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Structural elucidation of in vivo metabolites of phencynonate and its analogue thiencynonate in rats by HPLC–ESI-MS^n

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ABSTRACT

The structural elucidation of the metabolites of phencynonate and its analogue thiencynonate in rats was performed by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS^n) in positive ion mode, by comparing their changes in molecular masses (ΔM), retention times and spectral patterns with those of the parent drug. Phencynonate and thiencynonate were easily biotransformed in vivo by the pathways of N-demethylated, oxidative, hydroxylated and methoxylated to form seventeen metabolites that retained the some features of the two parent molecules. These metabolites included ten phencynonate metabolites (N-demethyphencynonate monoxide, N-demethyhydroxy phencynonate, phencynonate monoxide, hydroxyphencynonate, phencynonate dioxide, methoxyphencynonate, dihydroxyphencynonate, dihydroxyphencynonate, hydroxymethoxy phencynonate, trihydroxyphencynonate) and seven thiencynonate metabolites (N-demethy thiencynonate, N-demethythiencynonate monoxide, N-demethyhydroxythiencynonate, thiencynonate monoxide, hydroxythiencynonate, hydroxythiencynonate monoxide, dihydroxy thiencynonate). The described method has wide applicability to rapidly screen and provide structural information of these metabolites. The identifications of precise structures of these metabolites need to be confirmed by other techniques such as the ^{1}H and ^{13}C NMR.

1. Introduction

Drug metabolism investigation plays an important role in the drug discovery, drug design and synthesis, drug action and clinical application. Phencynonate is a novel anticholinergic drug developed by the Beijing Institute of Pharmacology and Toxicology of China. Pharmacological evaluation and clinical practice have proved that phencynonate effectively prevents the motion sickness, and has good effect in the Meniere's disease, Parkinson's disease and the epilepsy. Especially, phencynonate prevents motion sickness with higher efficacy and lower central inhibitory as the low side effect compared to the other drugs against motion sickness drugs, such as scopolamine and dimenhydrinate, etc. [1–9]. Thiencynonate, as the novel candidate drug or the lead compound of phencynonate, is similar in the structure and feature to phencynonate. It is also proved that thiencynonate has some potent effects for the hypnotics in coordination with sub-threshold dose of pentobarbital, and inhibition of tracheobronchial contractile response to guinea pig and salivation induced oxotremorine [10]. Researches about phencynonate and its analogue thiencynonate are mainly focused on the synthesis, in vitro and in vivo quantification and pharmacokinetics, pharmacological actions and clinical use [11–20], up to present, no paper has been found to deal with the structural elucidation of the metabolites of phencynonate and thiencynonate by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS^n) in detail.

LC–MS method has been increasingly used to rapidly determine and identify the drugs and their metabolites for the high sensitivity and specificity. The LC–MS system is suitable for the analysis of the thermolabile, highly polar and non-volatile metabolites owing to its soft-ionization technique, and the target compounds can be directly determined and identified in the mixtures [21]. ESI-MS, as a versatile technique, has been applied in all stages of drug discovery and development, including drug target identification, library verification, and drug analysis and drug toxicology, especially in drug metabolism [22,23]. And hyphenated MS techniques are frequently the initial choice for drug metabolite detection and identification because of their sensitivity and convenience compared with other methods [24–26]. In this paper, a sensitive and specific LC–ESI-MS^n method was presented to identify phencynonate and thiencynonate and their metabolites, from the rat urines after administration these drugs, ten metabolites of phencynonate and seven metabolites of thiencynonate were elucidated. The structures of these metabolites were characterized on the basis of their precursor ions, product ions, and HPLC retention times. Finally, the biotransformation pathways of phencynonate and thiencynonate

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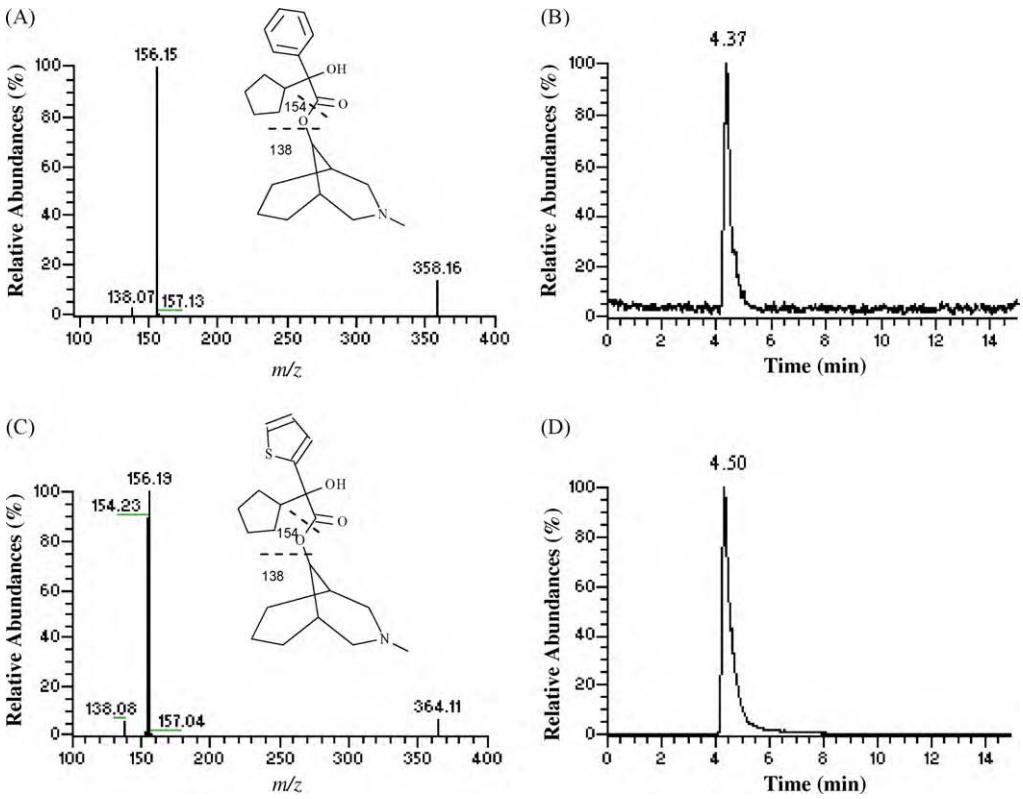

Fig. 1. (A) ESI-MS/MS product ion spectrum and the predominant fragmentation patterns of phencynonate; (B) LC–MS^2 chromatogram of phencynonate (parent ion at *m*/*z* 358); (C) ESI-MS/MS product ion spectrum and the predominant fragmentation patterns of thiencynonate; (D) LC–MS^2 chromatogram of thiencynonate (parent ion at *m*/*z* 364).

in rats were elucidated on the basis of in vivo metabolic studies. The data provides very important information for predicting the metabolic stability, developing a novel drug as well as lead compound and better use in clinical practice.

2. Experimental

2.1. Chemicals and reagents

Phencynonate {N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl)-2′-cyclopentyl-2′-hydroxyl-2′-phenylacetate} and its analogue thiencynonate {N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl)-2′-cyclopentyl-2′-hydroxyl-2′-thienylacetate} were synthesized and kindly supplied by the Beijing Institute of Pharmacology and Toxicology of China. The purity of phencynonate and thiencynonate were both more than 99%. Their chemical structures are shown in Fig. 1. Methanol was of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was of HPLC grade and purchased from Dikma Reagent Company (Beijing, China). Distilled water was prepared from demineralised water in our laboratory and used throughout this study. Other reagents and chemicals used were of analytical grade.

