The interaction between glimepiride and other drugs metabolized by CYP2C9 using an in vitro model

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Yajing Luo

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Advisor: David J. Greenblatt, MD

Abstract

Glimepiride is a second-generation sulfonylurea, which is generally used to treat diabetes mellitus type 2. The aim of this investigation was to study the in vitro biotransformation of glimepiride, and drug interactions of glimepiride with different inhibitors. In vitro interactions of glimepiride with different inhibitors were studied using human pooled liver microsomes, and with metabolite formation quantitated by using high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Trans-hydroxy glimepiride (THG) is one of the major metabolites of glimepiride. Data points were analyzed using the IC_{50} relationship equation. The model was used to study the different inhibitors affecting metabolism via CYP2C9: ketoconazole, quinidine, ticlopidine, fluvoxamine, and sulfaphenazole were tested. Basic on the inhibition formula, the IC_{50} for these compound was calculated to determine their inhibitory activity. Lower IC_{50} implies a higher inhibitory effect. The IC_{50} data (1.23 μM for THG) indicated that sulfaphenazole was the most potent inhibitor, confirming that CYP2C9 is the most important CYP isoform involved in glimepiride clearance.

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List of Abbreviations

CE: capillary electrophoresis

CID: collision-induced dissociation

CXP: collision cell potential

CYP: Cytochrome P450

DP: Declustering potential

F (Fluvo): Fluvoxamine

GMP: Glimepiride

HPLC: High-performance liquid chromatography

IS: Internal standards

K (Keto): Ketoconazole

META: Metabolite

MRM: Multiple reaction monitoring

MS: Mass spectrometry

NADPH: Nicotinamide adenine dinucleotide phosphate

Q1: The first quadrupole

Q2: The second quadrupole

Q3: The third quadrupole

Q (Quini): Quinidine

S (Sulfa): Sulfaphenazole

T (Ticlo): Ticlopidine

THG: Trans-Hydroxy Glimepiride

UV light: Ultraviolet light

Chapter 1: Introduction

Diabetes mellitus, commonly known as diabetes, is a very common metabolic disorder characterized by hyperglycemia and altered metabolism of lipids, proteins, and carbohydrates, and occurs due to absolute or relative deficiency of insulin, or insulin resistance.[1] Glimepiride is a widely used third-generation sulfonylurea suitable for once daily administration in treatment of type 2 diabetes mellitus (1). It is completely absorbed after oral administration and is eliminated mainly via metabolism by cytochrome P450 (CYP) 2C9. The oral bioavailability of glimepiride is close to 100% (2). The biotransformation of GMP is catalyzed by cytochrome P450 (CYP), mainly by 2C9 (4). The parent drug is metabolized to cyclohexyl hydroxymethyl derivative (Trans-hydroxy glimepiride, THG) by CYP and further metabolized to carboxyl derivative by other cytosolic enzymes (M2) (5). As a result of improved diabetes control and increased insulin sensitivity, the need for glimepiride may be reduced during treatment (3). Therefore, in order to avoid hypoglycemia, reducing the dose over time or using an inhibitor of the glimepiride might be necessary. If the patient's weight, lifestyle changes, or other factors increase sensitivity to hypoglycemia or hyperglycemia, dose adjustment should also be considered.

Many drug-drug interactions of clinical importance can be attributed to the pharmacokinetic and pharmacodynamic changes that occur due to alterations in hepatic drug metabolic pathways catalyzed by the CYP system (4). Use of high-performance liquid chromatography (HPLC) to analyze the metabolism of glimepiride in vitro might allow distinguishing the individual metabolites of glimepiride (5). Mass spectrometry (MS) allows greater sensitivity and specificity for quantitation of metabolite formation

rates (6). Drug-drug interactions are major concerns not only for physicians and patients, but also for the pharmaceutical industry during the development of new drugs (7). In order to avoid hypoglycemia during treatment with glimepiride, the potential hazards of coadministration of metabolic inhibitors need to be considered. In vitro study has become a critical first step in the assessment of drug interactions. Well-executed in vitro studies can be used as a screening tool for further in vivo assessment and can provide the basis for the design of subsequent in vivo drug interaction studies (8).

This study confirmed the experimental conditions for glimepiride metabolite formation in vitro using mass spectrometry, and also evaluated drug-drug interaction between different inhibitors and glimepiride. Ketoconazole, quinidine, ticlopidine, fluvoxamine, and sulfaphenazole were selected as index inhibitors to identify the principal CYP isoforms responsible for glimepiride biotransformation.

Chapter 2: Methods and Material

2.1 Drugs and chemical

Glimepiride and Trans-Hydroxy Glimepiride (THG) were obtained from Toronto Research Chemicals. Ketoconazole, quinidine, ticlopidine, fluvoxamine, and sulfaphenazole were used as index inhibitors. Erythromycin was internal standard, acetonitrile, methanol, 50mM phosphate buffer, 100mM phosphate buffer, magnesium chloride, reduced nicotinamide adenine dinucleotide phosphate (NADPH), DL-Isocitric acid trisodium salt, and isocitric dehydrogenase and human liver microsomes were used for the in vitro study. Human liver microsomes were kindly made by Ms. Su Xiang Duan.

