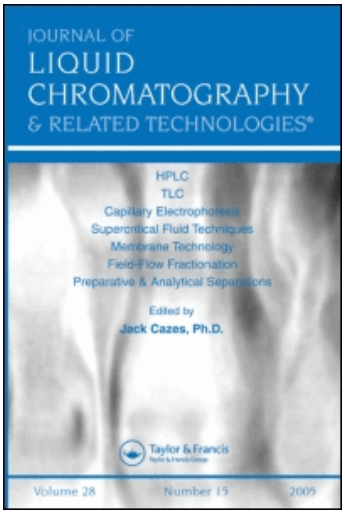


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Characterization of *in vivo* and *in vitro* Metabolic Pathway of Anisodamine by Liquid Chromatography-Tandem Mass Spectrometry

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**To cite this Article** Chen, Yong , Du, Peng , Han, Fengmei and Chen, Huaixia(2007) 'Characterization of *in vivo* and *in vitro* Metabolic Pathway of Anisodamine by Liquid Chromatography-Tandem Mass Spectrometry', Journal of Liquid Chromatography & Related Technologies, 30: 13, 1933 — 1949
**To link to this Article: DOI:** 10.1080/10826070701386538
**URL:** http://dx.doi.org/10.1080/10826070701386538

*Journal of Liquid Chromatography & Related Technologies*®, 30: 1933–1949, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701386538



# Characterization of *in vivo* and *in vitro* Metabolic Pathway of Anisodamine by Liquid Chromatography-Tandem Mass Spectrometry

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**Abstract:** *In vivo* and *in vitro* metabolisms of anisodamine were investigated using a highly specific and sensitive LC-$MS^n$ method. Feces, urine, and plasma samples were collected individually after ingestion of 25 mg/kg anisodamine to healthy rats. Rat feces and urine samples were cleaned up by a liquid-liquid extraction and a solid phase extraction procedure ($C_{18}$ cartridges), respectively. Methanol was added to rat plasma samples to precipitate plasma proteins. Anisodamine was incubated with homogenized liver and intestinal flora of rats *in vitro*, respectively. The metabolites in the incubation solution were extracted with ethyl acetate. Then, these pretreated *in vivo* and *in vitro* samples, were injected into a reversed-phase $C_{18}$ column with mobile phase of methanol/0.01% triethylamine solution (adjusted to pH 3.5 with formic acid) (60:40, v/v) and detected by an on-line $MS^n$ system. Identification and structural elucidation of the metabolites were performed by comparing their changes in molecular masses ($\Delta$M), retention times and full scan $MS^n$ spectra with those of the parent drug. Fifteen new metabolites (aponoranisodamine, apoanisodamine, methoxyanisodamine, hydroxy-methoxyanisodamine, trihydroxyanisodamine, dimethoxyanisodamine, dihydroxy-methoxyanisodamine, tetrahydroxyanisodamine, hydroxy-dimethoxyanisodamine, trihydroxy-methoxyanisodamine, dihydroxy-dimethoxyanisodamine, tetrahydroxy-methoxyanisodamine, trihydroxy-dimethoxyanisodamine, dihydroxy-trimethoxyanisodamine, and hydroxy-tetramethoxyanisodamine) were identified in rat urine after

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ingesting anisodamine. Seven metabolites (nor-6$\beta$-hydroxytropine, 6$\beta$-hydroxytropine, tropic acid, aponoranisodamine, apoanisodamine, noranisodamine, and anisodamine *N*-oxide) and the parent drug were detected in rat feces. Six metabolites (nor-6$\beta$-hydroxytropine, 6$\beta$-hydroxytropine, tropic acid, apoanisodamine, hydroxyanisodamine, and anisodamine *N*-oxide) and the parent drug are detected in rat plasma. Only apoanisodamine was detected in the homogenized liver incubation mixture. The hydrolyzed metabolites (6$\beta$-hydroxytropine and tropic acid) and the dehydrated metabolite of anisodamine were found in the rat intestinal flora incubation mixture.

**Keywords:** Anisodamine, LC-MS$^n$, Metabolite, *In vivo* metabolism, *In vitro* metabolism, Structural elucidation



## INTRODUCTION

Anisodamine, tropic acid-6$\beta$-hydroxide-3$\alpha$-tropic ester, was a kind of tropane alkaloid extracted from the leaves of traditional Chinese medicine *Anisodus tanguticus* (its synthetic form is called 654-2). It has widespread activities, such as spasmolytic, anaesthetic, acesodyne, and ophthalmic effects, and it is often used to treat transmissible shock, hepatitis, nephritis, sugar diabetes, etc.[1–3] In China, anisodamine was tentatively used to treat patients for SARS (Severe Acute Respiratory Syndrome) in 2003. Despite its important therapeutical values, its metabolism is not yet clear. Until now, studies only focused on the quantitative determination of anisodamine in rabbit and human serum by means of thin layer chromatography,[4] micellar liquid chromatography,[5] and reversed phase-high performance liquid chromatography.[6,7]

In the past, GC with electron capture detection or nitrogen phosphorus detection and HPLC with ultraviolet (UV) spectrophotometric detection, fluorescence detection, or electrochemical detection (ECD) were the main methods to detect drugs and their major metabolites *in vivo*. But these technologies cannot provide high enough sensitivity, specificity, and molecular structural information for the qualitative assay of drugs and their metabolites. The coupled GC-MS technology can overcome these insufficiencies, but it often requires time consuming derivatization of the target compound.[8,9] Therefore, this method is not suitable for the detection of drug metabolites.

