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Structural elucidation of *in vivo* and *in vitro* metabolites of anisodine by liquid chromatography–tandem mass spectrometry

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Abstract

Liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESIMS^n) was employed to investigate the *in vivo* and *in vitro* metabolism of anisodine. Feces, urine and plasma samples were collected after ingestion of 20 mg anisodine to healthy rats. Feces and urine samples were cleaned up by liquid–liquid extraction and solid-phase extraction procedures (C18 cartridges), respectively. Methanol was added to plasma samples to precipitate plasma proteins. Anisodine was incubated with homogenized liver and intestinal flora of rats *in vitro*, respectively, followed by extraction with ethyl acetate. LC–MS^n was used for the separation and identification of the metabolites using C18 column with mobile phase of methanol/0.01% triethylamine solution (2 mM, adjusted to pH 3.5 with formic acid) (60:40, v/v). The results revealed that five metabolites (norscopine, scopine, α-hydroxytropic acid, noranisodine and hydroxyanisodine) and the parent drug existed in feces. Three new metabolites (dimethoxyanisodine, tetrahydroxyanisodine and trihydroxy-methoxyanisodine) were identified in urine. Four metabolites (norscopine, scopine, hydroxyanisodine and anisodine *N*-oxide) and the parent drug were detected in plasma. Two hydrolyzed metabolites (scopine and α-hydroxytropic acid) were found in rat intestinal flora incubation mixture, and two metabolites (aponoranisodine and anisodine *N*-oxide) were identified in homogenized liver incubation mixture.

Keywords: Anisodine; LC–MS^n; Metabolite

1. Introduction

Anisodine, a tropane alkaloid extracted from the roots of Chinese traditional medicine *Anisodus tanguticus* (Maxim.) Pascher., has important physiological activities such as spasmolytic, anaesthetic, acesodyne and ophthalmic effects [1,2]. It is often used to treat transmissible shock, hepatitis, nephritis, and diabetes. It has smaller toxicity and side effects than other tropane alkaloids such as atropine, anisodamine and scopolamine. Despite its important therapeutic value, its *in vivo* or *in vitro* metabolism is not clear yet. Up till now, the works mainly focused on the quantitative determination of anisodine in plant [3,4] and only two recent publications from our group deal with the metabolism of anisodine [5,6].

Due to its high sensitivity and specificity LC–MS^n has been proven to be a powerful analytical tool for the identification of drug metabolites in biological matrices [7–9]. It is especially suitable for the analysis of thermolabile, highly polar and non-volatile metabolites. MS^n technique has made possible the acquisition of structurally informative data from protonated molecules of analytes of interest, even when they are not resolved chromatographically [10,11].

In the course of our recent study [5,6] 20 metabolites were detected and identified by LC–MS^n in rat urine and four metabolites in rat plasma. The major metabolic pathway of anisodine in rat was hydrolysis, demethylation, dehydration, hydroxylation, and sulfate and glucuronide conjugation.

For studying the metabolism of anisodine more comprehensively, this work presents the metabolism of anisodine in rat feces, urine and plasma. The present study also involves incubation of anisodine with intestinal flora and homogenized liver in order to clarify its *in vivo* and *in vitro* metabolic pathway. The parent drug and its five metabolites were detected in rat

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feces after ingesting 20 mg anisodine. Three new metabolites (dimethoxyanisodine, tetrahydroxyanisodine and trihydroxymethoxyanisodine) were identified in rat urine. Four metabolites were still found in rat plasma. Only two hydrolyzed products were identified in rat intestinal flora incubation mixtures, and another two metabolites were detected in homogenized liver incubation mixtures. The above-mentioned three metabolites detected in rat urine were newly discovered and characterized. The metabolites detected in rat feces, intestinal flora and homogenized liver incubation solutions were found for the first time, too.

2. Experimental

2.1. Chemicals

Anisodine hydrobromide was purchased from Sigma (St. Louis, MO, USA). Methanol was of HPLC grade (Fisher Chemical Co. Inc., CA, USA); water was deionized and double distilled; all other reagents were of analytical reagent grade.

