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Original article

Metabolism and antioxidant effect of malaxinic acid and its corresponding aglycone in rat blood plasma

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ABSTRACT

Malaxinic acid (MA) is a phenolic acid compound, found mainly in pear fruits (*Pyrus pyrifolia* N.), that is isoprenylated on the C-3 position of benzoic acid. Recently, the effects of prenylated phenolics on health have received much interest owing to their reported potent beneficial biological effects. We conducted a comparative study in rats to determine the metabolism, pharmacokinetics, and antioxidative activities of MA and its corresponding aglycone (MAA). MA and MAA were orally administered to rats (Sprague-Dawley, male, 6 weeks old) and their metabolites in plasma were analyzed. In addition, the MA metabolites in plasma were separated and the structures were confirmed via NMR and HR-MS analyses. The antioxidative activities of MA and MAA were evaluated by measuring their inhibitory effects on the 2,2′-azobis(2-amidinopropane)dihydrochloride- or copper ion-induced lipid peroxidation of rat plasma. MA was not absorbed in the intact form (the glucoside); both MA and MAA were absorbed as MAA and its metabolite form (glucuronide or sulfate). Moreover, the observed metabolite was the glucuronate of MAA rather than the glucuronide or sulfate. Concentrations of the free form of aglycone (MA administration, 4.6 ± 2.2 μM; MAA administration, 7.2 ± 2.3 μM) and total MAA (MA administration, 19.6 ± 4.4 μM; MAA administration, 21.7 ± 3.3 μM) in plasma reached a maximum at 15 min after the oral administration of MA and MAA, respectively. The relative inhibitory effects on the formation of cholesteryl ester hydroperoxides in plasma collected at 15 min after the oral administration of MA, MAA, and *p*-hydroxybenzoic acid (*p*-HBA) were as follows: MAA > MA ≥ *p*-HBA > control. Although the majority of MA and MAA is metabolized to conjugates, the compounds may contribute to the antioxidant defenses in the blood circulation owing to the presence of a phenolic hydroxyl group in the free form.

1. Introduction

Malaxinic acid [4-(*O*-β-D-glucopyranosyl)-3-(3′-methyl-2′-butenyl) benzoic acid (MA, Fig. 1)] is a glucosidic compound connected by an ether bond between β-glucose and 4-hydroxy-3-(3′-methyl-2′-butenyl) benzoic acid (MA aglycone, MAA), which is isoprenylated at the C-3 position of benzoic acid. MA has been isolated from *Prunus* species [1] and almond hull [2] and was identified in a previous investigation on the antioxidative active compounds present in pears (*Pyrus pyrifolia* N.) [3]. Asian pear fruits have an MA content of 1–6 mg/100 g fresh weight [3]; however, the corresponding aglycone (MAA) was not found in the pear fruits [4]. The isolation of MAA was recently reported from *Piper* species [5] and propolis [6]. Pharmacokinetic and metabolic studies on phenolic compounds including flavonoids and phenylpropanoids have been performed [7–11]. Studies on the absorption and metabolism of compounds present in food are very important because their structural differences affect bioavailability and bioactivity in vivo. MAA has been reported to exert various biological effects, such as HeLa cell cycle inhibition [12] and antifungal [13] and antibacterial [6] activities. However, the bioactivity of the glucoside form, MA, has not yet been studied. In addition, studies on the absorption and metabolism of MA and MAA have not been conducted.

