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Structural elucidation of *in vivo* metabolites of penehyclidine in rats by the method of liquid chromatography–mass spectrometry, gas chromatography–mass spectrometry and isotope ion cluster

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ABSTRACT

The structural elucidation of metabolites of penehyclidine in rats, a novel anti-cholinergic drug, by the method of liquid chromatography–electrospray ionization mass spectrometry, gas chromatography–mass spectrometry with electron impact ion source and stable isotope ion cluster was described. Identification and elucidation of the phase I and phase II metabolites were performed by comparing the daughter ion pairs of stable isotope cluster, changes of the protonated molecular masses, full scan MS^n spectra and retention times with those of the parent drug, penehyclidine and penehyclidine deuterium-labeled. Penehyclidine was easily biotransformed by the pathways of oxidative, hydroxylated, methoxylated and phase II conjugated reactions to form several metabolites that retained the some features of the parent molecules. Twelve metabolites (penehyclidine monoxide, hydroxypenehyclidine, penehyclidine dioxide, hydroxypenehyclidine monoxide, dihydroxypenehyclidine, dihydroxypenehyclidine (position isomer), dihydroxypenehyclidine monoxide, trihydroxypenehyclidine, methoxypenehyclidine dioxide, dimethoxypenehyclidine, trihydroxymethoxypenehyclidine and glucuronide conjugated hydroxypenehyclidine) were identified. The results from electrospray ion and electron impact ion data with the stable isotope cluster showed the qualitative differences in the mass spectral patterns, suggesting that these technologies should be used in parallel to ensure comprehensive metabolites detection and characterization. The described method has wide applicability to rapidly screen and provide structural information of metabolites.

1. Introduction

Penehyclidine is a novel anti-cholinergic drug developed by the Beijing Institute of Pharmacology and Toxicology of China. Pharmacological evaluation has proved that penehyclidine has obviously anti-muscarinic and anti-nicotinic activities, while retaining potent central and peripheral anti-cholinergic activities. And penehyclidine has lower toxicity and side effects than other tropane compounds such as atropine, anisodamine and scopolamine from the several toxic experiments [1–7]. The clinical results have demonstrated that penehyclidine has a positively preventive and curative effect for organic phosphorus pesticide poisoning and can be also used before general anesthesia [8,9]. In recent years, more and more pharmacological activities of penehyclidine have been investigated and noticed widely [10–12]. Compared with the comprehensive investigations of its pharmacological activities and therapeutical practices, the study on its metabolism *in vivo* is limited [13,14], although the further metabolic study of penehyclidine plays an important role in the development of a new drug and its better clinical application.

Because of development for soft ionization techniques, mass spectrometry has assumed an increasingly important role in pharmaceutics and biomedicine. Electrospray ionization multi-stage mass spectrometry, a versatile technique, is applied in all stages of drug development including chemical synthesis, drug target identification, library verification and toxicology, especially in drug metabolism and pharmacokinetics. Identification and quantification of drugs and their metabolites in biological matrices increasingly utilize MS technologies [15,16]. Hyphenated MS techniques are frequently the initial choice for drug metabolite detection and identification because of their sensitivity and convenience compared with other methods [17].

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This paper comprehensively presents the methods of liquid chromatography–tandem multi-stage mass spectrometry with electrospray ionization (LC–ESI–MS^n), gas chromatography–mass spectrometry with electron impact ion source (GC–EI–MS) and the stable isotope ion cluster for qualitative identification of the *in vivo* metabolites of penehyclidine. Xue et al. had only reported the four phase I metabolites metabolites of penehyclidine (monoxide, hydroxylated, monoxide and hydroxylated, and dihydroxylated product) in rat [13]. This paper has described the twelve penehyclidine metabolites which were both phase I metabolites and phase II conjugated metabolite. Structural assignments of the metabolites of penehyclidine were based on changes in molecular masses and spectral patterns of product ions. The analyzers of ion trap mass, electron impact ion mass and stable isotope ion cluster provided comparatively sufficient mass information for identifying the metabolites. And a superior sensitivity in full scan and MS^n mode in combination with mass fragmentation provided most structurally informative product ion spectra for the metabolites. This approach was found to be essential for more comprehensive detection and characterization of metabolites of penehyclidine by our method. The fragmentation pathway of penehyclidine and the metabolic pathways of the metabolites of penehyclidine were discussed. The results in this paper provided important information for developing a novel anti-cholinergic drug and for a better use in clinical practice.

2. Experimental

2.1. Chemicals and reagents

Penehyclidine [(3-(2-hydroxy-2-cyclopentyl-2-phenyl) quincycloxyethane), MW = 315] and penehyclidine deuterium-labeled at aromatic ring (penehyclidine-d_5, MW = 320) were prepared and provided from the Beijing Institute of Pharmacology and Toxicology of China, which the structures of two compounds were identified as pure compounds from the melting point, UV, IR, MS and NMR. The purities of penehyclidine and penehyclidine-d_5 were determined to be more than 99% by LC–MS, no impurities or degradation products were detected [18–20]. Methanol (HPLC grade) was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Formic acid (HPLC grade) was purchased from Dikma Reagent Company (Beijing, China). Water was triply distilled. All other reagents and chemicals were of analytical grade.

2.2. Liquid chromatography–mass spectrometry and conditions

The Liquid chromatography–mass spectrometry (LC–MS) system consisted of a HPLC system (Series 1100, Agilent technology, Palo Alto, CA, USA) including a HP 1100 G1312A binary pump, a G1379A vacuum degasser, and a G1313A autosampler, and coupled to Finngian LCQ Deca XP ion trap mass spectrometer (ITMS) equipped with electrospray ion source (ESI) working in positive or/and negative ion mode (Thermo Finnigan, San Jose CA, USA). The LC–MS system was controlled by the Thermo Finnigan Chemstation software (Xcalibur version 1.3).

Separations of analytes 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. The mobile phase was composed of methanol and water (40:60, v/v), containing 0.5‰ formic acid at pH 6, which was pumped at a flow rate of 0.2 ml/min during the whole run. The sample injection volume was 10 μl and the run time of samples was 10 min.

The effluent was on-line transferred to ESI–MS system without splitting. The mass spectrometer, working in positive or/and negative ion mode, was connected to the LC system. The first-order full scan mass spectra covered the range *m/z* 50–1000. Nitrogen was used as a sheath gas and ultra-high purity helium as the dampening gas in the ion-trap. The MS analyses were performed under automatic gain control conditions, using a sheath flow-rate of 34 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. The MS^n product ion spectra were produced by collision induced dissociation (CID) of the protonated molecular ion $[M+H]^+$ of all analytes at their respective HPLC retention times. Data acquisition was performed in full scan LC–MS and tandem MS modes.

