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# Determination of cryptotanshinone and its metabolite in rat plasma by liquid chromatography–tandem mass spectrometry

M. Song, T.J. Hang *, ZH. X. Zhang, R. Du, J. Chen

*Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, China*

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**Abstract**

A sensitive and selective LC–MS–MS method has been developed and validated for the determination of cryptotanshinone (CTS) and its active metabolite tanshinone II A (TS II A) in rat plasma using fenofibrate (FOFB) as internal standard. Liquid–liquid extraction was used for sample preparation. Chromatographic separation was achieved on a Waters symmetry ODS column using methanol and water (85:15) as mobile phase delivered at 1.0 mL/min. LC–MS–MS analysis was carried out on a Finnigan LC-TSQ Quantum mass spectrometer using atmospheric pressure chemical ionization (APCI) and positive multiple reaction monitoring. Ions monitored were *m/z* 297.0 → 251.0 for CTS, *m/z* 295.0 → 249.0 for TS II A, and *m/z* 361.1 → 233.0 for FOFB with argon at a pressure of 0.2 Pa and collision energy of 25 eV for collision-induced dissociation (CID). The assay was linear over the range 0.1–20 ng/mL for CTS and 0.2–15 ng/mL for TS II A. The average recoveries of CTS and TS II A from rat plasma were 93.7 and 94.7%, respectively. The established method has been applied in a pharmacokinetic study of CTS in rats.


*Keywords:* Cryptotanshinone; Tanshinone II A; Metabolism; Pharmacokinetic; LC–MS–MS

## 1. Introduction

*Salvia miltiorrhiza Bunge*, a well-known traditional Chinese medicinal herb, has attracted particular attention from medicinal chemists and clinicians for its reputed therapeutic effects in the treatment of coronary heart and cerebrovascular diseases as well as neurasthenic insomnia [1,2]. Compounds with antibacterial, anti-neoplastic, anti-fungal, anti-inflammatory and anti-platelet aggregation activities have been isolated from this medicinal herb [3–8]. The major active components of the herb are diterpenoid tanshinones, including cryptotanshinone (CTS, Fig. 1A) and tanshinone IIA (TS II A, Fig. 1B). Early studies showed that CTS could be metabolized to TS II A in vivo [9–10].

Earlier publication has described a method for analysis of CTS and TS II A in biological samples by using high-performance liquid chromatography (HPLC) and UV detection with the LODs for both CTS and TS II A were 25 ng/mL [11]. Because the diterpenoid tanshinones have low systemic bioavailability by oral administration, the reported method is not sensitive and specific enough for their pharmacokinetic study at the recommended therapeutic doses.

In this paper a new LC–MS–MS method was established for pharmacokinetic study of CTS and its metabolite. The method was fully validated and successfully applied to the plasma pharmacokinetic study of CTS and TS II A in rats after oral administration of CTS.

## 2. Experimental

### 2.1. Chemicals and solvents

Reference substances of CTS (Lot #852–9903) and TS II A (Lot #110766–200314) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). CTS for rat pharmacokinetic study was obtained from Sino-herb Bio-technology Co. Ltd. (Xi'an, China). Fenofibrate (FOFB, Fig. 1C) was provided by Xuzhou Enhua Pharmaceutical group Co. Ltd. (Xuzhou, China). Methanol (HPLC grade) was obtained from Merck KGaA (Darm stadt, German) and *tert*-butyl methyl ether was of HPLC grade from Tedia Company (Fairfield, USA). Water was prepared with double distillation.

* Corresponding author. Tel.: +86 25 8327 1090; fax: +86 25 8327 1090.
*E-mail address:* hangtj@cpu.edu.cn (T.J. Hang).

