Journal of Chromatography B, 976–977 (2015) 61–67

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry for rapid analysis of the metabolites of morroniside produced by human intestinal bacteria

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ARTICLE INFO

Article history:
Received 13 March 2014
Accepted 18 November 2014
Available online 29 November 2014

Keywords:
Morroniside
Human intestinal bacteria
UHPLC–Q-TOF-MS
Metabolites

ABSTRACT

Morroniside, the most abundant iridoid glycoside in the valuable traditional Chinese medicine Fructus Corni, exhibits various pharmacological activities and biological effects. Intestinal flora plays an important role in the metabolism of drug compounds, which might lead to the variation of ethnopharmacological profile of the medicine. However, little is known of the interactions of the morroniside with human intestinal bacteria. In this study, different pure bacteria were isolated from human feces and their capability to convert morroniside were investigated. The metabolites of morroniside were analyzed by ultra high performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UHPLC–Q-TOF-MS) technique using Metabolynx™ software. Parent compound and three metabolites were detected and tentatively identified based on the characteristics of their protonated ions. The parent is proposed to be metabolized by three main metabolic pathways including deglycosylation, dehydroxylation and methylation. Morroniside was firstly metabolized to its aglycone (M1), and then was further converted to dehydroxylated aglycone (M2) and methylated aglycone (M3). This is the first report of the metabolism of morroniside by human intestinal bacteria. These metabolites might influence the biological activities of morroniside *in vivo*, which could affect the clinical effects of medicines. Thus, the study on the metabolism of morroniside by human intestinal bacteria is very helpful to unravel how traditional medicines work.

1. Introduction

Cornus officinalis Sieb. et Zucc (Cornaceae) has been used to treat cerebrovascular disease and diabetes in traditional Chinese medicine for a long time [1]. Fructus Corni, deriving from the dry ripe sarcocarp of Cornaceae, has attracted wide interest as a popular and valuable herbal medicine in clinic practice. In Asian countries, it is commonly prescribed as a tonic formula exhibiting diuretic, anti-inflammatory and analgesic effects by suppressing cyclooxygenase-2 and inducible nitric oxide synthase expression through the down-regulation of nuclear factor-kappa B binding activity [2–4]. Fructus Corni suppresses hepatic gluconeogenesis related gene transcription, enhances glucose responsiveness of pancreatic beta-cells, and prevents toxin induced beta-cell death [5]. It also exhibits concentration-dependent relaxation effects of the corpus cavernosum [6], antimicrobial activity [7,8] and antidiabetic effect through its antihyperglycemic and beta-cell-protective actions [9].

The main ingredient of Cornaceae is a group of iridoid glycosides which might result in the hypoglycemic effects [10,11]. As we know, morroniside is the major active iridoid glycoside in Fructus Corni [12]. It shows neuroprotective activity against glutamate-induced toxicity in HT22 hippocampal cells [13] and oxidative stress-induced neurotoxic processes in SH-SY5Y cells [14]. Morroniside can notably protect the brain from damage induced by focal cerebral ischemia, which might be related to morroniside antioxidant and anti-apoptotic properties in the brain [1].

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http://dx.doi.org/10.1016/j.jchromb.2014.11.014
1570-0232/

Additionally, morroniside protects human umbilical vein endothelial cells against high ambient glucose induced injury, which suggests that morroniside can exert a beneficial effect on preventing diabetic angiopathies [15].

Most herbal medicines are administered orally and their components will inevitably contact with intestinal microflora in the alimentary tract. A lot of components are metabolized by the intestinal bacteria before absorption from the gastrointestinal tract [16]. The intestinal mucosa is recognized as a major determinant of the bioavailability of orally administered drugs. The parent compounds might be bio-transformed into metabolites with subsequent influence on efflux back to the intestinal lumen [17]. Nowadays it is clear that the complex microbial ecosystem in our intestines should be considered as a separate organ within the body, with a metabolic capacity which exceeds the liver by a factor 100. The intestinal microbiome is therefore closely involved in the first-pass metabolism of natural compounds [18]. The composition of the microbial community in the gut is governed by diet, age, environment and phylogeny [19] and the ecosystem contains all three domains of life: bacteria, archaea and eukarya, with the largest community residing in the colon. Indeed, the human colon harbors a highly complex microbial ecosystem of about 200 g living cells, at concentrations of 10^{12} microorganisms per gram gut content, the highest recorded for any microbial habitat [20]. Colon bacteria have a number of deconjugating enzyme activities, e.g. β-D-glucuronidases, β-D-glucosidases and α-L-rhamnosidases, which releases aglycones that are less polar and become more absorbable than their glycosides and glucuronides [21,22]. Other functions of intestinal microflora include metabolic activity, nutrients uptake, and host protection against invasion by foreign microorganisms. Based on the above facts, intestinal bacteria play an essential role in the development and homeostasis of the immune system [23].

