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Review

Identification of dauricine and its metabolites in rat urine by liquid chromatography–tandem mass spectrometry

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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 dauricine and its metabolites in rat urine. Six healthy rats were administrated a single dose (100 mg/kg) of dauricine by oral gavage. The urine were sampled from 0 to 24 h and purified by using a C18 solid-phase extraction (SPE) 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. At least eight metabolites (such as *N*-demethyl, dehydrogenate, demethoxyl, hydroxyl, glucuronide conjugated and sulfate conjugated metabolites) and the parent drug were found in rat urine.

Keywords: Dauricine; HPLC–MS^n; Metabolite

Contents

1. Introduction

HPLC–MS^n had been proved to be a modern powerful tool for the identification of drug metabolites in biological matrices [1–4]. This approach takes the advantage in sensitivity and

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specificity, and was considered less time consuming and less labor intensive than other methods, such as HPLC and GC–MS. In addition, MS^n technique had made it possible to acquire rich structural information of analytes of interest. The metabolites can retain 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. Therefore, the metabolites can be rapidly characterized by comparing their product ions with those of parent drug, even without standard for each metabolite [5–9].

Electrospray ionisation (ESI) mass spectrometry is useful for the analysis of thermo-labile, highly polar and non-volatile metabolites at trace levels compared with earlier ionization modes because of the relatively low internal energy imparted to the analytes. Ion trap analyzer (IT) can provide high sensitivity and rich mass spectral information, and is very suitable for the qualitative assay of analyte [7]. Therefore, the coupled HPLC/ESI-$ITMS^n$ method is a good technique for the structure elucidation of the metabolites.

Dauricine (Dau, Fig. 3), a kind of bisbenzyl-tetra-hydro-isoquinoline alkloid found in the root of Dauricum D.C., has good anti-arrhythmic effect, especially its character of use-dependence in prolonging APD in guinea myocardial, and was considered to be a promising new type anti-arrhythmic agent [10]. Many methods about the quantitative assay of dauricine in plants and pharmaceutical samples were established [11–15]. So far, only one metabolite of dauricine *in vivo* was found [11], others have not been reported yet. While the study on dauricine metabolism plays an important role in the development of new drugs and clinical application. In this work, a sensitive and specific HPLC/ESI-$ITMS^n$ method was presented for rapid identification of dauricine and its metabolites in rat urine. The parent drug and its eight metabolites (six phase I and two phase II metabolites) were found in rat urine. Except for the *N*-demethyl dauricine, the other seven metabolites were reported at the first time.

2. Materials and methods

2.1. Chemicals and reagents

Dauricine was isolated and purified by this laboratory with the purity >98%. Acetic acid was purchased from Sigma. Methanol was of HPLC grade (Fisher Chemical Co. Inc., CA, USA). Water was deionized and double distilled. Other reagents used were of analytical grade. Stock solution of dauricine 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 quadrupole ion trap mass spectrometer (Thermo Finnigan, Corp, San Jose, USA) with a modern TSP4000 HPLC pump 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. Rat urine samples were extracted on a C18 solid-phase extraction (SPE) 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. Chromatographic conditions

HPLC with a reversed-phase column (Zorbax Extend-C18, 3.0 mm × 100 mm I.D, 3.5 um, Agilent, USA) was used for separation dauricine and its metabolites. The mobile phase was composed 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 by filtration through a 0.45 μm filter (Nylon66), the flow rate was 0.2 mL/min and the injection volume was 20 μL.

2.4. Mass spectrometric conditions

Mass spectrometric detection was carried out using electrospray ionization in positive ion mode, and only the structures of phase II metabolites were validated 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 26 V and a heated capillary temperature of 250 °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 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 30% for both MS^2 and MS^3 works. Data acquisition was performed in full scan HPLC–MS and tandem MS modes.

