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Analysis of scopolamine and its eighteen metabolites in rat urine by liquid chromatography-tandem mass spectrometry

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Abstract

A rapid and sensitive method is described for the determination of scopolamine and its metabolites in rat urine by combining liquid chromatography and tandem mass spectrometry (LC–MS/MS). Various extraction techniques (free fraction, acid hydrolyses and enzyme hydrolyses) and their comparison were carried out for investigation of the metabolism of scopolamine. After extraction procedure, the pretreated samples were injected into a reversed-phase C18 column with 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 MS/MS 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 18 metabolites (norscopine, scopine, tropic acid, aponorscopolamine, aposcopolamine, norscopolamine, hydroxyscopolamine, hydroxyscopolamine *N*-oxide, *p*-hydroxy-*m*-methoxyscopolamine, trihydroxyscopolamine, dihydroxy-methoxyscopolamine, hydroxyl-dimethoxyscopolamine, glucuronide conjugates and sulfate conjugates of norscopolamine, hydroxyscopolamine and the parent drug) and the parent drug existed in urine after ingesting 55 mg/kg scopolamine to healthy rats. Hydroxyscopolamine, *p*-hydroxy-*m*-methoxyscopolamine and the parent drug were detected in rat urine for up 106 h after ingestion of scopolamine.

Keywords: Scopolamine; LC–MS/MS; Metabolite

1. Introduction

Scopolamine is a kind of tropane alkaloids separated from various solanaceous species [1], such as the roots of Chinese traditional medicine *Anisodus tanguticus* (*Maxim.*) *Pascher*. Scopolamine has widespread physiological activities such as spasmolytic, anaesthetic, acesodyne and ophthalmic effects [2,3]. In recent years, more and more pharmacological activities of scopolamine have been investigated and noticed widely. Compared with the comprehensive investigations of its therapeutical purpose, the study on its metabolism in vivo is limited, although the metabolic study of scopolamine plays an important role in the development of new drugs and its clinical application.

Some analytical assays have been developed for the quantification of scopolamine in plants and pharmaceutical samples or in urine based on capillary electrophoresis-diode array-electrospray mass spectrometry [4] and high-performance liquid chromatography (HPLC) [5–8]. Also, pharmacokinetic studies have been performed by gas chromatography-mass spectrometry (GC-MS) [9–12] and liquid chromatography-tandem mass spectrometry (LC–MS/MS) [13]. However, metabolic studies of scopolamine in vivo have not been reported except in three papers [14–16]. Werner and Schmidt [14] described the formation of metabolites such as 6-hydroxyatropine, scopine and scopolamine glucuronide. Wada and coworkers [15,16] found seven urinary metabolites (tropic acid, aponorscopolamine, aposcopolamine, norscopolamine, *p*-hydroxyscopolamine, *m*-hydroxyscopolamine, *p*-hydroxy-*m*-methoxyscopolamine) and the unchanged drug in several mammalian species by the combination

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of multi-approaches such as TCL, GC, GC-MS and NMR. Only three major metabolites (*p*-hydroxyscopolamine, *m*-hydroxyscopolamine and *p*-hydroxy-*m*-methoxyscopolamine) were detected in rat. Because scopolamine and its metabolites are highly polar and thermolabile and easy to undergo pyrolysis in the injector block of a gas chromatography, the used GC or GC–MS method is not satisfied in the analysis of metabolism of scopolamine. Besides, the urine samples were prepared using 7% HCl at 100 °C, which would make many metabolites decomposed.

LC–MS/MS has been proven to be a modern powerful tool for the identification of drug metabolites in biological matrices [17–20]. This approach has high sensitivity and specificity. Besides, it is considerably less time consuming and less labor intensive than other methods, such as HPLC and GC–MS. In addition, MS/MS technique has made it possible to acquire rich structural informative data from pseudomolecular ions of analytes of interest. The identification and structural elucidation of drug metabolites using LC–MS/MS are based on the premise that the drugs retain their basic structural features after biotransformation in vivo. Producing the MS^n product ions associated with these basic structural features as a substructural template by 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 [17,21–25].

Utilization of the electrospray LC–MS interface (LC–ESIMS), a soft ionization technique, allows for the consistent analysis of thermolabile, highly polar and non-volatile metabolites at trace levels compared to earlier ionization modes due to its low internal energy imparted to analytes. Ion trap analyzer (IT) can provide high sensitivity and rich mass spectral information, which makes it superior in qualitative assay [22]. Therefore, the coupled LC–ESI–IT–MS^n method is an initial choice for the structure elucidation of drug metabolites.

