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Identification of glyburide metabolites formed by hepatic and placental microsomes of humans and baboons

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ARTICLE INFO

Article history:
Received 11 July 2006
Accepted 18 August 2006

Keywords:
Gestational diabetes
Human placenta
Baboon placenta
Glyburide
Metabolism
Mass spectrometry
Pregnancy

ABSTRACT

Glyburide (glibenclamide) is a second-generation sulfonylurea used for treatment of type-2 and gestational diabetes mellitus. To date, two glyburide metabolites have been identified in maternal urine: namely, 4-*trans*-hydroxycyclohexyl glyburide and 3-*cis*-hydroxycyclohexyl glyburide. The use of glyburide to treat gestational diabetes prompted us to investigate its metabolism by the placenta. The metabolism of glyburide by microsomal preparations from human and baboon placenta was compared with metabolism by their livers. The metabolites formed by the microsomes of the four tissues were identified by high-performance liquid chromatography–mass spectrometry using retention times, ion current (extracted at *m/z* 510), and selected-ion monitoring. The data obtained revealed the formation of six distinct hydroxylated derivatives of glyburide by each of the four microsomal preparations. However, the amounts of the six metabolites formed by the placentas were a fraction of that formed by the livers. Moreover, the relative quantities of each metabolite formed differed between species as well as between the two tissues. Also, the structure of the unidentified metabolites was determined by comparison with synthesized standards. These metabolites were identified as the 4-*cis*-hydroxycyclohexyl glyburide, 3-*trans*-hydroxycyclohexyl glyburide, and 2-*trans*-hydroxycyclohexyl glyburide. Therefore, one glyburide metabolite remains to be identified, but the data we obtained allowed us to suggest its structure.

1. Introduction

Glyburide (glibenclamide; *N*-4-[β-(5-chloro-2-methoxybenzamidoethyl) benzenesulfonyl]-*N*′-[cyclohexyl] urea) is a second-generation sulfonylurea hypoglycemic drug that has been used successfully for controlling glucose levels in women with pregestational and gestational diabetes mellitus [1–5]. The pharmacokinetics (PK) of glyburide has been determined in healthy individuals [6–9], patients with impaired renal function [10], and those with type-2 diabetes [11] but not for women with gestational diabetes mellitus. The physiological changes associated with the onset of pregnancy in addition to various diseases have a significant effect on the bio-disposition of administered therapeutics. One of the factors affecting the PK of a drug during pregnancy is its disposition in the placenta. The latter includes transfer of the drug to the fetal circulation, metabolism by placental enzymes, and efflux by transporters from the tissue back to the maternal circulation. Data obtained from in vivo [5] and in vitro [1,3] investigations demonstrated a very low

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.08.024

transplacental transfer of glyburide. Other related investigations included the effect of human serum albumin on glyburide transfer and distribution [12] as well as the role of efflux transporters [13,14]. However, to the best of our knowledge, data on the metabolism of glyburide by placental enzymes are scarce to nonexistent.

Glyburide is extensively metabolized by the human liver and two major metabolites identified in the maternal urine: 4-*trans*-hydroxycyclohexyl glyburide and 3-*cis*-hydroxycyclohexyl glyburide. These two metabolites are pharmacologically active and are considered potent hypoglycemic agents [15,16]. Recent reports indicated that microsomes of human, rat, dog, and monkey metabolized glyburide and, depending on the species, up to four new metabolites were formed [17,18]. Currently, the baboon (*Papio cynocephalus*), a nonhuman primate, is being characterized as an animal model for investigating placental transfer and metabolism of drugs during pregnancy, which is not possible in humans due to safety considerations. During pregnancy, the human placenta plays an important role in the metabolism of endogenous compounds, xenobiotics, and environmental toxins [19]. However, the expression and activity of various P450 isoforms depends on placental gestational age and is lower than that in the liver [20,21].

Therefore, the aim of this investigation is to identify the metabolites of glyburide formed by placental microsomes of a human and a nonhuman primate (the baboon) and compare them with those formed by liver microsomes of both species. To achieve this goal, we developed a method to determine the metabolites formed utilizing HPLC–mass spectrometry and described recently in a preliminary report [22] from our laboratory. The newly synthesized standards of the anticipated metabolites were used as an analytical tool to identify those formed in vitro by the four microsomal preparations, namely, human and baboon placentas and livers.

