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Sensitive and specific liquid chromatographic–tandem mass spectrometric assay for atropine and its eleven metabolites in rat urine

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Abstract

A sensitive and specific method is described for the simultaneous determination of atropine and its metabolites in rat urine by combining liquid chromatography and tandem mass spectrometry (LC–MS^n). Various extraction techniques (free fraction, acid hydrolyses and enzyme hydrolyses) and their comparison were carried out for investigation of the metabolism of atropine. After extraction procedure the pretreated samples were separated on a reversed-phase C18 column using a mobile phase of methanol/ammonium acetate (2 mM, adjusted to pH 3.5 with formic acid) (70: 30, v/v) and detected by an on-line LC–MS^n system. Identification and structural elucidation of the metabolites were performed by comparing their changes in molecular masses (ΔM), retention-times and full scan MS^n spectra with those of the parent drug. The results revealed that at least eleven metabolites (*N*-demethyltropine, tropine, *N*-demethylatropine, *p*-hydroxyatropine, *p*-hydroxyatropine N-oxide, glucuronide conjugates and sulfate conjugates of *N*-demethylatropine, *p*-hydroxyatropine and the parent drug) and the parent drug existed in rat urine after ingesting 25 mg/kg atropine. *p*-Hydroxyatropine and the parent drug were detected in rat urine for up 106 h after ingestion of atropine.

Keywords: Atropine; LC–MS^n; Metabolite

1. Introduction

Atropine, a tropane alkaloid of medicinal interest, was found in plants of the Solanaceae family [1,2], such as *Atropa*, *Datura*, *Duboisia* and *Hyoscyamus*. Atropine has been widely utilized in clinic for many years as anticholinergic agents in premedication of anesthesia [3]. Currently, atropine is used for its antispasmodic activity on the gastrointestinal tract, as a preanesthesic agent and in ophthalmic solutions. Many methods have been developed for the quantitative determination of atropine in plants and pharmaceutical samples based on indirect atomic absorption spectrometry (AAS) [4], capillary electrophoresis (CE) [5–10], high-performance liquid chromatography (HPLC) [11–14] and liquid chromatography-mass spectrometry (LC–MS) [15]. Also, pharmacokinetic studies have been performed by gas chromatography (GC) [16], gas chromatography-mass spectrometry (GC–MS) [17] and liquid chromatography-tandem mass spectrometry (LC–MS^2) [18], respectively. But, little data is available on the metabolism and metabolites of atropine in vitro. The metabolic study of atropine will play an important role in the development of new drugs and its clinical application.

LC–MS^n has been proven to be a modern powerful analytical tool for the identification of drug metabolites in biological matrices [19,20]. This approach has superior sensitivity and specificity, and it is considerably less time consuming and less labor intensive than other methods, such as HPLC and GC–MS. In addition, it is especially suitable for the analyses of thermolabile, highly polar and non-volatile metabolites. MS^n technique has made possible the acquisition of

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structurally informative data from pseudomolecular ions of analytes of interest, even when they are not chromatographically resolved. Therefore, the coupled LC–MS^n method is an initial choice for the structure elucidation of drug metabolites.

The identification and structural elucidation of drug metabolites using LC–MS^n are based on the premise that the drugs retain their basic structural features after metabolism or biotransformation in vivo or in vitro. Producing the MS^n product ions associated with these basic structural features as a substructural template using parent drug, structures of metabolites may be rapidly characterized by comparing their product ions with those of parent drug even without standards for each metabolite [21,22]. Utilization of the electrospray LC–MS interface (LC-ESIMS), a soft ionization technique, allows for the consistent analysis of high labile and polar phase II metabolites at trace levels compared to earlier ionization modes due to its low internal energy imparted to analytes. In addition, ion trap analyzer (IT) can provide superior sensitivity and rich mass spectral information, and it is very suitable to qualitative assay for analytes [23].

This work presents a sensitive and specific LC-ESI-IT-MS^n method for rapid qualitative identification of atropine and its metabolites in rat urine. The LC–MS^n analyses of urine sampled from healthy rats after ingesting 25 mg/kg atropine revealed that the parent drug and its eleven metabolites (five phase I metabolites and six phase II metabolites) existed in rat urine. All these metabolites were detected for the first time. Various extraction procedures were also compared for the investigation of metabolism of atropine.

2. Experimental

2.1. Reagents and chemicals

Atropine and β-glucuronidase (from *E. coli*) were purchased from Sigma (St. Louis, MO, USA). 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.