2.3. Gas chromatography–mass spectrometry and conditions

Gas chromatography–mass spectrometry (GC–MS) experiments were performed with a HP 5971A quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) equipped with HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA). A HP-5 capillary column (length 12 m, 0.2 mm i.d., film thickness 0.33 μm) was employed with helium as carrier gas (flow 1.0 ml/min). A 1 μl volume of sample was injected into the GC. The column temperature was programmed as follows: first from 100 to 220 °C at 20 °C/min held for 6 min, then to 250 °C at a rate of 25 °C/min held for 10 min. The MS–EI ion source temperature was 200 °C, and the temperature of injection and the interface between the chromatograph and the spectrometer were set at 280 °C. The MS was operated in an electron impact ionization mode. The electron collision energy was 70 eV. The full scan range of the sample for the qualitative analysis was from *m/z* 50 to 1000. A HP ChemStation data system was used to control the GC–MS system and to collect the data.

2.4. Animal experiment and sample preparation

All studies on animals were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in China. Male Sprague–Dawley rats (BW 250 ± 20 g) were obtained from Animals Center of Capital Medical University (CCMU, Beijing, China). Rats were housed in metabolic cages for fasted, but freely available to water. Each rat was intraperitoneally given a dose of 10 mg/kg penehyclidine or administrated a dose of 5 mg/kg penehyclidine and 5 mg/kg penehyclidine-d_5 simultaneously. Urine samples were collected from rat at pre-dose and at 0–48 h post-dose, and stored at approximately −40 °C before the samples were extracted and analyzed.

An aliquot of 2 ml of mixed 0–48 h urine samples for LC–MS 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 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 20 μl was used for LC–MS analyses.

The urine mixture samples for GC–MS were centrifuged at 3000 × *g* for 10 min. The supernatant was extracted two times by adding two-fold volume of mixed solvent (ethyl ether–dichloromethane 2:1, v/v). After centrifugation, the upper organic layers were removed, collected and evaporated to dryness under nitrogen at 40 °C, the dried residues containing metabolites were dissolved in 100 μl of methanol, an aliquot of 1 μl into GC–MS for analysis. The blank experiment was carried out under the same conditions.

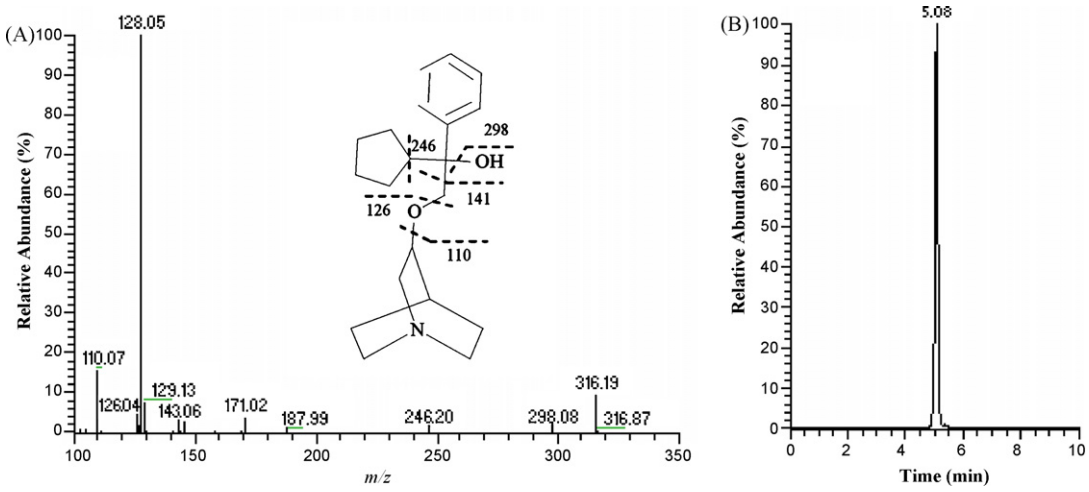

Fig. 1. (A) ESI–MS–MS product ion spectrum and the predominant fragmentation patterns of penehyclidine; (B) LC–MS^2 chromatogram of penehyclidine.

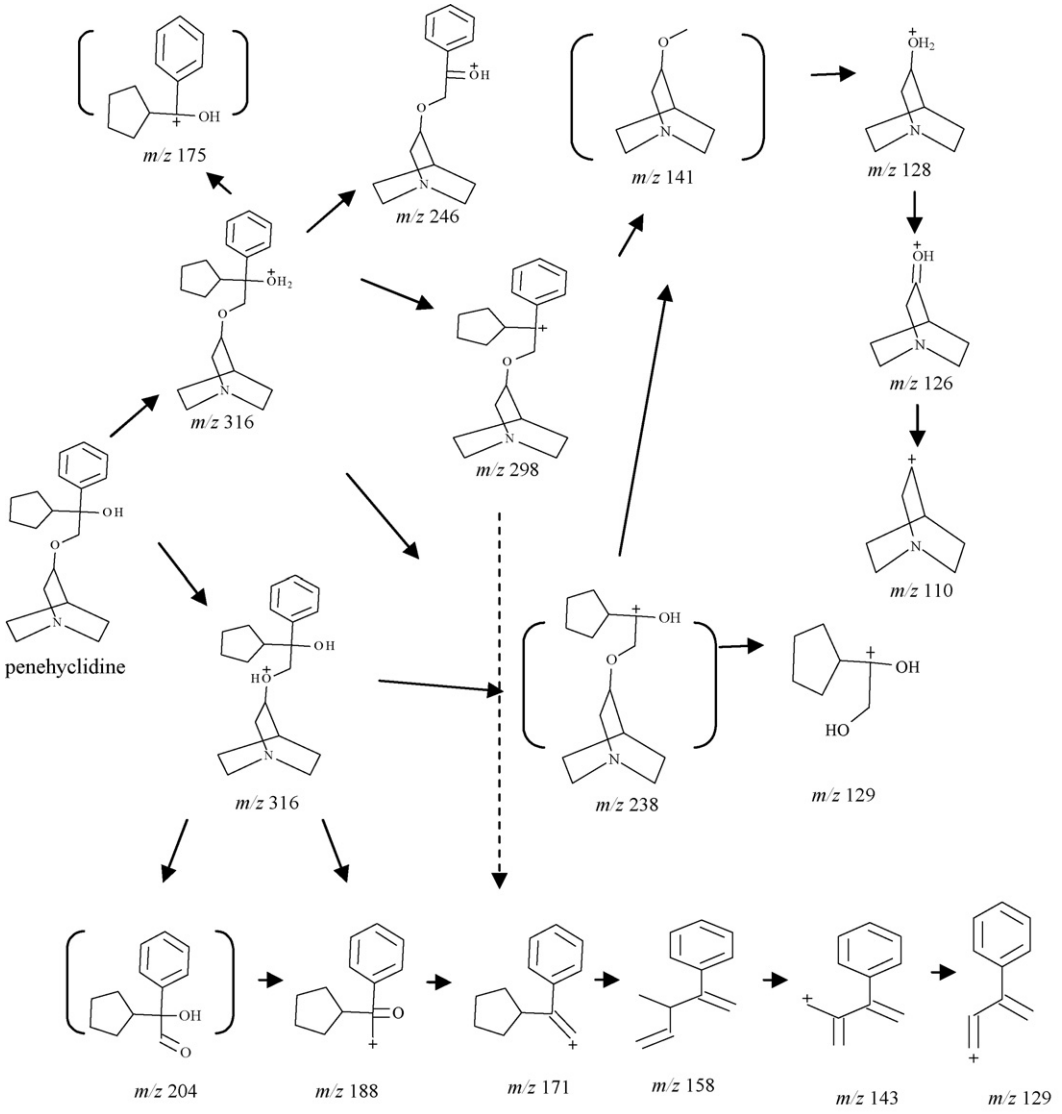

Fig. 2. Fragmentation pathways of penehyclidine in ESI–MS/MS.