2.5. Samples preparation

2.5.1. Administration

Six 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 Specific Pathogen Free (SPF) laboratory. Animals used in these experiments were managed according to the rules and regulations of the Institutional Animal Care and Use Committee at the HuBei University, which also approved the experimental protocol. The rats were provided standard laboratory food and water *ad libitum*. Before administered 100 mg/kg oral gavage doses of dauricine, the rats were fasted for 24 h but with access to water. 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 analytes.

2.5.2. Urine extraction

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 to elute impurity and 1 mL of methanol to elute the analytes in turn. The methanol elution solutions were filtered through 0.45 μm film and used for HPLC–MS^n analysis. Based on the experiment, the purified urine samples were stable for at least 2 months when preserved at 4 °C.

3. Results and discussion

3.1. LC–MS and LC–MS^n analysis of dauricine

The first step of this work involved the characterization of the chromatographic 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 structure of metabolites.

The HPLC–MS and MS^n analyses of dauricine were performed in positive ion detection mode because alkaloid molecules can be protonated easily under electrospray ionization condition. The chromatographic and mass spectrometric conditions were optimized using dauricine standard. The HPLC–MS^2 chromatogram and the MS^n spectra of dauricine are shown in Fig. 1. Dauricine was eluted at 2.68 min under the experimental conditions. The fragmentation of the molecular ion at *m/z* 625 led to five main product ions at *m/z* 206, 552, 566, 580 and 594. The product ions at *m/z* 580 were formed by simultaneous loss of one $-OCH_3$ group and one methylene group. The product ion at *m/z* 594 was formed by loss of one $-OCH_3$ group. The most abundant product ion at *m/z* 566 was formed by the neutral fragment loss of $C_2H_5NHCH_3$. The product ion at *m/z* 552 was produced by the simultaneous loss of one $-OCH_3$ group and one $-CH{=}N-CH_3$ group. The product ion at *m/z* 206 was the protonated product ion of AB ring or CD ring, and also presented in the MS^3 spectrum of the product ion at *m/z* 566. The product ions and the corresponding neutral fragment loss mentioned above were the characteristic structural information of dauricine, and form a sound basis for identification of metabolites of dauricine.

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

Possible metabolite structures were considered based on the parent structure and known common metabolic pathways, the known metabolite was *N*-demethyl dauricine. Then, the full scan mass spectrum of the purified rat urine after administration of dauricine was compared with those of blank rat urine sampled from the rats without administration of dauricine to find the possible metabolites. No impurity or endogenous interference has been found in the purified urine. Finally, the possible metabolites were analyzed by HPLC–MS/MS to elucidate their structures through comparing the changes in observed mass (Δm) and mass spectral patterns of product ions with those of dauricine. The parent drug can be detected for up to 72 h in urine sample of healthy rats after ingestion of dauricine, 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

