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An Unusual Metabolic Pathway of Sipoglitazar, a Novel Anti-Diabetic Agent: Cytochrome P450-Catalyzed Oxidation of Sipoglitazar Acyl Glucuronide

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Running title: Oxidation of Sipoglitazar Glucuronide *via* CYP2C8

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Abbreviations:

PPAR, peroxisome proliferator-activated receptor; CYP, cytochrome P450; UDPGA, uridine 5'-diphosphoglucuronic acid; UGT, uridine diphosphoglucuronosyltransferase; KPB, potassium phosphate buffer; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy.

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Abstract

Animal pharmacokinetic studies of sipoglitazar, a novel anti-diabetic agent, showed that the deethylated metabolite (M-I) and the glucuronide conjugate of sipoglitazar (sipoglitazar-G) appeared to be the key metabolites in elimination process. M-I was also measured as the main metabolite in the plasma of humans administered sipoglitazar. *In vitro* metabolic studies were carried out to investigate the metabolic pathways from sipoglitazar to M-I in humans. The metabolic profile with human hepatocytes and hepatic microsomes indicated that M-I was not formed directly from sipoglitazar and that sipoglitazar-G was involved in the metabolism from sipoglitazar to M-I. Further studies of the metabolism of sipoglitazar-G revealed that the properties of the glucuronide conjugate and its metabolism are as follows: HPLC, LC-MS/MS and NMR analyses showed that sipoglitazar-G was composed of two glucuronides; sipoglitazar-G1, a β-1-*O*-acyl and sipoglitazar-G2, an α-2-*O*-acyl glucuronide glucuronide. The stability study of these glucuronides suggested that sipoglitazar-G1 could be converted to sipoglitazar-G2 and sipoglitazar, but sipoglitazar-G2 could not be converted to sipoglitazar-G1. The oxidative metabolic study of sipoglitazar-G1 and -G2 with human hepatic microsomes and CYP-expressing microsomes revealed that M-I was formed only from sipoglitazar-G1, not from sipoglitazar-G2 and that CYP2C8 was mainly involved in this process. From these results, it is shown that the metabolic pathway from sipoglitazar to M-I is an unusual one, where sipoglitazar is initially metabolized to sipoglitazar-G1 by UGT and then sipoglitazar-G1 is metabolized to M-I by

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O-dealkylation by CYP2C8 and deconjugation. Sipoglitazar-G2 is sequentially formed by the migration of the β-site of sipoglitazar-G1.

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Introduction

Sipoglitazar, 3-[3-ethoxy-1-[[4-[(2-phenyl-1,3-thiazol-4-yl)methoxy]phenyl]methyl]pyrazol-4-yl]propanoic acid was attempted to be developed as a novel anti-diabetic with triple agonistic activities on the human peroxisome proliferator-activated receptors hPPAR-γ, hPPAR-α, and hPPAR-δ, although the development was terminated. Animal pharmacokinetic studies using ^{14}C-labeled sipoglitazar ([^{14}C]sipoglitazar) in rats and monkeys revealed that sipoglitazar was biotransformed to the deethylated metabolite (M-I), the hydroxyl metabolite (M-II), the glucuronide conjugate of sipoglitazar (sipoglitazar-G), the glucuronide of M-I (M-I-G) and other unidentified metabolites (unpublished observations). Among the metabolites, M-I and sipoglitazar-G were shown to be the key metabolites in the elimination process, because sipoglitazar was mainly excreted into the feces *via* biliary excretion as sipoglitazar-G in rats and M-I was detected as the main metabolite in the monkey plasma. Furthermore, metabolite M-I was also measured as the main metabolite in plasma of humans orally administered sipoglitazar.

In the present paper, to clarify the metabolic pathways from sipoglitazar to M-I in humans, *in vitro* metabolic studies using human hepatocytes and hepatic microsomes, identification of metabolites by LC-MS/MS and NMR and a stability study of the metabolites were carried out.

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Materials and Methods

Chemicals

Sipoglitazar and its metabolites (M-I, 3-(3-hydroxy-1-{4-[(2-phenyl-1,3-thiazol-4-yl)methoxy]benzyl}-1*H*-pyrazol-4-yl)propanoic acid and M-II, 3-[3-ethoxy-1-(4-hydroxybenzyl)-1*H*-pyrazol-4-yl]propanoic acid) were prepared by Takeda Pharmaceutical Company Limited (Osaka, Japan). [^{14}C]sipoglitazar with a specific radioactivity of 4.58 MBq/mg was synthesized by Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, UK). Glucose 6-phosphate and β-nicotinamide adenine dinucleotide (β-NADP$^+$) were purchased from Oriental Yeast Co., Ltd. (Osaka, Japan). Glucose-6-phosphate dehydrogenase, uridine 5'-diphospho-glucronic acid (UDPGA), gemfibrozil and β-glucuronidase (type H-1) were from Sigma Chemical Co. (St. Louis, MO), alamethicin from ICN Pharmaceuticals, Inc. (Costa Mesa, CA), and methanol, acetonitrile, and other reagents of analytical grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Other Materials

Cryopreserved primary human hepatocytes (Lot No. 69, from a 63-year-old female, Caucasian) were obtained from Celsis In Vitro Technologies (Baltimore, MD). Hepatic microsomes from humans (Lot No. 1299910008) were obtained from Tissue Transformation

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Technologies (Edison, NJ). Hepatic microsomes from dogs (Lot No. 9910064) were obtained from Xenotech, LLC (Lenexa, KS). Rat bile was collected from Crj:CD(SD)IGS rats, which was obtained from Charles River Japan Inc. (Yokohama, Japan). Human plasma (Lot No. MT116523) was purchased from Kojin Bio (Saitama, Japan).

Preparation of [^{14}C]sipoglitazar-G, [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2

In the course of this study, the glucuronide of sipoglitazar, sipoglitazar-G was revealed to be composed of two glucuronides, which were defined as sipoglitazar-G1 and sipoglitazar-G2.

For the *in vitro* metabolic study, [^{14}C]sipoglitazar-G, [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 were isolated from the glucuronidation reactant obtained by the incubation of [^{14}C]sipoglitazar (100 μmol/L) with 2 mg protein/mL of human hepatic microsomes at 37°C for 4 hours. After the termination of the reaction and centrifugation, the supernatant was concentrated under a nitrogen gas stream and applied into an HPLC. To isolate these glucuronides, the column temperature for HPLC analysis was set at 40°C for [^{14}C]sipoglitazar-G (the mixture of [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2) and the column temperature for HPLC analysis was set at 4°C for the isolation of [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2, respectively. The HPLC analytical conditions are described in "Analytical Procedures for [^{14}C]sipoglitazar and its Metabolites". Each eluate was dried under a nitrogen gas stream and resolved in 50% or 70% acetonitrile.

For the structural analysis, [^{14}C]sipoglitazar-G1 was prepared from the glucuronidation

reactant with 2 mg protein/mL of human hepatic microsomes or 1 mg protein/mL of dog hepatic microsomes, and [^{14}C]sipoglitazar-G2 was prepared from the bile of rats after single intraduodenal administration of [^{14}C]sipoglitazar (0.5 mg/kg). The HPLC analytical condition is described in "Analytical Procedures for [^{14}C]sipoglitazar and its Metabolites". The column temperature was set at 40°C for the structural analysis.

Incubation of [^{14}C]sipoglitazar with Human Hepatocytes

Cryopreserved hepatocytes were suspended using published procedures with minor modifications (Loretz et al., 1989; Li et al., 1999). Hepatocytes, suspended in 0.5 mL of DMEM were dispensed on 24-well culture plates at a density of 3 x 10^5 viable cells/well with 10 μmol/L of [^{14}C]sipoglitazar and incubated at 37°C for 6 hours.

Structural Analysis of [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 by LC-MS/MS and NMR

LC-MS and LC-MS/MS analyses were performed using an ion trap mass spectrometer (LCQ and TSQ-7000, Thermo Finnigan, CA, USA) equipped with an electrospray ionization (ESI) interface system. The spectra were obtained in the positive ion mode. The interface and mass spectrometer were operated under the following conditions: for LCQ (target analytes; sipoglitazar, M-I and M-II), heated capillary temperature; 200°C, spray voltage; 4.5 kV, sheath gas and auxiliary gas flow rate (arbitrary units); 80 and 15, respectively, and capillary

voltage; 3.0 V. The collisionally induced dissociation (CID) fragment ions were generated using helium as a target gas, and the CID energy was 40%. For TSQ-7000 (target analytes: sipoglitazar-G1 and -G2), heated capillary temperature; 230°C, spray voltage; 4.5 kV, and sheath gas pressure; 483 kPa. The CID fragment ions were generated within quadrupole 2, using argon as a target gas, and the CID energy (quadrupole 2 offset voltage) and the target gas pressure were -25 eV and 0.2 Pa, respectively.

