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Identification of tanshinones and their metabolites in rat bile after oral administration of TTE-50, a standardized extract of *Salvia miltiorrhiza* by HPLC–ESI–DAD–MS^n

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Abstract

TTE-50 is a standardized extract of *Salvia miltiorrhiza* which mainly consisted of tanshinones. A sensitive and specific method using liquid chromatography–diode array detection-electrospray ionization (ESI) ion trap mass spectrometry was established for the study of the constituents and metabolites of TTE-50 in rat bile sample after oral administration. The bile samples were extracted with ethyl acetate (EtOAc) of three-fold volume for three times. The chromatographic separation was carried out on a Zorbax Extend-C_{18} column with a gradient elution program whereas acetonitrile–water was used as mobile phase. Mass spectra were acquired in positive ionization mode and data-dependant scan was used for the identification of the tanshinones and metabolites in the bile samples. Identification and structural elucidation of the tanshinones and their metabolites in bile samples were performed by comparing their retention-times and full scan MS^n spectra with those of reference compounds and data in the literatures. Sixteen tanshinones in TTE-50 along with seventeen phase I metabolites were identified simultaneously. The metabolic modification could take place in the C-4 side chain of tanshinone IIA, from methyl to primary alcohol, then to aldehyde group was proposed for the first time. The established method was valuable for the study of the metabolism of complex system such as herbal extracts or traditional Chinese medicine (TCM) formula.
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Keywords: High performance liquid chromatography–tandem mass spectrometry; Tanshinone; Metabolism; Rat bile

1. Introduction

Salvia miltiorrhiza, Dan-Shen in Chinese, is a well-known traditional Chinese medicinal herb used for treatment of various kinds of diseases especially for cardiovascular diseases [1–5]. According to the chemical structures, the major bioactive constituents in *S. miltiorrhiza* could be classified into two groups: phenolic compounds such as danshensu, protocatechuic aldehyde, salvianolic acid, etc. and tanshinones such as tanshinone I, tanshinone IIA, dihydrotanshinone I and cryptotanshinone, which belong to a group of diterpenes with an abietane-type skeleton containing a 1,2- or 1,4-quinone in the C-ring. Modern pharmacological studies have demonstrated a variety of activities of tanshinones such as neuroprotective effects in transient focal cerebral ischemia mice [6], antioxidant effect on DNA damage by lipid peroxidation in liver cells [7], *in vitro* cytotoxicity and apoptosis induction of cancer cells [8–10]. TTE-50 is a standardized extract of *S. miltiorrhiza* which mainly consisted of different kinds of tanshinones and primarily used for the preparation of a series of Dan-Shen related therapeutic agents.

In recent years, the pharmacokinetic characteristics of total tanshinones, cryptotanshinone and tanshinone IIA were studied in animals to some extent. The oral bioavailability of cryptotanshinone in pigs was extremely poor as the plasma concentration was almost undetectable with the dosage of 40 mg/kg [11]. Using the HPLC–MS methods, the maximum plasma concentrations of cryptotanshinone and tanshinone IIA were below 20 ng/ml after an oral administration of total tanshinones (containing 12%

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of tanshinone IIA and cryptotanshinone) to rats at a dosage of 150 mg/kg [12]. The poor oral availability of cryptotanshinone could be partly explained by the P-glycoprotein (P-gp) mediated efflux as cryptotanshinone was demonstrated to be a substrate of P-gp by using single-pass rat intestinal absorption (SPIP), Caco2 and MDCK II models [13]. Cryptotanshinone can be metabolized to tanshinone IIA and tanshinone IIA can be further transformed to hydroxylated and dehydrogenated metabolites in the *in vivo* and *in vitro* studies [14–17].

In China, the drugs derived from TCM or herbal extracts are extensively used in clinical practice. Hence, it is important to understand the metabolic pathways of such complex systems consisting of multi-components in order to clarify the mechanism of pharmacological action of TCMs. The challenge for the *in vivo* metabolic study of the TCM extracts lies in lacking enough sensitive analytical methods for the identification of all the minor constituents in herbal extracts and the metabolites in trace amount.

Electrospray ionization tandem mass spectrometry (ESI-MS^n) has demonstrated its great advantages of high throughput, sensitivity, specificity and ease of coupling to online liquid chromatography systems for structural analysis of components in TCM extracts and drug metabolites via biotransformation. Our laboratory has carried out a number of studies on the identification of the chemical constituents in Chinese herbal medicines including Dan-Shen by HPLC–MS^n method [18–23]. Pan and his co-worker had established an HPLC–ESI–MS/MS method for analyzing absorbed and metabolic components in rat plasma samples after administration of 'Shuangdan' granule, which was a commonly used drug in China made from the aqueous extracts of *Radix S. miltiorrhizae* and *Cortex Moutan*, 16 components in the drug along with five metabolites were simultaneously identified [24].

Our preliminary experiment results indicated that the concentration of tanshinones in bile was much higher than that in plasma and in urine, which suggested that the tanshinones were mainly metabolized in liver. It also suggested that the hepatic first-pass effect of tanshinones is significant. Based on these observations, we focused our study on the identification of excretory tanshinones and their metabolites in rat bile to achieve a better understanding of the pharmacological action mechanisms of TTE-50. In this work, 16 tanshinones in TTE-50 along with 17 phase I metabolites were identified simultaneously (Fig. 1) by our established HPLC–DAD–ESI–MS^n method.

2. Experiment

2.1. Chemicals and reagents

TTE-50 was purchased from Masson Pharma Co. Ltd. (Guangzhou, China, Lot# 040701-2), which contained 50% of tanshinone IIA as its major constituent. Tanshinone IIA, cryptotanshinone, tanshinone I, 15,16-dihydrotanshinone I, tanshinone IIB, przewaquinone A, methyl tanshinonate, tanshindiol B, tanshindiol C, 3α-hydroxytanshinone IIA and tanshinaldehyde were isolated by the authors from the TTE-50. Their structures were fully characterized by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). The purities of tanshinone IIA and cryptotanshinone were over 99% while others were over 95% determined by HPLC/DAD analysis.

HPLC-grade acetonitrile was obtained from Fisher Scientific (Fisher, Fair Lawn, NJ, USA). Deionized water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA). High-purity nitrogen (99.999%) and helium (99.999%) were purchased from Gas Supplies Center of Peking University Health Science Center (Beijing, China). Ethyl acetate, other chemicals and solvents were all of analytical grade.