2.2 Laboratory method

2.2.1 HPLC

Varying concentrations of glimepiride were incubated with human liver microsomes. Erythromycin was the internal standard in the system used to profile the activity of CYP isoforms, and confirm the experimental conditions for metabolite formation from glimepiride using high-performance liquid chromatography.

The formation of THG was quantitated by high-performance liquid chromatography (HPLC). Incubations contained varying concentrations of the substrate glimepiride (ranging from 0 to 500μM). Besides the specific amount of drugs as design, the in-vitro system is based on the previous study (12). Briefly, the total 250 μL in-vitro system contained 50 mM KH_2PO_3 buffer at physiolologic pH, 50 mM $MgCl_2$, 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP), 3.75 mM Dl-isocitric acid, 1 unit/mL of isocitric dehydrogenase, and 0.1 mg/mL human liver microsomes.

Reactions were initiated by adding 25 µL of a 0.1 mg/mL concentration of protein from liver number 866 (human liver microsomal protein). After an incubation time of 20 min, the reactions were stopped by adding 100µL of stop solution (1 volume of erythromycin as internal standard and 2 volumes of acetonitrile) and cooling on ice.

The incubation mixture was centrifuged for 5 min, and the supernatant was transferred to an auto-sampling vial for HPLC analysis. The mobile phase consisted of the biphasic mobile system (A: B), reservoir A (0.1% trifluoroacetic acid in water) and reservoir B (0.1% trifluoroacetic acid in acetonitrile).

2.2.2 HPLC analysis

The HPLC condition was based on the previous article (8) and adjusted to our equipment. There is no data on the UV absorption of THG, so we did a full wavelength scan of THG. The HPLC apparatus consisted of a Kromasil C18, 250 × 4.6 mm, 5 µM column for the analysis. The Biphasic mobile phase system (A: B), reservoir A (0.1% trifluoroacetic acid in water) and reservoir B (0.1% trifluoroacetic acid in acetonitrile) was run at 30%: 70% with a total flow rate of 1 ml/min through the column to elute the analytes. Eluates were monitored using the Agilent HPLC-UV with Chemstation software at 237 nm. The run time for each sample was 5 min.

The THG metabolite was not detected by HPLC. The THG and M2 peaks were determined by a series of checks of each of the components of the system.

2.2.3 Mass Spectrometry (MS)

2.2.3.1 Confirmation of experimental conditions for metabolite formation from glimepiride by mass spectrometry

We used similar experimented conditions as in the HPLC study. Glimepiride was diluted to yield a final stock solution of concentration 250 μM.

Varying concentrations of glimepiride were incubated with human liver microsomes. Erythromycin was used as the internal standard.

The formation of THG was quantitated by mass spectrometry (MS). Incubations contained varying concentrations of 250mM as substrate glimepiride (ranging from 0 to 500μM). After the solvent was evaporated to dryness, we added 125μL 50mM phosphate buffer (pH 7.56), and a total of 100μL of mixtures containing 50mM phosphate buffer, 100mM phosphate buffer, magnesium chloride, b-NADP, DL-Isocitric acid trisodium salt, and isocitric dehydrogenase as a cofactor to a series of incubation tubes.

Reactions were initiated by addition of 25 μL human pooled liver microsomal protein. The incubation time was 20 min at 37°C, at 80 rpm in a shaking water bath.

To stop the reaction, 100μL of stop solution (1 volume of erythromycin as internal standards and 2 volumes of acetonitrile) was added to each incubation, and they were mixed and cooled on ice. The samples were centrifuged at 10,000 rpm for 5 min.

Supernatants were assayed for the presence of substrate using an isocratic mass spectrometry (MS) method. The supernatants were mixed with buffer and directly injected.

We varied the incubation times, the concentration of glimepiride, sources of human liver microsomal protein, and the concentrations of microsomal protein as described in the HPLC studies.

2.2.3.2 Defining the inhibition between glimepiride and the enzyme inhibitors

Based on previous experience, we selected appropriate concentrations of ketoconazole, quinidine, ticlopidine, fluvoxamine, and sulfaphenazole. Glimepiride was the substrate. We added 125μL 50mM phosphate buffer (pH 7.56), a total of 100μL of the cofactors, and 25 μL of microsomal protein. The mixtures were incubated for 20 min at 37°C in a shaking water bath.

100μL of 1 volume of erythromycin as internal standard and 2 volumes of acetonitrile was added to stop the reaction. Then, the mixture was centrifuged for 5 min, and the supernatants were directly injected into the MS for analyzed.

2.2.4 LC–MS studies

MS conditions are based on previous literature (9) and adjusted to fit our equipment. The MS and LC–MS studies were carried out using positive as well as negative electrospray ionization (+ESI and −ESI) modes on a Bruker Daltonics microTOF instrument (Bruker Daltonik GmbH, Bremen, Germany). The data were acquired and processed using microTOF control software ver. 2.0. The LC part of the LC–MS consisted of a 1100 series system (Agilent Technologies Inc., CA, USA), controlled by Hystar (ver. 3.1) software.