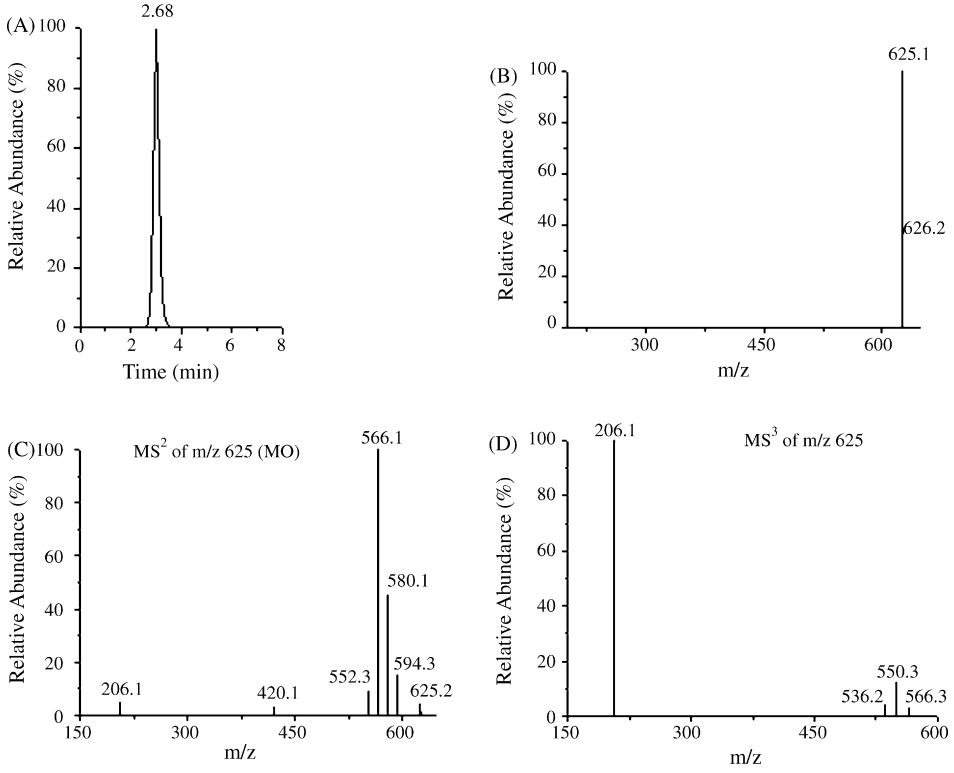

Fig. 1. The chromatogram and MS spectra of dauricine. (A) Chromatogram; (B) MS spectrum; (C) MS^2 spectrum and (D) MS^3 spectrum.

dauricine. Their molecular ions $[M+H]^+$ were at *m/z* 597, 609, 611, 621, 623, 625, 641, 787, 801, respectively. MS^n spectra of the motabolites, obtained by CID of their molecular ions in SRM mode, were 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* 625 (M0) were the same as those of dauricine. Therefore, M0 is the unchanged parent drug.

