Pharmacokinetics comparison of four major bio-active components in normal and blood stasis rats after administration of Naoxintong Capsule by UPLC-TQ/MS

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Research

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Abstract

Background Blood stasis is one major cause of cardiovascular and cerebrovascular diseases. Naoxintong capsule (NXTC), a Chinese patent medicine, has been widely employed in the prevention and treatment against cardiovascular and cerebrovascular diseases. However, the pharmacokinetics comparison of NXTC in normal and blood stasis rats were remain obscured.

Methods Acute blood stasis rats were induced by being placed in ice-cold water during the interval between two injections of adrenaline hydrochloride. Normal and blood stasis rats were administrated of NXTC suspension at the dosage of 5 g·kg -1 , and blood was collected at different time points after then. Concentrations of four main components including caffeic acid, ferulic acid, formononetin and tanshinone IIA in rat plasma were quantified by UPLC-TQ/MS. The pharmacokinetic parameters were calculated by Phoenix WinNonlin v6.2 software.

Results It was found that C max , AUC all , AUC INF_obs , Vz_F_obs and MRT last of ferulic acid, AUC all, Vz_F_obs and MRT last of caffeic acid in blood stasis rats were significantly different ($P < 0.05$) from normal rats. Compared with normal rats, C max of ferulic acid and formononetin decreased significantly in the plasma of acute blood stasis rats, AUC all of caffeic acid and ferulic acid decreased notably, AUC INF_obs of ferulic acid decreased remarkably, Vz_F_obs and MRT last of ferulic acid and caffeic acid increased reversely. It is suggested that the absorption of the four components of NXTC in acute blood stasis rats was weakened, and the elimination time was prolonged.

Conclusions The significant difference in some different parameters of the 4 NXTC components in normal and blood stasis rats might be caused by increasing of blood viscosity and slowing down of blood flow in acute blood stasis rats. The pharmacokinetic study under pathological condition provided important information for more rational use of NXTC in clinical situations.

Background

Naoxintong Capsule (NXTC), a Chinese patent medicine, is originated from a classic traditional Chinese prescription named Buyang Huanwu Decoction, which was recorded in the ancient medical book *Yi-Lin-Gai-Cuo* written by Qingren Wang in Qing Dynasty. It is composed of 16 traditional Chinese herbs (TCH), including 11 plant herbs Astragali Radix (Huangqi), Paeoniae Radix Rubra (Chishao), Salviae miltiorrhizae Radix et Rhizoma (Danshen), Angelicae Sinensis Radix (Danggui wei), Chuanxiong Rhizoma (Chuanxiong), Persicae Semen (Taoren), Carthami Flos (Honghua), Spatholobi Stem (Jixueteng), Achyranthis bidentatae Radix (Niuxi), Cinnamomi Ranulus (Guizhi), Mori Ramulus (Sangzhi), 2 resin herbs Olibanum (Ruxiang) and Myrrha (Moyao), and 3 animal herbs Pheretima (Dilong), Scorpio (Quanxie) and Hirudo (Shuizhi) [1]. NXTC is also one of commercial medicinal products approved by the China Food and Drug Administration and listed in the Chinese Pharmacopoeia 2015 with the effect of invigorating qi and activating blood circulation, removing blood stasis and dredging collaterals [2]. NXTC has gained the National Chinese Medicine Protection Certificate in 2014, and has been included in the National Basic Drug List (2012 edition). Modern researches show that the pharmacological effects of NXTC mainly focus on improvement of blood rheology and blood coagulation function, anti-myocardial ischemia and ischemia/reperfusion injury, anti-atherosclerosis, anti-myocardial fibrosis, anti-cerebral I/R injury, improvement on learning and memory functions [3]. It is commonly used for the treatment of coronary heart disease, angina pectoris, stroke, secondary prevention of myocardial ischemia, cerebral infarction, transient ischemic attack, vertebro-basilar insufficiency, carotid atherosclerosis, and other cardiovascular and cerebrovascular diseases [4].

Promoting blood circulation and removing blood stasis is the basic pharmacological action of NXTC in the treatment of cardiovascular and cerebrovascular diseases [5]. The active chemical ingredients of NXTC mainly include flavonoids, flavonoid glycosides, phenanthraquinones, phenolic acids, terpenoids, and polysaccharide, which are related to the pharmacological functions of NXTC on inhibition of platelet aggregation, anti-inflammation, reduction of apoptosis and ROS production, activation of lipid metabolism, promotion of angiogenesis and lesion plaque stability, attenuation of vascular calcification [1, 6–9]. Many researchers have been trying to explore and identify the major and bioactive constituents of NXTC, and many chemical constituents in NXTC *in vitro* were quantified [10–12].

Nowadays, it is of great significance to study the pharmacokinetics and metabolism of TCH components for promoting its modernization. Therefore, some studies were conducted to explore the pharmacokinetics and metabolites of the main components of NXTC. A total of 36 prototype compounds and 52 metabolites of NXTC were identified or tentatively characterized in beagle dog urine and feces [13]; the pharmacokinetic profiles of caffeic acid, ferulic acid, formononetin, cryptotanshinone and tanshinone IIA in healthy rats have already been reported [14]. However, it is generally known that when the body is in pathological state, the pharmacokinetic process of compounds will change, and even has a significant difference with the normal state [15, 16].