All experiments were performed under the same LC conditions as described below. The LC separation was performed using a Varian Omnisphere C18 column (100 × 2 mm i.d.,

3 μm; Varian, Sint-Katelijne-Waver, Belgium) [4] at a flow rate of 0.3 μL/min at 40℃. A 1 μL sample was injected into the instrument at 8℃.

The mobile phase consisted of water and methanol (MeOH) (A:B) both with 1 mM NH_4COOH and 0.001% HCOOH. The gradient program changed the percentage of mobile phase as follows: 0 min, 50%: 50%; 1 min, 50%: 50%; 1.2 min, 40%: 60%; 3.6 min 30%: 70%; 4 min, 50%: 50%.

MS/MS detection was carried out in the multiple reaction monitoring (MRM) mode in positive ionization mode. Scans in period is 333. Ion source is Turbo spray. The triple-quadrupole mass spectrometer, also known as QqQ, is a tandem MS method in which the first (Q1) and third (Q3) quadrupoles act as mass filters. The first quadrupole termed "Q1" can act as a mass filter and transmits a selected ion, and accelerates it towards "Q2" which is termed a collision cell. The pressure in Q2 is higher and the ions collide with neutral gas in the collision cell and are fragmented by collision-induced dissociation (CID). For glimepiride, the rotation time is 0.97, the resolution Q1 mass is 491, Q3 mass is 352; Declustering potential (DP) starts at 80 and stops at 80, capillary electrophoresis (CE) starts at 20 and stops at 20, collision cell potential (CXP) starts at 15 and stops at 15. For Trans-Hydroxy Glimepiride, the rotation time is 0.58, the resolution Q1 mass is 507, Q3 mass is 352; DP starts at 70 and stops at 70, CE starts at 20 and stops at 20, CXP starts at 15 and stops at 15. For erythromycin, the rotation time is 0.35, the resolution Q1 mass is 734 Q3 mass is 534; DP starts at 94 and stops at 94, CE starts at 21 and stops at 21, CXP starts at 27and stops at 27.

2.3 Analysis of pharmacokinetic parameters; kinetic model

2.3.1 Reaction model

Based on the mechanism of GMP metabolism, the reaction model we chose is the Michaelis-Menten model (9) (Equation 1).

$$V = \frac{Vmax * [S]}{km + [S]} \quad [Equation 1]$$

Iterated variables: V: reaction velocity, Vmax: maximum reaction velocity, [S]: substrate concentration, km: substrate concentration at 50% of Vmax. Metabolite formation rate was used to represent the velocity of the reaction. It is worth noticing that the CYP metabolite (THG) will be biotransformed further and we do not know the reaction activity for the second step, so we did a study of the incubation time versus THG to confirm the linearity of the reaction velocity and time. The incubation time was chosen to be in the linear phase. The sample needs to be run again with MS in the future.

2.3.2 Inhibition model

To determine the IC_{50} of an inhibitor, we used the Hill equation with modification to fit our design as previously established (10) (Equation 2).

$$Rv = 100 * (1 - \frac{Emax * [I]^r}{[I]^r + IC^r}) \quad [Equation\ 2]$$

Integrated variables: Rv: remain enzyme activity as a percentage of control velocity, Emax: maximum effect, [I]: inhibitor concentration, IC: inhibitor concentration at 50% of maximum effect, r: an exponent in the Hill equation. In Equation 2, the parameter IC is not IC_{50}. We take the result of Equation 2 to Equation 3 to calculate IC_{50}.

$$IC50 = \frac{IC}{(2Emax - 1)^{\frac{1}{r}}} \quad [Equation\ 3]$$

2.3.3 Mixed-enzyme model

Human liver microsomes are a mixture of enzymes that contains many CPYs that may catalyze GMP metabolism (cocktail). To determine the fraction of the metabolism of each enzyme in the system, we chose the 'index inhibitors' which specifically inhibited the activity of one (or a family) of the isoforms of main CYPs that may be associated with the biotransformation of GMP (15). We added the inhibitor in a fixed concentration that much higher than the ki (inhibition constant) reported in the literature (Table 1), to achieve complete inhibition of the isoform.

Index inhibitor	CYP isoforms	ki (uM)	Experimental concentration (μM)	Reference
Ketoconazole	CYP3A	0.007	2.5	(11)
Quinidine	CYP2D6	0.053	2.5	(12)
Ticlopidine	CYP2B6	0.2	5	(13)
Fluvoxamine	CYP1A2	2	5	(14)
Sulfaphenazole	CYP2C9	0.46	2.5	(15)

Table 2.1. Summary of the different index inhibitors.

The percentage of inhibition is calculated by the velocity of the inhibition group over the control group, and it approximately equals the fraction of GMP by the specific corresponding CYP as previously discussed.