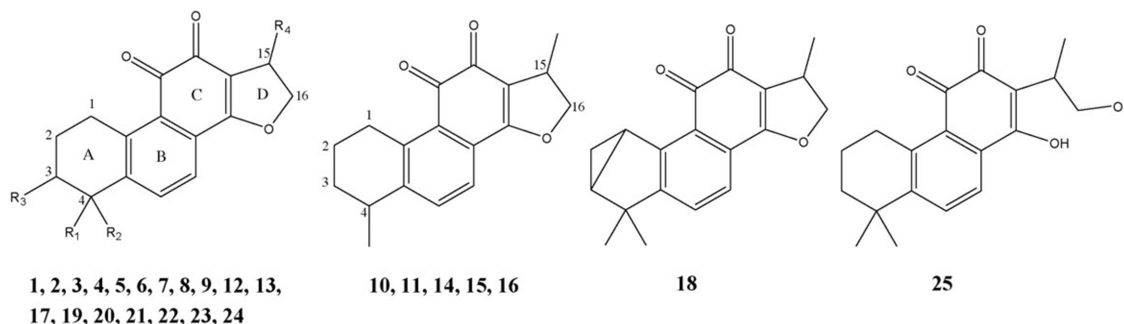
2.2. Instrumentation and conditions

HPLC analysis was performed on an Agilent 1100 Series HPLC system (Agilent, Waldbronn, Germany) consisting of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and a diode-array detector. The analytical column was Zorbax Extend-C_{18} column (250 mm × 4.6 mm, 5 μm) connected with a Zorbax Extend-C_{18} guard column (20 mm × 4 mm, 5 μm). The mobile phase consisted of acetonitrile (A) and water (B). A gradient program was listed in Table 1. The mobile phase flow rate was 1.0 mL/min, and the temperature of column was maintained at 30 °C. UV spectra were recorded from 190 to 400 nm and the detection wavelength was set at 270 nm.

HPLC–MS^n analysis was performed with an Agilent 1100 Series HPLC and FinniganLCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source, with Xcalibur 1.3 controlling software. Nitrogen (N_2) was used as the sheath and auxiliary gas, and helium (He) was used as the damping and collision gas. HPLC conditions were the same as described above. The LC effluent was introduced into the ESI source in a post-column splitting ratio of 2:1. Mass spectra were acquired with the optimized parameters in the positive ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas (N_2) pressure, 50 arbitrary units; auxiliary gas (N_2) pressure, 10 units; capillary temperature, 350 °C; capillary voltage, 30 V. For full-scan MS analysis, the spectra were recorded in the range of *m/z* 100–500. Data-dependent scan program was used in the liquid chromatography/tandem mass spectrometry analysis so that the three most abundant ions in each scan were selected and subjected to MS/MS and MS^n (n = 3–6) analyses. The collision-induced dissociation (CID) energy was adjusted to 45%. The isolation width of the precursor ions was 1.0 *m/z*.

Table 1
LC-gradient utilized to acquire the chromatographic separation of TTE-50 sample and the bile samples

Time (min)	Mobile phase (acetonitrile:water, v/v)
0	20:80
20	30:70
30	50:50
40	40:60
50	80:20
60	80:20



No.	Name	R1	R2	R3	R4	Double bonds	MW
1	Tanshindiol A	β-CH_2OH	α-OH	H	CH_3	$\Delta_{15,16}$	312
2	[a]Tanshindiol B	β-OH	α-CH_3	α-OH	CH_3	$\Delta_{15,16}$	312
3	[a]Tanshindiol C	β-OH	α-CH_3	β-OH	CH_3	$\Delta_{15,16}$	312
4	3α-Hydroxymethylenetanshinquinone	=CH_2	—	α-OH	CH_3	$\Delta_{15,16}$	294
5	Tanshinol B	OH	CH_3	H	CH_3	$\Delta_{15,16}$	296
6	[a]Tanshinone II B	β-CH_2OH	CH_3	H	CH_3	$\Delta_{15,16}$	310
7	[a]3α-Hydroxytanshinone II A	CH_3	CH_3	α-OH	CH_3	$\Delta_{15,16}$	310
8	[a]Tanshinaldehyde	CHO	CH_3	H	CH_3	$\Delta_{15,16}$	308
9	[a]Prewaquinone A	CH_3	CH_3	H	CH_2OH	$\Delta_{15,16}$	310
10	[a]15,16-Dihydrotanshinone I	—	—	—	—	$\Delta_{1,2}$,$\Delta_{3,4}$	279
11	1,2,15,16-tetrahydrotanshinone I	—	—	—	—	$\Delta_{1,2}$,$\Delta_{15,16}$	280
12	[a]Methyl tanshinonate	β-$COOCH_3$	α-CH_3	H	CH_3	$\Delta_{15,16}$	338
13	[a]Cryptotanshinone	CH_3	CH_3	H	β-CH_3	—	296
14	[a]Tanshinone I	—	—	—	—	$\Delta_{1,2}$,$\Delta_{3,4}$,$\Delta_{15,16}$	276
15	1,2-Dihydrotanshinone I	CH_3	CH_3	H	β-CH_3	$\Delta_{3,4}$,$\Delta_{15,16}$	278
16	3,4-Dihydrotanshinone I	—	—	—	—	$\Delta_{1,2}$,$\Delta_{15,16}$	278
17	[a]Tanshinone IIA	CH_3	CH_3	H	CH_3	$\Delta_{15,16}$	294
18	1,3-Dehydrotanshinone IIA	—	—	—	—	—	292
19	1,2-Dehydrotanshinone IIA	CH_3	CH_3	H	CH_3	$\Delta_{1,2}$,$\Delta_{15,16}$	292
20	2,3-Dehydrotanshinone IIA	CH_3	CH_3	H	CH_3	$\Delta_{2,3}$,$\Delta_{15,16}$	292
21	Tanshinacid	COOH	CH_3	H	CH_3	$\Delta_{15,16}$	325
22	1,2-Dehydrocrytotanshione	CH_3	CH_3	H	CH_3	$\Delta_{1,2}$	295
23	2,3-Dehydrocryptotanshinone	CH_3	CH_3	H	CH_3	$\Delta_{2,3}$	295
24	17-Hyroxycryptotanshione	CH_3	CH_3	H	CH_2OH	—	313
25	Tanshinone V	—	—	—	—	—	315

[a] identified by comparison with the reference compounds

Fig. 1. The structures of the tanshinones and metabolites identified in rat bile.

2.3. Animals, drug administration and bile sampling

Six male Sprague–Dawley rats weighing 250 ± 20 g were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, PR China) and they were kept in fully acclimatized room at constant temperature and humidity on a 12 h light/dark cycle. The animals were fixed on a wooden plate and anesthetized with ether following overnight fasting. An abdominal incision was made and the common bile duct was cannulated with PE-10 tubing (Id = 0.08 cm, Becton

Dickinson, USA) for collection of the bile samples, and closed by saturation. Oral doses of 100 mg/kg of TTE-50 were administered to four animals by oral gavage when the animals recovered conscious and the other two were administered with the same volume of 1% Tween-80 for blank. A heating lamp was used for maintaining the body temperatures during the experimental procedures to prevent hypothermic alterations of the bile flow. Bile samples were collected for 12 h and stored at −80 °C until additional extraction and analysis. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85–23, revised edition 1985).

2.4. Sample preparation

The 25 mg of TTE-50 was dispersed with appropriate volume of Tween-80 and then extracted with EtOAc at the ratio of 1:3 (v/v) for three times. Extraction was performed by vortex-mixing for 5 min, followed by centrifugation for 10 min at 4024 × *g*. The EtOAc fractions were affiliated and evaporated to dryness at 30 °C by rotary evaporation. The residue was reconstituted in 25 mL methanol. After filtration with 0.22 μm filter, 15 μL of the aliquot was injected for HPLC–MS^n analysis.