2.2. Instrumentation

LC–MS and LC–MS^n experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer (ThermoFinnigan Corp., San Jose, USA) with a TSP4000 HPLC pump and a TSP AS3000 autosampler using positive and negative electrospray as the ionization process. The software Xcalibur version 1.2 (Finnigan) was applied for system operation and data collection. A high-speed desk centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge samples. The urine samples were extracted on a C18 solid-phase extraction cartridge (3 ml/200 mg, AccuBond, Agilent). The intestinal incubation experiments were carried out in anaerobic incubation bags (AnaeroPouch^{TM}-Anaero 08G05A-23, Mitsubishi Gas Chemical Company Inc.) using anaerobic-generating bags (Mitsubishi Gas Chemical Company Inc.).

2.3. Sample preparation

2.3.1. In vivo samples

Five Wistar rats (180 ± 5 g, Hubei Experimental Animal Research Center, China) were housed in metabolic cages for the collection of feces, urine and plasma. The rats were fasted for 24 h but with access to water, and then they were administered 20 mg oral gavage doses of anisodine. Feces and urine were collected individually during the time period of 0–24 h. The samples were stored at −20 °C until analysis. Heparinized blood samples of 200 μl were collected at 0.25, 0.75, 2, 9, 18, 24 h from the ophthalmic veins of the rats by sterile capillary tube, then, shaken up and centrifuged at 2000 × *g* for 10 min. The supernatants were decanted, and immediately frozen at −20 °C until analysis.

The feces sample was homogenized with water. An aliquot of 500 μl feces homogenate was extracted twice with 1 ml of ethyl acetate after adding 50 μl of 0.001% Na_2CO_3 solution. The supernatant ethyl acetate layers were decanted, pooled and evaporated at 37 °C under nitrogen stream. The residue was redissolved in 500 μl of mobile phase and filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS^n analyses.

An aliquot of 1 ml of mixed 0–24 h urine samples was loaded onto a C18 solid-phase extraction cartridge that was preconditioned with 2 ml of methanol and 1 ml of water. Then, the SPE cartridge was washed with 2 ml of water and the analytes were eluted with 1 ml of methanol. The elution solutions were filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS^n analyses.

The plasma samples were added 300 μl of methanol to precipitate plasma proteins, and then centrifuged at 2000 × *g* for 10 min. The supernatant was filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS^n analyses.

2.3.2. In vitro samples

Preparation of anaerobic cultural solutions [12]. 37.5 ml A solution (0.78% K_2HPO_4), 37.5 ml B solution (0.47% KH_2PO_4, 1.18% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% $CaCl_2$, 0.25% $MgSO_4 \cdot H_2O$), 50 ml C solution (8% Na_2CO_3), 0.5 g L-cysteine, 2 ml 25% L-ascorbic acid, 1 g eurythrol, 1 g tryptone and 1 g nutrient agar were mixed together, and diluted to 1 l with distilled water. Two molars of HCl was used to adjust the solution to pH 7.5–8.0.

Metabolism in intestinal bacteria. The fresh intestinal contents were obtained from Wistar rat (200 g). Samples were homogenized with a glass rod in anaerobic cultural solution at the rate of 0.5 g:1.5 ml immediately. Then, the homogenate was filtrated using gauze. Anisodine was added into the above intestinal flora cultural solution to a final concentration of 50 μg/ml. The culture dishes were put in anaerobic incubation bags. The out bags of anaerobic-generating bags were opened, and put into anaerobic incubation bags immediately, then sealed. Incubations were carried out in a shaking water-bath at 37 °C anaerobically. The incubation was continued for 4 and 24 h, terminated and extracted (twice) with ethyl acetate. The organic extracts were merged and evaporated at 37 °C under nitrogen stream. The residues were reconstituted in 0.6 ml of mobile phase, centrifuged at 13,000 × *g* for 10 min. The supernatant was used for LC–MS^n analyses.