This work presents a sensitive and specific LC–ESI–IT–MS^n method for rapid and effective qualitative identification of metabolites of scopolamine in rat urine. The urine samples were pretreated using SPE cartridges, which makes the losses of metabolites decrease remarkably. The LC–MS/MS analyses of urine sampled from healthy rats after ingesting 55 mg/kg scopolamine revealed that the parent drug and its 18 metabolites (12 phase I metabolites and six phase II metabolites) existed in rat urine. Most of the metabolites were detected for the first time, which will be useful for future studies involving scopolamine, such as clinical therapy.

2. Experimental

2.1. Reagents and chemicals

Scopolamine hydrobromide 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); water was deionized and double distilled; all other reagents were of analytical reagent grade.

2.2. Apparatus

LC–MS and LC–MS^n experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer (ThermoFinnigan, Corp, San Jose, USA) with a modern TSP4000 HPLC pump and a TSP AS3000 autosampler using positive electrospray as the ionization process. The software Xcalibur version 1.2 (Finnigan) was applied for system operation and data collection. Rat urine samples were extracted on a C18 solid-phase extraction cartridge (3 ml/200 mg, AccuBond^{II}, Agilent). A high-speed desk centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge urine samples.

2.3. Sample preparation

2.3.1. Administration

Five wistar rats (180 ± 5 g, Hubei Experimental Animal Research Center, China) were housed in metabolic cages for the collection of urine. The rats were fasted for 24 h but with access to water, and then they were administered 55 mg/kg oral gavage doses of scopolamine. Urine samples were collected for a period of 120 h and centrifuged at 3000 × *g* for 10 min. The supernatants were stored at −20 °C until analyses.

2.3.2. Standard samples

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

2.3.3. Urine extraction

2.3.3.1. Free fraction. One millilitre of mixed 0–24 h urine samples was loaded onto a C18 solid-phase extraction cartridge that was preconditioned with 2 ml of methanol and 1 ml of water. Then, the SPE cartridge was washed with 2 ml of water and the analytes were eluted with 1 ml of methanol. The elution solutions were filtered through 0.45 μm film and an aliquot of 10 μl was used for LC–MS/MS analyses. The extracted urine samples were stable for at least 2 months at 4 °C. Free fraction was used for the comprehensive LC–MS/MS analyses of metabolites. The extracted solutions after acidic and enzymatic hydrolyses were used only 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 mm × 100 mm I.D., 3.5 μm, Agilent, USA) was connected with a guard column (cartridge 2.1 mm × 12.5 mm, 5 μm, Agilent) filled with the same packing material to separate scopolamine and its metabolites in rat urine. 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 spectrometric detection was carried out in positive ion mode, and only the structures of tropic acid and six phase II metabolites were validated by analyzing the LC–MS (MS^n) spectra of rat urine samples in negative ion detection mode. Nitrogen was used as the sheath gas (40 arbitrary units). The MS analyses were performed under automatic gain control conditions, using a typical source spray voltage of 4.5 kV, a capillary voltage of 21 V and a heated capillary temperature of 175 °C. The other parameters, including the voltages of octapole offset and tube lens offset, were also optimized for maximum abundance of the ions of interest by the automatic tune procedure of the instrument. The MS^n product ion spectra were produced by collision induced dissociation (CID) of the protonated 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.

3. Results and discussion

3.1. LC–MS and LC–MS/MS analyses of scopolamine

The first step in this work involved the characterization of mass spectral properties of the parent drug. The chromatographic and mass spectrometry conditions were optimized using scopolamine. Full scan mass spectral analysis of scopolamine showed protonated molecular ion of *m/z* 304. The

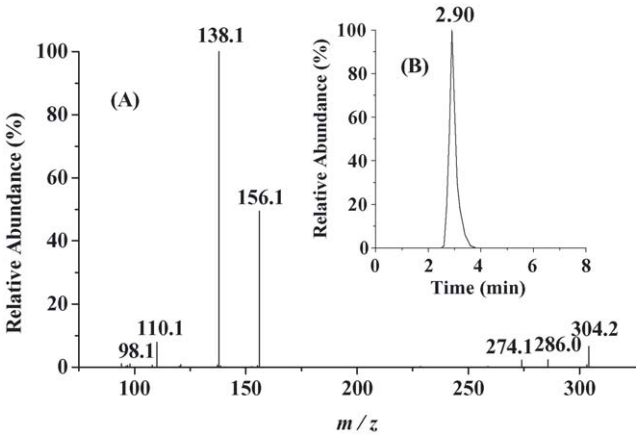

Fig. 1. (A) MS–MS product ion spectrum of scopolamine; (B) LC–MS^2 chromatogram of scopolamine.

