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Identification of amygdalin and its major metabolites in rat urine by LC–MS/MS

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Abstract

Amygdalin and its metabolites in rat urine were identified using liquid chromatography–electrospray ionization (ESI) tandem ion-trap mass spectrometry. The purified rat urine sample was separated using a reversed-phase C18 column with 10 mM sodium phosphate buffer (pH 3.1) containing 30% methanol as the mobile phase, amygdalin and its metabolites were detected by on-line mass detector in selected ion monitoring (SIM) mode. The identification of the metabolites and elucidation of their structure were performed by comparing the changes in molecular masses (ΔM), retention times and MS^2 spectral patterns of metabolites with those of parent drug. At least seven metabolites and the parent drug were found in rat urine after i.v. injection of 100 mg/kg doses of amygdalin. Among them, six metabolites were reported for the first time.

Keywords: Amygdalin; LC–MS/MS; Metabolite; Rat urine

1. Introduction

Amygdaline, the substance initially found in plants of the rosaceous family [1], such as *Prunus armeniaca* L. var. *ansu Maxim*, *Prunus sibirica* L., *Prunus mandshurica* (Maxim) *Koehne* and *P. armeniaca* L., has been widely used to treat asthma, aplastic anemia and tumors in oriental medicine [2]. Based on current knowledge, few studies about the major metabolites of amygdalin have been reported [3–5]. So the metabolic study of amygdalin will be playing an important role of demonstrating the in vivo process and its clinical application.

Chromatographic techniques coupled with mass spectrometry, such as GC–MS, LC–MS and LC–MS–MS, are basic analytical tools for analysis of drugs and their metabolites [6–10]. Among the above methods, the complex and time-consuming derivatization of analytes is often required for GC–MS due to the unfitness for thermolabile, highly polar, and non-volatile analytes [11,12], and a good chromatographic resolution, for LC–MS, is needed for the clear identification of metabolites based on the on-line mass detection [13]. The electrospray ionisation (ESI), a soft ionization technique, increases the possibility of analyzing high labile and polar phase II metabolites at trace levels [14–16]. Therefore, LC–MS–MS is a more powerful analytical tool for the identification of drug metabolites in biological matrices by comparing the changes in molecular masses (ΔM), retention times and MS^2 spectral patterns of metabolites with those of parent drug [17,18], even if the standard of metabolites were not obtained [19,20], or the metabolites are not achieved a good chromatographic resolution [21–23].

In this paper amygdalin and its metabolites were identified in rat urine using high-performance liquid chromatography–electrospray ionization (ESI) tandem ion-trap mass spectrometry after administered 100 mg/kg doses of amygdalin by i.v. The parent drug and its seven metabolites were found in rat urine. Among them six metabolites were detected for the first time.

2. Experimental

2.1. Reagents and chemicals

Amygdalin was purchased from Sigma (St. Louis, MO, USA). Methanol was of HPLC grade (Fisher Chemical Co., Inc., CA, USA), and distilled water was used. Other reagents were of analytical grade.

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2.2. Instrumentation

LC–MS and LC–MS^2 experiments were performed on a LCQ Duo quadruple 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 Thermo Fisher, Austin, TX, USA). The software Xcalibur version 1.2 (Thermo Fisher) 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 by ODS-18 solid-phase extraction cartridges (3 ml/200 mg, AccuBond, Agilent Technologies, Palo Alto, CA, USA).

2.3. In vivo study

All the animal studies were performed in the SPF laboratory authorized by Hubei province Gov. (China). Standard laboratory food and water ad libitum were provided to six Wistar rats obtained from Hubei Experimental Animal Research Center (China). The rats with weights ranged from 197 to 201 g in the experimental process were housed in metabolism cage and fasted for 24 h but with access to water, and then administered 100 mg/kg doses of amygdalin by mainline. Urine samples were collected at different times up to 48 h and centrifuged at 3000 × *g* for 10 min. The supernatant was stored at −20 °C until analyses.

