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Identification of new metabolites of morroniside produced by rat intestinal bacteria and HPLC-PDA analysis of metabolites in vivo

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Abstract

Morroniside, the most abundant iridoid glycoside of traditional Chinese medicines Fructus Corni, was shown to prevent diabetic angiopathies. During the course of our studies on its metabolism by intestinal bacteria, two metabolites (mor-1 and mor-2) were isolated and purified by thin layer chromatography (TLC) and preparative high performance liquid chromatography (HPLC), and then identified as nitrogen-containing compounds along with the known aglycones on the basis of mass spectrometry (MS), and by one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. Mor-1 and mor-2 were proved to be new compounds. The structures of the metabolites of morroniside detected in rat urine, bile, feces and contents of intestine after oral administration of morroniside proved to be identical with those of the microbial metabolites mor-1 and mor-2.

Keywords: Fructus Corni; Morroniside; Metabolite; Intestinal bacteria

1. Introduction

Traditional Chinese medicine (TCM) Fructus Corni is highly prized for its therapeutic abilities of reducing blood glucose, immunological regulation, antishock, antiarrhythmia, antibiosis, etc. [1,2]. The total iridoid glycosides of Fructus Corni containing loganin, morroniside and other minor constituents possess a number of pharmacological activities such as mitigating the vascular complications of diabetes [3,4], repress immunity [5] and against rheumatoid arthritis [6]. As the most abundant iridoid glycoside in Fructus Corni, morroniside has been proved to have stomachic function and to be effective in preventing diabetic angiopathies [7,8]. Its chemical structure is shown in Fig. 1 as "mor".

With the important effect of natural drugs in human health, pharmacokinetics of active constituents or fractions, simple or compound recipes of TCM is now becoming a powerful tool for identifying therapeutic basis and mechanism of action of TCM [9,10]. Being a major active constituent, morroniside is attracting increasing interest. Several methods for the analysis of morroniside had been reported in the literature, e.g. high performance liquid chromatography (HPLC) with UV detection, ESI-TOF-MS analysis and micellar electrokinetic capillary chromatography [11–13]. In our previous work, pharmacokinetics and tissue distribution of morroniside in rats were studied by HPLC [14], which showed that the absolute bioavailability of morroniside was about 5% and less than 2% was excreted in the urine after oral administration of morroniside. These results indicated that morroniside is metabolized, so it is necessary to focus on this to reveal physiological situation of morroniside.

The intestine was the site of metabolism. After being ingested in vivo, many glycosides are often hydrolyzed to their aglycones by hydrolytic enzymes produced by enteric microbial flora in digestive tract, and then aglycones are converted to other

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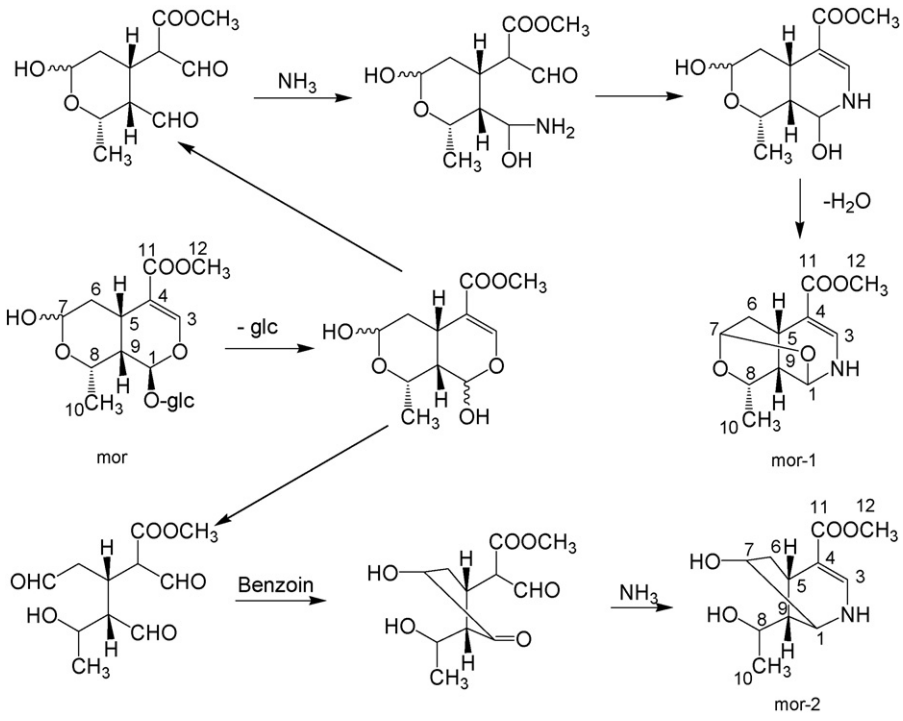

