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Quantitative determination of metformin, glyburide and its metabolites in plasma and urine of pregnant patients by LC-MS/MS

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Abstract

This report describes the development and validation of an LC-MS/MS method for the quantitative determination of glyburide (GLB), its five metabolites (M1, M2a, M2b, M3, and M4) and metformin (MET) in plasma and urine of pregnant patients under treatment with a combination of the two medications. The extraction recovery of the analytes from plasma samples ranged between 87% and 99%, and 85%–95% for urine samples. The differences in retention times among the analytes, and the wide range of the concentrations of the medications and their metabolites in plasma and urine patient samples required the development of three LC methods. The lower limit of quantitation (LLOQ) of the analytes in plasma samples was as follows: GLB, 1.02 ng/mL; its five metabolites, 0.100–0.113 ng/mL and 4.95 ng/mL for MET. LLOQ in urine samples was 0.0594 ng/mL for GLB, 0.984–1.02 ng/mL for its five metabolites and 30.0 µg/mL for MET. The relative deviation of this method was < 14% for intra-day and inter-day assays in plasma and urine samples, and the accuracy ranged between 86% and 114% in plasma, and 94% to 105% in urine. The method described in this report was successfully utilized for determining the concentrations of the two medications in patient plasma and urine.

Keywords

Metformin; Glyburide; metabolites; Gestational diabetes mellitus; LC–MS/MS

Introduction

Gestational diabetes mellitus (GDM), constitutes a major health problem in pregnancy, could lead to insufficient glucose tolerance, and is associated with increased risk of neonatal macrosomia and hypoglycemia (Singh *et al.*, 2008; Mitanchez *et al.*, 2010). Glyburide (GLB) and Metformin (MET) (Fig. 1) are oral hypoglycemic drugs FDA approved by the Food and Drug Administration of United States for treatment of type 2 diabetes in none pregnant patients (Nicholson *et al.*, 2010). The co-administration of GLB and MET provides

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better glycemic control than that reported for monotherapy with either medication. This is explained by their complementary mechanisms of actions namely, GLB enhances insulin secretion while MET decreases hepatic gluconeogenesis and glucose absorption, and increases sensitivity to insulin (Blonde *et al.*, 2002; Lamkanfi *et al.*, 2009; Slagle *et al.*, 2002). However, to the best of our knowledge, there are no reports on the use of the combination of GLB and MET in treatment of GDM.

Neonates of GDM patients are at risk of hypoglycemia and it is unclear whether trans-placental transfer of the oral hypoglycaemic medications and or their metabolites could be a contributing factor (Gandhi *et al.*, 2012; Flores-le Roux *et al.*, 2012). In addition, pregnancy is associated with physiological changes that affect the pharmacokinetics of medications including clearance (Frederiksen *et al.*, 2001; Unadkat *et al.*, 2007) and result in plasma levels that are different from those of non-pregnant patients (Naraharisetti *et al.*, 2007). Therefore, the obstetric-fetal pharmacology research units (OPRU) network of NICHD of which UTMB is a center, launched a clinical trial to determine the pharmacokinetics of the combination of GLB and MET in pregnant patients under treatment for GDM as well as several other parameters and outcomes.

Investigations of the *in vivo* metabolism of GLB revealed two major hydroxylated metabolites that are pharmacologically active namely, 4-trans-hydroxycyclohexyl glyburide (M1) and 3-cis-hydroxycyclohexyl glyburide (M2b) (Fig. 1) (Rydberg *et al.*, 1995). More recent investigations of the *in vitro* biotransformation of GLB revealed the formation of four other metabolites that represented 50% of the total metabolites formed namely, 4-cis-hydroxycyclohexyl glyburide (M2a), 3-trans-hydroxycyclohexyl glyburide (M3), 2-trans-hydroxycyclohexyl glyburide (M4) and ethyl hydroxycyclohexyl glyburide (M5) (Fig. 1) (Zharikova *et al.*, 2009; Zharikova *et al.*, 2007). On the other hand, metformin is eliminated primarily by the kidneys and no metabolites have been identified (Eyal *et al.*, 2010).