The bile sample (0–12 h) was similarly treated as the TTE-50 sample except that the residue was reconstituted in 500 μL methanol and 15 μL of the aliquot was injected for HPLC–MS^n analysis.

3. Results and discussion

3.1. Optimization of extraction, HPLC and HPLC–MS^n methods

Several sample preparation methods such as solid phase extraction with Waters Oasis HLB cartridges and extraction with different solvents (chloroform, dichloromethane and ethyl acetate) had been tried. Finally, liquid–liquid extraction with ethyl acetate was chosen in order to ensure the simultaneous extraction of the most target compounds and less interference from the co-eluted endogenous matrixes.

A number of HPLC or HPLC–MS methods for analysis of major tanshinones (tanshinone IIA, cryptotanshinone, tanshinone I and 15,16-dihydrotanshinone I) in Dan-Shen, it's preparation [25–27] and bio-fluids [17,28] have been reported. TTE-50 is a mixture of about 20 kinds of tanshinones, thus the metabolic study of such a complex system will be more challenging than that of single component. The existing methods reported for analysis of tanshinones were mostly optimized for quantitation of 1–4 major tanshinones in crude drugs or preparation with a relatively short analytical run, which could not provide enough resolution for TTE-50 sample. On the other hand, the methods reported for fingerprinting [29] were rather time-consuming for analysis of TTE-50 sample as the methods were developed for the determination of the two classes of constituents, tanshinones and salvianolic acids in Dan-Shen. According to the literatures [18,25], a Zorbax Extend C_{18} column and an acetonitrile–water (acid) mobile system were appropriate to separate tanshinones. The addition of small amount of acid, such as 0.03% (v/v) formic acid, in mobile phase could improve the ionization efficiency of the tanshinones in positive ionization mode. But the addition of acid will also increase the response intensity of endogenous matrix in bile, which will largely disturb the determination of minor tanshinones or metabolites in bile. However, there is not obvious improvement for resolution using the mobile phase with 0.03% formic acid. Based on the general consideration, an acetonitrile–water system was selected as mobile phase. In order to identify the minor tanshinones and metabolites and to avoid the interferences of endogenous matrixes in rat bile, a four-slope gradient elution method of 60 min was finally used, which could achieve maximum throughput and optimal resolution for TTE-50 and bile samples. Diode-array detector (DAD) was applied to select the optimized wavelength of constituents in the TTE-50 and bile samples and the HPLC chromatogram at 270 nm shows more peaks and better separation than that at other wavelengths.

ESI in both negative and positive ion modes was tried and the results showed that ESI in positive ion mode was more sensitive for tanshinones. The instrumental parameters (sheath gas pressure, auxiliary gas pressure, spray voltage, capillary temperature, capillary voltage, lens voltage and tube lens offset) were optimized by general analyzing four major tanshinones, tanshinone IIA, cryptotanshinone, tanshinone I and 15,16-dihydrotanshinone I for the maximum intensity. The optimized parameters in the positive ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas pressure, 50 arbitrary units; auxiliary gas pressure, 10 units; capillary temperature, 350 °C; capillary voltage, 30 V. To obtain the most abundant information of all the constituents in rat bile, the data-dependant scan was used in HPLC–MS^n analysis. The three most abundant ions in each scan were selected and subjected to MS/MS and MS^n (n = 3–6) analyses and the relative collision energy for CID was set at 45%, which could produce the satisfactory MS^n fragmentation information.

3.2. Identification of tanshinones in rat bile

A number of components in TTE-50 were observed in the bile sample following an oral administration to rat in comparison with the blank and the TTE-50 samples. In order to identify the majority of tanshinones in rat bile, 11 reference compounds were firstly analyzed by direct injection. The fragmentation pathways were proposed and applied for identification of compounds with tanshinone skeleton in rat bile and TTE-50 samples. The structures of the compounds in rat bile and TTE-50 were elucidated by a combined analysis of the UV, MS and MS^n spectra, as well as by mass and chromatographic spectral comparison with reference compounds.

The HPLC profile and total ion current (TIC) chromatograms of TTE-50 sample and rat bile sample were shown in Fig. 2. The profile of rat bile sample was greatly different from that of TTE-50 sample, which illustrated the changes of herbal components in the course of *in vivo* physiological disposition. Sixteen tanshinones in TTE-50 were identified and the data were listed in Table 2.

Table 2
HPLC/MS^n data of the tanshinones identified in rat bile after oral administration of TTE-50

Peak no.	T_R (min)	MS $[M+H]^+$	HPLC–ESI–MS^n m/z (% base peak)	UV λ_{max}	Identity
1	18.19	313	MS^2[313]: 295(100), 265(98), 247(21), 267(18), 247(21), 277(10), 249(4), 237(1)	270	Tanshindiol A
2[a]	19.84	313	MS^2[313]: 295(100), 277(8), 267(12) MS^3[313 → 295]: 267(100) MS^4[313 → 295 → 267]: 249(100) MS^5[313 → 295 → 267 → 249]: 221(100), 203(64)	270	Tanshindiol B
3[a]	24.73	313	MS^2[313]: 313(9), 295(100), 267(20) MS^3[313 → 295]: 267(100) MS^4[313 → 295 → 267]: 249(100), 221(15) MS^5[313 → 295 → 267 → 249]: 231(100)	270	Tanshindiol C
4	30.80	295	MS^2[295]: 277(100), 276(6), 267(66), 253(13), 251(12), 249(60) MS^3[295 → 277]: 262(6), 259(59), 249(100), 231(8)	280	3α-Hydroxymethylenetanshinquinone
5	31.60	297	MS^2[297]: 279(100), 269(20), 261(55) MS^3[297 → 279]: 261(100) MS^4[297 → 279 → 261]: 233(100), 205(3)	272	Tanshinol B
6[a]	34.30	311	MS^2[311]: 293(100), 265(11), 251(3) MS^3[311 → 293]: 278(5), 275(100), 265(11), 251(10), 249(5), 247(9), 219(6) MS^4[311 → 293 → 275]: 275(100), 247(99)	270	Tanshinone II B
7[a]	34.70	311	MS^2[311]: 293(67), 281(14), 275(100), 269(9), 263(9), 253(7), 251(13), 250(6), 227(7) MS^3[311 → 275]: 247(100), 229(3) MS^4[311 → 275 → 247]: 247(11), 232(13), 219(100), 218(2), 204(34) MS^5[311 → 275 → 247 → 219]: 219(100), 204(44)	270	3α-Hydroxytanshinone II A
8[a]	38.95	309	MS^2[309]: 281(100), 265(2), 263(6) MS^3[309 → 281]: 281(26), 266(14), 263(75), 253(4), 245(2), 239(35), 235(6), 211(100), 207(2), 197(2) MS^4[309 → 281 → 263]: 263(98), 248(12), 235(100), 232(4), 220(7), 207(55), 193(10) MS^5[309 → 281 → 263 → 235]: 235(100), 194(66), 193(100)	270	Tanshinaldehyde
9[a]	39.92	311	MS^2[311]: 293(100), 275(6), 247(2) MS^3[311 → 293]: 278(2), 275(100), 265(10), 247(31) MS^4[311 → 293 → 275]: 275(10), 260(45), 247(100), 233(4), 219(7) MS^5[311 → 293 → 275 → 247]: 247(20), 219(100), 260(61)	270	Przewaquinone A
10[a]	40.67	279	MS^2[279]: 261(100), 251(2), 237(3), 233(6), 209(2) MS^3[279 → 261]: 233(100), 215(2), 205(9) MS^4[279 → 261 → 233]: 233(5), 218(2), 215(2), 205(100), 190(5) MS^5[279 → 261 → 233 → 205]: 205(100), 191(18)	242	15,16-Dihydrotanshinone I
11	42.53	281	MS^2[281]: 263(100), 253(3), 235(78), 221(3) MS^3[281 → 263]: 263(14), 248(12), 245(6), 235(100), 221(20), 220(3), 217(23), 207(8), 169(4) MS^4[281 → 263 → 235]: 235(14), 220(7), 217(15), 207(100), 193(14), 192(4) MS^5[281 → 263 → 235 → 207]: 207(100), 192(98)	278	1,2,15,16-Tetrahydrotanshinone I
12[a]	43.11	339	MS^2[339]: 297(3), 279(100), 278(4), 261(4) MS^3[339 → 279]: 261(100) MS^4[339 → 279 → 261]: 233(100), 215(13), 205(37) MS^5[339 → 279 → 261 → 233]: 205(100), 179(79) MS^6[339 → 279 → 261 → 233 → 205]: 205(79), 190(100)	270	Methyl tanshinonate
13[a]	46.62	297	MS^2[297]: 282(12), 279(100), 268(16), 254(24), 251(82), 237(7) MS^3[297 → 279]: 279(19), 264(44), 261(36), 251(100), 250(13), 238(23), 237(75), 236(8), 223(14), 209(7) MS^4[297 → 279 → → 251]: 251(27), 236(47), 233(22), 223(100), 222(10), 209(34), 208(11), 197(6), 195(11), 181(14) MS^5[297 → 279 → 251 → 223]: 223(26), 208(78), 195(86), 193(47), 181(100)	264	Cryptotanshinone