Therefore, it is necessary to study the pharmacokinetic parameters changes in pathological state. In addition, the pharmacokinetic data obtained in pathological state will be more beneficial to clinical application than in normal state. Based on this, we employed the pharmacokinetics comparison of the main active components of NXTC between normal and blood stasis model rats, which will provide more valuable data for illuminating the variation mechanism of invigorating qi and activating blood circulation, removing blood stasis and dredging collaterals after oral administration of NXTC, and for better clinical applications of NXTC.

Materials And Methods

Instrument

Thermo TSQ Altis high performance liquid chromatography combined with triple quadrupole mass spectrometer (Thermo Company, USA), XS105 electronic balance (Metler-Toledo Group), JA5003 electronic balance (Shanghai Shunyu Hengping Scientific Instrument Co. Ltd.), Neofuge 1600R desktop cryogenic high-speed centrifuge (Shanghai Lishen Scientific Instruments Co. Ltd.), miVac Duo vacuum centrifuge concentrator (GeneVac Company, UK), VG 3 S25 vortexer (IKA Company of Germany); KQ-100DE digital ultrasonic cleaner (Kunshan Ultrasound Instrument Co., Ltd.); Elemental 18120 Molecular Ultra Pure Water System (Shanghai Moller Scientific Instrument Co., Ltd.).

Reagents And Materials

NXTCs (Shanxi Buchang Pharmaceutical Co., Ltd., batch number: 190193) and adrenaline hydrochloride injection (Suicheng Pharmaceutical Co., Ltd., specifications: 1mL: 1 mg, batch number: 61903011) both were purchased from pharmacy of the First Affiliated Hospital of Henan University of Chinese Medicine (Zhengzhou, China). Reference standards of caffeic acid, ferulic acid, stigmatin, tanshinone II A were purchased from Nanjing Liangwei Biological Technology Co., Ltd. with purity above 98% (Nanjing, China). Internal standards including clarithromycin and propione sulphonate were purchased from National Institutes for Food and Drug Control with purity above 98% (Beijing, China). Ultra-pure water was purified by Molecular waters purification system. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid (HPLC grade) was purchased from Dikma Technology Co., Ltd (Tianjin, China). Ultra-pure water was purified by using a Milli-Q system (Milford, MA, USA).

Ethical statement

Female Sprague–Dawley (SD) rats (6-8 weeks old, weighing 220 ± 20 g) were purchased by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Before the experiment, the rats were fed adaptively for one week, keeping the circadian rhythm for 12 hours and feeding and drinking freely. The temperature and humidity meet the requirements of animal feeding. Animal care and experimental protocols were performed based on 'Detailed Rules and Regulations for Administration and Implementation of Biomedical Animal Experiments' (No. 1998-55, Ministry of Public Health, China). The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Henan University of Chinese Medicine.

NXTC Solution Preparation

The content of NXTC was dispersed in 0.5% CMC-Na aqueous solution to produce the NXT suspension with the concentration of 0.33 g/mL.

Preparation of reference substance reserve solution, internal standard reserve solution and quality control sample

Caffeic acid, ferulic acid, anthocyanin and tanshinone II A were weighed accurately and then serially dissolved in methanol to obtain the reference stock solutions with different concentrations of 30.5, 30, 38 and 21.5 mg/mL respectively. Six series of mixed reference solutions were gained with appropriate individual reference stock solution mixed and diluted with methanol in volumetric flask: 0.5, 1, 5, 10, 50 and 100 ng/mL caffeic acid, 1, 5, 10, 50, 100 and 500 ng/mL ferulic acid, 0.05, 0.1, 0.5, 1, 5 and 10 ng/mL anthocyanin and 0.5, 1, 5, 10, 50 and 100 ng/mL tanshinone II A.

As internal standard substances, clarithromycin and probenecid were weighed precisely and dissolved with methanol to obtain the internal standard reserve solutions with concentrations of 532 and 536 mg/mL respectively. Appropriate amount of internal standard reserve solutions was diluted with methanol to provide the mixed internal standard reserve solution with clarithromycin of 106.4 ng/mL and probenecid of 107.2 ng/mL.

Quality control (QC) samples of caffeic acid with concentrations of 30, 50, 500 ng/mL, ferulic acid with concentrations of 10, 20, 200 ng/mL, anthocyanin with concentrations of 0.5, 1, 10 ng/mL and tanshinone II A with concentrations of 1, 5, 50 ng/mL were prepared with blank plasma.

Chromatography and mass spectrometry conditions

Liquid chromatographic analysis and mass spectrometry detection was performed on Thermo Scientific™ TSQ Altis™ (Thermo Company, USA). Chromatographic separation was carried out on an Acquity UPLC BEH C18 column (2.1 ´ 100 mm, 1.7 μm) maintained at 30°C. The mobile phase gradient conditions consisted of 0.1% aqueous formic acid (A) and methanol (B): 0–2 min, 40–100% B; 2–5 min, 100% B; at 5–5.1 min, 100–40% B; 5.1–8 min, 40% B. The flow rate was 0.2 mL/min. The autosampler was conditioned at 10 °C and the sample injection volume was 1 μL.