MS–MS product ion spectrum of the protonated molecular ion (*m/z* 304) and the LC–MS^2 chromatogram of scopolamine were showed in Fig. 1A and B. Scopolamine was eluted at 2.90 min under the experimental conditions. Fragmentation of protonated molecular ion of scopolamine in the ion trap lead to five main product ions *m/z*: 286, 274, 156, 138 and 110. The product ions at *m/z* 286 and 274 were formed by the loss of H_2O and HCHO from the molecular ion at *m/z* 304, respectively. The most abundant product ion at *m/z* 138 was formed by the loss of tropic acid ($C_9H_{10}O_3$, 166 Da). The ion at *m/z* 156 was produced by the loss of $C_9H_8O_2$ (148 Da). The fragment ions at *m/z* 156, 138 and 110 coexisted in the MS^3 spectra of *m/z* 286 and 274. It could be concluded that the ions at *m/z* 156 and 138 were a pair of characteristic product ions of scopolamine, and 148 and 166 Da were its characteristic neutral losses. These characteristic product ions and neutral losses were the sound bases to identify metabolites of scopolamine. The molecular structure of scopolamine and its proposed fragmentation pathway were showed in Fig. 2.

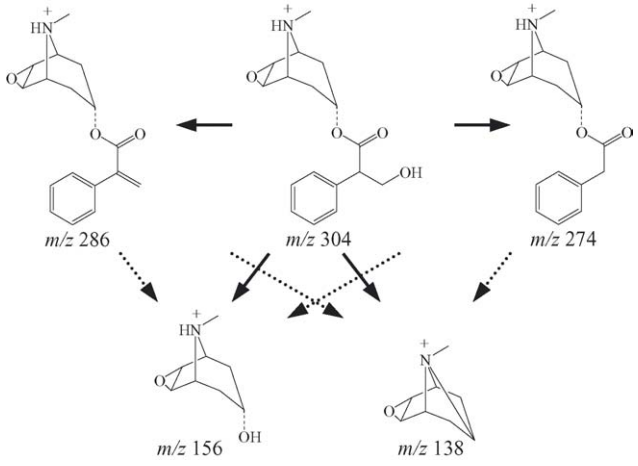

Fig. 2. Fragmentation pathway of scopolamine (solid lines represented fragmentation in MS^2; dash lines represented fragmentation in MS^3).

3.2. Chromatography and mass spectrometry identification of metabolites

In order to identify the metabolites, the possible structures of metabolites have been speculated according to the rule of drug metabolism firstly [26]. The full scan mass spectrum of free fraction of rat urine after administration of scopolamine was compared with those of blank rat urine samples and scopolamine solution to find out the possible metabolites in rat urine. Then, these compounds were analyzed by LC–MS/MS. Their retention times, changes in observed mass (ΔM) and spectral patterns of product ions were compared with those of scopolamine 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 scopolamine.

Based on the method mentioned above, the parent drug and its metabolites were found in rat urine after administration of scopolamine. Their molecular ions ($[M+H]^+$) were at *m/z* 142, 156, 272, 286, 290, 304, 320, 336, 350, 352, 366, 370, 380, 384, 400, 466, 480 and 496, respectively. Their retention times were given in Table 1. LC–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* 304 (M0) were the same as those of scopolamine. Therefore, M0 could be confirmed as the unchanged parent drug.

The MS^2 spectrum of *m/z* 156 (M1) was the same as the MS^3 spectrum of the molecular ion of scopolamine at *m/z* $304 \rightarrow 156$, and there were the characteristic product ions at *m/z* 98, 110, 138 in its MS^2 spectrum. So, M1 was identified as the hydrolysis product of scopolamine, and it was scopine [26].

