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Separation and identification of norcantharidin metabolites in vivo by GC–MS method

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ABSTRACT

Norcantharidin (NCTD), the demethylated analogue of cantharidin, inhibits the proliferation of a variety of human tumor cell lines, and appears to cause the least nephrotoxic and inflammatory side effects. Although NCTD has been used to treat human cancers in China for years, there is no report regarding its metabolism up to now. This is the first report to separate and identify the main metabolites of NCTD in vivo by GC–MS using TMS derivatives. Two hydrolyzed products and five phase I or phase II metabolites were found in rat by the chromatogram comparisons of the blank with incurred biological samples. Multiple stages of fragmentation patterns were used to confirm the metabolites characterizations. The established GC–MS method can also be applied to identifying unknown metabolites of the drugs containing hydroxyl or carbonyl groups in molecular structure.

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1. Introduction

Blister beetle (*Mylabris phalerata Pall.* or *M. cichoril* L.) has been used in traditional Chinese medicine for the treatment of malignant tumors by its main content cantharidin for more than 1000 years. Norcantharidin (NCTD, (1S,2R,3S,4R)-7-oxabicyclo-[2,2,1] heptane-2,3-dicarboxylic anhydride), the demethylated analogue of cantharidin, inhibits the proliferation of a variety of human tumor cell lines [1–11], and appears to cause the least nephrotoxic and inflammatory side effects. Moreover, NCTD is not a substrate for the P-glycoprotein pump, a known mechanism for developing cancer resistance [6,7]. It is noteworthy that NCTD can stimulate the bone marrow and increase the peripheral white blood cell significantly [12], which makes it more valuable in combination chemotherapy since most of the chemotherapeutic agents in use today have the side effect of bone marrow suppression.

Although NCTD was used to treat human cancers in China for years [13], there was no report describing the metabolic profiles of NCTD. Early studies hinted that the promoted biotransformation occurred in vivo after oral administration of NCTD [14].

Despite the recent advancement of various analytical tools, the metabolite identification for compounds undergoing multiple and unpredictable metabolism from biological matrix still remains great challenges [15]. In the past few years, mass spectrometry coupled with chromatographic separation has become a powerful and frequently used technique for metabolite separation and identification. GC–MS remains a technique of choice for the metabolic profiling of biological matrix due to a better discrimination of the compounds in the gas than in the liquid phase, capability for unknown compound identification based on the fragmentation pattern and well-established databases, which can offer the structure information of unknown with standard library retrieval to be applied to the identification and recognition of new compounds. In addition, the higher sensitivity of GC–MS compared to LC–MS could allow for the quantification of metabolites that are in small quantities. These, and other advantages, render GC–MS a useful tool for metabolites molecular diagnosis.

According to the chemical structure of NCTD (Fig. 1), the anhydride group and the oxo-bridge were easily hydrolyzed to carboxyl and hydroxyl metabolites, which can react with silylation reagents to form more volatile and thermo-stable silane derivatives. In this study, the rat serum and bile samples after dosing of NCTD were