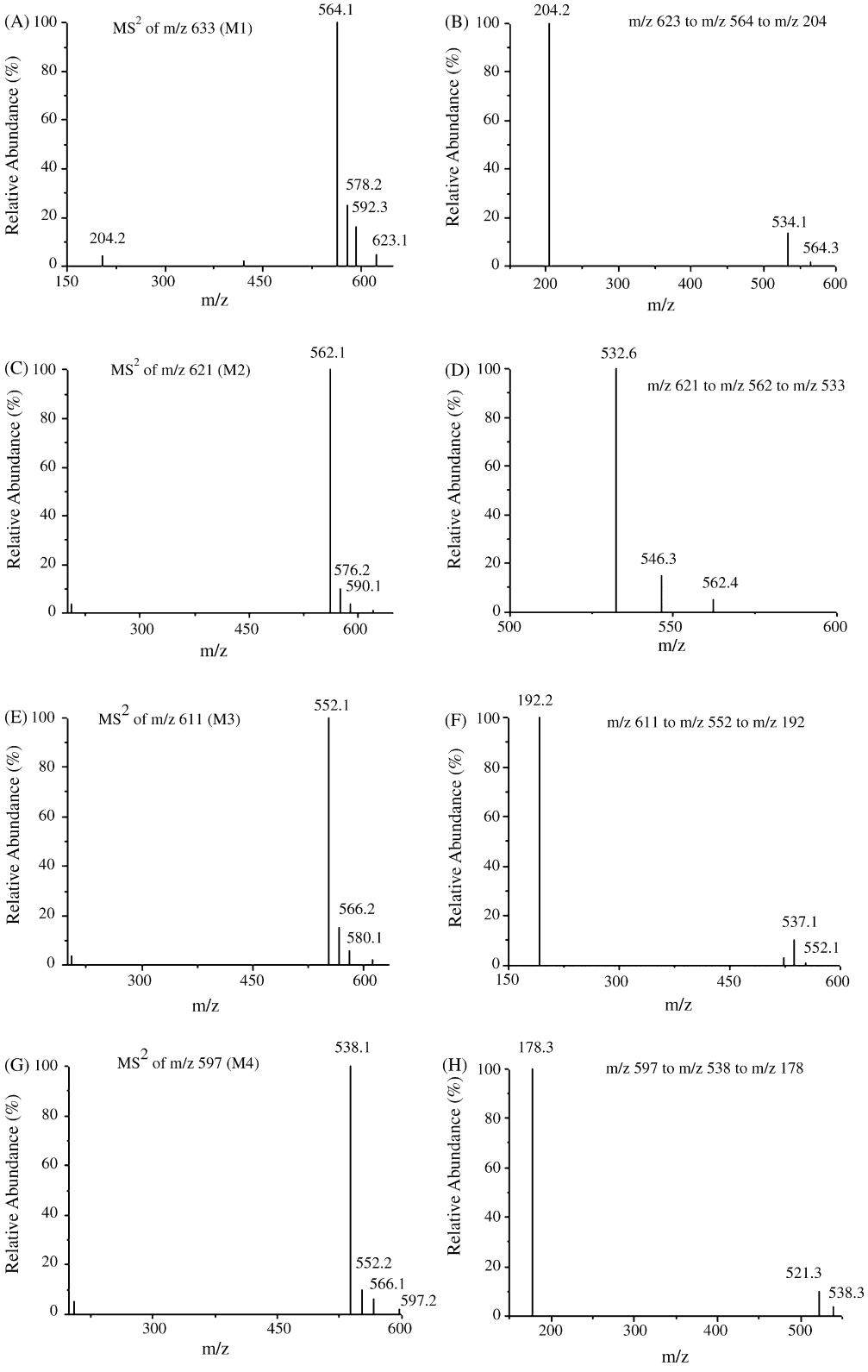

Fig. 2. The MS^2 and MS^3 spectra of the metabolites of dauricine in rat urine.