Table 2 (*Continued*)

Peak no.	T_R (min)	MS $[M+H]^+$	HPLC–ESI–MS^n m/z (% base peak)	UV λ_{max}	Identity
14[a]	47.39	277	MS^2[277]: 259(6), 249(100), 231(16), 221(7)	246	Tanshinone I
15	49.59	279	MS^2[279]: 261(100) MS^3[279 → 261]: 233(100) MS^4[279 → 261 → 233]: 205(100), 190(12)	292	1,2-Dihydrotanshinone I/3,4-Dihydrotanshinone I
16[a]	52.67	295	MS^2[295]: 277(100), 249(13) MS^3[295 → 277]: 277(21), 262(17), 249(100) MS^4[295 → 277 → 249]: 249(10), 234(41), 221(100), 207(15), 206(47) MS^5[295 → 277 → 249 → 221]: 221(100), 206(95)	270	Tanshinone IIA

[a] Identified by comparison with the reference compounds.

The UV spectra of peak 1 suggested that it has a tanshinone IIA nucleus with maxima absorbance at 270 nm and the mass spectrum gave a $[M+H]^+$ ion at 313. According to the literature [30], it was identified as tanshindiol A. Peaks 2, 3, 6, 7, 8, 9, 10, 12, 13, 14 and 16 were unambiguously identified as tanshindiol B, tanshindiol C, tanshinone IIB, 3α-hydroxytanshinone IIA, tanshinaldehyde, przewaquinone A, 15,16-dihydrotanshinone I, methyl tanshinonate, cryptotanshinone, tanshinone I and tanshinone IIA, respectively, by comparing their retention times and mass fragmentation behaviors with those of the reference compounds.

Peak 11 were tentatively designated as 1, 2, 15, 16-tetrahydrotanshinone I (Trijuganone B) by comparing the UV and MS^n data reported in the literature [18]. Peak 15 was eluted as a single sharp peak with the LC method described and was identified as 1,2-dihydrotanshinone I [18], but it appears to be a pair of isomers with the very similar UV spectra absorption and mass fragmentation. The MS^n fragmentations of these two isomers were almost identical. Hence this peak was tentatively identified as 1,2-dihydrotanshinone I or 3,4-dihydrotanshinone I. The current data cannot distinguish these two compounds.

3.3. Identification of metabolites in rat bile

To clarify the origin of the metabolites, two major constituents of TTE-50, tanshinone IIA and cryptotanshinone were orally administered to rats, respectively. The bile samples were collected and handled using the method described above. Based on that, the origin of metabolites was confirmed by a general comparison between the blank bile and bile samples after orally administered reference compounds and TTE-50 sample. Seventeen metabolites were identified and the data were listed in Table 3.

Early publications indicated that hydroxylation and dehydrogenation metabolisms are the major metabolic pathways of cryptotanshinone and tanshinone IIA [15,17]. Comparing the prototype in bile, some metabolites were in trace amount and were hardly to be detected in the UV spectra. Hence, the extracted ion chromatograms were used in the further identifi-

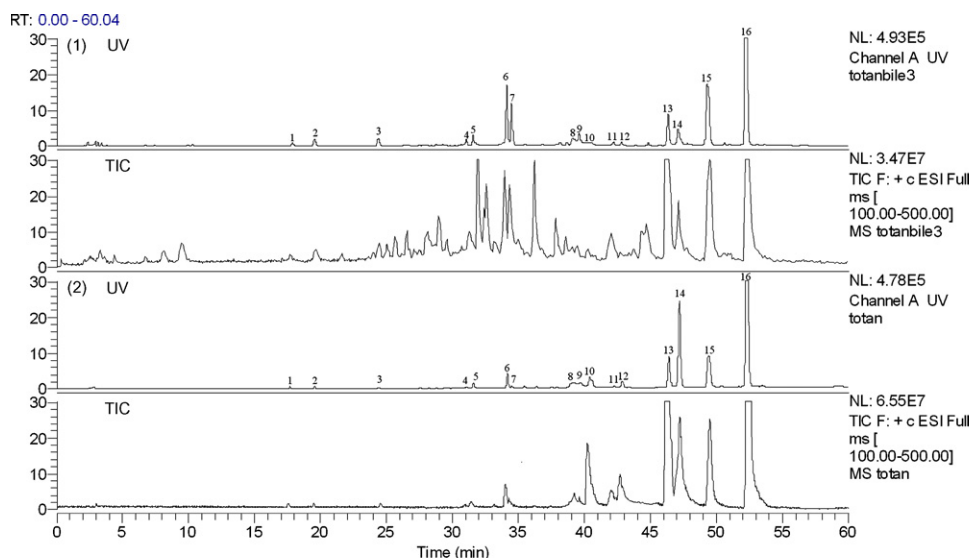


Fig. 2. UV (270 nm) and total ion current (TIC) chromatograms of rat bile sample and TTE-50 sample: (1) rat bile sample after oral administration of TTE-50 and (2) TTE-50 sample. Peak numbers were consistent with those shown in Table 2.

