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Chromatographic tandam mass spectrometric detection of papaverine and its major metabolites in rat urine

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Abstract

A rapid, sensitive and specific liquid chromatographic-electrospray ionization (ESI) tandem ion trap mass spectrometric method has been developed for identification of papaverine and its metabolites in rat urine. Six healthy rats were administrated a single dose (100 mg/kg) of papaverine by oral gavage. The urine were sampled for 0–24 h and purified by using a C18 solid-phase extraction cartridge, then the purified urine samples were separated on a reversed-phase C18 column using methanol/2 mmol/L ammonium acetate (70:30, v/v, adjusted to pH 3.5 with formic acid) as mobile phase and detected by an on-line MS detector. Identification and structural elucidation of the metabolites were performed by comparing their changes in molecular mass (Δm) and full scan MS^n spectra with those of the parent drug. The results indicated that there were 14 metabolites in rat urine, such as de-methoxyl, hydroxyl, glucuronide and sulfate conjugated metabolites and so on. All these metabolites were reported for the first time.

Keywords: Papaverine; HPLC-MS^n; Metabolite; Rat

1. Introduction

HPLC-MS^n, which takes the advantage in sensitivity and specificity, and less time consuming and less labor intensive comparing GC and HPLC had been proved to be a modern powerful tool for the identification of drug metabolites in biological matrices [1–4]. In addition, ESI-MS^n technique had made it possible to analyze thermo-labile, highly polar and non-volatile metabolites and acquire rich structural information of interesting analytes. The metabolites can keep the basic structural features of parent drug after biotransformation, so the product ions of parent drug associated with their basic structural features obtained by MS^n technique can be used as a substructural template of metabolite's analysis [5–8].

Papavarine (4-(3′,4′-dimethoxybenzyl)-6,7-dimethoxy-quinoline, m.w. 340), as one of benzyl isoquinoline alkaloids, was used clinically as a bronchodilator to relaxes various smooth muscles, smooth musculature of the larger blood vessels, especially coronary, systemic peripheral and pulmonary arteries to increase cerebral blood flow [9]. Despite its important therapeutical values, its metabolism both *in vivo* and *in vitro* is not clear yet. Up till now, the works only focused on the quantitative determination of papaverine. The methods have been reported for the determination of papaverine including TLC [10], spectrophotometry [11–13], GC [14], HPLC [15,16], and MS [17].

In this work, a sensitive and specific HPLC/ESI-$ITMS^n$ method was presented for rapid identification of papaverine and its metabolites in rat urine. The HPLC/ESI-$ITMS^n$ analyses of urine sampled from healthy rats after ingesting 100 mg/kg papaverine revealed that the parent drug and its 14 metabolites existed in rat urine. All these metabolites were reported for the first time.

2. Materials and methods

2.1. Chemicals and reagents

Papaverine·HCl was purchased from TianJin YiFang Co., Ltd. (TianJin, China) with a certificate of analysis indicating

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an estimated purity >99%. Methanol is of HPLC grade (Fisher Chemical Co. Inc., CA, USA). Acetic Acid was purchased from Sigma Co. Water was deionized and double distilled. Other reagents used are of analytical grade.

Stock solution of papaverine was prepared by dissolving accurately weighed pure substances in methanol to yield a concentration of 10 μg/mL.

2.2. Apparatus

HPLC-MS^n experiments were performed on an LCQ Duo quadru-pole ion trap mass spectrometer (Thermo Finnigan, Corp, San Jose, USA) with a modern Quat HPLC pump and an auto-sampler (Agilent 1100 series) and a TSP AS3000 auto-sampler using positive electrospray as the ionization process. The software Xcalibur version 1.2 (Finnigan) was applied for system operation and data collection. A high-speed desktop centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge urine samples.

2.3. Chromatographic conditions

The Agilent 1100 HPLC was equipped with a reversed-phase column (Zorbax Extend-C_{18}, 3.0 mm × 100 mm i.d., 3.5 um, Agilent, USA), including a Phenomenex 4 mm × 2 mm phenyl-propyl guard column. The mobile phase was consisted of methanol and 2 mmol/L ammonium acetate (70:30, v/v, adjusted to pH 3.5 with formic acid). Before use, the mobile phase was filtrated through a 0.45 μm filter, the flow rate was 0.2 mL/min and the injection volume was 20 μL.