Preparation and incubation of homogenated liver. Wistar rats (200 g) were fasted for 24 h and killed by decapitation between 10 a.m. and noon. A weighed amount of liver was rapidly placed on ice. It was rinsed twice with saline and immediately minced with scissors and homogenized in ice-cold Krebs–Henseleit buffer (pH 7.4) [13] after sterilization to yield liver homogenate (0.4 g/ml). All the above steps were carried out at 0–4 °C. The concentration of P450 was detected by spectrophotometry [14]. Anisodine was added to liver homogenate at a concentration of 50 μg/ml. The mixture was incubated at 37 °C with shaking. The incubation time was varied from 0, 30, 60, 90, 120 to 240 min. The gas phase was oxygen. After the termination of incubation the mixture was extracted twice with equal volumes of ethyl acetate. The organic extracts were merged and evaporated at 37 °C under a slow stream of nitrogen. The residues were recon-

stituted in 0.6 ml of mobile phase, centrifuged at 13,000 × *g* for 10 min. The supernatant was used for LC–MS^n analyses. The blank experiment was carried out under the same conditions by replacing the liver homogenate with Krebs–Henseleit buffer.

2.4. Chromatography and mass spectrometry

A reversed-phase column (Aichrom ReliAsil C18, 5 μm, 2 mm × 150 mm i.d., Agilent) was connected with a guard column (cartridge 4.6 mm × 12.5 mm, 5 μm, Agilent) filled with the same packing material to separate anisodine and its metabolites. The temperature of the column was set at 40 °C. The mobile phase consisted of methanol and 0.01% triethylamine solution (adjusted pH to 3.5 with formic acid) (60:40, v/v). The flow rate was 0.2 ml/min during the whole run. Mass spectral analyses were carried out in positive ion detection mode, and only the structures of α-hydroxytropic acid and phase II metabolites were validated by the LC–MS (MS^n) data in negative ion detection mode. Nitrogen was used as the sheath gas (40 a.u.). The MS analyses were performed under automatic gain control conditions, using a typical source spray voltage of 5.0 kV, a capillary voltage of 45 V and a heated capillary temperature of 200 °C. The other parameters, including the voltages of octapole offset and tube lens offset, were also optimized for maximum abundance of the ions of interest by the automatic tune procedure of the instrument. The MS^n product ion spectra were produced by collision induced dissociation (CID) of the protonated molecule $[M+H]^+$ of all analytes at their respective HPLC retention times. Data acquisition was performed in full scan LC–MS and tandem MS modes.

3. Results and discussion

3.1. LC–MS and LC–MS^n analyses of substrate

The chromatographic and mass spectrometry conditions were optimized with the use of anisodine. Full scan mass spectral analysis of anisodine showed protonated molecule of *m/z* 320 (Fig. 1A). The MS–MS product ion spectrum of the protonated molecule (*m/z* 320) and the predominant fragmentation patterns of anisodine are shown in Fig. 1B. Anisodine was eluted at 3 min (Fig. 1C). Fragmentation of protonated molecule of anisodine in the ion trap lead to five product ions at *m/z*: 302, 290, 156, 138 and 110. The subordinate product ions at *m/z* 302 and *m/z* 290 were formed by the loss of H_2O and HCHO from the molecular ion at *m/z* 320, respectively. The product ion at *m/z* 138 was formed by the loss of anisodic acid ($C_9H_{10}O_4$, 182 Da). The most abundant product ion at *m/z* 156 was produced by the loss of $C_9H_8O_3$ ($C_9H_{10}O_4{\cdot}H_2O$, 164 Da). The fragment ions at *m/z* 156, 138 and 110 coexisted in the MS^3 spectra of *m/z* 302 and *m/z* 290, and the MS^3 spectrum of *m/z* 156 showed fragment ions at *m/z* 138 and 110. The MS^3 spectrum of *m/z* 138 gave fragment ion at *m/z* 110. It could be concluded that the ions at *m/z* 156 and 138 were a pair of characteristic product ions of anisodine,