The ESI source was set in both positive and negative ionization mode. The parameters in the source were set in the following manner: positive ion scanning voltage, 3500 V; negative ion scanning voltage, 2500 V; sheath gas velocity, 25 arb; auxiliary air velocity, 7 arb; collision gas pressure, 1.5 mTorr; ion transmission tube temperature, 325 °C; ion source temperature, 400 °C. The scanning mode was set multiple reaction monitoring (MRM) mode and the selected monitor ion were *m/z* 178.96®135.04 for caffeic acid, *m/z* 192.95®134.00 for ferulic acid, *m/z* 267.00®251.97 for

anthocyanin, *m/z* 295.05®277.20 for tanshinone IIA, *m/z* 748.35®590.29 for clarithromycin, and *m/z* 284.00®240.05 for propafenone. The collision energy and RF-lens were optimized for precursor/product ion pairs of each analyte and the selected values are presented in Table 1.

Modeling, drug administration and preparation of plasma samples

In this experiment, twenty rats were randomly divided into blank administration group and model administration group with 10 rats in each group respectively. After 7 days of acclimation, the rats in the model administration group were given adrenaline hydrochloride injection (0.8 mg/kg) subcutaneously. After 2 h, the rats were placed in 0°C ~ 2°C ice water to swim for 4 minutes, the first time 4 hours after the administration of epinephrine hydrochloride injection, subcutaneous injection of epinephrine hydrochloride injection 0.8 mg/kg was performed again, resulting in a model of acute blood stasis in rats. All rats fasted for 12 hours and drank freely. NXTC suspension was administrated by gavage at a dose of 5g/kg. At 0.083, 0.167, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10 and 12 hours after administration, blood samples were collected from the eye orbital venous plexus of rats and placed in a heparinized centrifuge tube. Plasma was separated by 3000 r/min centrifugation for 10 min and stored at -80 °C for determination.

Plasma Sample Preparation

100 mL of plasma sample was precisely pipetted and 20 mL of 10% formic acid water, 10 mL of internal standard solution were added, the mixture was vortexed for 3 min, then 300 mL of methanol was added and vortexed for 5 min additionally. Subsequently, the sample was centrifugated at 14 000 r/min for 10 min. The supernatant was centrifugally concentrated to dryness under vacuum condition. The residue was re-dissolved in 100 mL methanol, agitated on a vortexer for 3 min and centrifugated at 14000 r/min for 10 min, then the supernatant was obtained for analysis.

Methodological Investigation

Specificity

The blank plasma of rats, blank plasma of rats added with reference solution and internal standard solution, and the plasma samples obtained from rats of model group were taken 100 mL respectively, the samples were processed applying the sample processing procedure in item 2.5. The ion chromatograms of the ingredients were acquired abiding by the corresponding chromatographic and mass spectrometric methods in item 2.3.

linearity, limit of detection (LOD) and lower limit of quantification (LLOQ)

Six duplicates of 100 mL blank rat plasma were added with a series of mixed reference solutions with different concentrations. The samples were processed according to the sample processing procedure in item 2.5 and conducted LC-MS/MS analysis. Taking the concentration of each substance to be measured as the abscissa (x), the ratio of the peak area of the substance against the internal standard as the ordinate (y), and the reciprocal of the concentration (1/x) as the weighting coefficient for linear regression, the regression equations of the calibration curves of each ingredient in rat plasma were established. By comparing the results of samples with known analyte concentration and those of blank samples, the signal-to-noise ratio (S/N) of 3:1 was determined to be the LOD, the S/N of 10:1 was determined to be the LLOQ, which is the lowest concentration point of the standard curve.

Precision and accuracy

Certain duplicates of 100 mL blank plasma were respectively added with different concentrations of mixed reference solution and internal standard solution to prepare the lower limit of quantification sample and quality control samples with low, medium and high concentrations. Six samples were prepared in parallel with each concentration, which were processed according to the procedure displayed in item 2.5. The measured concentration of QC sample was ~~were~~ calculated according to the accompanying standard curve. Measurements for 3 consecutive days were implemented to investigate the accuracy and precision of the established analysis method.

Recovery of extraction and matrix effect

Quality control samples of low, medium and high concentrations prepared from 100 mL blank plasma were processed according to the sample processing method above. The peak area of each component was recorded as A1. Other duplicates of 100 mL blank plasma were also processed according to the method above. The residues were respectively re-dissolved in reference solutions of low, medium and high concentration containing internal standard. The peak area of each component was recorded as A2. The low, medium and high concentration reference solutions containing internal standard were also respectively injected into the analysis system. The peak area of each component was recorded as A3 here. Each sample was prepared in 6 duplicates. The recovery (%) was the ratio of A1 to A3, and the matrix effect (%) was the ratio of A2 to A3.

Stability

The quality control samples with low, medium and high concentration were prepared and placed at 4 °C for 0, 2, 4, 8, 12 and 24 h for analysis. The quality control samples were also frozen at -80 °C and thawed at room temperature for 3 cycles with 6 duplicates for each concentration respectively. According to the established calibration curve, the measured concentration of QC sample was calculated, and compared with the labeled concentration to investigate the stability of each component in rat plasma.

Data processing and analysis

The data collection and sample analysis were controlled by Tracefinder 4.1 software and the data were processed with Microsoft Excel. The content of each compound was expressed as $x \pm s$. The obtained data of content were processed with Phoenix Winnonlin (version 6.2, Pharsight Corporation, USA) pharmacokinetic software, and the pharmacokinetic parameters were calculated applying non-compartment model.

Results

Contents of four components in Naoxintong Capsule

The LC-MS/MS analysis of the sample solution revealed that the contents of caffeic acid, ferulic acid, anthocyanin and tanshinone IIA in Naoxintong capsule were determined as 0.011, 0.099, 0.060 and 0.148 mg×g^{-1} respectively. The chemical structure formulas of the four compounds are shown in Figure 1.