2.4. Mass spectrometric conditions

The mass spectrometer was tuned for positive ion spectra by direct infusion of 10 μg mL^{-1} papaverine. The peak shape and intensity of the mono-protonated papaverine *m/z* 340 ion were optimized by adjustment of source spray voltage, capillary voltage, capillary temperature, voltages of octa-pole offset and tube lens offset. The source spray voltage was 4.5 kV; a capillary voltage of 26 V and a heated capillary temperature 250 °C were left. The voltages of octa-pole 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 molecular ion $[M+H]^+$ of all analytes in selected reaction monitoring mode (SRM). The collision energy for each ion transition was optimized to produce the highest intensity of the selected ion peak. The optimized CID energy was 40% for both MS^2 and MS^3 works. Data acquisition was performed in full scan HPLC-MS and tandem MS^n modes.

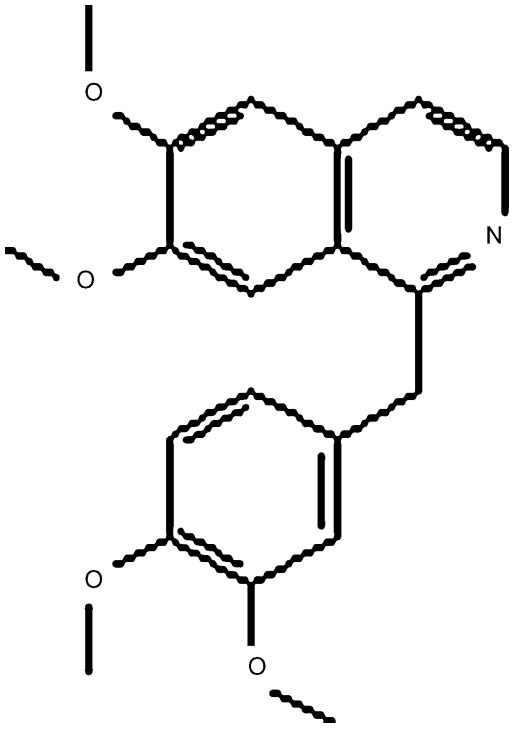

Fig. 1. Structure of papaverine.

2.5. Samples preparation

2.5.1. Administration

Six male Wistar rats (180 ± 5 g, Hubei Experimental Animal Research Center, China) were housed in metabolic cages for the collection of urine. All the animal studies were performed in the SPF laboratory. The rats were provided standard laboratory food and water *ad libitum*. The rats were fasted for 24 h but with access to water, and then administered 100 mg/kg oral gavage doses of papaverine. Urine samples were collected for a period of 0–48 h and centrifuged at 3000 × *g* for 10 min. The supernatant was collected and stored at −20 °C until used to purify the metabolites Table 1.

2.5.2. Urine extraction

Solid phase extraction (SPE) with a C18 cartridge (3 mL/200 mg, AccuBond^{II}, Agilent) was used to purify the

Table 1
The retention time of the metabolites

	m/z						
	326 (**M1**)	312 (**M2**)	298 (**M3**)	342 (**M4**)	328 (**M5**)	314 (**M6**)	356 (**M7**)
t_R(min)	2.59	2.25	3.53	2.97	3.92	3.01	2.81
	406 (**M8**)	502 (**M9**)	392 (**M10**)	488 (**M11**)	518 (**M12**)	504 (**M13**)	532 (**M14**)
t_R(min)	3.21	2.59	2.25	3.53	2.97	3.92	3.01