2.2. Apparatus

LC–MS and LC–MS^n experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer with a modern TSP4000 HPLC pump and a TSP AS3000 autosampler using positive electrospray as the ionization process (all components from Finnigan, Austin, TX, USA). 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).

2.3. Sample preparation

2.3.1. Administration

Six wistar rats were obtained from Hubei Experimental Animal Research Center (China) 6 days before the experiment. Our laboratory is an SPF laboratory authorized by Hubei province gov. (China). All the animal studies were performed in the SPF laboratory. The rats were provided standard laboratory food and water ad libitum. The rats weighed 196–203 g at the time of the experiment. The rats were housed in metabolism cages and fasted for 24 h but with access to water, then they were administered 25 mg/kg oral gavage doses of atropine. Urine samples were collected at different time-points up to 110 h and centrifuged at 3000 × *g* for 10 min. The supernatants were stored at −20 °C until analyses.

2.3.2. Standard samples

Stock atropine solutions were prepared by dissolving atropine in methanol (1 mg/ml) and diluting to the desired concentration with methanol.

2.3.3. Urine extraction

2.3.3.1. Free fraction. An aliquot of 1 ml of mixed 0–24 h urine samples was loaded onto a C18 solid-phase extraction cartridge which 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 film and an aliquot of 10 μl was used for LC–MS^n analyses. Free fraction was used for the comprehensive LC–MS^n analyses of metabolites. The extracted solutions after acidic and enzymatic hydrolyses were only used for assistant investigation of phase II metabolites.

2.3.3.2. Acidic hydrolysis. After optimizing the acidity and the heated time, 0.8 ml of 6 M HCl and 50 mg of cysteine were added to 1 ml of mixed 0–24 h urine samples. The mixture was heated at 100 °C for 60 min. After cooling to room temperature, it was neutralized to pH 8 with 6 M NaOH and extracted by SPE cartridge immediately, according to the procedure described in Section 2.3.3.1.

2.3.3.3. Enzymatic hydrolysis. After optimizing the acidity, temperature, enzymatic content and the time of hydrolysis, 1 ml of mixed 0–24 h urine samples was adjusted to pH 5.0 with a few drops of glacial acetic acid. Then, 0.5 ml of acetate buffer (pH 5.0) and 0.2 ml of β-glucuronidase from *E. coli* (10,000 units/ml) were added to the solution prior to enzymatic hydrolyses. It took 5 h at 55 °C. After cooling, the solution was adjusted to pH 8 with 6 M NaOH and extracted by SPE cartridge immediately, according to the procedure described in Section 2.3.3.1.

2.4. Chromatographic conditions

A reversed-phase column (Zorbax extend C18, 3.0 × 100 mm i.d., 3.5 μm, Agilent Technologies) was connected with a guard column (cartridge 2.1 × 12.5 mm, 5 μm, Agilent Technologies) filled with the same packing material to separate atropine and its metabolites in rat urine. After optimizing the column temperature and the acidity of the mobile phase using atropine standard, the temperature of the column was set at 40 °C. The mobile phase consisted of methanol and 2 mM ammonium acetate (adjusted to pH 3.5 with formic acid) (70:30, v/v). The flow rate was 0.2 ml/min during the whole run.

2.5. Mass spectrometry conditions

Mass spectral Analyses were carried out in positive ion detection mode, and only the structures of phase II metabolites were validated by the LC–MS (MS^n) data of rat urine samples in negative ion detection mode. Nitrogen was used as the sheath gas (40 arbitrary units). A typical source spray voltage of 4.5 kV, a capillary voltage of 21 V and a heated capillary temperature of 175 °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 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 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.

3. Results and discussion

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

The first step of this work involved the characterization of the chromatogram and mass spectral properties of the parent drug. The characteristic product ions and neutral losses of the parent drug were the substructural 'template' for interpreting the structures of metabolites.

The LC–MS and LC–MS^n analyses of atropine were in positive ion detection mode because alkaloid molecules can be protonated easily under electrospray ionization condition. The chromatographic and mass spectrometry conditions were optimized using atropine standard. Full scan mass spectral analysis of atropine showed protonated molecular ion of *m*/*z* 290 (Fig. 1A). The MS^2 product ion spectrum of the protonated molecular ion (*m*/*z* 290) and the predominant fragmentation patterns were showed in Fig. 1B. Atropine was eluted at 3.21 min under the experimental conditions (Fig. 1C). Fragmentation of protonated molecular ion of