3. Results and discussion

3.1. LC–MS/MS analysis of penehyclidine

This investigation involved the chromatographic and mass spectral properties of the parent drug. The chromatographic and mass spectrometry conditions were optimized for maximum abundances of the ions of the interests of penehyclidine standard by the automatic tune procedure of the instrument. The first step in this investigation involved the characterization of mass spectral properties of penehyclidine, full scan mass spectral analyses of penehyclidine showed protonated molecule ions of *m/z* 316 from LC–ESI–MS. The MS–MS product ion spectrum of the protonated molecular ion, the predominant fragmentation patterns and the LC–MS^2 chromatogram of penehyclidine were showed in Fig. 1A and B. Penehyclidine was eluted at 5.08 min under the experimental conditions. Fragmentation of the protonated molecular ion of penehyclidine in the ion trap led to form the main ion series at *m/z*: 298, 246, 188, 171, 158, 143, 129, 128, 126 and 110. The product ions at *m/z* 298 and 246 were generated via the loss of the neutral fragment H_2O and cyclopentyl group (C_5H_{10}, 70 Da) from the molecular protonated ion at *m/z* 316, respectively. The most abundant protonated product ion at *m/z* 128 was formed by the loss of cyclopentyl group (C_5H_9, 69 Da), phenyl group (C_6H_5, 77 Da) and the fragment ion C_2H_2 group (26 Da) from the ion at *m/z* 298. The protonated ion at *m/z* 126 and 110 was produced by the loss of two protons and H_2O (18 Da) from the ion at *m/z* 128, respectively. The product ion at *m/z* 188 was formed by the loss of quiniuclidinol ring ($C_7H_{14}ON$, 128 Da) from the molecular ion at *m/z* 316. The fragment ion at *m/z* 171 was formed by the loss of OH (17 Da) from the ion at *m/z* 188, and the ion at *m/z* 143 was formed by cyclopentane ring cleavage and loss of C_2H_4 (28 Da) from the ion at *m/z* 171. The characteristic fragment ion series at *m/z* 128, 126 and 110 coexisted in the MS^3 spectrum of *m/z* 298. It was easy to conclude that the ions at *m/z* 128, 126 and 110 were the series of characteristic product ions of penehyclidine. The molecular structure of penehyclidine and its proposed fragmentation pathways were showed in Fig. 2. Determination of the structures of penehyclidine metabolites was facilitated by the fact that the compounds produced the extensive and characteristic fragment ions under MS^n conditions. The mass spectral patterns served as templates in the elucidation of the structures of the proposed metabolites.

3.2. GC–MS analysis of penehyclidine

The rat urine samples were collected and extracted after each rat was given an equal dose of penehyclidine and penehyclidine-d_5 simultaneously. The first step elucidation of main metabolites was carried out by the method of stable isotope ion cluster and mass spectrometry. Penehyclidine-d_5 that five deuterium were labeled at phenyl ring of penehyclidine was as the useful tracer for screening and recognizing the metabolites. Parent drug and its metabolites formed by the labeled penehyclidine-d_5 and the non-labeled penehyclidine in rats showed the series of characteristic stable isotope ion clusters which named as the equal abundance daughter peaks and there were the differences of five mass units for each pair of these compounds. From the characteristic ion cluster data the initial metabolites were easily screened and recognized. The characteristic daughter peaks of stable isotope ion clusters and the predominant fragmentation patterns of penehyclidine and penehyclidine-d_5 in EI–MS were presented in Fig. 3.

3.3. Identification of in vivo metabolites of penehyclidine

In order to identify the metabolites of penehyclidine, the possible structures of metabolites have been speculated according to the rule of drug metabolism [7]. At first, the full scan mass spectra of free fractions in rat urine were compared with those of blank rat urine sample and penehyclidine standard to explore the possible metabolites after an equal dose coadministration of penehyclidine and penehyclidine-d_5, and the initial metabolites could be screened and recognized easily from the equal abundance and characteristic stable isotope ion cluster data. Then, these compounds were further analyzed by LC–ESI–MS^n and GC–EI–MS. Their retention times, changes in observed mass and spectral patterns of product ions were compared with those of penehyclidine to identify metabolites and elucidate their structures.

The several pairs of characteristic daughter peaks of stable isotope ion cluster of penehyclidine, penehyclidine-d_5 and their metabolites in mass spectra were obviously presented and screened easily from the mass spectra data in rat urine after coadministration of equal dose labeled and no-labeled drug in rats. These characteristic and equally abundant daughter peaks of metabolites could be recognized effectively from these complicated ion clusters in rat urine samples. Only two pairs of the metabolites of penehyclidine and penehyclidine-d_5 could be detected using GC–EI–MS method with the stable isotope ion cluster, M3–M12 could be not detected because it was difficult that these compounds were gasified in GC–MS systems, which were more polar molecules. Thirteen characteristic daughter peaks of penehyclidine, penehyclidine-d_5 and their metabolites in LC–ESI–MS were also presented and screened obviously. And the ion cluster data from ESI–MS were more comprehensive and sound. The stable isotope ion cluster comparison of penehyclidine, penehyclidine-d_5 and their metabolites with LC–ESI–MS and GC–EI–MS in rats was shown in Table 1.

Based on the methods mentioned above, the parent drug and its metabolites were found in rat urine after coadministration of penehyclidine and penehyclidine-d_5. The LC–MS^2 chromatograms, the MS–MS molecular ions and the product ions of penehyclidine and its metabolites in rats were showed in Figs. 4 and 5. These 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* 316 (M0) were the same as those of the parent drug. Therefore, M0 could be confirmed as the unchanged parent drug, penehyclidine.

The mass spectra of M1, which was detected at a retention time of 4.03 min, gave a protonated ion $[M+H]^+$ at *m/z* 330 that was increased by 14 Da compared to that of the parent compound. Series of characteristic product ions at *m/z* 246, 128, 126 and 110 of M1 appeared in the MS^2 spectra. It indicated that there were no

Table 1
Stable isotope ion cluster comparison of penehyclidine, penehyclidine-d_5 and their metabolites with LC–ESI–MS and GC–EI–MS in rats

Peak	Molecular ion cluster	
	ESI–MS $[M+H]^+$	EI–MS $[M]^+$
M0	316, 321	315, 320
M1	330, 335 (+14, =O)	329, 334(+14, =O)
M2	332, 337 (+16, —OH)	331, 336(+16, —OH)
M3	344, 349 (28, 2O)	–
M4	346, 351 (30, O, —OH)	–
M5	348, 353 (32, 2—OH)	–
M6	348, 353 (32, 2—OH)	–
M7	362, 367 (46, O, 2—OH)	–
M8	364, 369 (48, 3—OH)	–
M9	374, 379 (58, 2O, —OCH_3)	–
M10	376, 381 (60, 2—OCH_3)	–
M11	394, 399 (78, 3—OH, —OCH_3)	–
M12	508, 513 (16, 176, —OGlu[a])	–

[a] Glu = glucuronic acid.