There are several analytical methods for determining the concentrations of GLB, MET, GLB and its two major metabolites (M1, or both M1 and M2b), or GLB and MET in human plasma or urine. These methods are as follows: GLB was determined in plasma or urine using HPLC-UV (Rydberg *et al.*, 1995; Gedeon *et al.*, 2008), LC-MS/MS (Albu *et al.*, 2007; Hsieh *et al.*, 2002; Chen *et al.*, 2004; Georgita *et al.*, 2007) or micellar electrokinetic chromatography (Núñez *et al.*, 1995). The reported LLOQ of GLB ranged between 0.25 and 50 ng/mL (Rydberg *et al.*, 1995; Albu *et al.*, 2007; Hsieh *et al.*, 2002; Chen *et al.*, 2004; Gedeon *et al.*, 2008; Núñez *et al.*, 1995). There are also two reports on the simultaneous quantitative determination of GLB and its metabolites in human plasma and urine using HPLC-UV and HPLC-MS/MS (Naraharisetti *et al.*, 2007; Rydberg *et al.*, 1995). However, these two methods are not adequate for determining the pharmacokinetics of GLB in plasma and urine samples because of their limited sensitivity (LLOQ of the metabolites > 0.4 ng/mL). In addition, the retention of MET on reverse-phase chromatography was poor and to overcome the problem the following methods have been developed: Ion-pair chromatography (Zarghi *et al.*, 2003; Liu *et al.*, 2009), hydrophilic interaction chromatography (Huttunen *et al.*, 2009), and nitrile-modified reverse-phase liquid chromatography (Georgita *et al.*, 2007). Nevertheless, these methods suffered from low sensitivity (Chen *et al.*, 2004; Zarghi *et al.*, 2003; Liu *et al.*, 2009) or poor retention times

(Georgita *et al.*, 2007). Moreover, to the best of our knowledge, there are no reports on the simultaneous quantitative determination of GLB, its six metabolites (M1, M2a, M2b, M3, M4, and M5) and MET in human biological fluids (plasma and urine).

Therefore, the aim of this investigation is to develop and validate an LC–MS/MS method for the quantitative determination of GLB, its five metabolites and MET in plasma and urine of GDM patients under treatment with a combination of the two medications. This method was crucial for determining the PK of the combination of the two medications used for treating GDM in an ongoing clinical trial conducted by OPRU.

Experimental

Chemicals and reagents

Metformin (MET), glyburide (GLB), and glipizide were purchased from Sigma (Poole, England). Glyburide-d₁₁ and metformin-d₆ hydrochloride were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). The metabolites of glyburide: 4-trans-hydroxycyclohexyl glyburide (M1) (purity ~ 99.9%), 4-cis-hydroxycyclohexyl glyburide (M2a) (purity ~ 96.0 %), 3-cis-hydroxycyclohexyl glyburide (M2b) (purity ~ 98.9%), 3-trans-hydroxycyclohexyl glyburide (M3) (purity ~ 97.8%), and 2-trans-hydroxycyclohexyl glyburide (M4) (purity ~ 96.0 %) were synthesized by Dr. Hill and made available to our laboratory (Hill *et al.*, 2003). Acetonitrile, methanol, ammonium acetate, ethyl acetate and acetic acid were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, USA). Blank human plasma was purchased from Innovative Research (Peary Court, USA). Blank human urine was collected from 6 healthy volunteers.

Preparation of the stock solutions, calibration standards and quality control (QC) samples for plasma analysis

Glyburide-d₁₁, glipizide, and metformin-d₆ hydrochloride were chosen as internal standards (IS) for the quantification of GLB, its metabolites (M1, M2a, M2b, M3, and M4), and MET, respectively. The IS stock solutions were prepared by dissolving standards in methanol to achieve the following concentrations: Glyburide-d₁₁, 2.00 µg/mL; glipizide, 1.00 µg/mL and metformin-d₆ hydrochloride, 4.00 µg/mL. A stock solution of the seven analytes was prepared in methanol in the following concentrations: GLB, 16.4 µg/mL; M1, 6.56 µg/mL; M2a, 6.96 µg/mL; M2b, 6.44 µg/mL; M3, 6.40 µg/mL; M4, 7.20 µg/mL and MET, 119 µg/mL. Appropriate dilutions of the stock solutions with methanol were used to prepare the working standard solutions. Calibration standards were prepared freshly by spiking 10 µL of the working standard into 400 µL of blank human plasma to yield the following ranges of concentrations: GLB, 1.02–410 ng/mL; M1, 0.102–164 ng/mL; M2a, 0.109–174 ng/mL; M2b, 0.101–161 ng/mL; M3, 0.100–160 ng/mL; M4, 0.113–180 ng/mL and MET, 4.95–2.97 × 10³ ng/mL. The QC samples for low, medium and high concentrations were prepared as described for calibration standards and were as follows: GLB, 2.05, 205, 410 ng/mL; M1, 0.818, 81.8, 164 ng/mL; M2a, 0.872, 87.2, 174 ng/mL; M2b, 0.806, 80.6, 161 ng/mL; M3, 0.800, 80.0, 160 ng/mL; M4, 0.900, 90.0, 180.0 ng/mL and MET, 14.9, 1.49 × 10³, 2.97 × 10³ ng/mL. All standard solutions were stored at 4 °C.