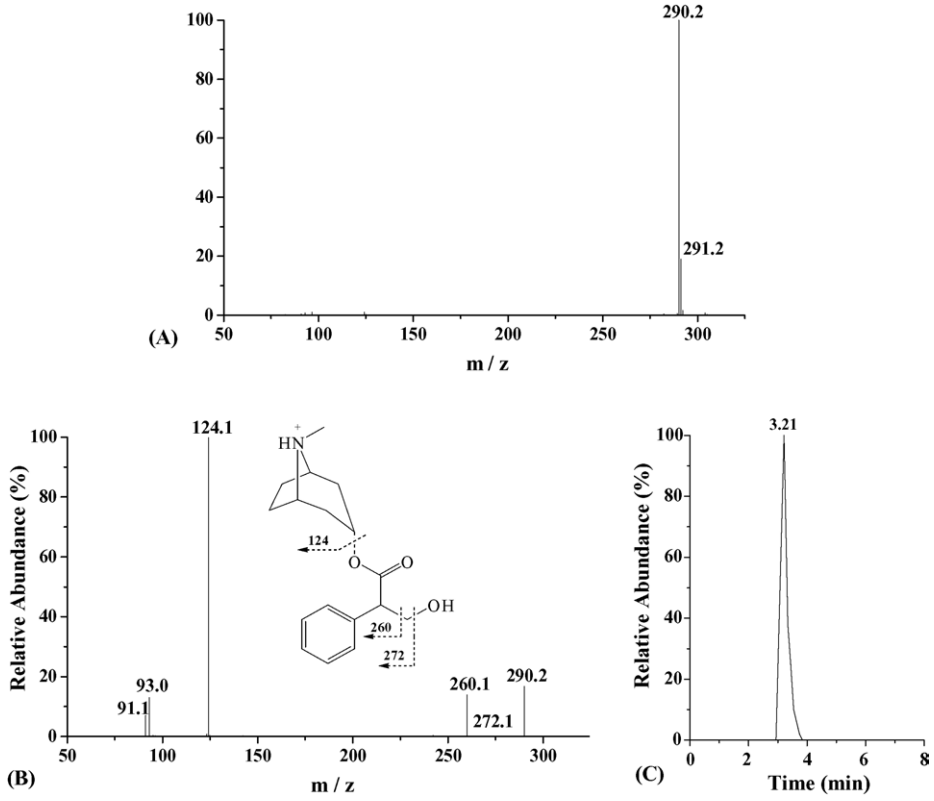

Fig. 1. (A) Full scan MS spectrum; (B) full scan MS^2 product ions spectrum and the predominant fragmentation patterns; (C) LC–MS^2 chromatogram of atropine.

atropine in the ion trap lead to five product ions at *m/z*: 272, 260, 124, 93 and 91. The product ions at *m/z* 272 and *m/z* 260 were formed by the loss of H_2O and HCHO from the molecular ion at *m/z* 290, respectively. The most abundant product ion at *m/z* 124 was formed by the loss of tropic acid ($C_9H_{10}O_3$, 166 Da). The ion at *m/z* 93 was inferred to be produced by the loss of NH_2CH_3 (31 Da) from the *m/z* 124 ion. The fragment ions at *m/z* 124, 93 and 91 coexisted in the MS^3 spectra of *m/z* 272 and *m/z* 260, and the MS^3 spectra of *m/z* 124 gave fragment ions at *m/z* 93 and *m/z* 91. It could be concluded that the ions at *m/z* 124, 93 and 91 were the characteristic product ions of atropine, and 30 and 166 Da were the characteristic neutral losses. These characteristic product ions and neutral losses were the sound bases to identify metabolites of atropine in vitro.

3.2. Chromatography and mass spectrometry identification of metabolites

Firstly, the possible structures of metabolites have been speculated according to the metabolism rule of drugs in vitro [24]. The full scan mass spectrum of free fraction of rat urine after ingesting atropine was compared with that of blank urine sample to find out the probable metabolites in rat. Then, these compounds were analyzed by LC–MS^n. Their retention-times, changes in observed mass (ΔM) and MS^n spectra were compared with the substructural 'template' of atropine standard to identify metabolites and elucidate their structures. Various extraction techniques (free fraction, enzyme hydrolyses and acid hydrolyses) and their comparison were carried out for investigation of the metabolism of atropine.

Based on the method mentioned above, the parent drug and its main metabolites were found in rat urine after ingesting atropine. Their molecular ions ($[M+H]^+$) were at *m/z* 128, 142, 276, 290, 306, 322, 356, 370, 386, 452, 466 and 482, respectively. Their LC–MS^2 chromatograms were presented in Fig. 2. It is obvious that the retention times of all metabolites are shorter than that of parent drug, which accords with the rule of drug metabolism.