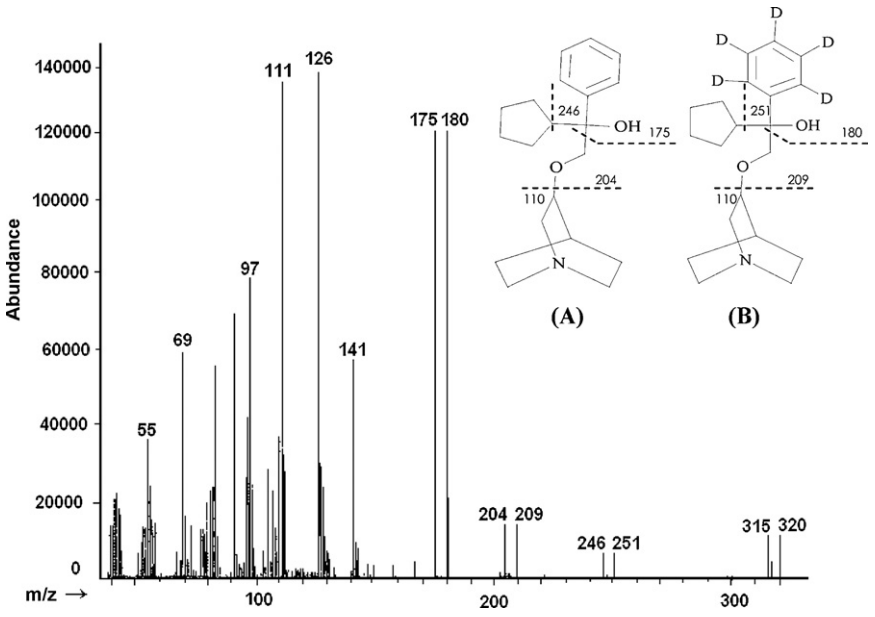

Fig. 3. The characteristic daughter peaks of stable isotope ion clusters and the predominant fragmentation patterns of penehyclidine (A) and penehyclidine-d_5 (B) in EI–MS.

changes at the phenyl group and quiniuclidinol ring of the parent drug. The product ion at *m/z* 312 was generated via the loss of the neutral fragment H_2O from the molecular protonated ion at *m/z* 330. The product ion at *m/z* 246 was formed via the loss of the cyclopentene (C_5H_7, 67 Da, dehydration from cyclopentanone) from the protonated ion at *m/z* 312. The appearance of product ion at *m/z* 185 in the MS^2 spectrum at *m/z* 330 indicated that the cyclopentyl part was oxidized to form cyclopentanone, and the fragment ion at *m/z* 185 was generated via the loss of H_2O and quiniuclidinol ring ($C_7H_{14}ON$, 128 Da) from the parent ion at *m/z* 330. In the MS^2 spectrum, the product ions at *m/z* 143 and 141 were produced via the loss of 42 and 44 Da and rearrangement from the ion at *m/z* 330, respectively. 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) were detected in the EI–MS spectrum by coadministrating of equal dose of penehyclidine and penehyclidine-d_5. The fragmentation feature has been validated in our experiment [13]. Based on these data, M1 was identified as penehyclidine monoxide at the cyclopentane ring.

The mass spectra of M2 that was detected at a retention time of 4.40 min gave an ion $[M+H]^+$ at *m/z* 332. The protonated molecular ion at *m/z* 332 was increased by 16 Da compared to that of the unchanged drug. The characteristic product ions of M2 were the series ions of *m/z* 246, 128, 126 and 110, which were similar to the characteristic ions of penehyclidine. It indicated that there were no changes at the phenyl group and quiniuclidine group of the parent drug. The product ions at *m/z* 314, 246 and 204 were generated via the loss of neutral fragment H_2O, hydroxycyclopentane (hydroxylation in cyclopentane, C_5H_9O, 85 Da) and quiniuclidinol ring ($C_7H_{14}ON$, 128 Da) from the molecular protonated ion at *m/z* 332, respectively. The fragment ion at *m/z* 187 was formed by the loss of quiniuclidinol group ($C_7H_{14}ON$, 128 Da) from the ion at *m/z* 314. The characteristic fragment ion at *m/z* 175 was produced by the loss of CO group from the neutral fragment ion at *m/z* 204. The neutral fragment ion at *m/z* 169 was formed via the loss of phenyl group (C_6H_5, 77 Da) from the ion at *m/z* 246. The ion at *m/z* 157 was formed via the loss of quiniuclidinol group and OH group from the ion at *m/z* 314. Based on the appearances of these characteristic fragment ions in its MS^2 spectrum, M2 was identified as the hydroxypenehyclidine at cyclopentane ring [13].

The mass spectra of M3, which was detected at a retention time of 4.06 min, gave a protonated ion at *m/z* 344 that was increased by 28 Da compared to that of the parent compound. Series of characteristic product ions at *m/z* 246, 128, 126 and 110 of the ion at *m/z* 344 appeared in the MS^2 spectra. It indicated that there were no changes at the phenyl group and quiniuclidine group of the parent drug. The protonated ion at *m/z* 296 was generated via the loss of three oxygen fragments (two oxygen came from cyclopentadinone lead to form cyclopentadiene) and rearrangement from the molecular protonated ion at *m/z* 344. The protonated ion at *m/z* 262 was produced via the loss of these three neutral fragments ($2CO + C_2H_2$, 82 Da) and rearrangement from the molecular protonated ion at *m/z* 344. The most abundant product ion at *m/z* 246 was formed by the loss of cyclopentadinone ($C_5H_5O_2$, 97 Da) from the molecular protonated ion at *m/z* 344 or by the loss of methyl group from the protonated ion at *m/z* 262. The appearance of protonated ion at *m/z* 173 in the MS^2 spectrum at *m/z* 344 also indicated that the cyclopentyl part was oxidized and this neutral fragment (*m/z* 172) was generated via the loss of oxygen group in cyclopentadinone, OH, CH_2, and quiniuclidinol group ($C_7H_{14}ON$, 128 Da) from the parent ion at *m/z* 344. Based on the data above, M3 was deduced as penehyclidine dioxide at the cyclopentane.