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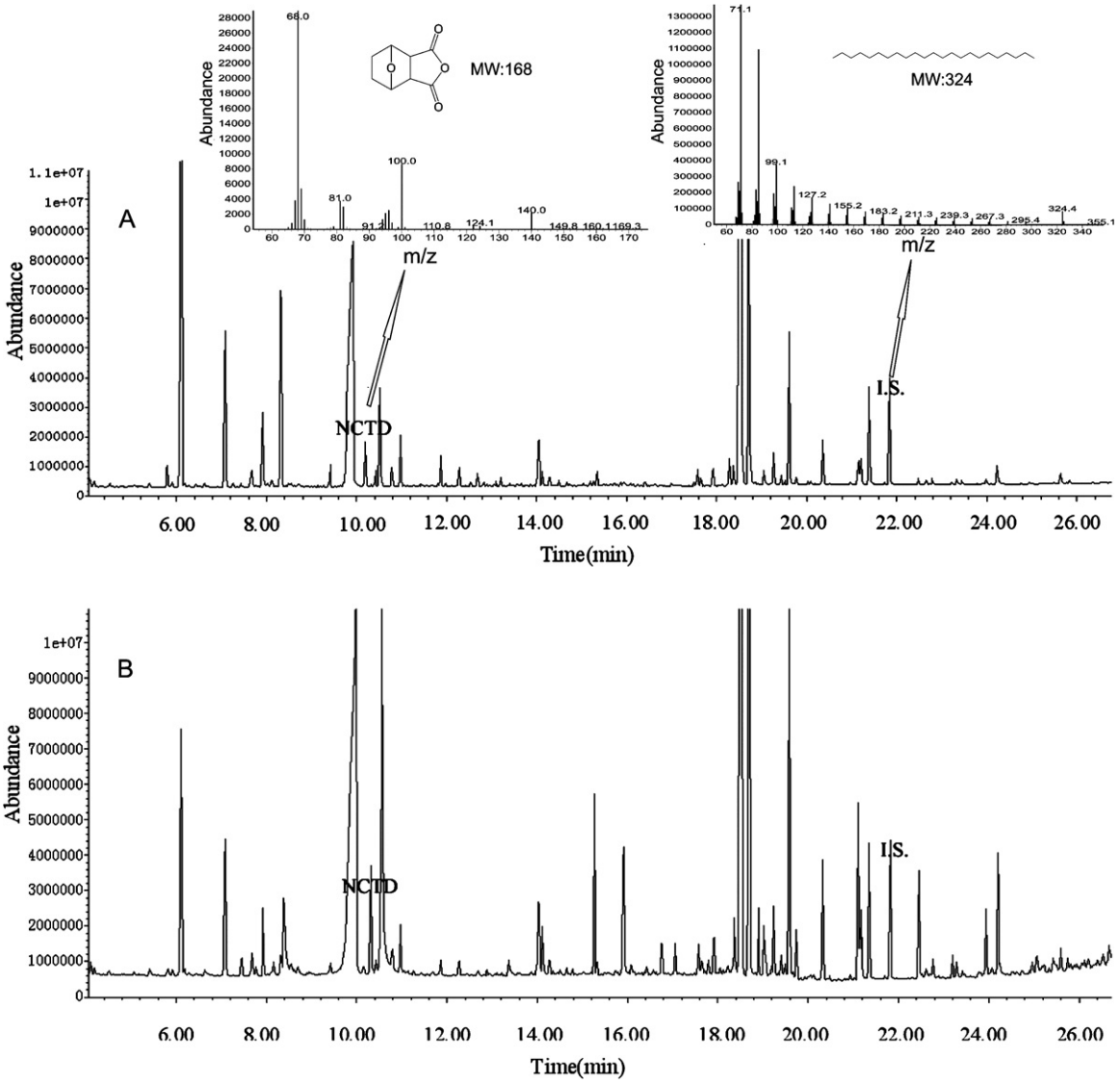

Fig. 1. The total ion chromatograms of rat serum (A) and bile (B) samples spiked with NCTD and I.S. (n-tricosane), and the mass spectrum of NCTD and I.S.

separated and identified by GC–MS technology to investigate the characteristics of NCTD metabolites in vivo. It revealed that seven main metabolites existed in rat, and some of them were newly detected. The results could be useful for future studies involving NCTD, such as clinical therapy and new formulation development.

2. Materials and methods

2.1. Chemical reagents

NCTD (99.92%) was obtained from *Junan Pharmaceutical Factory* (Shandong, China). Trimethyl-chlorosilane (TMCS) was from *Sinopharm Chemical Reagent Ltd.* (Shanghai, China). O-methylhydroxylamine hydrochloride (99%) was from *J&K Chemical Ltd*. and n-tricosane (99%) was from *Johnson Matthey Company* (USA). N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, 97%) and n-heptane (99%) were from *Acros Organic* (NJ, USA). Pentobarbital sodium was purchased from *Shanghai Kefeng Chemical Reagents Co. Ltd*. (China), and pyridine was from *Tianjin Kermel Chemical Reagent Co. Ltd.* (China). HPLC grade chloroform, ethyl acetate and acetonitrile were purchased from *J.T. Baker* (USA).

2.2. Animals

Sprague-Dawley rats (200–250 g) were obtained from *the Experimental Animal Center of Shandong University* and housed with unlimited access to food and water except for fasting 12 h before experiment. All procedures were pre-approved by the Institutional Animal Use Committee.

2.3. Drug administration and sample collection

Eighteen rats were divided into three groups. 100, 150 mg kg^{-1} of NCTD solution (10 mg mL^{-1}) and 3 mL saline were intragastrically given at fasting conditions. All rats were anaesthetized by subcutaneous injection of 3% pentobarbital sodium solution (1 mL/100 g). 200–400 μL bile samples were drained at pre-dose and 0–2 h, 2–4 h, 4–6 h post-dose. 800 μL of blood were taken from sinus jugularis before and 15 min, 30 min, 45 min, 1 h and 1.5 h