^{1}H-NMR spectrometry was carried out using a Varian Gemini-300 (Varian Instruments Ltd., Palo Alto, CA). The conditions for measurement were as follows: spectral width, 4500.5 Hz; acquisition time, 3.498 s; measurement solvent, deuterated acetonitrile and deuterium oxide (7:3, by vol.); temperature, ambient. Chemical shifts (ppm) were referenced to the residual solvent peak at 1.95 ppm. Signals of the ^{1}H-NMR spectrum were assigned by the observed coupling patterns.

Stability of [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2

[^{14}C]sipoglitazar-G1 or [^{14}C]sipoglitazar-G2 (final concentration: 10 μmol/L) was incubated at 37°C in 50 mmol/L potassium phosphate buffer (KPB) (pH 7.4), acetate buffer (pH 4.4), rat bile and human plasma. An aliquot was withdrawn from the reaction mixture after incubation for 0, 5, and 30 minutes and 1, 2, and 4 hours for sipoglitazar-G1 and after incubation for 0, 1, 2, and 4 hours for sipoglitazar-G2.

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Oxidative Metabolism of [^{14}C]sipoglitazar, [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 by Human Hepatic Microsomes

An acetonitrile solution of 1 mmol/L [^{14}C]sipoglitazar, [^{14}C]sipoglitazar-G1 or [^{14}C]sipoglitazar-G2 was added to the incubation mixture, consisting of 1 mg protein/mL human hepatic microsomal protein, 5 mmol/L $MgCl_2$, and an NADPH-generating system with a final concentration of 5 mmol/L glucose-6-phosphate, 0.5 mmol/L β-$NADP^+$ and 1.5 unit/mL glucose-6-phosphate dehydrogenase in 50 mmol/L KPB with a final volume of 0.25 mL. The incubation was conducted at 37°C for 1 hour ([^{14}C]sipoglitazar) or 10 minutes ([^{14}C]sipoglitazar-G1 or [^{14}C]sipoglitazar-G2). The final concentration of the substrate was 10 µmol/L.

Glucuronidation of [^{14}C]sipoglitazar by Human Hepatic Microsomes

The acetonitrile solution of 1 mmol/L [^{14}C]sipoglitazar was added to the incubation mixture, consisting of 1 mg protein/mL human hepatic microsomal protein, 5 mmol/L $MgCl_2$, 50 µg/mL alamethicin, and 5 mmol/L of UDPGA in 50 mmol/L KPB (pH 7.4) with a final volume of 0.25 mL. The incubation was conducted at 37°C for 1 hour. The final concentration of [^{14}C]sipoglitazar was 10 µmol/L.

Identification of the CYP Isoform Involved in the Metabolism from Sipoglitazar-G1 to M-I

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To identify the CYP isoform involved in the metabolism from sipoglitazar-G1 to M-I, a CYP metabolic study using 80 pmol P450/mL CYP-expressing microsomes and a correlation study using 0.5 mg protein/mL hepatic microsomes from 16 humans provided as the Reaction Phenotyping Kit (Version 5.0) were performed. The correlation analysis was carried out with SAS System (SAS Institute). The biochemical activity data for the specific CYP enzymes provided with the kit were used for the correlation analysis. In the CYP metabolic study, [^{14}C]sipoglitazar-G was used and in the correlation study, [^{14}C]sipoglitazar-G1 was used as the substrate. An acetonitrile solution of 0.5 mmol/L [^{14}C]sipoglitazar-G or 1 mmol/L [^{14}C]sipoglitazar-G1 was added to the incubation mixture, consisting of the respective microsomal protein, 5 mmol/L $MgCl_2$, and an NADPH-generating system as described above in 50 mmol/L KPB (pH 7.4) or Tris-HCl buffer (pH7.4) with a final volume of 0.25 mL. The incubation was conducted at 37°C for 15 minutes (the CYP metabolic study) or 10 minutes (the correlation study). The final concentration of the substrate was 10 μmol/L in the both studies.

Incubation of [^{14}C]sipoglitazar and Gemfibrozil with Human Hepatic Microsomes in the Presence of UDPGA with/without an NADPH-generating System

The acetonitrile solution of 1 mmol/L [^{14}C]sipoglitazar was added to the incubation mixture, consisting of 0.5 mg protein/mL human hepatic microsomal protein, 5 mmol/L $MgCl_2$, 50 μg/mL alamethicin, 5 mmol/L of UDPGA with/without an NADPH-generating system and

gemfibrozil in 50 mmol/L KPB (pH 7.4) with a final volume of 0.25 mL. Gemfibrozil was prepared to solve in acetonitrile and its final concentration was 0, 10, 30, 100, or 300 μmol/L. The incubation was conducted at 37°C for 30 minutes. The final concentration of [^{14}C]sipoglitazar was 10 μmol/L.

Analytical Procedures for [^{14}C]sipoglitazar and its Metabolites

The incubation procedures described above were terminated by the addition of acetonitrile at a volume equivalent to the volume of the reaction mixture and centrifuging at approximately 1500x*g* for 10 minutes. The supernatant was analyzed by an HPLC (LC-10 gradient system; Shimadzu Corp., Kyoto, Japan) with an on-line RI detector (D505TR Flow Scintillation Analyzer, Packard Instrument Company, Meriden, CT). The typical HPLC analytical method was as follows: separation of the metabolites was carried out on an Inertsil ODS-3 (5-μm particle size, 150 or 250 x 4.6 mm I.D.; GL Sciences, Tokyo, Japan) at 40°C or 4°C, following gradient elution using acetonitrile, H_2O and trifluoroacetic acid (TFA). The acetonitrile concentration was increased from 34% to 66% over a period of 40 minutes and held at 86% for 10 minutes. Throughout the elution, the flow of 0.1% of TFA was continued and the flow rate was 0.5 or 1 mL/minutes. On-line RI detection was carried out using Ultima-Flo as the flow scintillation cocktail.

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Results

Metabolic Profiles of [^{14}C]sipoglitazar with Hepatocytes and Hepatic Microsomes from Humans

The metabolic profile of [^{14}C]sipoglitazar was examined using hepatocytes and hepatic microsomes from humans (Figure 1). In the reactions with hepatocytes, the deethylated metabolite (M-I) and the glucuronide conjugate of sipoglitazar (sipoglitazar-G) were the main metabolites and the hydrolyzed metabolite (M-II) was also detected as a minor metabolite. To separate the reactions of oxidation and glucuronidation, metabolic studies using hepatic microsomes with/without their cofactors, NADPH-generating system and UDPGA were conducted. When both an NADPH-generating system and UDPGA were added, the metabolites were detected with similar profiles in the reactions with hepatocytes. Addition of only an NADPH-generating system to the reaction mixture resulted in the formation of M-II, and a limited amount of M-I. When only UDPGA was added to the microsomal reaction mixture, sipoglitazar-G was formed extensively.

Preparation of Sipoglitazar-G1 and Sipoglitazar-G2

In the course of metabolic analysis of sipoglitazar-G formed by hepatic microsomes, sipoglitazar-G in the reaction mixture was revealed to consist of two isomers. In the HPLC analysis with the column set at 40°C, sipoglitazar-G was detected as one peak (Figure 2A),

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but two separate peaks originating from sipoglitazar-G were detected, when the temperature of the column for HPLC analysis was changed to 4°C and these were named sipoglitazar-G1 and sipoglitazar-G2 according to the elution order (Figure 2B).

LC-MS/MS Analysis of Sipoglitazar-G1 and Sipoglitazar-G2

To elucidate the properties of the two types of sipoglitazar glucuronides, [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 were each analyzed by LC-MS/MS. The full ion mass spectrum of sipoglitazar-G1 gave the $[M+H]^+$ at m/z 642 (Figure 3A), which was 176 amu higher than that of the parent compound. The characteristic fragment ions in the product ion mass spectrum at m/z 642 were detected at m/z 466, 282 and 174 (Figure 3B). The ion at m/z 466 was derived by diagnostic loss of the sugar moiety (-176 amu). The ions at m/z 282 and 174 were the same as those of unchanged sipoglitazar. The full ion mass spectrum of sipoglitazar-G2 gave the $[M+H]^+$ at m/z 642 (Figure 4A), which was 176 amu higher than that of the parent compound. The characteristic fragment ions in the product ion mass spectrum at m/z 642 were detected at m/z 624, 282 and 174 (Figure 4B). The ion at m/z 624 was a dehydrated ion. The ions at m/z 282 and 174 were the same as those of the parent compound. Alkaline hydrolysis (0.1 mol/L sodium hydroxide solution, at 37°C for 2 hours) of the peaks at m/z 642 of sipoglitazar-G1 and -G2 gave sipoglitazar in the LC-MS, while enzymatic hydrolysis [5 w/v% of sulfatase containing β-glucuronidase (type H-1), at 37°C for 2 hours] of the peaks at m/z 642 of sipoglitazar-G1 gave sipoglitazar in the LC-MS, but that

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of sipoglitazar-G2 could not show the cleave (data not shown). From these results, sipoglitazar-G1 and sipoglitazar-G2 are shown to be isomers with respect to the site of glucuronidation.