Since the introduction of atmospheric pressure ionization (API) interfaces, LC-MS has been increasingly used to determine drugs and their metabolites for preclinical and clinical studies.[10–12] LC-MS allows for the analyses of thermolabile, highly polar, and non-volatile metabolites, owing to its soft ionization technique and high sensitivity, and the target compounds can be directly determined in mixtures without complicated sample preparation or derivatization. Compared with LC-MS, LC-MS$^n$ can give us the maximum amount of structural information and high specificity for qualitative analysis at trace levels. It has been proven to be a powerful approach for the metabolic identification of drugs.[13–17] Therefore, the LC-MS$^n$ technique is frequently the initial choice for metabolite detection and identification.

Structural elucidation of drug metabolites using LC-$MS^n$ is based on the premise that metabolites retain the substructures of the parent drug molecule. MS-MS product ion spectrums of each metabolite provide detailed substructural information of its structure. Using the product ion spectrum of a parent drug as a substructural template, metabolites presented in crude mixtures may be rapidly identified and detected based on their changes in molecular masses ($\Delta M$) and spectral patterns of product ions, even without standards for each metabolite.[18–20]

We have applied the LC-$MS^n$ technique to identify the metabolites of anisodamine in rats.[21] Eleven metabolites (nor-6$\beta$-hydroxytropine, 6$\beta$-hydroxytropine, tropic acid, noranisodamine, hydroxyanisodamine, anisodamine *N*-oxide, hydroxyanisodamine *N*-oxide, glucuronide conjugated noranisodamine, sulfate conjugated and glucuronide conjugated anisodamine, sulfate conjugated hydroxyanisodamine) were found in the rat urine. The results showed that the major metabolic pathway of anisodamine in the rat was hydrolysis, demethylation, hydroxylation, and sulfate and glucuronide conjugates.

For studying the metabolism comprehensively, this work presents the metabolites of anisodamine in rat feces, urine, and plasma after administration. The present study also involves incubation of anisodamine with intestinal flora and homogenized liver in order to clarify its *in vivo* and *in vitro* metabolic pathway. Fifteen new metabolites were found in rat urine after ingesting 25 mg/kg anisodamine. The parent drug and its 7, 6 metabolites were found in rat feces and plasma, respectively. Only 1 and 3 metabolites of anisodamine were identified in rat homogenized liver and intestinal flora incubation mixtures *in vitro*, respectively. For the first time, these metabolites detected in rat feces, urine, plasma, intestinal flora, and homogenized liver incubation solutions. To the best of our knowledge, the metabolism of anisodamine has not been studied as extensively before.



## EXPERIMENTAL

### Chemicals and Reagents

Anisodamine was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). Methanol was of HPLC grade (Fisher chemical Co., Inc, CA, USA). Distilled water, prepared from demineralised water, was used throughout the study. Other reagents used were of analytical grade.

### Instrumentation, Chromatographic and Mass Spectrometric Conditions

A reversed-phase column (AICHROM$^{TM}$ ReliAsil C18, 5 $\mu$m, 2 $\times$ 150 mm I.D., Agilent Technologies, Palo Alto, CA, USA) was connected with

a guard column (cartridge 4.6 mm × 12.5 mm, 5 μm, Agilent Technologies) filled with the same packing material to separate the anisodamine and its metabolites in rat urine. After optimizing the column temperature and the acidity of the mobile phase using the anisodamine standard, the temperature of the column was set at 40°C. The mobile phase consisted of methanol and 0.01% triethylamine solution (adjusted to pH 3.5 with formic acid) (60:40, v/v), which was eluted at a flow rate of 0.2 mL/min during the whole run.

LC-MS and LC-$MS^n$ experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer with a TSP4000 HPLC pump and a TSP AS3000 autosampler (all components from Finnigan, Austin, TX, USA). Mass spectral analyses were carried out using electrospray ionization (ESI) in positive ion detection mode, and only the structures of nor-6$\beta$-hydroxytropine, 6$\beta$-hydroxytropine, and phase II metabolites were validated by LC-MS ($MS^n$) in the negative ion detection mode. Nitrogen was used as the sheath gas (40 arbitrary units). A typical source spray voltage of 5.0 kV, a capillary voltage of 45 V, and a heated capillary temperature of 200°C were obtained as optimal control conditions. The other parameters, including the voltages of octapole offset and tube lens offset, were optimised for maximum abundance of the ions of interest. The $MS^n$ product ion spectra were produced by collision induced dissociation (CID) of the protonated molecular ion $[M + H]^+$ of analytes at their respective HPLC retention times utilizing helium in the ion trap, and the isolation width ($m/z$) was 1. The collision energy for each ion transition was optimized to produce the highest intensity of the selected ion peak. The optimized relative collision energy of 30% was used for all $MS^n$ works.