2.2. Apparatus and conditions

The liquid chromatography–mass spectrometry system consisted of an Agilent HPLC 1100 system (Palo Alto, CA, USA) and an LCQ Deca XP ion trap mass spectrometer was used for the analysis, which included an HP 1100 G1312A binary pump, G1379A vacuum degasser, and G1313A autosampler. Separations and determination of the analystes were achieved using a BetaMax Acid C_{18} reversed-phase column (150 mm × 2.1 mm i.d., 5 μm; Thermo Electron, CA, USA) at ambient temperature. After optimizing the analytical condition, the mobile phase used was a mixture of methanol/water containing 0.5‰ formic acid employing gradient elution (from10:90 to 25:75, v/v) at a flow rate of 0.1 ml/min during the whole run. The sample injection volume was 10 μl and the run time of samples was 15 min. The effluent was on-line transferred to the ESI/MS system without splitting.

Mass spectrometric experiments were performed on an LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an electrospray ion source working in positive ion mode. The instrument was connected to the LC system outlet. Nitrogen was used as a sheath gas and an aux/sweep gas in the ion trap. And ultra-high purity helium was used as the dampening gas in the ion trap. The LC–MS system was controlled by the Thermo Finnigan Chemstation software (Xcalibur version 1.3).

Mass spectral analyses were performed under the optimizing and automatic gain control conditions, using a sheath flow rate of 241.3 kPa, a typical source spray voltage of 5 kV, a capillary voltage of 41 V and a heated capillary temperature of 320 °C. The other parameters were also optimized for maximum abundances of the ions of the interests by the automatic tune procedure of the instrument. Selective reaction monitoring (SRM) was used to preselect the chromatographic peaks as the potential metabolites. The MS^n product ion spectra were produced by collision induced dissociation (CID) of the protonated molecule $[M+H]^+$ of all the analytes at their respective HPLC retention times utilizing helium in the ion trap, and the isolation width (*m*/*z*) was 1. The optimized relative collision energy of 30–35% was used for all MS^n works. The data acquisition was performed in full scan LC–MS and tandem MS modes.

2.3. Animal experiment and sample preparation

Male Sprague–Dawley rats (250 ± 20 g) were obtained from the Laboratory Animal Center of the Capital Medical University (LAC-

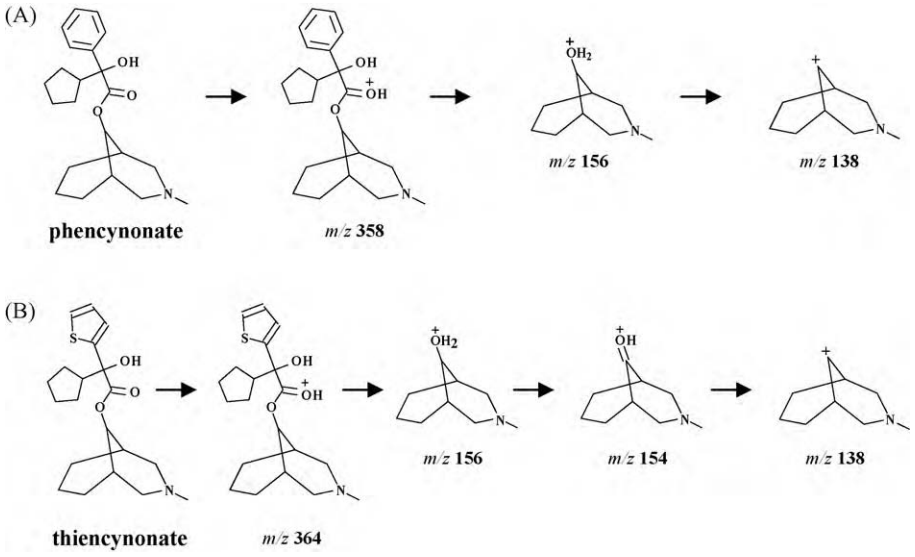

Fig. 2. Fragmentation pathways of phencynonate (A) and thiencynonate (B) in ESI-MS/MS.

CMU, Beijing, China). All studies on animals were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in China. The animal experimental protocols were approved by the LAC of CCMU. Rats were housed in metabolic cages for fasted, but freely available to water. Rats were intraperitoneally given a dose of 10 mg/kg of phencynonate and thiencynonate, respectively. Urine samples were collected from rats at pre-dose and 0–24 h post-dose, and stored at −20 °C before the sample extracted and analyzed.

An aliquot of 2 ml of mixed 0–24 h urine samples for LC–MS was loaded onto a C_{18} solid-phase extraction cartridge (SPE cartridge, 100 mg/ml, Abel Industries, USA) that was preconditioned with 2 ml of methanol and 1 ml of water. Then, the C_{18} SPE cartridge was washed with 4 ml of water and the analytes were eluted with 2 ml of methanol. The elution solutions were filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS analyses.

3. Results and discussion

The mass spectral patterns of the parent drugs served as the templates in elucidation of the structures of the proposed metabolites of phencynonate and its analogue thiencynonate. Determination of the metabolite structure was facilitated by the fact that the parent compound undergoes extensive and well definable fragmentation under MS–MS conditions.

3.1. LC–MS analyses of phencynonate and thiencynonate

This investigation involved the chromatographic and mass spectral properties of the parent drugs. The chromatographic and mass spectrometry conditions were optimized for maximum abundances of the ions of the interests of the parent drugs by the automatic tune procedure of the instrument. The first step in this work involved the characterization of chromatographic and mass spectral properties of phencynonate and thiencynonate, full scan mass spectral analyses for these two parents showed protonated molecules of *m*/*z* 358 and 364 from LC–ESI-MS, respectively. The MS/MS product ion spectra of the protonated molecules, the predominant fragmentation patterns and the LC–MS^2 chromatograms of phencynonate and thiencynonate were shown in Fig. 1A–D. Phencynonate and thiencynonate were eluted at 4.37 and 4.50 min under the experimental conditions. Fragmentation of the protonated molecule of phencynonate in the ion trap led to form the main ion series at *m*/*z*: 358, 156, and 138. And the main ion series of thiencynonate were at *m*/*z*: 364, 156, 154 and 138. Among these ion series the ions at *m*/*z* 156 and 138 were a pair of the characteristic product ions for both phencynonate and thiencynonate. The most abundant product ion at *m*/*z* 156 was the protonated N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl)-hydroxy fragment, which was produced via the loss of the neutral fragment $C_{13}H_{14}O_2$ (202 Da) for phencynonate, and the loss of the neutral fragment $C_{11}H_{12}O_2S$ (208 Da) for thiencynonate, respectively. The fragment ion ($C_{13}H_{14}O_2$) was consisted of the cyclopentyl group (C_5H_9, 69 Da), the phenyl group (C_6H_5, 77 Da) and the two neutral fragments (2CO, 56 Da) from the protonated molecule at *m*/*z* 358, phencynonate. The fragment ion ($C_{11}H_{12}O_2S$) was consisted of the cyclopentyl group (C_5H_9, 69 Da), the thienyl group (C_4H_4S, 84 Da) and the two neutral fragments (2CO, 28 + 28 Da) from the protonated molecule at *m*/*z* 364, thiencynonate. The ion at *m*/*z* 154 was formed by the loss of two protons from the ion at *m*/*z* 156. The ion at *m*/*z* 138 was generated by the loss of H_2O from the ion at *m*/*z* 156 or by the loss of O from the ion at *m*/*z* 154. The proposed fragmentation pathways of phencynonate (A) and thiencynonate (B) were shown in Fig. 2A and B. These characteristic product ions and neutral losses were the basis to identify the metabolites of these two drugs.