2.4. Urine extraction

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 eluted solution, stable at least 2 months at 4 °C, was filtered through a 0.45 μm filter and an aliquot of 10 μl was used for LC–MS^2 analyses of metabolites.

2.5. Chromatography and mass spectrometry

To separate amygdalin and its metabolites in rat urine, a reversed-phase column (Zorbax extend C18, 3.0 mm × 100 mm i.d., 3.5 μm, Agilent Technologies) was connected to a guard column (cartridge 2.1 mm × 12.5 mm, 5 μm, Agilent Technologies) which has been filled with the same packing material. Using amygdalin standard, the column temperature and the acidity of the mobile phase were optimized. Then the temperature of the column was set at 40 °C. The mobile phase was a 10 mM sodium phosphate buffer (pH 3.1) containing 30% methanol, while the flow rate was 0.2 ml/min.

Mass spectral analyses were carried out in positive 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 20 V and a heated capillary temperature of 250 °C were obtained as optimal control conditions. The other parameters, including the voltages of octapole offset and tube lens offset, were optimized for maximum abundance of the ions of interest by the automatic tune procedure of the instrument. The sodium adduct molecular ions of amygdalin and its metabolites were detected by on-line mass detector in selected ion monitoring (SIM) mode, and their MS^2 product ion spectra were obtained by collision-induced dissociation (CID) utilizing helium in the ion trap with isolation width (*m*/*z*) of 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^2 works.

3. Results and discussion

3.1. LC–MS and LC–MS^2 analyses of amygdalin

Because metabolites can retain base substructure of the parent drug, so the first important step in the analysis of the metabolites of amygdalin in rat urine is to obtain its chromatogram and mass spectrum characterization. All these data were the substructural 'template' for interpreting the structures of the metabolites.

The conditions of chromatography and mass spectrometry were optimized using amygdalin standard. Under the condition of electrospray ionization, amygdalin molecules ($C_{20}H_{27}NO_{11}$, MW 457.16 Da) can easily form sodium adduct molecular ion of *m*/*z* 480 and 502 ($[M + 2Na–H]^+$) in positive ion detection mode, but the protonated molecular ion *m*/*z* 458 were not detected (Fig. 1A). Amygdalin was eluted after 3.1 min under above experimental conditions (Fig. 1B). The MS^2 product ion spectrum of the sodium adduct molecular ion of amygdalin and its predominant fragmentation patterns were illustrated in Fig. 1C and D. Fragmentation of sodium adduct molecular ion of amygdalin in the ion trap led to four product ions at *m*/*z* 453, 374, 363 and 347. The product ions at *m*/*z* 453 and 363 were formed by the loss of neutral fragments HCN and C_8H_7N from the ion at *m*/*z* 480. The most abundant product ion at *m*/*z* 347 was formed by the loss of neutral fragments mandelonitrile (C_8H_7NO, 133 Da) from the ion at *m*/*z* 480. The product ion at *m*/*z* 374 was inferred to be produced by the loss of C_7H_8N (106 Da) from the ion at *m*/*z* 480. So it can be concluded that the ions at *m*/*z* 453, 374, 363 and 347 were the characteristic product ions of amygdalin; 27 and 133 Da were its characteristic neutral losses. These characteristic product ions and neutral losses were the sound bases to identify metabolites of amygdalin in vivo.

3.2. Identification of metabolites by chromatography and mass spectrometry

Firstly, the possible structures of metabolites have been speculated according to the metabolism rule of drugs in vitro [3]. In order to find out the possible metabolites, the full scan mass spectrum of purified rat urine after i.v. injection of amygdalin was compared with that of blank urine sample. Then, the metabolites were analyzed by LC–MS^2 in selected ion monitoring (SRM) mode. Finally, the retention times, changes in observed mass (ΔM) and MS^2 spectra of metabolites were compared with those of amygdalin to identify metabolites and elucidate their structures.