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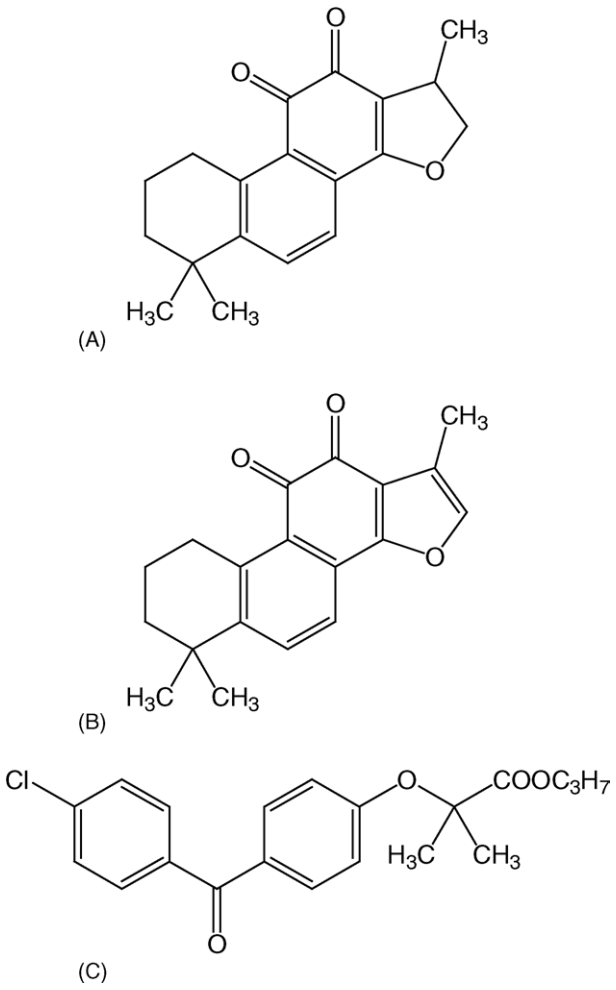


Fig. 1. Chemical structures of CTS (A), TS II A (B) and FOFB (C, internal standard).

### 2.2. Standards

Standard stock solution of CTS, TS II A, and FOFB each with the known concentration of 1 mg/mL were accurately prepared in methanol. The stock solutions were diluted stepwise with methanol to form less concentrated working standards of 20, 200, and 1000 ng/mL for CTS and TS II A, respectively. A 100 ng/mL solution for internal standard (FOFB) was also prepared in methanol. The stock solutions were stored in refrigerator at 4 °C as these compounds are unstable at room temperature when exposed to light.

### 2.3. Instrumentation and conditions

The LC–MS–MS system consists of a Surveyor LC pump, a Surveyor auto-sampler, a TSQ Quantum Ultra AM triple-quadrupole tandem mass spectrometer, and Xcalibur 2.1 software for data acquisition and analysis (Thermo Finnigan, San Jose, USA). The separation was performed on a Waters Symmetry ODS column (4.6 mm × 250 mm, 5.0 μm, Waters, Milford, USA) preceded by an ODS guard column (2 mm × 4 mm). Separation conditions were as follows: auto-sample temperature, 4 °C; column temperature, 30 °C; sample injection volume, 20 μL. The analysis was isocratic at 1.0 mL/min flow with methanol–water (85:15) as mobile phase.

The atmospheric pressure chemical ionization source (APCI) was used as interface. The mass spectrometer was operated in the positive ion multiple reaction monitoring (MRM) mode with the discharge current set at 6.0 μA. Vaporizer temperature was 500 °C, nitrogen sheath and auxiliary gas were set at $2.07 \times 10^5$ Pa and $3.45 \times 10^4$ Pa, respectively. The heated capillary temperature was set at 350 °C. The collision energy of 25 eV was used with argon at a pressure of 0.2 Pa for collision-induced dissociation (CID). The total run time for a LC–MS–MS analysis was 12.5 min. The flow was directed to mass spectrometer 5.5 min after the start of the run.

### 2.4. Rat plasma pharmacokinetic study design

All studies in animals were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of Jiangsu, China. Sprague–Dawley rats (250 ± 10 g, half male and half female) were provided by the experimental animal center of Nanjing Medical University. The rats were fasted 12 h before administration of drug with free access to water. Oral doses of 5.7 mg/kg for CTS were administered in adequate volumes of CTS suspension of 0.57 mg/mL in 1.0% Tween 80. Blood samples (0.5 mL) were collected from the ophthalmic venous plexus in 1.0 mL heparinized Ependroff tubes before dosing and at 5, 10, 15, 20, 30, 40, 60, 120, 240, 360, 480, 720, and 1440 min post-dose. Plasma was separated by centrifugation and stored at −20 °C until analysis. Aliquots of 0.2 mL plasma samples were processed and analyzed for CTS and its major metabolite TS II A concentrations.