LC–MS^n spectra of the metabolites of atropine were obtained via fragmentation of protonated molecular ions that used for more precise structural identification of metabolites. The MS^2 product ion spectra of these analytes were shown in Fig. 3. Among them, the retention time, the MS and MS^2 spectra of the molecular ion at *m/z* 290 (M0, Figs. 2D and 3D) were the same as those of atropine. Therefore, M0 can be confirmed as the unchanged parent drug.

The characteristic product ions at *m/z* 124, 93, 91 of the parent drug all appeared in the MS^2 spectrum of *m/z* 142 (M2, Fig. 3B). The MS^3 spectrum of *m/z* 142 → 124 was the same as that of *m/z* 290 → 124 (atropine). So, M2 was identified as the hydrolysis product of atropine, and it was called tropine [24].

The molecular ion at *m/z* 128 (M1) and its daughter ions at *m/z* 110 and 84 (Fig. 3A) were all 14 Da less than the molecular ion at *m/z* 142 (M2) and its daughter ions at *m/z* 124 and 98. These results indicated that M1 should be the *N*-demethyl product of M2 (*N*-demethyltropine).

The characteristic fragment ions at *m/z* 124, 93 and 91 coexisted in the MS^2 spectrum of *m/z* 276 (M3, Fig. 3C). The *m/z* 276 ion and its daughter ions at *m/z* 246 and 110 were all 14 Da less than the molecular ion of parent drug (*m/z* 290) and its daughter ions at *m/z* 260 and 124. Thus, M3 can be identified as the *N*-demethyl product of atropine (*N*-demethylatropine).

The protonated molecular ion at *m/z* 306 (M4) was increased by 16 Da compared to that of the unchanged atropine. Because of the appearances of the characteristic fragment ions at *m/z* 124, 93, 91 and characteristic neutral losses 30 Da (*m/z* 306 → 276), 182 Da (166 + 16) (*m/z* 306 → 124) in its MS^2 spectrum (Fig. 3E), M4 should be the hydroxylation product of atropine, and the localization of the hydroxyl group was on the tropic acid substructure. The presence of the *m/z* 288 ion, which was produced by the loss of H_2O, indicated that the benzyl carbon was not hydroxylated. So, the hydroxylation occurred at the aromatic ring. According to the oxidation metabolic rule of aromatic ring and the steric hindrance effect [24], the phenolic hydroxyl was produced at the para position of the substituent group of benzene ring (*p*-hydroxyatropine).

The characteristic product ions at *m/z* 124, 93 and 91 appeared in the MS^2 spectrum of the molecular ion at *m/z* 322 (M5, Fig. 3F) which was increased by 32 Da compared to that of the parent drug. The product ion at *m/z* 140 (124 + 16) and two pairs of product ions at *m/z* 305 (322-17), 304 (322-18) and *m/z* 123 (140-17), 122 (140-18) coexisted in the MS^2 spectrum of the molecular ion at *m/z* 322. All these results indicated that M5 was the N-oxide of M4 (*p*-hydroxyatropine N-oxide) because it is the cleavage feature of N-oxides to loss 17 and 18 Da [25], just like the characteristic MS^2 spectrum of oxymatrine we have studied.

The *m/z* 276 ion appeared in the MS^2 spectrum of the molecular ion at *m/z* 356 (M6, Fig. 3G), and the MS^3 spectrum of *m/z* 356 → 276 was the same as the MS^2 spectrum of the protonated molecular ion of M3. The product ion at *m/z* 276 was produced by neutral loss of 80 Da diagnostic of SO_3 [26,27]. Based on these data, M6 was identified as the sulfate conjugate of M3. This deduction can be validated further by the fact that there was *m/z* 354 ion in the negative ion full scan LC–MS spectrum of the urine samples.

There were the characteristic product ions at *m/z* 272 and 124 of the parent drug in the MS^2 spectrum of the molecular ion at *m/z* 370 (M7, Fig. 3H). The *m/z* 370 ion lost neutral fragment 80 Da (SO_3) to produce its predominant product ion at *m/z* 290, and the MS^3 spectrum of *m/z* 370 → 290 was the same as the MS^2 spectrum of atropine. There was molecular ion at *m/z* 368 in the negative ion full scan LC–MS spectrum of the urine samples. So M7 should be the sulfate conjugate of atropine.