3.2. Identification of in vivo metabolites of phencynonate

In order to identify the metabolites of phencynonate, firstly, the possible structures of the metabolites have been speculated according to the metabolism rules of these drugs [27–29]. The full scan mass spectra of the free fractions in rat urine after administration of phencynonate were compared with those of blank rat urine samples and the drug standard solution to explore the possible metabolites. Selective reaction monitoring (SRM) was used to preselect the chromatographic peaks as potential metabolites. Identification and structural elucidation of these metabolites were performed by comparing their changes in molecular masses (ΔM), retention times and spectral patterns with those of the parent drug. Based on the method mentioned above, phencynonate and its metabolites were found in rat urine after administration of the drug. The LC–MS^2 chromatograms of phencynonate metabolites in rat urine were shown in Fig. 3. The eleven major protonated molecules ($[M+H]^+$) in ESI-MS were at *m*/*z* 358, 358, 360, 372, 374,

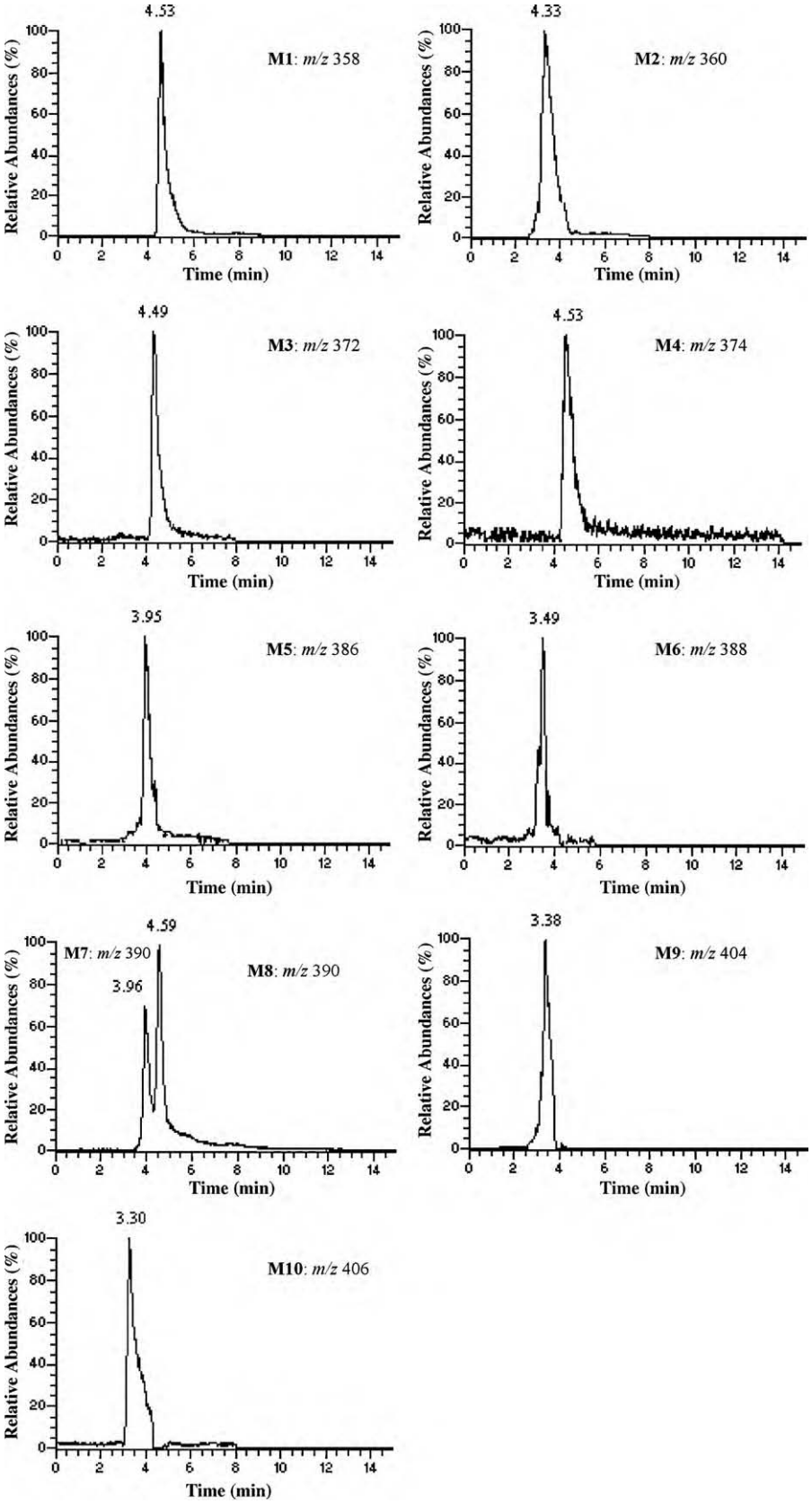

Fig. 3. LC–MS^2 chromatograms of phencynonate metabolites in rat urine.

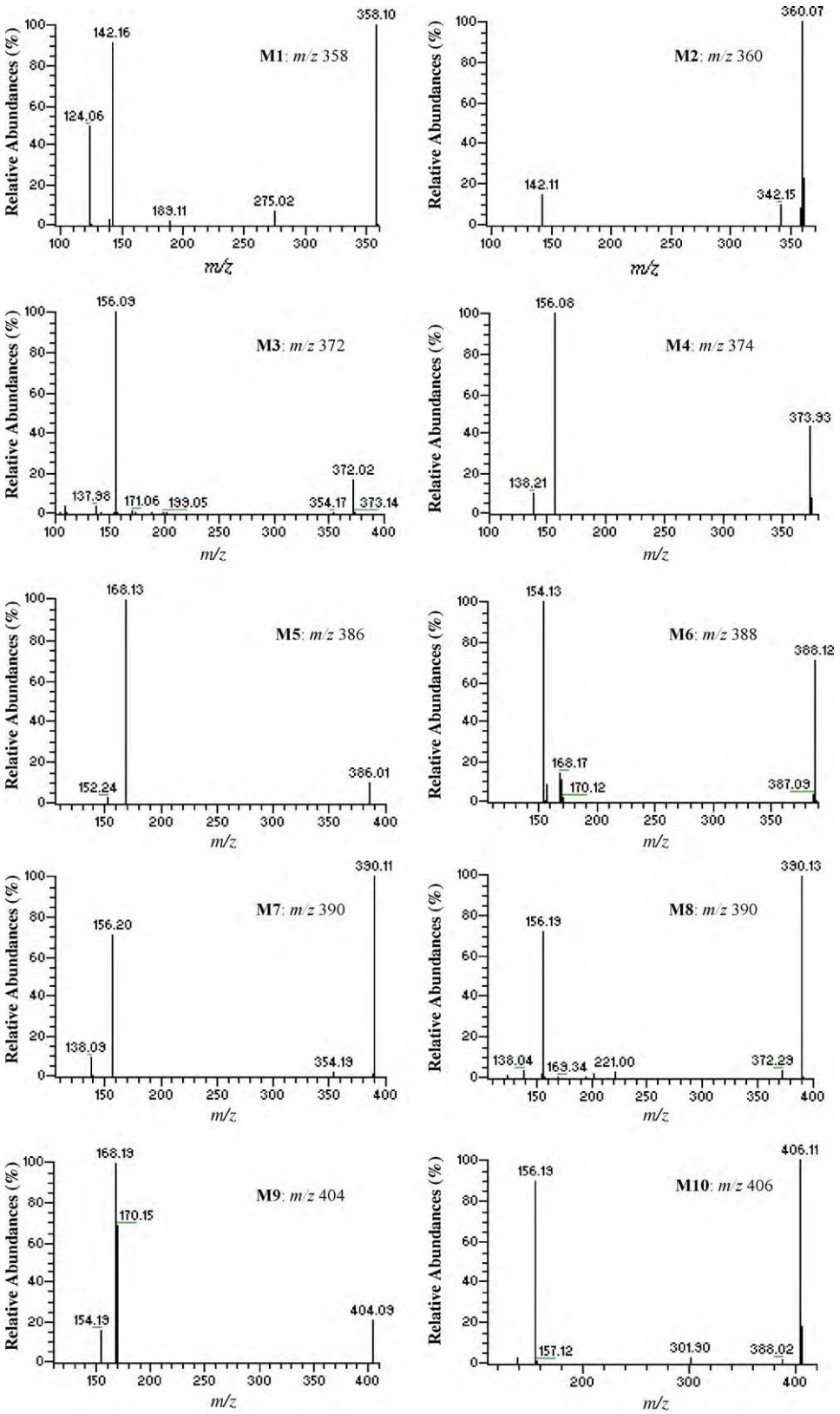

Fig. 4. MS–MS product ion spectra of phencynonate metabolites in rat urine.