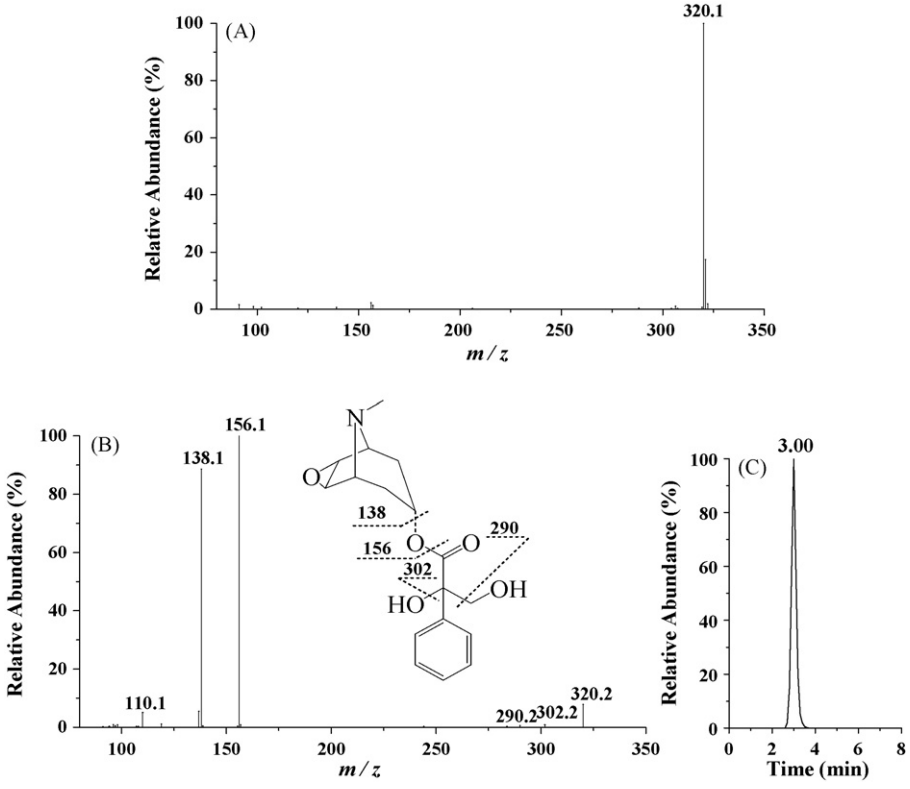

Fig. 1. (A) ESI mass spectrum of anisodine; (B) MS–MS product ion spectrum and the predominant fragmentation patterns of anisodine; (C) LC–MS^2 chromatogram of anisodine.

Table 1
MS–MS product ions of anisodine and its metabolites in rat feces and plasma

Analyte	$[M+H]^+$	R_t (min)	Product ions (*m/z*, relative abundance/%)	Plasma	Feces
M1	142	2.54	124 (100), 114 (60), 110 (8), 96 (10), 84 (15)	+	+
M2	156	2.99	138 (100), 28 (6), 110 (10), 98 (8), 84 (10)	+	+
M3	306	2.94	288 (10), 276 (8), 142 (100), 124 (10)	−	+
M0	320	3.01	302 (2), 290 (3), 156 (86), 138 (100), 110 (6)	+	+
M4	336	2.53	156 (100), 138 (78), 110 (6)	+	+
M5	336	3.21	172 (100), 155 (8), 154 (15), 136 (6), 119 (5)	+	−
M6	181$[M-H]^-$	2.03	163 (3), 151 (20), 137 (100), 113 (8), 97 (2)	−	+

+: found; −: not found.

and 164, 182 Da were its characteristic neutral losses. These characteristic product ions and neutral losses were the basis to identify metabolites of anisodine.

3.2. In vivo metabolism study

3.2.1. Identification of metabolites in rat feces

The full scan mass spectrum of rat feces after administration of anisodine was compared with that of blank feces sample and anisodine solution to find the possible metabolites. Then, these compounds were analyzed by LC–MS^n. Their retention times, changes in observed mass (ΔM) and spectral patterns of product ions were compared with those of anisodine to identify metabolites and elucidate their structures.