Methodological investigation

Specificity test

The endogenous substances in blank plasma did not interfere with the determination of caffeic acid, ferulic acid, anthocyanin, tanshinone IIA, clarithromycin and probenecid. The extracted ion flow chromatograms of each component are shown in Figure 2.

Linearity, LOD and LLOQ

The calibration curve and lower limit of quantification of the four components are displayed in Table 2. The linearity of each component is good within the corresponding concentration range, and the correlation coefficient is greater than 0.99, meeting the requirements of in vivo drug analysis method.

Precision and accuracy

Three QC samples of different mass concentrations with six duplicates for each were analyzed for three consecutive days. The results showed that the RSD of intra-day precision, intra-day precision and accuracy of each sample could meet the requirements (Table 3).

Recovery and matrix effect

The results of matrix effect and extraction recovery of each component could meet the requirements of biological sample detection (Table 4).

Stability

The stability of the plasma sample is good after 24 hours of cold storage (4 °C) or 3 cycles of freeze-thaw (Table 5).

Comparative analysis of blood concentration-time curves for four blood components

The blood concentration-time curves of caffeic acid, ferulic acid, formonetin and tanshinone IIA in NXTC in normal and acute blood stasis model rats are shown in Figure 3.

Comparative analysis of pharmacokinetic parameters

The statistical analysis results of main pharmacokinetic parameters of caffeic acid, ferulic acid, formononetin and tanshinone IIA in normal and acute blood stasis model rats intragastrically administrated with NXTC are shown in Table 6. As the pharmacokinetic parameters of caffeic acid, ferulic acid, formononetin and tanshinone IIA exhibited above, the peak time (T_{max}) of the four components was 0.12 ± 0.05, 0.11 ± 0.04, 0.08 ± 0.00 and 0.13 ± 0.05 h, respectively, which indicated that the above four components could be absorbed into the blood rapidly. Concluded from the results of AUC_{all} and AUC_{INF_obs} and the peak concentrations (C_{max}) of the four components being 5.01±0.74, 71.37±15.56, 5.70±1.19 and 2.08±0.57 mg/L, ferulic acid was absorbed best in body of normal rats, with C_{max} being 12-35 times of the other three components. Tanshinone IIA exhibited the

lowest C_{max}, indicating that its absorption being worse than the other three components. However, the apparent distribution volume (VZ_F_obs) and clearance rate (CL_F_obs) of caffeic acid and ferulic acid were smaller than those of formononetin. With the largest VZ_F_obs and CL_F_obs, tanshinone IIA possessed a widespread in vivo distribution. The average retention time of four components was 4.40 ± 0.46 ~ 5.78 ± 0.29 h.

Discussion

In this experiment, compared with the normal group, the T_{max} of the four components of NXTC were prolonged in acute blood stasis model rats with no statistical difference and the C_{max} of the four components were decreased, with the decrease of ferulic acid and formononetin being statistically significant ($P < 0.05$), which suggested that the blood circulation disorder caused by acute blood stasis hindered the absorption of the four components. The AUC_{all} of caffeic acid and ferulic acid were significantly reduced ($P < 0.05$) in model group. The AUC_{INF_obs} of ferulic acid was also remarkable reduced ($P < 0.01$). It was observed that compared with the normal group, the clearance rate of tanshinone IIA in the model group was reduced, which allowed more tanshinone IIA to remain in rats body and play its role in the treatment of blood stasis. Significant elevations of the plasma concentration of ferulic acid and caffeic acid ($P < 0.05$) and increased in the mean retention time (MRT_{last}) of caffeic acid and ferulic acid ($P < 0.05$) increased as well. The difference of the above results might be due to the characteristics of "viscosity, concentration, coagulation and aggregation" of the blood in acute blood stasis model rats, which makes the blood viscosity increase, the blood flow slow down, the absorption of components slow down, T_{max} and MRT_{last} prolonged.

Blood stasis syndrome is a pathological state of blood circulation, which is described as slow or accumulation of blood due to *xinqi* disorder in traditional Chinese medicine (TCM) and TCM believes that the "sadness" of seven emotions (*qiqing*) and the "cold evil" of six evils (*liuxie*) are the main causes of acute blood stasis [17]. Now blood stasis is usually understood as a blood system disease, pathological studies show that blood stasis is mainly characterized by cardiovascular and cerebrovascular diseases, such as cerebral infarction, myocardial infarction, coronary heart disease, hypertension and so on [17-19]. The efficacy of NXTC capsule is replenishing *qi* and activating blood, removing blood stasis and dredging collaterals. It is often used in the treatment of cardiovascular and cerebrovascular diseases, which is closely related to its main components caffeic acid, ferulic acid, formononetin and tanshinone IIA. Numerous studies have demonstrated that caffeic acid and its derivatives have pharmacological effects such as antioxidation, immune regulation, anti-cancer, regulation of cardiovascular and cerebrovascular diseases and protection of brain tissue damage [20-22]. Ferulic acid is a metabolite of caffeic acid methylation, which can promote bone marrow hematopoiesis, enhance immunosuppression, protect cardiovascular system, reduce blood lipid, resist arteriosclerosis and inhibit platelet aggregation [23-25]. Formononetin has pharmacological actions on improving atherosclerosis and inhibiting the proliferation of vascular smooth muscle cells [26, 27]. In recent years, tanshinone IIA has attracted the attention of much more researchers in cardiovascular and cerebrovascular aspects. The protective effects of tanshinone IIA on the heart include preventing the formation of atherosclerosis, preventing myocardial injury and hypertrophy, expanding coronary arteries and related mechanisms of action have been reported [28-31].