The mass spectra of M4 that was detected at a retention time of 5.05 min gave an ion $[M+H]^+$ at *m/z* 346. The protonated molecular ion at *m/z* 346 was increased by 30 Da compared to that of penehyclidine and there would be oxidation (14 Da) and hydroxylation (16 Da) reactions for penehyclidine. The characteristic product ions of M4 were also existed at series ions of *m/z* 262, 246, 204, 128, 126 and 110, which were similar to the characteristic ions of the parent drug, M1 and M2, and these data also indicated that there were no changes at the phenyl group and quiniuclidinol ring of the parent drug. The protonated ion at *m/z* 189 was formed by the loss of quiniuclidinol ring ($C_7H_{14}ON$, 128 Da), H_2O, CH_2 group and oxygen atom at cyclopentane from the protonated molecular ion at *m/z* 346, and the ion at *m/z* 189 indicated that oxidation and hydroxylation existed at cyclopentyl group. The neutral fragment ion at *m/z* 144 was produced via the hydroxylcyclopentane cleavage and the loss of CO and CH_2 fragments from the ion at *m/z* 189. Based on the appearances of these characteristic fragment ions in its MS^2

spectrum, M4 was identified as hydroxypenehyclidine monoxide at cyclopentyl group [13].

The mass spectra of M5 and M6 that were detected at a retention time of 3.81 and 4.56 min, respectively, and both gave the protonated molecular ion at *m/z* 348. The molecular ion $[M+H]^+$ at *m/z* 348 was increased by 32 Da compared to that of the unchanged parent drug and there would be dihydroxylation (16 + 16 Da) reactions for penehyclidine. The characteristic product ions of M5 were also the ions of *m/z* 246, 128 and 110, which were similar to the characteristic ions of penehyclidine and it indicated that there were no changes at the benzene ring and quiniuclidinol group of the parent drug. The product ion at *m/z* 330 was generated via the loss of neutral fragment H_2O at dihydroxylcyclopentane ring (*m/z* 101 → 83) from the molecular protonated ion at *m/z* 348. The neutral fragment ion at *m/z* 158 (1-dihydroxycyclopentyl ethyl-diketone) was formed by the loss of quinuclidine ring ($C_7H_{12}N$, 110 Da) and phenyl group and rearrangement from the molecular protonated ion at *m/z* 348. The neutral fragment ion at *m/z* 155 was formed by the loss of phenyl group (C_6H_5, 77 Da), C_5H_7O fragment (83 Da) and OH group from the ion at *m/z* 330, what the fragment ion at *m/z* 155 existed further indicated that there was not change at the quinuclidine ring. It also indicated that there was not reaction at the benzene ring from the fragment ions at *m/z* 158 and 146 existed. Based on the appearances of these characteristic fragment ions in its MS^2 spectrum and according to the investigation and reference [7,13], M5 was identified as dihydroxypenehyclidine at cyclopentane ring.

M6 also gave the protonated molecular ion $[M+H]^+$ at *m/z* 348, but there were some differences at the ion series between M6 and M5. The fragment ion at *m/z* 262 was formed by the addition of OH group from the ion at *m/z* 246 and it indicated there was hydroxylation at the phenyl or quinuclidine group. Because of the protonated ion at *m/z* 144 (hydroxyquiniuclidinol group) and

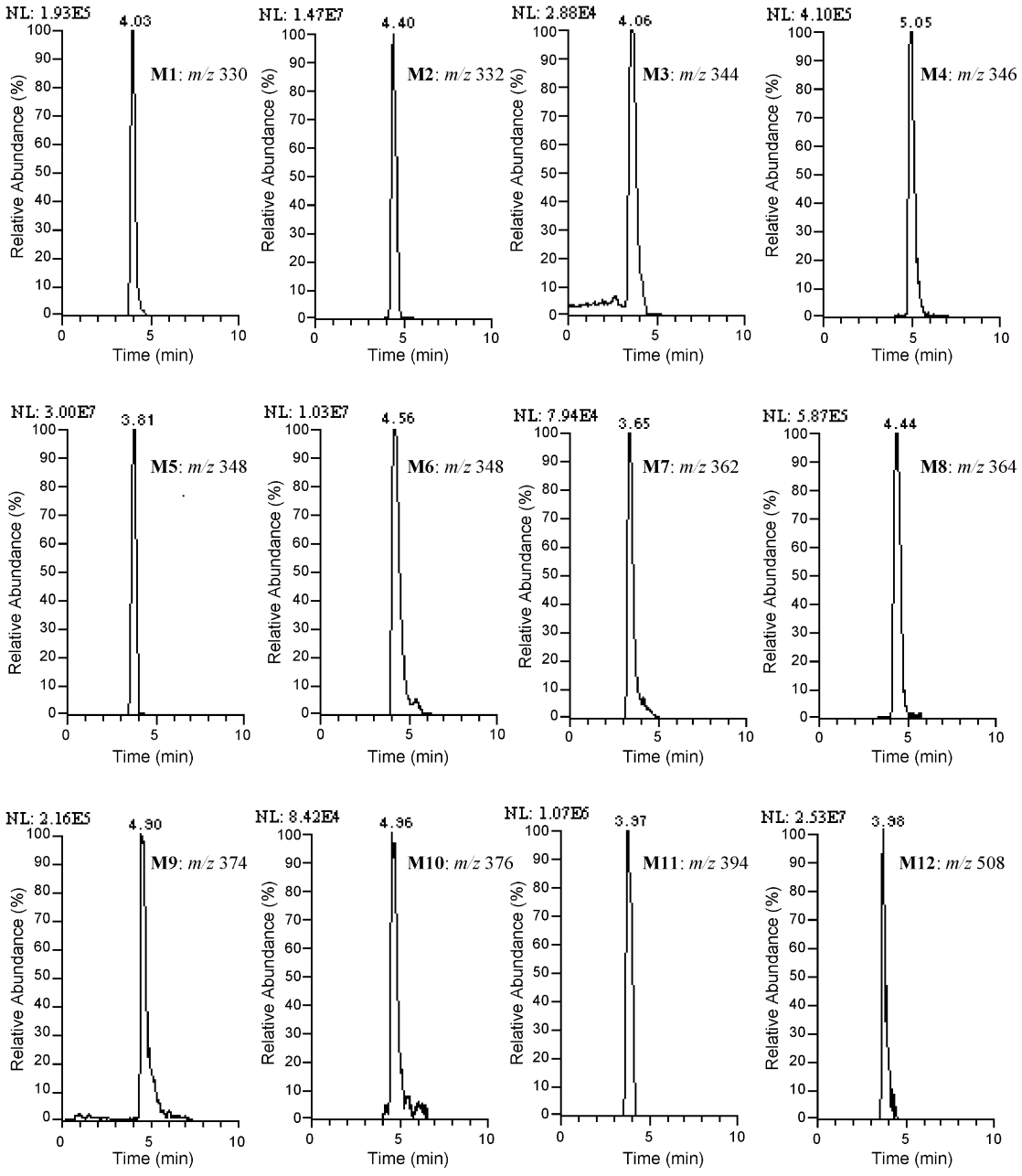

Fig. 4. LC–MS^2 chromatograms of penehyclidine metabolites in rat urine.