Fig. 1. The possible metabolic pathway of morroniside.

compounds. Hence, intestinal bacteria system was often considered a reasonable model to reflect the metabolism of natural or synthetic compounds [15–18]. The model has the advantages of saving the cost of large numbers of animals, isolating and identifying new metabolites easier, etc.

In the present report, metabolites of morroniside in vitro were prepared by anaerobic culture with intestinal bacteria and identified by their mass spectra and NMR spectra. In vivo metabolites of morroniside were also investigated by high performance liquid chromatography-photodiode array detector (HPLC-PDA) analysis and compared with the microbial in vitro metabolites.

2. Experimental

2.1. Materials and reagents

The reference standard of morroniside was extracted and purified from Fructus Corni that was purchased from Anguo T.C.M. market, Hebei, China, and identified by Prof. Fengzhi Nie (Department of Pharmacognosy, Hebei medical University, Shijiazhuang) in our laboratory. Its chemical structure was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and its purity was over 99% by HPLC-PDA area normalization. The purity of morronoside administrated to rats was over 95%. Acetonitrile and methanol were of HPLC grade and obtained from Tedia (Tedia, Fairfield, USA).

Male Sprague-Dawley rats (210 ± 10 g) were obtained from the Hebei Laboratory Animal Center (Shijiazhuang, People's Republic of China). They were kept in an environmentally controlled breeding room for 5 days before starting the experiments and fed with standard laboratory food and water *ad libitum*. All rats were dosed following an overnight fast (except for water).

2.2. Instruments and conditions

2.2.1. HPLC-PDA conditions

HPLC analysis was carried out on a Waters Series (a Model 1525 pump and 2996 photodiode array detector; Waters Assoc., Milford, MA, USA) HPLC system. Data were collected by a HPLC chromatography workstation (Empower software). The analytical column was a Diamonsil C_{18} reversed-phase column (250 mm × 4.6 mm, 5 μm). Solvent A was acetonitrile and

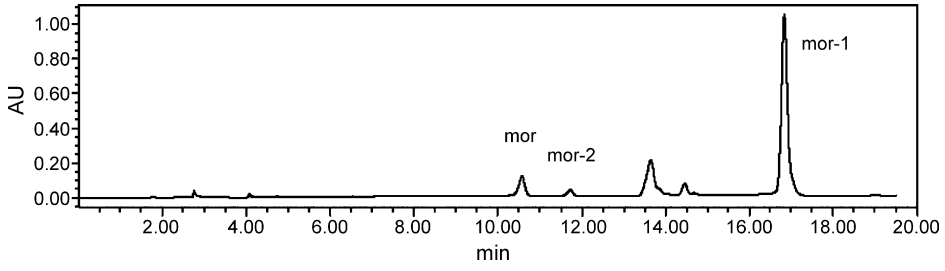

Fig. 2. The HPLC-PDA chromatograms of morroniside and its metabolites in vitro.

Table 1
^{1}H NMR, ^{13}C NMR, DEPT, HMBC and ^{1}H–^{1}H-COSY spectral data for mor-1

Position	δ_H (J, Hz)	δ_C	DEPT	HMBC	^{1}H–^{1}HCOSY
1	5.11 (br s)	71.8	CH	3, 5	9, NH
3	7.25 (d, J = 5.5)	138.7	CH	1, 4, 5, 11	NH
4		102.1	C		
5	2.85 (br d, J = 9.5)	23.8	CH	1, 3, 4, 6, 7, 9, 11	6a, 6b
6a	1.23 (br d, J = 14.0)	37.5	CH_2	4, 7	5, 6b, 7
6b	2.25 (br d, J = 14.0)	37.5	CH_2	4, 5, 7	5, 6a, 7
7	4.63 (br s)	84.6	CH	1, 5, 8	6a, 6b
8	4.05 (q, J = 6.5, 13.0)	68.7	CH	1, 5, 9	10
9	1.35 (br s)	31.6	CH	1, 5, 6, 10	1
10	1.18 (d, J = 6.5)	19.0	CH_3	8, 9	8
11		166.9	C=O		
12	3.56 (s)	50.4	CH_3	11	
NH	7.84 (br s)			1, 3, 4, 9, 11	1, 3

solvent B was water. For the detection of the metabolites of morroniside, the mobile phase consisted of a gradient elution for 0–5 min with 10–20% solvent A, 5–15 min with 20–30% solvent A, followed by 15–18 min with 30–10% solvent A at a flow rate of 1 ml/min. The temperature of column was kept at 25 °C and the spectra of the peaks were scanned from 210 nm to 400 nm.