Preparation of the stock solutions, calibration standards and quality control (QC) samples for urine analysis

The IS stock solutions were prepared in methanol containing: 0.800 µg/mL glyburide-d₁₁, 4.11 µg/mL glipizide, and 80.0 µg/mL metformin-d₆ hydrochloride. The stock solutions of the seven analytes in methanol were prepared in the following concentrations: GLB, 0.952 µg/mL; M1, 19.8 µg/mL; M2a, 19.7 µg/mL; M2b, 20.4 µg/mL; M3, 20.1 µg/mL; M4, 19.7 µg/mL and MET, 1.80 mg/mL. The working standard solutions were prepared in methanol by serial dilutions of the stock to the desired concentrations. Calibration standards were freshly prepared by spiking 10 µL of the working standard solution in 400 µL of blank human urine to yield the following concentration ranges: GLB, 0.0594–23.8 ng/mL; M1, 0.989–494 ng/mL; M2a, 0.984–492 ng/mL; M2b, 1.02–511 ng/mL; M3, 1.00–503 ng/mL and M4, 0.984–492 ng/mL. MET samples were freshly prepared by spiking 10 µL of working standards solution into 20 µL of blank human urine to concentrations of 30.0–899 µg/mL. The QC samples of low, medium and high concentrations were prepared as described above and were as follows: GLB, 0.297, 11.9, 23.8 ng/mL; M1, 4.94, 198, 494 ng/mL; M2a, 4.92, 197, 492 ng/mL; M2b, 5.11, 204, 511 ng/mL; M3, 5.03, 201, 503 ng/mL; M4, 4.92, 197, 492 ng/mL and MET, 6.00×10^4 , 6.00×10^5 , 8.99×10^5 ng/mL. All standard solutions were stored at 4 °C.

Sample preparation

Preparation of plasma samples—The plasma samples were prepared by protein precipitation method as follows: IS solution (10 µL) was added to calibration standards samples or 400 µL of patient plasma samples, vortexed for 1 min followed by the addition of 1.2 mL of acetonitrile containing 0.1% (v/v) formic acid. The solution was vortexed again for 3 min and centrifuged at $12,000 \times g$ for 15 min at 4 °C. The supernatant was transferred to a tube and dried under air stream at 40 °C. The residue was reconstituted with 120 µL of aqueous ammonium acetate buffer (5 mM, pH = 5.0)–methanol solution (50:50, v/v) and filtered.

GLB and M5 determination: Aliquots (10 µL) of the reconstituted samples were injected into the LC-MS/MS.

M1, M2a, M2b, M3 and M4 determination: Aliquots (50 µL) of the reconstituted samples were injected into the LC-MS/MS.

MET determination: The above reconstituted sample (10 µL) was further diluted with 400 µL of aqueous ammonium acetate buffer (20 mM, pH = 4.0)–acetonitrile solution (15:85, v/v), and 10 µL of the diluted sample was injected into the LC-MS/MS.

Preparation of urine samples

GLB and its metabolites determination: The urine samples were prepared by liquid-liquid extraction method as follows: IS solution (10 µL) was added to calibration standard samples or 400 µL of patient urine samples, vortexed for 1 min followed by the addition of 1.0 mL of ethyl acetate. They were vigorously shaken for 3 min and centrifuged at $12,000 \times g$ for 15 min at 4 °C. The extraction procedure was repeated once more. The two extracts were

combined and evaporated to dryness in an analytical evaporator. The residue was reconstituted in 120 μ L of an aqueous solution made of (50:50, v/v) 5 mM ammonium acetate buffer (pH = 5.0) and methanol. Aliquots of the samples were injected into the LC-MS for analysis of the following: GLB and its metabolite M5 (50 μ L); the other five metabolites of GLB (M1, M2a, M2b, M3 and M4) (10 μ L).

MET determination: The urine samples were prepared by direct dilution as follows: The IS solution (10 μ L) was added to 20 μ L of urine sample and vortexed for 1 min. The solution was diluted with aqueous ammonium acetate buffer (20 mM, pH = 4.0)-acetonitrile solution (15:85, v/v) to achieve a final dilution of 1: 5,000. Then, 10 μ L aliquots of the samples were injected into the LC-MS/MS.

HPLC-MS/MS conditions

—Analysis of human plasma and urine samples were carried out using an Agilent HPLC 1200 series system coupled with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The HPLC system consisted of a degasser, binary pump delivery system, and Hip-ALS auto-sampler controlled by AnalystTM 1.5 Software (MDS Inc. and Applera Corporation, USA).

Separation of GLB and M5: HPLC was performed at 40 °C on an Agilent Eclipse XDB-C₁₈ column (150 mm $\times$ 4.6 mm, 5 μ m) preceded by a Phenomenex C₁₈ guard column (4 mm $\times$ 3.0 mm). The mobile phase consisted of an aqueous ammonium acetate buffer (5 mM, pH = 5.0) and methanol. Samples were eluted isocratically by 70% methanol for 10 min at a flow rate of 0.8 mL/min. The LC effluent was introduced into ESI source using a post-column splitting ratio of 1:3 (retained/waste).