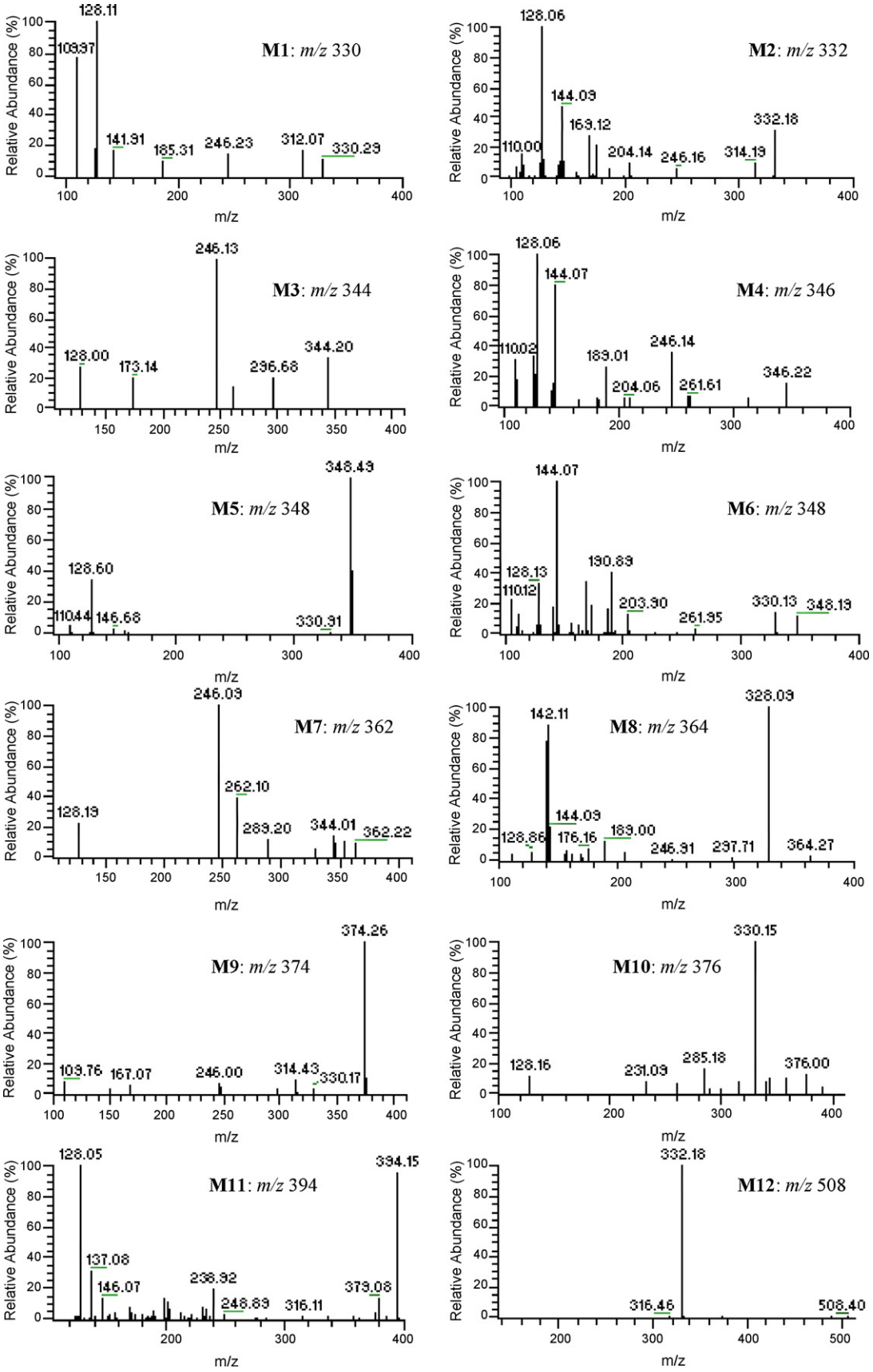

Fig. 5. MS/MS product ion spectra of penehyclidine metabolites in rat urine.

the neutral fragments at *m/z* 162, 146, 142, 105 (benzene derivative series) existed, it further indicated the hydroxylation occurred at the quinuclidine ring not at benzene ring. The fragment ions at *m/z* 191 and 187 were generated via the loss of $C_8H_{14}O_2N$ group (156 Da) and $C_7H_{12}O_2N$ + OH (142 + 17 Da) from the ion at *m/z* 348, respectively. Both fragment ions at *m/z* 173 and 169 were produced via the loss of H_2O from the ion at *m/z* 191 and 187, respectively, it indicated that there was hydroxylation at cyclopentyl group. The other neutral fragment ion at *m/z* 169 was formed by the loss of phenyl group (C_6H_5, 77 Da), hydroxycyclopentyl and OH group from the molecular ion at *m/z* 348. Based on the appearances of these characteristic fragment ions in its MS^2 spectrum, M6 was elucidated as dihydroxypenehyclidine at cyclopentane and quinuclidine ring.

The mass spectra of M7 that was detected at a retention time of 3.65 min gave an ion $[M+H]^+$ at *m/z* 362. The protonated molecular ion at *m/z* 362 was increased by 46 Da compared to that of penehyclidine and there would be oxidation (14 Da) and dihydroxylation (16 + 16 Da) reactions at penehyclidine. The characteristic product ions of M7 were also existed at series ions of *m/z* 262, 246, 128, 126 and 110, which were partially similar to the characteristic ions of the parent drug, M1 and M6. There were the features of M3 and M4 which oxidation and hydroxylation existed from the characteristic product ions of *m/z* 344 and 346 and these data also indicated that there were no changes at the phenyl group of the parent drug. The protonated fragment ion at *m/z* 328 was formed by the loss of H_2O and hydroxyl group (18 + 16 Da) from the protonated molecular ion at *m/z* 362, and the neutral fragment at *m/z* 289 was produced via the cyclopentene ring cleavage and the loss of C_3H_4 fragment from the ion at *m/z* 328. Based on the appearances of these characteristic fragment ions in its MS^2 spectrum, M7 was elucidated as dihydroxypenehyclidine monoxide that one hydroxylation occurred at cyclopentyl group and the other hydroxylation at quinuclidine group.