NMR Analysis of Sipoglitazar-G1 and Sipoglitazar-G2

To determine the site of glucuronidation of sipoglitazar-G1 and sipoglitazar-G2, an ^{1}H-NMR spectrum analysis was performed (Table 1). In the ^{1}H-NMR spectrum of sipoglitazar-G1, proton signals assigned to H-10 and H-11 shifted downfield (δ 2.48 to 2.54-2.62 ppm) compared with those of sipoglitazar. This metabolite showed novel signals indicative of a glucuronic acid moiety (five protons, δ 3.38-5.45 ppm) and the split of the anomeric proton signal (δ 5.45 ppm, d, J=7.7 Hz) showed an β-glucuronide. The ^{1}H-NMR spectrum of sipoglitazar-G2 was similar to that of sipoglitazar-G1 except for a glucuronic acid moiety, namely, (a) the anomeric proton signal shifted downfield (δ 5.45 to 5.20 ppm) and the coupling constants were 3.6 Hz, (b) proton signal assigned to H-2' shifted upfield (δ 3.38 to 4.57 ppm). From these results, sipoglitazar-G1 was estimated to be the β-1-*O*-acylglucuronide of sipoglitazar and sipoglitazar-G2 was the α-2-*O*-acylglucuronide.

Stability and Interconversion of Sipoglitazar-G1 and Sipoglitazar-G2

To investigate the stability and interconversion between [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2, these compounds were incubated in KPB (pH 7.4), acetate buffer (pH

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4.4), rat bile and human plasma (Figure 5). In acetate buffer (pH 4.4), sipoglitazar-G1 and sipoglitazar-G2 were stable and neither degradation nor interconversion was observed. In both KPB (pH 7.4) and rat bile, similar composition change was shown and sipoglitazar-G1 was gradually eliminated to form sipoglitazar-G2 and sipoglitazar. Sipoglitazar-G2 was slightly eliminated to form sipoglitazar, but conversion from sipoglitazar-G2 to -G1 was not observed. More significant deglucuronidation and sipoglitazar-G1-to-G2 conversion were also observed in rat bile. In human plasma, sipoglitazar-G1 was converted to sipoglitazar-G2 immediately after the mixing with human plasma and thereafter both sipoglitazar-G1 and sipoglitazar-G2 were gradually eliminated to form sipoglitazar. Sipoglitazar-G2 was gradually eliminated to form sipoglitazar, but conversion from sipoglitazar-G2 to -G1 was not observed. These results indicate that sipoglitazar-G1 could be converted to sipoglitazar-G2 and then to sipoglitazar from both sipoglitazar-G1 and -G2.

Oxidative Metabolisms of Sipoglitazar-G1 and Sipoglitazar-G2

[^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 were metabolized by human hepatic microsomes with or without an NADPH-generating system (Figure 6A-D). Sipoglitazar was formed from both [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 regardless of the presence of an NADPH-generating system. M-I was formed only when [^{14}C]sipoglitazar-G1 was metabolized with an NADPH-generating system (Figure 6A). In this study, HPLC analysis was performed under conditions where sipoglitazar-G1 and

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sipoglitazar-G2 could not detected separately, therefore the interconversion between sipoglitazar-G1 and sipoglitazar-G2 could not be evaluated.

Identification of the CYP Isoform Involved in the Metabolism of [^{14}C]sipoglitazar-G1

The CYP isoform involved in the formation of M-I from sipoglitazar-G1 was identified by a metabolic study using microsomes expressing human CYP isoforms and a correlation study using hepatic microsomes from 16 individual humans (Figure 7 and Table 2). CYP2C8-expressing microsomes showed extensive activity to form M-I (Figure 7). In the correlation study, the elimination rate of [^{14}C]sipoglitazar-G1 and the formation rate of M-I correlated most strongly with paclitaxel 6α-hydroxylase activity (CYP2C8) (Table 2). Sipoglitazar-G1 elimination and M-I formation were also correlated with the *S*-mephenytoin *N*-demethylation activity (CYP2B6) and chlorzoxazone 6-hydroxylation activity (CYP2E1). However, sipoglitazar-G1 was hardly metabolized by CYP2B6 and CYP2E1 enzymes (Figure 7). From these results, CYP2C8 is considered to be the main CYP isoform involved in the formation of M-I from sipoglitazar-G1.

The Effect of Gemfibrozil on the *in vitro* Oxidative Metabolism and/or Glucuronidation of [^{14}C]sipoglitazar

In the presence of both an NADPH generating system and UDPGA, sipoglitazar was metabolized to M-I, M-II, sipoglitazar-G1, sipoglitazar-G2, and unidentified metabolites.

Elimination of sipoglitazar and formation of M-I, and M-II were inhibited by gemfibrozil, and the relative activities at 300 μmol/L of gemfibrozil were 58.7%, 9.1%, and 13.4%, respectively (Figure 8). However, sipoglitazar-G1 formation was increased rather than inhibited and the relative activities at 10, 30, 100, and 300 μmol/L of gemfibrozil were 103.5%, 110.3%, 130.3%, and 125.9%, respectively.

In the presence of UDPGA without an NADPH generating system, sipoglitazar was mainly metabolized to sipoglitazar-G1. Elimination of sipoglitazar and formation of sipoglitazar-G1 were inhibited by gemfibrozil and the relative activities at 300 μmol/L of gemfibrozil were 66.8% and 65.7%, respectively (Figure 8).

Discussion

Generally, drug metabolism reactions are divided into the reactions of such functional groups as oxidation, reduction and hydrolysis (phase I) and conjugative reactions such as glucuronidation and sulfation (phase II). In *in vivo* animal ADME studies and *in vitro* studies using hepatocytes, the deethylated metabolite of sipoglitazar (M-I), hydrolyzed metabolite of sipoglitazar (M-II), and glucuronide conjugate of sipoglitazar (sipoglitazar-G) were observed and M-I was also measured as the main metabolite in the plasma of humans orally administered sipoglitazar (unpublished observations). Therefore, sipoglitazar M-I and M-II were considered to be formed by phase I oxidative enzymes such as CYP and sipoglitazar-G was formed by UGT, a phase II metabolic enzyme. To separate the reactions of oxidation and glucuronidation, an *in vitro* metabolic study was conducted using human hepatic microsomes with and without an NADPH-generating system and UDPGA (Yan et al., 2003) (Figure 1), because an NADPH-generating system and UDPGA are essential cofactors for CYP and UGT, respectively. When both the cofactors were added to the *in vitro* reaction mixture, M-I, M-II and sipoglitazar-G were formed from sipoglitazar. However, in oxidative conditions, formation of M-II was observed and M-I could be hardly detected, while only sipoglitazar-G was formed in the glucuronidation condition. These results indicated that M-I formation requires both CYP and UGT, and also suggested that sipoglitazar-G could be an intermediate in the formation of M-I from sipoglitazar.

To examine the metabolism of sipoglitazar-G in detail, isolation of sipoglitazar-G from an *in*

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vitro sample was performed. Interestingly, under an appropriate HPLC condition, sipoglitazar-G prepared by hepatic microsomes was revealed to consist of two isomers (Figure 2). The main isomer observed in the *in vitro* reactant, was named sipoglitazar-G1. The other sipoglitazar-G isomer with the longer retention time, named sipoglitazar-G2, indicated the same retention time in the HPLC and LC-MS/MS spectrum as the sipoglitazar-G detected in the bile of [^{14}C]sipoglitazar-administered rats. Further analysis of sipoglitazar-G1 and sipoglitazar-G2 by LC-MS/MS and ^{1}H-NMR revealed that sipoglitazar-G1 was a β-1-*O*-acyl glucuronide and sipoglitazar-G2 was an α-2-*O*-acyl glucuronide (Table 1, Figures 3 and 4). These results showed that the sipoglitazar-G1 has been detected only in *in vitro* studies, while the sipoglitazar-G detected in the *in vivo* studies was sipoglitazar-G2.