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, China) was used to centrifuge urine samples. The urine samples were extracted on ODS-18 solid-phase extraction cartridges (3 mL/200 mg, AccuBond$^{II}$, Agilent Technologies, Palo Alto, CA, USA). The intestinal incubation experiments were carried out in anaerobical incubation bags (AnaeroPouch$^{TM}$-Anaero 08G05A-23, Mitsubishi Gas Chemical Company, Inc.) using anaerobical generating bags (Mitsubishi Gas Chemical Company, Inc.).

## *In Vivo* Sample Preparation

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 25 mg/kg oral gavage doses of anisodamine. 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 tubes, 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 in it. The supernatant ethyl acetate layers were decanted, pooled, and evaporated at 37°C under nitrogen. The residue was redissolved in 500 μL of mobile phase and filtered through a 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 $C_{18}$ 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 membranes and an aliquot of 10 μL was used for LC-$MS^n$ analyses.

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



## *In Vitro* Sample Preparation

### Preparation of Anaerobical Cultural Solutions[22]

Solution A of 37.5 mL (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-ascorbie acid, 1 g eurythrol, 1 g tryptone, and 1 g nutrient agar were mixed together, and diluted to 1 L with distilled water. 2 M 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 rats (200 g). Samples were homogenized with a glass rod in anaerobical cultural solution at the rate of 0.5 g:1.5 mL immediately. Then, the homogenates were filtrated using gauze. Anisodamine was added into above intestinal flora cultural solution to a final concentration of 50 μg/mL. The culture dishes were put in anaerobical incubation bags. The out bags of anaerobical generating bags were opened, and put into anaerobical 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. The residues were reconstituted in 0.6 mL of mobile phase, centrifuged at 13000 *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 AM and noon. A weighed amount of liver was rapidly placed on ice. It was rinsed twice with saline and immediately minced with scissors and homogenated in ice cold Krebs-Henseleit buffer (pH 7.4)[23] 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 a spectrophotometer.[24] Anisodamine was added to the liver homogenate to the 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 in all experiments. The incubation was terminated and extracted (twice) with equal volume of ethyl acetate. The organic extracts were merged and evaporated at 37°C under a slow stream of nitrogen. The residues were reconstituted in 0.6 mL of mobile phase, centrifuged at 13000 *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.



## RESULTS AND DISCUSSION

The *in vivo* and *in vitro* metabolism pathway of anisodamine was investigated. Blank samples and substrate were analyzed for identifying the metabolites in biological samples.

### LC-MS and LC-$MS^n$ Analyses of Substrate

The chromatographic and mass spectrometry conditions were optimized using anisodamine standards. Full scan mass spectral analysis of anisodamine showed a protonated molecular ion of $m/z$ 306 (Figure 1A). The MS-MS product ion spectrum of the protonated molecular ion ($m/z$ 306) and the predominant fragmentation patterns were shown in Figure 1B. Anisodamine was eluted at 3.29 min (Figure 1C). Fragmentation of the protonated molecular ion of anisodamine in the ion trap leads to five main product ions at $m/z$: 288, 276, 140, 122, and 91. The product ions at $m/z$ 288 and 276 were formed by the loss of $H_2O$ and HCHO from the parent ion at $m/z$ 306, respectively. The most abundant product ion at $m/z$ 140 was formed by the loss of tropic acid ($C_9H_{10}O_3$, 166 Da). The $m/z$ 122 ion was inferred to be produced by the loss of 184 Da ($C_9H_{10}O_3 + H_2O$). The product ion at $m/z$ 91 was formed by

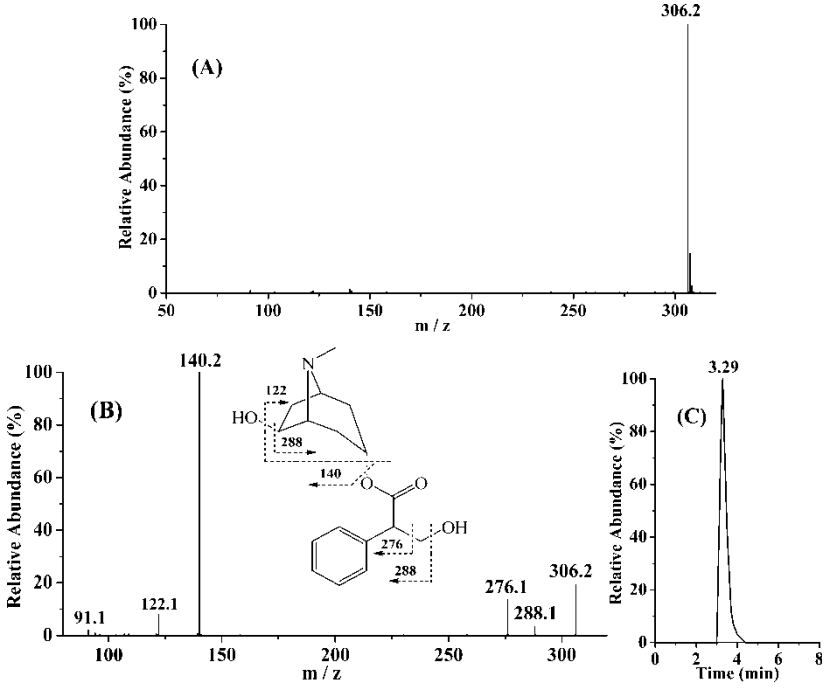


***Figure 1.*** (A) Full scan MS spectrum; (B) Full scan $MS^2$ product ions spectrum and the predominant fragmentation patterns; (C) LC-$MS^2$ chromatogram of anisodamine.