2.2.2. Preparative HPLC conditions

The mobile phase consisted of acetonitrile–water (20:80 and 22:78), and other conditions were similar to those at analytical HPLC-PDA.

2.2.3. Preparative TLC conditions

The stationary phase was silica gel GF_{254}, mobile phase was MeOH–$CHCl_3$ (1:9 and 2:8).

2.2.4. ESI-MS apparatus

ESI-MS (electrospray ionization-mass spectrometry) analysis was performed with a ZMD mass spectrometer (Micromass, England) equipped with ESI interface.

2.2.5. NMR apparatus

^{1}H and ^{13}C NMR spectra were recorded at 500 MHz on a VARIAN INOVA-500 NMR spectrometer in $CDCl_3$ at ambient temperature with tetramethylsilane (TMS) as the internal standard. Standard pulse sequences were used for DEPT, HMQC, HMBC and ^{1}H–^{1}H-COSY experiments.

2.3. Separation and identification of biotransformation products

2.3.1. Preparation of intestinal bacteria culture solution

One gram feces from rats was transferred into a baffled flask containing 50 ml incubation buffer [15]. After mixing thoroughly, cultures were incubated in an anaerobic sack (MGC Company, Japan) under a N_2 atmosphere at 37 °C for 30 min, and then culture solution of intestinal bacteria was prepared.

2.3.2. Separation of biotransformation products

Morroniside (500 mg) was added in 200 ml culture solution of intestinal bacteria, and mixed well. The mixture was incubated in condign time with anaerobic culture at 37 °C, and then extracted with equal volumes of ethyl acetate three times. The ethyl acetate extract was evaporated to dryness in a rotary evap-

Table 2
^{1}H NMR, ^{13}C NMR, DEPT, HMBC and ^{1}H–^{1}H-COSY spectral data for mor-2

Position	δ_H (J, Hz)	δ_C	DEPT	HMBC	^{1}H–^{1}HCOSY
1	4.62 (br s)	55.3	CH		7, 9, NH
3	7.45 (d, J = 6.0)	143.7	CH	1, 4, 5, 11	NH
4		97.2	C		
5	2.78 (br s)	29.6	CH		6a, 6b, 9
6a	1.74 (m)	36.1	CH_2		5, 6b, 7
6b	1.87 (m)	36.1	CH_2		5, 6a, 7
7	5.13 (m)	69.6	CH		1, 6a, 6b, 7-OH
8	4.38 (m)	64.4	CH		9, 10, 8-OH
9	1.49 (dt, J = 3.0, 9.5)	44.7	CH		1, 5, 8
10	1.18 (d, J = 6.0)	21.0	CH_3	8, 9	8
11		166.7	C=O		
12	3.52 (s)	50.2	CH_3	11	
NH	7.73 (t, J = 5.0, 5.5)			1, 3, 4, 9	1, 3
7-OH	5.95 (d, J = 7.5)			6, 7	7
8-OH	4.67 (d, J = 6.0)			8, 9	8

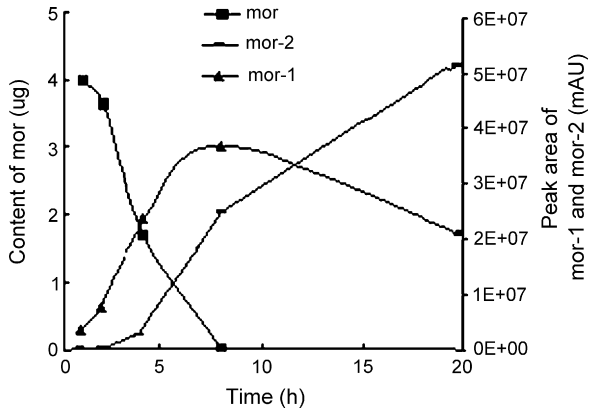

Fig. 3. Time course of microbial transformation of morroniside.

orator and dissolved in methanol. A sufficient quantity of this was diluted for HPLC-PDA analysis and the rest was separated by preparative TLC and purified by preparative HPLC.