To clarify the reason why only sipoglitazar-G2 was detected in *in vivo* studies, the stability and interconversion of the sipoglitazar glucuronides were examined. The different properties of these isomers between sipoglitazar-G1 and sipoglitazar-G2 were caused by irreversible internal migration of the glucuronides (Figure 5). In the neutral condition, sipoglitazar-G1 was converted to sipoglitazar-G2, while sipoglitazar-G1 was not formed from sipoglitazar-G2. Moreover, sipoglitazar was formed by deconjugation from both sipoglitazar-G1 and sipoglitazar-G2. These conversions were significantly observed in rat bile and human plasma. From these results, it is believed that sipoglitazar-G1 is rapidly converted to sipoglitazar-G2 and sipoglitazar and is biotransformed to M-I simultaneously in

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in vivo conditions. Therefore, it is considered that of the two isomers, only sipoglitazar-G2 was detected *in vivo*. Such conversion is generally known with acyl glucuronides where β-1-*O*-acyl glucuronide is irreversibly converted to 2-*O*-isomer (Skordi et al., 2005). The rates of acyl migration depend on the temperature and pH, with such compounds being more stable in cold acidic conditions (Dickinson et al., 1984; Smith et al., 1985; Hyneck et al., 1988), and also depending on the solvent (Smith et al., 1985; Mortensen et al., 2002) and the nature of the aglycon (Nicholls et al., 1996; Vanderhoeven et al., 2004). These properties of the acylglucuronides would support the clarification of the metabolic pathway of sipoglitazar.

To elucidate the metabolic pathways of sipoglitazar, [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 were metabolized by human hepatic microsomes with and without an NADPH-generating system (Figure 6). In either condition, deglucuronidation of both sipoglitazar-G1 and sipoglitazar-G2 were observed. In contrast, formation of M-I was observed only from sipoglitazar-G1 and the reaction was dependent on the presence of NADPH, while M-I was not formed from sipoglitazar-G2. These results indicate that CYP may be involved in the formation of M-I from sipoglitazar-G1. Further investigations to identify the CYP isoform revealed that CYP2C8 was involved in the oxidation of sipoglitazar-G1 (Table 2 and Figure 7). In total, it is considered that M-I formation is *via* the oxidation of sipoglitazar-G1 by CYP2C8 and it was clarified that the glucuronide is a substrate for oxidative metabolism by CYP.

This unusual metabolic pathway has been already reported that glucuronides of diclofenac,

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β-estradiol and MRL-C could be a substrate for oxidative metabolism by CYP (Kumar et al., 2002; Delaforge et al., 2005; Kochansky et al., 2005). CYP2C8 is also involved in the oxidation of these compounds. These compounds are supposed to have easier access to the active site of CYP2C8 by the polarization of the compound by glucuronidation. This finding is a common feature with CYP2C8 substrates, revealed from the pharmacophore analysis of CYP2C8; CYP2C8 substrates have a long hydrophobic chain with an acidic or polar group and a hydrophobic or aromatic moiety and another structural feature of CYP2C8 likely causing this is an active site, Arg (Schoch et al., 2004; Melet et al., 2004; Johnson et al., 2005). Although sipoglitazar also has a similar structural property, there was a big difference between sipoglitazar and these compounds. Interestingly, M-I was formed only when sipoglitazar was metabolized *via* glucuronidation, while the position subject to oxidation in the glucuronides of diclofenac and MRL-C could be also oxidized by other CYPs and their glucuronidation was not always essential to form their oxidative metabolites. This difference suggests that the metabolism of sipoglitazar-G1 is more CYP2C8-selective than that of the other compounds, and makes the metabolic pathway of sipoglitazar very unique. Furthermore, gemfibrozil-1-*O*-β-glucuronide is well known as CYP2C8 inhibitor and could cause drug-drug interactions at the level of transporters (Ogilvie et al., 2006; Baer et al., 2009; Shitara et al., 2004). Sipoglitazar also has a similar property with gemfibrozil in terms of the structure and the involvement of CYP2C8 in its metabolic pathway. The effects of gemfibrozil on the *in vitro* oxidative metabolism and/or glucuronidation of

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sipoglitazar were investigated based on the metabolite profile of sipoglitazar (Figure 8). In this study, gemfibrozil could inhibit the glucuronidation of sipoglitazar concentration-dependently in the presence of UDPGA and M-I formation in the presence of an NADPH-generating system and UDPGA, resulting sipoglitazar-G1 accumulation. These results indicated the oxidation is affected more extensively than its glucuronidation by gemfibrozil, which might be due to the gemfibrozil and gemfibrozil-1-*O*-β-glucuronide as an inhibitor of CYP2C8, and support that the formation of M-I from sipoglitazar-G1 depended on CYP2C8. Therefore, sipoglitazar has a possibility to increase the plasma level by the inhibition of the oxidation metabolism when gemfibrozil is coadministrated.

From the results of this study, the metabolic pathway of sipoglitazar is postulated as follows (Figure 9). Sipoglitazar is metabolized *via* glucuronidation by UGT to give sipoglitazar-G1 as a first step, and sipoglitazar-G1 is deethylated by CYP2C8 to form M-I. Part of sipoglitazar-G1 is metabolized *via* deglucuronidation to sipoglitazar or is subsequently converted by acyl migration to sipoglitazar-G2. M-II was formed directly from sipoglitazar. Considering this metabolic pathway of sipoglitazar in detail, the deethylated sipoglitazar-G1, namely the acyl glucuronide of M-I, is expected to exist as the intermediate between sipoglitazar-G1 and M-I. Moreover, in the deglucuronidation of acyl glucuronide, an interesting metabolic pathway was reported for statins, namely hydroxymethylglutaryl-CoA reductase inhibitors (Prueksaritanont et al., 2001 and 2002). Statin acyl glucuronide could be lactonized spontaneously and metabolized *via* deglucuronidation to the formed statin

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lactone when hydrolyzed to the open acid chemically or enzymatically. When taking the structural similarity into consideration, the deethylated sipoglitazar-G1 has the possibility to be metabolized to M-I through the lactone or to be induced chemically, because the structure of deethylated sipoglitazar-G2 could be superimposed on the structure of statin acyl glucuronide (Figure 10). Unfortunately, a lactone-form intermediate could not be detected in the *in vitro* [^{14}C]sipoglitazar-G2 reactant. Too rapid hydrolysis of the lactone-form intermediate might be one of the reasons for this. Further investigation for M-I formation is also necessary.

In conclusion, the present study demonstrates the unusual deethylation pathway of sipoglitazar which is metabolized *via* glucuronidation by UGT, a phase II metabolic enzyme, as a first step to form an unstable β-1-*O*-acyl glucuronide, and this acyl glucuronide is deethylated by CYP2C8, a phase I metabolic enzyme. The deethylated sipoglitazar acyl glucuronides are immediately metabolized *via* deglucuronidation spontaneously or enzymatically to form M-I, deethylated sipoglitazar.

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Authorship Contributions

Participated in research design: Nishihara, Sudo, Kawaguchi, Takahashi, Kiyota, Kondo, and Asahi

Conducted experiments: Nishihara, Sudo, and Kiyota

Performed data analysis: Nishihara, Sudo, Kawaguchi, Takahashi, and Kiyota

Wrote or contributed to the writing of the manuscript: Nishihara, Kawaguchi, Takahashi, Kondo, and Asahi

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Footnotes

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Legends for Figures

Figure 1. Composition of metabolites of [^{14}C]sipoglitazar formed by hepatocytes and hepatic microsomes from human.

The composition ratio represents the ratio of sipoglitazar and its metabolites, when the total radioactivity in the incubation mixture is regarded as 100%. The composition ratio of others was calculated by subtracting the sum of sipoglitazar and its metabolites from 100%. Data represent the means of duplicate determinations.

Figure 2. Representative HPLC-radiochromatograms of the incubation mixture of sipoglitazar with human hepatic microsomes in the presence of UDPGA.

This HPLC-radiochromatogram is for the preparation of [^{14}C]sipoglitazar-G (A) and for that of [^{14}C]sipoglitazar-G1 and [^{14}C]sipoglitazar-G2 (B). [^{14}C]sipoglitazar (100 μmol/L) was incubated with human hepatic microsomes (2 mg protein/mL) in the presence of UDPGA (5 mmol/L) at 37°C for 4 hours. Separation of the metabolites was carried out on an Inertsil ODS-3 (5-μm particle size, (A) 150 x 4.6 mm I.D., (B) 250 x 10 mm I.D.; GL Sciences, Tokyo, Japan) at (A) 40°C and (B) 4°C, following gradient elution using acetonitrile, H_2O and trifluoroacetic acid (TFA). The acetonitrile concentrations were as follows: (A) it was increased from 34% to 66% over a period of 40 minutes and held at 86% for 10 minutes. (B) it was held at 45.2% for 30 minutes, increased from 45.2% to 90% over a period of 10

minutes and held at 90% for 10 minutes. Throughout the elution, the flow of 0.1% TFA was continued and the flow rate was (A) 1.0 mL/minutes and (B) 2.4 mL/minutes.

Figure 3. Full ion mass spectrum of sipoglitazar-G1 (A), and its product ion mass spectrum of the precursor ion at m/z 642 (B).

Figure 4. Full ion mass spectrum of sipoglitazar-G2 (A), and its product ion mass spectrum of the precursor ion at m/z 642 (B).