386, 388, 390, 390, 404 and 406, respectively. The characteristic MS–MS product ion spectra of phencynonate metabolites in rat urine were presented in Fig. 4. LC–ESI-MS/MS mass spectra were obtained via fragmentation of protonated molecular ions that used for more precise structural identification of metabolites. Among them, the retention time, the MS and MS^2 spectra of the molecular ion at m/z 358 (M0) were the same as those of the parent drug. Therefore, M0 could be confirmed as the unchanged parent drug,

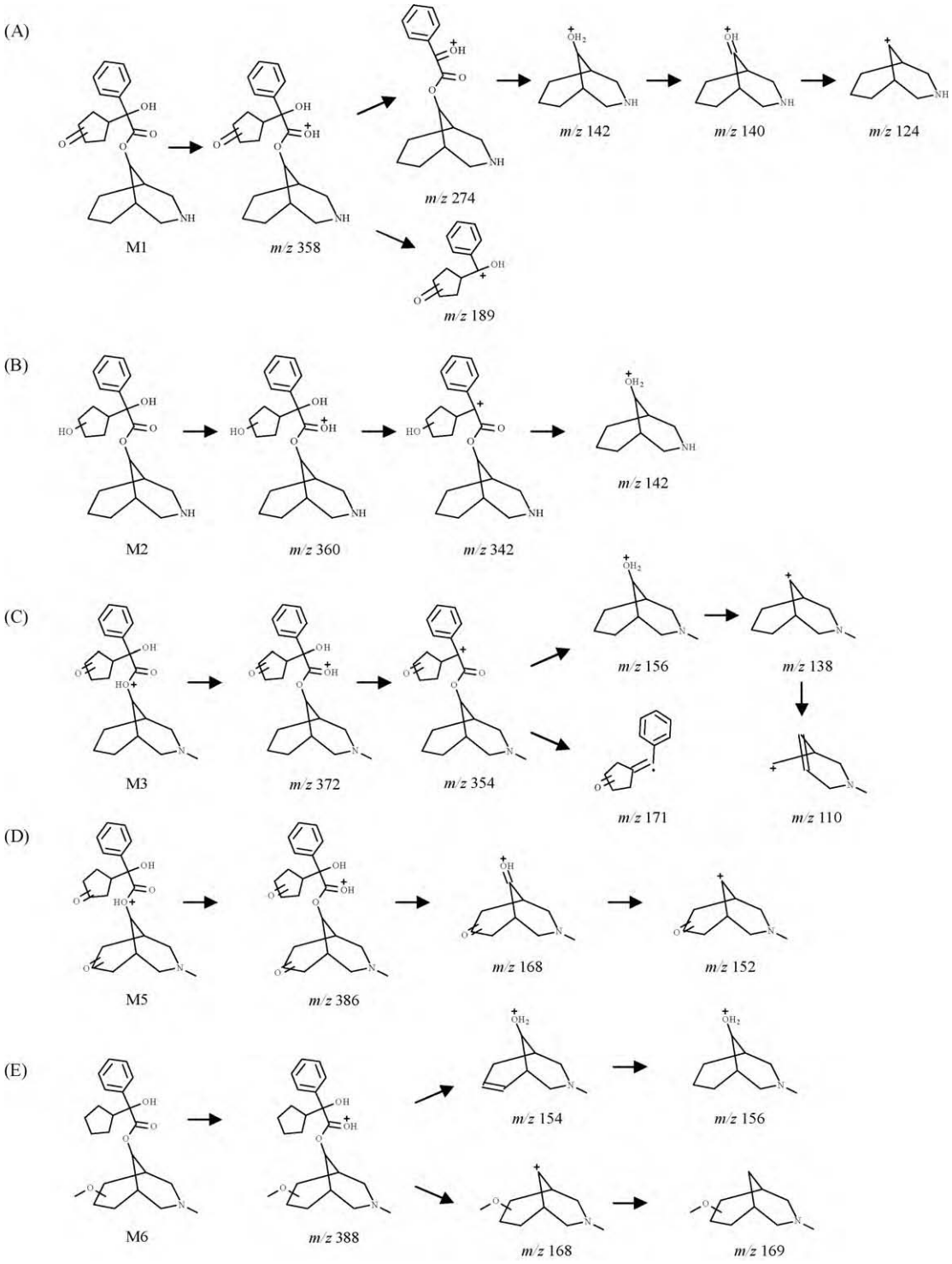

Fig. 5. Fragmentation pathways of phencynonate metabolites M1 (A), M2 (B), M3 (C), M5 (D) and M6 (E) in ESI-MS/MS.

phencynonate [18]. The fragmentation pathways of some metabolites M1 (A), M2 (B), M3 (C), M5 (D) and M6 (E) of phencynonate in ESI-MS/MS were presented in Fig. 5.

The mass spectrum of M1 was detected at a retention time of 4.53 min, and gave a protonated molecule $[M+H]^+$ at m/z 358 that had the same molecular weight to the parent compound. The protonated product ion series of M1 were m/z 275, 189, 142 and 124, and the characteristic product ions were m/z 142 and 124 in the MS^2 spectra, which were noticeably different from the protonated product ion series of the parent compound. It indicated that there were some changes at the cyclopentyl group of M1 compared to the parent drug. The product ion at m/z 275, which could be a radical that contains one nitrogen and unstable and in CID, was generated via loss of the cyclopentyl group (69 Da) and the O fragment at the cyclopentyl group from the protonated molecular ion at m/z 358. The product ion at m/z 189 was a carbocation, which was formed via loss of the azabicyclononanyl group (124 Da), the O fragment and the CO fragment from the protonated molecule at m/z 358. The product ion at m/z 142 was the protonated hydroxyl azabicyclononanyl fragment ion and the ion

was produced via the loss of the ion at *m/z* 189, the CO fragment and the O fragment from the protonated molecule at *m/z* 358. The product ion at *m/z* 124 was formed via the loss of one H_2O group from the ion at *m/z* 142 (see Fig. 5A). It indicated that the N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) skeleton lost one methyl group at the nitrogen atom site to produce the product ion at *m/z* 142 and 124, respectively, and the monooxidation reaction occurred in the cyclopentyl group (+14 Da). Besides, there was the evidence for supporting the cyclopentanone oxidation from the equal abundant product ions at *m/z* 189 (175 + 14) and *m/z* 194 (180 + 14) detected in the EI–MS spectra by coadministrating of equal dose of phencynonate and phencynonate-d_5 [27]. Based on these data and our works published [28,29], M1 was deduced as the N-demethylphencynonate monoxide that occurred at the cyclopentane ring.

M2 was detected at the retention time of 4.33 min and gave an protonated molecule $[M+H]^+$ at *m/z* 360. The ion at *m/z* 360 was increased by 2 Da compared to that of unchanged phencynonate. There also was the characteristic product ion at *m/z* 142 existed which the fragmentation feature was similar to that of M1 and formed by the loss of the ion at *m/z* 189, the CO fragment and the O fragment from the protonated molecular ion at *m/z* 358 (see Fig. 5B). The product ion at *m/z* 342 was 18 Da less than that of *m/z* 360 and was formed by the loss of H_2O from the ion *m/z* 360. The protonated parent ion at *m/z* 358 produced the metabolite M2 ion at *m/z* 360 by adding a hydroxyl group at the cyclopentyl part and losing a methyl group at the N site from the N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) skeleton. Based on these data and our works published [27–29], M2 was identified as the N-demethylhydroxyphencynonate at the cyclopentyl group.