The molecular ion at *m/z* 142 (M2) and its daughter ions at *m/z* 124, 114, 96, 84 and 70 were all 14 Da less than *m/z* 156 (M1) and its daughter ions at *m/z* 138, 128, 110, 98 and 84, respectively. These results indicated that M2 was the *N*-demethyl product of M1 (norscopine).

The characteristic product ions of *m/z* 110 and 138 appeared in the MS^2 spectrum of the protonated molecular ion at *m/z* 286 (M4), which was decreased by 18 Da compared to that of the unchanged scopolamine. The result indicated that M4 was the dehydrated metabolite of scopolamine (aposcopolamine), which accorded to the result of Wada and coworkers [15,16].

The molecular ion at *m/z* 272 (M3) and its daughter ions at *m/z* 254, 124 and 96 were all 14 Da less than *m/z* 286 and its daughter ions *m/z* 268, 138 and 110, respectively. Therefore, M3 could be confirmed as the *N*-demethyl product of M4 (aponorscopolamine). This was also in accordance with the Refs. [15,16].

The fragment ions at *m/z* 142 and 124 were produced by losing neutral fragments 148 Da and 166 Da from the parent ion at *m/z* 290 (M5), which were the same as the neutral losses of the parent drug. It was obvious that the *m/z* 290 ion and its daughter ions at *m/z* 272, 260, 142, 124 and 96 were all 14 Da less than the molecular ion of parent drug (*m/z* 304) and its daughter ions at *m/z* 286, 274, 156, 138 and 110. Thus, M5 could be affirmed as the *N*-demethyl product of scopolamine (norscopolamine).

The protonated molecular ion at *m/z* 320 (M6) was increased by 16 Da compared to that of the unchanged scopolamine. Because of the appearances of the characteristic fragment ions at *m/z* 156, 138 and characteristic neutral losses

Table 1
Comparison between different extraction procedures

Analyte	$[M+H]^+$	RT (min)	$LC–MS^2$ chromatographic peak area ($\times 10^5$)		
			Free fraction	Acidic fraction	Enzymatic fraction
M2	142	2.25	1.8	2.1	1.9
M1	156	2.35	7.2	7.9	7.7
M3	272	3.15	10.6	10.0	10.1
M4	286	3.05	37.1	35.6	37.0
M5	290	2.39	15.4	23.0	18.1
M0	304	2.91	65.2	79.8	71.3
M6	320	2.32	208.3	214.7	212.4
M7	336	2.30	3.8	4.3	4.1
M8	350	2.31	95.8	98.1	95.9
M9	352	2.28	0.75	0.80	0.78
M10	366	3.27	2.4	2.1	2.4
M11	370	2.37	2.5	ND[a]	1.9
M12	380	2.33	1.2	1.2	1.1
M13	384	2.36	9.8	Trace	8.3
M14	400	2.28	2.1	ND	2.7
M15	466	2.23	1.9	ND	ND
M16	480	2.26	5.7	Trace	Trace
M17	496	2.27	3.4	ND	Trace

[a] Not found.

164 Da (148 + 16) (*m/z* 320 → 156), 182 Da (166 + 16) (*m/z* 320 → 138) in its MS^2 spectrum, M6 should be the hydroxylation product of scopolamine hydroxylated at the tropic acid part. The *m/z* 302 ion was produced by the loss of H_2O from the parent ion at *m/z* 320. The result indicated that the benzyl hydrogen still existed in M6. Therefore, M6 presented *p*-hydroxyscopolamine and *m*-hydroxyscopolamine according to the study of Wada and coworkers [15,16]. Because the attempt to identify chromatographically the isomers failed in our experiment, we could not ascertain the hydroxylated site and isomer number.

The characteristic product ions at *m/z* 156, 138 and 110 appeared in the MS^2 spectrum of the molecular ion at *m/z* 336 (M7) that was increased by 32 Da compared to that of the parent drug. The appearance of the predominant product ion at *m/z* 172 (156 + 16) in the MS^2 spectrum of the molecular ion at *m/z* 336 indicated that the scopine part was oxidized, and the other oxidation should occur at trapic acid part. Besides, in the MS^2 spectrum of *m/z* 336, a pair of product ions at *m/z* 155 and 154 (more abundant than *m/z* 155) was produced by the loss of 17 and 18 Da from the ion at *m/z* 172, respectively. Chong [27] theoretically expounded the fragmentation feature of N-oxide: losing 17, 18 Da from the parent molecule. The fragmentation feature has been validated using oxymatrine in our experiment. Base on these data, M7 was deduced as N-oxide of scopolamine. The *m/z* 318 ion was produced by the loss of H_2O from its parent ion at *m/z* 336. The result indicated that the benzyl hydrogen still existed in M7. So, M7 should be the hydroxyscopolamine N-oxide.

