% to 30%. The flow rate is 1 mL/min and the wavelength is 340 nm.

LC-MS system is Agilent 1100 series system, equipped with a 4000 QTrap hybrid QqQ_{LIT} mass spectrometer with positive and negative electronspray ionization source (Applied Biosystems, Foster City, CA, USA). The samples were separated by Agilent ZORBAX XDB-C18 (50 × 2.1 mm,3.5 μm)at 30℃. The mobile phase is acetonitrile and water, which is gradiently eluted by acetonitrile from 50% to 90% within 20 minutes at a flow rate of 0.5 mL/min.

2.3. Animals and Sample Preparation

Sprague-Dawley (SD) male rats, weighing 200-250 g, were purchased from Guangzhou University of Chinese Medicine. They were housed in a animal room with constant temperature (22 ± 4 °C) and humidity (60 ± 10%) with free food and water.

CT-011 was dissolved in 10% DMSO, 10% absolute ethanol, 35% polyethylene glycol 400 (PEG 400) and 35% saline. After intravenous administration of three doses of CT-011 (10 mg/kg, 30 mg/kg, 90 mg/kg), plasma samples were collected at 5, 10, 20, 30, 45, 60 and 90 min. Tissue samples were collected at the same time point as plasma samples after intravenous administration of 90 mg/kg.

Acetonitrile (600 μL) was added to 600 μL of plasma sample. After adding 50 μL of the internal standard solution, immediately add 3 mL of ethyl acetate to vortex for 5 minutes, centrifuge at 3000 rpm for 5 minutes, and then take

the supernatant, blow dry in a nitrogen stream at 45 °C. Reconstitute the residue with 50 μL of methanol, centrifuge at 12000 rpm for 10 minutes, then take 20 μL into HPLC.

Tissue samples (heart, liver, spleen, lung, kidney, brain, stomach) were homogenized with methanol at 1:1.5 (weight/volume), take 600 μL of the homogenate, after adding 50 μL of the internal standard solution, immediately add 3 mL of ethyl acetate to vortex for 5 minutes, centrifuge at 3000 rpm for 5 minutes, and then take the supernatant, blow dry in a nitrogen stream at 45 °C. Reconstitute the lung tissue residue with 500 μL of methanol, and other tissue residues were reconstituted with 50 μL of methanol. Reconstituted liquid was centrifuged at 12000 rpm for 10 minutes, then take 20 μL into HPLC.

2.4. Working Solution Preparation

The stock solution of CT-011 and the internal standard (IS) solution were accurately weighed to 10 mg respectively, and dissolved in 10 mL of acetonitrile solution to obtain a mother liquor concentration of 1 mg/mL. The stock solution of CT-011 and the internal standard solution were further diluted with acetonitrile to obtain working solutions at several concentration levels. Calibration curve and quality control (QC) sample in plasma and tissue were prepared by diluting the corresponding working solutions with blank rat plasma and blank rat tissue. The final plasma calibration curve concentration is 0.02, 0.1, 0.5, 1, 2.5, 5, 10, 20, 40 μg/mL and the final tissue calibration curve concentration is 0.025, 0.1, 0.25, 0.5, 1.25, 2.5 μg/g (heart, liver, spleen, kidney, brain, stomach) and 3.125, 6.25, 12.5, 25, 50, 100 μg/g (lung). The concentrations of QC samples in plasma were 0.1, 0.5, 2.5, 32 μg/mL and the concentrations of QC samples in tissue were 0.075, 0.25, 2 μg/g (heart, liver, spleen, kidney, brain, stomach) and 5, 20, 80 μg/g (lung). The all working solutions were stored at -20°C until analyzed.

2.5. Method Validation

Method validation includes selectivity, linearity, accuracy, precision, recovery and stability [17].

The selective evaluation of endogenous impurity interference was performed by comparing the blank blood, blank blood with CT-011 and IS, and blood after administration.

The linearity was evaluated by calibration curve, and calibration curves were established by a series linear regression of the peak area ratios of the CT-011 and IS. Take the concentration as abscissa, and the ratio of CT-011 to internal standard peak area as ordinate, the weighted least squares method ($1/X^2$) is used for linear regression. The signal-to-noise (S/N) was not less than 10 as the limit of quantification (LOQ).