Separation of M1, M2a, M2b, M3, and M4: Chromatographic separation was carried out on an Agilent Eclipse XDB-C₁₈ column (150 mm $\times$ 4.6 mm, 5 μ m) for 19 min. Elution was isocratic using 50% methanol at 40 °C with a flow rate of 0.8 mL/min. Following each injection, the HPLC column was washed with 90% methanol (7 min) to remove phospholipids residue. The column was then re-equilibrated with 50% methanol for 9 min. The aqueous mobile phase and splitting ratio for the mass spectrometer were as described above.

Separation of MET: HPLC was performed at 40 °C on a Waters Atlantis HILIC Silica column (150 mm $\times$ 2.1 mm, 3 μ m) connected to a Phenomenex C₁₈ guard column (4 mm $\times$ 3.0 mm). The aqueous mobile phase consisted of ammonium acetate buffer (20 mM, pH = 4.0) and acetonitrile. Samples were eluted at a flow rate of 0.5 mL/min using isocratic elution with 85% acetonitrile for 15 min. The LC effluent was introduced into the ESI source without splitting.

The API 4000 triple quadrupole mass spectrometer was equipped with a Turbo (V) ion source (ESI) and operated in positive mode. Optimal MS parameters are shown in Table 1. The compound-dependent parameters for each multiple reaction monitoring (MRM) transition of analytes and deuterium-labeled internal standards are shown in Table 2.

Method validation

The method was validated for selectivity, matrix effects, linearity, sensitivity, accuracy, precision, extraction recovery, and stability according to the US Food and Drug Administration Guidelines for Industry (FDA, 2001). The selectivity was evaluated by preparing and analyzing six blank human plasma and urine samples to assess the potential of interfering compounds that co-elute with the analytes or IS. The matrix effect of the analytes and IS was evaluated by matrix factor by comparing the analyte peak areas of the post-extracted samples with those of standards at three QC concentrations in six different blank human plasma and urine samples. The efficiency for extraction of the analytes from human plasma and urine was carried out by comparing the peak areas of analytes in QC samples with those in post-extraction spiked samples at three QC concentrations in six replicates. Linearity was evaluated by calculation of a regression line using a weighted linear least-squares regression model. Precision and accuracy were determined by analyzing five sets of spiked plasma and urine QC samples at LLOQ, low, medium, and high concentrations on the same day and over three days. Accuracy of the method was evaluated by comparing the calculated concentration using calibration curves, with the added concentration. The stability of the analytes in plasma and urine was investigated at low and high concentrations; the tested conditions included 4 h at room temperature (22–25 °C), 30 days at 70°C and three freeze–thaw cycles.

Cross-validation against the previous method was carried out using the incurred plasma samples from 10 % of patients; deviations between the new and original value should be less than 20% for at least two-thirds of the samples.

Results and discussion

Quantification ranges of analytes in patient samples

Reports on the concentrations of GLB, two of its metabolites and MET in biological fluids indicated the following: the concentration of GLB in patients plasma samples ranged between 1 and 380 ng/mL (Rydberg *et al.*, 1995; Albu *et al.*, 2007; Hsieh *et al.*, 2002; Georgita *et al.*, 2007); M1 and M2b, ranged between 1 and 15 ng/mL (Naraharisetti *et al.*, 2007; Rydberg *et al.*, 1995) while for MET it was between 5 and 1700 ng/mL (Georgita *et al.*, 2007). In addition, GLB was not detected in patients' urine but its M1 and M2b metabolites were present in significant concentrations (Rydberg *et al.*, 1995). It is apparent that there is a wide difference in concentrations among GLB, its metabolites and MET in plasma and urine samples. Thus these analytes can't be analyzed by one LC injection. Therefore the samples were divided according to the ranges of concentrations of the analytes in plasma as follows: Low range for GLB metabolites, medium (GLB) and high (MET). For urine samples, the three ranges were low for GLB, higher for GLB metabolites and highest for MET. Consequently, three different injection volumes of analytes were developed.

In the method reported here, the sensitivity for determining the metabolites of GLB in plasma and GLB in urine was considerably improved by using 400 µL of each sample and increasing the injection volume. The resulted LLOQ for the five metabolites of GLB (M1, M2a, M2b, M3 and M4) in plasma were 0.100–0.113 ng/mL and GLB in urine was 0.0594

ng/mL. The quantification of high levels of MET in plasma, following measurement of GLB and its metabolites, was possible by further dilution of the reconstituted samples with 85% acetonitrile. Similarly, further dilution of urine samples achieved a similar result for quantification of MET.