2.4. Time course of biotransformation of morroniside

Twelve test tubes were taken and each was put into 5 ml culture solution of intestinal bacteria and then incubated at 37 °C for 30 min. Two hundred microlitres of aqueous solution of morroniside (2 mg/ml) was added to each test tube and incubated for 0, 1, 2, 4, 8 and 20 h, respectively. Samples were prepared in duplicate.

One millilitre of the above sample was extracted with equal volumes of ethyl acetate three times. The ethyl acetate extract was combined, followed by evaporation to dryness. The remainder slag was dissolved in 100 μl methanol, 20 μl of the solution were injected into the HPLC-PDA system for analysis.

2.5. Sample preparation and identification of in vivo metabolites

2.5.1. Sample collection

After oral administration at a dose of 100 mg/kg water solution of morroniside (15 mg/ml) in rat: (1) rats were executed at 4 h, and then the intestine and its contents were harvested and cut in to pieces; (2) 300 μl plasma samples were obtained from fossa orbitalis vein at 1 h and 4 h; (3) rats were injected urethane 1.5 g kg^{-1} in abdominal cavity to anesthetize followed by biliary cannulation. Bile sample was collected in 15 h; (4) rats were in the metabolic cages without any food except water. Urine was collected in 6 h; (5) Rats were in the metabolic cages without any food except water. Feces sample was collected in 24 h.

Rats were administrated with the same dosage of water as the samples to be the control group and the other sequences were the same as for the sample group.

2.5.2. Sample preparation

Plasma, bile, urine samples were extracted with equal volumes of ethyl acetate three times. The ethyl acetate extract was evaporated to dryness in a rotary evaporator and dissolved in a sufficient quantity of methanol. Twenty microlitre of the solution were injected into the HPLC-PDA system for analysis. The peaks of morroniside and its metabolites were validated by comparing their retention times and the maximum wavelengths of UV spectra with the reference standards.

Intestine and its contents and feces samples were extracted twice by sonication with sufficient quantities of methanol for 15 min. The extract was filtered and evaporated, and then suspended with water. The other sequences were the same as in the course of plasma, bile and urine sample preparations.

3. Results and discussion

3.1. Separation and identification of biotransformation products

3.1.1. In vitro HPLC-PDA analysis

HPLC-PDA analysis detected morroniside and its metabolites eluting at 10.5, 16.7 and 11.7 min (Fig. 2). The maximum wavelengths of peaks were at 239 nm, 273 nm and 292 nm, respectively.

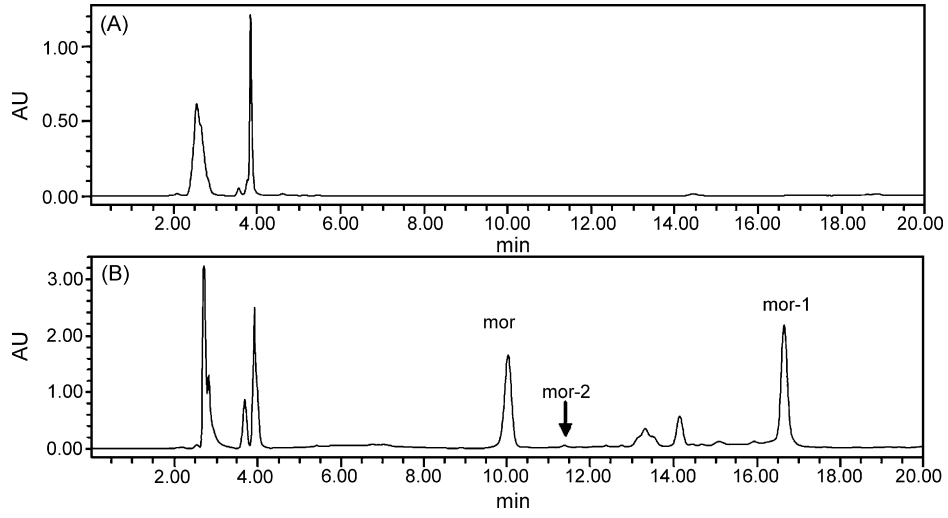

Fig. 4. The HPLC-PDA chromatograms of intestine and its contents in rat: (A) blank and (B) sample after administration of morroniside.