In the pathological state, the pharmacokinetic of drug is different from the normal state due to changes in the physiological state and biochemical response of the body. Given that NXTC is usually used for the treatment of patients with blood stasis, we studied the pharmacokinetics of NXTC in rats with acute blood stasis. The acute state of blood stasis may be caused by abnormal hemorheology, such as the increase of blood viscosity and coagulation degree, blood aggregation, vascular obstruction and so on [32, 33]. In the model group, it can be observed from the overall point of view that the T_{max} and C_{max} of the four components were prolonged and decreased, respectively, parameters of AUC_{all}, Vz_F_obs and MRT_{last} were changed as well, indicting that the absorption and metabolism of NXTC were affected in acute blood stasis. These results provide important information for guiding the clinical rational use of drugs.

Conclusion

Herein, we have established the analytical method to probe the pharmacokinetic properties of the four main ingredients caffeic acid, ferulic acid, formononetin and tanshinone IIA of NXTC in acute blood stasis rats compared with the corresponding profile in normal rats. The parameters demonstrated that the C_{max} of ferulic acid and formononetin decreased statistically significantly, the AUC_{all}/AUC_{INF_obs} of ferulic acid and the AUC_{all} of caffeic acid were all significantly reduced. The plasma concentration and mean retention time (MRT_{last}) of ferulic acid and caffeic acid all increased. The alteration of the pharmacokinetics was consistent with the change of the rheological characteristics of the blood in acute blood stasis rats. These investigations may provide experimental basis for the plan adjustment of clinical medication of NXTC for patients with blood stasis.

Abbreviations

NXTC: Naoxintong Capsule; QC: quality control; ESI: electrospray ion source; MRM: multi response monitoring; IS: internal standard; LOD: limit of detection; LLOQ: lower limit of quantification; S/N: signal-to-noise; Cmax: peak concentrations; Tmax: peak time; VZ_F_obs: apparent distribution volume; CL_F_obs: clearance rate; MRTlast: the mean retention time.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

WXL and PPC contributed equally to this work. JFT and XLL conceived and designed the study. HZ was mainly responsible for the instrument operation. WXL and SQZ conducted the data analysis and wrote the manuscript. PPC and MML revised the manuscript. XYW and LN conducted the animal experiment. CXL provided the advice for the study. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Animal care and experimental protocols were performed based on 'Detailed Rules and Regulations for Administration and Implementation of Biomedical Animal Experiments' (No. 1998-55, Ministry of Public Health, China). All experiments involving animals were approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Henan University of Chinese Medicine.

Consent for publication

All of authors consent to publication of this study in journal of Chinese Medicine.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Tables

Table 1
Collision Energy and Ion Lens Voltage Detection Parameters of Four Main Components and Internal Standard Compounds in Naoxintong Capsules

compound	$[M+H]^+$ (M/Z)	$[M-H]^-$ (M/Z)	Parent ion→daughter ion	Collision energy(eV)	RF-lens(V)
caffeic acid		178.96	178.96→135.04	15.88	49
ferulic acid		192.95	192.95→134.00	14.7	44
formononetin		267.00	267.00→251.97	20.27	68
tanshinone IIA	295.05		295.05→277.20	19.78	67
clarithromycin(IS)	748.35		748.35→590.29	17.7	74
Probenecid (IS)		284.00	284.00→240.05	14.97	57

Table 2
Calibration curve and quantitative LLOQ for four components in NXTC.

component	calibration curve	R	linear range /ng·ml^{-1}	LOD/ ng·ml^{-1}	LLOQ/ng·ml^{-1}
caffeic acid	$Y = 3.195\cdot10^{-3}X + 2.034\cdot10^{-3}$	0.9995	0.5–100	0.1	0.5
ferulic acid	$Y = 2.136\cdot10^{-4}X + 2.514\cdot10^{-5}$	0.9979	1-500	0.5	1
formononetin	$Y = 4.326\cdot10^{-3}X + 1.843\cdot10^{-3}$	0.9992	0.05-10	0.01	0.05
tanshinone IIA	$Y = 4.102\cdot10^{-2}X$-$8.833\cdot10^{-3}$	0.9994	0.5–100	0.1	0.5

Table 3
Precision and accuracy of the analysis method for four components in NXTC.

component	concentration of reference compound solution(ng/mL)	intraday			interday		
		content (ng/mL)	RR%	RSD%	content (ng/mL)	RR%	RSD%
caffeic acid	30	30.42±1.61	101.40±5.73	5.31	31.77±1.12	105.90±3.73	3.54
	50	55.73±6.06	111.46±12.12	10.88	52.24±4.35	104.48±8.70	8.31
	500	496.61±37.80	99.32±7.56	7.61	480.47±23.24	96.09±4.65	4.84
ferulic acid	10	9.49±0.46	94.90±4.60	4.86	9.68±0.27	96.80±2.70	2.76
	20	25.91±1.98	129.55±9.90	7.64	23.00±2.11	115.00±10.55	9.16
	200	197.66±14.03	98.83±7.01	7.10	211.76±16.95	105.88±8.48	8.00
formononetin	0.5	0.55±0.07	110.00±14.00	12.08	0.58±0.05	116.00±10.00	8.66
	1	1.14±0.12	114.00±12.00	10.13	1.17±0.06	117.00±6.00	4.91
	10	11.04±1.57	110.40±15.70	1.24	10.95±0.48	109.50±1.09	4.35
tanshinone IIA	1	1.46±0.20	146.00±20.00	13.95	2.11±0.56	211.00±56.00	26.32
	5	4.59±0.53	91.80±10.60	11.51	4.84±0.44	96.80±8.80	9.13
	50	55.61±3.93	111.22±7.86	7.07	56.62±7.46	113.24±14.92	13.17