The precision and accuracy of QC plasma samples were assessed by the determination of QC plasma samples in five replicates with concentration levels of 0.1, 0.5, 2.5, 32 μg/mL, while tissues (heart, liver, spleen, kidney, brain, stomach) at 0.075, 0.25, 2 μg/g and lung tissue at concentration level of 5, 20, 80 μg/g. Precision is further subdivided into intra-day and inter-day precision, which is evaluated using relative standard deviation (RSD %), and accuracy is estimated using relative error (RE %).

The extraction recovery of CT-011 was evaluated by comparing the peak area ratios of extracted QC sample with standard solutions.

The stability of CT-011 in rat plasma samples was studied by measuring the concentration of QC plasma samples under different storage conditions. Combined with the specific requirements of the experiment, the stability of CT-011 QC plasma samples was investigated at room temperature, 4°C and -20°C.

3. Result and Discussion

3.1. Selectivity

Typical chromatograms of blank rat blood sample, blank rat blood with CT-011 and IS, and rat blood samples collected after intravenous administration of CT-011 are shown in Figure 2. There were no endogenous interferences was observed at the retention time of the CT-011 and IS.

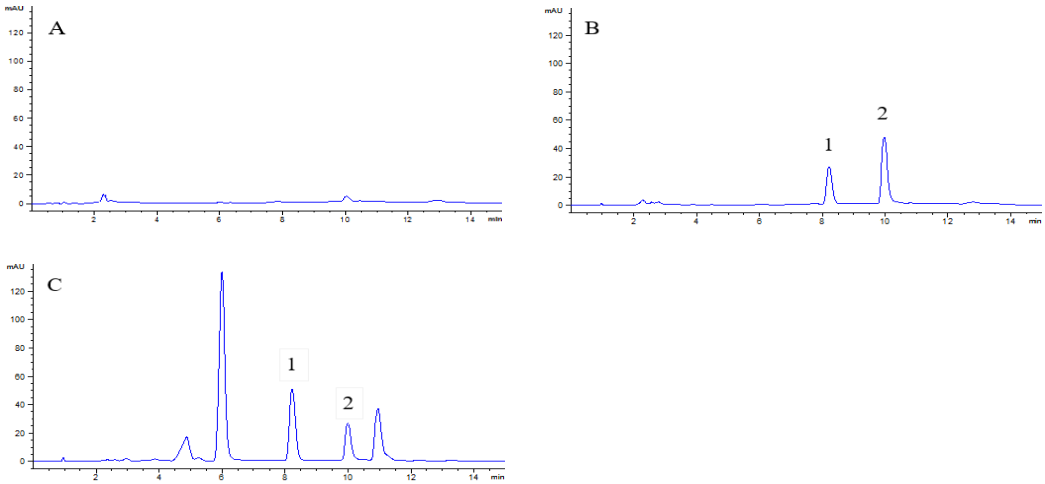

Figure 2: Representative chromatograms of CT-011and IS in rat blood samples: (A) a blank blood sample, (B) a blank blood sample spiked with CT-011 and IS, (C) a rat blood sample collected at 30 min after i.v. administration of 90 mg/kg and added IS. 1: IS, 2: CT-011

3.2. Linearity and LOQ

The linear regression of peak area ratio and concentration is fitted in the concentration range of 0.02-40 μg/mL for CT-011 in rat plasma, 0.025-2.5 μg/g or 3.125-100 μg/g in rat tissues. The calibration curve for plasma samples were y=5.311x-0.1009, r^2=0.9999 (0.02-1 μg/mL) and y=5.6337x-0.0755, r^2=0.9995 (1-40 μg/mL). The results of calibration curves for tissue samples are listed in Table 1. The LOQ of CT-011 in plasma and tissue was 0.02 μg/mL.