2. Materials and methods

2.1. Chemicals and supplies

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned. Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Glyburide metabolites were synthesized according to the procedure reported by Hill et al. [23] and made available to our laboratory.

2.2. Human and baboon tissues

A pool of 15 donor human liver microsomes was purchased from Cellz Direct (Austin, TX). The pool was made up of livers from males and females between the ages of 24 and 74 years. Human placentas were obtained from the labor and delivery ward of the John Sealy Hospital, Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston, TX according to a protocol approved by the Institutional Review Board. All placentas were obtained from term uncomplicated pregnancies. Human placental microsomal fractions were prepared for this investigation by differential centrifugations as previously described in detail by Zharikova et al. [24]. A pool of 12 preparations was used in the experiments cited in this report. Baboon placentas were obtained from animals undergoing abdominal delivery, and livers were obtained from animals being sacrificed for herd reduction. All tissues were obtained according to an approved protocol by the Institutional Animal Care and Use Committee of the Southwest National Primate Research Center, San Antonio, TX.

2.3. Enzyme-catalyzed reactions

The metabolic activity of microsomal preparations was determined in a reaction solution (1 ml total volume) made of the following components at their respective final concentrations: glyburide, 50 μM; microsomal protein, 1 mg/ml; 0.1 M potassium phosphate (pH 7.4). The contents were preincubated for 5 min at 37 °C, and the reaction initiated by the addition of an NADPH regenerating system (0.4 mM NADP, 4 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase and 2 mM $MgCl_2$). The reaction was incubated for 60 min at the same temperature, terminated by the addition of 10 μl trichloroacetic acid (10%, w/v), and placed on ice. Samples were centrifuged at 10,000 × *g* for 12 min and the pellets were discarded. The metabolites in the supernatant were extracted by a dichloromethane/hexane (1:1) solution. The organic phase containing the metabolites was siphoned off and evaporated to dryness; the residue reconstituted in 200 μl of the HPLC mobile phase.

2.4. Experimental conditions for HPLC

The samples were analyzed using a Waters μbondapack, C18 (Milford, MA), 3.9 mm × 100 mm column packed with 10 μm particles (125 Å, average pore size). Isocratic elution (Waters 600 pump; Milford, MA) was carried out at a flow rate of 1 ml/min with a mobile phase of acetonitrile:water 35:65. Acetic acid was used to adjust the pH of the mobile phase to 3.5. The sample injection volume was 100 μl (Waters 717 auto sampler; Milford, MA). The effluent from the column was transferred to the LCMS interface with postcolumn splitting; approximately 700 μl of the effluent was directed to waste and 300 μl to the mass spectrometer.

2.5. Experimental conditions for mass spectrometry

The spectrometer (Waters EMD 1000 single-quadrupole; Milford, MA) was equipped with an electrospray ion source (ESI) operated in positive-ion mode and maintained at a temperature of 120.0 °C; liquid nitrogen was the desolvation gas with a flow rate of 650 l/h and temperature of 300 °C. The mass resolution was 1 Da (1 amu). The capillary voltage was 3.2 kV, and the cone voltage was 35 V. Radio frequency and direct current voltages were 2 and 0 V, respectively. The mass range of 100–1000 Da was scanned at a rate of 1 scan/s. Selected-ion monitoring (SIM) at *m/z* 510 [MH+] enabled mass spectral characterization of the metabolites detected in the total ion current (TIC) chromatograms.

2.6. Identification of the standard compounds (metabolites) by HPLC–MS

The metabolites of glyburide were synthesized by Dr. Hill according to the methods described previously [23]. Each of the synthesized standards (M1, M2a, M2b, M3, and M4) was dissolved in acetonitrile/water (50:50). M1 is 4-*trans*-hydroxycyclohexyl glyburide; M2a, 4-*cis*-hydroxycyclohexyl glyburide; M2b, 3-*cis*-hydroxycyclohexyl glyburide; M3, 3-*trans*-hydroxycyclohexyl glyburide; M4, 2-*trans*-hydroxycyclohexyl glyburide. Solution compositions were similar to that of hepatic and placental microsomes. The HPLC and MS experimental conditions for analysis are detailed in Sections 2.4 and 2.5.