the loss of $NH_2CH_3$ (31 Da) from the $m/z$ 122 ion. It could be concluded that the ions at $m/z$ 140, 122, and 91 were the characteristic product ions of anisodamine, and $C_9H_{10}O_3$ (166 Da) and $C_9H_{12}O_4$ (184 Da) were the characteristic neutral losses. These characteristic product ions and neutral losses were the key features that allowed identification of metabolites.

## *In Vivo* Metabolism Studies

In order to identify the metabolites, the possible structures of the metabolites have been speculated, firstly, according to the metabolism rule of drugs.[25] The full scan mass spectrum of rat feces after administration of anisodamine was compared with those of blank feces samples and anisodamine solution to find the possible metabolites in rat urine. Then, these compounds were analyzed by LC-$MS^n$. Their retention times, changes in observed mass ($\Delta M$), and spectral patterns of product ions, were compared with those of the anisodamine standard to identify metabolites and elucidate their structures.

Identification of Metabolites in Rat Feces

Based on the method mentioned above, the parent drug and its metabolites were found in rat feces after administration of anisodamine. Their protonated molecular ions ($[M + H]^+$) were at $m/z$ 144, 158, 274, 288, 292, 306, and 322, respectively. The retention times and MS-MS product ion spectra of these analytes were shown in Figure 2. Among them, the retention time, the MS and MS-MS spectra of the protonated molecular ion at $m/z$ 306 (M0, Figure 2F) were the same as those of the anisodamine standard. Therefore, M0 can be confirmed as the unchanged parent drug.

The characteristic product ions at $m/z$ 140 and 122 of the parent drug appeared in the $MS^2$ spectrum of $m/z$ 158 (M2, Figure 2B). The $MS^3$ spectra of $m/z$ 158 → 140 and $m/z$ 158 → 122 was the same as those of $m/z$ 306 → 140 and $m/z$ 306 → 122 (anisodamine standard), respectively. Therefore, M2 was identified as the hydrolysis product of anisodamine (6$\beta$-hydroxytropine).

The protonated molecular ion at $m/z$ 144 (M1) and its daughter ions at $m/z$ 126, 116, 98, and 84 (Figure 2A) were all 14 Da less than the protonated

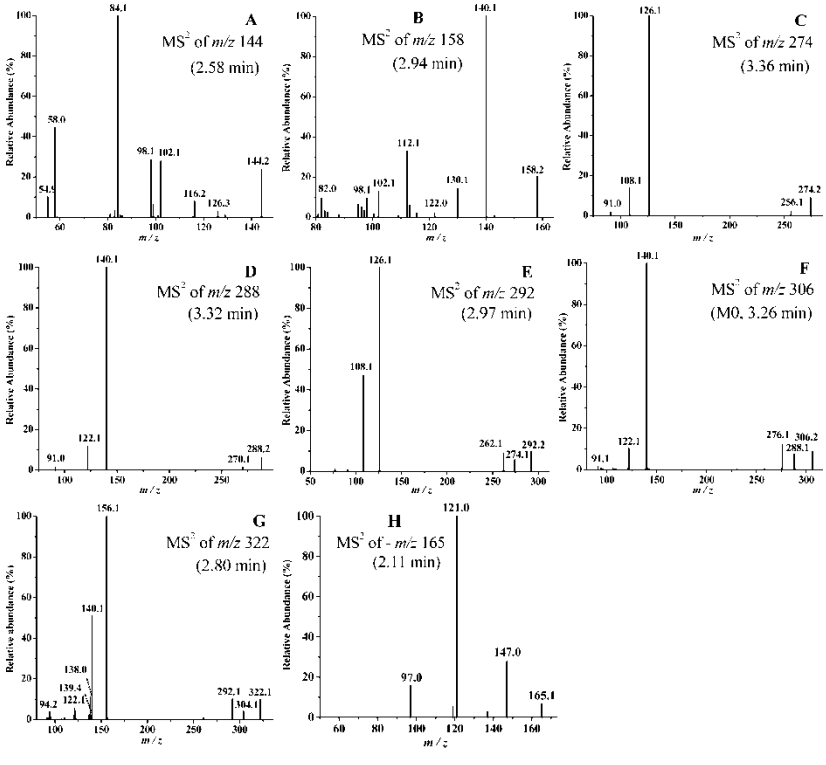


***Figure 2.*** MS-MS product ion spectra of anisodamine and its metabolites in rat feces.

molecular ion at $m/z$ 158 (M2) and its daughter ions at $m/z$ 140, 130, 112, and 98, respectively. These results indicated that M1 should be the *N*-demethyl product of M2 (nor-6$\beta$-hydroxytropine).