Table 1: Calibration curves for determination of CT-011 in different biological samples

Biological samples	Calibration curves	Correlation coefficients (r^2)	Linear ranges
Plasma	y=5.311x-0.1009	0.9999	0.02-1 (μg/mL)
	y=5.8664x-1.6194	0.9993	1-40 (μg/mL)
Lung	y=0.1726x-0.1239	0.9992	3.125-100 (μg/g)
Heart	y=3.4852x-0.1065	0.9999	0.025-2.5 (μg/g)
Liver	y=4.2744x-0.1308	0.9998	0.025-2.5 (μg/g)
Spleen	y=2.3831x-0.1194	0.9993	0.025-2.5 (μg/g)
Kidney	y=3.7226x-0.0435	0.9997	0.025-2.5 (μg/g)
Brain	y=3.0298x-0.0921	0.9998	0.025-2.5 (μg/g)
Stomach	y=3.9407x=0.0078	0.9998	0.25-2.5 (μg/g)

3.3. Precision, Accuracy and Extraction Recovery

The accuracy, precision and extraction recovery of the method are shown in Table 2. Both intraday and interday precision were <9.08%. The accuracy ranges from 95.81% to 109.70%, and extraction recovery ranges from 79.27% to 86.12% in plasma and 70.01% to 85.53% in tissues.

Table 2: Precision, accuracy and extraction recovery of CT-011 in different biological samples (n=5)

Biological samples	Concentration (μg/g)	Accuracy (Mean ± SD, %)	Precision (%)		Extraction recovery (Mean ± SD,%)
			Intraday	Interday	
Plasma	0.1	98.07 ± 0.34	2.33	6.83	86.12 ± 4.75
	0.5	96.69 ± 1.58	1.74	7.35	79.27 ± 4.21
	2.5	101.68 ± 3.65	1.28	1.70	80.06 ± 4.40
	32	101.91 ± 5.16	1.41	1.93	79.09 ± 1.33
Heart	0.075	105.37 ± 0.24	1.88	6.29	74.38 ± 2.34
	0.25	98.48 ± 0.67	1.03	6.70	76.60 ± 1.94
	2	99.89 ± 2.70	1.29	1.08	85.53 ± 1.28
Liver	0.075	107.56 ± 0.08	0.67	1.94	70.01 ± 5.52

	0.25	100.53 ± 0.10	0.35	0.45	71.60 ± 3.31
	2	102.03 ± 2.43	0.95	1.91	76.80 ± 1.65
Spleen	0.075	109.70 ± 0.14	0.83	3.83	71.76 ± 1.94
	0.25	101.01 ± 0.28	1.03	1.18	77.62 ± 3.87
	2	102.18 ± 3.60	1.25	3.29	79.09 ± 1.33
Lung	5	98.63 ± 3.23	4.68	6.54	82.31 ± 4.02
	20	101.54 ± 4.75	4.51	4.57	75.74 ± 2.37
	80	102.21 ± 6.27	5.95	6.31	77.36 ± 6.67
Kidney	0.075	105.16 ± 0.53	6.08	7.48	73.42 ± 2.61
	0.25	102.51 ± 1.46	4.55	9.08	77.10 ± 5.83
	2	100.20 ± 8.15	3.91	2.91	80.23 ± 1.76
Brain	0.075	108.23 ± 0.22	1.87	5.26	79.59 ± 7.32
	0.25	95.81 ± 0.76	2.59	4.86	83.02 ± 3.65
	2	103.26 ± 7.20	1.66	8.11	75.69 ± 4.32
Stomach	0.075	96.08 ± 1.63	1.72	2.70	74.19 ± 3.33
	0.25	97.83 ± 4.78	1.27	2.01	81.96 ± 4.35
	2	101.03 ± 5.28	6.84	7.09	77.64 ± 1.57

3.4. Stability

The stability of CT-011 at room temperature, 4°C and -20°Cwere shown in Table 3. Because the concentration is biased within ±15% of the normal concentration, the established method can be applied to pharmacokinetic studies.