3. Results

3.1. Extracted ion current for the six metabolites of glyburide formed by human and baboon liver microsomes

Each of the six glyburide metabolites is referred to in this text by an "M" followed by a number that corresponds to its elution order, i.e., M1 for the metabolite with lowest retention time, M2 for the metabolite with the next lowest retention time, and so on. The six metabolites of glyburide were formed upon incubation with human liver (Fig. 1a) and baboon liver (Fig. 1b) microsomes eluted with identical retention times, suggesting that they are most likely the same compounds. However, the quantities of the metabolites formed by human liver microsomes, relative to each other, were significantly different from those produced by the baboon liver microsomes. For the human liver microsomes, metabolites M1 and M5 were formed in large quantities; whereas, metabolite M5 was predominant for the baboon liver microsomes.

For human liver microsomes, the ion current extracted at *m*/*z* 510 indicated the formation of six monohydroxylated metabolites as identified by their retention times. The retention times for the first five metabolites were as follows: M1, 16.6 min; M2a, 19.2 min; M2b, 19.8 min; M3, 21.8 min; M4, 28.3 min; M5, 45.2 min. Assuming a reasonable mass-proportionality of TIC peak area response, the amounts of metabolites formed by the human liver were calculated relative to that for M1. Accordingly, the metabolites formed relative to M1 (100%) were M2a, 46%; M2b, 49%; M3, 18%; M4, 11%; M5, 98%. Therefore, the observed rank order for the metabolites formed by human liver microsomes was M1 ≥ M5 > M2b ≥ M2a > M3 > M4.

For the baboon liver microsomes, the amount of M5 was the highest and was set as 100%. The other metabolites are presented as a percentage of M5: M1, 46%; M2a, 44%; M2b, 40%; M3, 27%; M4, 8% (i.e., M5 > M1 ≥ M2a ≥ M2b > M3 > M4).

3.2. Mass spectra of glyburide metabolites formed by human liver microsomes

The mass spectrum of each metabolite was obtained by LC–MS to elucidate its structure (Fig. 2). The mass spectrum for each of the six metabolites indicated a molecular weight at *m*/*z* 510, which corresponds to the mono-oxygenation of glyburide. The metabolites M1, M2a, M2b, M3 and M4 display a predominant daughter ion peak at *m*/*z* 369; whereas, M5 did not display this peak, but rather displayed a daughter ion peak at *m*/*z* 385. M3 displays a prominent peak at *m*/*z* 492.

3.3. Selected-ion monitoring at m/z 510 for synthesized glyburide metabolites (standards)

To assign the structures of the hydroxylated metabolites of glyburide, several of the possible metabolites were synthesized according to the procedures reported by Hill et al. [23]. The position of the hydroxyl group substitution on the cyclohexyl ring of glyburide had a significant effect on the chromatographic retention time. The retention times of the synthetic standards were as follows: 4-*trans*-hydroxycyclohexyl glyburide (M1), 16.6 min; 4-*cis*-hydroxycyclohexyl glyburide (M2a), 19.2; 3-*cis* (M2b), 19.8 min; 3-*trans* (M3), 21.8 min; 2-*trans* (M4), 28.1 min (Fig. 3). These retention times were used to tentatively identify the unknown metabolites formed during the metabolism of glyburide, and these identities were then confirmed by comparison of the mass spectra.

3.4. Metabolism of glyburide by human placental microsomes

Glyburide was metabolized by full-term human placental microsomes, as observed in liver microsomes, i.e., six compounds were formed but one of them (M2a) was formed in a minute amount (Fig. 4b). The quantities of the monohydroxylated metabolites were a fraction of that formed by human liver microsomes. Each metabolite formed under comparable incubation conditions by human placental

