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Determination of the metabolic profile of gentianine after oral administration to rats by high performance liquid chromatography/electrospray ionization-trap mass spectrometry

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ABSTRACT

We investigated the metabolic fate of gentianine after oral administration to Wistar rats for the first time. Liquid chromatography/ion trap mass spectrometry detected four metabolites secogentianoxide, gentiandiol, gentianepoxide and gentianoxide in rat plasma together with the original compound gentianine. The structures of the metabolites were identified by comparing the retention times, as well as MS (mass) and MS/MS (tandem mass) spectra with those of authentic compounds, which were synthesized from gentianine or isolated from the urine. Three of the metabolites, secogentianoxide, gentianepoxide and gentianoxide, are novel compounds. The major *in vivo* metabolic processes associated with gentianine include *N*-oxide, epoxidation, dihydroxylation of double bond and hydrolysis of lactone. Gentianine together with the metabolites in plasma were quantified using gentianone as the internal standard. The mean C_{max} of G0, G1, G2 and G3 are 425.76, 287.56, 188.45 and 85.05 ng/mL, respectively. The mean T_{max} of G0, G1, G2 and G3 are 1.16, 3.87, 6.23 and 4.28 h, respectively. The mean $T_{1/2}$ of G0, G1, G2 and G3 are 5.23, 12.34, 7.78 and 5.64 h, respectively. A comprehensive metabolic pathway was proposed. The new metabolites may shed light on clinical efficacy of gentianine.

1. Introduction

Gentianine (G0, Fig. 1) is the major alkaloid of *Gentiana macrophylla* Radix which has been used for the treatment of inflammatory diseases such as osteoarthritis and rheumatoid arthritis in oriental medicine [1–4]. Up to now, pharmacological studies of G0 have revealed various biological activities including anti-ulcerogenic activity, anti-inflammatory activity and anti-analgesic activity [5–7].

While investigating the metabolism of the iridoid compound swertiamarin by human intestinal bacteria [8], we discovered that one of the metabolic intermediates of swertiamarin was further transformed by human intestinal bacteria to the biologically active metabolite G0. It was reported that the anti-diabetic effect of swertiamarin is due to G0 [9], and G0 exerts better depression of the central nervous system and anti-ulcerogenic action, as well as inhibitory action against gastric secretion than swertiamarin [10–14]. In our investigation of *in vivo* metabolism of swertiamarin after oral administration to rats, instead of G0, the further biotransformation product of G0 by liver, gentiandiol (G1, Fig. 1), was detected in plasma [3], which indicated that G0 can be metabolized rapidly *in vivo* and may exhibit its pharmacological effect through its metabolites. To further understand the mechanism of action of G0, it is important to characterize the metabolites of G0 *in vivo*. The present work aimed at study of the metabolic profile of G0 *in vivo* using high performance liquid chromatography/electrospray

Abbreviations: LC/MS, liquid chromatography–mass spectrometry; ESI, electrospray ionization; IS, internal standard; TIC, total ion current.

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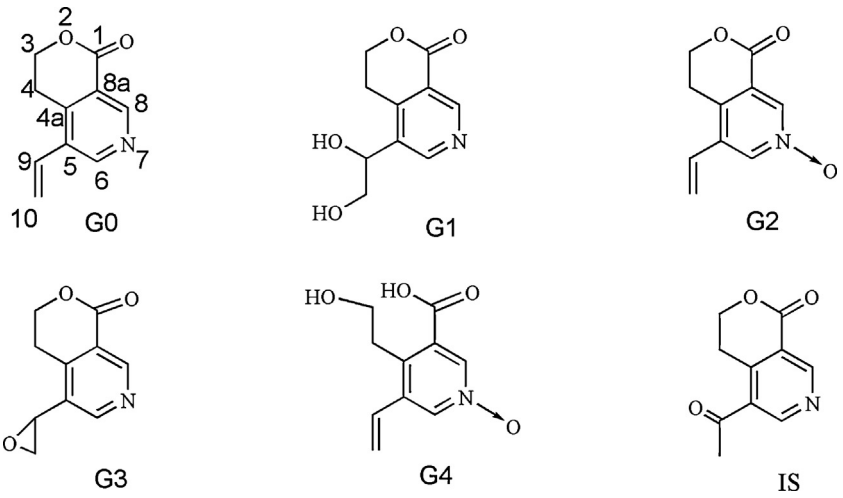

Fig. 1. Chemical structures of G0, G1, G2, G3, G4 and internal standard.

ionization-trap mass spectrometry and the principal metabolic pathway was also proposed.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and all other organic solvents of analytical HPLC (high performance liquid chromatography) grade were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Metachloroperbenzoic acid (m-CPBA) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Triethylamine and glucose were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Dichloromethane, 2-propanol, NH4Cl, β-NADH (Nicotinamide adenine dinucleotide), β-NADP (nicotinamide adenine dinucleotide phosphate), glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical (St. Louis, MO, USA). Male Wistar rat microsomes were purchased from Charles River Laboratories (Yokohama, Japan).