The fragment ions at *m/z* 286, 274, 156, 138 and 110 of the parent drug appeared in the MS–MS spectrum of *m/z* 350 (M8) that was increased by 46 Da compared to that of the parent drug. Therefore, considering results obtained by Wada and coworkers [15,16], M8 should be *p*-hydroxy-*m*-methoxy-scopolamine.

The characteristic product ions at *m/z* 156, 138 and 110 of the parent drug appeared in the MS–MS spectra of *m/z* 352 (M9), 366 (M10) and 380 (M12), and there were not the *m/z* 172 (156 + 16) ion in their MS^2 spectra. The dehydrated fragment ions ($[M + H–H_2O]^+$) at *m/z* 334, 348 and 362 existed in the MS^2 spectra of M9, M10 and M12, respectively. So, they were phenolic metabolites. According to their changes in molecular masses (ΔM), M9, M10 and M12 could be affirmed as trihydroxyscopolamine, dihydroxy-methoxyscopolamine and hydroxyl-dimethoxyscopolamine, respectively.

The MS^2 spectrum of the molecular ion at *m/z* 370 (M11) showed daughter ions at *m/z* 290, 142 and 124, and the MS^3 spectrum of *m/z* 290 was the same as the MS^2 spectrum of the protonated molecular ion of M5 (*m/z* 290). The product ion at *m/z* 290 was produced by neutral loss of 80 Da diagnostic of SO_3 [18,28]. Based on these data, M11 was identified as the sulfate conjugate of M5. This deduction can be validated further by the fact that there was *m/z* 368 ion in the negative ion full scan LC–MS spectrum of the urine samples.

There were the characteristic product ions at *m/z* 156 and 138 of the parent drug in the MS^2 spectrum of the molecular ion at *m/z* 384 (M13). The *m/z* 384 ion lost neutral fragment 80 Da to produce its predominant product ion at *m/z* 304, and the MS^3 spectrum of *m/z* 384 → 304 was the same as the MS^2 spectrum of scopolamine. There was molecular ion at *m/z* 382 in the negative ion full scan LC–MS spectrum of the urine samples. So M13 could be confirmed as the sulfate conjugate of scopolamine.

The protonated molecular ion at *m/z* 400 (M14) lost neutral fragment 80 Da (SO_3) to produce the daughter ion at *m/z* 320, and the MS^3 spectrum of *m/z* 400 → 320 was the same as the MS^2 spectrum of M6 (*m/z* 320). There was the molecular ion at *m/z* 398 in the negative ion full scan MS spectrum of the urine samples also. So, M14 was identified as the sulfate conjugate of M6. Phenol has stronger affinity and higher speed than alcohol in the esterification reactions according to the rule of drug metabolism [26], and the appearance of the product ion at *m/z* 382 ($[M + H–H_2O]^+$) indicated that M14 still retained the alcoholic hydroxyl. So, M14 should be the sulfate conjugate of M6 esterified at its phenolic hydroxyl position.

The MS^2 spectrum of *m/z* 466 (M15) gave the abundant daughter ion at *m/z* 290, which was produced by neutral loss of 176 Da, and the MS^3 spectrum of *m/z* 466 → 290 was the same as the MS^2 spectrum of M5 (*m/z* 290). Besides, there was the molecular ion at *m/z* 464 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* 464 → 175. This fragmentation (*m/z* 464 → 175 → 113) is the cleavage feature of glucuronide conjugates [29,30]. Consequently, M15 was identified as the glucuronide conjugate of M5.

In the MS^2 spectrum of *m/z* 480 (M16), the parent ion lost neutral fragment 176 Da to give its daughter ion at *m/z* 304, and the MS^3 spectrum of *m/z* 480 → 304 was the same as the MS^2 spectrum of scopolamine. Besides, there was the molecular ion at *m/z* 478 in the negative ion full scan LC–MS spectrum of the urine samples, which gave the fragmentation of *m/z* 478 → 175 → 113 in its tandem MS spectra. So, M16 was the glucuronide conjugate of scopolamine.