The protonated molecular ion at *m/z* 386 (M8) lost neutral fragment 80 Da (SO_3) to produce the daughter ion at *m/z* 306

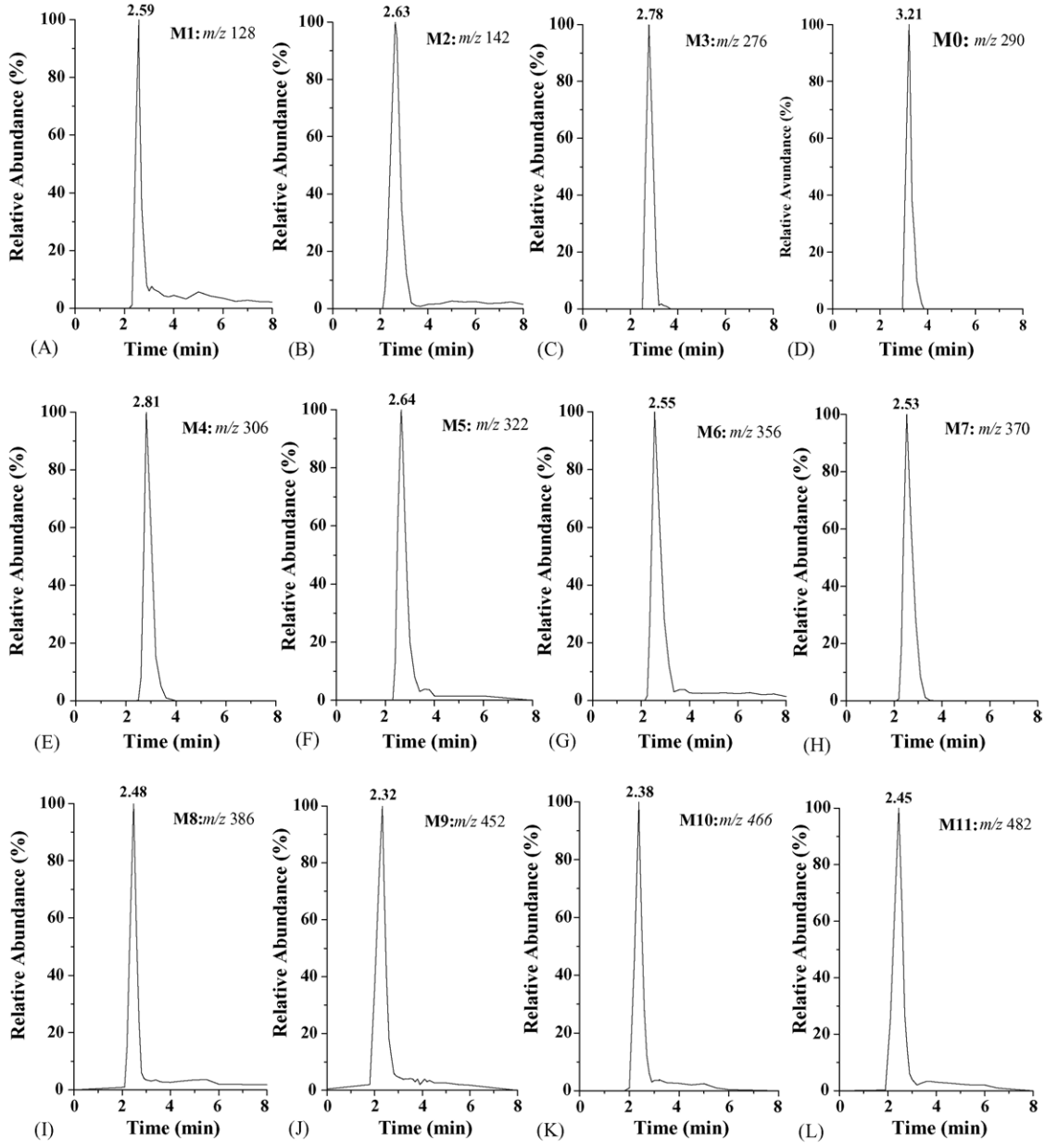

Fig. 2. LC–MS^2 chromatograms of atropine and its metabolites in rat urine.

(Fig. 3I), and the MS^3 spectrum of m/z 386 → 306 was the same as the MS^2 spectrum of the molecular ion of M4. There was the molecular ion at m/z 384 in the negative ion full scan MS spectrum of the urine samples. Consequently, M8 was identified as the sulfate conjugate of M4. Because phenolic hydroxyl has stronger affinity and higher speed than alcoholic hydroxyl in the sulfate esterifying reactions according to the rule of drug metabolism [24], and the selectivity of this conjugated reaction has been validated by many studies, M8 should be the sulfate conjugate of M4 conjugated at its phenolic hydroxyl position.