Figure 5. Stability and interconversion of sipoglitazar-G1 and sipoglitazar-G2 in KPB (pH 7.4), acetate buffer (pH 4.4), rat bile and human plasma.

Each ratio of radioactivity of sipoglitazar (●), sipoglitazar-G1 (□) and sipoglitazar-G2 (▲) was expressed, while the total radioactivity in the incubation mixture is regarded as 100%.

Figure 6. Representative HPLC-radiochromatograms of the incubation mixture of sipoglitazar-G1 or sipoglitazar-G2 by human hepatic microsomes with or without an NADPH-generating system.

Figure 7. Formation of M-I from [^{14}C]sipoglitazar-G by specific human CYP-expressing microsomes.

Data represent the means of duplicate determinations.

Figure 8. The Effect of gemfibrozil on the *in vitro* oxidative metabolism and/or glucuronidation of [^{14}C]sipoglitazar.

Data represent the means of duplicate determinations.

Figure 9. Postulated metabolic pathways of sipoglitazar.

Figure 10. Possible metabolic pathway of sipoglitazar-G1 *via* lactone-form and metabolic pathway of statin glucuronide.

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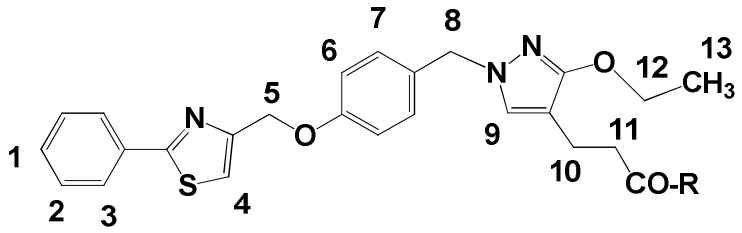
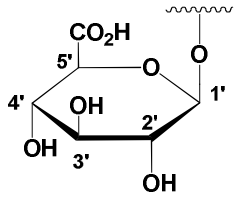
Table 1. ^{1}H Chemical shifts of sipoglitazar and its metabolites.

H-No.	δ(ppm), J(Hz)		
	Sipoglitazar	Sipoglitazar-G1	Sipoglitazar-G2
1	7.45-7.48 (m)	7.45-7.49 (m)	7.45-7.50 (m)
2	7.45-7.48 (m)	7.45-7.49 (m)	7.45-7.50 (m)
3	7.90 (m)	7.90 (m)	7.91 (m)
4	7.22 (s)	7.25 (s)	7.26 (s)
5	5.16 (s)	5.17 (s)	5.17 (s)
6	6.97 (d, J=8.7)	6.97 (d, J=8.5)	6.97 (d, J=8.6)
7	7.15 (d, J=8.7)	7.14 (d, J=8.5)	7.16 (d, J=8.6)
8	4.94 (s)	4.92 (s)	4.94 (s)
9	7.49 (s)	7.50 (s)	7.51 (s)
10	2.48 (m)	2.54-2.62 (m)	2.54-2.58 (m)
11	2.48 (m)	2.54-2.62 (m)	2.54-2.58 (m)
12	4.07 (q, J=7.0)	ca. 4.1 (hidden)	4.08 (q, J=7.0)
13	1.25 (t, J=7.0)	1.25 (t, J=7.0)	1.26 (t, J=7.0)
1'	---	5.45 (d, J=7.7)	5.20 (d, J=3.6)
2'	---	3.38 (m)	4.57 (dd, J=9.6,3.6)
3'	---	3.45-3.52 (m)	3.80 (m)
4'	---	3.45-3.52 (m)	3.55 (m)
5'	---	3.89 (d, J=8.8)	4.20 (d, J=10.2)



Sipoglitazar: R=H

Sipoglitazar-G1: R=



Sipoglitazar-G2: R=

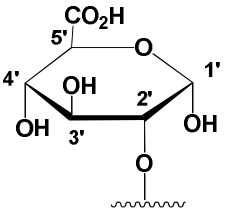


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Table 2. Correlation coefficients for the metabolism of [^{14}C]sipoglitazar-G1 with CYP isoform-specific activities.

Isoform-Specific Activity	CYP enzymes	Correlation Coefficient (*r*)	
		Sipoglitazar-G1 elimination	M-I formation
7-Ethoxyresorufin *O*-dealkylation	CYP1A2	-0.189	-0.062
Coumarin 7-hydroxylation	CYP2A6	0.498	0.287
S-Mephenytoin *N*-demethylation	CYP2B6	0.769	0.704
Paclitaxel 6α-hydroxylation	CYP2C8	0.896	0.977
Diclofenac 4'-hydroxylation	CYP2C9	0.486	0.326
S-Mephenytoin 4'-hydroxylation	CYP2C19	0.078	0.076
Dextromethorphan *O*-demethylation	CYP2D6	0.152	0.353
Chlorzoxazone 6-hydroxylation	CYP2E1	0.698	0.609
Testosterone 6β-hydroxylation	CYP3A4/5	0.475	0.439
Lauric acid 12-hydroxylation	CYP4A9/11	0.351	0.310

Data represent the means of duplicate determinations.

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Figure 1.

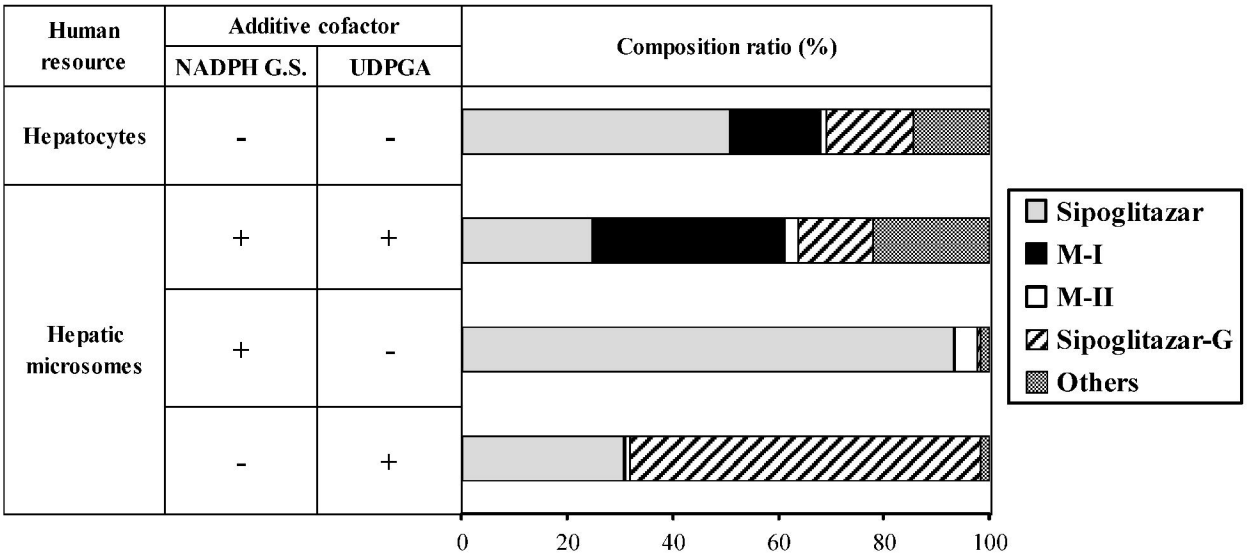

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Figure 2.