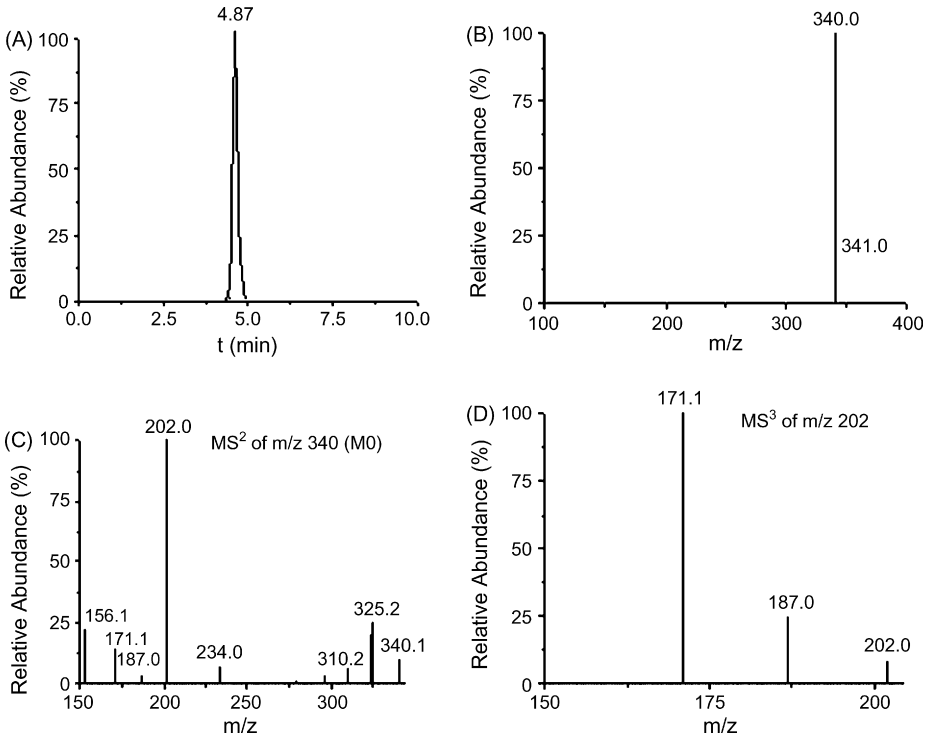

Fig. 2. The chromatogram and MS spectra of papaverine, (A) chromatogram; (B) MS spectrum; (C) MS^2 spectrum; (D) MS^3 spectrum.

above supernatants of urine sample, for LC–MS^n analysis. Before use, SPE columns were conditioned by adding sequentially 2 mL the mixture solution consisted of methanol and 2 mmol/L ammonium acetate (70:30,v/v, adjusted to pH 3.5 with formic acid), 2 mL methanol, 2 mL deionized water. Then the selected supernatant sample was loaded, and the column was washed with 3 mL deionized water to elute the impurity and 2 mL methanol to elute the analytes in turn. The eluent containing the metabolites were centrifuged again at $3000 \times g$ for 10 min. The decanted liquid was transferred to a microinjection vial and sealed. Based on this experiment, the purified samples were stable for at least 2 months at 4 °C Fig. 1.

3. Results and discussion

3.1. LC-MS and LC-MS^n analysis of papaverine

On dissolution in methanol: 0.05% formic acid (aq.), Papaverine readily produced an *m/z* 340 $[M+H]^+$ protonated