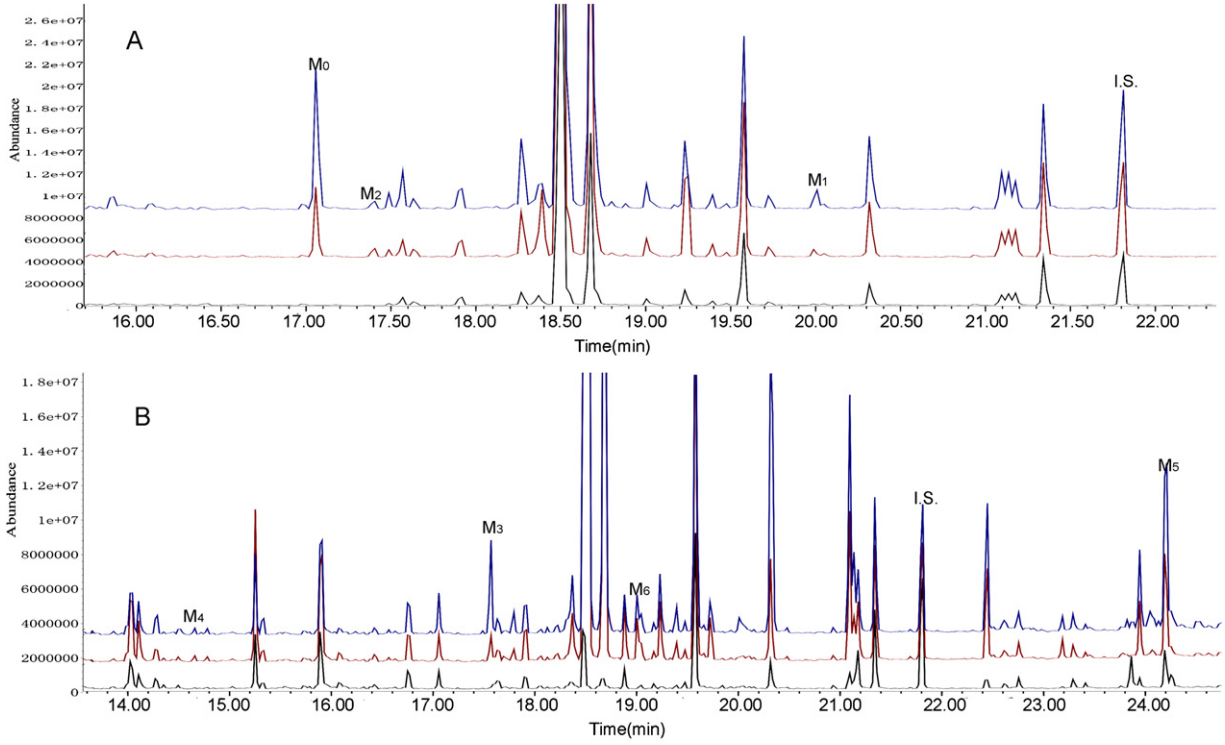

Fig. 2. The total ion chromatograms of blank (nether) and incurred rat serum (A) or bile (B) after100 mg kg^{-1} (middle) and150 mg kg^{-1} (upper) of NCTD.

after dosing. Serum was isolated after being deposited for 0.5 h, centrifuged at 1700 × *g* for 10 min of the blood sample.

2.4. Instruments

Agilent 7890A-5975C GC–MS (Agilent Technologies Inc., USA), AX205 Metteller-Toledo scale (Metteller-Toledo Instrument (Shanghai) Co. Ltd.), LDZ4-08 centrifuge (Beijing Medical Centrifuge Factory), PK514BP ultrasound Cleaner (BANDEL Electronic, Germany), tweezers and surgical scissors (Shanghai Boxun Industry & Commerce Co. Ltd.), medical syringes (1 mL, Shandong Weigao Group Medical Polymer Co. Ltd., China), and gavage needles (No. 16, Beijing Midwest Great Technology Co. Ltd., China) were used.

2.5. Sample processing

250 μL of acetonitrile was added into 100 μL of serum or bile samples (ice-bath for 15 min), then centrifuged (10 000 × *g*, 10 min, 4 °C). 150 μL of supernatant was transferred to a GC vial and evaporated under a stream of nitrogen gas to dryness. Methoxymation was carried out at 70 °C for 1 h after 50 μL of O-methylhydroxylamine hydrochloride (15 mg mL^{-1} in pyridine) was added. 50 μL of MSTFA (with 1% TMCS as the catalyst) was added and kept at 70 °C for another hour, and then vortexed after adding 150 μL of n-tricosane (0.10 mg mL^{-1} in n-heptane) as internal standard.

2.6. Analytical conditions

2 μL of the derivative sample was injected into GC–MS. An HB-5 MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent, USA) was used for the separation with helium carrier gas at flow rate of 1.0 mL min^{-1}. The GC oven was initially set at 85 °C for 5 min, increased to 300 °C at a rate of 10 °C min^{-1}, and then kept at 300 °C for 5 min. Splitless mode injection was used. MS detection was conducted with EI mode with electron energy of 70 eV and full scan mode with *m*/*z* of 60–600.