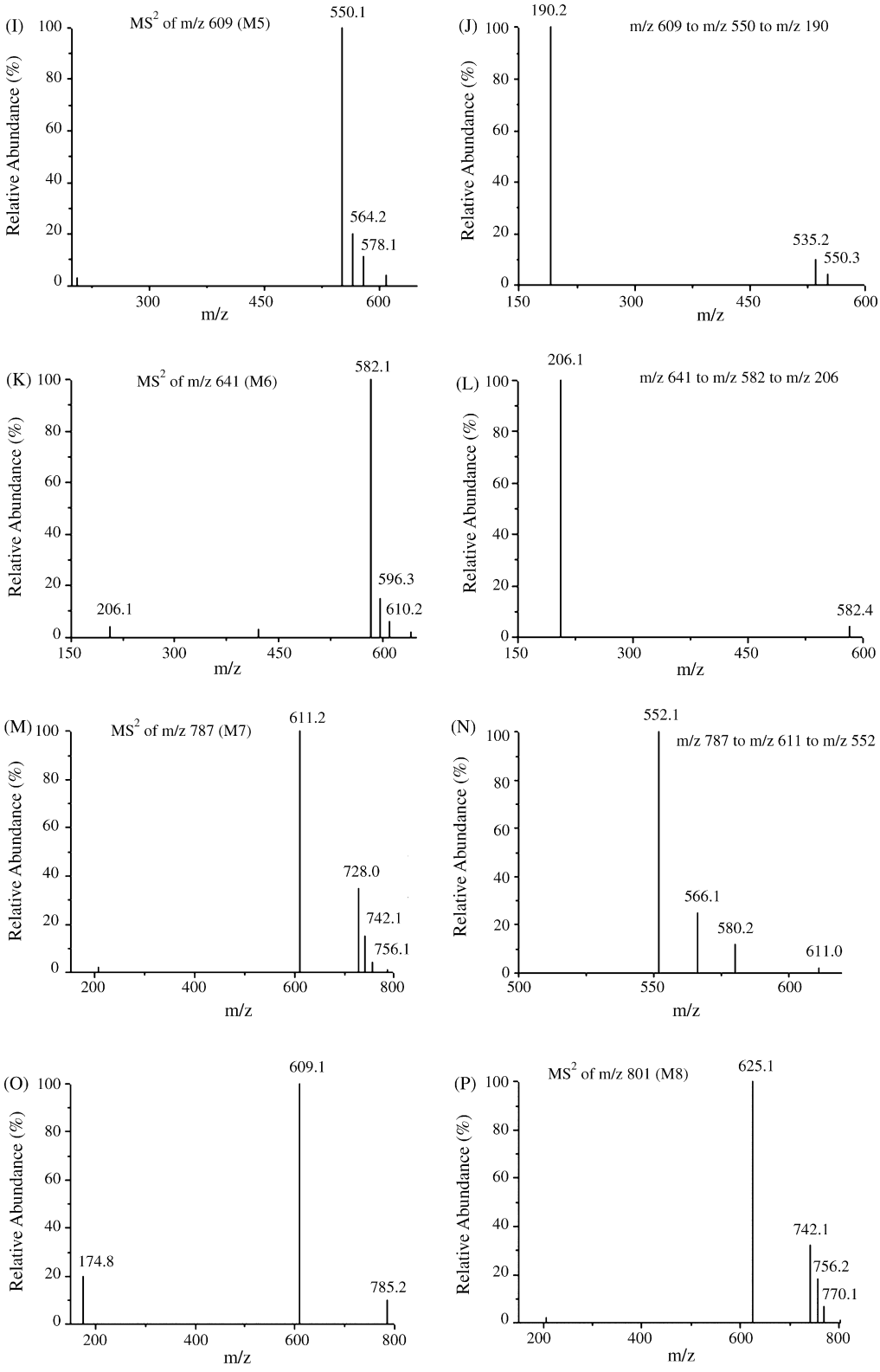

Fig. 2. (*Continued*)

The molecular ion at *m/z* 623 (M1) and its MS^2 ions at *m/z* 204, 564, 578, 592 (Fig. 2A) were all 2 Da less than those of dauricine, respectively. The MS^3 spectrum of *m/z* 623 → 204 (Fig. 2B) was similar as that of *m/z* 625 → 206. These results indicated that M1 was the di-dehydrogenating product of dauricine, and the dehydrogenating position is located at the saturated 3,4-carbon atoms of A ring or 3′,4′-carbon atoms of D ring, respectively.

The molecular ion at *m/z* 621 (M2) and its daughter ions at *m/z* 562, 576 and 590 (Fig. 2C) were all 4 Da less than those of