Chapter 3: Results

3.1 From HPLC study

Analysis with HPLC-UV

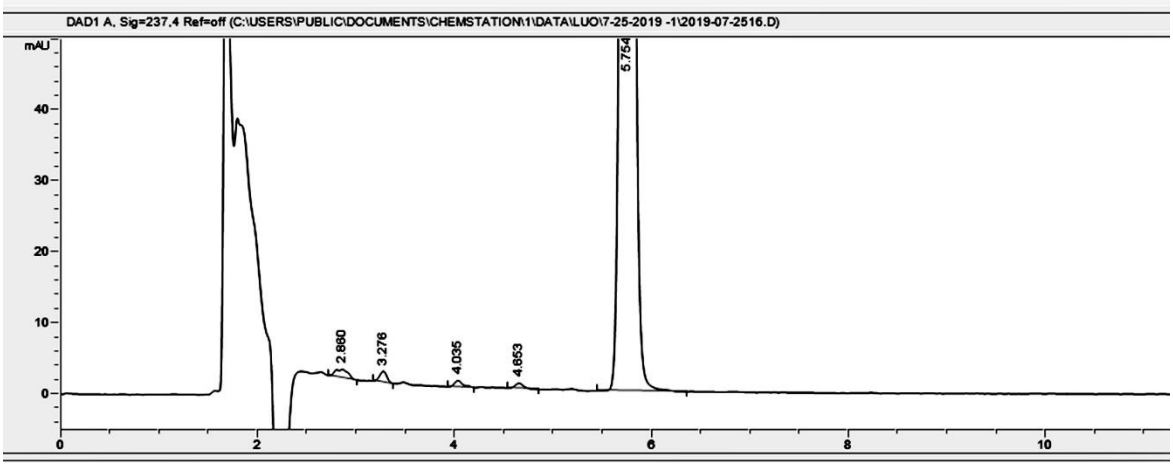

Figure 3.1. HPLC-UV analysis of incubates. No metabolite peak was found. The X-axis is the appearing time (min).

Results of the glimepiride in vitro HPLC study are shown in Figure 3.1 (16). The figure shows 4 different unknown peaks around 2.9 min- 4.7 min. (Figure 3.1) However, those 4 unknown peaks are too small, and hard to define. Due to the results of UV spectroscopy in Figure 3.2, we cannot tell all those peaks were due to metabolism or not. So, we decided to use mass spectrometry to analyze the formation of metabolites from glimepiride in vitro.

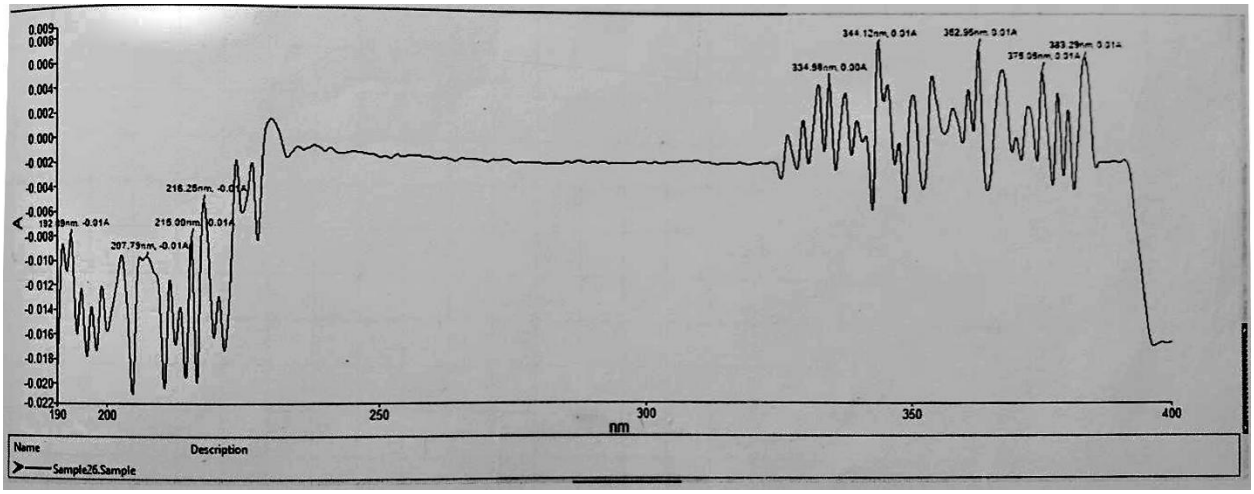

Figure 3.2. UV absorption of THG. the X-axis is the wavelength (nm), the Y-axis is absorption (AU). No significant absorption wavelength.

3.2 From MS study

3.2.1 Confirmation of experimental conditions for metabolite formation from glimepiride by mass spectrometry

The results of metabolite formation from glimepiride in vitro form the MS study are shown in figure 3.3 (17).