The characteristic product ions of $m/z$ 140, 122, and 91 appeared in the $MS^2$ spectrum of the protonated molecular ion at $m/z$ 288 (M4, Figure 2D), which was decreased by 18 Da compared to that of the unchanged anisodamine. The result indicated that M4 should be the dehydrated metabolite of anisodamine (apoanisodamine).

The characteristic product ions at $m/z$ 91 appeared in the $MS^2$ spectrum of the protonated molecular ion at $m/z$ 274 (M3), and the molecular ion at $m/z$ 274 and its daughter ions at $m/z$ 256, 126, and 108 (Figure 2C) were all 14 Da less than $m/z$ 288 and its daughter ions $m/z$ 270, 140, and 122, respectively. Therefore, M3 could be idendifed as the *N*-demethyl product of M4 (aponoranisodamine).

The $m/z$ 292 ion (M5, Figure 2E) and its daughter ions at $m/z$ 274, 262, 126, and 108 were all 14 Da less than the protonated molecular ion of the parent drug ($m/z$ 306) and its daughter ions at $m/z$ 288, 276, 140, and 122. Thus, M5 should be the *N*-demethyl product of anisodamine (noranisodamine).

The protonated molecular ion at $m/z$ 322 was increased by 16 Da compared to that of the unchanged anisodamine. The appearances of the predominant product ion at $m/z$ 156 (140 + 16) and a pair of product ions at $m/z$ 139 (156−17), $m/z$ 138 (156−18) in the $MS^2$ spectrum of the protonated molecular ion of M6 (Figure 2G) indicated that M6 should be the *N*-oxidation product of the parent drug (anisodamine *N*-oxide), because it is the cleavage feature of *N*-oxides to lose 17 Da and 18 Da. P.Z. Chong[26] theoretically expounded the fragmentation feature of *N*-oxide: losing 17, 18 Da from the parent molecule. The fragmentation feature has been validated by using oxymatrine in our experiment.

The $m/z$ 165 ion (M7) appeared in the negative ion full scan LC-MS spectrum of the feces samples. The appearances of the product ions at $m/z$ 147 ($[M\text{-}H\text{-}H_2O]^-$) and 121 ($[M\text{-}H\text{-}CO_2]^-$) indicated that M7 should be the hydrolysis product of anisodamine (tropic acid). No sulfate or glucuronide conjugate of M7 was found in rat feces.

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

## Identification of Metabolites in Rat Urine

We have identified 11 metabolites (nor-6$\beta$-hydroxytropine, 6$\beta$-hydroxytropine, noranisodamine, tropic acid, hydroxyanisodamine, anisodamine *N*-oxide, hydroxyanisodamine *N*-oxide, glucuronide conjugated noranisodamine,