The mass spectrum of M3, which was detected at a retention time of 4.49 min, gave a protonated molecule $[M+H]^+$ at *m/z* 372 that was increased by 14 Da compared to that of the unchanged drug. The characteristic product ions *m/z* 156 and 138 appeared in the MS^2 spectra of the M3. It indicated that there were no changes at the N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) skeleton. The product ion at *m/z* 354 was generated via the loss of the neutral fragment H_2O from the protonated molecular ion at *m/z* 372. The appearance of carbon radical at *m/z* 171 in the MS^2 spectrum at *m/z* 372 also indicated that the cyclopentyl part was oxidized. The carbon radical (*m/z* 171) was formed via the loss of the H_2O in the carbocation *m/z* 189 that was also existed in the structure of M1. This carbon radical (*m/z* 171) was also unstable (see Fig. 5C). We had the evidence for supporting the cyclopentanone oxidation from the equal abundant product ions at *m/z* 189 (175 + 14) and *m/z* 194 (180 + 14) detected in the EI–MS spectra by coadministrating of equal dose of phencynonate and phencynonate-d_5 [27]. Based on these data, M3 was identified as phencynonate monoxide at the cyclopentyl group.

M4, eluted at 4.53 min, gave rise to the protonated molecule $[M+H]^+$ at *m/z* 374. The molecular ion at *m/z* 374 was increased by 16 Da compared to that of the parent compound. The pair of ions of *m/z* 156 and 138 also existed in the molecular ions as the characteristic product ions of M4, which were the same as the characteristic ions of phencynonate. It indicated that there were no changes at the N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) skeleton. Based on these data and our works published [29], M4 was identified as monohydroxy phencynonate at cyclopentane ring.

The mass spectrum of M5, which was detected at a retention time of 3.95 min, gave a protonated molecule at *m/z* 386 that was increased by 28 Da (14 + 14 Da, dioxide product) compared to that of phencynonate. The MS^2 spectra at *m/z* 168 and 152 were the characteristic ion series of M5 (see Fig. 5D), the product ion at *m/z* 168 generated firstly via the increase of oxygen group in the N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) skeleton, and then the loss of the cyclopentyl group (69 Da), the phenyl group (77 Da) and the two fragments CH_2OH and CO, the product ion at *m/z* 152 was formed via the loss of one oxygen atom from the ion at *m/z* 168. Based on these data and our works published [27–29], M5 was deduced as phencynonate dioxide that occurred at the cyclopentane and the azabicyclononanyl skeleton.

M6 was observed as a protonated molecule $[M+H]^+$ at *m/z* 388, with a retention time of 3.49 min. The ion at *m/z* 388 was increased by 30 Da compared to that of the parent drug, and there would be single methoxy reaction (30 Da) in phencynonate. Series of characteristic product ions at *m/z* 169, 168, 156, and 154 of the molecular ion at *m/z* 388 appeared in the MS^2 spectra. The neutral fragment at *m/z* 169 and product ion at *m/z* 168 were generated firstly via adding a methoxy group in the N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) skeleton, and then the loss of the cyclopentyl group (69 Da), the phenyl group (77 Da) and the three fragments CH_2OH, CO and O atom (see Fig. 5E). The ion at *m/z* 154 was formed by loss of the methoxy group in N-methyl-9α-(3-azabicyclo [3,3,1]nonanyl) skeleton and rearranging to form a double bond in the azabicyclononanyl ring. This ion was also formed via the loss of the cyclopentyl group, the phenyl group and the two fragments CH_2OH and CO. The product ions at *m/z* 156 were the hydrogen-added product of the ion at *m/z* 154. Based on the data above, M6 was deduced as methoxyphencynonate that the methoxylation occurred at the azabicyclononanyl group.

The mass spectra of M7 and M8, eluted at the retention time of 3.96 and 4.59 min, gave the protonated molecule $[M+H]^+$ at *m/z* 390. There were some differences in the product ion series between M7 and M8 (seen in Fig. 4). The protonated molecule at *m/z* 390 was increased by 32 Da compared to that of the unchanged parent drug, and there would be dihydroxylation (16 + 16 Da) reactions existed at the different sites from phencynonate. The characteristic product ions of M7 were also the ions of *m/z* 354, 156 and 138, which were similar to the characteristic ions of phencynonate. It indicated that there were no changes at the azabicyclononanyl part of the parent drug. And the product ion at *m/z* 354 was generated via the loss of two neutral fragments H_2O (18 + 18) from the molecular protonated ion at *m/z* 390. Therefore, M7 could be identified as dihydroxyphencynonate at cyclopentane ring.

The product ion series of M8 was partly similar to that of M7 such as the ions *m/z* 156 and 138. The fragment ions ($C_{13}H_{17}O_3$) at *m/z* 221 was formed by the loss of the hydroxyl N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) hydroxyl group ($C_9H_{15}O_2N$, 169 Da) from the protonated molecular ion at *m/z* 390, which consisted of the hydroxycyclopentyl group (C_5H_9-OH, 85 Da), the phenyl group (C_6H_5, 77 Da), the fragment CHO and the fragment CH_2OH. The fragment ion ($C_{13}H_{15}O_2$) at *m/z* 203 was produced by the loss of H_2O from the product ion at *m/z* 221. The product ions at *m/z* 169 were just generated via the loss of the fragment ions ($C_{13}H_{17}O_3$) at *m/z* 221 and could be formed by the hydroxylation at the azabicyclononanyl group. Based on these data and our works published [29], M8 was also identified as dihydroxyphencynonate occurred at the cyclopentane ring and the azabicyclononanyl group.

M9, eluted at a retention time of 3.38 min, gave rise to the protonated molecule $[M+H]^+$ at *m/z* 404 that was increased by 46 Da compared to that of phencynonate. There would be methoxylation (30 Da) and hydroxylation (16 Da) reactions existed at the phencynonate. The protonated product ion series of M9 were *m/z* 170, 169, 168 and 154. The product ions at *m/z* 170, 169 and 168 could produce three kinds of N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) ion fragments, which were formed firstly via adding a methoxy group in the N-methyl-9α-(3-azabicyclo[3,3,1]nonanyl) skeleton, and then the loss of the cyclopentyl group, the phenyl group and the three fragments CH_2OH, CO and O atom, respectively. The product ions at *m/z* 154 were formed via the loss of the hydroxycyclopentyl group, the phenyl group and the two fragments CH_2OH and CO. Based on the data above, M9 could be deduced as the hydrox-