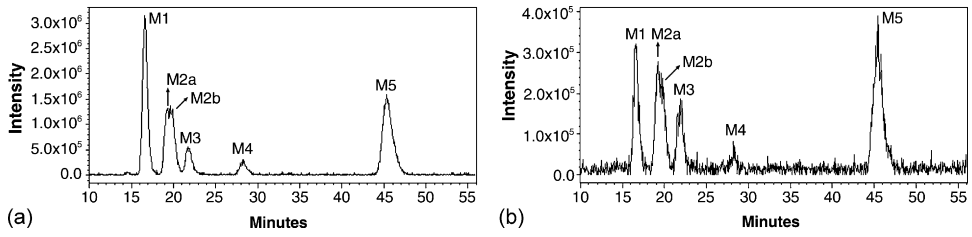

Fig. 1 – Extracted ion chromatogram of *m*/*z* 510 from high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis for glyburide metabolites formed by (a) human liver microsomes; (b) baboon liver microsomes; M1-4, *trans*-hydroxycyclohexyl glyburide; M2a, 4-*cis*-hyroxycyclohexyl glyburide; M2b, 3-*cis*-hydroxycyclohexyl glyburide; M3, 3-*trans*-hydroxycyclohexyl glyburide; M4, 2-*trans*-cyclohexyl glyburide; M5, ethyl hydroxycyclohexyl glyburide.

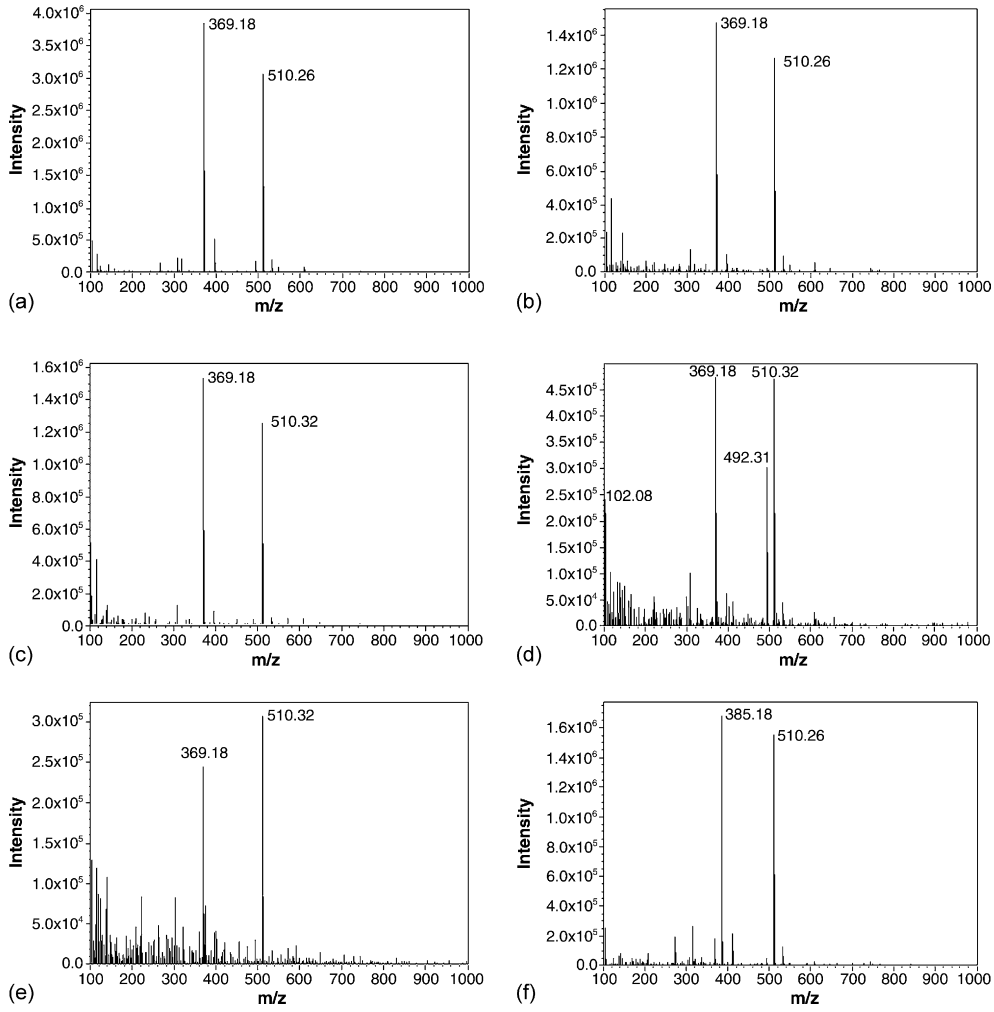

Fig. 2 – Mass spectra for the six metabolites of glyburide formed by human liver microsomes: (a) 4-*trans*-hydroxycyclohexyl glyburide, (b) 4-*cis*-hydroxycyclohexyl glyburide, (c) 3-*cis*-hydroxycyclohexyl glyburide, (d) 3-*trans*-hydroxycyclohexyl glyburide, (e) 2-*trans*-hydroxycyclohexyl glyburide, and (f) ethyl hydroxycyclohexyl glyburide.