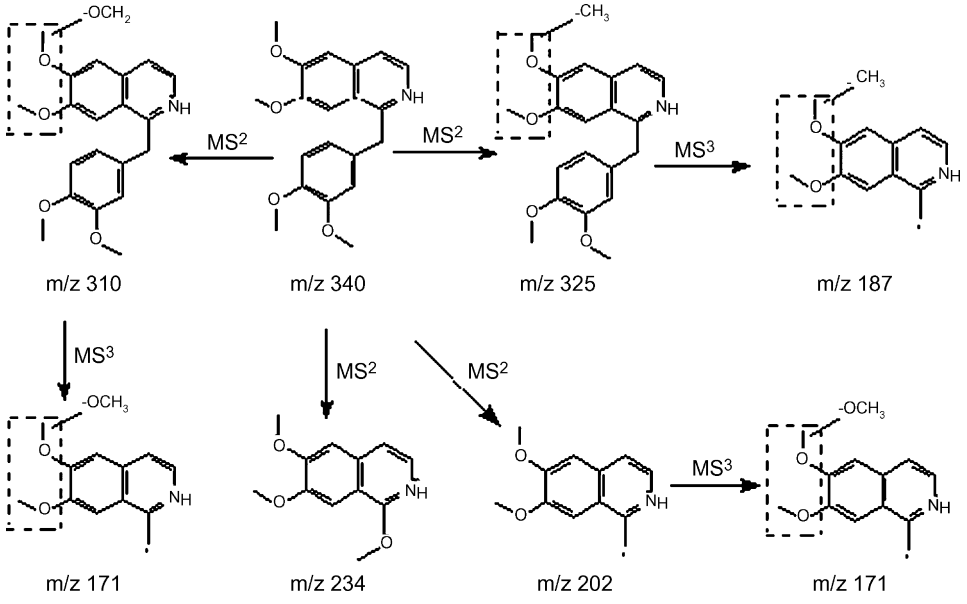

Fig. 3. Fragmentation pathway of papaverine.

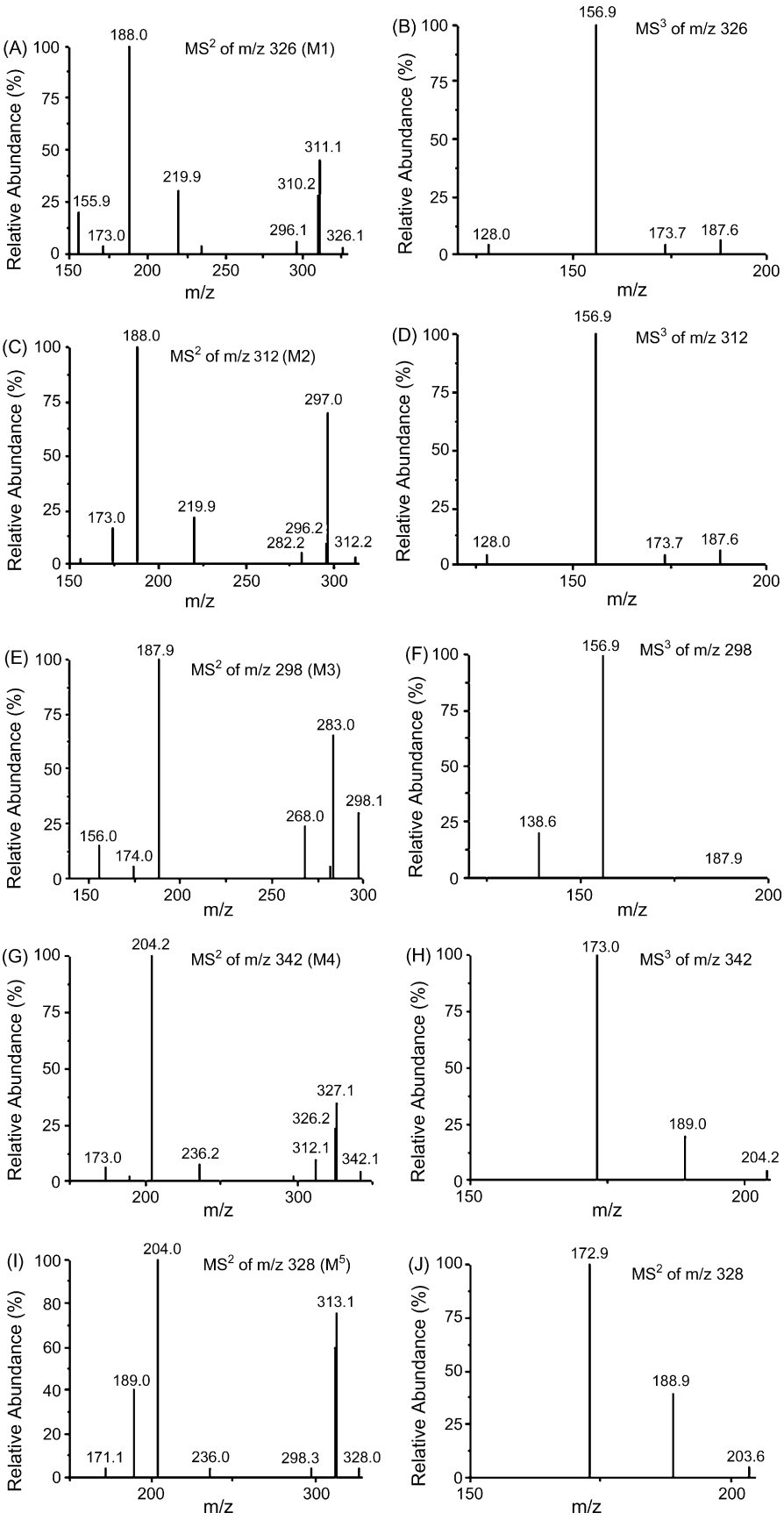

Fig. 4. The MS^2 and MS^3 spectra of the metabolites of papaverine in rat urine.