### 2.5. Blanks and plasma standards for validation

Blank rat plasma was extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent response at the retention time of CTS, TS II A, and FOFB was compared to the lower limit of quantification. To prepare plasma standards for validation, the blank plasma (0.2 mL) was spiked with known concentrations of CTS and TS II A, and then followed by internal standard. The standard curves consisted of samples containing 0.1, 0.5, 1.0, 2.0, 5.0, 10 and 20 ng/mL of CTS, and 0.2, 0.4, 1.0, 2.0, 4.0, 10 and 15 ng/mL of TS II A, respectively. To determine the precision, accuracy and recovery of the assay and to evaluate the stability of the compounds after three cycles of freeze–thaw, control standards were prepared at the concentration of 1.0, 5.0 and 20 ng/mL for CTS, 0.4, 2.0 and 10 ng/mL for TS II A.

### 2.6. Sample preparation

Sample preparation was carried out under subdued light. Blood samples collected in heparinized Ependroff tubes were

immediately centrifuged at 3500 × *g* force for 10 min. The resulting supernatant plasma was separated and an aliquot of 0.2 mL was mixed with 40 μL of a 100 ng/mL FOFB solution in methanol and 0.3 mL of saline solution. The aqueous phase was vortex-mixed with 3 mL of *tert*-butyl methyl ether for 3 min. After phase separation by centrifugation, an aliquot of 2.5 mL of the organic layer was taken and evaporated to dryness under a gentle stream of nitrogen flow at 40 °C. The residue was reconstituted with 100 μL of mobile phase and kept in cold and shaded auto-sampler and an aliquot of 20 μL was injected into the LC–MS–MS system.

## 3. Result and discussion

### 3.1. Mass spectral analysis

Diterpenoid tanshinones are much more easily and positively ionized in atmospheric pressure chemical ionization (APCI) source than in electron spray ionization (ESI) source due to their weak polarity and strong lipophilicity. Therefore, APCI source was used as interface. Multiple reaction monitoring (MRM) afforded by tandem mass spectrometry has great advantage in reducing interference and enhancing sensitivity over the selected ion monitoring (SIM). MRM was set for the detection of CTS, TS II A and FOFB in this study. The full product scan mass spectra of CTS, TS II A, and FOFB and their fragmentation schemes of product ions from the parent ions $[M + H]^+$ are shown in Fig. 2, respectively.

The MRM transitions selected for the detection of CTS, TS II A and FOFB are *m/z* 297 → *m/z* 251, *m/z* 295 → *m/z* 249, and *m/z* 361.1 → *m/z* 233, respectively. The discharge current, vaporizer temperature, nitrogen sheath gas, the heated capillary temperature, and the collision energy were selected to optimize specificity and sensitivity for the detection.

### 3.2. Separation and specificity

Typical chromatograms are shown in Fig. 3. Observed retention times were 8.2, 9.2 and 11.2 min for CTS, FOFB and TS II A, respectively, with a total run time of 12.5 min. Blank plasma was tested for endogenous interference. A representative chromatogram of the plasma blank is shown in Fig. 3A. No additional peaks due to endogenous substances were observed that would interfere with the detection of compounds of interest. Fig. 3B shows the chromatograms of calibration standard containing 5.0 ng/ml of CTS, 4.0 ng/mL of TS II A and 20 ng/ml of FOFB in plasma. A typical chromatogram of plasma sample is shown in Fig. 3C which was collected 10 min after oral administration of CTS (5.7 mg/kg) to a rat and the corresponding concentration of CTS and TS II A was found to be 1.2 and 3.2 ng/mL, respectively.

### 3.3. Linearity and limit of quantitation

Calibration curves were calculated using peak area ratios (*Y*) for both CTS and TS II A to FOFB. Calibration curves for CTS and TS II A in plasma were linear in the concentration (*C*,