2.2. Instrumentation

^{1}H and ^{13}C NMR spectra were measured on a Varian UNITY 500 spectrometer. IR spectra were measured with a Jasco (Tokyo, Japan) FT/IR-230 infrared spectrometer. HR-EIMS were measured with a JMX-AX 505 HAD mass spectrometer (Jeol Co., Tokyo, Japan). Chromatograms and UV spectra were obtained using an Agilent 1100 series HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) comprising a binary pump, thermostated column compartment and diode array detector (DAD). Characteristic peaks were identified using an Esquire 3000 mass spectrometer system with electrospray ionization (ESI) source (Bruker Daltonik GmbH, Bremen, Germany). Samples were separated on a TSK gel ODS-80 Ts column (2.0 mm × 150 mm; i.d., 5 μm) with a flow rate of 0.5 mL/min at 30 °C in a stepwise gradient increasing from 10% (v/v) to 40% acetonitrile for 15 min, then to 100% acetonitrile for 5 min. Thereafter, the column was washed with 100% acetonitrile for 10 min and equilibrated with the starting eluant for 10 min The ESI-MS conditions were as follows: drying gas N_2, 10.0 L/min; temperature, 300 °C; nebulizer pressure, 50 psi; isolation width, 4; fragment amplification, 1.0; scan range, 50–1000 mass units.

2.3. Synthesis and isolation of metabolites and internal standard

2.3.1. Synthesis of G0 and G1

G0 was synthesized from swertiamarin according to the method of Kubota and Kamikawa [15] and the structure was identified by NMR and mass spectrum. G1 was synthesized as we have reported before [3].

2.3.2. Synthesis of N-oxide-3, 4-dihydro-5-vinylpyrano [3,4-c] pyridin-1-one (G2)

G0 (168 mg, 0.96 mmol) was dissolved in 25 mL dichloromethane, and m-CPBA (182 mg, 1.05 mmol) was added under stirring at 0 °C. The mixture was stirred for 3 h at 0–5 °C. The solvent was washed with 5% $NaHCO_3$ and saturated saline solution in turn. The organic fraction was removed under reduced pressure after dryness by anhydrous sodium sulfate, and then the crude product was subjected to a preparative TLC with $CHCl_3$–MeOH (15:1) to yield gentianoxide (G2, 32 mg, 0.168 mmol, 17.5% yield). Gentianoxide (G2): Colorless oil. ^{1}H NMR ($CDCl_3$) δ: 8.73 (1H, s, H-8), 8.42 (1H, s, H-6), 4.59 (2H, t, J = 6.0 Hz, H-3), 3.04 (2H, t, J = 6.0 Hz, H-4), 5.68 (1H, d, J = 11.2 Hz, H-10), 5.82 (1H, d, J = 17.2 Hz, H-10), 6.68 (1H, dd, J = 17.2 Hz, 11.2 Hz, H-9). ^{13}C NMR ($CDCl_3$) δ: 197.7 (C-9), 162.8 (C-1), 154.3 (C-8), 154.0 (C-6), 148.9 (C-4a), 129.7 (C-5), 122.4 (C-8a), 66.3 (C-3), 29.1 (C-10), 25.7 (C-4). HR-EI-MS m/z 191.0580 $[M]^+$ (Calcd for $C_{10}H_9NO_3$ 191.0582).

2.3.3. Synthesis of 5-acetyl-3, 4-dihydropyrano [3,4-c] pyridin-1-one (IS)

A mixture of G0 (618 mg, 3.51 mmol), [5,10,15,20-tetrakis(4-methoxyphenyl)-21*H*,23*H*-porphinato]cobalt(II) (500 mg, 0.63 mmol), and triethylsilane (418 mg, 3.60 mmol) in 200 mL of 2-propanol-dichloromethane (1:1) was stirred at 28 °C under oxygen at atmospheric pressure for 24 h. The solvent was removed under reduced pressure, and then the crude product was subjected to a silica gel column with $CHCl_3$-acetone (20:1) to yield gentianone (IS, 606 mg, 3.17 mmol, 90% yield). IS: Colorless needles. mp 166 °C. ^{1}H NMR ($CDCl_3$) δ: 9.38 (1H, s, H-8), 9.22 (1H, s, H-6), 4.53 (2H, t, J = 6.6 Hz, H-3), 3.47 (2H, t, J = 6.6 Hz, H-4), 2.71 (3H, s, H-10). ^{13}C NMR ($CDCl_3$) δ: 197.7 (C-9), 162.8 (C-1), 154.3 (C-8), 154.0 (C-6), 148.9 (C-4a), 129.7 (C-5), 122.4 (C-8a), 66.3 (C-3), 29.1 (C-10), 25.7 (C-4). HR-EI-MS m/z 191.0564 $[M]^+$ (Calcd for $C_{10}H_9NO_3$ 191.0582).