3. Results

3.1. Separation of NCTD and its metabolites in rat serum or bile

Blank rat serum or bile spiked with NCTD standard solutions to get 0.5 μg mL^{-1} QC sample was analyzed for method validation.

The total ion chromatograms of serum and bile samples spiked with NCTD and I.S. (n-tricosane) were shown in Fig. 1. The retention time of NCTD and internal standard were 10.30 min and 21.81 min under above GC–MS condition. The mass spectrum of NCTD and I.S. was also shown in Fig. 1.

The identification ions of *m*/*z* 169, 140 and 100 for NCTD were extracted from the total ion chromatograms of rat serum and bile samples after 100 and 150 mg kg^{-1} of NCTD, but no peaks were eluted out at about 10.30 min, indicating that few of original NCTD existed in rat serum and bile, and most of NCTD might be metabolized in rat after oral administration. Compared with the GC–MS chromatograms of blank serum (or bile), five new peaks at retention time of 17.06, 17.41, 17.57, 19.00, 24.21 min and two area-increasing peaks at 14.78 and 19.99 min were found in the incurred samples. The total ion chromatograms of serum and bile samples were shown in Fig. 2. The results provided strong evidence to the deduction of NCTD metabolites.

3.2. Identification of NCTD metabolites

Seven NCTD metabolites were identified in rat serum and bile samples after fragmentation pattern analysis and the mass spectrum comparison of the compounds with those in the instrument library (NIST 05). The mass spectrum of M_0–M_6 and their fragmentation patterns were shown in Figs. 3 and 4. The retention times, formula and the chemical names for the seven metabolites were reported in Table 1.

NCTD metabolites were derivatized and the molecular weight of each compound derivative increased due to the linkage of the $Si(CH_3)_3$ from MSTFA.