Optimization of the chromatographic system

The separation and quantification of GLB and its metabolites (M1, M2a, M2b, M3, and M4) were achieved using conventional reverse phase LC column, Agilent Eclipse XDB C₁₈ column. However, this column resulted in poor retention and peak symmetry for MET. Accordingly, the following HPLC columns were tested: Atlantis HILIC Silica, Waters Atlantis T3 C₁₈, Waters Symmetry C₁₈, Phenomenex Gemini-NX C₁₈, and Thermo BDS HYPERSIL Cyano columns. Good retention was achieved using Atlantis HILIC and Thermo BDS HYPERSIL Cyano columns. However, the Thermo BDS HYPERSIL Cyano column resulted in peak tailing and consequently inaccurate quantification. Therefore, Atlantis HILIC Silica column was used to separate MET and acceptable peak shape and retention time were achieved with an optimal mobile phase of aqueous ammonium acetate buffer (20 mM, pH = 4.0) and acetonitrile (15:85, v/v).

In summary, the apparent incompatible concentrations and polarities of target compounds in plasma and urine samples of the pregnant patients required the development and utilization of three separate LC methods for quantitative analysis of GLB, its five metabolites, and MET.

Selection of IS

The isotope labeled M1 and M2b, 4-trans-hydroxy glyburide-d₃ and 3-cis-hydroxy glyburide-d₃ were first chosen as IS for quantification of M1, M2a, M2b, M3, and M4. However, in MRM mode, the isotopic peak of the analytes interfered the peak area of IS. Therefore, glipizide was finally used as IS due to the good result of linear regression and precision for the analytes. Two stable isotope-labeled analytes, glyburide-d₁₁ and metformin-d₆ hydrochloride were chosen as the internal standards for GLB and MET. No interference from endogenous compounds was observed.

Optimization of mass spectrometric conditions

The mass spectrometric parameters of GLB, its metabolites, and MET were optimized by direct infusion of their neat solutions in initial mobile phase under both positive and negative ion modes of ESI. All analytes exhibited higher sensitivity in positive ion mode than those in negative ion mode. The chosen MRM transition of analytes and IS were shown in Table 2. The MS² spectra of the analytes and IS were shown in Fig. S1. Other MS parameters optimized to enhance the sensitivity of the analytes are summarized in Table 1 and Table 2.

Qualitative and semi-quantitative analysis of M5

Due to the lack of commercially available M5 metabolite, only qualitative/semi-quantitative determination of its concentration in patient samples was possible using LC–MS/MS. Crude M5 was prepared by biotransformation of GLB by human placental microsomes according

to a previous report from our laboratory (Ravindran et al., 2006). The identification of M5 in patient samples was based on the MS fragmentation and was further confirmed by comparing its retention time with crude M5 (Fig. 3).

We reported here the semi-quantitative analysis of M5 in patients' plasma and urine samples using peak area ratios of M5 to the internal standard glyburide-d₁₁ (Supplementary data, Table S1).

Method validation

Selectivity

The typical MRM chromatograms of plasma blank samples and urine as well as those spiked with the seven analytes at low concentrations are shown in Fig. 2 and Fig. S2. No significant interferences from endogenous substances with the analytes and IS were detected in blank samples.

Matrix effects and extraction recovery

The matrix factor of analytes and IS were evaluated in six blank/control human plasma and urine samples at low, medium, and high concentrations.

The matrix factor for the six analytes in plasma samples, except for M2b, ranged between 78% and 109%, with a relative standard deviation of < 15%. These results indicated that the ion suppression or enhancement of the analytes, except for M2b, in the plasma matrix was negligible. Although the ion suppression effects of M2b was high (range between 55% and 69%), it was acceptable because of the variation < 15% and sufficient sensitivity (LLOQ, 0.872 ng/mL) for quantification analysis. The matrix factor for urine samples was also acceptable and in agreement with the FDA guidelines.

The extraction recovery of GLB, its metabolites, and MET from plasma ranged between 87% and 99%, with a variation of < 15% at low, medium, and high concentrations (Table 3). The extraction recovery was consistent and reproducible. Therefore, the single-step protein precipitation procedure and extraction of the analytes used in this analytical method was efficient and simple. The extraction recovery for urine samples was acceptable and also met FDA guidelines (Supplementary data, Table S2).