The MS^2 spectrum of m/z 452 (M9) gave abundant daughter ion at m/z 276 (Fig. 3J), which was produced by neutral loss of 176 Da, and the MS^3 spectrum of m/z 452 → 276 was the same as the MS^2 spectrum of the molecular ion of M3. Besides, there was the molecular ion at m/z 450 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^2 spectrum. Furthermore, the m/z 113 ion appeared in the MS^3 spectrum of m/z 450 → 175. This fragmentation (m/z 450 → 175 → 113) is the cleavage feature of glucuronide conjugates [28,29]. Thus, M9 was identified as the glucuronide conjugate of M3.

In the MS^2 spectrum of m/z 466 (M10), the parent ion lost neutral fragment 176 Da to give its daughter ion at m/z 290 (Fig. 3K), and the MS^3 spectrum of m/z 466 → 290 was the same as the MS^2 spectrum of the molecular ion of atropine. Besides, there was the molecular ion at m/z 464 in the negative ion full scan LC–MS spectrum of the urine samples,

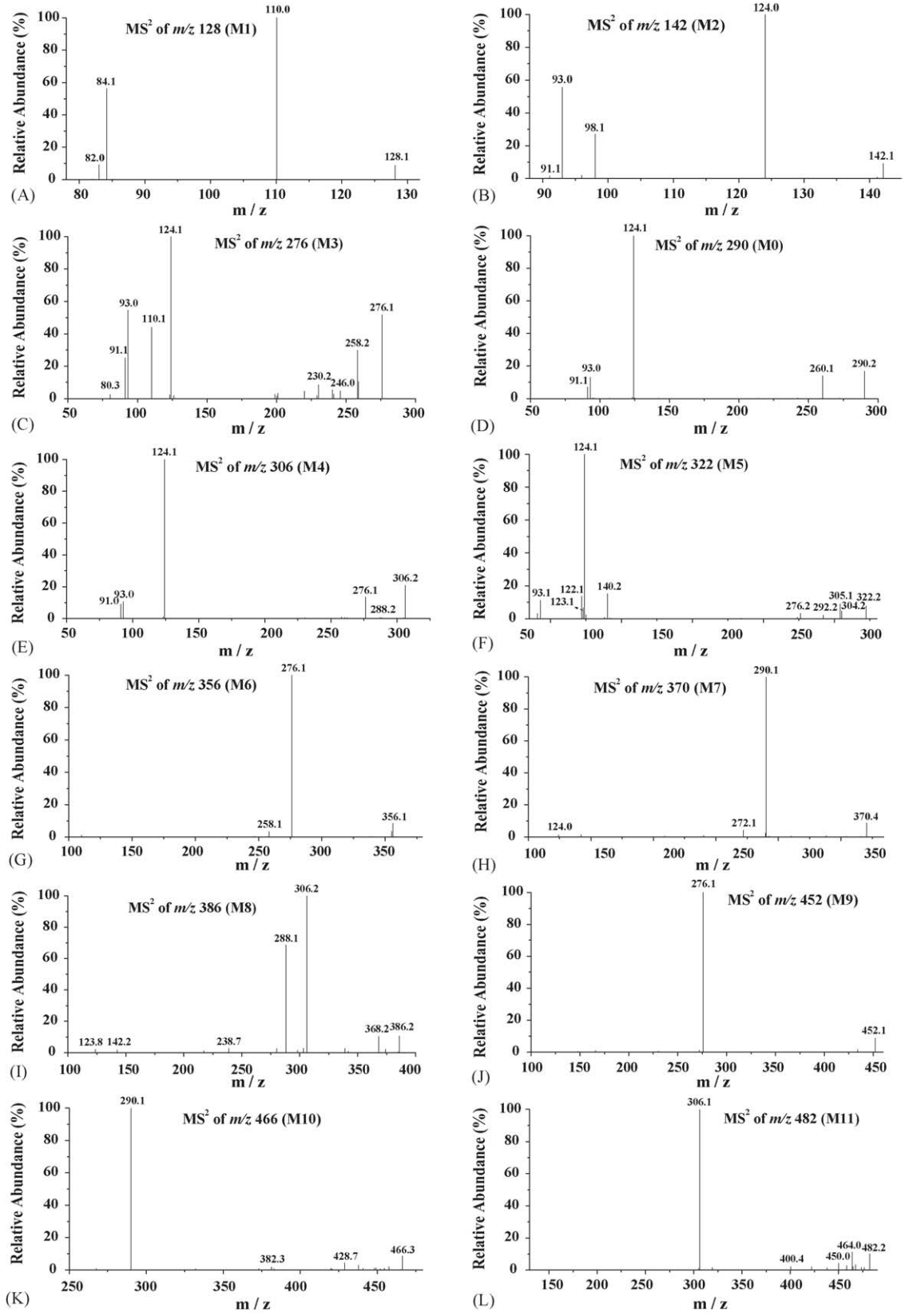

Fig. 3. MS^2 product ion spectra of atropine and its metabolites in rat urine.

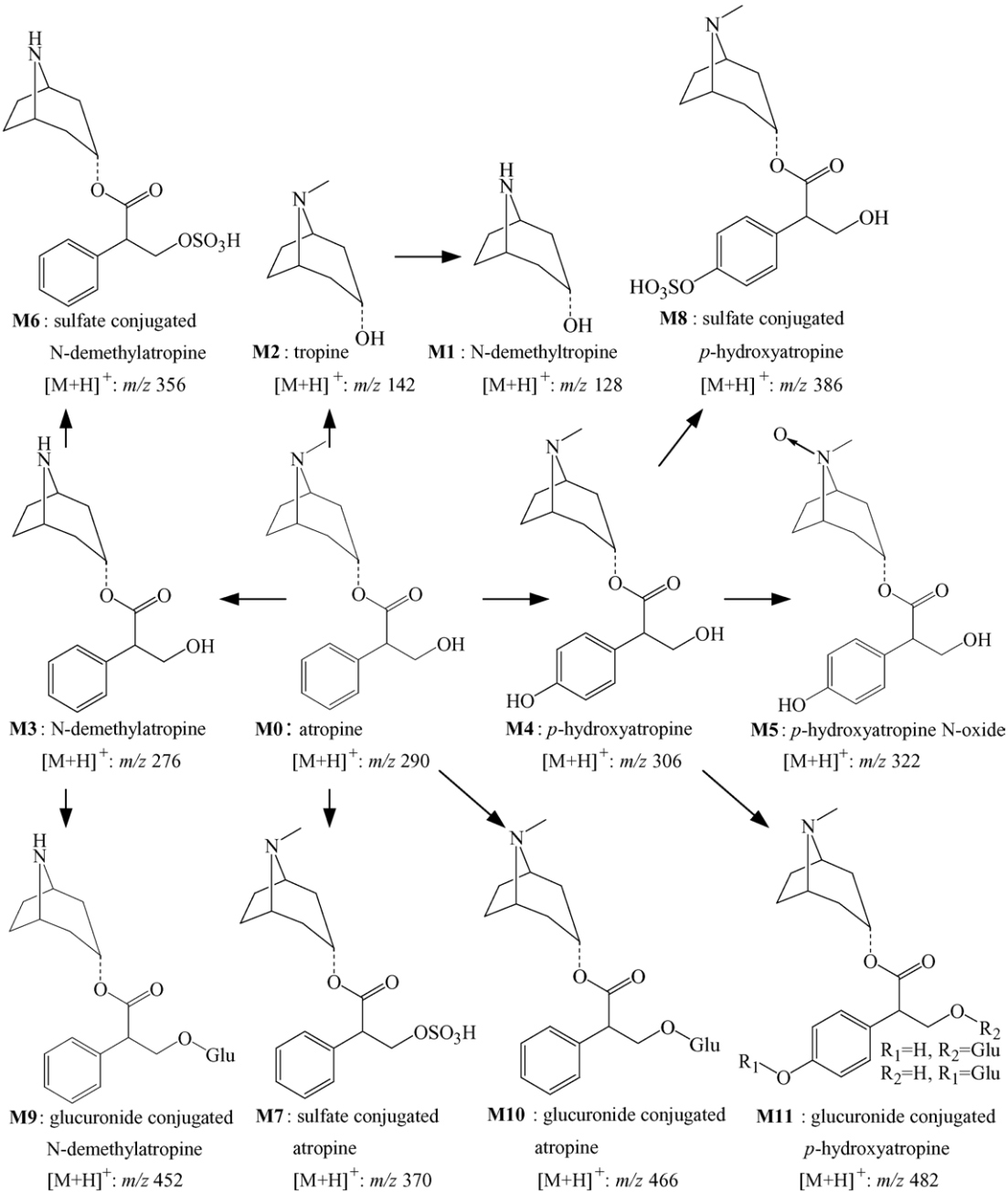

Fig. 4. Proposed major metabolic pathway of atropine in rats (*Glu*: flucuronic acid).

which gave the fragmentation of m/z 464 → 175 → 113 in its tandem MS spectra. So, M10 should be the glucuronide conjugate of atropine.