Table 3: Stabilities of CT-011 under various storage conditions in rat plasma (n=5)

Storage conditions	**Concentration (μg/ml)**		**RE (%)**	**RSD (%)**
	Nominal	**Found**		
Room temperature storage for 12 h	0.1	0.106 ± 0.006	6.49	6.06
	2.5	2.521 ± 0.056	0.83	2.22
	32	33.357 ± 0.976	4.24	2.93
Storage at 4°C for 24 h	0.1	0.953 ± 0.006	-4.74	5.80
	2.5	2.606 ± 0.026	4.25	0.98
	32	33.156 ± 1.215	3.61	3.67
Storage at -20°C for 24 h	0.1	0.108 ± 0.004	7.66	3.95
	2.5	2.504 ± 0.074	0.16	2.95
	32	31.514 ± 0.781	-1.52	2.48

3.5. Pharmacokinetic and metabolism studies

The pharmacokinetic parameters were analyzed by DAS 3.0 software. The mean plasma concentration-time profiles of the three doses of CT-011 (10 mg/kg, 30 mg/kg, 90 mg/kg) after intravenous administration were shown in Figure 3 and pharmacokinetic parameters were calculated by non-compartment model were listed in Table 4. After intravenous administration of CT-011(90 mg/kg), the concentration of tissues were shown in Figure 4.

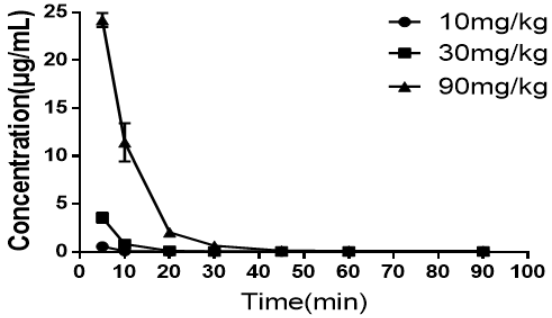

Figure 3: Mean plasma concentration-time profiles of CT-011 after intravenous administration in rats (n=3)

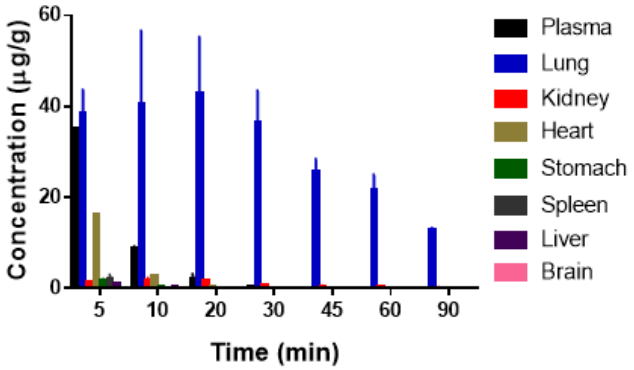

Figure 4: Distribution of CT-011 in tissues and plasma after intravenous administration of CT-011 at 90 mg/kg in rats (n=3)

Table 4: The main pharmacokinetic parameters of CT-011 after intravenous administration in rats (n=3)

Parameters	**Unit**	**Intravenous administration**		
		10 mg/kg	**30 mg/kg**	**90 mg/kg**
$T_{1/2z}$	min	23.329±3.289	22.833±0.332	42.257±3.786
CLz	L/min/kg	0.659±0.085	0.433±0.049	0.242±0.008
Vz	L/kg	22.293±5.079	14.266±1.798	14.712±1.090
$AUC_{(0\rightarrow\infty)}$	mg/L·min	15.365±2.145	69.935±7.743	372.802±11.587
$AUC_{(0\rightarrow t)}$	mg/L·min	14.596±2.197	69.075±7.750	369.152±11.097
$MRT_{(0\rightarrow\infty)}$	min	7.202±1.626	4.437±0.501	7.758±0.193

After intravenous administration of CT-011, the plasma concentration decreased and eliminated rapidly, and the half-life of terminal elimination ranged from 22.833 to 42.257 min. The possible reason is that CT-011 contains an ester bond, which is easily hydrolyzed in rats, the initial drop sharply in plasma concentration may also indicate that the compound leaves the blood and rapidly distributes into tissues. The concentration in the lung tissue is much higher than that of other tissues, indicating that the lung tissue may be its target organ and may have a potential role in the treatment of lung disease.