Linearity and lower limit of quantification (LLOQ)

Calibration curves used for analysis of plasma samples, consisted of eight standard solutions in the linear ranges as follows: GLB, 1.02–410 ng/mL; M1, 0.102–164 ng/mL; M2a, 0.109–174 ng/mL; M2b, 0.101–161 ng/mL; M3, 0.100–160 ng/mL; M4, 0.113–180 ng/mL, and MET, $4.95\text{--}2.97 \times 10^3$ ng/mL. For urine samples, the linear ranges were as follows: GLB, 0.0594–23.8 ng/mL; M1, 0.989–494 ng/mL; M2a, 0.984–492 ng/mL; M2b, 1.02–511 ng/mL; M3, 1.00–503 ng/mL; M4, 0.984–492 ng/mL, and MET, 30.0–899 µg/mL. Linear curves were fit by using weighted (1/x) least-squares linear regression analysis of the peak area ratio of each analyte to IS. The calibration curves exhibited good linear regressions ($r^2 > 0.99$) over the test range. The LLOQ in plasma for GLB was 1.02 ng/mL, and for its metabolites were M1, 0.102 ng/mL; M2a, 0.109 ng/mL; M2b, 0.101 ng/mL; M3, 0.100

ng/mL; M4, 0.113 ng/mL, and MET, 4.95 ng/mL, while in urine, they were GLB, 0.0594 ng/mL; M1, 0.989 ng/mL; M2a, 0.984 ng/mL; M2b, 1.02 ng/mL; M3, 1.00 ng/mL; M4, 0.984 ng/mL and MET, 30.0 µg/mL. At LLOQ concentrations of the seven analytes in plasma and urine, the relative deviation was less than 20% and 16% for intra-day and inter-day, and the accuracy ranged from 81% to 120% for plasma, and 89% to 105% for urine (Table 4 and Supplementary data, Table S3).

Precision and accuracy

Precision and accuracy were validated at LLOQ, low, medium, and high concentrations of the analytes (Table 4). The intra-day accuracy for the seven analytes in samples of plasma ranged between 86% and 114% with precision of < 12%, and the inter-day accuracy ranged between 91% and 110% with precision of < 14%. All of the values were within acceptable range, indicating that the method was accurate, precise, and reproducible. The precision, accuracy and reproducibility for urine samples were acceptable according to the FDA guidelines (Supplementary data, Table S3).

Stability

The stability of the analytes under the following conditions of storage and processing was studied: short- and long-term storage as well as freeze-thaw cycles. Short-term stability was performed for 4 h at room temperature (22–25 °C). The accuracy ranged between 85% and 112% with precision of < 15% at the two QC levels (low and high). The results indicated that the seven analytes in plasma samples were stable for at least 4 h at room temperature (Table 5). The freeze-thaw stability following three cycles was between 85% and 115% with precision of < 15%, indicating that the seven analytes were stable under these conditions (Table 5). The seven analytes in human plasma samples were stable at 70 °C for 30 days (Table 5). Similar data were obtained on the stability of the compounds in urine samples and are shown in Table S4.

Samples from 10 % of the patients, including those with C_{max} and lowest concentrations of GLB were analyzed following their storage for 32 weeks at –70 °C, 78% of the samples deviated by less than 20% from the original value, and none deviated by more than 30%. (Table S5).

Method application

The validated methods were used to determine the concentrations of GLB, its metabolites (M1, M2a, M2b, M3, and M4), and MET as well as the semi-quantitative determination of M5 in pregnant patients under treatment with a dose of 500 mg MET and 2.5 mg GLB twice per day upon reaching steady-state levels for the medications. Plasma and urine samples were collected according to the time points. QC samples at low, medium, and high concentrations were prepared daily and analyzed together with patient samples. Up to now, 150 samples from 13 pregnant patients enrolled in the clinical trial were analyzed. The concentrations of GLB in plasma ranged between 1.25 ng/mL and 60.8 ng/mL, its five metabolites ranged between 0.100 ng/mL and 13.6 ng/mL, and MET ranged between 125 ng/mL and 1.93×10^3 ng/mL. In patient urine, the concentrations of GLB ranged between 0.0617 ng/mL and 1.00 ng/mL, its five metabolites ranged between 1.00 ng/mL and 412

ng/mL, and MET ranged between 42.6 µg/mL and 1.13×10^3 µg/mL. The sensitivity and calibration range of this method were adequate for profiling during the 12 h sampling period.

Conclusions

Glyburide and metformin are used for treatment of type 2 diabetes mellitus. The OPRU network is currently conducting a multi-center clinical trial to evaluate the combination of GLB and MET in treatment of GDM. We reported here the development of an LC-MS/MS method for the quantitative determination of MET, GLB and its metabolites (M1, M2a, M2b, M3, and M4) as well as semi-quantitation of M5 in the plasma and urine of pregnant patients enrolled in the clinical trial. The analytical methods reported here for the three types of analytes consisted of sample pretreatment followed by three sensitive, specific, and rapid LC-MS/MS methods. The validity, low LLOQ, and wide linearity range of the method renders it valuable for providing the data necessary for determining the pharmacokinetics of GLB and MET combination therapy for treatment of GDM patients. The method was successfully applied for the analysis of approximately 150 samples from pregnant patients receiving either a combination of GLB and MET or monotherapy with MET.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

GLB	glyburide
MET	metformin
M1	4-trans-hydroxycyclohexyl glyburide
M2a	4-cis-hydroxycyclohexyl glyburide
M2b	3-cis-hydroxycyclohexyl glyburide
M3	3-trans-hydroxycyclohexyl glyburide
M4	2-trans-hydroxycyclohexyl glyburide
M5	ethyl hydroxycyclohexyl glyburide
PK	pharmacokinetic
GDM	gestational diabetes mellitus
MRM	multiple reaction monitoring

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Figure 1.