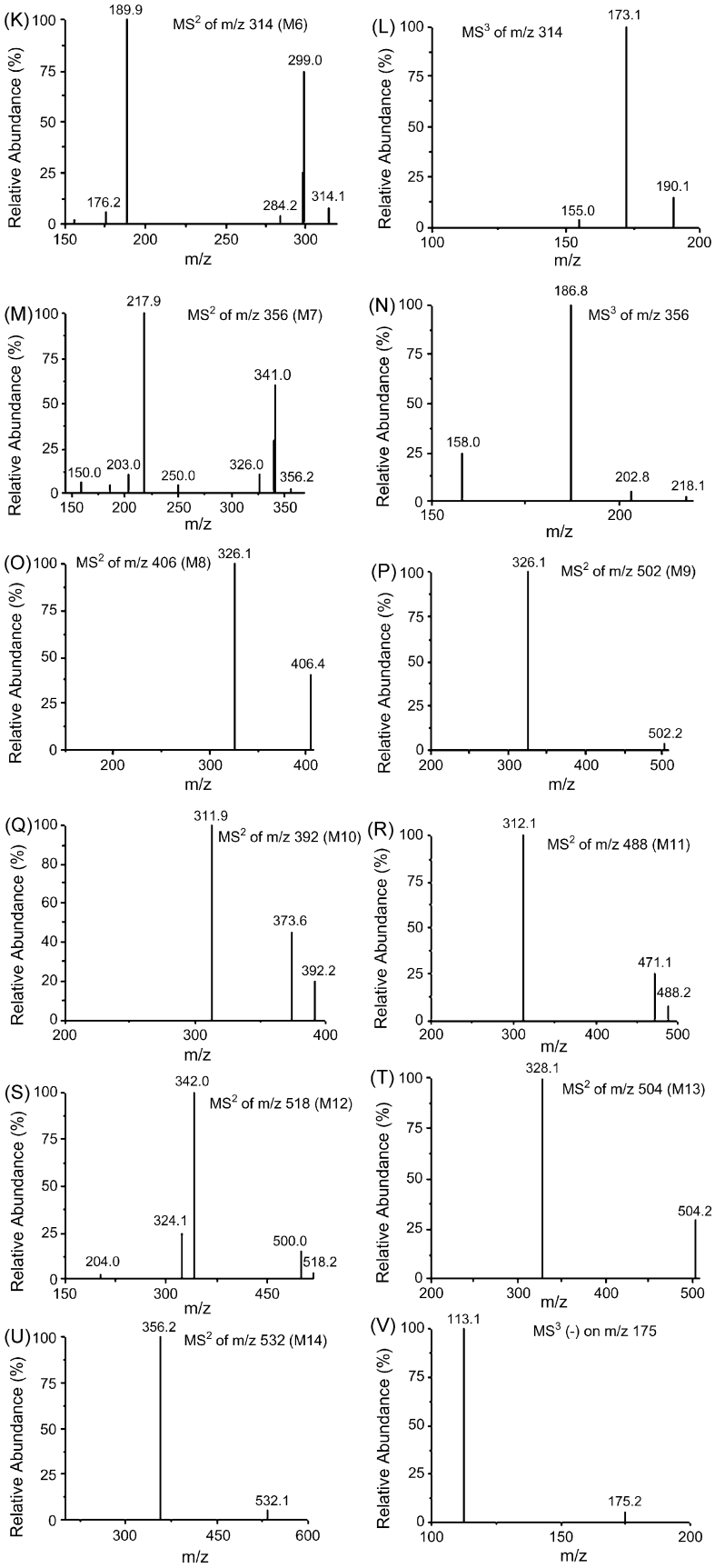

Fig. 4. (*Continued*).