The mass spectra of M8 that was detected at a retention time of 4.44 min gave an ion $[M+H]^+$ at *m/z* 364. The protonated molecular ion at *m/z* 364 was increased by 48 Da compared to that of the unchanged penehyclidine and there would be trihydroxylation reactions (16 + 16 + 16 Da) for penehyclidine. The characteristic product ions of M8 were the series of ions of *m/z* 246, 189, 171, 144, 142 and 128, which were partially similar to the characteristic ions of penehyclidine and its metabolites above. The most abundant product ion at *m/z* 328 was produced easily via the loss of two neutral fragments ($2H_2O$, 36 Da) from the parent protonated ion at *m/z* 364, which there were two dehydration reactions in cyclopentane to form a cyclopentadiene group (*m/z* 101 → 65) and it could be elucidated that the dihydroxylation reactions existed at cyclopentyl group. The protonated ion at *m/z* 207 was generated via the loss of quiniuclidinol ring ($C_7H_{12}ON$, 126 Da), OCH_2 and OH group from the molecular protonated ion at *m/z* 364, and the product ion at *m/z* 207 was composed of cyclopentadinol, phenyl and $CHCH_3$ group (neutral fragment, 1-phenyl, 1-dihydroxycyclopentyl ethane). The protonated ion at *m/z* 189 was produced by the loss of quiniuclidinol group ($C_7H_{12}ON$, 126 Da), and OH group from the protonated ion at *m/z* 328, which formed the fragment, 1-phenyl, 1-cyclopentadiene ethane. And because of the appearances of the characteristic series ions of phenyl groups in its MS^2 spectrum, it further indicated that there were dihydroxylation reactions existed at the cyclopentyl ring and the other hydroxylation occurred at quiniuclidine ring. Based on the appearances of these characteristic fragment ions in its MS^2 spectrum, M8 was deduced as trihydroxypenehyclidine of two hydroxylation occurred at cyclopentyl group and one hydroxylation at quiniuclidine ring.

The mass spectra of M9, which was detected at a retention time of 4.90 min, gave a protonated ion at *m/z* 374 that was increased by 58 Da compared to that of the parent drug. There would be dioxidation (14 + 14 Da) and single methoxy reaction (30 Da) in penehyclidine. Series of characteristic product ions at *m/z* 246, 128, and 110 of the molecular ion at *m/z* 374 appeared in the MS^2 spectra. The protonated ion at *m/z* 330 was generated via the loss of one oxygen atom and methoxy group from the protonated ion at *m/z* 374. The protonated ion at *m/z* 314 was produced via the loss of OH fragment from the protonated ion at *m/z* 330. The neutral fragments at *m/z* 167, 149, and 105 (benzene derivative series) existed, it indicated the reaction occurred at the cyclopentane or quinuclidine ring but not at benzene ring. The product ion at *m/z* 246 was formed via the loss of cyclopentadinone ($C_5H_5O_2$, 97 Da) and methoxy group from the molecular protonated ion at *m/z* 374. Based on the data above, M9 was deduced as methoxypenehyclidine dioxide that two oxidation reactions occurred at cyclopentane to form cyclopentadinone and methoxylation at quinuclidine ring.

The mass spectra of M10, which was detected at a retention time of 4.96 min, gave a protonated ion at *m/z* 376 that was increased by 60 Da compared to that of the parent drug, and there would be dimethoxy reactions (30 + 30 Da) in penehyclidine. Series of characteristic product ions at *m/z* 246, 128, and 110 appeared in the MS^2 spectrum of the molecular ion at *m/z* 376. The protonated ions at *m/z* 358, 346, 344 were generated via the loss of H_2O, two methyl and methoxy group from the molecular protonated ion at *m/z* 376, respectively. The fragment ions at *m/z* 330 and 315 were produced via the loss of one oxygen atom from the protonated ion at *m/z* 344 and 330, respectively. The neutral fragment ion at *m/z* 299 was produced via the loss of H_2O from the ion at *m/z* 315. The series of ions at *m/z* 285, 260 and 231 were formed via cyclopentane cleavage and the loss of relevant fragment ions from the molecular protonated ion at *m/z* 376. Based on the data above, M10 was deduced as the dimethoxypenehyclidine of dimethoxylation at cyclopentane ring.

The mass spectra of M11 that was detected at a retention time of 3.97 min gave an ion $[M+H]^+$ at *m/z* 394. The protonated molecular ion at *m/z* 394 was increased by 78 Da compared to that of the unchanged drug and there would be trihydroxylation (16 + 16 + 16 Da) and monomethoxy (30 Da) reactions in penehyclidine. The characteristic product ions of M11 were the series of ions of *m/z* 316, 248, 189, 169, 158, 146 and 128, which were similar to the characteristic ions of penehyclidine. The protonated product ions at *m/z* 379 and 376 were produced via the loss of methyl group and H_2O from the molecular protonated ion at *m/z* 394, respectively. Neutral fragment (H_2O) was lost from the parent protonated ion at *m/z* 394 which there was a dehydration reaction in cyclopentane to form a cyclopentene group and it could elucidate that the hydroxylation reaction existed at the cyclopentyl group. The protonated product ion at *m/z* 337 was produced via the loss of C_3H_3 group (by cyclopentene cleavage) from the protonated ion at *m/z* 376, and the protonated product ion at *m/z* 278 was produced by the loss of $C_2H_3O_2$ fragment (also by cyclopentene cleavage) from the protonated ion at *m/z* 337. The neutral product ion at *m/z* 199 was produced by the loss of phenyl group from the protonated ion at *m/z* 337. The ion at *m/z* 140 was all existed at the series ions of *m/z* 394, 376, 337, 278 and 199, which was exact quiniuclidine with a methoxy group (110 + 30 Da) and it could elucidate that the methoxylated reaction existed at the quiniuclidine ring. Because of the appearances of the characteristic phenyl ion series at *m/z* 203, 189, 169, 158 and 146 in its MS^2 spectrum, it could elucidate that there was no change at benzene ring. Based on the appearances of these characteristic fragment ions in its MS^2 spectrum, M11 was deduced as trihydroxymethoxypenehyclidine that trihydroxylation occurred at cyclopentyl group and monomethoxylation at quiniuclidine ring.

The mass spectra of M13 that was detected at a retention time of 3.98 min gave a protonated molecular ion at *m/z* 508 which was increased by 176 Da compared to that of the monohydroxylated

metabolite of penehyclidine (M2, *m/z* 332). The MS^2 spectrum of *m/z* 508 gave the most abundant daughter ion at *m/z* 332, which was produced by neutral loss of 176 Da, and the MS^3spectrum of *m/z* 508 → 332 was the same as the MS^2spectrum of M2 (*m/z* 332). Besides, there was the molecular ion at *m/z* 506 in the negative ion full scan LC–MS spectrum of the urine samples, which gave the daughter ion at *m/z* 175 in its MS^2spectrum. Furthermore, the ion at *m/z* 113 appeared in the MS^3 spectrum of *m/z* 508 → 175. This fragmentation (*m/z* 506 → 330 → 175 → 113) is the cleavage feature of glucuronide conjugates [21]. Consequently, M12 was identified as the glucuronide conjugate of M2, hydroxylpenehyclidine.

From our results, a superior sensitivity in full scan and MS^n mode combined with mass fragmentation provided most structurally informative product ion spectra for the metabolites. This approach was found to be essential for more comprehensive detection and characterization of metabolites of penehyclidine by our method. The fragmentation pathway of penehyclidine and the metabolic pathways of the metabolites of penehyclidine were discussed. The proposed major metabolic pathways of penehyclidine in rats were shown in Fig. 6. This investigation contributed with new information on penehyclidine metabolism which is essential for understanding the safety and efficacy of this drug and development of a novel drug.