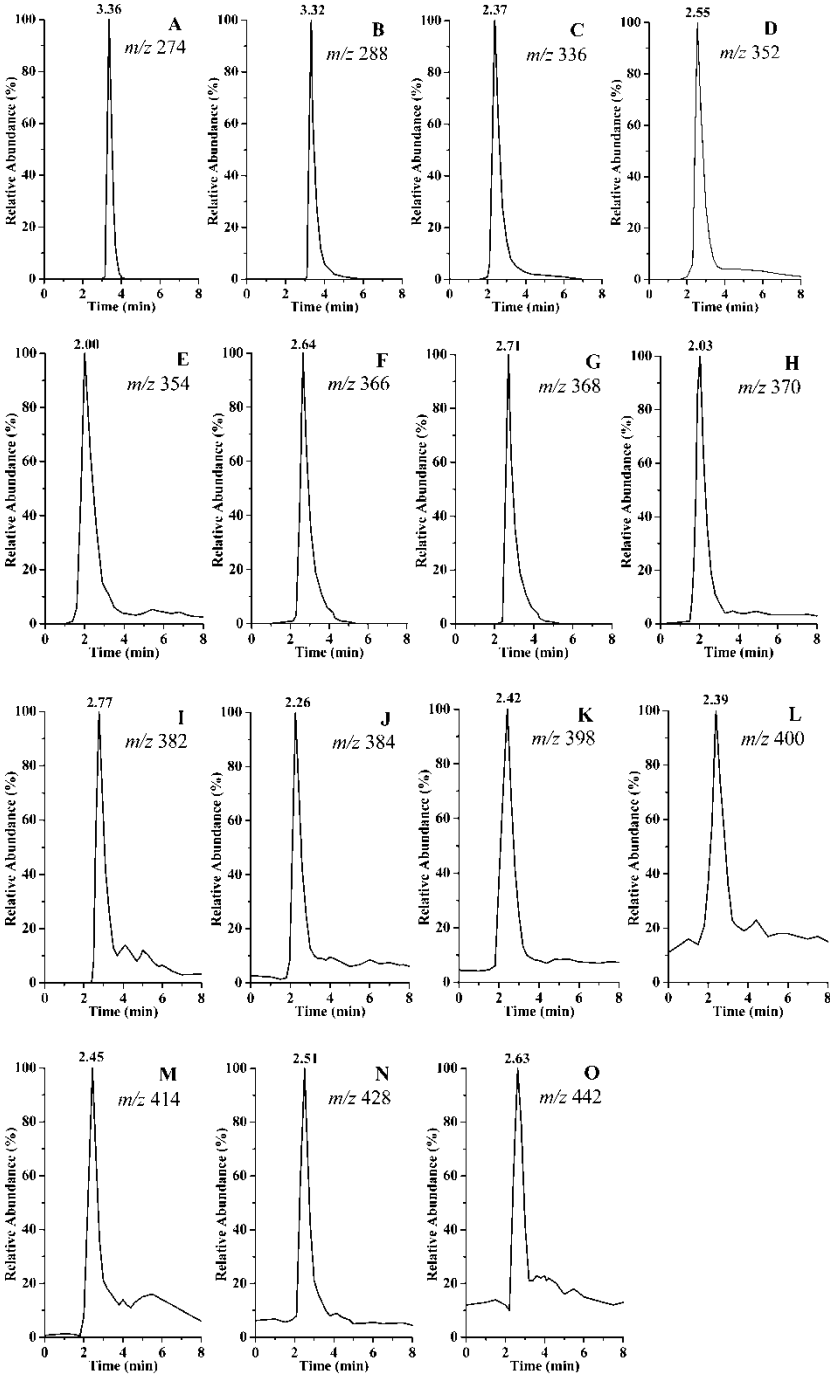


***Figure 3.*** LC-MS$^2$ chromatograms of 15 new metabolites in rat urine.

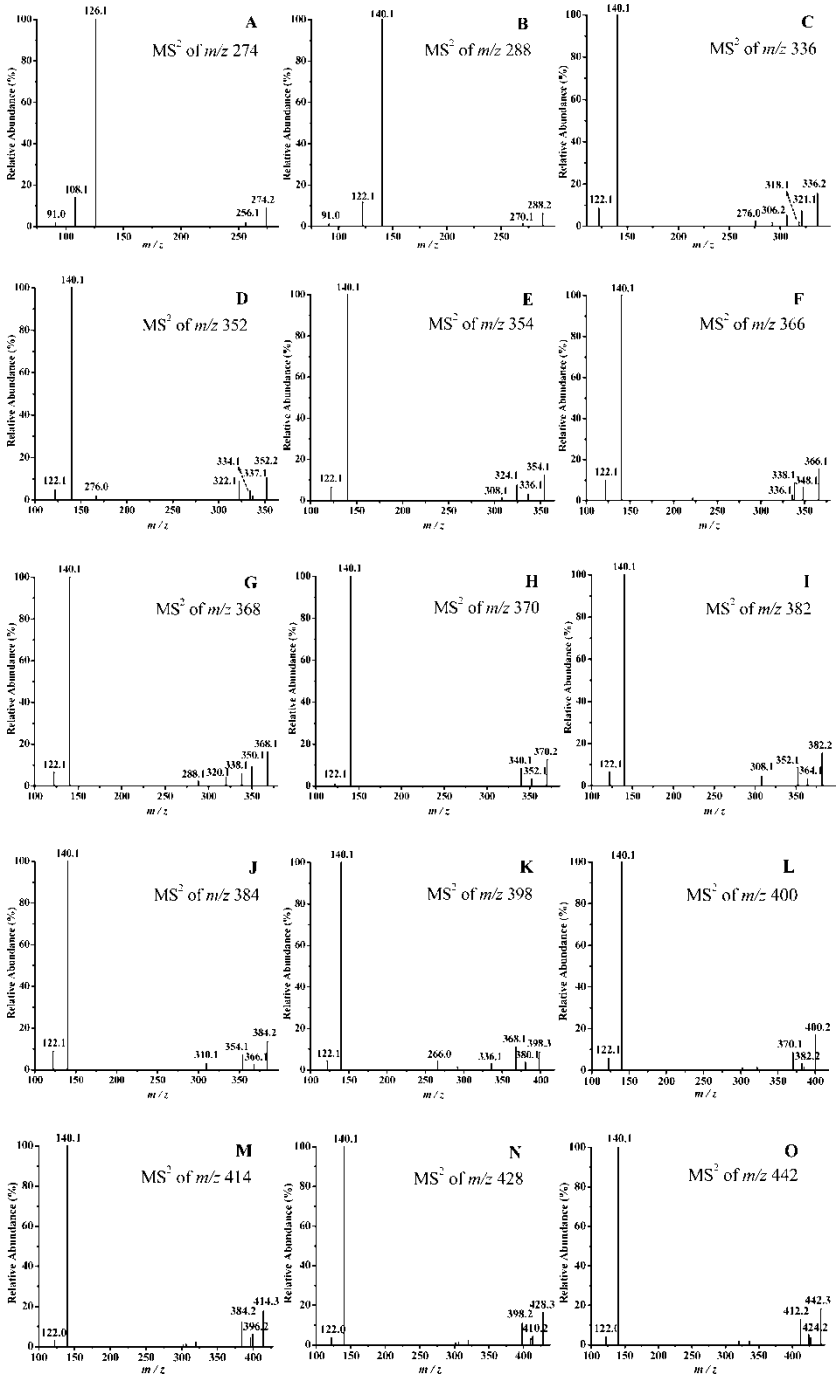


***Figure 4.*** MS-MS product ion spectra of 15 new metabolites in rat urine.

sulfate conjugated and glucuronide conjugated anisodamine, sulfate conjugated hydroxyanisodamine) and the parent drug in rat urine, after ingesting anisodamine to healthy rats. In our study, 15 new metabolites were found in rat urine. Their protonated molecular ions were at $m/z$ 274, 288, 336, 352, 354, 366, 368, 370, 382, 384, 398, 400, 414, 428, and 442, respectively. LC-$MS^2$ chromatograms and MS-MS spectra of these analytes were presented in Figure 3 and Figure 4, respectively. Among them, $m/z$ 274, 288 were identified as aponoranisodamine and apoanisodamine based on the above method.