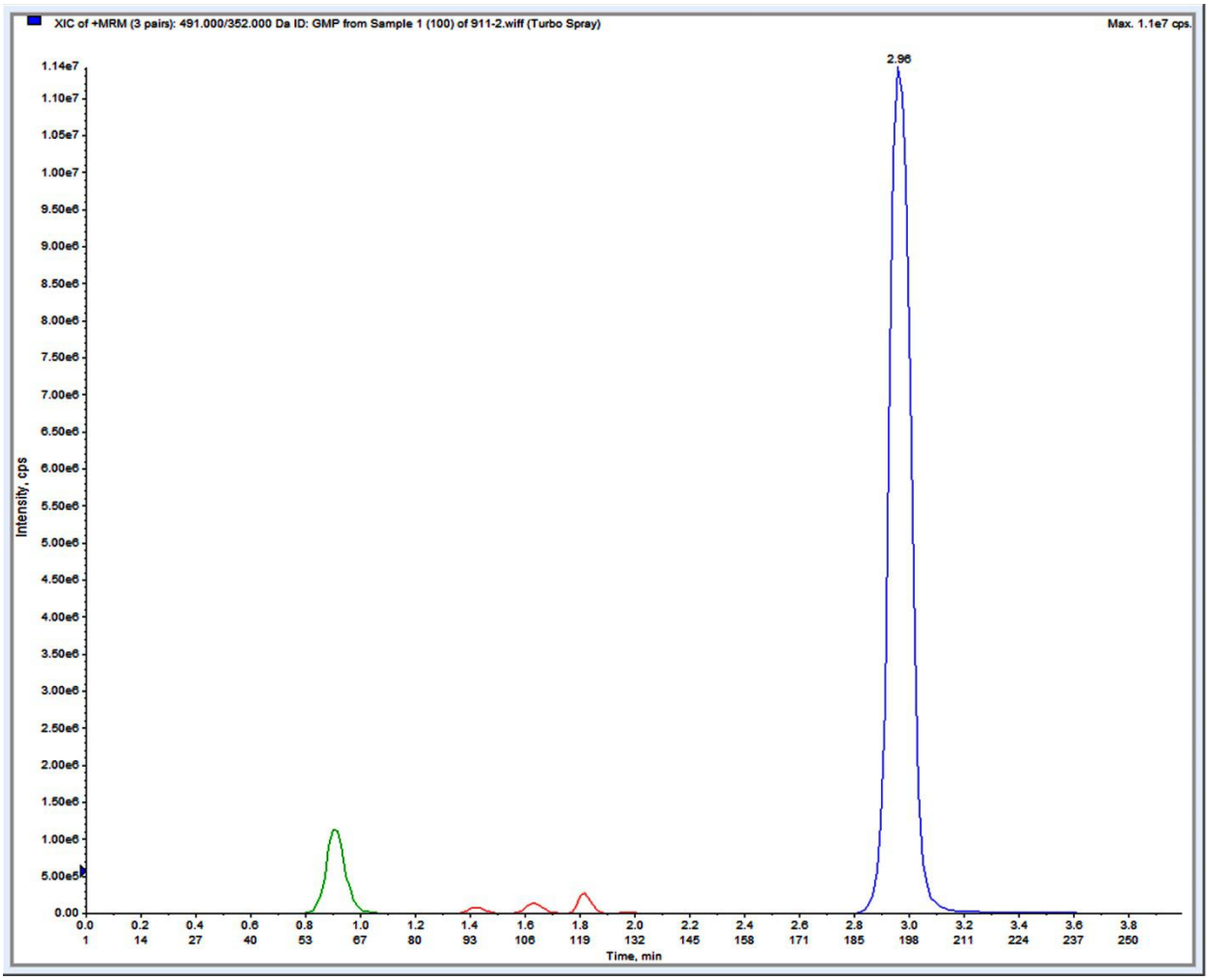

Figure 3.3. MS determination of metabolites from glimepiride. The X-axis is the run time (min). The green line peak indicates erythromycin, near 0.9 min. The red line peak indicates the metabolites near 1.4 min. The blue line peak indicates glimepiride near 3.0 min. All those three red LC-MS peaks indicate the metabolites, which is the THG.

When we injected pure THG, we got 3 peaks corresponding to THG, ecause under the collision-induced dissociation (CID) mass spectrometry method, THG will be broken into three pieces. We selected the second peak to analyze.

Through changing the incubation time (20min, 60min), and the source of protein(Liver 866, 870, 871), we got results as follows. In Figure 3.4- 3.7, the X-axis is the concentration of GMP (μM). Each point on Y-axis is the metabolite peak height converted to amount of metabolite formed using a standard curve, then divided by the incubation time. Equation was used 1 for analysis. The calculate results are shown in table 3.1.