Based on the method mentioned above, amygdalin and its main metabolites were found in rat urine after i.v. injection of

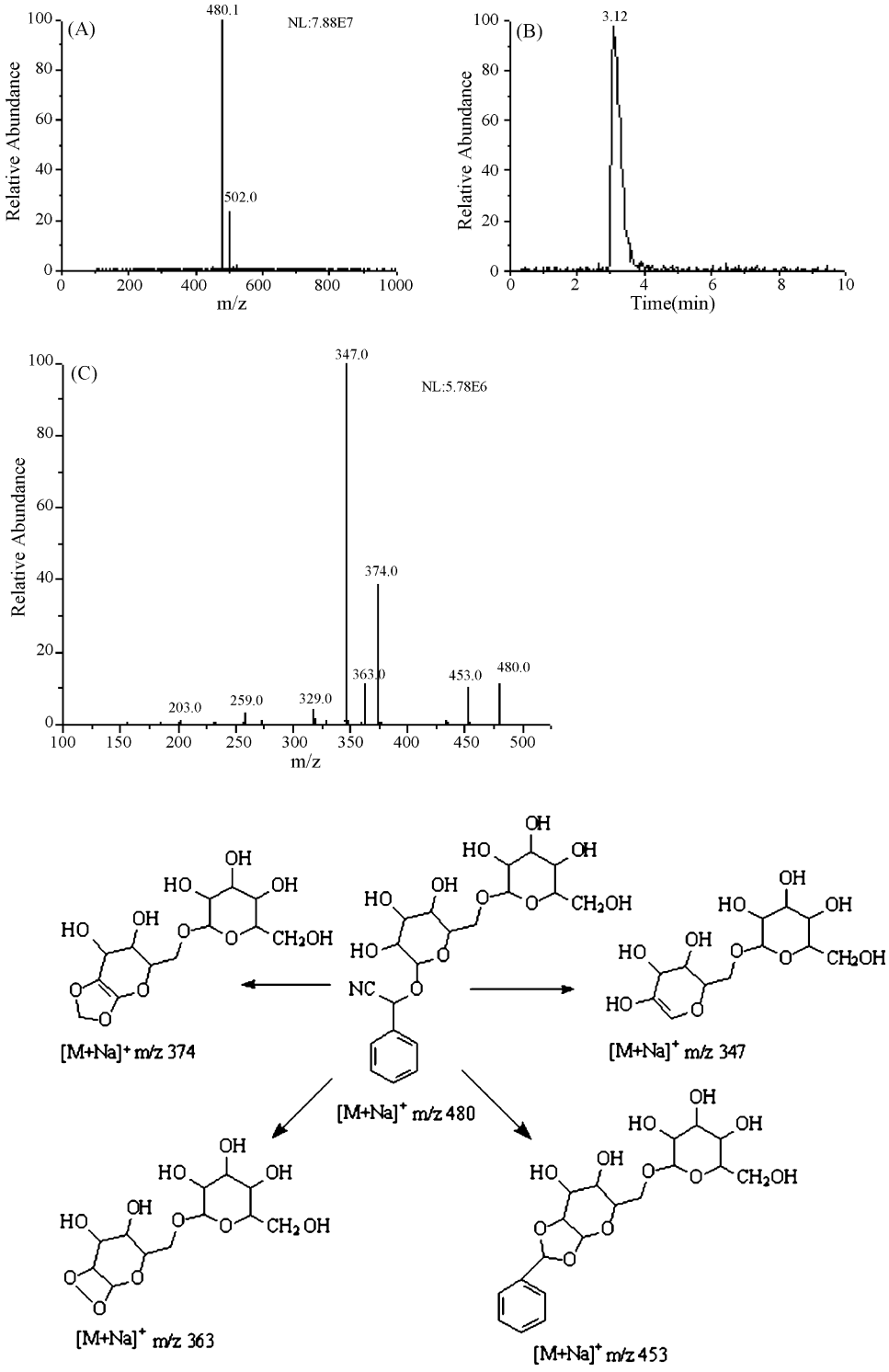

Fig. 1. (A) Full scan MS spectrum; (B) LC–MS^2 chromatogram of amygdalin; (C) full scan MS^2 product ions spectrum; (D) the predominant fragmentation patterns.