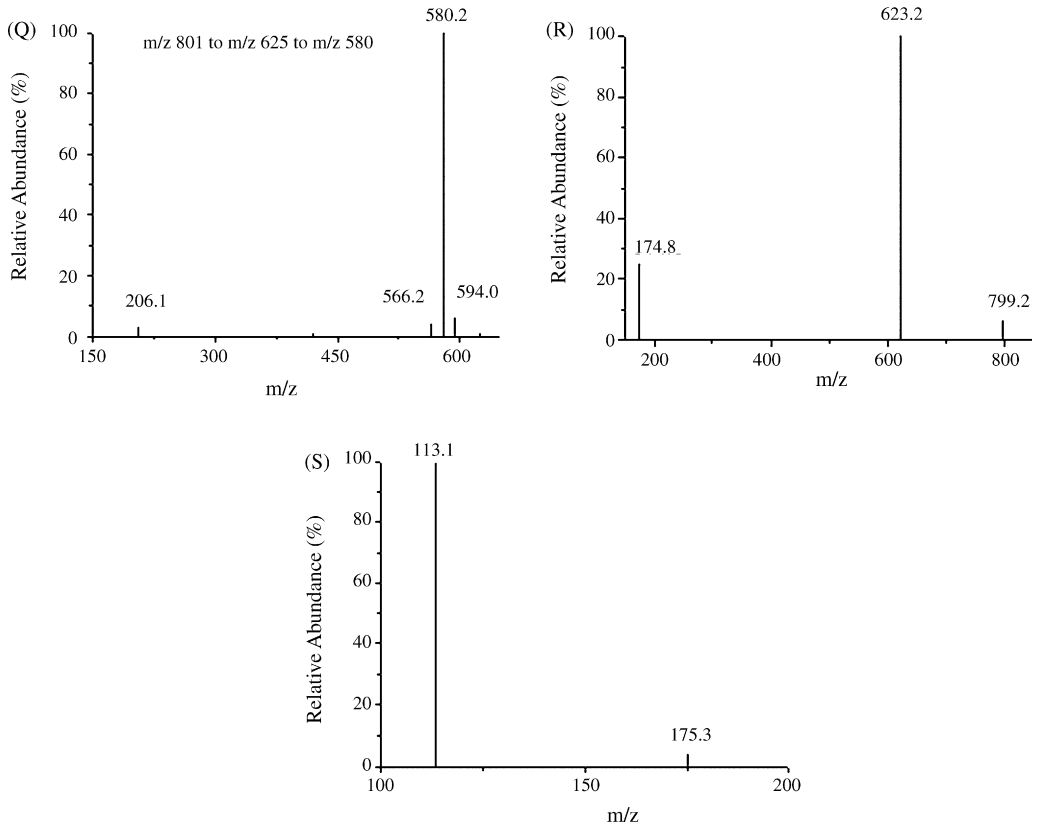

Fig. 2. (*Continued*)

dauricine, respectively. These results indicated that M2 was the tetra-dehydrogenating product of dauricine. Because no characteristic product ion related to *m/z* 206 of dauricine was presented in the MS^2 and MS^3 spectra of M2 (Fig. 2D), so the dehydrogenating position should be located at the saturated 1,11-carbon atoms and 1′,11′-carbon atoms. This is impossible for dehydrogenation at A ring and D ring.

The molecular ion of M3 (*m/z* 611) and its characteristic product ions at *m/z* 580, 566 and 552 (Fig. 2E) were all 14 Da less than those of dauricine, respectively. The characteristic fragment ion at *m/z* 192 (206–14 = 192 Da) was presented in the MS^3 spectrum of *m/z* 552 (Fig. 2F). According to the result of Chen and her coworkers [11], M3 should be the *N*-demethyl metabolite of dauricin at A ring or D ring, respectively.

The fragment ions at *m/z* 538, 552 and 566 were produced by the loss of neutral fragments 59, 45 and 31 Da from the molecular ion at *m/z* 597 (M4, Fig. 2G), which were similar as the neutral fragments loss of dauricine. It was obvious that the molecular ion of M4 and its daughter ions at *m/z* 538, 552 and 566 were all 28 Da less than the molecular ion of dauricine and its daughter ions at *m/z* 566, 580 and 594. Thus, M4 can be affirmed as the di-demethyl product of dauricine. The MS^3 spectrum of *m/z* 538 → 178 (Fig. 2H) was similar as that of *m/z* 566 → 206. It is indicated that the demethyl position should be at either B ring or C ring.

The molecular ion at *m/z* 609 (M5) and its daughter ions at *m/z* 550, 564 and 578 (Fig. 2I) were all 16 Da less than the molecular ion of dauricine and its daughter ions at *m/z* 566, 580 and 594, respectively. The MS^3 spectrum of *m/z* 550 → 190 (Fig. 2J) was similar as that of *m/z* 566 → 206. Thus, M5 could be affirmed as the simultaneously *N*-demethyl and dehydrogenating product of dauricine at either A ring or D ring, respectively. If the dehydrogenating position is located at the saturated carbon atom between A and E ring or between D and F ring, it is impossible for the product ion at *m/z* 550 to form the MS^3 ion at *m/z* 190.