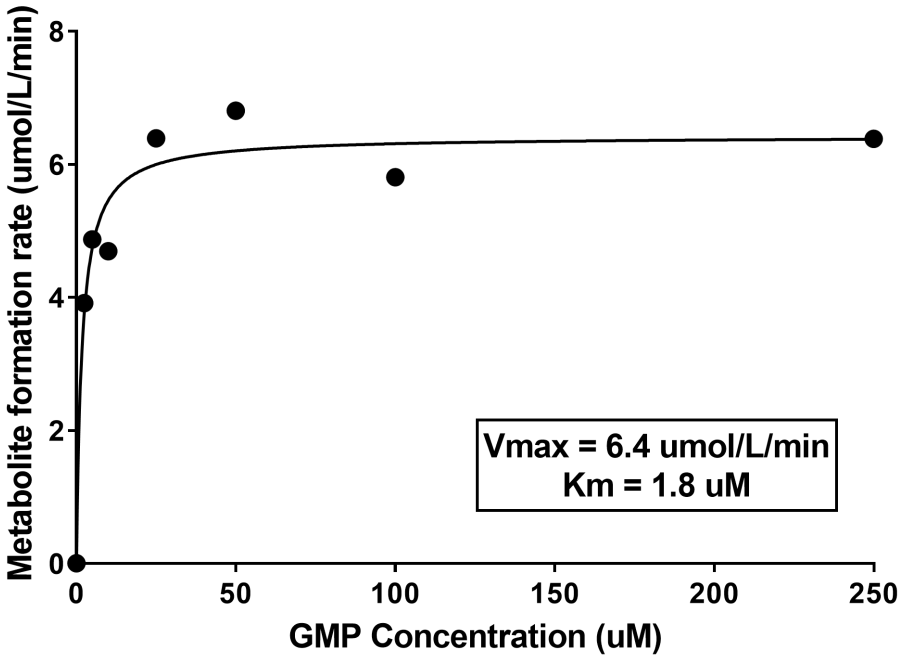

Figure 3. 4. P866 60 minutes incubation. R square = 0.96

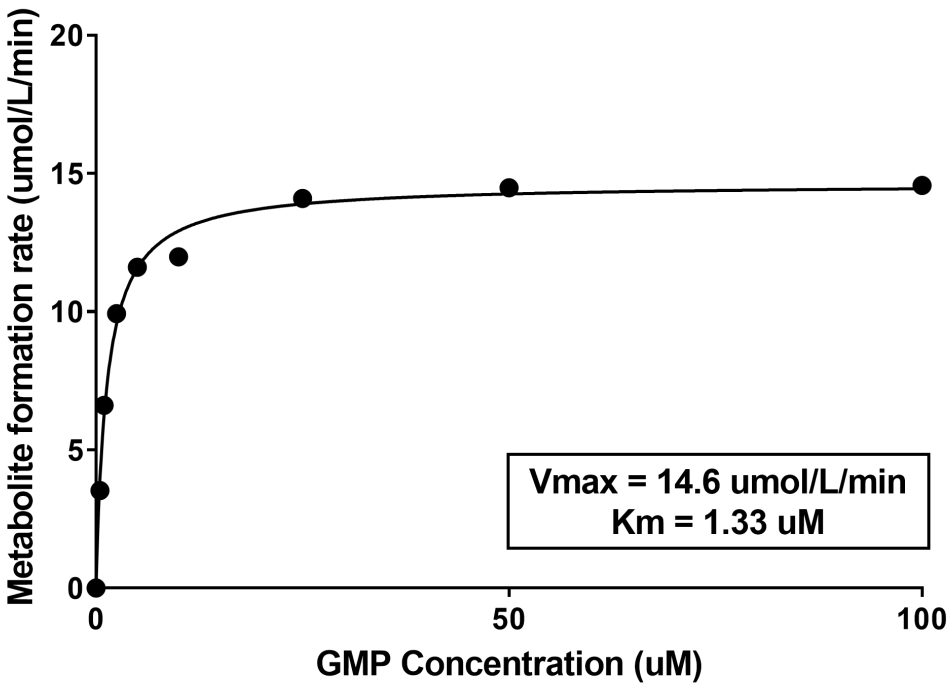

Figure 3.5. P870 20 minutes incubation. R square = 0.99

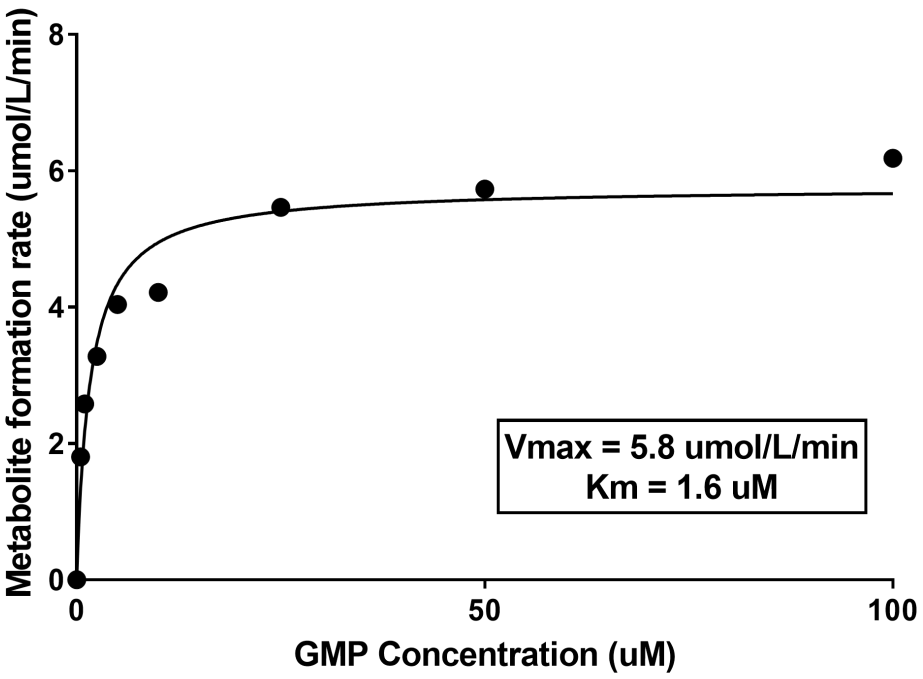

Figure 3.6. P871 20 miutes incubation. R squre = 0.96

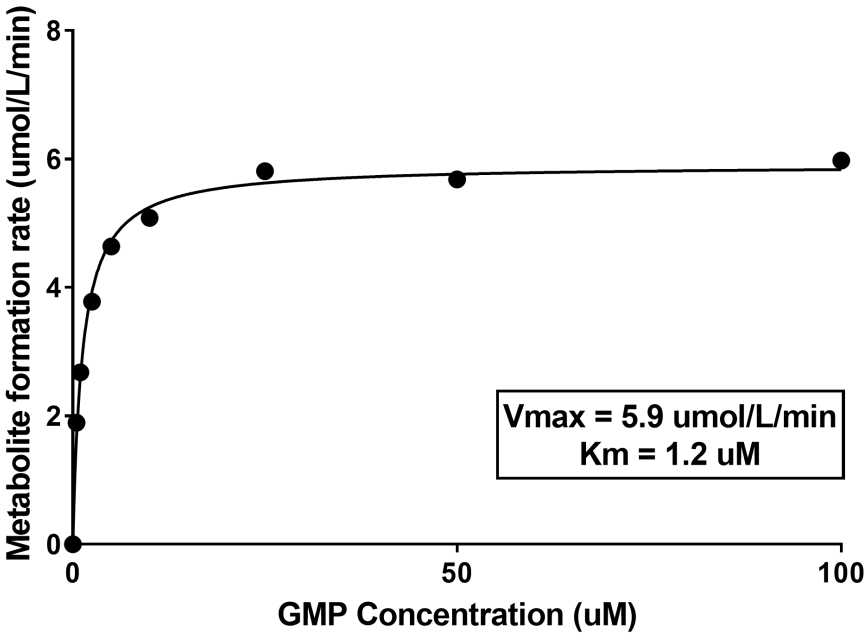

Figure 3.7. P866 20 miutes incubation. R squre = 1.00

	P870		P871		P866	
Incubation time (minutes)	Vmax (umol/L/ min)	km (uM)	Vmax (umol/L/ min)	km (uM)	Vmax (umol/L/ min)	km (uM)
60	/	/	/	/	6.4	1.8
20	14.6	1.33	5.8	1.6	5.9	1.2

Table 3.1.Summary of the result of different incubation times.