As described above, MA and MAA contain an isoprenyl group as part of their structure. The isoprenyl group is a starting compound for the biosynthesis of terpenoids. In addition, isoprenyl group is bound to various phenolic compounds [14–18] including MA and MAA as an intermediate structure. The effects of prenylated phenolic compounds on human health have been thoroughly investigated in recent years because they have been reported to exert potent beneficial antioxidant, antibacterial, and estrogenic activities [15,19]. A study on the

Abbreviations: CE-OOH, cholesteryl ester hydroperoxide; ESI-MS, electrospray ionization mass spectrometry; MA, malaxinic acid; MAA, malaxinic acid aglycone; NMR, nuclear magnetic resonance; *p*-HBA, *p*-hydroxybenzoic acid; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride

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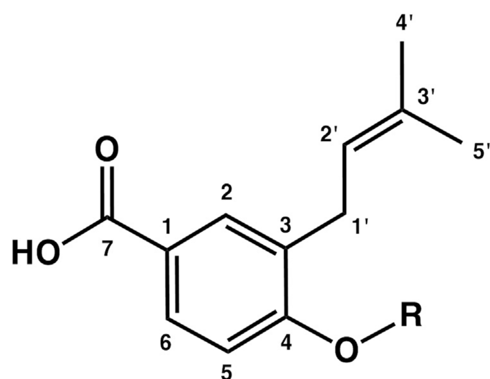

R: H (MAA)
R: β-D-glucose (MA)

Fig. 1. Structure of MA and MAA. MA, malaxinic acid; MAA, malaxinic acid aglycone.

pharmacokinetics of quercetin and 8-isoprenylquercetin in Caco-2 cells showed enhanced cellular uptake of the isoprenylated compound, which was attributed to an increase in lipophilicity; however, in the accompanying in vivo study in rats, the bioavailability of the isoprenylated compound diminished [14]. Therefore, the introduction of the isoprenyl group to phenolic compounds may alter their in vivo bioavailability and bioactivity.

Therefore, by studying the absorption and metabolism of MA and MAA we may be able to elucidate their biological applications, and those of other isoprenylated phenolic compounds in vivo. Standards preparations of MA and MAA are not commercially available and the supply of large-scale, high purity plant sources is also limited [1–6]. Therefore, in the present study, we chemically synthesized MA and MAA [20]. Our objective was to compare the metabolism, pharmacokinetics, and antioxidative activities of MA and MAA in rat plasma following a single administration of the compounds.

2. Materials and methods

2.1. General experimental procedures

Column chromatography was performed with silica gel resin (Kieselgel 60, 70–230 mesh; Merck, Darmstadt, Germany). Nuclear magnetic resonance (NMR) spectral data were recorded in CD_3OD (Acros Organics, New Jersey, USA) by Varian ^{unity}INOVA 500 and ^{unity}INOVA 600 spectrometers (Varian, Walnut Creek, CA, USA) using tetramethylsilane as an internal standard (Acros Organics, New Jersey, USA).

2.2. Chemicals

Ethyl-*p*-hydroxyl benzoate, 1-bromo-3-methyl-2-butene, acetobromo-α-D-glucose, propylene glycol, *p*-hydroxybenzoic acid (*p*-HBA), 2,6-di-*tert*-butyl-4-methyl-phenol (BHT), β-glucuronidase type H-1 (from *Helix pomatia*, EC 3.2.1.31), D-saccharic acid 1,4-lactone, and α-tocopherol (α-Toc) were purchased from Sigma Chemical Co. (St. Louis, USA). Copper (II) sulfate ($CuSO_4$) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals and solvents were HPLC grade.

2.3. Chemical synthesis of MAA and MA

The synthesis of MAA was conducted using the modified method described by Kim et al. [12]. First, ethyl 4-hydroxybenzoate was reacted under the conditions indicated in Scheme 1. Ethyl 4-hydroxybenzoate (exactly 7 g; 0.04 mol) and 1 g sodium (0.04 mol) were added to dry toluene (130 mL) and the mixture was stirred for 5 h at 120 °C. 1-Bromo-3-methyl-2-butene (7 mL) was added slowly to the mixture and then stirred successively for 15 h at room temperature and 30 min at 50 °C. After filtration, the organic layer was evaporated in vacuo at 35 °C. The concentrate was purified using silica gel (Kieselgel 60, Darmstadt, 2.5 × 85 cm, *n*-hexane/EtOAc = 2:0.5, v/v) column chromatography. The synthetic compound was identified as ethyl 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoate (EHMB) via ^{1}H NMR analysis. Subsequently, EHMB (600 mg) was added to 0.1 N NaOH (200 mL) and the mixture was stirred for 2 h at 70 °C. After cooling to room temperature, the solution (200 mL) was acidified to pH 3.0 with dilute hydrochloric acid solution and partitioned three times with ethyl acetate (EtOAc; 200 mL) to yield MAA. After concentration, the structure of the concentrated compound was confirmed using ^{1}H NMR spectroscopy and ESI-MS.