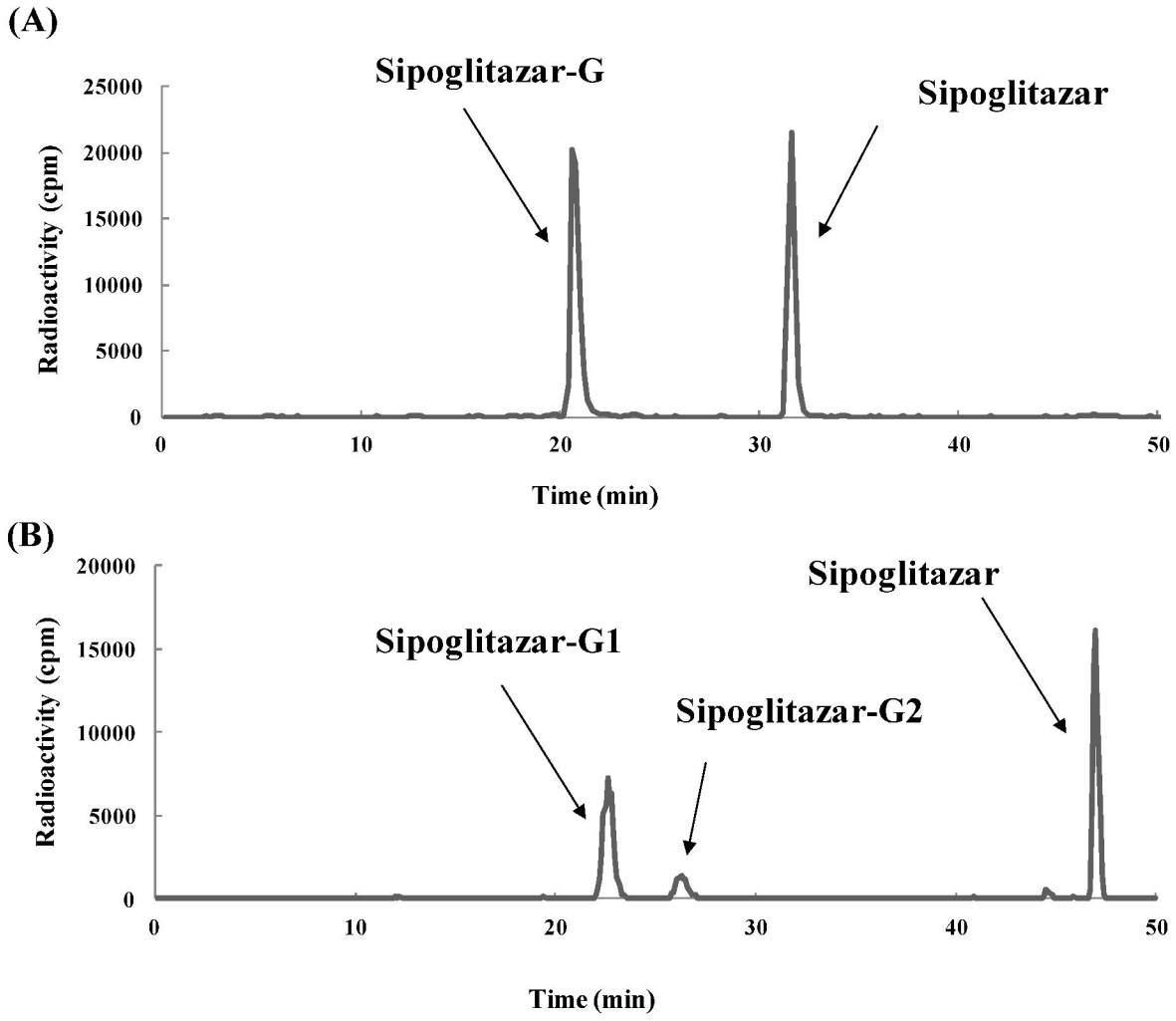

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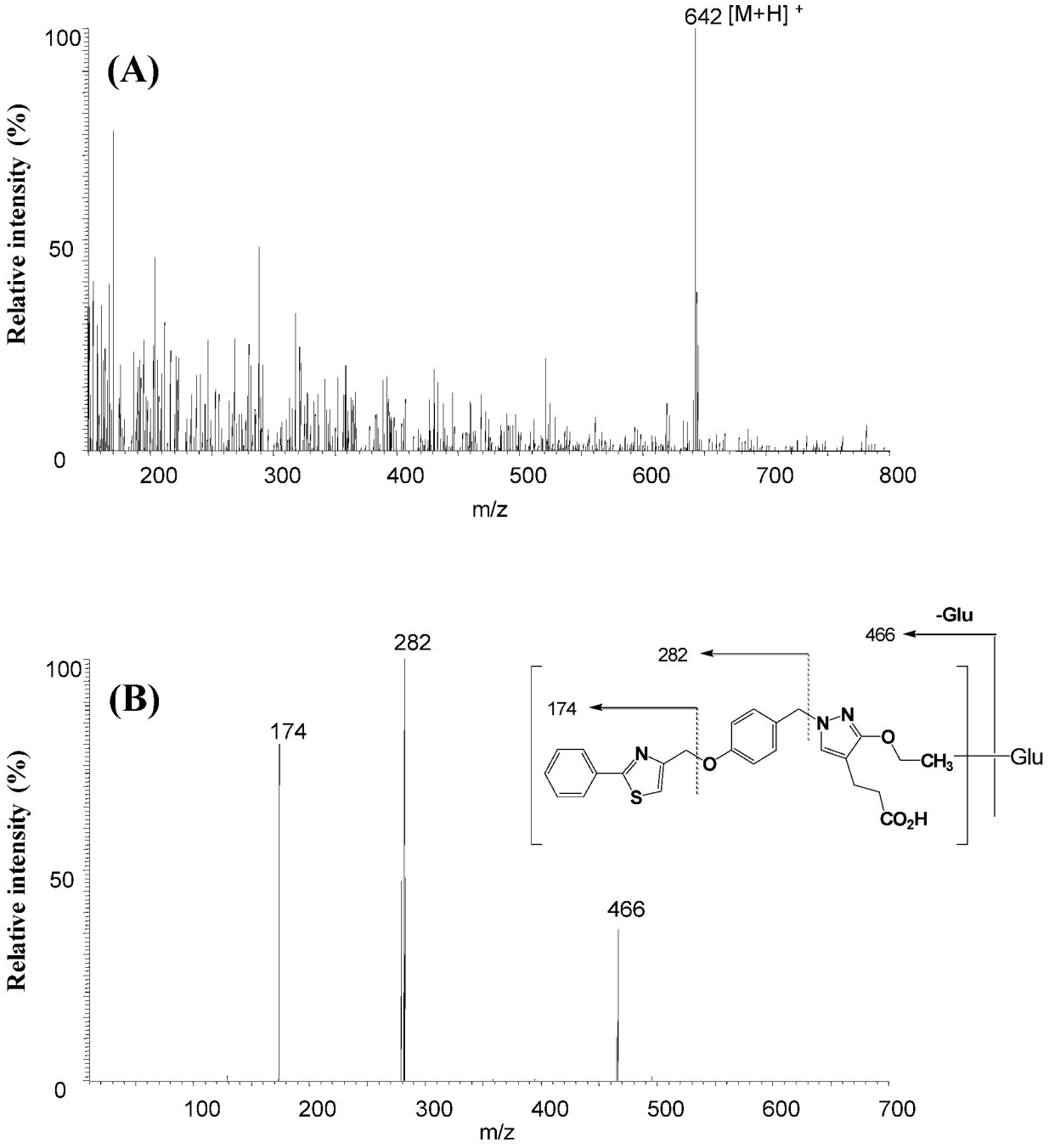
Figure 3.



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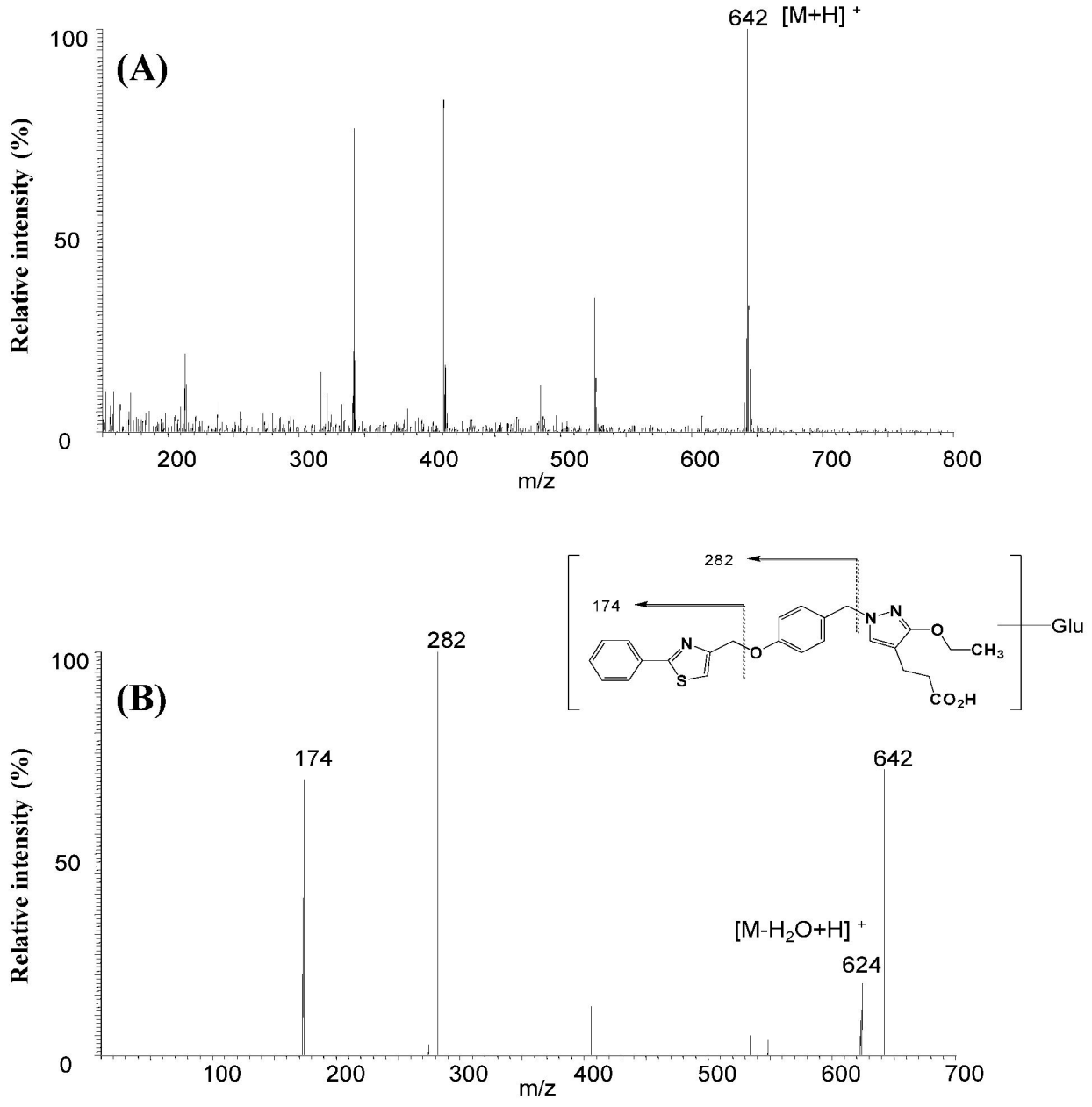