Comparing the chromatograms of CT-011 between after administration and blank blood, two new peaks can be observed at 6 min and 11 min, respectively. It shows that CT-011 can be easily metabolized in rats. We used LC-MS/MS to speculate the metabolites of CT-011.In addition to the protonated ion peaks of CT-011 at 400.1[M+H], we can also observe four distinct protonated ion peaks and marked as M1, M2, M3 and M4, the m/z was 414.7[M+H], 238.1[M+H], 192.9[M-H] and 178.8[M-H], respectively. The protonated ion of M1 increased by 14 Da compared with CT-011, We speculated that the hydroxyl group on the benzene ring was methylated. Further fragment ions of M2 and M4 at m/z 238.1, 182.0, 164.0 and 178.9, 134.9, respectively (Figure 6), which are the same as those of standard 2-N-tert-butyl-nitrone-5-hydroxymethyl-3,6-dimethylpyrazine (5-OH-TBN) and standard caffeic acid, respectively. Compared to M3, the protonated ion of M4 is reduced by14 da, and we speculate that M3 loses a methylene group to obtain M4.

3.6. Kinetics and metabolism of CT-011 in rat and human blood in vitro

In order to find the metabolic difference about CT-011 in rats and humans, the rat and human blood were used for incubation in vitro, and CT-011 was added to fresh rat and human blood to evaluate concentration-time profile. A single-chamber model was used to examine the kinetics regularity. The results show a good linear relationship between the logarithm of the concentration-time curve (Figure7). The half-life of CT-011 shows significant difference in rat and human blood, which was 45.89 min and 238.97 min *in vitro*, respectively. Under vitro incubation conditions, we also found that the metabolites of CT-011 were 5-OH-TBN and caffeic acid. Based on *in vivo* and *in vitro* results, we proposed a metabolic pathway for CT-011, as shown in Figure 5.

3.7. Discussion

In vitro blood stability experiments, we found that CT-011 was easily hydrolyzed in rat blood, which may be the presence of carboxylesterase in rat blood. In order to reduce the degradation of CT-011, the acetonitrile was added to the blood samples immediately. A liquid-liquid extraction was used to reduce the interference of endogenous impurities and improve the precision. We chose n-hexane, ethyl acetate, ether and dichloromethane as extracting solvents. The results showed that ethyl acetate as extracting solvent could obtain good recovery and precision. This method can shorten sample preparation time and simplify operation.

The pharmacokinetic parameters showed that CT-011 had a short half-life in rats. In order to explain the metabolic difference of CT-011 between rats and humans, we chose to incubate CT-011 with rat and human blood in vitro, and the results showed that CT-011 was more stable in human blood than in rat blood, and there are significant differences. The reason may be that there is an active carboxylesterase in rats, but not in humans [18,19]. Current evidence suggests that carboxylesterase plays an important role in the metabolism of CT-011. It also suggests that blood incubation *in vitro* can be used to investigate the metabolic differences of drugs between rats and humans.