Table 4
Recovery rate and matrix effect of the analysis method for four components in NXTC.

component	concentration of reference compound solution (ng/mL)	recovery rate (%)	RSD (%)	matrix effect (%)	RSD (%)
caffeic acid	30	81.90 ± 6.75	9.21	72.00 ± 3.97	6.19
	50	74.84 ± 6.27	9.37	119.00 ± 4.32	4.06
	500	69.09 ± 8.96	10.01	71.81 ± 7.20	12.27
ferulic acid	10	97.20 ± 13.85	15.94	100.39 ± 10.58	12.17
	20	83.76 ±5.25	7.01	118.25 ± 6.22	5.88
	200	83.97 ± 4.86	6.47	96.15 ± 3.18	4.05
formononetin	0.5	78.56 ± 4.81	6.84	116.10 ± 15.39	14.82
	1	89.35 ± 11.61	14.52	110.34± 10.98	11.12
	10	71.24 ± 1.87	2.93	90.31± 9.32	11.30
tanshinone IIA	1	22.01 ± 1.97	10.33	52.08 ± 4.70	10.09
	5	35.56± 1.43	4.49	45.55 ± 3.64	8.94
	50	30.10 ± 2.01	7.48	37.17 ± 4.15	13.67

Table 5
Investigation of the stability for four components in NXTC.

component	concentration of reference compound solution (ng/mL)	short term		freeze-thaw	
		content (ng/mL)	RSD %	content (ng/mL)	RSD %
caffeic acid	30	36.73 ± 5.51	15.00	8.34 ± 0.56	8.25
	50	52.11 ± 3.56	6.83	23.62 ± 1.14	5.29
	500	498.57 ± 40.88	8.20	255.89 ± 23.64	10.33
ferulic acid	10	11.79 ± 0.90	7.65	9.89 ± 0.85	9.40
	20	21.38 ± 1.03	4.81	25.91 ± 1.81	7.64
	200	226.27 ± 7.07	3.12	197.66 ± 12.81	7.10
formononetin	0.5	0.62 ± 0.03	5.54	0.55± 0.06	12.08
	1	1.23 ± 0.06	4.93	1.14 ± 0.11	10.13
	10	11.48 ± 0.31	2.67	11.04 ± 1.44	14.24
tanshinone IIA	1	1.81 ± 0.07	3.74	1.48 ± 0.18	13.05
	5	4.78 ± 0.38	7.99	1.92 ± 0.20	11.51
	50	53.49 ± 2.24	4.19	21.04 ± 3.43	18.22

Table 6
Pharmacokinetic parameters of four components in NXTC applying non-compartment model in normal and acute blood stasis model rats.

compound	□□	*n*	Rsq_adjusted	HL_Lambda_z	T_{max}	C_{max}	AUC_{all}	AUC_{INF_obs}	Vz_F_obs	Cl_F_obs	MRT_{last}
				h	h	μg·L^{-1}	h·μg·L^{-1}	h·μg·L^{-1}	L·kg^{-1}	L·h^{-1}·kg^{-1}	h
caffeic acid	normal	7	0.77 ± 0.12	5.15 ± 1.01	0.12 ± 0.05	5.01 ± 0.74	18.98 ± 1.98	22.81 ± 3.40	18.14 ± 3.23	2.27 ± 0.73	4.69 ± 0.39
	model	6	0.54 ± 0.10*	11.19 ± 4.93	0.18 ± 0.08	4.91 ± 1.20	14.79 ± 2.32*	30.75 ± 10.02	28.21 ± 7.89*	2.29 ± 1.22	5.38 ± 0.43*
ferulic acid	normal	7	0.78 ± 0.08	5.29 ± 1.42	0.11 ± 0.04	71.37 ± 15.56	160.99 ± 33.48	217.45 ± 44.39	23.14 ± 10.84	3.22 ± 1.69	4.40 ± 0.46
	model	6	0.52 ± 0.34	6.11 ± 0.54	0.12 ± 0.05	30.82 ± 19.53**	63.44 ± 16.66**	96.45 ± 18.49**	46.87 ± 19.00*	4.95 ± 1.00	5.41 ± 0.85*
formononetin	normal	7	0.86 ± 0.21	9.96 ± 4.21	0.08 ± 0.00	5.70 ± 1.19	15.17 ± 4.69	24.10 ± 7.98	115.36 ± 41.76	13.62 ± 4.54	5.06 ± 0.15
	model	6	0.76 ± 0.11	5.03 ± 2.62	0.37 ± 0.35	3.82 ± 0.84*	11.87 ± 1.41	18.05 ± 6.97	132.21 ± 45.96	14.65 ± 4.39	4.78 ± 0.63
tanshinone IIA	normal	7	0.72 ± 0.21	7.17 ± 5.71	0.13 ± 0.05	2.08 ± 0.57	8.55 ± 1.14	11.45 ± 2.75	442.23 ± 123.76	56.72 ± 21.18	5.78 ± 0.29
	model	6	0.34 ± 0.12*	19.63 ± 18.62	0.50 ± 0.35	1.67 ± 0.29	9.68 ± 0.73	27.74 ± 8.26	530.32 ± 209.61	37.33 ± 23.67	5.79 ± 0.31