The molecular ion at *m/z* 641 (M6) and its MS^2 ions at *m/z* 582, 596 and 610 (Fig. 2K) were all 16 Da more than the molecular ion of dauricine and its daughter ions at *m/z* 566, 580 and 594. Because the characteristic product ion at *m/z* 206 was presented in the MS^3 spectrum of the product ion at *m/z* 582 (Fig. 2L), M6 should be the hydroxylated product of dauricine at either E or F ring, respectively.

The molecular ion at *m/z* 787 (M7) and its MS^2 ions at *m/z* 728, 742 and 756 (Fig. 2M) were all 176 Da more than the molecular ion of M3 and its daughter ions at *m/z* 552, 566 and 580. The most abundant daughter ion at *m/z* 611 of M7 was produced by neutral fragment loss of 176 Da. The MS^3 spectrum of *m/z* 787 → 611 → 552, 566 (Fig. 2N) was similar as the MS^2 spectrum of *m/z* 611 → 552, 566 of M3. Besides, the de-protonated molecular ion at *m/z* 785 was presented in the negative ion full scan HPLC–MS spectrum of the urine samples (Fig. 2O), and the fragmentation of *m/z* 785 was *m/z* 785 → 175 → 113 (Fig. 2S), this fragmentation is the cleavage feature of glucuronide conjugates [16,17]. Consequently, M7 was identified as the glucuronide conjugate of M3.

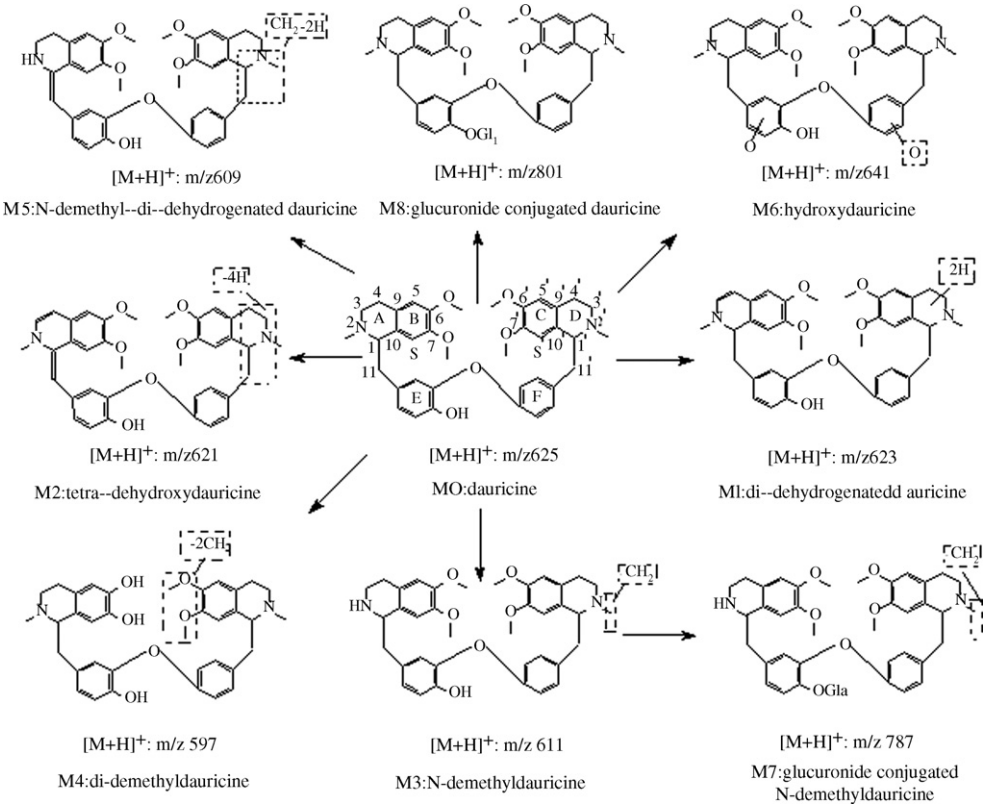

Fig. 3. The proposed *in vivo* metabolic pathways of dauricine in rat.