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Figure 5.

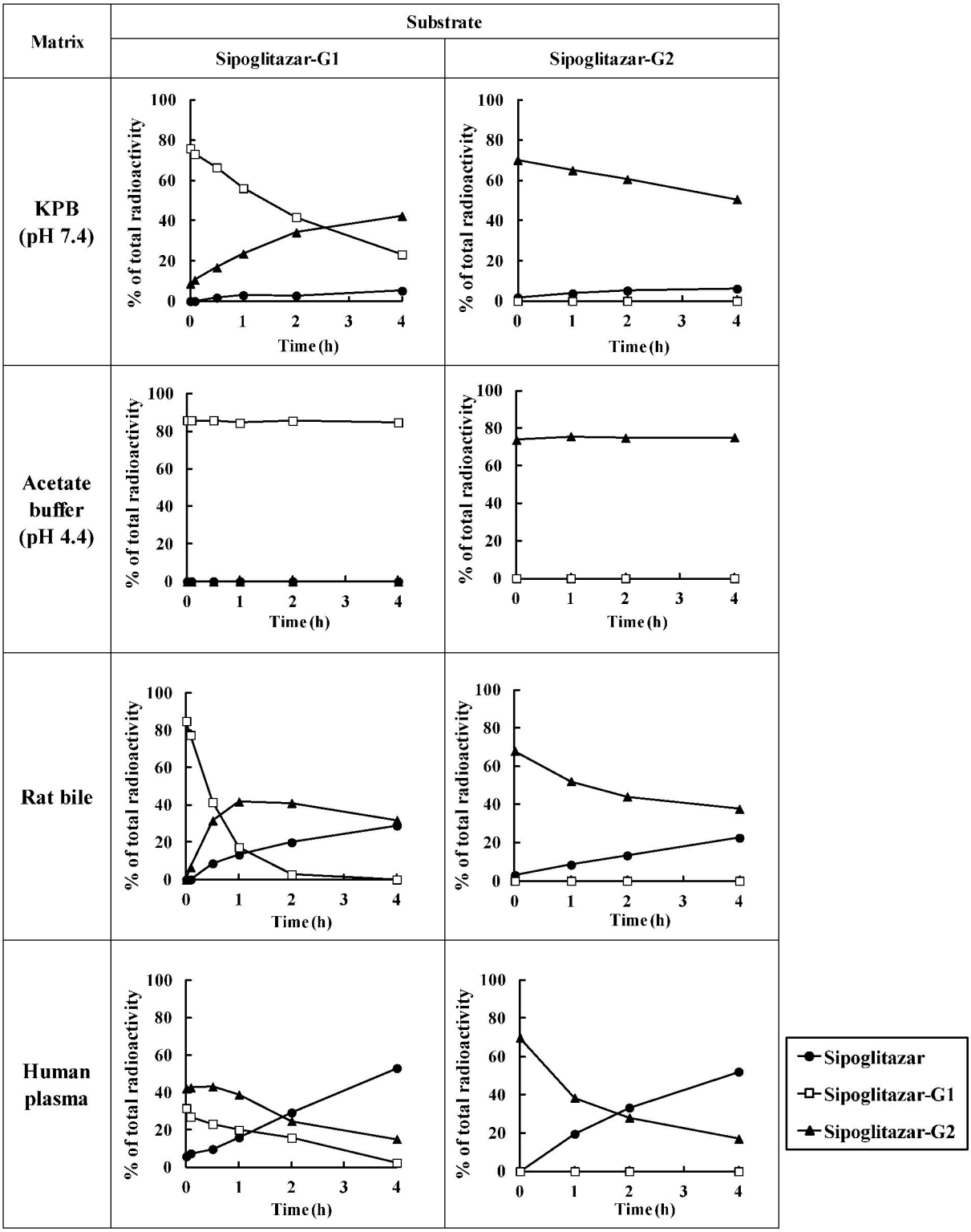

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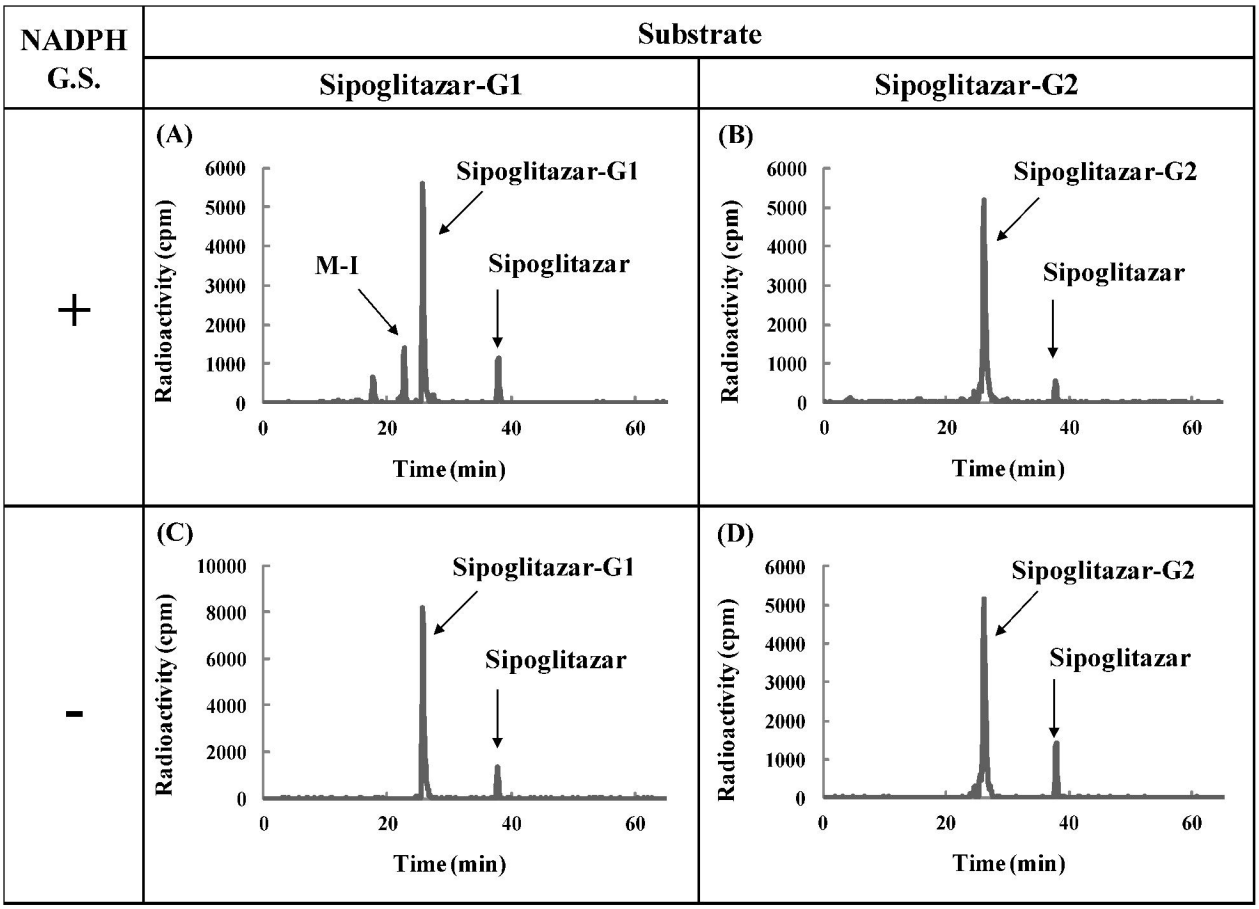
Figure 6.