Footnote: Rsq_adjusted represented a parameter to predict the fitting effect with the actual value, same as adjustment r^2;HL_Lambda_z represented elimination phase half-life:T_{max} represented time to peak:C_{max} represented peak concentration:UC_{all} represented AUC spanned from the beginning of administration to the last point:UC_{INF_obs} represented AUC spanned from the beginning of administration to the infinite time by extrapolation:z_F_obs represented apparent distribution volum:Cl_F_obs represented clearance rate: MRT_{last} represented average residence time.
Compared with the normal group, * $P < 0.05$; ** $P < 0.01$.

Figures

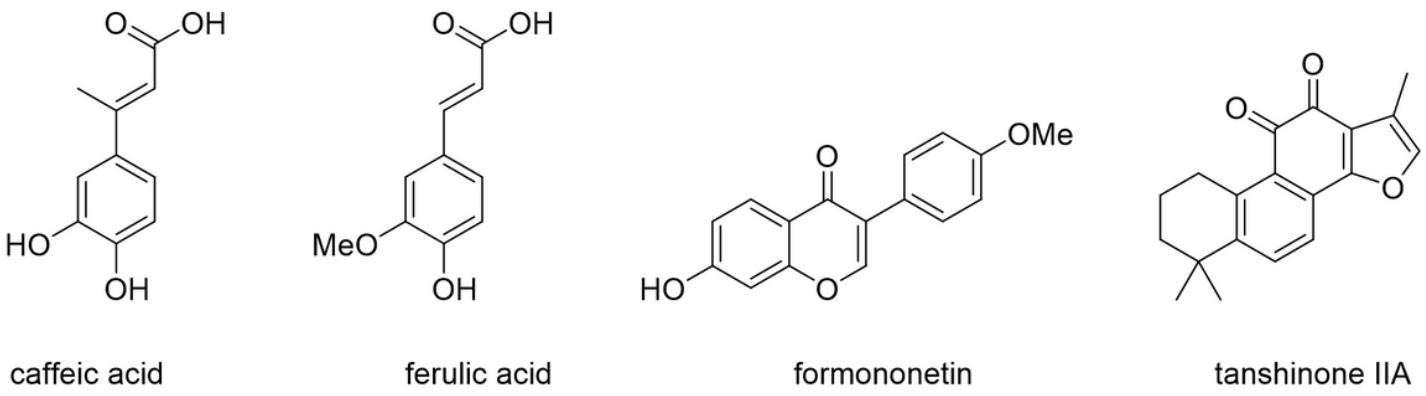

Figure 1

Chemical structures of four main components in Naoxintong Capsule.