amygdalin. The sodium adduct molecular ions of the metabolites were at *m/z* 318, 334, 484, 496, 500, 512 and 514, respectively. Their MS^2 spectra (Fig. 2) obtained by fragmentation of sodium adduct molecular ions were used for more precise structural identification of metabolites. Among them, the retention time, the MS and MS^2 spectra of the sodium adduct molecular ion at *m/z* 480 (M0, Fig. 2A) were the same as those of amygdalin. Therefore, M0 can be confirmed as the unchanged parent drug.

The sodium adduct molecular ion of M1 (*m/z* 318, Fig. 2B) and its main daughter ions at *m/z* 291 and 185 were all 162 Da less than the sodium adduct molecular ion of M0 and its main daughter ions at *m/z* 347 and 453. The daughter ions at *m/z* 291 and 185 were produced by the loss of neutral fragments 27 and

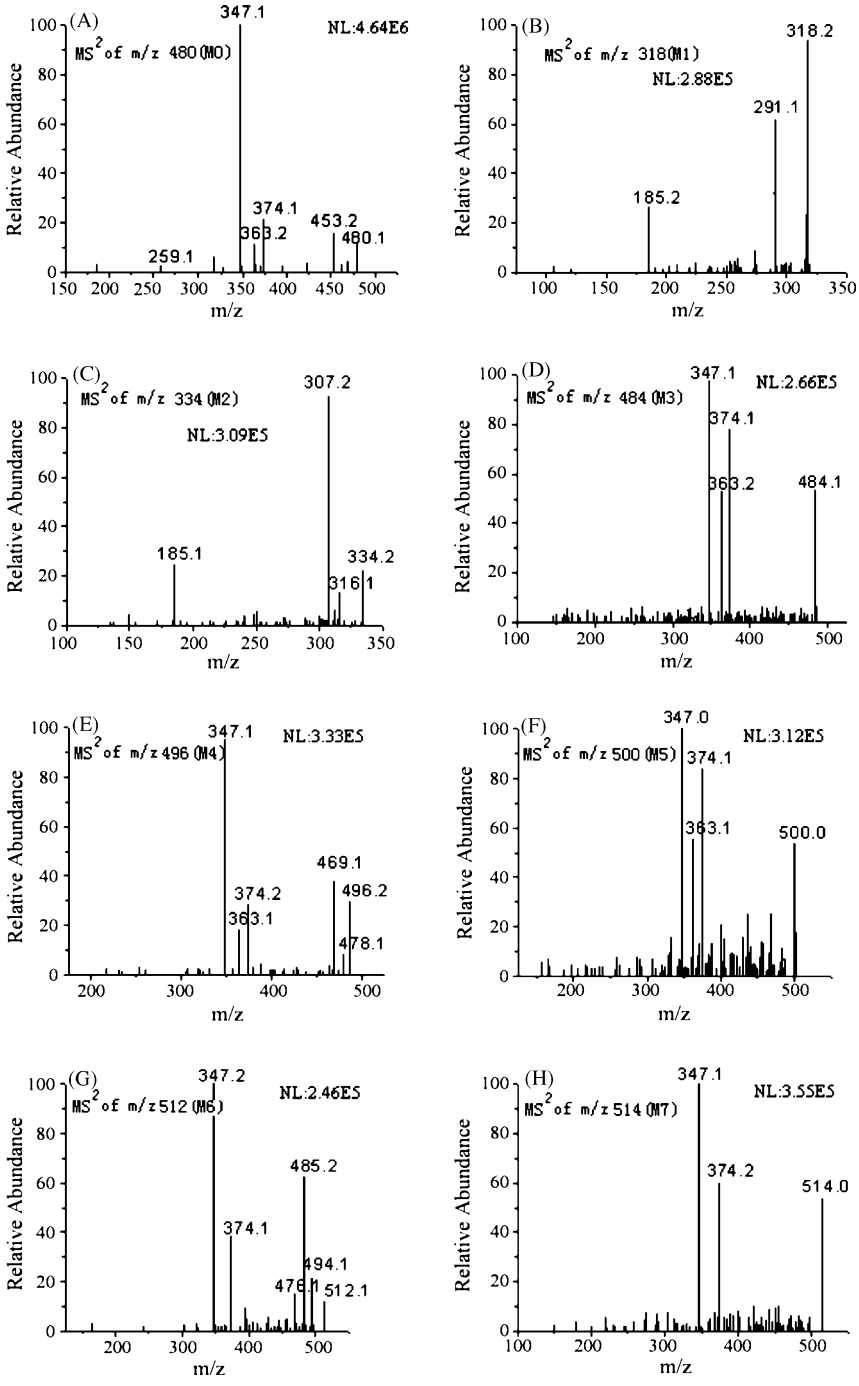

Fig. 2. MS^2 product ion spectra of amygdalin and its metabolites in rat urine.

133 Da from the ion at *m/z* 318. These results indicated that M1 may be prunasin [3].

The sodium adduct molecular ion at *m/z* 334 (M2, Fig. 2C) and its daughter ions at *m/z* 307 were all 16 Da more than the sodium adduct molecular ion of M1 (*m/z* 318) and its daughter ion at *m/z* 291. The other daughter ions of M2 at *m/z* 316, 307 and 185 were formed by the loss of neutral fragments 18, 27 and 149 (133 + 16) Da from the sodium adduct molecular ion of M2, respectively. Thus, M2 can be identified as the hydroxylation product of prunasin. Because of the appearances of neutral losses 27 and 149 (133 + 16) Da in the MS^2 fragmentation of M2, the localization of the hydroxyl group should be at the aromatic ring of M2.