The predominant product ion at m/z 306 was formed by the loss of neutral fragment 176 Da from the parent ion at m/z 482 (M11, Fig. 3L), and the MS^3 spectrum of m/z 482 → 306 was the same as the MS^2 spectrum of M4 (m/z 306). There was also molecular ion at m/z 480 in the negative ion full scan LC–MS spectrum of the urine samples. The fragmentation of m/z 480 → 175 → 113 existed in the tandem MS spectra of the negative ion at m/z 480. Consequently, M11 was identified as the glucuronide conjugate of M4.

The proposed major metabolic pathway of atropine in rats was shown in Fig. 4.

These metabolites can be investigated further by comparing various extraction techniques. Compared with free fraction, the peak areas of M3, M0 and M4 increased, and those of M6–M11 decreased after acidic hydrolyses. The peak areas of M3, M0 and M4 increased, and those of M9, M10 and M11 decreased after enzymatic hydrolysis (Table 1). These results revealed that *N*-demethylatropine (M3), the unchanged atropine (M0) and the abundant *p*-hydroxyatropine (M4) excreted from rat urine as the free, sulfate conjugated and glucuronide conjugated forms.

Table 1
Comparison between different extraction procedures

Analyte	$[M+H]^+$	RT (min)	Peak area ($\times 10^5$)		
			Free SPE fraction	Acidic fraction	Enzymatic fraction
M1	128	2.59	1.3	Trace	Trace
M2	142	2.63	13.4	12.8	14.2
M3	276	2.78	12.6	45.3	30.9
M0	290	3.21	424.0	481.6	457.2
M4	306	2.81	421.4	428.1	423.7
M5	322	2.64	102.3	110.0	103.0
M6	356	2.55	12.3	ND[a]	11.4
M7	370	2.53	23.1	Trace	23.9
M8	386	2.48	5.0	ND	4.7
M9	452	2.32	17.6	ND	Trace
M10	466	2.38	39.0	Trace	Trace
M11	482	2.45	2.7	ND	ND

[a] Not found.

The time of excretion of atropine and its metabolites was detected using the tandem MS technique, all the phase I metabolites (M1–M6) and the parent drug (M0) were detected in 0–1 h rat urine, but all the phase II metabolites (M7–M10) were not found. M1, M2, M3 and M5 could be detected for up 76–96 h. M0 and M4 could be detected for up 106 h. M6 and M7 were detected in 1–2 h urine and excreted completely in 26 and 80 h, respectively. M8 and M10 appeared in 2–3 h urine, and disappeared in 50 and 26 h, respectively. M9 and M11 were detected in 3–4 h urine, and disappeared in 26 h.

In this work, the sensitivity of the method was determined using atropine spiked in blank urine, and its limit of detection (LOD) was lower than 5 ng/mL by LC–MS^n. The mean recoveries ($n = 5$) were 76.3% at concentrations of 10 ng/ml with the precision (R.S.D.) of 3.7%. The specificity of the assay was evaluated by analyzing blank solution and blank urine samples of rats, no impurity or endogenous interferences were found. Therefore, the proposed method is a highly sensitive and special method for the qualitative determination of atropine and its metabolites. The extracted urine samples were stable at least two months at 4 °C.

4. Conclusions

For the first time, the method using an LC–MS^n ion trap with electrospray ionization in the positive ion mode has been developed for the analysis of atropine and its metabolites in rat urine. Atropine and its eleven major metabolites were identified through comparing their chromatographic retention times, changes in observed mass (ΔM) and tandem MS spectra with those of the parent drug. These metabolites included five phase I metabolites (*N*-demethyltropine, tropine, *N*-demethylatropine, *p*-hydroxyatropine and *p*-hydroxyatropine N-oxide) and six phase II metabolites (glucuronide conjugates, sulfate conjugates of *N*-demethylatropine, atropine and *p*-hydroxyatropine). Various extraction techniques and their comparison validated the presence of six phase II metabolites. *p*-Hydroxyatropine and the parent drug could be detected for up 106 h in urine sampled from healthy rats after ingesting 25 mg/kg atropine.

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