microsomes is expressed as a percentage of the amount formed upon incubation with human liver microsomes; M1, 0.9%; M2a, 0.18%; M2b, 0.71%; M3, 2.6%, M4, 4.4%; M5, 13.3%. SIM was used to confirm the metabolites formed by human placental microsomes (Fig. 4). The metabolites formed by placental microsomes (Fig. 4b) and human liver microsomes (Fig. 4a) had comparable retention times. Therefore, it is most likely that the metabolites formed by placental microsomes are identical to those formed by human liver microsomes (the small quantities made it impossible to acquire definitive mass spectra).

3.5. *Metabolism of glyburide by baboon placental microsomes*

The amounts of monohydroxylated metabolites formed upon incubation with baboon placental microsomes were less than those formed by human placental microsomes except for M4 (Fig. 5). The amounts of metabolites of glyburide formed by baboon placental microsomes expressed as a percent of the amounts of the corresponding metabolites formed by baboon liver microsomes were as follows: M1, 5.0%; M2a, 0.46%; M2b, 1.9%; M3, 1.3%; M5, 4.2%. The predominant metabolite formed by baboon placental microsomes was M4 (2-*trans*-hydroxycyclohexyl glyburide) and its quantity was greater than that observed for baboon liver microsomes. The amount of the latter was 77% of that formed by baboon placental microsomes.

The retention times for the metabolites formed by baboon placenta were similar to those of human placenta, human liver, and baboon liver. Ion current chromatograms extracted at *m*/*z* 510 for the synthesized standards and the comparability of mass spectra confirm (except for chirality issues) the identities of the metabolites formed upon metabolism of glyburide by human and baboon liver microsomes, and human and baboon placental microsomes.

4. Discussion

Glyburide is a hypoglycemic drug that could be used for treatment of gestational diabetes; this drug is currently in

