Drug Metabolism and Pharmacokinetics 30 (2015) 305–313

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics

Regular article

Role of cytochrome P450 and UDP-glucuronosyltransferases in metabolic pathway of homoegonol in human liver microsomes

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ARTICLE INFO

Article history:
Received 17 February 2015
Received in revised form
26 May 2015
Accepted 26 May 2015
Available online 5 June 2015

Keywords:
Homoegonol
4-*O*-demethylation
Glucuronidation
Human liver microsomes
Cytochrome P450s
UDP-Glucuronosyltransferases

ABSTRACT

Homoegonol is being evaluated for the development of a new antiasthmatic drug. Based on a pharmacokinetic study of homoegonol in rats, homoegonol is almost completely eliminated via metabolism, but no study on its metabolism has been reported in animals and humans. Incubation of homoegonol in human liver microsomes in the presence of the reduced form of nicotinamide adenine dinucleotide phosphate and UDP-glucuronic acid resulted in the formation of five metabolites: 4-*O*-demethylhomoegonol (M1), hydroxyhomoegonol (M2 and M3), 4-*O*-demethylhomoegonol glucuronide (M4), and homoegonol glucuronide (M5). We characterized the cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) enzymes responsible for homoegonol metabolism using human liver microsomes, and cDNA-expressed CYP and UGT enzymes. CYP1A2 played a more prominent role than CYP3A4 and CYP2D6 in the 4-*O*-demethylation of homoegonol to M1. CYP3A4 was responsible for the hydroxylation of homoegonol to M2. The hydroxylation of homoegonol to M3 was insufficient to characterize CYP enzymes. Glucuronidation of homoegonol to M5 was mediated by UGT1A1, UGT1A3, UGT1A4, and UGT2B7 enzymes, whereas M4 was formed from 4-*O*-demethylhomoegonol by UGT1A1, UGT1A8, UGT1A10, and UGT2B15 enzymes.

1. Introduction

Homoegonol, 3-(2-(3,4-dimethoxyphenyl)-7-methoxybenzofuran-5-yl)propan-1-ol, is a biologically active neolignan isolated from *Styrax* species such as *Styrax camporum*, *Styrax japonicus*, and *Styrax pohlii* [1–3], and it exhibits an antiasthmatic effect in an ovalbumin-induced murine asthma model [4], along with cytotoxic [2,5], antimicrobial [6,7], and anti-inflammatory [1,8] activities. Asthma is a complex chronic inflammatory airway disease with a substantial increase in incidence. New drug development for the treatment of asthma is urgently needed, and thus homoegonol is currently being evaluated for its development as an antiasthmatic drug.

The clearances of homoegonol remained unchanged over the dose range examined, i.e., the values were 130.3 ± 9.6, 112.7 ± 25.3, and 133.0 ± 36.9 mL/min/kg at 1, 2.5, and 5 mg/kg intravenous doses in rats, respectively [9]. The fraction of renal clearance in the overall homoegonol clearance was almost negligible; the percentage of homoegonol excreted in the urine during 24 h after dosing as an intact drug was 0.001–0.006% for 1, 2.5, and 5 mg/kg doses. These results indicate that homoegonol is almost completely metabolized. Although the metabolism of homoegonol is important in its pharmacodynamics and toxicity, no studies have reported the *in vitro* and *in vivo* metabolism of homoegonol in animals and humans. The metabolite identification and characterization of specific cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) enzymes responsible for the drug metabolism are necessary to reveal interindividual variations in drug metabolism and pharmacokinetics, along with potential drug interactions [10–15].

The purpose of the present study was to identify the metabolites of homoegonol formed from *in vitro* incubation of homoegonol with human liver microsomes using liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-QTOF) and to characterize the CYP and UGT enzymes responsible for homoegonol metabolism in human liver microsomes using LC–tandem mass spectrometry (LC-MS/MS).

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http://dx.doi.org/10.1016/j.dmpk.2015.05.005

2. Materials and methods

2.1. Materials

Homoegonol was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). 4-*O*-demethylhomoegonol, 3-*O*-demethylhomoegonol, and 7-*O*-demethylhomoegonol were kindly given by Professor Kyoung Lee at Dongguk University (Ilsan, Korea). NADPH, Trizma® base, Trizma® HCl, UDPGA, and alamethicin (from *Trichoderma viride*) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were acquired from Burdick and Jackson Inc. (Muskegon, MI, USA). Udenafil (used as an internal standard) was a gift from Dong-A Pharmaceutical Co. (Yongin, Korea). The other chemicals were of the highest quality available.

Pooled and individual human liver microsomes (coded H003, H056, HFC205, HFC208, HG32, HH18, HH581, HK37, HG43, HH47, HG64, and HG74), human cDNA-expressed CYP enzymes (CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) produced using a baculovirus–insect cell expression system, human cDNA-expressed UGT enzymes (UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17), and human selective antibodies for the immunoinhibition of human CYP1A2, CYP2D6, and CYP3A4 such as anti-CYP1A2 (catalog number, 458312; mouse monoclonal), anti-CYP2D6 (catalog number, 458366; mouse monoclonal), and anti-CYP3A4 (catalog number, 458334; mouse monoclonal) were purchased from Corning Life Sciences (Woburn, MA, USA).