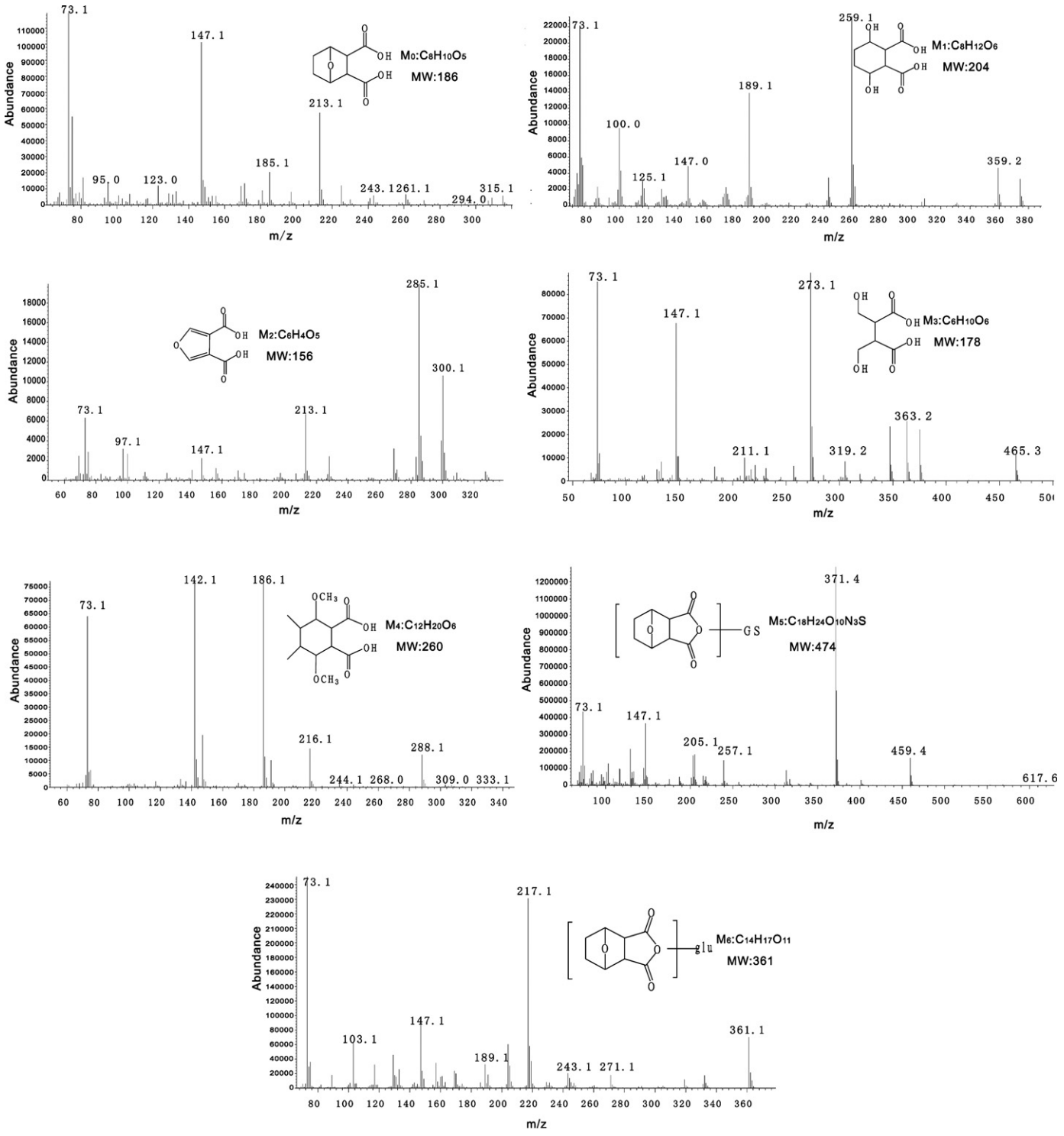

Fig. 3. The mass spectrum of M_0–M_6.

As an anhydride, it is easy for NCTD to turn into diacid (M_0). The *m*/*z* of 330 was a result of the reaction between M_0 and the MSTFA reaction group. The loss of CH_3 produced the fragment of *m*/*z* 315.1, the fragment *m*/*z* 213.1 was originated by the loss of $CO_2Si(CH_3)_3$, the subsequent loss of C_2H_4 on the left hexatomic ring generated the fragment of *m*/*z* 185.1.

M_1 was the hydrolysate of M_0 at oxo-bridge. For M_1, the molecular weight increased to be 492 after derivatization, the loss of $OSi(CH_3)_3$ and three CH_3 produced the fragment of *m*/*z* 359.2, the loss of two $CO_2Si(CH_3)_3$ produced the fragment of *m*/*z* 259.1. The trimethyl-silylation of M_1 after loss of two COOH generated the fragment of *m*/*z* 189.1.

Similarly, for M_2, the degradation product of M_0 via the loss of C_2H_4 on the left hexatomic ring, the molecular weight increased to be 300.1 after derivatization, the fragment of *m*/*z* 285.1 was originated by loss of CH_3, and the subsequent loss of $Si(CH_3)_3$ produced the *m*/*z* of 213.1.

M_3 was the degradation product of M_1 via the loss of C_2H_4 on the left hexatomic ring. The molecular weight of M_3 increased to be 465.3 after derivatization, the loss of $CH_2OSi(CH_3)_3$ produced the