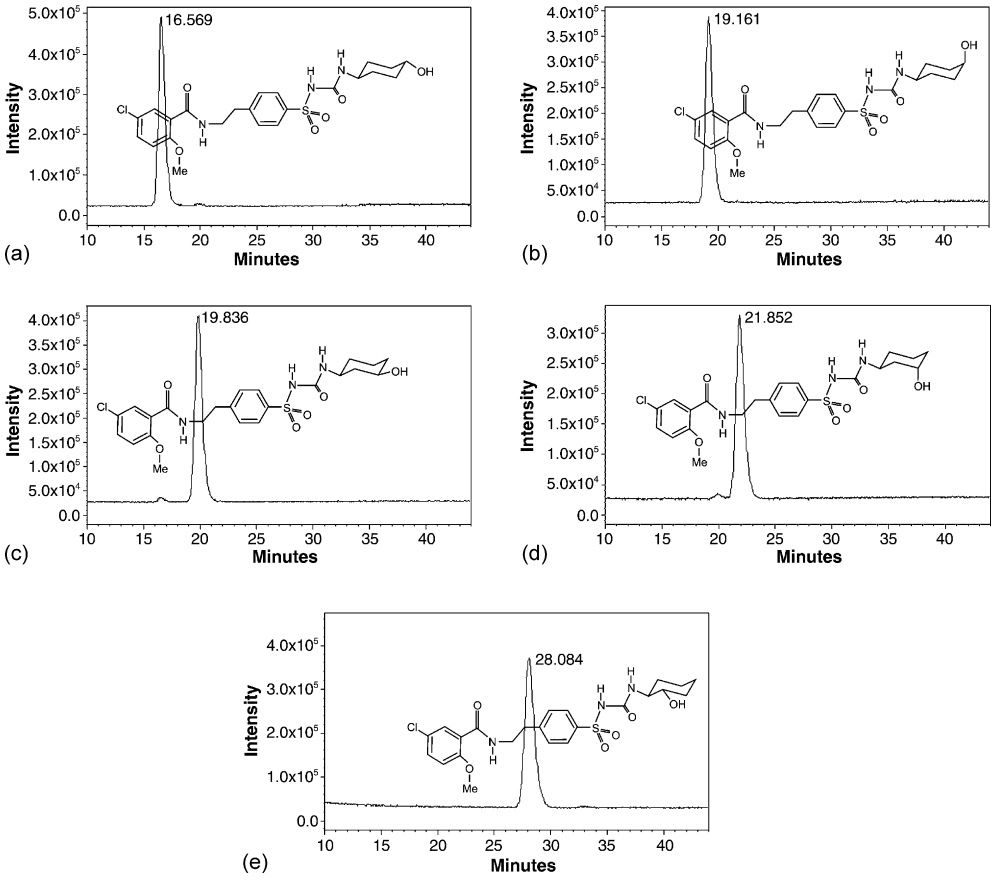

Fig. 3 – The reconstructed ion chromatogram of *m/z* 510 for the synthesized standards: (a) 4-*trans*-hydroxycyclohexyl glyburide, (b) 4-*cis*-hydroxycyclohexyl glyburide, (c) 3-*cis*-hydroxycyclohexyl glyburide, (d) 3-*trans*-hydroxycyclohexyl glyburide, and (e) 2-*trans*-hydroxycyclohexyl glyburide in the high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis.

clinical trial to determine its efficacy and pharmacokinetics. Human placental disposition of a drug is one of the factors affecting the changes in its PK observed during pregnancy. Therefore, the aim of this investigation was to identify the metabolites of glyburide formed by human placental microsomal preparations and compare them with those formed by hepatic microsomes. Moreover, ongoing investigations in our laboratory have focused on the PK of glyburide in pregnant baboons—a nonhuman primate having 98% DNA homology with humans and also known to suffer from gestational diabetes. Therefore, the metabolites formed by human hepatic and placental microsomes were compared with those formed by baboon livers and placentas.

HPLC–MS is more sensitive than HPLC–ultraviolet (UV) to identify and quantify the metabolites formed in lower quantities. The amount of each glyburide metabolite formed by placental microsomes was significantly lower than that formed by the liver microsomes. The results obtained in the process of identifying the monohydroxylated metabolites produced by liver microsomes were very useful in assisting the identification of those formed in much lesser quantities by placental microsomes. Earlier reports on the in vivo metabo-

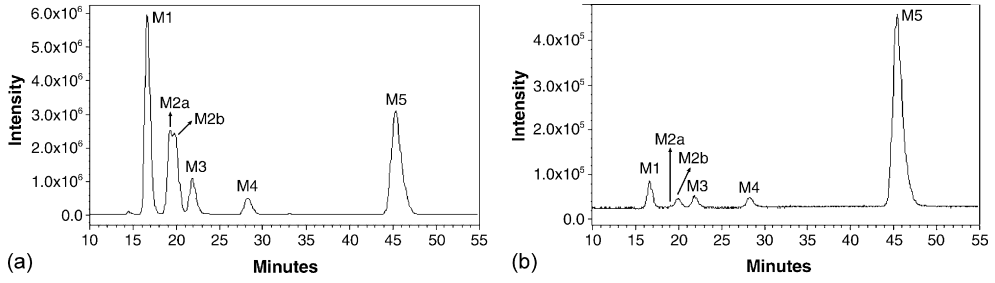

Fig. 4 – The reconstructed ion chromatogram of *m/z* 510 from high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis for glyburide metabolites formed by (a) human liver microsomes and (b) human placental microsomes.

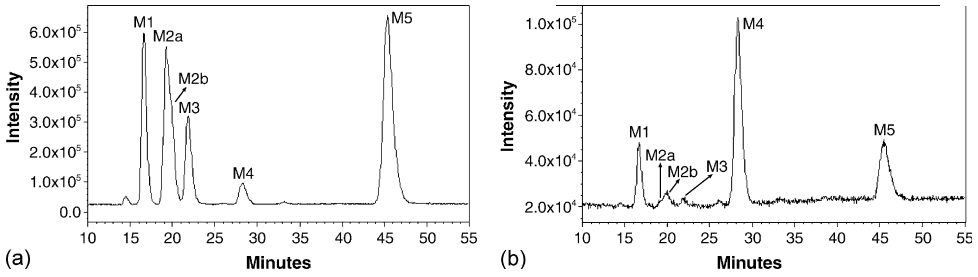

Fig. 5 – The reconstructed ion chromatogram of *m/z* 510 from high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis for glyburide metabolites formed by (a) baboon liver microsomes and (b) baboon placental microsomes.