pseudomolecular ion. This species produced one prominent daughter ion at *m/z* 202 on collision-induced dissociation in the MS^2 spectrum (Fig. 2C). This ion arises by cleavage of the C–C bond at the methylene bridge between dimethoxybenzyl and isoquinoline ring systems to release neutral 3′,4′-dimethoxybenzene, m.w. 137 with shift of a proton. Besides, The fragment ions at *m/z* 325, 310, 234, 187, 171 and 156 were also detected in the MS^2 spectrum (Fig. 2C). The product ions at *m/z* 325 and 310 were formed by loss of one methyl group and loss of one neutral fragment OCH_2, respectively. The product ion at *m/z* 234 was produced by the methoxyl long-range rearrangement to isoquinoline. The product ion at *m/z* 187 was isoquinoline ring. The product ion at *m/z* 171 was formed by demethoxyl of the fragment ion at *m/z* 202, which also exist in MS^3 (Fig. 2D) of *m/z* 340 → 202, and the product ion at *m/z* 156 was produced by demethyl of the fragment ion at *m/z* 171. The product ions and the corresponding neutral fragment loss mentioned above which were the similar with Wickens et al. [18], were the characteristic structural information of papaverine and were the sound bases to identify metabolites of papaverine. The mass fragmentation rule of papaverine was shown in Fig. 3.

3.2. LC-MS and LC-MS^n analysis of metabolites

In order to identify the metabolites, the possible structures of metabolites were speculated according to the rule of drug metabolism and the structure of parent drug firstly. Then, the full scan mass spectrum of the purified rat urine after administration of papaverine was compared with those of blank rat urine free of papaverine to find the possible metabolites. No impurity or endogenous interference has been found in the presented method. Finally, the possible metabolites were analyzed by HPLC-MS^n to elucidate their structures through comparing their retention times, changes in observed mass (Δm) and mass spectral patterns of product ions with those of papaverine. The parent drug can be detected for up to 72 h in urine sample of healthy rats after ingestion of papaverine, but its metabolites cannot be monitored beyond 24 h.

Based on the method mentioned above, the parent drug and its metabolites were found in rat urine after administration of papaverine. Their molecular ions ($[M+H]^+$) were at *m/z* 326, 312, 298, 342, 328, 314, 356, 406, 502, 392, 488, 518, 504, and 532, respectively. MS^n spectra of motabolites, obtained by CID of their molecular ions in SRM mode, were used for more