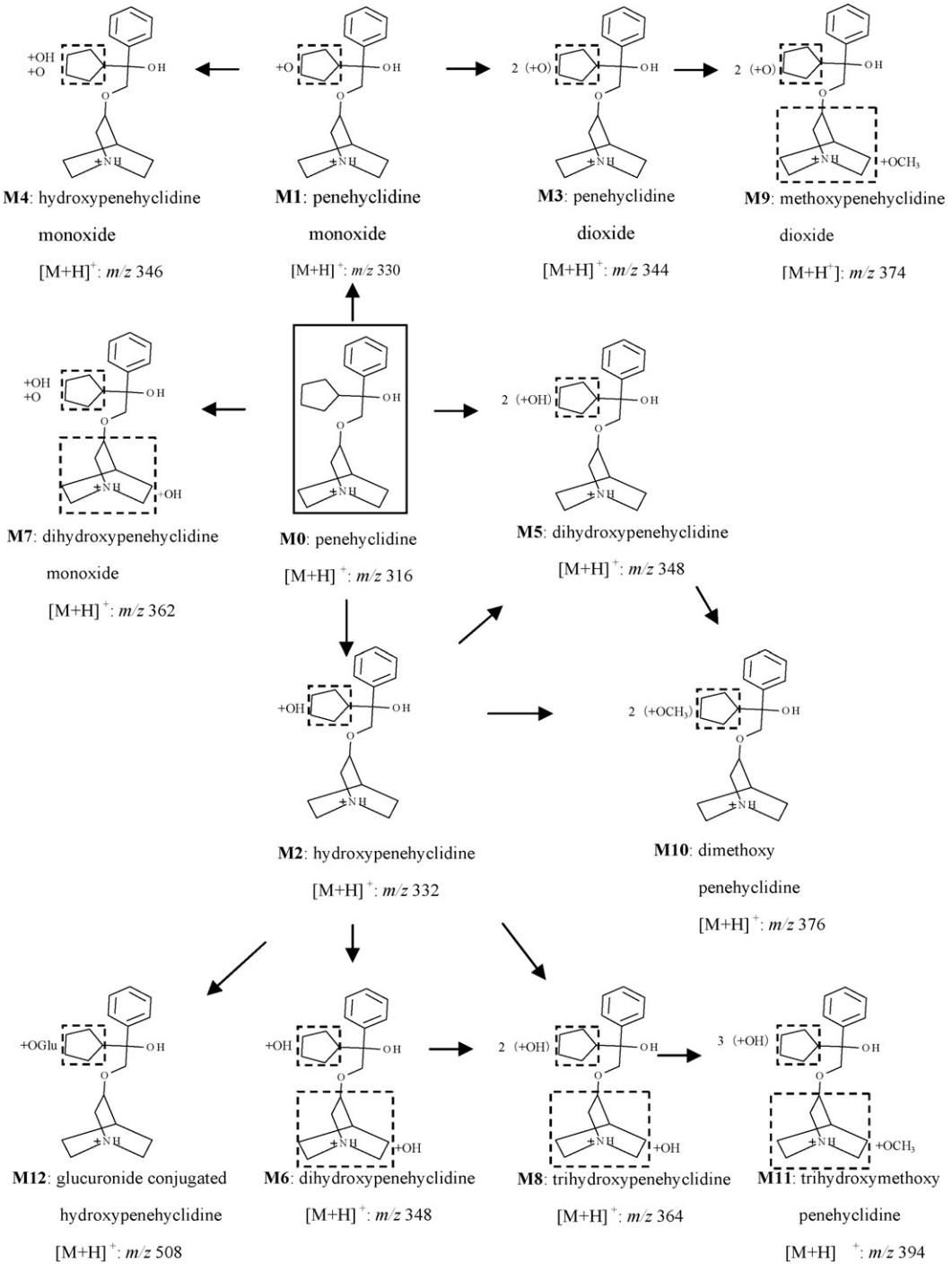

Fig. 6. Proposed major metabolic pathways of penehyclidine in rats (Glu = glucuronic acid).

The utility of MS on an ESI–MS^n was well documented for a variety of applications, including structural analyses of biopolymers such as DNA, proteins, oligosaccharides, and especial metabolites of drugs. It was useful to recognize that mass spectrometry with electron impact ion was not always suitable for drug metabolic investigation, especially for the polar molecules. Our work further demonstrated some of the limitations in GC–EI–MS capabilities of instrumentation. The examples given here had illustrated that the electrospray ionization mass spectrometry was well suited for full scan MS^n analysis due to full of sensitivity and specificity for the polar compounds. And it was widely accepted that considerable variation in sensitivity and specificity existed not only among different instrument models but also among different manufacturers of mass spectrometers. These data suggested that ESI–MS should be used exclusively for detection and identification of metabolites of drugs. Certainly, the precise identifications of metabolite structures need to further use the method of ^1H and ^{13}C NMR and metabolite synthesis. Different mass analyzers should be used to complement each other and to take advantage of specific properties of individual MS instruments. It was true that the stable isotope techniques as the template or the marker could increase specificity and efficiency of determination. Without complementary use of the stable isotope ion cluster, the metabolites of drug would be overlooked and/or incompletely identified. The systems should be regarded as synergistic methods when these techniques were used together to offer significant advantages. The results from electrospray ion and electron impact ion data with the stable isotope cluster showed the qualitative differences in the mass spectral patterns, suggesting that these techniques should be used in parallel to ensure comprehensive metabolites detection and characterization. The described method had wide applicability to screen drug metabolites rapidly and provide comprehensive structural information.

4. Conclusions

The method using a LC–MS^n ion trap with electrospray ionization, a GC–MS with electron impact ion source and stable isotope ion cluster has been developed for the rapid analysis of penehyclidine and its metabolites in rat urine. The proposed method is highly sensitive and specific for the qualitative determination of penehyclidine and its metabolites. Penehyclidine and its twelve metabolites were identified in rat urine. Elucidation of the phase I and phase II metabolites were performed by comparing the daughter ion pairs of stable isotope cluster, changes of the protonated molecular masses, full scan MS^n spectra and retention times with those of the parent drug, penehyclidine and penehyclidine five deuterium-labeled at phenyl group. These metabolites included eleven phase I metabolites (penehyclidine monoxide, hydroxypenehyclidine, penehyclidine dioxide, hydroxypenehyclidine monoxide, dihydroxypenehyclidine, dihydroxypenehyclidine, dihydroxypenehyclidine monoxide, trihydroxypenehyclidine, methoxypenehyclidine dioxide, dimethoxypenehyclidine, trihydroxymethoxypenehyclidine) and two phase II metabolites (the sulfate conjugates of penehyclidine and the glucuronide conjugates of hydroxypenehyclidine). The results from electrospray ion and electron impact ion data with the stable isotope cluster showed the qualitative differences in the mass spectral patterns, suggesting that these techniques should be used in parallel to ensure comprehensive metabolites detection and characterization. The described method had wide applicability to rapidly screen and identify the metabolites.

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