The predominant product ion at *m/z* 320 was formed by the loss of neutral fragment 176 Da from the parent ion at *m/z* 496 (M17), and the MS^3 spectrum of *m/z* 496 → 320 was the same as the MS^2 spectrum of M6 (*m/z* 320). There was also molecular ion at *m/z* 494 in the negative ion full scan LC–MS spectrum of the urine samples. The characteristic cleavage of *m/z* 494 → 175 → 113 existed in the tandem MS spectra of the negative ion at *m/z* 494, too. The appearance of the product ion at *m/z* 478 ($[M + H–H_2O]^+$) in the MS^2 spectrum of *m/z* 496 indicated that M17 still retained the alcoholic hydroxyl. So, M17 was confirmed as the glucuronide conjugate of M6 conjugated at the phenolic hydroxyl site.

The *m/z* 165 ion (M18) appeared in the negative ion full scan LC–MS spectrum of the urine samples. The appearances

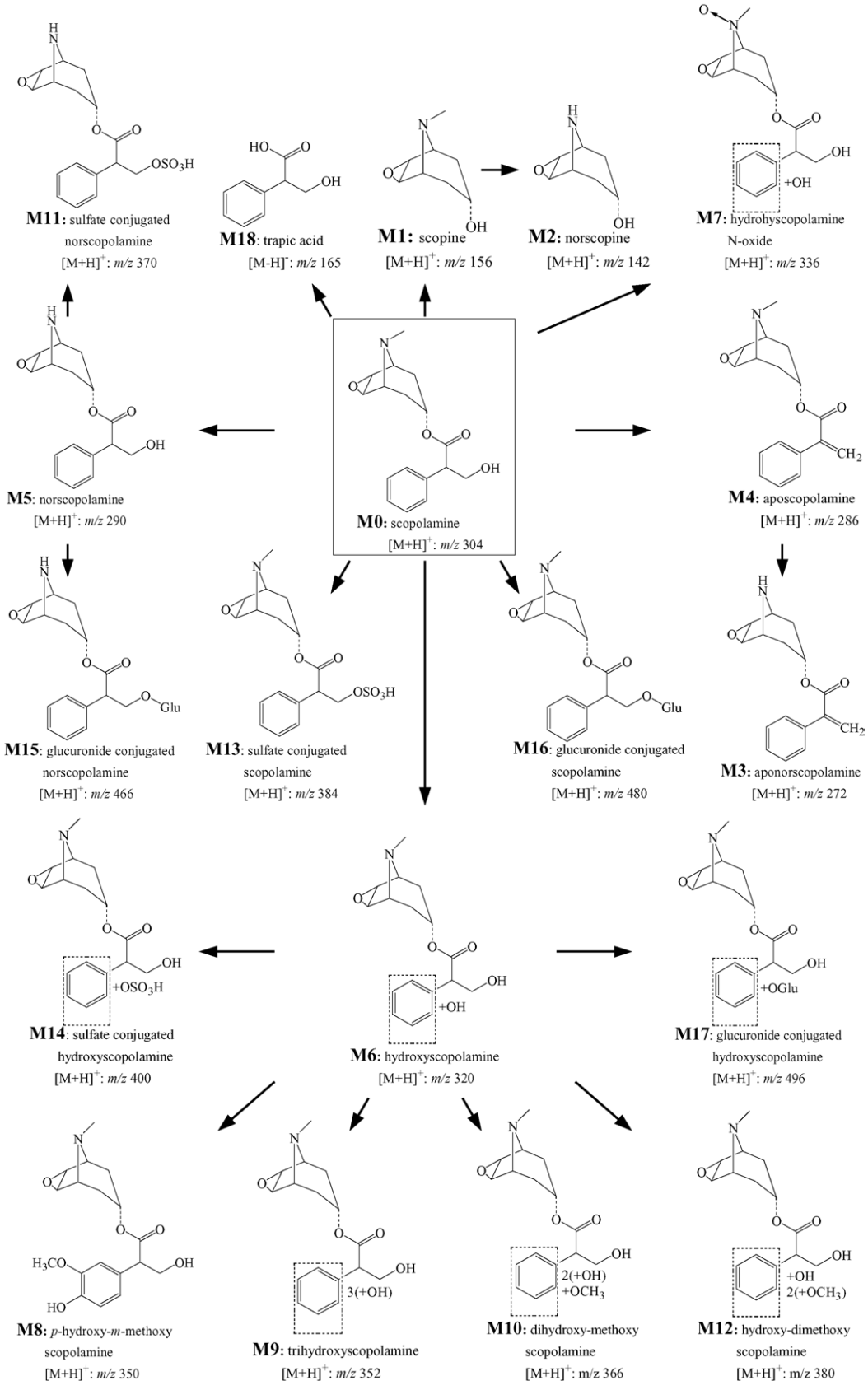

Fig. 3. Proposed major metabolic pathway of scopolamine in rats (Glu = flucuronic acid).

of the product ions at m/z 147 ($[M–H–H_2O]^-$) and 121 ($[M–H–CO_2]^-$) indicated that M18 was the hydrolysis product of scopolamine (tropic acid), which accorded to the result of Wada and coworkers [15,16]. No sulfate or glucuronide conjugate of M18 was found in rat urine.

The structures of M6, M7, M9, M10, M12, M14 and M17 could not be determined conclusively by mass spectrometry alone, but partial identification was made.

The proposed major metabolic pathway of scopolamine in rats was shown in Fig. 3. Most of the metabolites were found for the first time. Werner and Schmidt [14] reported that rats excreted 6-hydroxyatropine as a major metabolite of scopolamine, although the identification method was not described in detail [14]. Wada and coworkers did not found this metabolite [15,16], so did we. This inconsistency may due either to a difference of age of the rats used, or to the dose (152 mg/kg for Werner and Schmidt), or some other unknown factors.

These metabolites can be investigated further by comparing various extraction techniques. Compared with free fraction, the peak areas of M5, M0 and M6 increased, and those of M11, M13–M17 decreased after acidic hydrolyses; The peak areas of M5, M0 and M6 increased, and those of M15, M16 and M17 decreased after enzymatic hydrolysis (Table 1). These results revealed that norscopolamine (M5), the unchanged scopolamine (M0) and the abundant hydroxyscopolamine (M6) excreted from rat urine as the free, sulfate conjugated and glucuronide conjugated forms.