The molecular ion at *m/z* 801 (M8) and its MS^2 ions at *m/z* 728, 742, 756 and 770 (Fig. 2P) were all increased by 176 Da compared with the molecular ion of dauricine and its daughter ions at *m/z* 552, 566, 580 and 594, respectively. The most abundant daughter ion at *m/z* 625 of M8 was produced by the neutral fragment loss of 176 Da. the MS^3 spectrum of *m/z* 801 → 625 (Fig. 2Q) was similar as the MS^2 spectrum of dauricine. Besides, there was the de-protonated molecular ion at *m/z* 799 in the negative ion full scan HPLC–MS spectrum (Fig. 2R) of the urine samples, and *m/z* 799 led to the fragment ions of *m/z* 799 → 175 → 113 in its tandem MS spectra. So, M8 was the glucuronide conjugate of dauricine.

Based on the above discussion, the metabolites of dauricine in rat urine can be shown in Fig. 3.

4. Conclusions

In the paper, the metabolites of dauricine in rat urine was analyzed by the presented method. Dauricine and its eight metabolites were identified through comparing the changes in observed mass (Δm) and tandem MS spectra with those of the parent drug. These metabolites include six phase I and two phase II metabolites. Except for the *N*-demethyl dauricine, the other seven metabolites were reported at the first time.

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References

[1] 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.
[2] P. Rudewicz, K.M. Straub, Anal. Chem. 58 (1986) 2928.
[3] X. Yu, D.H. Cui, M.R. Davis, J. Am. Soc. Mass Spectrom. 10 (1999) 175.
[4] W. Lam, R. Ramanatyhan, J. Am. Soc. Mass Spectrom. 13 (2002) 345.
[5] Q.G. Dong, J.K. Gu, D.F. Zhong, J.P. Fawcett, D.F. Chu, J. Chromatogr. B 772 (2002) 369.
[6] E. Gangl, H. Utkin, N. Gerber, P. Vouros, J. Chromatogr. A 974 (2002) 91.
[7] E.H. Kerns, R.A. Rourich, K.J. Volk, M.S. Lee, J. Chromatogr. B 698 (1997) 133.
[8] E.W. Chung, E.M. Ho, D.K.K. Leung, F.P.W. Tang, K.C.H. Yin, T.S.M. Wan, Chromatographia 59 (2004) S29.
[9] S.A.A. Appolonova, A.V. Shpak, V.A. Semenov, J. Chromatogr. B 800 (2004) 281.
[10] C.J. Hu, F.D. Zeng, Chin. Native Med. Pharmacol. Clin. Res. Prog. 1 (1992) 26.
[11] S.J. Chen, Y.M. Yang, Z.S. Dai, F.D. Zeng, J. Tongji Med. Univ. 20 (2000) 253.
[12] H. Li, G. Chen, F.K. Chen, Chin. Tradit. Patent Med. (2001) 839.
[13] Y. Chen, H.X. Chen, Chin. J. Anal. Chem. 34 (2006) 675.
[14] S.J. Chen, Y.M. Yang, Y.M. Yang, Y.M. Liu, B. Zhang, X.B. Pang, F.D. Zeng, Zhongguo Yaolixue Tongbao 17 (2001) 225.
[15] S.X. Hou, Z.S. Dai, F.J. Wang, L.J. Guo, C.J. Hu, Yaoxue Xuebao 17 (1982) 863.
[16] J.K. Gu, D.F. Zhong, X.Y. Chen, Fresenius J. Anal. Chem. 365 (1993) 533.
[17] X.Y. Chen, D.F. Zhong, J.K. Gu, Acta Pharm. Sin. 33 (1998) 849.