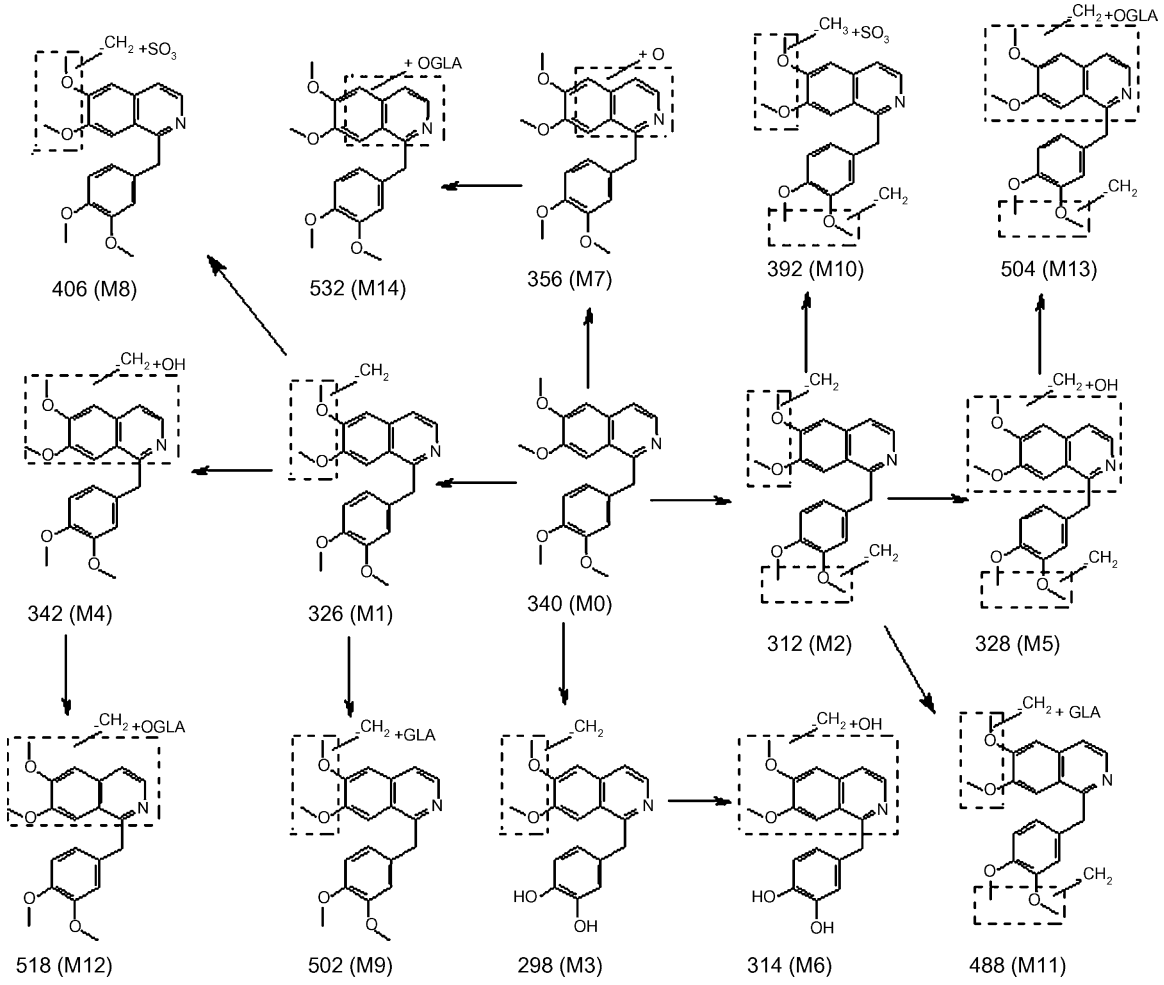

Fig. 5. The proposed *in vivo* metabolic pathways of papaverine in rat.

precise structural identification of metabolites. Among them, the retention time, the MS^2 and MS^3 spectra of the molecular ion at *m/z* 340 (M0) were the same as those of papaverine. Therefore, M0 is the unchanged parent drug.

The molecular ion at *m/z* 326 (M1) and its daughter ions at *m/z* 311, 296, 220, 188 and 173 (Fig. 4A) were all 14 Da less than the molecular ion of parent drug (*m/z* 340) and its daughter ions at *m/z* 325, 310, 234, 202 and 187. These results indicated that M1 was the demethyl product of papaverine. The major ion at *m/z* 157 existed in MS^3 spectrum of *m/z* 326 → 188 (Fig. 4B). These results indicated that M1 was the demethyl product of papverine, and the demethyl position is located at the isoquinoline cycle.

The protonated molecular ion of *m/z* 312 (M2) and its characteristic product ions at *m/z* 297, 282, 220, 188, and 173 (Fig. 4C) were 28 and 14 Da less than those of the unchanged papaverine and its characteristic product ions at *m/z* 325, 310, 234, 202, and 187, respectively. The characteristic fragment ion at *m/z* 157 was presented in the MS^3 spectrum of *m/z* 312 → 188 (Fig. 4D). So M2 was de-dimethyl metabolite of the parent drug, and the locations of de-methyl were in isoquinoline ring and dimethoxybenzyl ring, simultaneously.

The fragment ions at *m/z* 283 and 268 were produced by the loss of neutral fragments 15 and 30 Da from the molecular ion at *m/z* 298 (M3, Fig. 4E), which were the same as the fragments loss of papaverine. It was obvious that the molecular ion of M3 and its daughter ions at *m/z* 283 and 268 were all 42 Da less than the molecular ion of papaverine and its daughter ions at *m/z* 325 and 310. Thus, M3 can be affirmed as the de-trimethyl product of papaverine. The MS^3 fragment ion at *m/z* 157 of M3 (*m/z* 298 → 188) (Fig. 4F) was 14 Da less than the MS^3 fragment ion at *m/z* 171 of M0 (*m/z* 340 → 202). These results indicated that M3 was formed by lossing one methyl of isoquinoline ring and two methyls of dimethoxybenzyl ring from M0.