The time of excretion of scopolamine and its metabolites has been assessed using the tandem MS technique. Norscopolamine (M5) and its conjugated products (M11, M15) could be detected for only 26–30 h. M9 and M18 could be detected for up 50 h. M1–M4, M12 and M16 could be detected for up about 70–86 h. M7, M10, M13, M14 and M17 could be detected for up 96–100 h. The unchanged scopolamine (M0), hydroxyscopolamine (M6) and *p*-hydroxy-*m*-methoxyscopolamine (M8) could not be detected for up 106 h.

In this work, the sensitivity of the method was determined using scopolamine. The limit of detection (LOD) was lower than 5 ng/ml by LC–MS/MS. For the free fraction of rat urine, the mean recoveries ($n = 5$) were 67.6 and 79.2% at concentrations of 20 and 50 ng/ml, respectively. The specificity of the assay was evaluated by analyzing blank solution and blank urine samples of rats. No impurity or endogenous interferences were found.

4. Conclusions

For the first time, the method using a LC–MS/MS ion trap with electrospray ionization in the positive ion mode has been developed for the analysis of scopolamine and its metabolites in rat urine. The proposed method is highly sensitive and special for the qualitative determination of scopolamine and its metabolites. Scopolamine and its 18 metabolites were identified in rat urine. Compared to the works of Werner and Wada, the metabolites identified are much more. These metabolites included 12 phase I metabolites (norscopine, scopine, tropic acid, aponorscopolamine, aposcopolamine, norscopolamine, hydroxyscopolamine, hydroxyscopolamine N-oxide, *p*-hydroxy-*m*-methoxyscopolamine, trihydroxyscopolamine, dihydroxy-methoxyscopolamine and hydroxyldimethoxyscopolamine) and six phase II metabolites (glucuronide conjugates, sulfate conjugates of norscopolamine, scopolamine and hydroxyscopolamine). The investigation of different extraction procedures confirmed the existence of phase II metabolites further. Hydroxyscopolamine, *p*-hydroxy-*m*-methoxyscopolamine and the parent drug could be detected for up 106 h in urine sampled from healthy rats after ingesting 55 mg/kg scopolamine.