Chemical structures of glyburide, its metabolites (**M1**, 4-trans-hydroxycyclohexyl glyburide; **M2a**, 4-cis-hydroxycyclohexyl glyburide; **M2b**, 3-cis-hydroxycyclohexyl glyburide; **M3**, 3-trans-hydroxycyclohexyl glyburide; **M4**, 2-trans-hydroxycyclohexyl glyburide; **M5**, ethyl hydroxycyclohexyl glyburide), metformin, glyburide-d₁₁ (IS of glyburide), glipizide (IS of metabolites of glyburide), and metformin-d₆ hydrochloride (IS of metformin).

Figure 2.

Representative MRM chromatograms of glyburide, its metabolites (M1, M2a, M2b, M3, and M4), and metformin in human plasma: (A) blank human plasma; (B) blank human plasma spiked with glyburide (2.05 ng/mL), M1 (0.818 ng/mL), M2a (0.872 ng/mL), M2b (0.806 ng/mL), M3 (0.800 ng/mL), M4 (0.900 ng/mL), metformin (14.9 ng/mL), and IS; (C) human plasma of pregnant patient prescribed 500 mg of metformin and 2.5 mg of glyburide tablet twice a day. Peaks: (1) glyburide; (2) glyburide-d₁₁ (IS of GLB); (3) **M1**, 4-trans-hydroxycyclohexyl glyburide; (4) **M2a**, 4-cis-hydroxycyclohexyl glyburide; (5) **M2b**, 3-cis-hydroxycyclohexyl glyburide; (6) **M3**, 3-trans-hydroxycyclohexyl glyburide; (7) **M4**, 2-trans-hydroxycyclohexyl glyburide; (8) glipizide (IS of metabolites of glyburide); (9) metformin; (10) metformin-d₆ hydrochloride (IS of metformin).

Figure 3. Identification of **M5**. (A) MRM chromatogram of **M5**; (B) MS² product ion spectra of **M5** and proposed fragmentation pathway.

Table 1

MS parameters for quantitative analysis of GLB, its metabolites and MET

MS parameters ^a	Method 1	Method 2	Method 3
Ion spray voltage (V)	4500	4500	4000
Curtain gas (L/h)	20	20	20
Ion source Gas1 (L/h)	40	40	50
Ion source Gas2 (L/h)	50	60	50
Temperature (°C)	450	500	350
Collision Gas (L/h)	10	10	10

^a Method **1**, for the quantitative determination of GLB and qualitative analysis of M5; Method **2**, for the quantitative determination of metabolites of GLB; Method **3**, for the quantitative determination of MET.

Table 2

The compound dependent parameters for the MRM transition of GLB, its metabolites (M1, M2a, M2b, M3, M4 and M5), and MET

Compound dependent parameters	GLB	M1	M2a	M2b	M3	M4	M5	MET
Retention time of analyte (min)	5.5	8.6	10.5	11.6	13.5	18.9	3.9	8.5
Analyte MRM transition (m/z)	494.2→369.2	510.0→369.0	510.0→369.0	510.0→369.0	510.0→369.0	510.0→369.0	510.0→385.0	130.0→71.0
Retention time of internal standard (min)	5.4	14.6	14.6	14.6	14.6	14.6	5.4	8.6
Internal standard MRM transition (m/z) ^a	505.0→369.0	446.0→321.0	446.0→321.0	446.0→321.0	446.0→321.0	446.0→321.0	505.0→369.0	136.0→77.0
Declustering potential (V)	45	45	45	45	45	45	45	39
Entrance potential (V)	5	5	5	5	5	5	5	4
Collision energy (V)	20	20	20	20	20	20	20	29
Collision cell exit potential (V)	10	10	10	10	10	10	10	14

^aThe internal standards are glyburide-d11, glipizide, and metformin-d6 hydrochloride for determination of GLB, its metabolites (M1, M2a, M2b, M3, and M4), and MET, respectively. (GLB, glyburide; M1, 4-trans-hydroxycyclohexyl glyburide; M2a, 4-cis-hydroxycyclohexyl glyburide; M2b, 3-cis-hydroxycyclohexyl glyburide; M3, 3-trans-hydroxycyclohexyl glyburide; M4, 2-trans-hydroxycyclohexyl glyburide; M5, ethyl hydroxycyclohexyl glyburide; MET, metformin).