lism of glyburide revealed the formation of two hydroxylated compounds: namely, the 4-*trans*-hydroxycyclohexyl glyburide and 3-*cis*-hydroxycyclohexyl glyburide. These compounds were identified in human urine and plasma [7,25–30] and have also been shown to be pharmacologically active, i.e., exhibit hypoglycemic effects [15,16]. In this investigation, our data confirmed that these two metabolites were formed in vitro by human and baboon liver microsomes and were referred to in this report as M1 and M2b on the basis of their HPLC retention times being 16.5 and 19.8 min, respectively.

Two more recent reports on the in vitro metabolism of glyburide by human hepatic microsomes [17,18], and by rats, dogs, and monkeys [17] indicated the formation of at least eight hydroxylated metabolites. These metabolites were not identified ambiguously; the results clearly indicated that for at least six metabolites, the cyclohexyl ring of glyburide has been hydroxylated at different positions. In the substitution of a hydrogen in the cyclohexyl ring by a nonhydrogen substituent (in this case, a sulfonylurea moiety), the other eleven positions become nonequivalent. This is depicted in Fig. 6. The chair conformer of the cyclohexane moiety (as shown) predominates because it allows the large sulfonylurea substituent to be disposed equatorially. Of the 11 positions where hydroxylation can occur in this conformer, 6 are axial and 5 are equatorial. It should be noted that the cyclohexyl carbons 2 and 6, as well as carbons 3 and 5, are chemically equivalent but chirally distinct. For example, hydroxyl substitution at the C-3 equatorial position versus the C-5 equatorial constitutes a pair of enantiomers (which would not be chromatographically distinguishable except with a suitable chiral chromatographic system). Therefore, seven chromatographically distinguishable hydroxycyclohexyl isomers are possible: namely, C-1, C-2 (axial, equatorial), C-3 (axial, equatorial), and C-4 (axial, equatorial), which include C-2 (*cis*, *trans*), C-3 (*trans*, *cis*), and C-4 (*cis*, *trans*).

In this investigation, the mass spectra for glyburide metabolites formed by human liver microsomes were similar to those reported earlier [18]. The first five metabolites (M1, M2a, M2b, M3, and M4) displayed a parent ion peak at *m/z* 510 and a daughter ion peak at *m/z* 369 (Fig. 2). This data suggests that the substitution of the hydroxyl group in these metabolites occurs in the cyclohexane ring. However, the sixth metabolite (M5) exhibited a daughter ion at *m/z* 385, which, together with the mass spectral fragmentation reported by Tiller et al. [17] and Zhang et al. [18], allows the following to be concluded: M5 is formed by hydroxylation of one of the carbon atoms in the ethyl moiety located between the central aromatic ring and the benzamide group. At this time, it is impossible to identify which of these two carbons has been hydroxylated.

Metabolite M3 exhibits an additional fragment signal at *m/z* 492 due to the loss of a water molecule (*m/z* 18) from the hydroxycyclohexyl glyburide and suggests that it was a hydroxycyclohexyl metabolite having an axial hydroxyl group with sulfonylurea group in an equatorial position. This suggestion was confirmed by its retention time and mass spectral fragmentation pattern being identical to that of the synthesized 3-*trans*-hydroxycyclohexyl glyburide. In earlier reports [17,18], the fragmentation patterns obtained from the mass spectra distinguished metabolite M5 from the others but