Newly ingested bacteria cannot necessarily colonize and proliferate in the intestine. Therefore, an individual's intestinal microflora in feces are believed to be fairly stable over time in the absence of disease and/or antimicrobial therapy [24]. Although the degree to which composition and function of the fecal microflora differ from mucosal microflora remains unclear, fecal samples are often used to investigate the intestinal microflora because they are easily collected [25].

It is well known that microbial transformation frequently influences the biological activities of natural products [26]. However, the roles of bacteria in the human intestinal tract on the metabolic processes and biological activities of morroniside are not clear. In this work, we attempted to isolate different pure strains of bacteria from healthy human feces and carried out research on their abilities and characteristics in the metabolism of morroniside. To clarify the metabolic profile, ultra high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC–Q-TOF-MS) with automated data analysis (MetaboLynx^{TM}), a selective, sensitive and rapid method [27], was established to identify morroniside metabolites in the human intestinal bacterial samples.

2. Materials and methods

2.1. Chemicals

The HPLC-grade acetonitrile was purchased from TEDIA Company Inc. (Fairfield, USA). The formic acid was obtained from Merck KGaA (Darmstadt, Germany). The distilled water was purified by an EPED super purification system (Nanjing, China). Other reagents were of analytical grade. AnaeroPack Rectangular Jars were purchased from Mitsubishi Gas Chemical Company INC (Japan). Morroniside standard (purity, >98%) was purchased from Shanghai Winherb Medical S&T Development Co. Ltd (Shanghai, China).

2.2. Organism

Fresh human feces (4.0 g) was weighed and suspended in a centrifuge tube covered with 20 mL sterile physiological saline, then was homogenized adequately by a vortex-mixer. The mixture was centrifuged at 2000 × *g* for 10 min and the suspension was used as the human intestinal bacterial mixture.

The bacterial mixture was diluted serially in sterile water and each of the dilutions was spread on GAM agar plates which were then incubated under anaerobic condition in anaerobic jars for 48 h at 37 °C. At last, about one hundred different types of bacterial colonies developed on plates were observed [28]. Each bacterium picked up from GAM agar plate was inoculated into 1.0 mL GAM broth and anaerobically incubated at 37 °C for 24 h.

2.3. Growth media and anoxic techniques

The general anaerobic medium (GAM) used in the fermentation experiment contained the following compounds per liter: 10.0 g tryptone, 10.0 g proteose peptone, 13.5 g digestibility serum powder, 5.0 g yeast extract, 3.0 g soya peptone, 3.0 g glucose, 3.0 g NaCl, 5.0 g soluble starch, 2.5 g KH_2PO_4, 2.2 g beef extract, 0.3 g L-cysteine hydrochloride, 0.3 g sodium thioglycolate, 1.2 g beef liver extract powder, and 1000 mL distilled water. After these above being well mixed, its pH was adjusted to approximately 7.3 with NaOH aqueous solution. Then the GAM was autoclaved at 121 °C for 20 min.

2.4. Preparation of morroniside for analysis

The standard solution was prepared by dissolving accurately weighed morroniside in methanol to give a final concentration of 3.0 mg/mL. 1 mM morroniside was inoculated into 0.9 mL of GAM broth with 0.1 mL precultured bacteria and anaerobically incubated at 37 °C for 24 h. The sample was extracted twice with ethyl acetate. The organic layer was dried under vacuum and dissolved with 0.3 mL methanol. Then the ethyl acetate exhausted liquors was extracted twice with butanol. The butanol layer was also dried under vacuum and dissolved with 0.3 mL methanol which combined with the ethyl acetate layer. The mixture was centrifuged at 12,000 × *g* for 10 min and the supernatant was filtered through a 0.22 μm membrane before UHPLC/MS analysis.

2.5. UHPLC/MS spectrometry

Morroniside and its metabolites were separated and identified on a Waters ACQUITY UHPLC system (Waters Corp., Milford, MA, USA) fitted with a Syncronis C 18 column (100 mm × 2.1 mm i.d., 1.7 μm; Thermo, USA). The chromatographic conditions were optimized to obtain good separation of the target compounds and avoid the interferences. The column temperature was maintained at 35 °C. 0.1% formic acid in water (A) and acetonitrile (B) served as the mobile phase in a gradient mode (B increased from 5 to 90% in 11 min, and was held for 1 min) with a flow rate of 0.4 mL/min. Injection volume was 5 μL. The retention time of morroniside was 2.92 min. The standard stock solution of morroniside was prepared in methanol and then diluted with methanol to appropriate concentrations. A series of diluted standard solutions were prepared to determine the limits of detection (LOD). The value was determined at signal to noise (S/N) ratios of 3. The LOD of morroniside was about 0.58 ng/mL.

For analysis, a Waters ACQUITY Synapt mass spectrometer (Waters Corp., Manchester, UK) connected to the UHPLC system via an electrospray ionization (ESI) interface was used. ESI in both