In table 3.1, The Vmax and Km values are different in 870, 871, and 866 because different people have different metabolic abilities. In 866, the Vmax and Km values are similar at 60 min and 20 min because the change is a linear relationship when we increase time.

3.2.2 Defining the inhibition between glimepiride and the enzyme inhibitors.

Ketoconazole, quinidine, ticlopidine, fluvoxamine, and sulfaphenazole are the inhibitory drugs selected for the glimepiride. Sulfaphenazole showed the greatest inhibitory effect. (Figure 3.8) (Table 3.2).

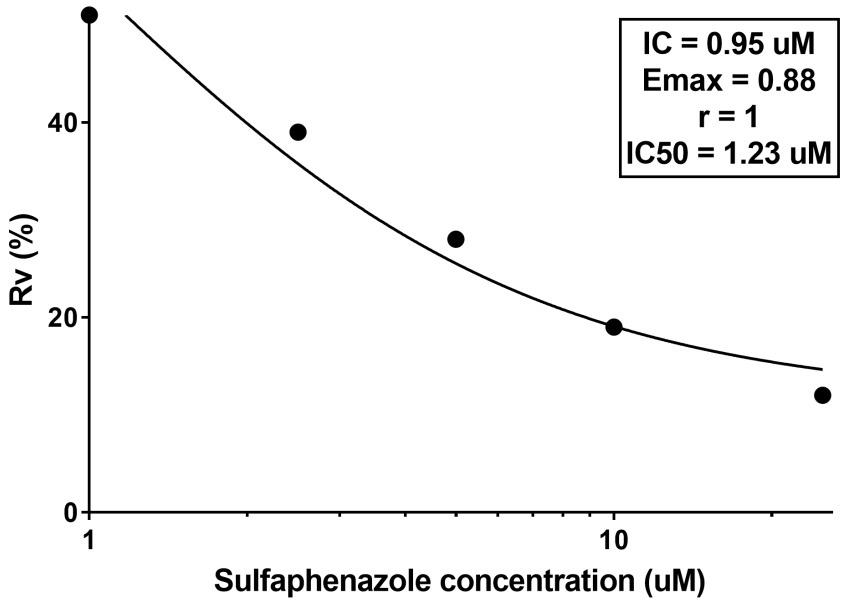

Figure 3.8. IC_{50} of sulaphenazole. R_v is the reaction velocity divided by the control velocity without inhibitor, expressed in percent. R square = 0.99