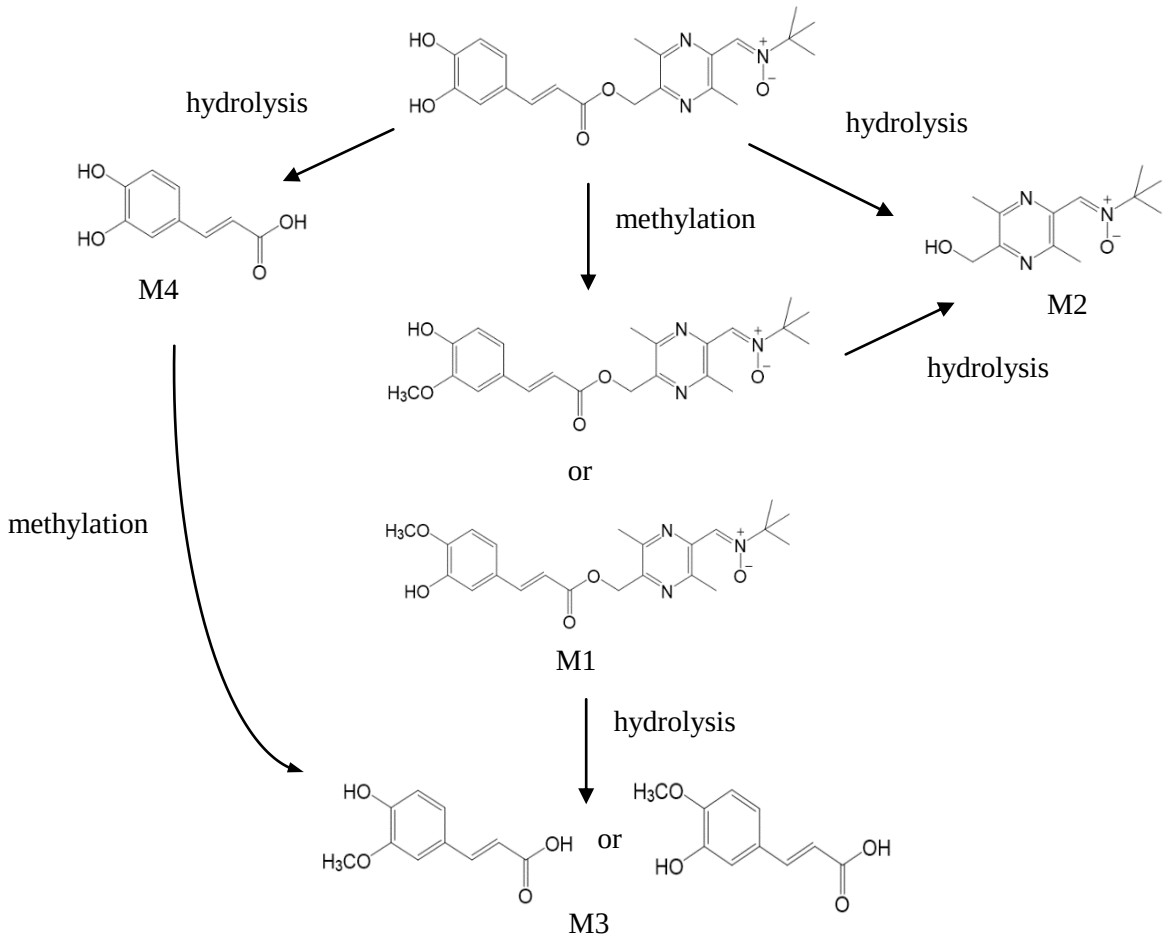

Figure 5: Proposed metabolic pathways of CT-011 in rats

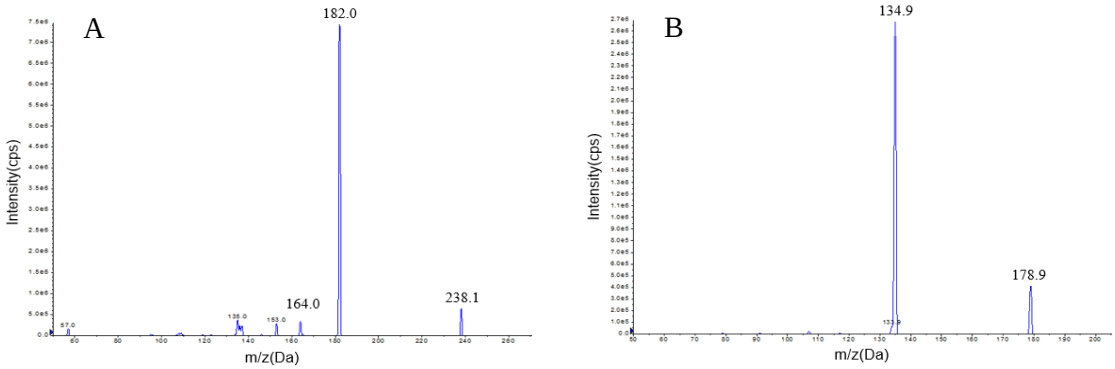

Figure 6: Product ion mass spectrum (A) 5-OH-TBN in the positive ionization mode and (B) caffeic acid in the negative ionization mode