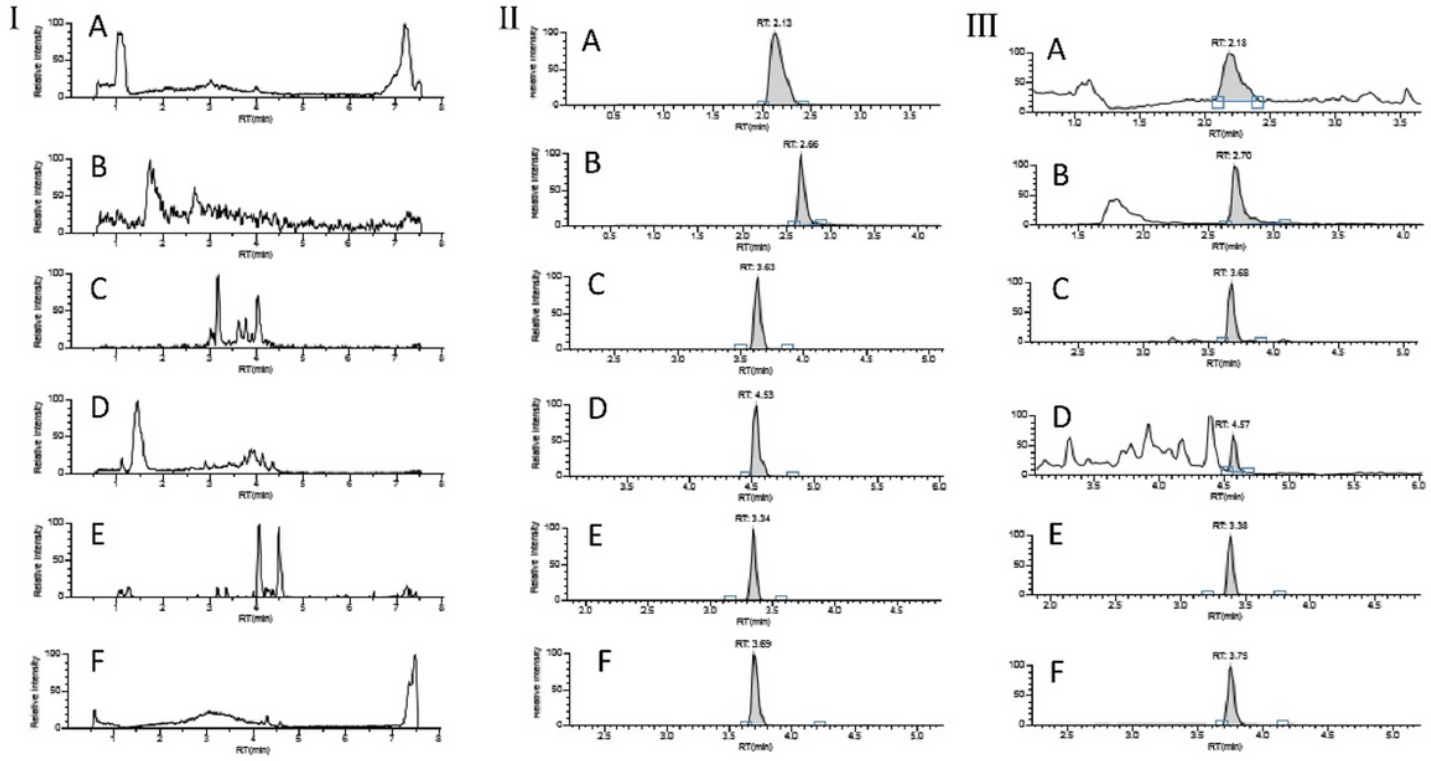

Figure 2

Typical MRM chromatograms of various components in rat plasma.

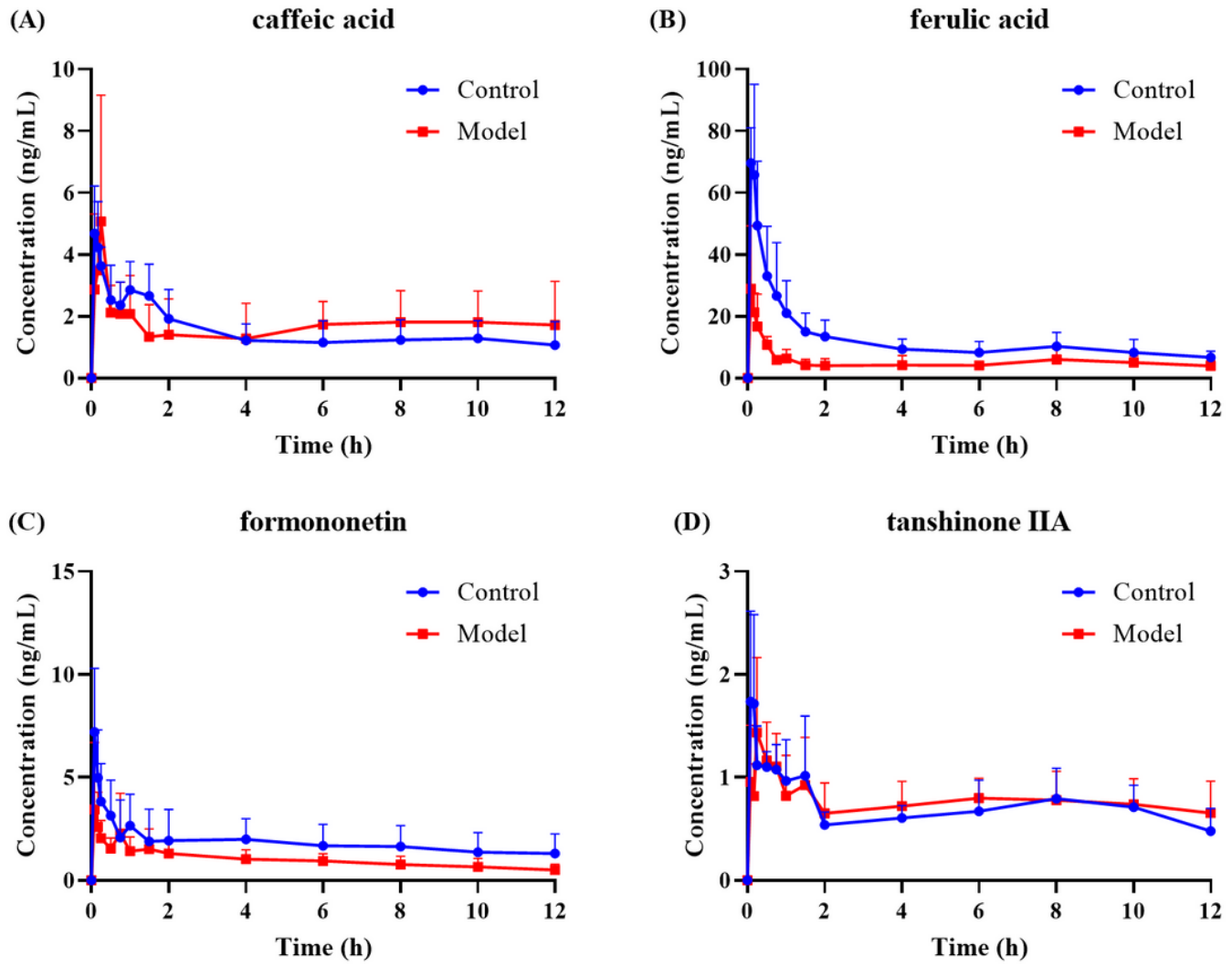

Figure 3

Blood concentration-time curves of caffeic acid, ferulic acid, menthol and tanshinone IIA in normal and acute blood stasis model rats intragastric administration of NXTC.