Index inhibitor	CYP isoforms	Experimental concentration (μM)	Rv (%)	Inhibition (%)
Ketoconazole	CYP3A	2.5	85%	15%
Quinidine	CYP2D6	2.5	99%	1%
Ticlopidine	CYP2B6	5	90%	10%
Fluvoxamine	CYP1A2	5	101%	-1%
Sulfaphenazole	CYP2C9	2.5	50%	50%

Table 3.2. Summary of index inhibition.

In table 3.2, Rv is the remain enzyme activity, the percentage of inhibition is used as 1 minus the Rv value. Then we can get the percentage of each inhibitor it accounts for in the process of inhibiting GMP.

Fluvoxamine shows small, negative inhibition, which is not experimentally different from zero inhibition.

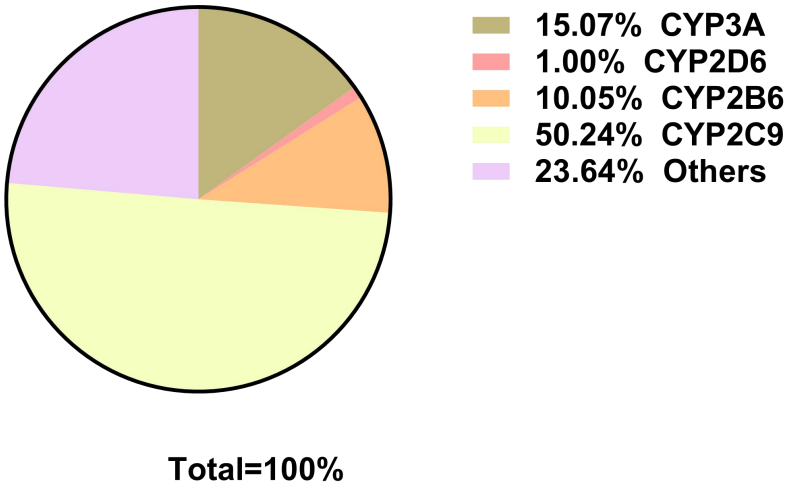

Figure 3.9. Metabolic enzyme fractions for the specific experimental conditions.

In Figure 3.9, Inhibitors suppressed these specific enzymes, CYP2C9 accounted for 50.%, CYP3A accounted for 15.%, CYP2B6 accounted for 10%, CYP2D6 accounted for 1%, and other unknown enzymes accounted for 24%. Each enzyme should use Equation 2 for nonlinear regression, which will make the results more accurate.

Statement: All the results in chapter 3 are based on my own experimental results and analysis.

In conclusions, first, we used rhe HPLC method, but the results were not conclusive. All chromatographic peaks were too small to analyze. So we used MS for follow-up data analysis.

Second, to calculate the metabolic parameters, we used different human liver microsomal preparations in the Michaelis-Menten model, and determined the Km and Vmax value from each.

Then, we found that GMP is mainly metabolized by CYP2C9. The contribution of CYP2D6 is low.

Finally, sulfaphenazole has the smallest value of IC50 for GMP, which indicates that sulfaphenazole has the strongest inhibitory effect.

Chapter 4: Discussion

The experimental conditions for glimepiride metabolism in vitro were confirmed in the previous HPLC study, and the analytic conditions for MS are also confirmed. The results gave an insight into the inhibitory effect of ketoconazole (CYP3A) , quinidine (CYP2A6), ticlopidine (CYP2C19), fluvoxamine (CYP1A2 and CYP2C19), and sulfaphenazole (CYP2C9). All of those drugs are potent inhibitors of the correcponding isoforms. The metabolic pathway of glimepiride is mostly mediated by CYP2C9. Comparing the results, sulfaphenazole is the most potent inhibitor of glimepiride metabolism. When coadministered with sulfaphenazole, glimepiride biotransformation was significantly inhibited. This confirms that sulfaphenazole acts as an inhibitor of CYP2C9 activity.

The results of this study support that both the ketoconazole and sulfaphenazole are strong enzyme inhibitors when given in combination with glimepiride, but sulfaphenazole is the most potent inhibitor for glimepiride.

Further, the method should be repeated with another drug that uses the same metabolic pathway as glimepiride, to check for drug-drug interactions. Drug interactions can lead to changed systemic exposure, resulting in variations in the response to co-administered drugs. This may have significant implications in clinical practice. As such, further investigation of drug interactions between different drugs with glimepiride is necessary. Appropriate changes in doses and dose regimens of the substrate and/or inhibitor may be warranted.

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