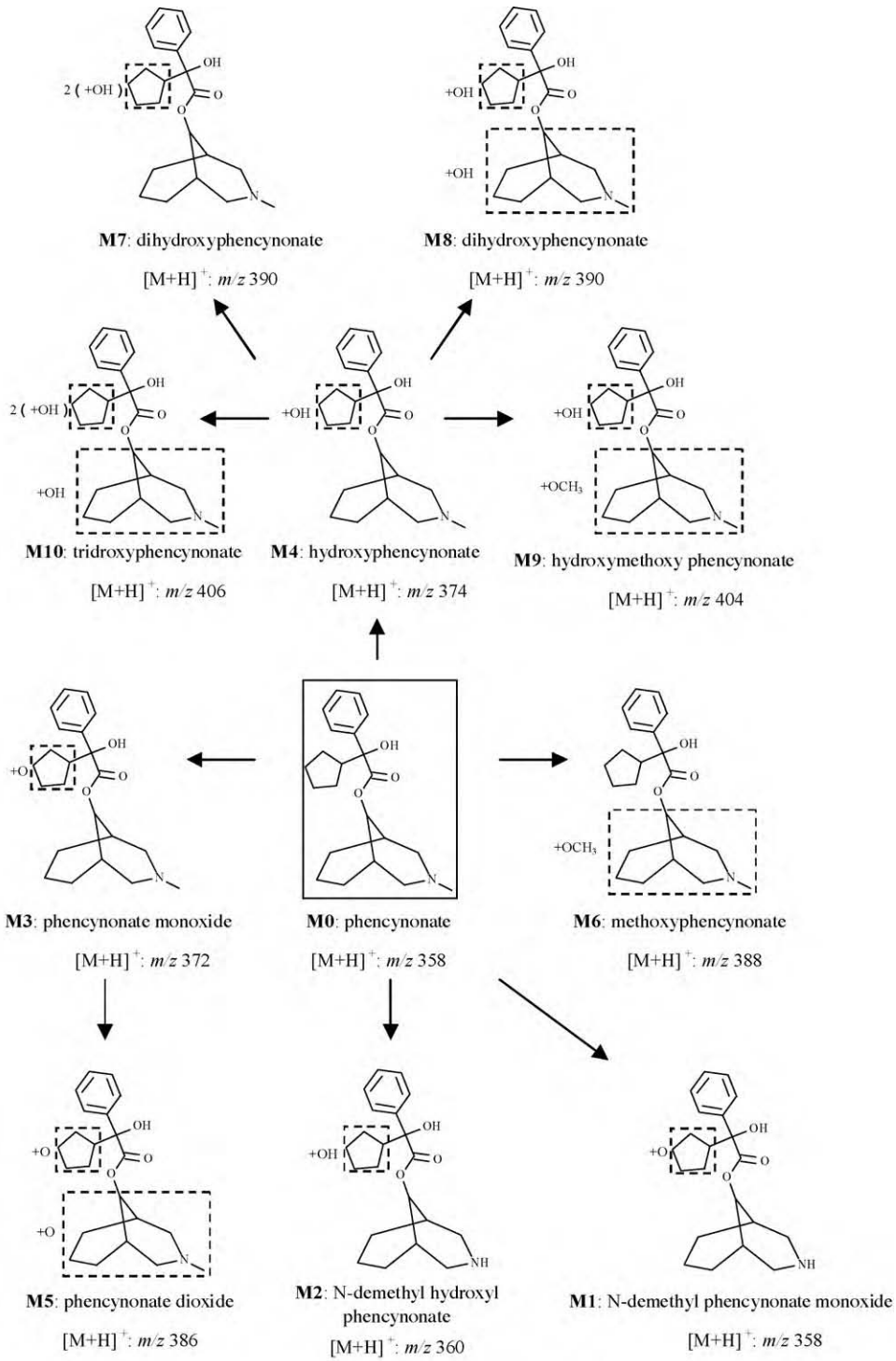

Fig. 6. Proposed major metabolites and metabolic pathways of phencynonate in rats.

ymethoxyphencynonate that one methoxylation occurred at the azabicyclononanyl group and one hydroxylation at the cyclopentane ring.

The mass spectrum of M10 that was detected at a retention time of 3.30 min gave a protonated molecule $[M+H]^+$ at m/z 406. The ion at m/z 406 was increased by 48 Da compared to that of the unchanged phencynonate and there would be trihydroxylation reactions (16 + 16 + 16 Da) for phencynonate. The characteristic product ions of M10 were the series of m/z 388, 302, 172, 156 and 138, which were partially similar to that of phencynonate. The product ion at m/z 388 was formed by the loss of H_2O at the cyclopentyl group from the protonated molecular ion at m/z 406. The product ion at m/z 305 was produced by the loss of dihydroxycyclopentyl group fragment ($C_5H_9O_2$, 101 Da) from the protonated molecular ion at m/z 406. The product ion at m/z 302 was generated by the loss of dihydroxycyclopentyl group fragment ($C_5H_9O_2$, 101 Da) from the protonated molecular ion at m/z 406. The product ion at m/z 172 was formed by adding one hydroxyl to the characteristic product ion m/z 156. Based on the appearances of these characteristic fragment ions in its MS^2 spectrum, M10 was deduced

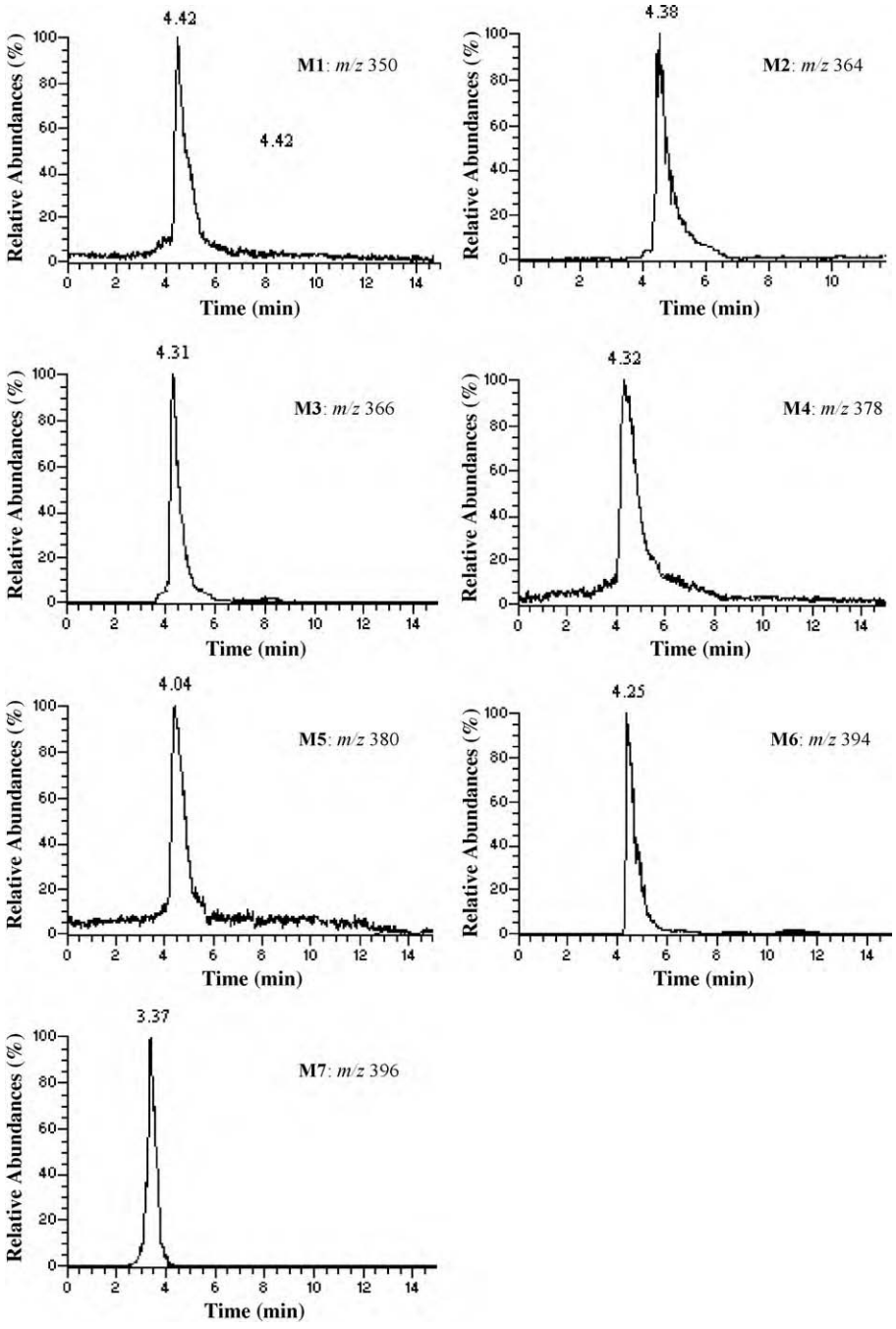

Fig. 7. LC–MS^2 chromatograms of thiencynonate metabolites in rat urine.

as trihydroxy phencynonate of dihydroxylation occurred at the cyclopentyl ring and one hydroxylation at the azabicyclononanyl group.

3.3. Identification of in vivo metabolites of thiencynonate

Likewise, thiencynonate and its metabolites were also found in rat urine after administration of the parent drug. The LC–MS^2 chromatograms of thiencynonate metabolites in rat urine were shown in Fig. 7. The characteristic MS–MS product ion spectra of thiencynonate metabolites in rat were presented in Fig. 8. The eight major protonated molecules ($[M+H]^+$) in ESI-MS were at *m*/*z* 350, 364, 364, 366, 378, 380, 394 and 396, respectively. Among them, the retention time, the MS and MS^2 spectra of the molecular ion at *m*/*z* 364 (M0) were the same as those of the parent drug. Therefore, M0 could be confirmed as the unchanged parent drug, thiencynonate [19].