Based on the method described above, the parent drug and its metabolites were found in rat feces after ingesting anisodine. Their protonated molecules ($[M+H]^+$) were at *m/z* 142, 156, 306, 320, 336 (2.53 min), respectively, and the deprotonated molecule ($[M-H]^-$) at *m/z* 181. LC–MS^n spectra of the metabolites of anisodine were obtained via fragmentation of protonated molecules and were used for more precise structural identification of metabolites. The retention times and MS–MS product ions of these analytes are presented in Table 1. Among them, the retention time, the MS and MS^2 spectra of the molecular ion at *m/z* 320 (M0) were the same as those of anisodine. Therefore, M0 was confirmed as the unchanged parent drug.

The MS^2 spectrum of *m/z* 156 (M2) was the same as the MS^3 spectrum of the molecular ion of anisodine standard at *m/z* 320 → 156, and the characteristic product ions were at *m/z* 138 and 110 in its MS^2 spectrum. M2 was identified as the hydrolysis product of anisodine (scopine).

The molecular ion at *m/z* 142 (M1) and its product ions at *m/z* 124, 114, 96 and 84 were all 14 Da less than *m/z* 156 (M2) and its product ions at *m/z* 138, 128, 110 and 98. These results indicated that M1 should be the *N*-demethyl product of M2 (norscopine).

The fragment ions at *m/z* 142 and 124 were produced by losing neutral fragments 164 and 182 Da from their parent ion at *m/z* 306 (M3, Table 1), and the *m/z* 306 ion and its product ions at *m/z* 288, 276, 142 and 124 were all 14 Da less than the molecular ion of parent drug and its product ions at *m/z* 302, 290, 156 and 138. Thus, it should be the *N*-demethyl product of anisodine (noranisodine).

The protonated molecule at *m/z* 336 (M4) was increased by 16 Da compared to that of the unchanged anisodine. The appearance of the characteristic fragment ions at *m/z* 156, *m/z* 138 and characteristic neutral losses 180 Da (164 + 16) (*m/z* 336 → 156), 198 Da (182 + 16) (*m/z* 336 → 138) in the MS^2 spectrum of molecular ion of M4 indicated that M4 should be the hydroxylation product of anisodine with the hydroxyl group at the benzene ring (hydroxyanisodine).

The *m/z* 181 ion (M6) appeared in the negative ion full scan LC–MS spectrum of the urine samples. The appearance of the product ions at *m/z* 163 ($[M-H-H_2O]^-$), 151 ($[M-H-HCHO]^-$) and 137 ($[M-H-CO_2]^-$) (see Table 1) indicated that M6 should be the hydrolysis product of anisodine (α-hydroxytropic acid).

3.2.2. Identification of metabolites in rat urine

We have identified 20 metabolites (norscopine, scopine, α-hydroxytropic acid, aponoranisodine, apoanisodine, noranisodine, anisodine *N*-oxide, hydroxyanisodine, hydroxyanisodine *N*-oxide, methoxyanisodine, hydroxyl-methoxy anisodine, trihydroxyanisodine, dihydroxy-methoxy anisodine, hydroxyl-dimethoxy anisodine, glucuronide conjugates and sulfate conjugates of noranisodine, hydroxyanisodine and the parent drug) in rat urine after ingesting anisodine to healthy rats. In this study, three new metabolites were also found in rat urine. Their retention times and MS–MS product ions are presented in Table 2.

The protonated molecule at *m/z* 380 (M7), 384 (M8) and 398 (M9) was increased by 60 Da (30 × 2), 64 Da (16 × 4) and 78 Da (16 × 3 + 30) compared to that of unchanged anisodine. The characteristic fragment ions at *m/z* 156, 138 and 110 appeared in their MS^2 spectra, and the *m/z* 172 (156 + 16) ion

Table 2
MS–MS product ions of three new metabolites of anisodine in rat urine

Analyte	$[M+H]^+$	R_t (min)	Product ions (*m/z*, relative abundance/%)
M7	380	2.52	362 (10), 350 (4), 156 (100), 138 (76), 110 (3)
M8	384	2.20	366 (6), 354 (2), 156 (100), 138 (59), 110 (5)
M9	398	2.36	380 (6), 368 (3), 156 (90), 138 (100), 110 (3)

was not present in their MS^2 spectra. This showed that the tropine structure was retained in these metabolites. The appearance of their dehydrated fragment ions ($[M+H-H_2O]^+$) at *m/z* 362, 366 and 380 in the MS^2 spectra showed that they were phenolic metabolites. So, the three metabolites could be identified as dimethoxyanisodine, tetrahydroxyanisodine and trihydroxy-methoxyanisodine.