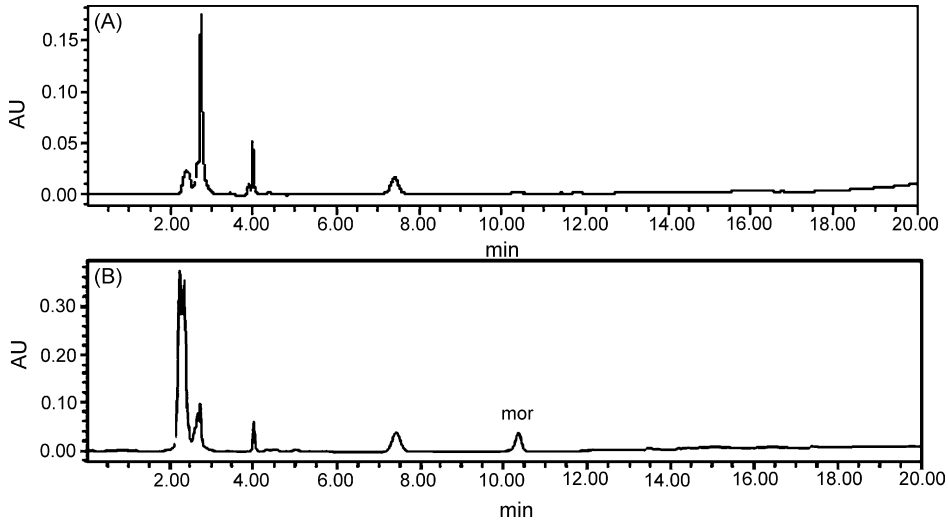

Fig. 5. The HPLC-PDA chromatograms of plasma in rat: (A) blank plasma and (B) plasma sample after administration of morroniside.

3.1.2. Identification of metabolites

Mor-1: molecular formula was $C_{11}H_{15}NO_4$, *m/z* 224 was distinguishable quasi-molecular ion $[M-H]^-$, high resolution electron ionization (HREI) *m/z* 225.1001 coincided with molecular weight of theoretical calculation, λ_{max}MeOH 273 nm, the data of ^{1}H NMR, ^{13}C NMR, DEPT, HMBC and ^{1}H–^{1}H-COSY are shown in Table 1. Mor-2: molecular formula was $C_{11}H_{17}NO_4$, *m/z* 227 was distinguishable molecular ion [*M*], λ_{max}MeOH 292 nm, the data of ^{1}H NMR, ^{13}C NMR, DEPT, HMBC and ^{1}H–^{1}H-COSY are shown in Table 2. Chemical structures of mor-1 and mor-2 are shown in the reaction scheme in Fig. 1.

Two new nitrogen-containing metabolites of morroniside, mor-1 and mor-2, were first obtained in the course of the metabolism of morroniside by intestine bacteria. It was reported that geniposide and gardenoside were transformed to new nitrogen-containing compounds, genipinine and gardenine, respectively, along with the known aglycones [18]. In common with geniposide and gardenoside, morroniside is also an iridoid glycoside. A possible metabolic pathway for these two compounds is proposed in Fig. 1. When morroniside was hydrolyzed by glucosidase, its aglycone was released. After the cleavage of B-ring of the aglycone, two steps of nucleophilic addition reaction took place to produce an N-heterocycle derivative, and this derivative further dehydrated to form mor-1; and the product from the cleavage of A- and B-ring of the aglycone would produce mor-2 via a step of benzoin condensation and two steps of nucleophilic addition reaction.

Several traditional dosage forms of TCM are administered orally, and their effective constituents are often metabolized by intestine bacteria. Anaerobic culture with intestinal bacteria in vitro proved to be a powerful tool for preparation of the metabolite and for the study of drug metabolism. Anaerobic culture with intestinal bacteria in vitro has several advantages, e.g. it was convenient to control metabolic conditions, purify the metabolites and to solve complicated problems easily and rapidly.