The molecular ion at *m/z* 342 (M4), 328 (M5), 314 (M6) and 356 (M7) were all 16 Da more than the molecular ions at *m/z* 326 (M1), 312 (M2), 298 (M3), 340 (M0), respectively, and their fragment loss were the same as papaverine. The MS^3 fragment ion at *m/z* 173 of M4 (*m/z* 342 → 204), M5 (*m/z* 328 → 204), M6 (*m/z* 314 → 204) was 16 Da more than the MS^3 fragment ion at *m/z* 157 of M1 (326 → 188), M2 (312 → 188), M3 (298 → 188), and the MS^3 fragment ion at *m/z* 187 of M7 (*m/z* 356 → 218) (Fig. 4H, J, L) was also 16 Da more than the MS^3 fragment ion at 171 of M0 (*m/z* 340 → 202) (4N), respectively. Thus, M4, M5, M6, M7 could be affirmed as the mono-hydroxylation product of M1, M2, M3, and M0, respectively. The protonated molecular ion at *m/z* 406 (M8) and *m/z* 392 (M10) lost neutral fragment 80 Da (SO_3) to produce the daughter ion at *m/z* 326 (M1) and *m/z* 312 (M2), respectively (Fig. 4O and Q). The MS^3 spectrums of *m/z* 406 → 326, 392 → 312 were the same as the MS^2 spectrum of the protonated molecular ion of M1, M2. In addition, there were the molecular ions at *m/z* 404 and 390 in the negative ion full scan MS spectrum of the urine samples. Consequently, M8, M10 were identified as the sulphated conjugate of M1, M2 according to the rule of drug metabolism. The selectivity of this conjugated reaction has been validated by many studies [19].

The MS^2 spectrum of *m/z* 502 (M9), 488 (M11), 518 (M12), 504 (M13) and 532 (M14) gave abundant daughter ion at *m/z* 326, 312, 342, 328 and 356, respectively (Fig. 4P and R, S–U), which were all produced by the neutral loss of 176 Da (glucuronic acid), and the MS^3 spectra of *m/z* 502 → 326, 488 → 312, 518 → 342, 504 → 328, 532 → 356 were the same as the MS^2 spectra of M1, M2, M4, M5 and M0, respectively. Besides, there were the molecular ion at *m/z* 500, 486, 516, 502 and 530 in the negative ion full scan mass spectrum of the urine sample and product ion at *m/z* 175 in the MS^2 spectra of the metabolites mentioned above. Furthermore, the fragment ion at *m/z* 113 appeared in the MS^3 spectrum of *m/z* 500 → 175, 486 → 175, 516 → 175, 502 → 175, 530 → 175. This fragmentation (500 → 175 → 113, 486 → 175 → 113, 516 → 175 → 113, 502 → 175 → 113, 530 → 175 → 113) (Fig. 4V) is the cleavage feature of glucuronide conjugates [20,21]. Thus, M9, M11, M12, M13 and M14 were identified as the glucuronide conjugate of M1, M2, M4, M5 and M7, respectively. The product ion at *m/z* 471.1 in Fig. 4R was formed by loss of 1 OH group from *m/z* 488.2. And in Fig. 4S, the product ion at *m/z* 500 was produced by the loss H_2O from *m/z* 518.2; *m/z* 324.1 was formed by the neutral fragment loss of H_2O from *m/z* 342. The fragment ion at *m/z* 204 may be the both demethyl and oxidation on isoquinoline of 342.

Based on the above discussion, the proposed major metabolic pathway of papaverine *in vivo* was shown in Fig. 5.

4. Conclusions

For the first time, the metabolites of papaverine in rat urine were analyzed by the presented method. Papaverine and its metabolites in rat urine were identified through comparing their chromatographic retention times, changes in observed mass (Δm) and tandem MS spectra with those of the parent drug. The results indicated that there were 14 metabolites in rat urine, such as de-methoxyl, hydroxyl, and glucuronide conjugated and sulfate conjugated metabolites and so on. All these metabolites were reported for the first time.

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