The sodium adduct molecular ion of M3 (*m/z* 484) was increased by 4 Da when compared with that of the unchanged amygdalin. The characteristic MS^2 product ions at *m/z* 347, 363 and 374 of M0 were appeared in the MS^2 spectrum of M3 (Fig. 2D), but the characteristic neutral losses of HCN (27 Da) disappeared in the MS^2 fragmentation of M3. All above results indicated that the hydrogenation reaction was occurring in the –CN group of amygdalin. Hence, M3 could be identified as the amination product of amygdalin.

Table 1
ESI MS/MS data for amygdalin or its metabolites in rat urines

Amygdalin or its metabolites	t_R (min)	Ion polarity	MS, m/z $[M+Na]^+$	Ion, m/z in MS/MS
M0	3.12	Positive	480	453, 374, 363, 347
M1	3.32	Positive	318	291, 185
M2	3.30	Positive	334	316, 307, 185
M3	3.25	Positive	484	374, 363, 347
M4	3.21	Positive	496	478, 469, 374, 363, 347
M5	3.22	Positive	500	374, 363, 347
M6	3.20	Positive	512	494, 485, 476, 374, 347
M7	3.23	Positive	514	374, 347

The sodium adduct molecular ion of M4 (m/z 496, Fig. 2E) and its daughter ion at m/z 469 were all increased by 16 Da when compared with that of the sodium adduct molecular ion of M0 and its daughter ion at m/z 453. The characteristic MS^2 product ions at m/z 363, 374 and 347, and characteristic neutral loss 27 Da (HCN) of M0 were all appeared in the MS^2 spectrum of M4 (Fig. 2E). So M4 may be the hydroxylation product of amygdalin, and the localization of the hydroxyl group should be at the aromatic ring of M4 (Table 1).