The characteristic product ions at $m/z$ 140, 122 of the parent drug appeared in the MS-MS spectra of $m/z$ 336 (Figure 4C), 352 (Figure 4D), 354 (Figure 4E), 366 (Figure 4F), 368 (Figure 4G), 370 (Figure 4H), 382 (Figure 4I), 384 (Figure 4J), 398 (Figure 4K), 400 (Figure 4L), 414 (Figure 4M), 428 (Figure 4N), and 442 (Figure 4O), respectively. Also, there was no $m/z$ 140 $(124 + 16)$ ion in their $MS^2$ spectra. The dehydrated fragment ions ($[M + H\text{-}H_2O]^+$) at $m/z$ 318, 334, 336, 338, 350, 352, 364, 366, 380, 382, 396, 410, and 424 existed in their $MS^2$ spectra, respectively. Therefore, they were phenolic metabolites. They were increased by 30 Da, 46 $(16 + 30)$ Da, 48 $(16 \times 3)$ Da, 60 $(30 \times 2)$ Da, 62 $(16 \times 2 + 30)$ Da, 64 $(16 \times 4)$ Da, 76 $(16 + 30 \times 2)$ Da, 78 $(16 \times 3 + 30)$ Da, 92 $(16 \times 2 + 30 \times 2)$ Da, 94 $(16 \times 4 + 30)$ Da, 108 $(16 \times 3 + 30 \times 2)$ Da, 122 $(16 \times 2 + 30 \times 3)$ Da, and 136 $(16 + 30 \times 4)$ Da compared to that of unchanged anisodamine. Thus, they could be affirmed as methoxyanisodamine, hydroxy-methoxyanisodamine, trihydroxyanisodamine, dimethoxyanisodamine, dihydroxy-methoxyanisodamine, tetrahydroxyanisodamine, hydroxy-dimethoxyanisodamine, trihydroxy-methoxyanisodamine, dihydroxy-dimethoxyanisodamine, tetrahydroxy-methoxyanisodamine, trihydroxy-dimethoxyanisodamine, dihydroxy-trimethoxyanisodamine and hydroxy-tetramethoxyanisodamine, respectively.

## Identification of Metabolites in Rat Plasma

According to the method mentioned above, the parent drug and its 6 metabolites were identified in rat plasma after administration of atropine. They