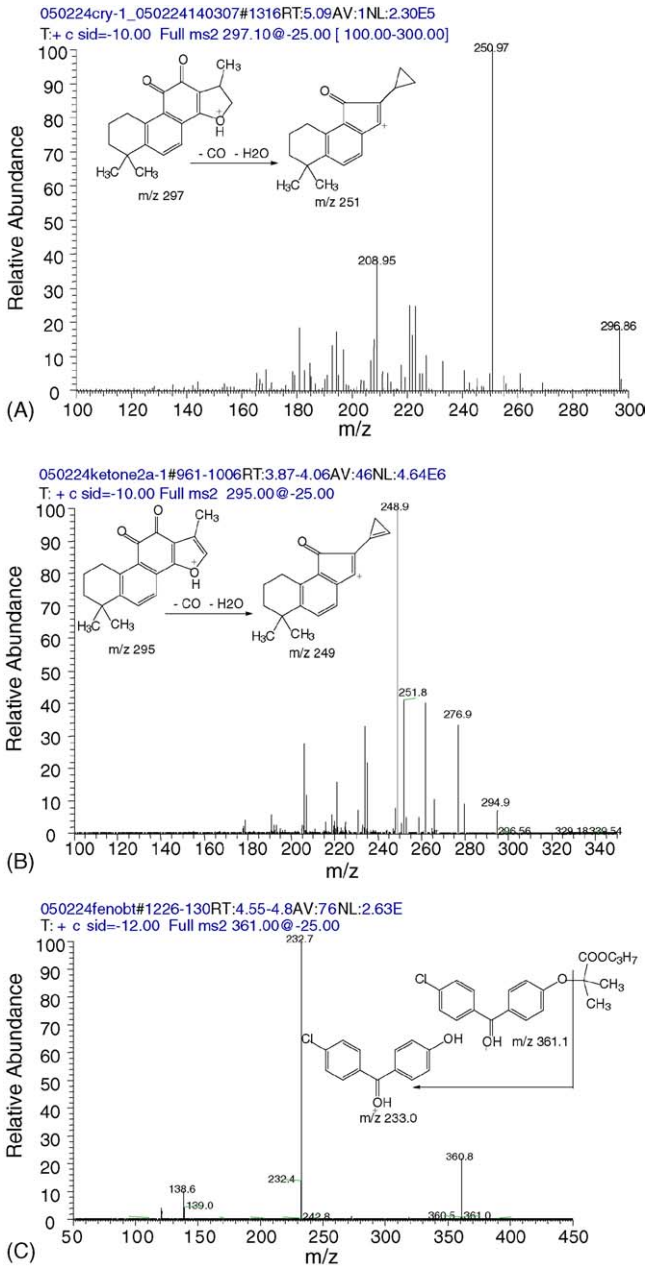


Fig. 2. Structures and product ion spectra of CTS (A), TS II A (B) and FOFB (C).

ng/mL) range of 0.1–20 and 0.2–15 ng/mL, respectively. The typical calibration curve for CTS is $Y = 0.02943C + 0.00633$, the correlation coefficient was 0.999. The typical calibration curve for TS II A is $Y = 0.04077C + 0.01149$, the correlation coefficient was 0.999. LOQ of 0.1 ng/mL for CTS and 0.2 ng/mL for TS II A were determined according to FDA guidelines as a control value where the R.S.D. is <15% and analyte signal-to-noise is >3.

### 3.4. Recovery

The mean absolute recoveries were measured at three different concentration levels for both CTS (1.0, 5.0 and 20 ng/mL)

and TS II A (0.4, 2.0 and 10 ng/mL) in triplicate by dividing the areas found for the spiked plasma samples with that of the corresponding pure control. The mean recoveries for CTS and TS II A were 93.7% (% CV 6.9) and 94.7% (% CV 4.1), respectively. The mean recovery for internal standard was 57.7% (% CV 6.4). The data of recovery obtained are shown in Table 1. No significant matrix effect was observed for the analytes in the plasma samples.