Table 3
HPLC/MS^n data of the metabolites identified in rat bile after oral administration of TTE-50

Metabolites	T_R (min)	MS $[M+H]^+$	HPLC/ESI-MS^n m/z (% base peak)	UV λ_{max}	Identity
M1[a]	34.30	311	Listed in Table 1	270	Tanshinone IIB
M2[a]	34.70	311	Listed in Table 1	270	3α-Hydroxytanshinone IIA
M3[a]	39.92	311	Listed in Table 1	270	Przewaquinone A
M4	45.13	293	MS^2[293]: 278(6), 275(100), 265(15), 251(6), 247(34) MS^3[293 → 275]: 275(10), 247(100) MS^4[293 → 275 → 247]: 247(64), 232(58), 219(100), 204(31), 203(22)	272	1,3-Dehydrotanshinone IIA
M5	47.56	293	MS^2[293]: 275(100), 265(8), 251(3), 247(28) MS^3[293 → 275]: 275(17), 260(6), 259(2), 247(100), 229(3) MS^4[293 → 275 → 247]: 248(7), 247(7), 232(14), 219(100), 218(4), 217(11), 204(22), 193(9)	240	1,2-Dehydrotanshinone IIA or 2,3-Dehydrotanshinone IIA
M6	49.79	293	MS^2[293]: 293(2), 279(2), 278(4), 275(100), 265(10), 252(2), 251(3), 247(23), 233(2), 205(2) MS^3[293 → 275]: 275(16), 247(100) MS^4[293 → 275 → 247]: 248(24), 232(33), 221(10), 219(100), 204(79)	252	1,2-Dehydrotanshinone IIA or 2,3-Dehydrotanshinone IIA
M7[a]	38.95	309	MS^2[309]: 291(2), 281(100), 263(6), 235(2) MS^3[309 → 281]: 281(26), 266(14), 263(75), 253(4), 245(2), 239(35), 235(6), 211(100), 207(2), 197(2) MS4[309 → 281 → 263]: 263(98), 248(12), 235(100), 232(4), 220(7), 207(55), 193(10) MS^5[309 → 281 → 263 → 235]: 235(100), 194(66), 193(100)	270	Tanshinaldehyde
M8	48.01	295	MS^2[295]: 277(100), 249(7) MS^3[295 → 277]: 262(31), 259(13), 249(100), 241(10), 235(12), 234(6), 145(11) MS^4[295 → 277 → 249]: 249(25), 234(100), 233(12), 231(8), 221(62), 207(10) MS^5[295 → 277 → 249 → 234]: 234(58), 219(100), 206(79)	288	1,2-Dehydrocryptotanshinone IIA or 2,3-Dehydrocryptotanshinone IIA
M9[a]	52.67	295	MS^2[295]: 277(100), 249(13) MS^3[295 → 277]: 277(21), 262(17), 249(100) MS^4[295 → 277 → 249]: 249(10), 234(41), 221(100), 207(15), 206(47) MS^5[295 → 277 → 249 → 221]: 221(100), 206(95)	270	Tanshinone IIA
M10	27.95	313	MS^2[313]: 295(100), 277(7), 267(25), 253(3), 249(3) MS^3[313 → 295]: 280(8), 277(100), 267(19), 253(11), 249(9), 221(6), 211(6) MS^4[313 → 295 → 277]: 277(100), 249(49)	262	Hydroxycryptotanshione
M11	28.86	313	MS^2[313]: 295(89), 283(11), 277(100), 270(9), 267(8), 265(13), 249(92), 235(11), 225(49) MS^3[313 → 277]: 277(92), 249(100)	264	Hydroxycryptotanshione
M12	29.08	313	MS^2[313]: 295(100), 277(89), 267(92), 249(80), 239(20), 229(12), 200(30) MS^3[313 → 295]: 277(91), 249(100)	–	Hydroxycryptotanshione
M13	29.35	313	MS^2[313]: 295(100), 283(10), 282(5), 277(40), 271(6), 270(3), 269(3), 267(34), 265(10), 255(2), 253(7), 252(7), 249(44), 245(4), 237(11), 229(6), 223(2) MS^3[313 → 295]: 280(4), 277(100), 267(37), 266(2), 262(2), 253(4), 252(3), 249(48), 235(3), 234(2), 225(14), 185(2) MS^4[313 → 295 → 249]: 234(71), 221(100), 220(10), 206(15) MS^5[313 → 295 → 249 → 221]: 221(100), 179(60)	266	Hydroxycryptotanshione
M14	35.60	313	MS^2[313]: 295(100), 252(2) MS^3[313 → 295]: 277(100), 263(10), 249(10), 239(7), 225(6) MS^4[313 → 295 → 277]: 262(44), 249(100), 235(14), 217(13), 207(11) MS^5[313 → 295 → 277 → 249]: 234(100), 207(43), 205(49)	264	17-Hydroxycryptotanshinone
M15	40.81	327	MS^2[327]: 312(43), 295(100) MS^3[327 → 295]: 277(100), 267(16), 261(4), 249(29), 235(7), 225(8), 207(5) MS^4[327 → 295 → 277]: 277(66), 262(77), 250(100), 249(73), 248(49), 235(40), 222(48)	–	Methoxylated metabolite of cryptotanshinone

Table 3 (*Continued*)

Metabolites	T_R (min)	MS $[M+H]^+$	HPLC/ESI-MS^n *m*/*z* (% base peak)	UV λ_{max}	Identity
M16	36.29	315	MS^2[315]: 297(100) MS^3[315 → 297]: 279(100), 264(20), 254(17), 251(46), 233(11) MS^4[315 → 297 → 279]: 279(21), 251(53), 237(100), 233(50)	248	Tanshinone V
M17	26.57	325	MS^2[325]: 307(58), 297(3), 289(2), 281(100), 279(21), 263(19), 261(11), 235(6), 217(2) MS^3[325 → 281]: 281(43), 263(100), 253(19), 235(95), 207(15) MS^4[325 → 281 → 263]: 263(68), 235(100), 220(51) MS^5[325 → 281 → 263 → 235]: 235(76), 207(100)	272	Tanshinacid

[a] Identified by comparison with the reference compounds.

cation of the metabolites. The extracted ion chromatograms of blank bile, rat bile sample collected after oral administration of TTE-50 and rat bile collected after administered tanshinone IIA and cryptotanshinone were shown in Figs. 3 and 4, respectively. The results showed that the metabolites in rat bile after administration of TTE-50 was very similar with that of reference compounds, which further confirmed that M1–M17 were metabolites.

3.3.1. The metabolites of tanshinone IIA origin

Tanshinone IIB (M1), 3α-hydroxytanshinone IIA (M2) and przewaquinone A (M3) were identified in TTE-50 and in the bile samples after administering TTE-50. They were also the three hydroxylated metabolites of tanshinone IIA. This was confirmed by administering pure tanshinone IIA. M2, which was considered as a chiral isomer of tanshinone IIB in the literature [17], was identified as 3α-hydroxytanshinone IIA by comparing the retention time and the MS^n fragmentation behavior with those of the reference compound.