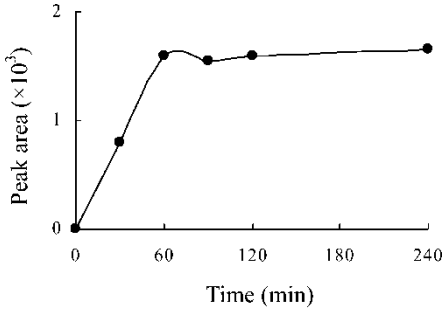


***Figure 5.*** The proposed metabolic pathway of anisodamine in rats.

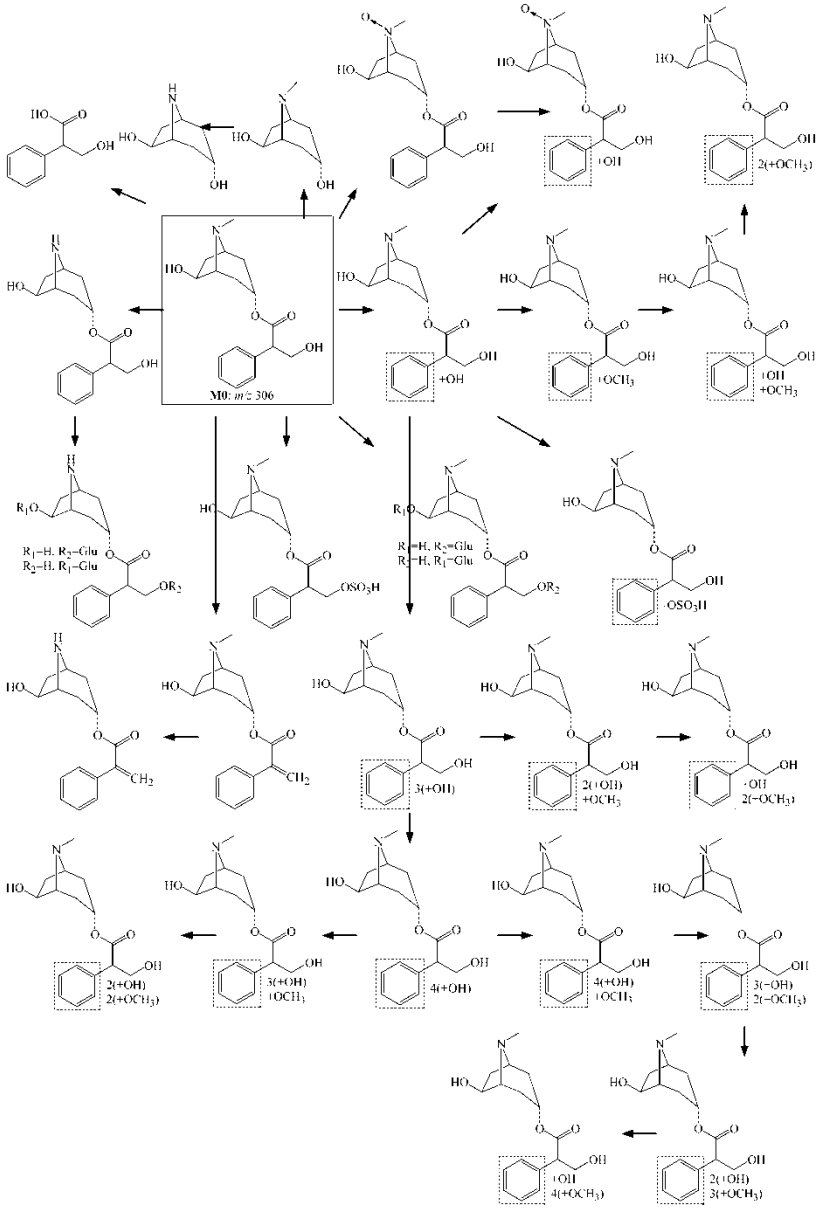


***Figure 6.*** Time-area curves of metabolites of anisodamine in homogenized liver of rats.

were nor-6$\beta$-hydroxytropine, 6$\beta$-hydroxytropine, tropic acid, apoanisodamine, hydroxyanisodamine, and anisodamine *N*-oxide.

The LC-MS$^2$ analysis of plasma samples showed that the parent drug and nor-6$\beta$-hydroxytropine, 6$\beta$-hydroxytropine, apoanisodamine existed in

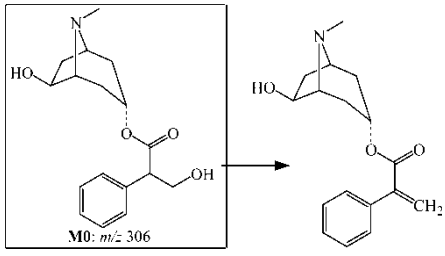


***Figure 7.*** Proposed metabolic pathway of anisodamine in rat intestinal flora.

plasma between 0.25 and 24 h after administration. However, hydroxyanisodamine, anisodamine *N*-oxide, and tropic acid only appeared in plasma between 0.5 and 24 h.

Based on the above discussion and combining the results we have reported,[21] the proposed metabolic pathway of anisodamine in rats was represented in Figure 5.

## *In Vitro* Metabolism Studies

The substrate was incubated with liver homogenate and intestinal fractions of rats; substrate was observed. However, only apoanisodamine was detected in the rat liver homogenate incubation. Figure 6 presented the time area curves of metabolite of anisodamine in the homogenized liver of rats. 6$\beta$-Hydroxytropine, apoanisodamine, and tropic acid were found in the intestinal part. The result showed that the metabolism of atropine was very weak in rat intestinal bacteria. The metabolic pathways of anisodamine in rat homogenated liver and intestinal bacteria were shown in Figure 7 and Figure 8.

In this work, the sensitivity of the method was determined using anisodamine standard, and its limit of detection (LOD) was lower than

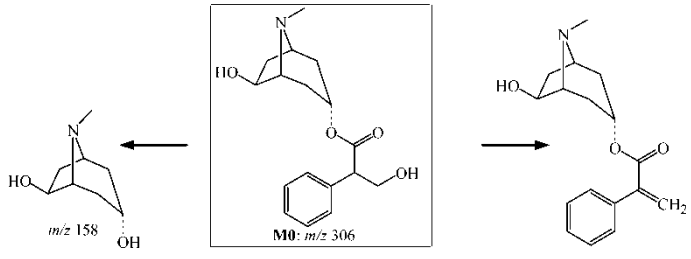


***Figure 8.*** Proposed metabolic pathway of anisodamine in rat intestinal homogenized liver.

6 ng/mL by LC-$MS^n$. The specificity of the assay was evaluated by analyzing blank urine samples of rats; no impurity or endogenous interferences were found.

## CONCLUSIONS

In the present study, in vivo and in vitro metabolism of atropine has been studied extensively. Metabolites were resolved, identified, and characterized using the highly specific and selective technique of LC-$MS^n$ ion trap with electrospray ionization in the positive ion mode. For the first time, 10 new metabolites were found in rat urine after ingestion of atropine, and 9, 5, 2, 3 metabolites were identified in rat feces, plasma, liver homogenate, intestinal bacteria. Various extraction solvents were compared for investigation of the metabolism of atropine.



## ACKNOWLEDGMENTS

This paper was supported by the grant 020091130 (ky2006004) of specialist fund of Hubei University. The authors would like to thank their colleagues for their valuable technical assistance.

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Received December 1, 2006
Accepted December 22, 2006
Manuscript 6993