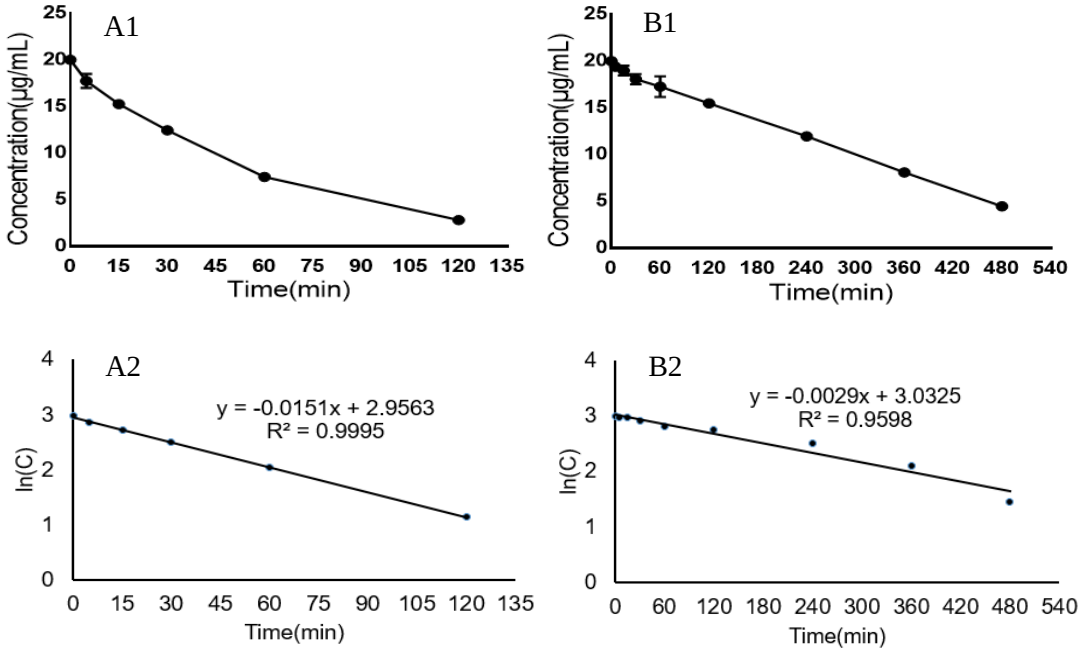

Figure 7: A1 and B1 show the concentration-time relationship of CT-011in rat and human blood in vitro. A2 and B2 represent the corresponding logarithmic concentration-time relationship

4. Conclusion

A rapid and sensitive HPLC-UV method for the determination of CT-011 in rat plasma and tissues was developed and validated. LC-MS was used to speculate the metabolites of CT-011 in rats *in vivo* and *in vitro*. In order to elucidate the metabolic differences of CT-011 in rats and humans, the CT-011 was incubated with fresh rat and human blood in vitro. Different half-life in rats and humans blood in vitro indicated that ester drugs may have different hydrolysis rates in rats and humans in vivo. The achieved pharmacokinetic and metabolite studies can be useful for further study of the bioactive mechanism of CT-011.

References

[1]. Li, S. Y., Jia, Y. H., Sun, W. G., Tang, Y., An, G. S., Ni, J. H., & Jia, H. T.(2010). Stabilization of mitochondrial function by tetramethylpyrazine protects against kainate-induced oxidative lesions in the rat hippocampus. Free Radical Biology & Medicine, 48(4):597-608.

[2]. Wang, P., Luo, C. H., Wang, Q. X., Li, Q. Y., Li, P., & Yuan, X. H.(2013). Antithrombotic effect of

ligustrazine hydrochloride injection on the model of induced arteriovenous shunt thrombosis. Journal of Research in Medical Sciences the Official Journal of Isfahan University of Medical Sciences, 18(8):704-706.

[3]. Wang, Y., Zhu, H., Tong, J., & Li, Z. (2016). Ligustrazine improves blood circulation by suppressing Platelet activation in a rat model of allergic asthma. Environmental Toxicology and Pharmacology, 45:334-339.