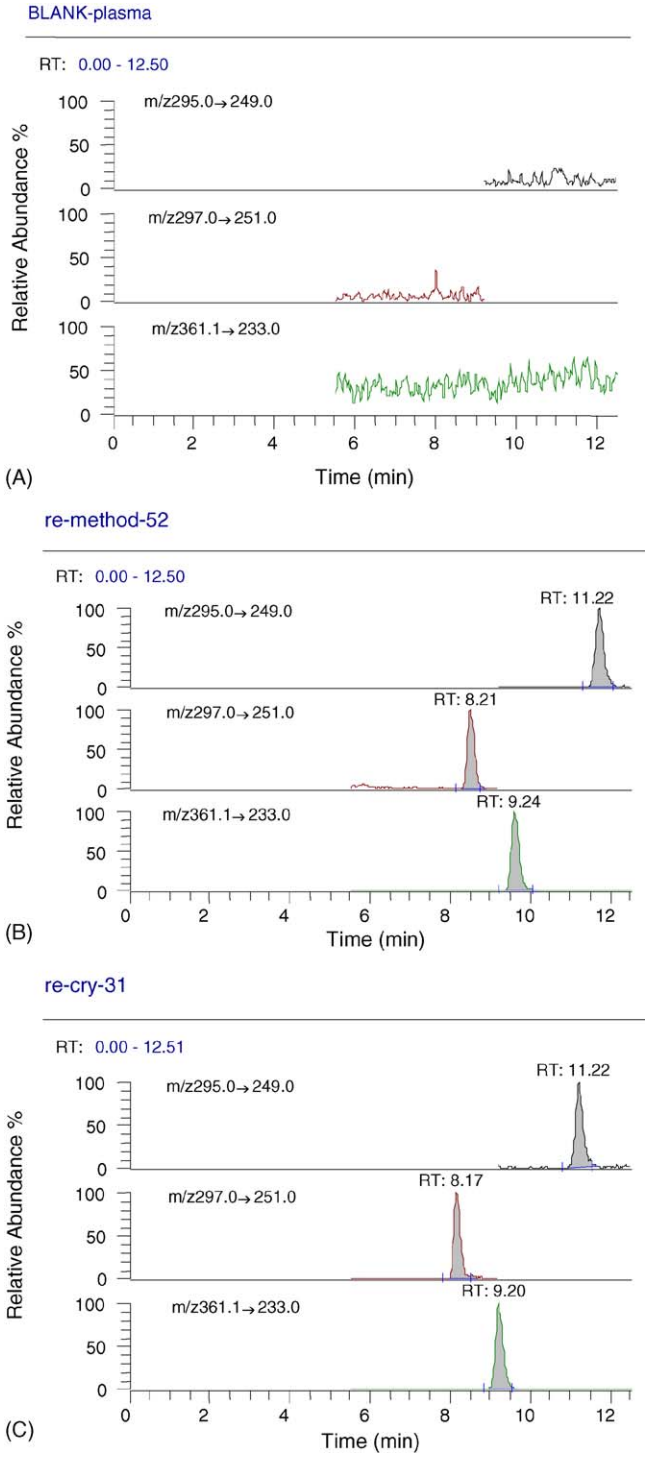


Fig. 3. Representative chromatograms: (A) blank plasma; (B) calibration standard containing 5.0 ng/ml of CTS ($t_R$ = 8.21 min), 4.0 ng/mL of TS II A ($t_R$ = 11.22 min) and 20 ng/ml of FOFB ($t_R$ = 9.24 min) in plasma; (C) rat plasma sample taken 10 min after oral administration of CTS 5.7 mg/kg, the concentration of CTS ($t_R$ = 8.17 min) and its metabolite TS II A ($t_R$ = 11.22 min) were found to be 1.2 and 3.2 ng/ml, respectively.

Table 1
Recovery for CTS and TS II A

| Analyte | CTS | | | TS II A | | | FOFB |
|---|---|---|---|---|---|---|---|
| Spiked *C* (ng/ml) | 1.0 | 5.0 | 20 | 0.4 | 2.0 | 10 | 20 |
| Mean found *C* (ng/ml) (*n* = 5) | 0.99 | 4.60 | 17.6 | 0.37 | 1.92 | 9.7 | 11.5 |
| CV (%) | 6.3 | 3.0 | 5.5 | 3.3 | 4.8 | 3.2 | 6.4 |
| Overall recovery (%) | 98.6 | 92.0 | 88.0 | 91.7 | 96.0 | 96.5 | 57.7 |

Table 2
Intra- and inter-day precision and accuracy for CTS

| Spiked *C* (ng/ml) | 1.0 | 5.0 | 20 |
|---|---|---|---|
| Intra-day run | | | |
| Mean found *C* (ng/ml) (*n* = 5) | 0.94 | 5.19 | 19.80 |
| S.D. | 0.05 | 0.34 | 0.89 |
| CV (%) | 5.4 | 6.5 | 4.5 |
| Accuracy (%) | −6.5 | 3.8 | −1.0 |
| Inter-day run | | | |
| Mean found *C* (ng/ml) (*n* = 5) | 0.97 | 5.18 | 19.82 |
| S.D. | 0.04 | 0.29 | 0.65 |
| CV (%) | 4.5 | 5.5 | 3.3 |
| Accuracy (%) | −2.6 | 3.5 | −0.9 |

### 3.5. Accuracy and precision

Plasma control samples (0.2 mL) of 1.0, 5.0 and 20 ng/mL for CTS or 0.4, 2.0 and 10 ng/mL for TS II A were analyzed for accuracy and precision. The data obtained for CTS and TS II A are shown in Tables 2 and 3, respectively. The data obtained both for CTS and TS II A were within the acceptable limits to meet the guidelines for bioanalytical methods.