The sodium adduct molecular ion of M5 (m/z 500, Fig. 2F) was increased by 16 Da when compared with that of the sodium adduct molecular ion of M3. The characteristic product ions at m/z 347, 363 and 374 of M0 were also appeared in the MS^2 spectrum of the ion at m/z 500. Thus, M5 could be conformed as the hydroxylation product of M3.

The sodium adduct molecular ion at m/z 512 (M6, Fig. 2G) was increased by 32 Da when compared with that of the unchanged amygdalin, and the characteristic MS^2 product ions at m/z 374, 347 and characteristic neutral losses 27 Da of M0 were all presented in the MS^2 spectrum of M6, so M6 may be the dihydric product of amygdalin, and the localization of the hydroxyl group was considered on the aromatic ring of M6.

The characteristic MS^2 product ions at m/z 347 and 374 of M0 were presented in the MS^2 spectrum of the sodium adduct molecular ion of M7 (m/z 514, Fig. 2H), but the characteristic loss of neutral fragment 27 Da (HCN) disappeared in the MS^2 fragmentation of M7. It indicated that the biotransformation of amygdalin could be occurred in the –CN group of amygdalin. Hence, M7 should be the nitryl product of amygdalin.

The proposed major metabolic pathway of amygdalin in rats was shown in Fig. 3.

The time of excretion of amygdalin and its metabolites was detected by the tandem MS technique. All the metabolites (M1–M7) and the parent drug (M0) were detected in 0–6 h rat urine, and disappeared in the following 48 h time.

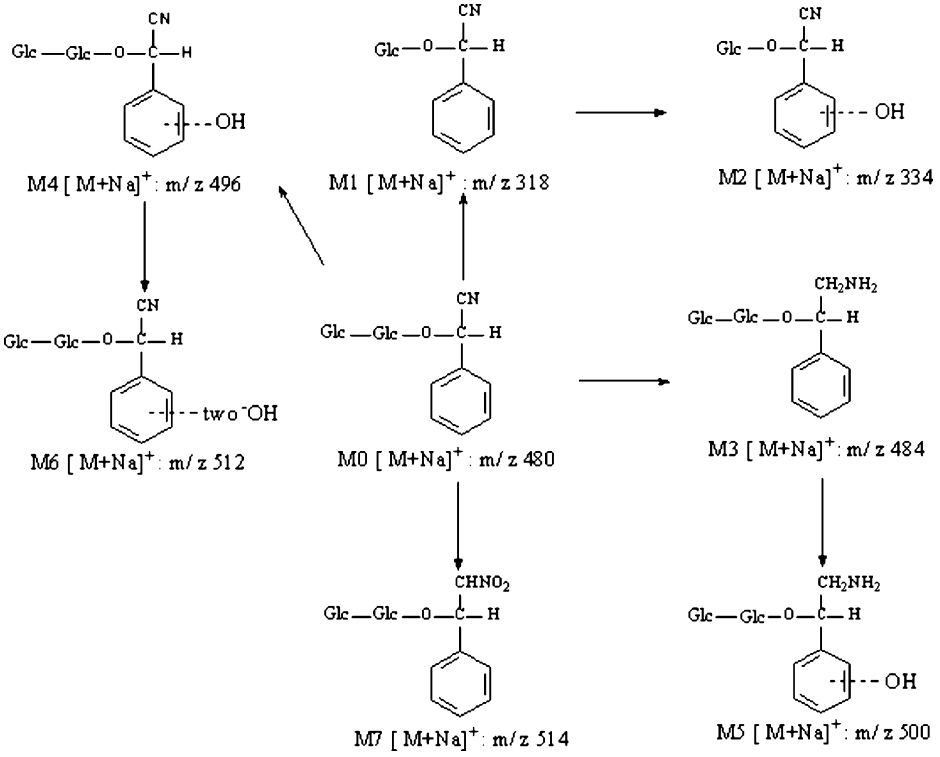

Fig. 3. Proposed major metabolic pathway of amygdalin in rats (Glu = glucose).