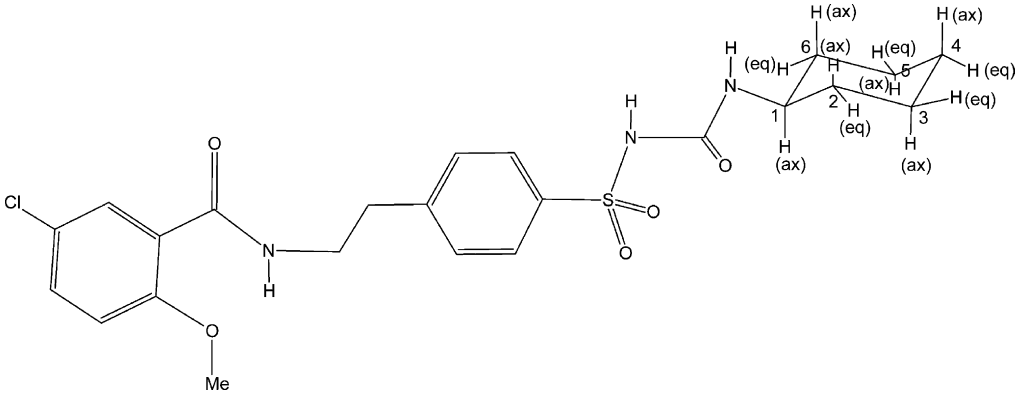

Fig. 6 – Structure of glyburide (cyclohexane ring in the chair form is shown with axial and equatorial positions).

did not differentiate between the hydroxylation positions in M1, M2a, M2b, M3, and M4. The structures of M1 and M2b were confirmed by comparison to synthesized standards, but not for the remaining metabolites (M2a, M3, and M4) (Figs. 4a and 5a) to the best of our knowledge. Accordingly, these metabolites were synthesized [23], and their retention times and mass spectral characteristics were determined. The data obtained allowed unambiguous identification (excepting chirality) of the metabolites M2a, M3, and M4 as 4-*cis*-hydroxycyclohexyl glyburide, 3-*trans*-hydroxycyclohexyl glyburide, and 2-*trans*-hydroxycyclohexyl glyburide, respectively.

The mass spectra obtained for glyburide metabolites formed by baboon liver microsomes exhibited fragmentation pattern similar to those metabolites formed by human liver microsomes. For human and baboon placental microsomes, the mass spectra for all the metabolites observed in the TIC chromatograms had a prominent signal at *m*/z 510. Also, the quantities of the metabolites formed were much lower than those formed by hepatic microsomes and consequently a clear fragmentation pattern could not be obtained. However, the use of SIM allowed comparison of the data for the metabolites formed by placental microsomes with those data for human liver microsomes. Moreover, the retention times of all the metabolites formed were identical, regardless of the source of the microsomal fractions. Therefore, it is reasonable to assume that glyburide metabolites, irrespective of the primate and tissue, are the same except that chirality issues remain unresolved.

Six glyburide metabolites were formed by human placental and hepatic microsomes. The metabolites M1, M2b, M3, and M4 were formed by placenta in small and approximately equal amounts with a minute amount of M2a, while M5 was higher in quantity. At this time, it is unclear whether the greater amount of M5 formed by placenta than by the liver is due to a difference in the activity of the same enzyme in the two tissues or the presence of two different isozymes. The identification of the responsible enzyme(s) is currently under investigation.

The metabolism of glyburide by baboon placental microsomes revealed the formation of six metabolites (Fig. 5b) with the amount of M4 being higher than M5; whereas, M5 had the highest amount of those formed by human placental microsomes.

In summary, HPLC–MS proved to be a valuable tool in identifying the structure of the metabolites of glyburide formed in vitro by human and baboon hepatic and placental microsomes. The metabolites formed were identified on the basis of their retention times and fragmentation patterns being identical to those of the synthesized standards. The previously reported unknown metabolites have been identified as 4-*cis*-hydroxycyclohexyl glyburide, 3-*trans*-hydroxycyclohexyl glyburide, and 2-*trans*-hydroxycyclohexyl glyburide. It is also apparent that the amounts of metabolites formed by baboon and human placental microsomes are a fraction of those formed by the liver microsomes, except for M4. The amounts of M4 formed by baboon placental microsomes are higher than that formed by baboon liver. Moreover, the metabolites formed by human and baboon microsomes are identical, but their relative quantities are different. The latter may be attributed to different levels of expression of the same enzyme between human and baboons, or by two different isozymes. Currently, we are investigating the identification of the enzyme(s) responsible for the metabolism of glyburide by microsomes of human and baboon tissues.

Acknowledgements

This investigation was supported by the Obstetric-Fetal Pharmacology Research Units Network (OPRU/NICHD). The assistance of the following personnel is greatly appreciated: the medical staff of the labor and delivery ward of the John Sealy hospital; the Perinatal Research Division; the Publication, Grant, & Media Support Office in the Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston, TX.

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