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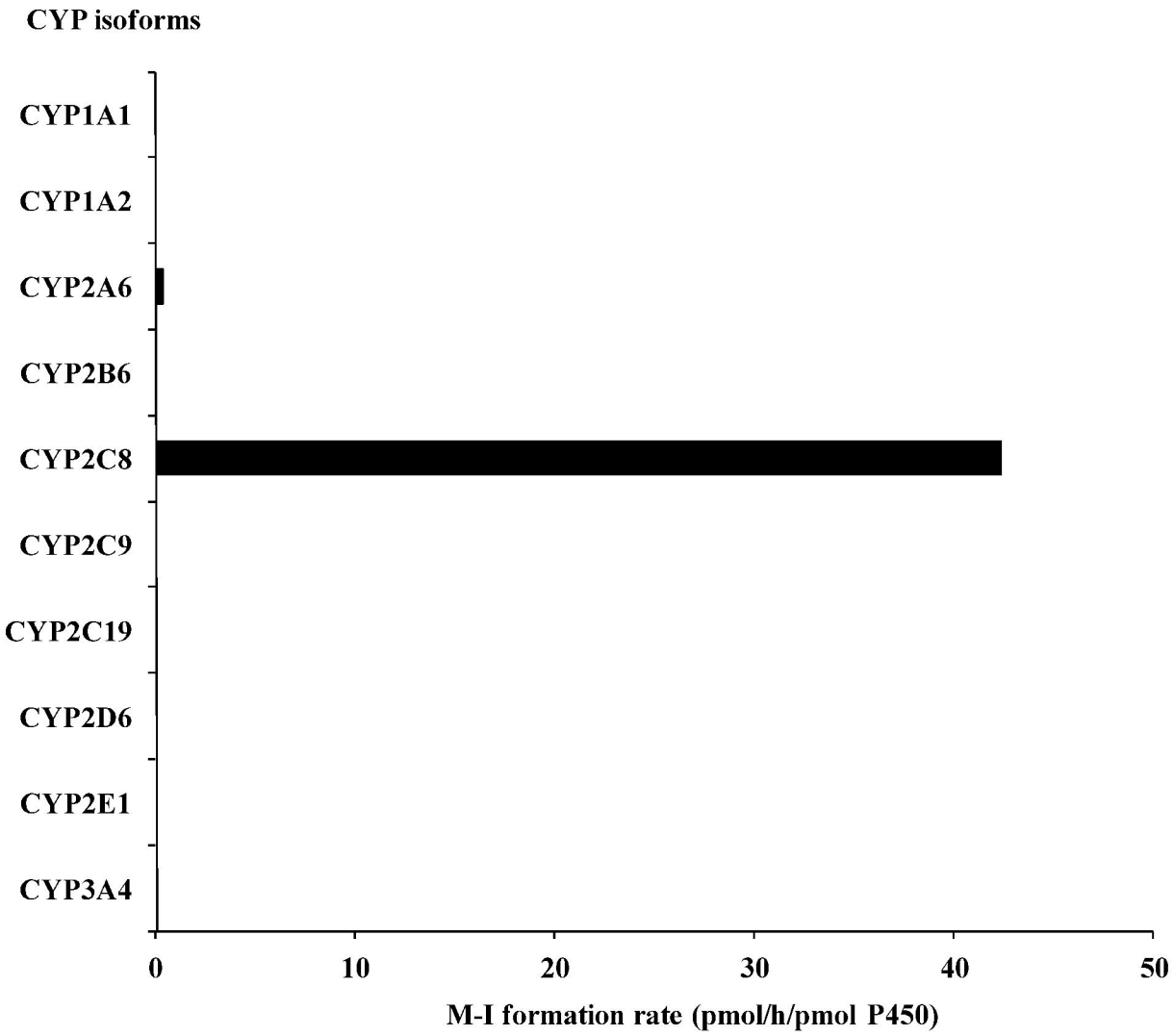

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Figure 8.

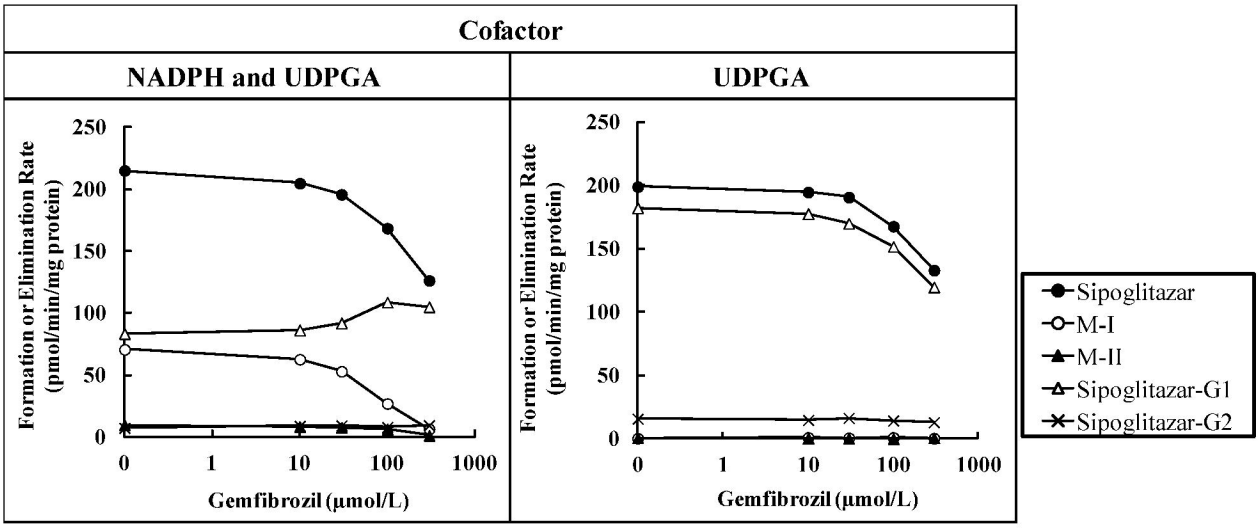

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Figure 9.

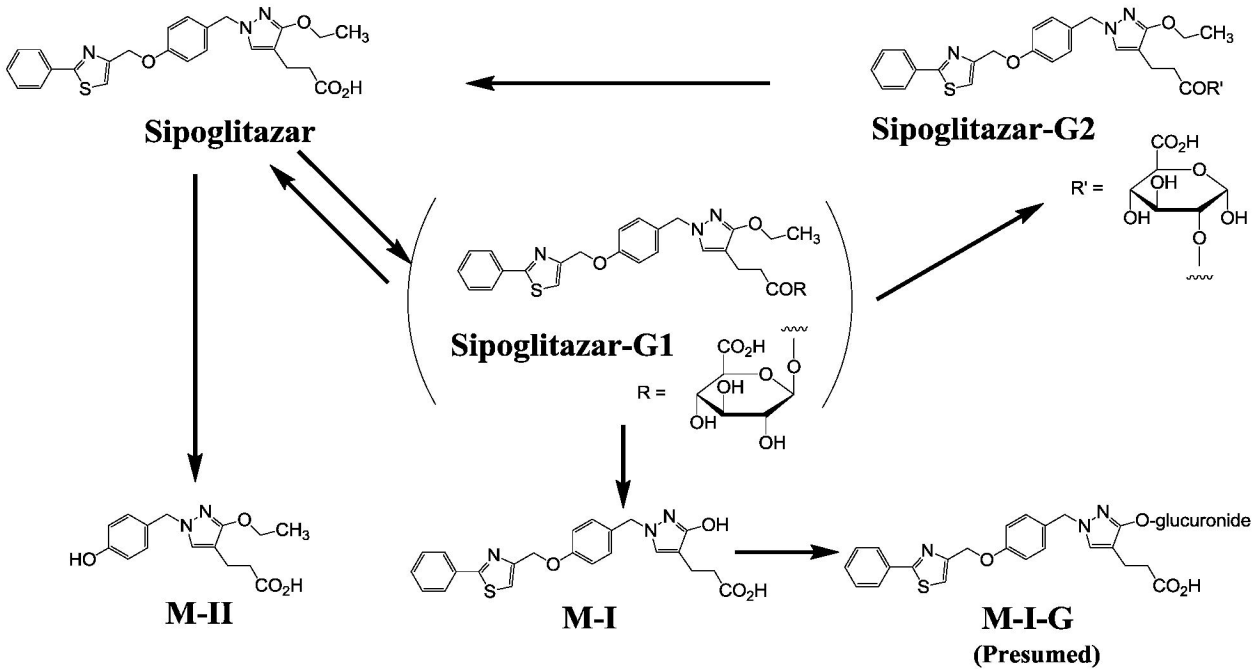

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Figure 10.