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References

[1] M. Lounasmaa, T. Tamminen, in: G.A. Cordell (Ed.), The Alkaloids, 44, Academic Press, New York, 1993.
[2] S. Pompeia, J.M. Rusted, H.V. Curran, Hum. Psychopharmacol. Clin. Exp. 17 (2002) 51.
[3] L.E. Shutt, J.B. Bowes, Anaesthesia 34 (1979) 476.
[4] L. Mateus, S. Cherkaoul, P. Christen, J.L. Verthey, Electrophoresis 20 (1999) 3402.
[5] H.L. Yi, G.D. Zhang, Y.Y. Tong, K. Sagara, T. Oshima, T. Yoshida, J. Chromatogr. 481 (1989) 428.
[6] S. Auriola, A. Nartinsen, K.M. Oksman-Caldentey, T. Naaranlahti, J. Chromatogr. 562 (1991) 737.
[7] R. Whelptonand, P.R. Hurst, Method Surveys Biochem. Anal. 20 (1990) 279.
[8] B. Drager, J. Chromatogr. A 978 (2002) 1.
[9] J. Deutsch, T.T. Soncrant, N.H. Greig, S.I. Rapoport, J. Chromatogr. 528 (1990) 325.
[10] R. Oertel, K. Richter, U. Ebert, W. Kirch, J. Chromatogr. B 682 (1996) 259.
[11] U. Ebert, M. Siepmann, R. Oertel, K.A. Wesnes, W. Kirch, J. Clin. Pharmacol. 38 (1998) 720.
[12] W.F. Bayne, F.T. Tao, N. Crisologo, J. Parm. Sci. 64 (1975) 288.
[13] R. Oertel, K. Richter, U. Ebert, W. Kirch, J. Chromatogr. B 750 (2001) 121.
[14] G. Werner, K.-H. Schmidt, Hoppe-Seyler's Zeitschrift für Physiologische Chemie 349 (1968) 741.
[15] S. Wada, T. Yoshimitsu, N. Koga, H. Yamada, K. Oguri, H. Yoshimura, Xenobiotica 21 (1991) 1289.
[16] H. Yamada, S. Wada, T. Yoshimitsu, T. Shimizudani, M. Yamamoto, S. Mitsunaga, K. Oguri, N. Koga, H. Yoshimura, Japanese J. Forens. Toxicol. 10 (1992) 96.
[17] S.A. Chan, M.J. Chen, T.Y. Liu, M.R. Fuh, J.F. Deng, M.L. Wu, S.J. Hsieh, Talanta 60 (2003) 679.
[18] P. Rudewicz, K.M. Straub, Anal. Chem. 58 (1986) 2928.

[19] X. Yu, D.H. Cui, M.R. Davis, J. Am. Soc. Mass Spectrom. 10 (1999) 175.
[20] W. Lam, R. Ramanathan, J. Am. Soc. Mass Spectrom. 13 (2002) 345.
[21] Q.G. Dong, J.K. Gu, D.F. Zhong, J.P. Fawcett, D.F. Chu, J. Chromatogr. B 772 (2002) 369.
[22] E. Gangl, H. Utkin, N. Gerber, P. Vouros, J. Chromatogr. A 974 (2002) 91.
[23] E.H. Kerns, R.A. Rourich, K.J. Volk, M.S. Lee, J. Chromatogr. B 698 (1997) 133.
[24] E.W. Chung, E.N.M. Ho, D.K.K. Leung, F.P.W. Tang, K.C.H. Yiu, T.S.M. Wan, Chromatographia 59 (2004) S29.
[25] S.A. Appolonova, A.V. Shpak, V.A. Semenov, J. Chromatogr. B 800 (2004) 281.
[26] W.S. Zhang, A.L. Li, Medicinal Chemistry, Higher education press, China, 1999, pp. 43, 67 and 235.
[27] P.H. Chong, The Application of Mass Spectroscopy in the natural Organic Chemistry, Science Press, China, 1997, p. 406.
[28] R.F. Stack, P.J. Rudewicz, J. Mass Spectrom. 30 (1995) 857.
[29] J.K. Gu, D.F. Zhong, X.Y. Chen, Fresen. J. Anal. Chem. 365 (1993) 533.
[30] X.Y. Chen, D.F. Zhong, J. Hao, J.K. Gu, Acta Pharm. Sin. 33 (1998) 849.