M4, M5 and M6 all gave rise to protonated molecules $[M+H]^+$ at *m*/*z* 293, but the UV spectra were quite different. The UV absorbance of M4 was similar to that of tanshinone IIA, suggesting that the saturation of A ring remained. Considering its molecular weight being 2 Da less than that of tanshinone IIA,

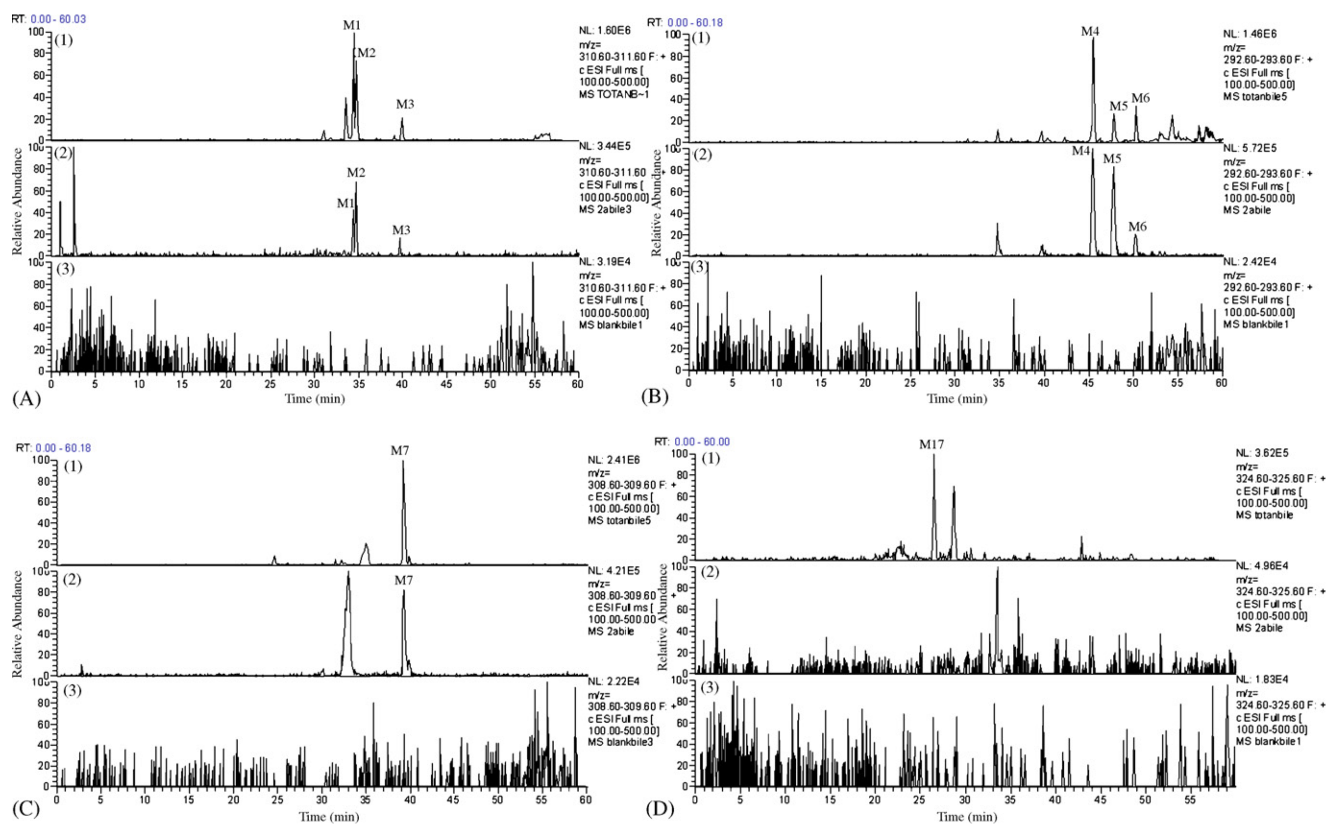


Fig. 3. Extracted ion chromatograms of the metabolites: (1) rat plasma sample after oral administration of TTE-50, (2) rat bile sample after oral administration of tanshinone IIA and (3) blank rat bile sample. (A) Extracted ion chromatogram of *m*/*z* at 311, (B) extracted ion chromatogram of *m*/*z* at 293, (C) extracted ion chromatogram of *m*/*z* at 309 and (D) extracted ion chromatogram of *m*/*z* at 325.