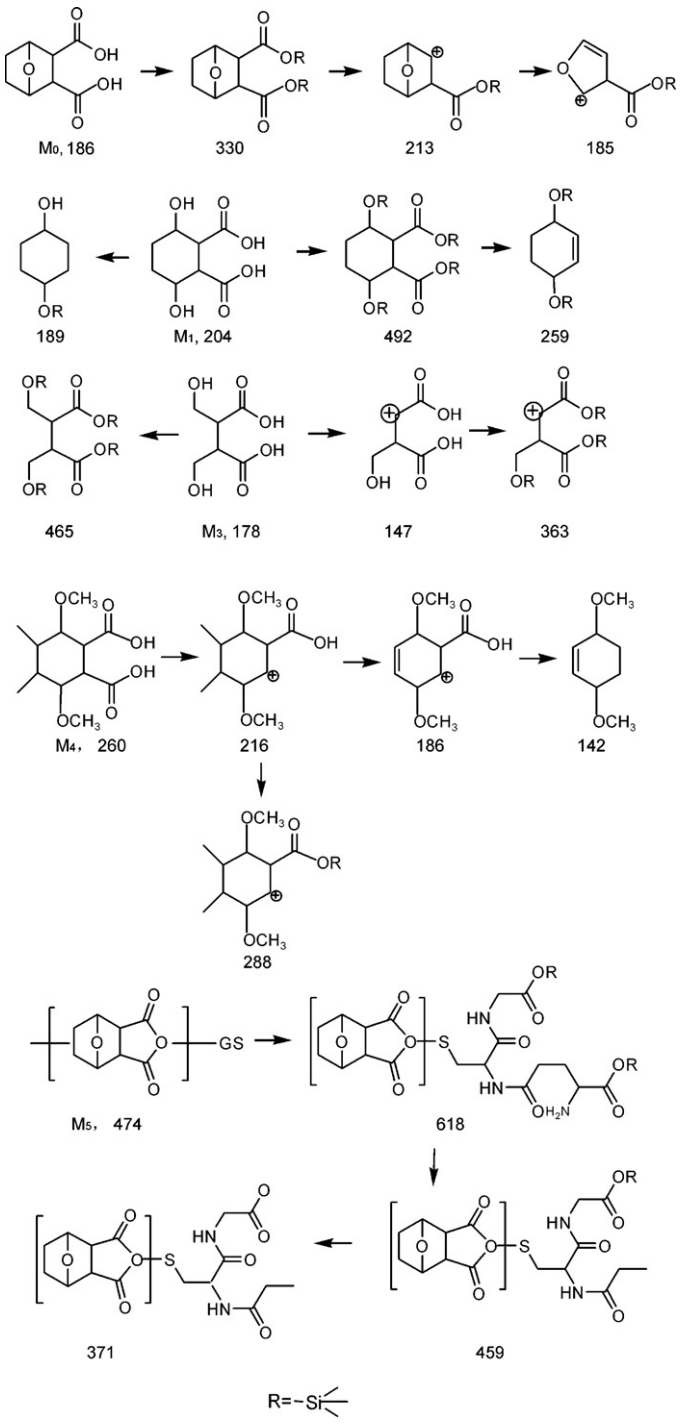

Fig. 4. The fragmentation pattern of M_0, M_1, M_3, M_4, and M_5. M_0-derivate (TMS) with MW of 330, produced the fragment ion of *m*/*z* 315.1 by loss of CH_3, of 213.1 by loss of $CO_2Si(CH_3)_3$, and of 185.1 by subsequent loss of C_2H_4 on the left hexatomic ring. M_1-derivate (TMS) with MW of 492, produced the fragment ion of 259.1 by loss of two $CO_2Si(CH_3)_3$, and the trimethyl-silylation of M_1 after loss of two COOH generated the fragment of *m*/*z* 189.1. M_3-derivate (TMS) with MW of 465, produced the fragment ion of *m*/*z* 363.2 by loss of $CH_2OSi(CH_3)_3$, and the fragment of 147.1 was produced by the loss of CH_2OH from M_3. M_4 with MW of 260, produced the fragment ion of *m*/*z* 216.1 and its derivate product ion of 288.1 by loss of COOH, of 186.1 by loss of two CH_3, and of 142.1 by subsequent loss of another COOH. M_5-derivate (TMS) with MW of 618, produced the fragment ion of *m*/*z* 459.4 by loss of $NH_2CHCO_2Si(CH_3)_3$, and of 371.4 by subsequent loss of $Si(CH_3)_3$.

Table 1
The possible NCTD metabolites in rat serum and bile samples.

t_R (min)	Formula (MW)	Chemical names
17.057	$C_8H_{10}O_5$ (186)	M_0, (1S,2R,3S,4R)-7-oxabicyclo-[2,2,1] heptane-2,3-dicarboxylic acid
17.406	$C_8H_{12}O_6$ (204)	M_1, 3,6-dihydroxycyclohexane-1,2-dicarboxylic acid
17.569	$C_6H_4O_5$ (156)	M_2, furan-3,4-dicarboxylic acid
19.004	$C_6H_{10}O_6$ (178)	M_3, 2,3-bis(hydroxymethyl) succinic acid
24.210	$C_{12}H_{20}O_6$ (260)	M_4, 3,6-dimethoxy-4,5-dimethylcyclo-hexane-1,2-dicarboxylic acid
14.782	$C_{18}H_{24}O_{10}N_3S$ (474)	M_5, norcantharidin-glutathione conjugate
19.988	$C_{14}H_{17}O_{11}$ (361)	M_6, norcantharidin-glucuronic acid conjugate

fragment at *m*/*z* 363.2, and the loss of $COSi(CH_3)_3$ and three CH_3 produced the fragment at *m*/*z* 319.2, the subsequent loss of six CH_3 generated the fragment at *m*/*z* 273.1.

M_4, the tetramethyl product of M_1, with the molecular weight of 260, produced the fragment ion of *m*/*z* 216.1 by loss of COOH, of 186.1 by loss of two CH_3, and of 142.1 by subsequent loss of another COOH.

M_5 and M_6 were the glutathione and glucuronic acid conjugate of NCTD. M_5-derivate (TMS) with MW of 618, produced the fragment ion of *m*/*z* 459.4 by loss of $NH_2CHCO_2Si(CH_3)_3$, and of 371.4 by subsequent loss of $Si(CH_3)_3$. The molecular weight of M_6 was 361, and the fragment ion of *m*/*z* 271.1 was produced by loss of $(CHOH)_3$.

Three (M_0, M_1, M_2) in serum and four (M_3, M_4, M_5, M_6) in bile metabolites were identified. Trace amount of M_2 in bile and M_4 in serum were found by extracting the identification ions. Two hydrolysate metabolites (M_0 and M_1) were only in serum, the glutathione and glucuronic acid conjugates (M_5 and M_6) were found only in bile.

3.3. Semi-quantification of the metabolites

For semi-quantification of the metabolites, the selected fragment ions shown in Table 2 were integrated and analyzed.