3.2.3. Identification of metabolites in rat plasma

The parent drug and its four metabolites were detected in rat plasma after administration of anisodine. Their molecular ions ($[M+H]^+$) were at *m/z* 142, 156, 320 and 336, respectively (see Table 1). *m/z* 142, 156, 320 and 336 (2.53 min) were identified as norscopine (M1), scopine (M2), anisodine (M0) and hydroxyanisodine (M4).

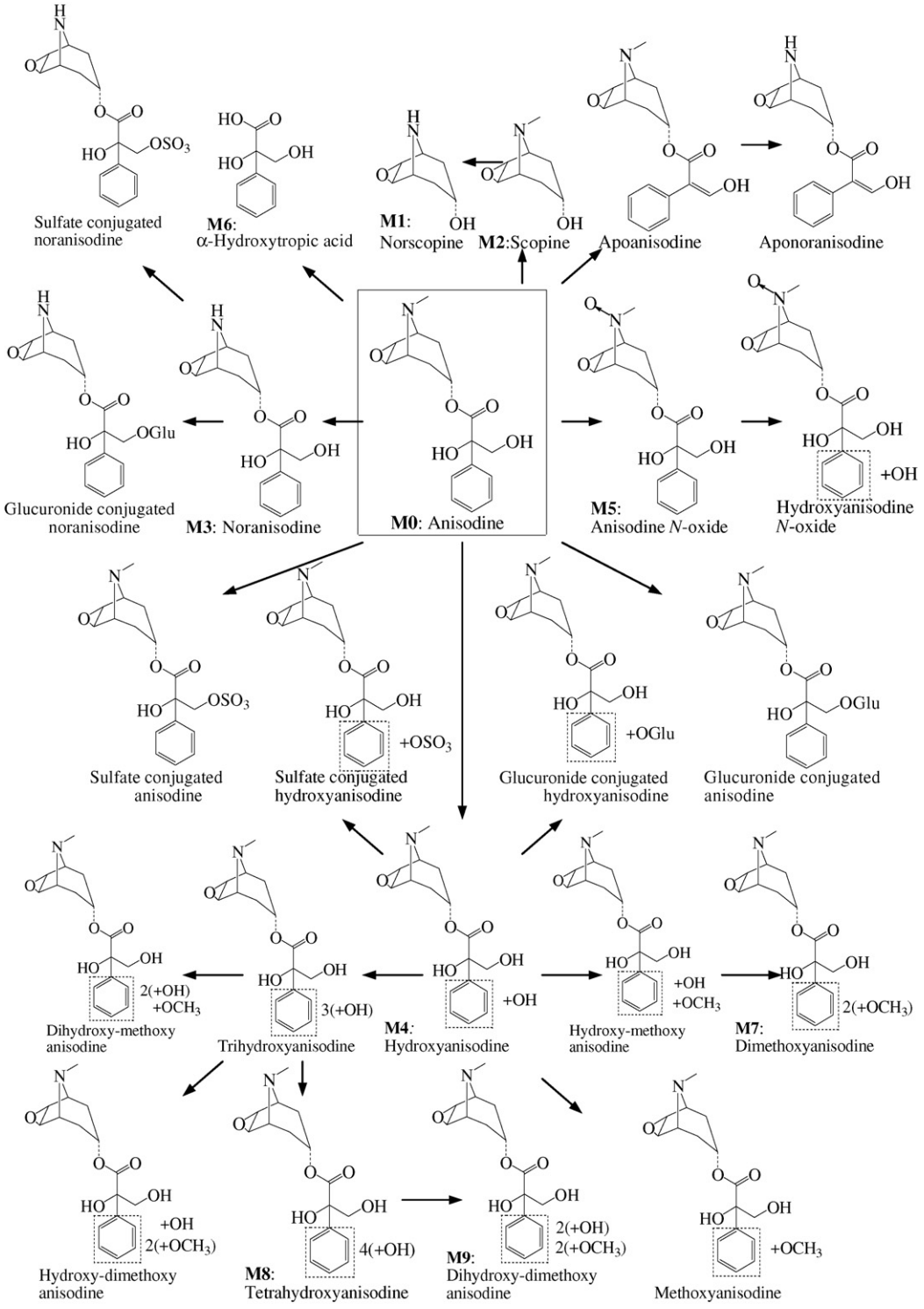

Fig. 2. The proposed major metabolic pathway of anisodine in rats.

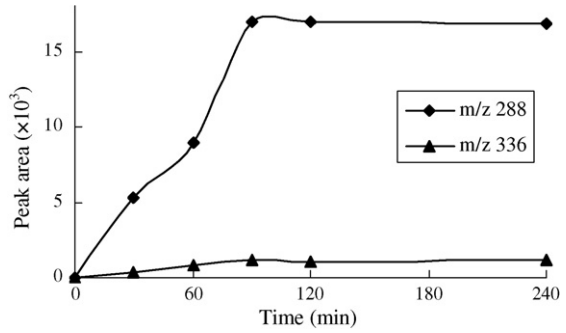

Fig. 3. Time–area curves of metabolites of anisodine in homogenized liver of rats.

Two chromatographic peaks of *m/z* 336 can be found in the LC–MS^2 chromatogram with retention times 2.53 min (M4) and 3.21 min (M5), respectively. A predominant product ion at *m/z* 172 (156 + 16) and a pair of product ions at *m/z* 155 (172 − 17), *m/z* 154 (172 − 18) (stronger than *m/z* 155) appeared in the MS^2 spectrum of the molecular ion of M5. All these results indicated that M5 should be the *N*-oxidation product of the parent drug (anisodine *N*-oxide) because it is the cleavage feature of *N*-oxides to lose 17 and 18 Da [15].

The LC–MS^2 analysis of plasma samples showed that the parent drug and its four metabolites (M1, M2, M4, M5) all existed in plasma between 0.25 and 24 h after administration.

No sulfate or glucuronide conjugate of anisodine and its phase I metabolites were found in rat plasma or feces.

Based on the above discussion and combining the results we have reported [5], the proposed metabolic pathway of anisodine in rats is presented in Fig. 2.

3.3. In vitro metabolism study