4. Conclusions

The proposed method in this paper is a suitable method for the qualitative determination of amygdalin and its metabolites due to its some advantages in sensitivity, high speed and accuracy. At least seven metabolites and the parent drug were found in rat urine after i.v. injection of 100 mg/kg doses of amygdalin. Among them, six metabolites (M2–M7) were reported for the first time.

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References

[1] Chinese Pharmacopoeia, vol. 1, 2000, p. 160.
[2] Y.P. Zhu, Chin. Mater. Med. 1998 (1998) 496.
[3] X.W. Yang, J. Res. Dev. Chin. Med. Chang Bai Shan 5 (1996) 50.
[4] A.G. Rauws, L.G. Gramberg, M. Olling, Pharm. Weekbl. Sci. 4 (6) (1982) 172.
[5] G.J. Strugala, A.G. Rauws, R. Elbers, Biochem. Pharmacol. 35 (13) (1986) 2123.
[6] C. Sauer, F.T. Peters, R.F. Staack, G. Fritschi, H.H. Maurer, J. Mass Spectrom. 41 (2006) 1014.
[7] R.H. Lowe, A.J. Barnes, E. Lehrmann, W.J. Freed, J.E. Kleinman, T.M. Hyde, M.M. Herman, M.A. Huestis, J. Mass Spectrom. 41 (2006) 175.
[8] C. Soler, B. Hamilton, A. Furey, K.J. James, J. Manes, Y. Pico, Rapid Commun. Mass Spectrom. 20 (2006) 2151.
[9] B.D. Davis, P.W. Needs, P.A. Kroon, J.S. Brodbelt, J. Mass Spectrom. 41 (2006) 911.
[10] D. Wang, T. Hang, C. Wu, W. Liu, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 829 (2005) 97.
[11] Z.H. Zhang, H.T. Chen, K.K. Chan, T. Budd, R. Ganapathi, J. Chromatogr. B 728 (1999) 85.
[12] M.H. Spyridaki, E. Lyris, I. Georgolakis, D. Kouretas, M. Konstantinidou, C.G. Georgakopoulos, J. Pharm. Biomed. Anal. 35 (2004) 107.
[13] J.L. Wolfender, K. Ndjoko, K. Hostettmann, J. Chromatogr. A 1000 (2003) 437.
[14] A. Tolonen, M. Turpeinen, J. Uusitalo, O. Pelkonen, Eur. J. Pharm. Sci. 25 (2005) 155.
[15] K. Hiraoka, Y. Asakawa, Y. Yamamoto, M. Nakamura, K. Ueda, Rapid Commun. Mass Spectrom. 15 (2001) 2020.
[16] J.Y. A, G.J. Wang, M.S. Wu, J.H. Liu, Acta Pharmacol. Sin. 23 (2002) 1045.
[17] Y. Li, D.F. Zhong, S.W. Chen, I. Maeba, Acta Pharmacol. Sin. 26 (2005) 1519.
[18] A. Li, M.P. May, J.C. Bigelow, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 836 (2006) 129.
[19] S.A. Appolonova, A.V. Shpak, V.A. Semenov, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 800 (2004) 281.
[20] E. Molden, B.G. Helen, H. Christensen, L. Reubsaet, J. Pharm. Biomed. Anal. 332 (2003) 75.
[21] R.L. Fitzgerald, J.D. Rivera, D.A. Herold, Clin. Chem. 45 (1999) 1224.
[22] M. Sato, M. Hida, H. Nagase, Forensic Sci. Int. 128 (2002) 146.
[23] M. Katagi, M. Nishikawa, M. Tatsuno, A. Miki, H. Tsuchihashi, J. Chromatogr. B: Biomed. Sci. Appl. 751 (2001) 177.