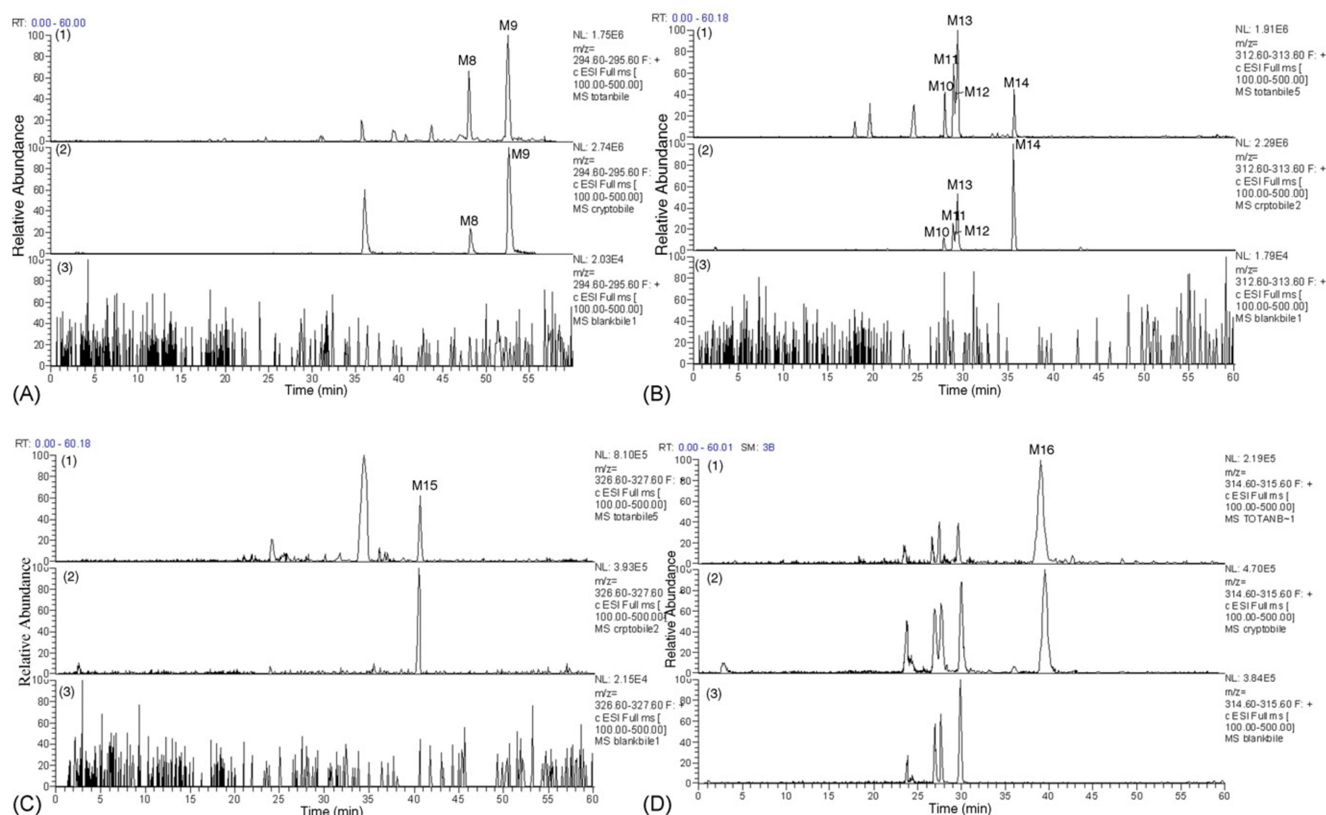


Fig. 4. Extracted ion chromatograms of the metabolites: (1) rat plasma sample after oral administration of TTE-50, (2) rat bile sample after oral administration of cryptotanshinone and (3) blank rat bile sample. (A) Extracted ion chromatogram of *m/z* at 295, (B) extracted ion chromatogram of *m/z* at 313, (C) extracted ion chromatogram of *m/z* at 327 and (D) extracted ion chromatogram of *m/z* at 315.

M4 was deduced to be 1,3-dehydrotanshinone IIA, a dehydrogenated metabolite of tanshinone IIA, which has been reported in the literature [17]. The UV absorbance of M5 and M6 suggested the alteration of the A ring from saturated ring to unsaturated ring and the substitution of double bond may take place at 1,2 or 2,3 position. These two metabolites were tentatively identified as 1,2-dehydrotanshinone IIA or 2,3-dehydrotanshinone IIA.

M7 was found in both the bile sample of TTE-50 and bile sample of tanshinone IIA with the quasi-molecule ion $[M+H]^+$ at *m/z* 309. The MS^n ($n=2$–5) spectra gave prominent ions at *m/z* 281 [M + H–CO], 263 [M + H–CO–H_2O], 235 [M + H–CO–2H_2O] and 193 [M + H–CO–2H_2O–42] by sequential losses of CO, H_2O, H_2O and propylene. The retention time 38.95 min and fragmentation behavior of M7 was identical to that of tanshinaldehyde. Hence M7 was designated as tanshinaldehyde. The metabolic modification could take place in the C-4 side chain of tanshinone IIA, from methyl to primary alcohol, then to aldehyde group. This metabolic pathway was proposed for the first time.

M17, eluted at 26.57 min, was found in the bile sample of TTE-50 and gave the protonated molecule $[M+H]^+$ at *m/z* 325. In negative ion mode, the MS^2 spectrum showed a prominent loss of CO_2 ($[M+H\text{-}44]^+$), which indicated the existence of carboxyl group. This compound might be produced by the oxidation of tanshinaldehyde and M17 was therefore proposed as tanshinacid (named by author), which was probablely a metabolite produced by the further oxidation of tanshinaldehyde. The metabolic pathways of tanshinone IIA were shown in Fig. 5.

3.3.2. *The metabolites of cryptotanshinone origin*

M8 and M9 both gave $[M+H]^+$ ions at *m/z* 295. M9 was identified as tanshinone IIA undoubtedly by comparing with the reference compound. M8 was another dehydrogenated metabolite of cryptotanshinone with the similar fragmentation behavior of tanshinone IIA. The UV absorbance of M9 was different from that of cryptotanshinone or tanshinone IIA, therefore this metabolite was tentatively characterized as 1,2-dehydrocryptotanshione or 2,3-dehydrocryptotanshinone.

M10, M11, M12, M13 and M14, eluted at 27.95, 28.86, 29.08, 29.35 and 35.60 min, respectively, all gave $[M+H]^+$ ion at *m/z* 313, which are 16 Da heavier than that of cryptotanshinone. The fragmentation pathway of these five compounds showed obvious loss of $[M+H\text{-}18]^+$. The fragment ion at *m/z* 295 was formed via the elimination of H_2O from the parent ion *m/z* 313, hence they are the hydroxylated metabolites of cryptotanshinone. The MS^n spectra of M14 was very similar to that of przewaquinone A, except that the corresponding ions were each heavier by 2 Da

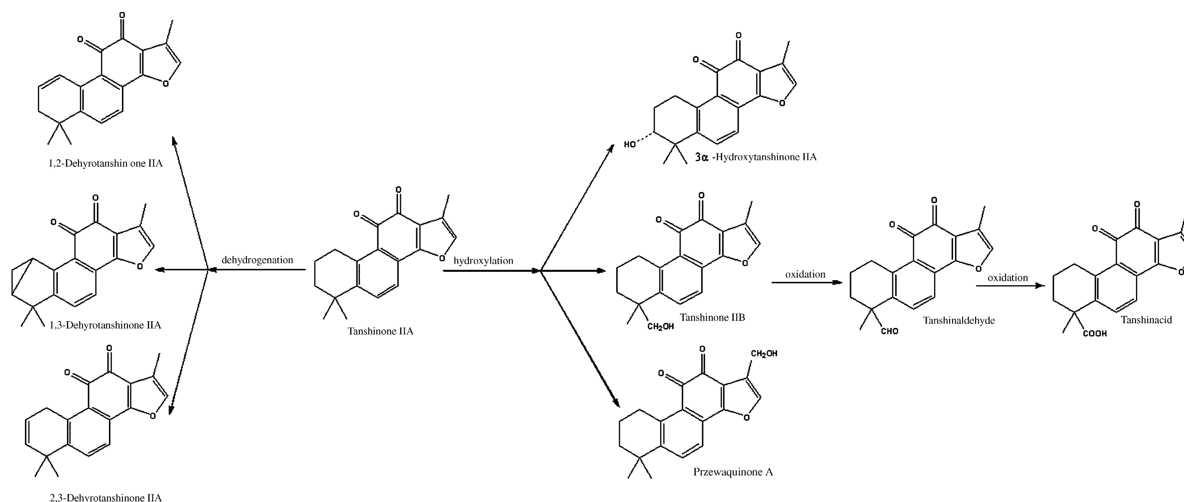


Fig. 5. Proposed metabolic pathways of tanshinone IIA in rat.

(shown in Fig. 6). Considering the similar mass fragmentation behaviors of M14 and przewaquinone A, M14 was identified as 17-hydroxycrytotanshinone.

M15 gave the protonated molecule $[M+H]^+$ at m/z 327, the MS^2 spectrum showed prominent ions at m/z 312 ($[M+H–CH_3]^+$) and m/z 295($[M+H–CH_3OH]^+$). The loss of methyl and CH_3OH confirmed that M15 was a methoxylated metabolite of cryptotanshinone. M15 was suspected to be an artificial metabolite at the first glance for methanol was used to reconstitute the samples in the experiment. To examine whether M15 was an artifact, acetonitrile was used to reconstitute the bile sample. As M15 was still identified in this sample, it was ensured that M15 was a native metabolite of cryptotanshinone.