2.2. Identification of homoegonol metabolites in human liver microsomes

Incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4), 250 mM magnesium chloride, pooled human liver microsomes (125 μg protein), 1 mM NADPH, 2 mM UDPGA, and 10 μM homoegonol were prepared in a total incubation volume of 250 μL. Control incubations were conducted in the absence of NADPH. The reaction mixtures were incubated for 60 min at 37 °C in a shaking water bath, and the reaction was terminated by the addition of 500 μL of methanol on ice. The reaction mixture was then centrifuged at 13,000 ×*g* for 4 min at 4 °C, and the supernatant was evaporated. The residue was dissolved in 100 μL of 50% methanol, and an aliquot (5 μL) was analyzed by LC-QTOF using the mass scan and product scan modes for identification of the metabolites.

2.3. Enzyme kinetics of homoegonol metabolism to 4-*O*-demethylhomoegonol (M1) and hydroxyhomoegonol (M2) in human liver microsomes

Preliminary experiments showed that the metabolism of homoegonol to 4-*O*-demethylhomoegonol (M1) and hydroxyhomoegonol (M2) was linear with respect to both incubation time over 30 min and liver microsomal protein concentration (0.1–0.3 mg/mL). Therefore, a 20-min incubation time and 0.2 mg/mL microsomal protein concentration were selected for subsequent experiments.

Incubation mixtures containing pooled human liver microsomes (20 μg protein), various concentrations of homoegonol (1, 2, 5, 10, 20, 40, and 60 μM; final acetonitrile concentration not exceeding 0.5%, v/v), 10 mM magnesium chloride, and 50 mM potassium phosphate buffer (pH 7.4) were preincubated for 3 min at 37 °C. The reaction was initiated by the addition of 1 mM NADPH, and the mixtures were further incubated (final volume of 100 μL) for 20 min at 37 °C in a shaking water bath. The reactions were terminated by adding 100 μL of udenafil in acetonitrile (internal standard, 10 ng/mL). The mixtures were vortexed and centrifuged at 13,000 ×*g* for 4 min at 4 °C. Then 50 μL of the supernatant was diluted with 50 μL of deionized water, vortexed for 2 min, and transferred to an injection vial; a 5-μL aliquot was injected into the LC-MS/MS system.

2.4. Metabolism of homoegonol in human cDNA-expressed CYP enzymes

Incubation mixtures containing 10 different human cDNA-expressed CYP enzymes (CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5; 4 pmol), homoegonol (2 or 20 μM), 10 mM magnesium chloride, and 50 mM potassium phosphate buffer (pH 7.4) were prewarmed for 3 min at 37 °C. The reaction was initiated by addition of an NADPH-generating system (1.3 mM $NADP^+$, 3.3 mM glucose 6-phosphate, and 0.5 U glucose 6-phosphate dehydrogenase), and the mixtures were further incubated (final volume of 100 μL) for 20 min at 37 °C in a shaking water bath.

For the enzyme kinetic study, the various concentrations of homoegonol (1, 2, 5, 10, 20, 40, and 60 μM; final acetonitrile concentration not exceeding 0.5%, v/v) were incubated with human cDNA-expressed CYP enzymes (CYPs 1A2, 2D6, and 3A4; 3 pmol), the NADPH-generating system, and 250 mM magnesium chloride in 50 mM potassium phosphate buffer (pH 7.4) for 20 min at 37 °C.

2.5. Correlation of homoegonol metabolism with probe substrate activities in human liver microsomes

The comparative metabolic rates of homoegonol in 12 different human liver microsomes were investigated by incubating 2 or 20 μM homoegonol with 0.2 mg/mL microsomal proteins and a NADPH-generating system for 20 min at 37 °C. The correlation between specific CYP activities in human liver microsomes (as reported by Corning Life Sciences) and the formation rates of 4-*O*-demethylhomoegonol (M1) and hydroxyhomoegonol (M2) were evaluated using the Pearson product–moment correlation calculation (SigmaStat Software version 2.0; Systat Software Inc., San Jose, CA, USA). A p value of less than 0.05 was considered to be significant.

2.6. Immunoinhibition of homoegonol metabolism with CYP antibodies in human liver microsomes

Immunoinhibition studies were performed by incubating pooled human liver microsomes with various amounts of human CYP selective antibodies such as anti-CYP1A2, anti-CYP2D6, and anti-CYP3A for 15 min on ice before the addition of 50 mM potassium phosphate buffer (pH 7.4), 2 μM homoegonol, 10 mM magnesium chloride, and the NADPH-generating system. As controls, comparable incubations were performed using microsomes and 25 mM Tris buffer instead of a selective antibody, which was prepared in this buffer.

2.7. Metabolism of homoegonol and 4-*O*-demethylhomoegonol in human cDNA-expressed UGT enzymes

To screen UGT enzymes involved in the glucuronidation of homoegonol and 4-*O*-demethylhomoegonol, reaction mixtures (final volume of 100 μL) containing 12 different human cDNA-expressed UGT enzymes (UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17; 0.1 mg protein/mL), 2 mM UDPGA, 0.25 mg/mL alamethicin, 50 mM Tris buffer (pH 7.4), and homoegonol or 4-*O*-demethylhomoegonol (5 or 20 μM) were incubated for 20 min at 37 °C in a shaking water bath. The reactions were terminated by adding 100 μL of udenafil in acetonitrile (internal