The substrate was incubated with intestinal fractions and liver homogenate of rats, respectively. The substrate and two hydrolysis product of anisodine (scopine and α-hydroxytropic acid) were detected in rat intestinal part and, aponoranisodine and anisodine *N*-oxide were found in liver homogenate part. The results were almost the same when intestinal incubation solutions at 4 and 24 h were investigated. The results showed that the metabolism of anisodine was very weak in rat intestinal bacteria and homogenized liver. Fig. 3 presented the time–area curves of metabolites of anisodine in homogenized liver of rats.

Various solvents were used for the liquid–liquid extraction of anisodine and its metabolites in rat feces. The analytical results were almost the same when ethyl acetate was substituted by chloroform or methylene dichloride. But the ethyl acetate layers were supernatant and easy to decant, and hence ethyl acetate was used for the liquid–liquid extraction of anisodine and its metabolites in rat feces.

The sensitivity of the LC–MS^2 method was determined using anisodine standard, and its limit of detection (LOD) was 8 ng/ml. The specificity of the assay was evaluated by analyzing blank samples of rats and no impurity or endogenous interferences were found. Therefore, the proposed method can be the basis for a highly sensitive and specific method for the quantitative determination of anisodine and its metabolites.

4. Conclusion

For the first time, *in vivo* and *in vitro* metabolism of anisodine was studied extensively. Metabolites were resolved, identified and characterized using LC–MS^n ion trap with electrospray ionization in the positive ion and negative ion modes. Three new metabolites were found in rat urine and four, five, two, two metabolites were identified in rat plasma, feces, intestinal bacteria and liver homogenate, respectively.

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