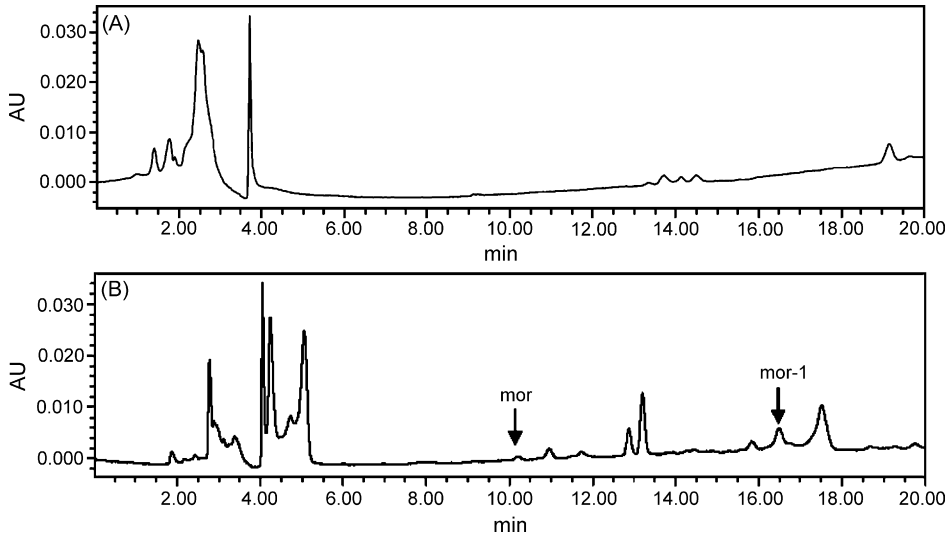

Fig. 6. The HPLC-PDA chromatograms of bile in rat: (A) blank bile and (B) bile sample after administration of morroniside.

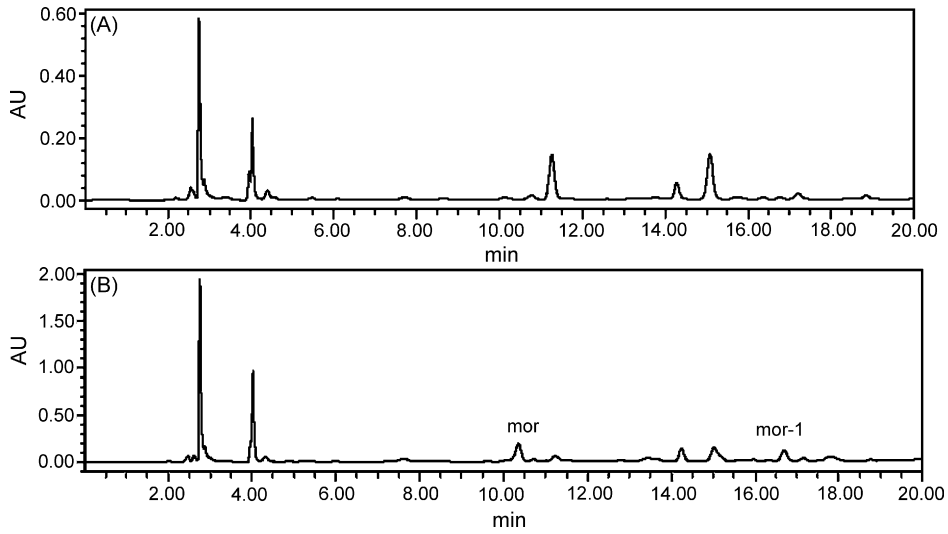

Fig. 7. The HPLC-PDA chromatograms of urine in rat: (A) blank urine and (B) urine sample after administration of morroniside.

3.2. Time course of biotransformation of morroniside

Correlation coefficients greater than 0.9998 were obtained in the content range of 0.0162–5.05 μg, and standard curve of morroniside was $Y = 1.328 \times 10^6 C + 2.750 \times 10^4$, where Y was the peak area of morroniside, and C was the content of morroniside. The contents of morroniside were calculated from standard curve of morroniside, and the contents of metabolites were calculated by their peak area at 273 nm and 292 nm (maximum wavelengths of mor-1 and mor-2), respectively.

The time courses of the two metabolites and the substrate are shown in Fig. 3. The concentration of morroniside decreased and vanished within 8 h. Mor-1 was detected at 1 h following addition of substrate and its concentration reached maximum after 8 h of incubation followed by a marked decrease. Mor-2 appeared at 4 h and its concentration increased until the end of investigation (20 h). This suggested that mor-2 might be formed from mor-1. So optimum incubation times for mor-1 and mor-2 were 8 h and 20 h, respectively.