All the metabolites in rat after 150 mg kg^{-1} NCTD were more than that of 100 mg kg^{-1}, according to the corresponding peak areas ratio vs. I.S., and M_0, M_1, M_3, M_5 expressed good correlations between concentration and dosage, but M_2, M_4 and M_6 after 150 mg kg^{-1} NCTD were less proportionate than that of 100 mg kg^{-1}. The time to maximum serum concentration of M_0 and

Table 2
The selected fragment ions for semi-quantification of the metabolites and I.S.

Compounds	Quantitative ion (*m*/*z*)	Qualitative ions (*m*/*z*)
M_0	213	185, 213
M_1	259	359, 189
M_2	285	300, 213
M_3	273	465, 363
M_4	142	288, 186
M_5	459	618, 371
M_6	361	217, 271
I.S.	324	155, 127

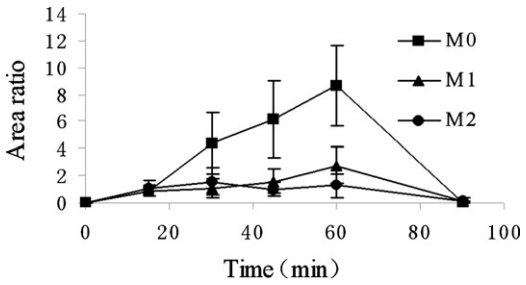

Fig. 5. The distribution curves of metabolites M_0, $M_1$and M_2 in rat serum after 150 mg kg^{-1} NCTD (n = 6).

M_1 were about 1 h and of M_2 was 0.5 h, shown as the area ratio-time curves at Fig. 5. The same tendency of the two dosages was obtained.

The time to maximum bile concentration of M_3 was about 4–6 h, of M_4 and M_5 were 1–2 h, and of M_6 was 2–4 h, shown as Fig. 6.

3.4. The possible metabolic pathway of NCTD in vivo

According to the chemical structures of NCTD and its metabolites, the possible metabolic pathway was proposed. Fig. 7 depicted the metabolic pathways of NCTD, including the hydrolysis and de-ethyl products, the glutathione, glucuronic acid and methyl conjugation of both parent compound and the hydrolysis products.

4. Discussion

Acid anhydride is chemically active, easily reacts with water, alcohols, ammine, acyl chlorides, primary amines, ammonia and phenol, and can be actually turned to anhydride by dehydrating acid. With the vivid chemical properties of anhydride and the big pulling force of the oxo-bridge on the left ring, NCTD was hydrolyzed easily in gastric fluid to form hydroxyl or carboxyl metabolites, all of which can be analyzed by GC–MS after derivatization.

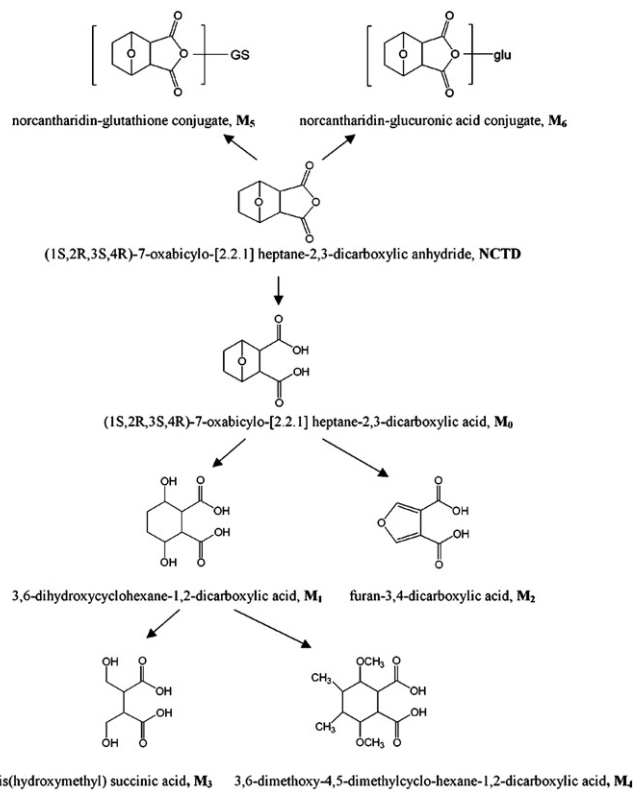

Fig. 7. The possible metabolic pathways of NCTD.