M16 gave the protonated molecule $[M+H]^+$ at m/z 315, the MS^2 and MS^3 spectra showed consecutive loss of H_2O by comparison with the MS^n data in the literature [19], M16 was identified as tanshinone V, a hydrolyzated metabolite of cryptotanshinone produced in the D-ring (furan ring) cleavage. The proposed metabolic pathways of cryptotanshinone were shown in Fig. 7.

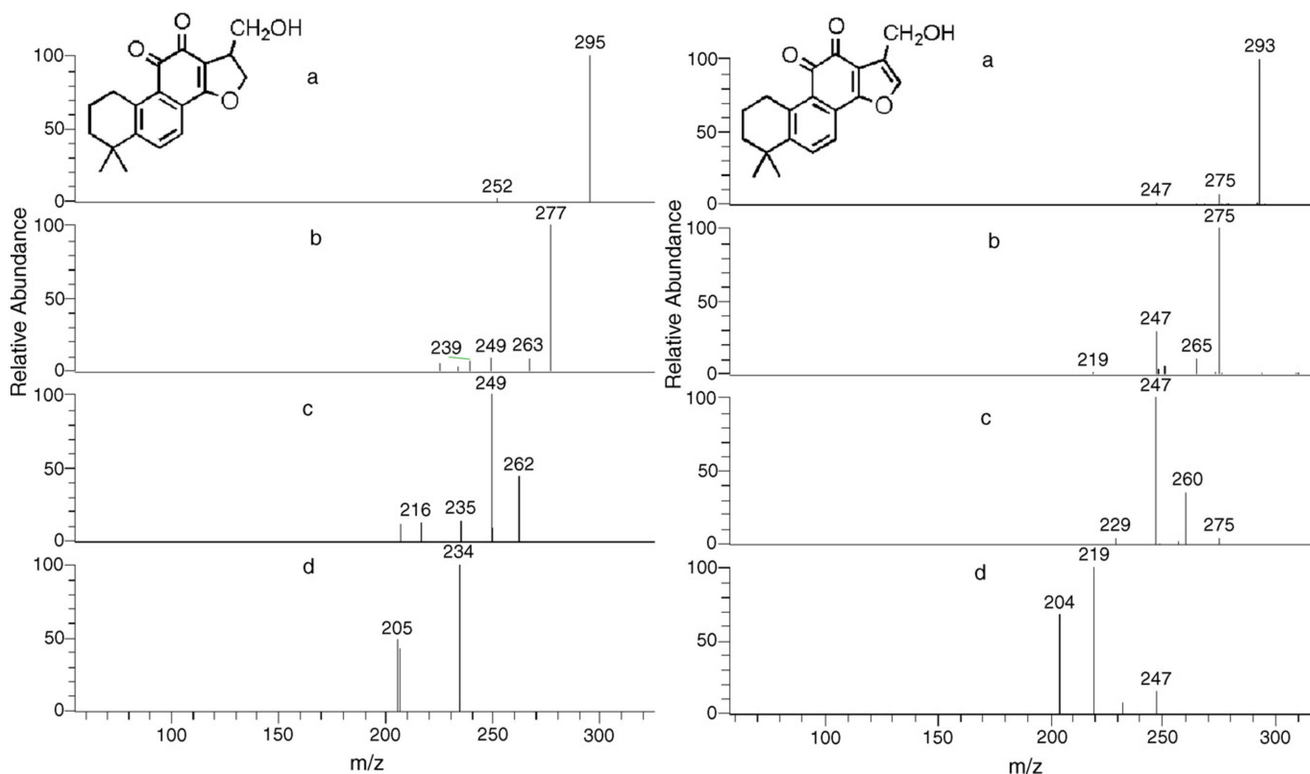


Fig. 6. MS^n ($n=2$–5) fragmentation of M17 (a. MS^2[313], b. MS^3[313 → 295], c. MS^4[313 → 295 → 277] and d. MS^5[313 → 295 → 277 → 249]) and przewaquinone A (a. MS^2[311], b. MS^3[311 → 293], c. MS^4[311 → 293 → 275] and d. MS^5[311 → 293 → 275 → 247]).

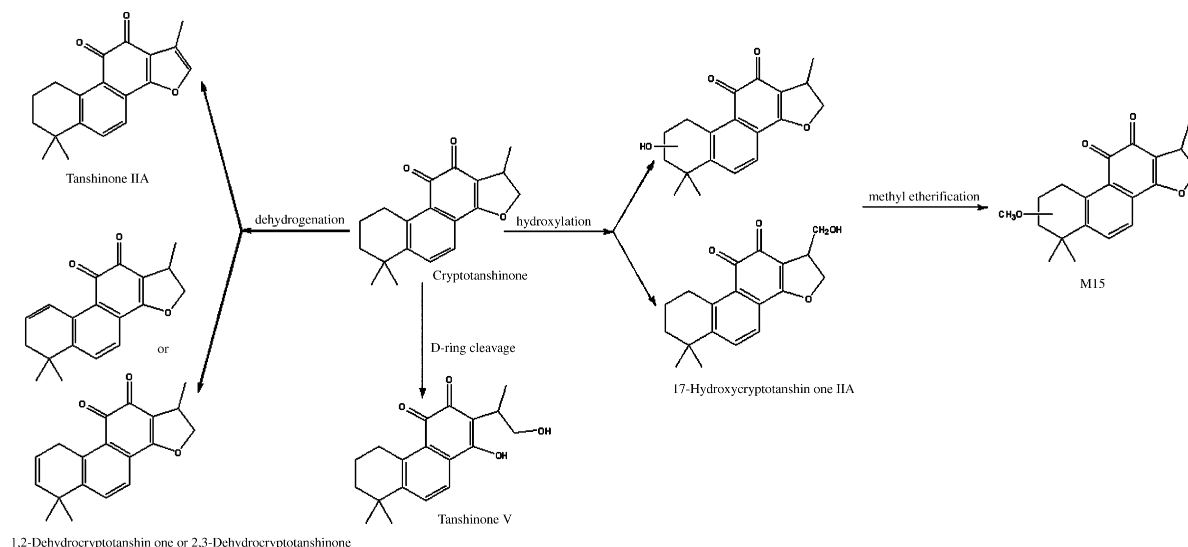


Fig. 7. Proposed metabolic pathways of cryptotanshinone in rat.

4. Conclusion

In this study, a HPLC–DAD–ESI–MS^n method was developed for the analysis of the rat bile after oral administration of TTE-50. Sixteen tanshinones from TTE-50 and 17 metabolites were simultaneously identified. In addition, the origin of the metabolites was confirmed by comparing the mass spectra and chromatographic behavior with those of rat bile sample obtained after administration of reference compounds of two major constituents in TTE-50. The developed method was simple, reliable and sensitive, which revealed it to be appropriate for rapid screening and structural characterization of tanshinones and their metabolites after oral administration of TTE-50 in rat bile. Though the structures of some metabolites could not be determined conclusively by HPLC–MS^n, the present method was helpful to a better understanding for the *in vivo* metabolism of TTE-50 and it is valuable and dependable for the further study of the metabolism of complex systems such as herbal extracts or TCM formula.

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