[4]. Liu, X. J., & Wu, W. T. (1999). Effects of ligustrazine, tanshinone II A, ubiquinone, and idebenone on mouse water maze performance. Acta pharmacologica Sinica, 20(11):987-990.

[5]. Dai, X. Z., & Bache, R. J. (1985). Coronary and Systemic Hemodynamic Effects of Tetramethylpyrazine in the Dog. Journal of Cardiovascular Pharmacology, 7(5):841-849.

[6]. Liao, S. L., Kao, T. K., Chen, W. Y., Lin, Y. S., Chen, S. Y., Raung, S. L., ... & Chen, C. J. (2004). Tetramethylpyrazine reduces ischemic brain injury in rats. Neuroscience Letters, 372(1):40-45.

[7]. ZHU, H. Z., ZHAN, Z. L., HE, W. Y., & GOU, X. (2004). Effects of Dexamethasone and tetramethylpyrazine on c-Fos, Bcl-2, ICAM-1 protein expression after renal ischemia-reperfusion. Chinese Journal of Pathophysiology, 20(5):893-895

[8]. Gülçin, İ. (2006). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology, 217(2):213-220.

[9]. Russo, A., Longo, R., & Vanella, A. (2002). Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. Fitoterapia, 73: S21-S29.

[10]. Bailly, F., & Cotelle, P. (2005). Anti-HIV Activities of Natural Antioxidant Caffeic Acid Derivatives: Toward an Antiviral Supplementation Diet. Current Medicinal Chemistry, 12(15):1811-1818.

[11]. Im, S. E., Yoon, H., Nam, T. G., Heo, H. J., Lee, C. Y., & Kim, D. O. (2010). Antineurodegenerative Effect of Phenolic Extracts and Caffeic Acid Derivatives in Romaine Lettuce on Neuron-Like PC-12 Cells. Journal of Medicinal Food, 13(4):779-784.

[12]. Lee, D. H., Kim, H. H., Cho, H. J., Bae, J. S., Yu, Y. B., & Park, H. J. (2014). Antiplatelet effects of caffeic acid due to Ca2+ mobilizationinhibition via cAMP-dependent inositol-1, 4, 5-trisphosphate receptor phosphorylation. Journal of atherosclerosis and thrombosis, 23(1):23-27.

[13]. Prasad, N. R., Jeyanthimala, K., & Ramachandran, S. (2009). Caffeic acid modulates ultraviolet radiation-B induced oxidative damage in human blood lymphocytes. Journal of Photochemistry and Photobiology B: Biology, 95(3): 196-203.

[14]. Floyd, R. A., Neto, H. C. C. F., Zimmerman, G. A., Hensley, K., & Towner, R. A. (2013). Nitrone-based therapeutics for neurodegenerative diseases: Their use alone or in combination with lanthionines. Free Radical Biology and Medicine, 62:145-156.

[15]. Guo, B., Xu, D., Duan, H., Du, J., Zhang, Z., Lee, S. M., & Wang, Y. (2014). Therapeutic Effects of Multifunctional Tetramethylpyrazine Nitrone on Models of Parkinson's Disease *in vitro* and *in vivo*. Biological and Pharmaceutical Bulletin, 37(2):274-285.

[16]. Sun, Y., Zhang, G., Zhang, Z., Yu, P., Zhong, H., Du, J., & Wang, Y. (2012). Novel multi-functional nitrones for treatment of ischemic stroke. Bioorganic & Medicinal Chemistry, 20(12): 3939-3945.

[17]. Brodie, R. R., & Hill, H. M. (2002). Validation issues arising from the new FDA guidance for industry on bioanalytical method validation. Chromatographia, 55(1):S91-S94.

[18]. Redinbo, M. R., & Potter, P. M.(2005). Keynote review: Mammalian carboxylesterases: From drug targets to protein therapeutics. Drug discovery today, 10(5):313-325.

[19]. Li, B., Sedlacek, M., Manoharan, I., Boopathy, R., Duysen, E. G., Masson, P., & Lockridge, O. (2005). Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. Biochemical Pharmacology, 70(11):1673-1684.