The fragmentation pattern analysis and the mass spectrum comparison of the compounds with those in the instrument library are very useful for the metabolite identification and characterizations. In this study, the NCTD metabolites were proposed and verified by GC–MS method with standard spectrum library retrieval. Two hydrolyzed products and five phase I or phase II metabolites were found by the chromatogram comparisons of the blank

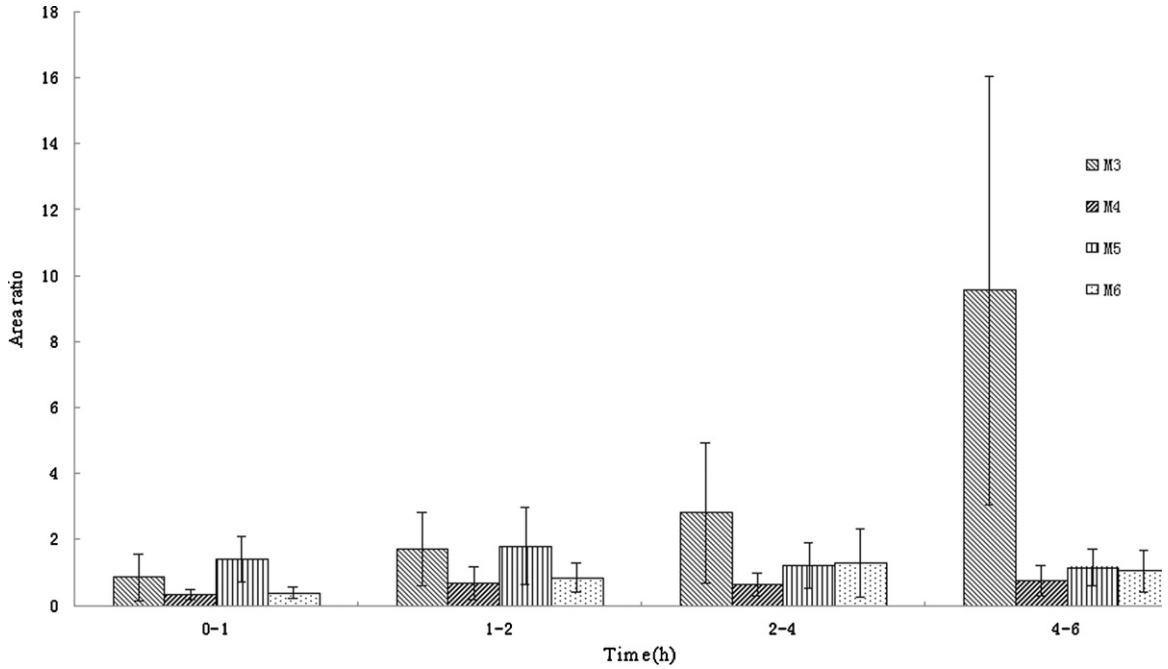

Fig. 6. The distribution histograms of metabolites M_3, M_4, M_5 and M_6 in rat bile after dosing of 150 mg kg^{-1} NCTD (n = 6).

with incurred biological samples. Multiple stages of fragmentation patterns were used to confirm the metabolites characterizations. The established GC–MS method can also be applied to identifying unknown metabolites of the drugs containing hydroxyl or carbonyl groups in molecular structure.

Due to the higher sensitivity, better discrimination and more capability than LC, GC (–MS) was used in this study. Few of NCTD was monitored in rat serum or bile, instead of being detected in human plasma by HPLC–MS/MS in our early studies [16]. One possible reason was the impurities co-eluting with NCTD at retention time of 10.30 min, but no identification ions (m/z 169, 140, 100) peaks of NCTD were extracted from the total ion chromatograms of rat serum and bile after dosing, indicating that most of original NCTD was metabolized to diacid firstly in rat after oral administration. More procedures, such as using different columns with different polarity to control the impurities should be considered in the future study. The availability of suitable internal standard, such as labeled analyte or molecule similar to the analyte with the same chemical and physical characteristics, is essential for the development of quantification method. Since authentic reference compounds were unavailable, a simple, rapid method for simultaneous SIM semi-quantification of NCTD metabolites was developed using n-tricosane as internal standard. In some metabolomics research, n-alkane usually was added as internal standard just before injection into GC [17], similarly as the process in this study, but as for more precise quantitative analysis, the internal standard should be firstly added before any treatment of the sample.

From the semi-quantification results of this study, all the metabolites in rats after dosing of 150 mg kg^{-1} NCTD were more than that of 100 mg kg^{-1}, but M_2, M_4 and M_6 did not express as good correlation as that of other metabolites. The possible reason may involve in the enzyme equilibrium or the further metabolism of the metabolites.

Although these results and the method are important for the research and clinical use of NCTD, further studies on metabolism of NCTD incubated with recombined CYP450 enzymes and researches on ascertaining the structures of metabolites by TOF or NMR need to be conducted to make clear the enzymes mediated NCTD metabolism and the possible existed genotype or phenotype polymorphisms, in order to fully understand the relativity of NCTD effect or toxicity with the dose and to develop organ-targeting formula and new structure modified compounds.

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