In addition, EHMB was dissolved in acetone (60 mL), mixed with acetobromo-α-D-glucose (4.38 g) and 1 N NaOH (10.65 mL), and stirred for 2 h at room temperature. Then, the solution was stirred again for 2 h at 70 °C and cooled to room temperature. The solution (70.65 mL) was acidified to pH 3.0 with diluted hydrochloric acid solution and partitioned three times with EtOAc (200 mL). The EtOAc layers were concentrated and purified using silica gel column chromatography (Kieselgel 60, Darmstadt, 2.5 × 91 cm, EtOAc/$CHCl_3$/EtOH = 1:1:0.5, EtOAc/$CHCl_3$/EtOH = 1:1:1, v/v) to yield MA. The compound structure was confirmed via ^{1}H NMR and ESI-MS analyses. Mass spectral data were obtained via ESI-MS (LCMS-8030, Shimadzu). The mass spectrometer was operated in negative ion mode with a dwell time of 0.1 s per transition. The optimal ion source parameters were as follows: Q1, 15.0 eV; Q3, 26.0 eV; collision energy, 15 eV; ESI source voltage, 3.5 kV; detector voltage, 45 V; heat block temperature, 400 °C; desolvation line temperature, 250 °C; nebulizing gas flow, 3.0 L/min, drying gas flow, 15.0 L/min; and collision gas (argon) pressure, 230 kPa.

2.4. Animals and administration of MA and MAA

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (no. CNU IACUC-YB-2013-54). Six-week-old Sprague-Dawley male rats weighing 180–200 g were supplied by Samtako Bio Korea (Osan, Korea). The animals were kept in an environmentally controlled animal facility with a 12 h dark/light cycle at 20 ± 1 °C and 55 ± 5% humidity with free access to distilled water and normal feed (Harlan Rodent diet, 2018S, Samtako Bio Korea). The rats were acclimatized to the environment for 1 day before the experiments commenced. Prior to compound administration, all rats were fasted for 15 h and deprived of water for 3 h. MA and MAA were dissolved in propylene glycol (1 mL) and then orally administered at 50 μmol/kg body weight (MA, 18.4 mg; MAA, 10.3 mg). Before (control) and 15, 30, 60, 120, 240, 480 min after administration, blood was withdrawn from the abdominal aorta of rats under light anesthesia with diethyl ether and placed into heparinized tubes. Plasma was obtained via centrifugation (5000 rpm, 10 min, 4 °C) and stored at –80 °C until use.

2.5. Determination of MA and MAA in rat plasma

MA and MAA were analyzed via HPLC after extraction from the blood plasma. Plasma (200 μL) was mixed with of 0.1 M pH 5.0 sodium acetate buffer (200 μL) and a 19:1 (v/v) mixture of methanol/acetic acid (500 μL). The mixture was vortexed for 30 s, sonicated for 30 s, and centrifuged (5000 rpm, 10 min, 4 °C). The supernatant was collected and the residue was mixed with a 19:1 (v/v) mixture of acetone/acetic acid (500 μL), vortexed, sonicated, and centrifuged as mentioned before. After centrifugation, the supernatants were collected, concentrated, and dissolved in 2% acetic acid aqueous solution (1 mL). The acidic aqueous solution was extracted three times with an equal volume

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