3.3. Identification of in vivo metabolites

After rats were taken orally morroniside, samples of plasma, urine, bile, feces and contents of intestine were pretreated and analyzed by HPLC-PDA for revealing the metabolic pathway of morroniside. Metabolites in these samples and in those prepared from intestinal bacteria in vitro had identical retention times and UV maxima. This identity shows that metabolites might be prepared by anaerobic culture with intestinal bacteria. By using microbial transformation methods, two new transformed products were confirmed to be major in vivo rat metabolites. Fig. 4 shows the HPLC-PDA chromatograms of intestine and its contents in rat. It can be seen that morroniside and its metabolites were detected in intestine and its contents,

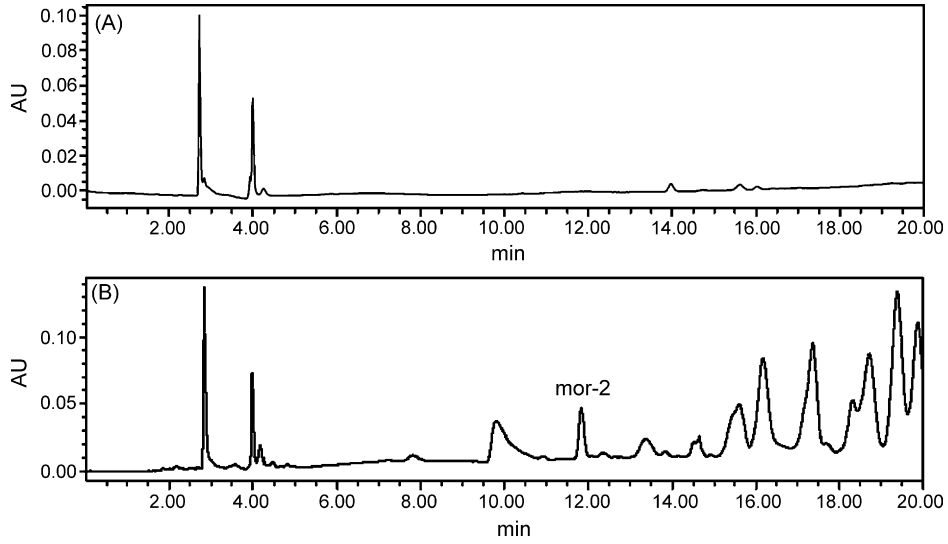

Fig. 8. The HPLC-PDA chromatograms of feces in rat: (A) blank feces and (B) feces sample after administration of morroniside.

indicating that the intestine was the metabolic site where morroniside was transformed into its metabolites. Fig. 5 shows the HPLC-PDA chromatograms of rat plasma. It can be seen that morroniside was detected and its metabolites were undetected in plasma. Fig. 6 shows the HPLC-PDA chromatograms of rat bile. It can be seen that morroniside and mor-1 were detected in bile and mor-2 was not. Fig. 7 shows the HPLC-PDA chromatograms of urine in rat. It can be seen that morroniside and mor-1 were found in urine but mor-2 was not. Thus, bile and urine excretion were two ways for the elimination of morroniside and mor-1. Fig. 8 shows the HPLC-PDA chromatograms of rat feces. It can be seen that only mor-2 was found in feces. Hence, mor-1 is not the end product of metabolism but could be transformed into mor-2 and mor-2 was excreted at last.

Therefore, physiological situation of morroniside could be inferred as follows. After administrating morroniside in rat, morroniside is absorbed in blood and distributed in most tissues [14]. Although mor-1 was not detected in plasma, which might be because mor-1 was at a concentration less than limit of HPLC-PDA detection, it was present in urine and bile, thus mor-1 could be absorbed in blood and transported to other tissues or organs. Morroniside could be transformed to mor-1 and mor-2 by intestinal bacteria in intestine. Morroniside and mor-1 was excreted by urine and bile, and mor-2 by feces.

4. Conclusions

Two metabolites (mor-1 and mor-2) were prepared by anaerobic culture with intestinal bacteria and identified as nitrogen-containing compounds along with the known aglycones. Mor-1 and mor-2 were proved to be new compounds. In vivo metabolites of morroniside were proved to be identical with mor-1 and mor-2 by HPLC-PDA analysis. Mor-1 and mor-2 were detected in contents of intestine, Mor-1 was found in urine and bile and mor-2 was observed in feces. Anaerobic culture with intestinal bacteria in vitro is a useful and valuable means for investigating metabolism of bioactive natural products from TCM.

Acknowledgments

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