The mass spectra of M1, which was detected at a retention time of 4.42 min, gave a protonated molecule $[M+H]^+$ at *m*/*z* 350 that was decreased by 14 Da compared to that of the parent compound. The product ion at *m*/*z* 332 was formed by the loss of H_2O from the protonated molecular ion at *m*/*z* 350. The molecular ion at *m*/*z* 350 (M1) and its product ions at *m*/*z* 142 and 124 were also 14 Da less than that of the protonated molecular ion *m*/*z* 364 (the parent drug) and its product ions at *m*/*z* 156 and 138, respectively. And the features of the product ions at *m*/*z* 142 and 124 were the same to that of N-demethyl product of phencynonate. Therefore, compared with the data of N-demethyl product of phencynonate, M1 should be identified as the N-demethyl product of thiencynonate.

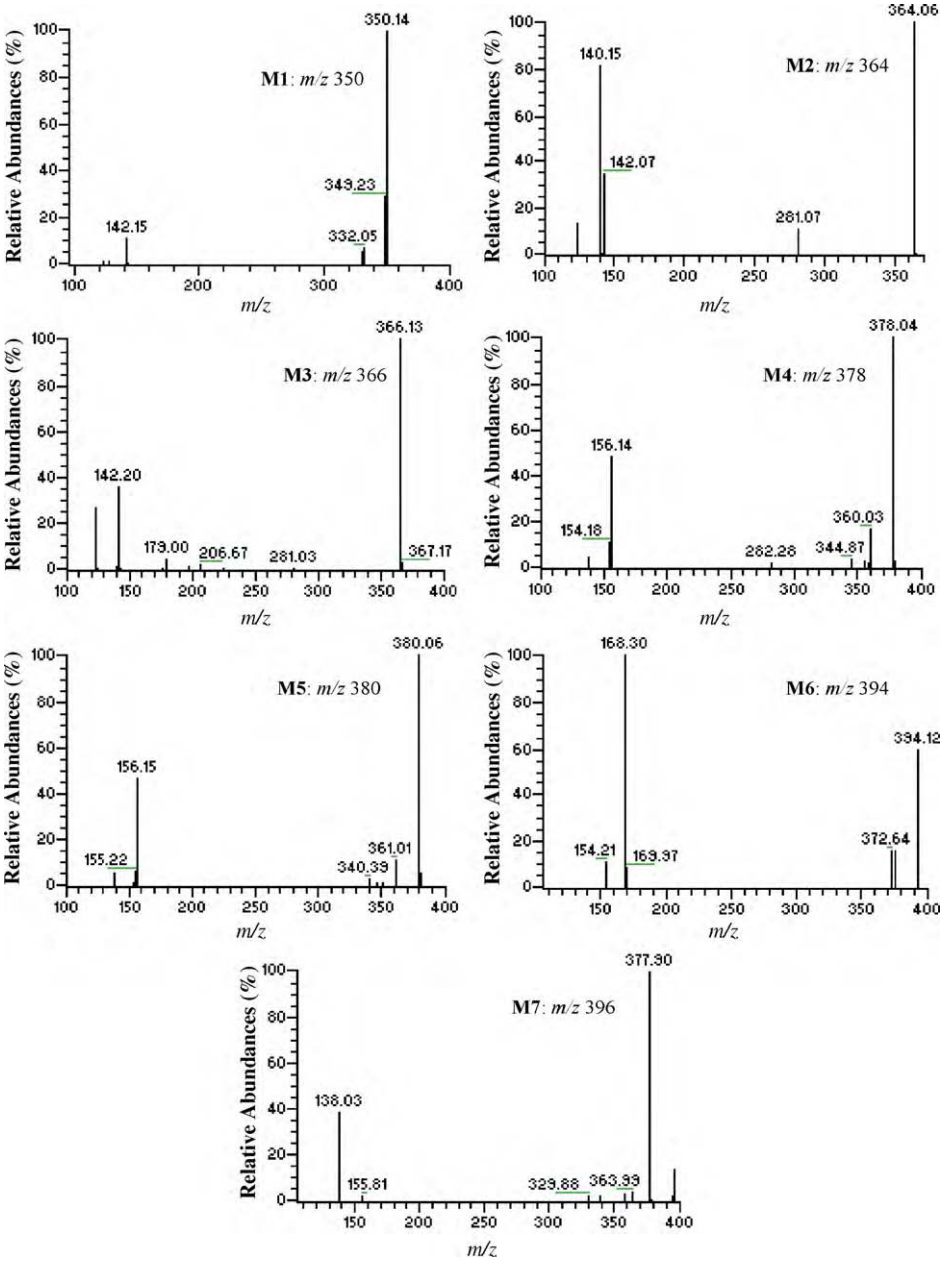

Fig. 8. MS–MS product ion spectra of thiencynonate metabolites in rat urine.

M2, eluted at a retention time of 4.38 min, gave rise to a protonated molecule $[M+H]^+$ at *m*/*z* 364. The characteristic product ions of M2 were also the ions of *m*/*z* 142, 140 and 124, which were less 14 Da than that of thiencynonate. And the features of the product ions at *m*/*z* 142 and 124 were also the same to that of N-demethyl product of phencynonate. It indicated that N-methyl group lost at the azabicyclononanyl group of the parent drug. The product ion at *m*/*z* 281 was generated via the loss of thiophene fragment (C_4H_3S, 83 Da) from the molecular ion *m*/*z* 364. Based on the data above, M2 could be identified as the N-demethylthiencynonate monoxide occurred at the cyclopentyl group.

The mass spectra of M3 gave a protonated molecule $[M+H]^+$ at *m*/*z* 366, which was detected at a retention time of 4.31 min. The protonated molecular ion at *m*/*z* 366 was increased by 2 Da compared to that of thiencynonate. The product ion at *m*/*z* 281 was generated via the loss of protonated thiophene fragment (C_4H_4S, 85 Da) from the molecular ion *m*/*z* 366. The product ion at *m*/*z* 225 was produced via the loss of the protonated hydroxyazabicyclononanyl group from the molecular ion *m*/*z* 366, which consisted of hydroxycyclopentyl, thiophene group and two fragments CHO and CO. The product ion at *m*/*z* 207 was formed by the loss of H_2O from the product ion *m*/*z* 225. The product ion at *m*/*z* 179 was produced by the loss of CO from the product ion *m*/*z* 207. Similarly, the features of the product ions at *m*/*z* 142 and 124 were also the same to that of N-demethyl product of thiencynonate above. Therefore, M3 was identified as the N-demethyl hydroxythiencynonate at cyclopentyl ring.