### 3.6. Storage stability at −20 °C, freeze–thaw stability

Plasma samples spiked with known concentrations of 1.0, 5.0 and 20 ng/mL for CTS, or 0.4, 2.0 and 10 ng/mL for TS II

Table 3
Intra- and inter-day precision and accuracy for TS II A

| Spiked *C* (ng/ml) | 0.4 | 2.0 | 10 |
|---|---|---|---|
| Intra-day run | | | |
| Mean found *C* (ng/ml) (*n* = 5) | 0.36 | 1.91 | 10.4 |
| S.D. | 0.03 | 0.03 | 0.11 |
| CV (%) | 9.6 | 1.6 | 1.1 |
| Accuracy (%) | −10.9 | −4.4 | 4.3 |
| Inter-day run | | | |
| Mean found *C* (ng/ml) (*n* = 5) | 0.37 | 1.91 | 10.2 |
| S.D. | 0.03 | 0.10 | 0.23 |
| CV (%) | 8.2 | 5.3 | 2.2 |
| Accuracy (%) | −8.0 | −4.6 | 1.8 |

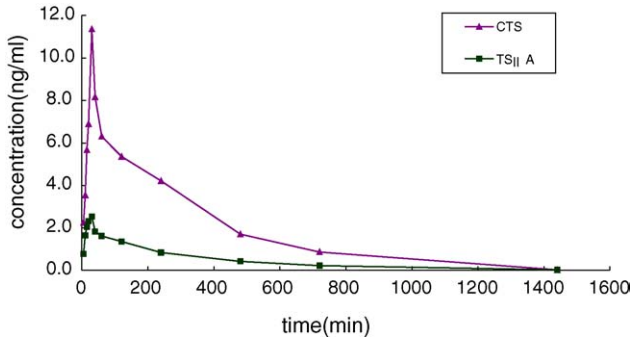


Fig. 4. Mean plasma concentration-time profiles of CTS and TS II A after oral administration of CTS 5.7 mg/kg in rats.

A, respectively were stored at −20 °C and analyzed at regular intervals to evaluate and establish the long-term storage conditions and stability. The freeze thaw (in 37 °C water bath) stability was determined for samples that underwent three freeze–thaw cycles. Standard calibration curve was freshly prepared on the day of analysis.

The results of long-term storage stability data indicated that the plasma samples were stable at −20 °C for at least 30 days.

The freeze–thaw stability was determined with the R.S.D.% for CTS calculated were 11.3, 9.1, and 6.3% and R.S.D.% for TS II A were 10.5, 8.6, and 4.5%, respectively. These results suggested that plasma samples could be thawed and refrozen without compromising the integrity of the sample in three freeze–thaw cycles.

### *3.7. Pharmacokinetic studies*

The described method was applied to a pharmacokinetic study in rats. The mean plasma concentration-time curves after an oral dose of 5.7 mg/kg CTS are shown in Fig. 4. The maximum plasma concentration of CTS and TS II A detected in the rats were 11.1 and 2.5 ng/mL, respectively. The time to peak was 30 min for both CTS and TS II A. The result indicated that CTS was absorbed and metabolized to TS II A rapidly in rat.

## 4. Conclusion

A sensitive and selective LC–MS–MS method was developed for pharmacokinetic study of CTS and its metabolite in rats. The assay uses FOFB as internal standard, the extraction procedure is simple allowing large sample throughput to be applied to clinical pharmacokinetic studies of CTS and TS II A. The assay has been validated, and the results of validation have shown that the method is sensitive, accurate and reproducible. This LC–MS–MS method requires only 0.2 mL of plasma, with the limit of detection being 0.1 ng/mL for CTS and 0.2 ng/mL for TS II A, which is superior to other analytical methods and essential for pharmacokinetic studies.

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