M4 was observed as a protonated molecule $[M+H]^+$ at *m*/*z* 378, with a retention time of 4.32 min. The molecular ion at *m*/*z* 378 was increased by 14 Da compared to that of the unchanged thiencynonate and there would be oxidation (14 Da) at thiencynonate. The characteristic product ions of M4 were the series of ions of *m*/*z* 360, 345, 282, 156, 154 and 138, which were partially similar to the characteristic ions of thiencynonate. The protonated ion at *m*/*z* 360 was generated via the loss of neutral fragment H_2O from the metabolite ion *m*/*z* 378. The ion at *m*/*z* 360 could produce the

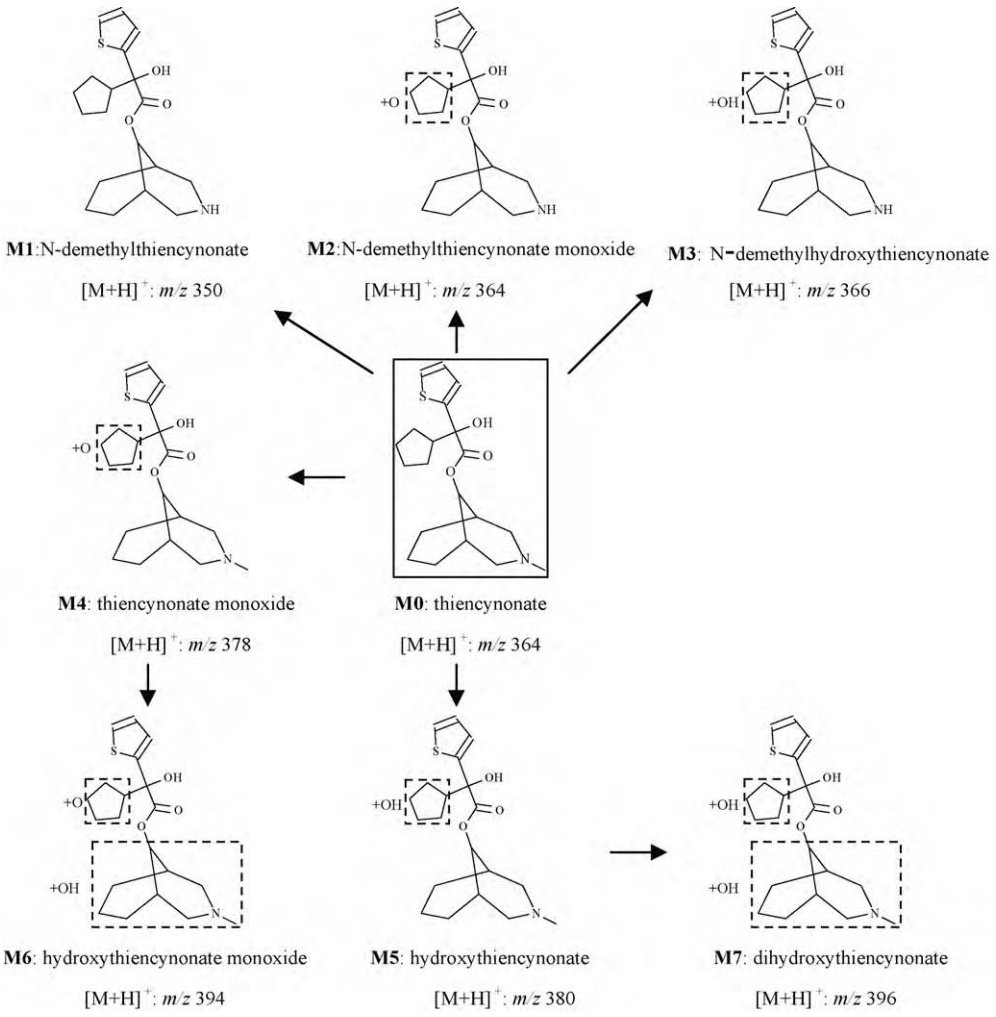

Fig. 9. Proposed major metabolites and metabolic pathways of thiencynonate in rats.

ion at *m*/*z* 345 by losing the CH_3 group at the azabicyclononanyl group. The protonated ion at *m*/*z* 282 was produced by the loss of the fragment C_5H_7O-CH (96 Da) from the metabolite ion *m*/*z* 378. It indicated that the oxidation reaction existed at the cyclopentyl ring. And there was no change at the azabicyclononanyl group owing to the ion series 156, 154 and 138. Based on the data above, M4 was identified as thiencynonate monoxide at cyclopentyl ring.

M5, eluted at 4.04 min, gave rise to a protonated molecule $[M+H]^+$ at *m*/*z* 380. The molecular ion at *m*/*z* 380 was increased by 16 Da compared to that of thiencynonate and there would be hydroxylation reaction (16 Da) existed at thiencynonate. The characteristic product ions of M5 were the ions *m*/*z* 362, 361, 340, 156 and 138. The molecular ion at *m*/*z* 380 and 379 lost a neutral fragment H_2O (18 Da) could produce the ion at *m*/*z* 362 and 361. The protonated molecular ion at *m*/*z* 380 could produce the fragment ion at *m*/*z* 340 by losing the fragment ion $C_3H_4^{+\bullet}$ (40 Da) from the cleavage of thiophene ring. Therefore, M5 was elucidated as monohydroxythiencynonate that the hydroxylation occurred at cyclopentyl group.

The mass spectra of M6, which was detected at a retention time of 4.25 min, gave a protonated molecule $[M+H]^+$ at *m*/*z* 394 that was increased by 30 Da compared to that of the parent drug and there would be one oxidation and one hydroxylation reaction (14 + 16 Da) in thiencynonate. Series of characteristic product ions at *m*/*z* 376, 169, 168, and 154 of the molecular ion at *m*/*z* 394 appeared in the MS^2 spectra. The protonated ion at *m*/*z* 376 was generated via the loss of one neutral fragment H_2O from the protonated ion at *m*/*z* 394. The product ion series at *m*/*z* 170, 169 and 168 were just generated via the loss of the fragment ions ($C_{13}H_{17}O_3$) at *m*/*z* 224, 225 and 226 and could be formed by the hydroxylation at the azabicyclononanyl group. Based on these data and our works published [27,29], M6 could be identified as hydroxyphencynonate monoxide that oxidation occurred at the cyclopentane ring and hydroxylation at the azabicyclononanyl group.

M7 was detected as a protonated molecule $[M+H]^+$ at *m*/*z* 396, with a retention time of 3.37 min. The molecular ion at *m*/*z* 396 was increased by 32 Da compared to that of the parent compound and there would be dihydroxylation (16 + 16 Da) reactions occurred at the thiencynonate skeleton. The characteristic product ions of M7 were the series ions of *m*/*z* 378, 364, 339, 330, 156 and 138, which were partially similar to that of thiencynonate. The molecular ion at *m*/*z* 396 by losing a neutral fragment H_2O produced the product ion at *m*/*z* 378. The ion at *m*/*z* 378 could also produce the fragment ions at *m*/*z* 339 and 330 by losing the fragment ions $C_3H_3^+$ (39 Da) and CH_3SH (48 Da) from the cleavage of thiophene ring, respectively. The ion at *m*/*z* 396 by losing two oxygen fragments produced the ion at *m*/*z* 364, it further proved the dihydroxylation reactions existed. Based on these data above, M7 was identified as dihydroxyphencynonate that one hydroxylation occurred at the cyclopentane ring and the other at the azabicyclononanyl group.

Phencynonate and thiencynonate were the analogues of the tropane alkaloid compounds, and their chemical structures, bio-

logical activities and the metabolic commonness were partially similar to that of scopolamine, anisodamine and anisodine [30–33]. The major metabolic pathways of these two compounds in rats were N-demethylation, oxidation, hydroxylation and methoxylation. The proposed major metabolites and metabolic pathways of phencynonate and its analogue thiencynonate in rats were shown in Figs. 6 and 9. Although the structures of the metabolites cannot be determined conclusively by LC–MS^n alone, the present method is still very valuable and dependable for the further study of the metabolic mechanism and intermediate process of these compounds. The data may also provide very important information for predicting the drug metabolic stability, developing a novel drug as the candidate or lead compound.

4. Conclusions

The method using a sensitive and specific LC–MS/MS ion trap with electrospray ionization has been achieved for the rapid analysis of phencynonate, thiencynonate and their metabolites in rat. Identification and elucidation of these metabolites were performed by comparing the changes of the protonated molecular masses, the full scan MS^n spectra and the retention times with those of the parent drug. Phencynonate and thiencynonate were mainly biotransformed by the pathways of the N-demethylated, oxidative, hydroxylated and methoxylated reactions. These metabolites included ten phencynonate metabolites and seven thiencynonate metabolites. The described method had wide applicability to rapidly screen and identify these metabolites. The identifications of precise structures of these metabolites need to be confirmed by other techniques such as the ^1H and ^{13}C NMR. This investigation contributes with new information on phencynonate and its analogues metabolism which is essential for understanding the safety and efficacy of these drugs and developing a novel drug.

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