Table 3

Extraction recovery of GLB, its five metabolites (M1, M2a, M2b, M3, and M4), and MET in human plasma (n = 3)

Analyte ^a	Concentration (ng/mL)	Mean recovery ^b (%)	CV (%)
GLB	2.05	95.2	7.6
	205	91.3	13.5
	410	98.9	4.3
M1	0.818	95.7	4.6
	81.8	93.3	4.2
	164	96.5	6.8
M2a	0.872	94.4	3.9
	87.2	93.8	1.7
	174	95.3	2.8
M2b	0.806	95.3	4.0
	80.6	96.2	3.7
	161	94.4	4.5
M3	0.800	97.4	1.9
	80.0	95.3	12.6
	160	94.1	6.7
M4	0.900	95.8	4.5
	90.0	96.7	5.7
	180	93.2	4.8
MET	14.9	93.0	7.5
	1.49×10^3	89.3	8.8
	2.97×10^3	87.3	7.3

^a GLB, glyburide; M1, 4-trans-hydroxycyclohexyl glyburide; M2a, 4-cis-hydroxycyclohexyl glyburide; M2b, 3-cis-hydroxycyclohexyl glyburide; M3, 3-trans-hydroxycyclohexyl glyburide; M4, 2-trans-hydroxycyclohexyl glyburide; MET, metformin.

^b Recovery = (peak area of QC sample/peak area of post-extract sample) $\times$ 100%.

Table 4

Intra-day and inter-day precision and accuracy of the method for human plasma

Analyte ^a	Concentration (ng/mL)	Intra-day (n = 5)			Inter-day (n = 3)		
		Mean (ng/mL)	Accuracy ^b (%)	CV (%)	Mean (ng/mL)	Accuracy ^b (%)	CV (%)
GLB	1.02	0.830	81	11.6	0.983	96	16.9
	2.05	2.07	101	8.3	2.09	102	5.0
	205	175	85	3.7	200	98	7.2
	410	350	86	8.9	401	98	13.2
M1	0.102	0.110	108	12.8	0.103	101	11.2
	0.818	0.890	109	10.0	0.882	108	5.6
	81.8	82.1	100	3.3	80.5	98	3.0
	164	153	94	6.4	151	92	5.4
M2a	0.109	0.110	101	19.5	0.0980	90	11.8
	0.872	0.990	114	6.5	0.963	110	5.7
	87.2	93.0	107	2.2	88.2	101	6.0
	174	165	94	5.2	160	92	6.7
M2b	0.101	0.120	118	18.2	0.105	104	16.5
	0.806	0.850	106	10.2	0.825	103	6.8
	80.6	81.6	101	1.3	79.1	98	5.3
	161	155	97	5.5	154	96	4.9
M3	0.100	0.120	120	15.3	0.105	105	18.5
	0.800	0.840	105	10.3	0.830	104	4.3
	80.0	81.5	102	3.0	79.7	100	3.1
	160	153	96	8.2	153	95	5.4
M4	0.113	0.130	115	10.3	0.114	101	17.8
	0.900	0.960	107	12.0	0.930	103	7.5
	90.0	92.1	102	3.1	91.9	102	8.2
	180	183	102	3.7	181	101	12.8
MET	4.95	4.87	97	18.1	4.56	92	6.4
	14.9	14.3	96	11.9	15.0	101	5.8
	1.49×10^3	1.50×10^3	100	2.6	1.49×10^3	100	2.1

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Analyte ^a	Concentration (ng/mL)	Intra-day (n = 5)			Inter-day (n = 3)		
		Mean (ng/mL)	Accuracy ^b (%)	CV (%)	Mean (ng/mL)	Accuracy ^b (%)	CV (%)
	2.97 × 10 ³	2.84 × 10 ³	96	6.2	2.94 × 10 ³	99	5.6

^a GLB, glyburide; M1, 4-trans-hydroxycyclohexyl glyburide; M2a, 4-cis-hydroxycyclohexyl glyburide; M2b, 3-cis-hydroxycyclohexyl glyburide; M3, 3-trans-hydroxycyclohexyl glyburide; M4, 2-trans-hydroxycyclohexyl glyburide; MET, metformin.

^b Accuracy = (obtained concentration/added concentration) × 100%.

Table 5
Stability of GLB, its metabolites (M1, M2a, M2b, M3, and M4) and MET in human plasma

Analyte ^a	Concentration (ng/mL)	Freeze and thaw stability (3 cycles, -70 °C)			Short-term stability (4h, 22-25 °C)			Long-term stability (30 days, -70 °C)		
		Mean (ng/mL)	Mean accuracy ^b (%)	CV (%)	Mean (ng/mL)	Mean accuracy ^b (%)	CV (%)	Mean (ng/mL)	Mean accuracy ^b	CV (%)
GLB	2.05	2.31	113	0.3	2.29	112	3.3	2.11	103	13.9
	410	424	103	6.9	406	99	7.1	414	101	13.7
M1	0.818	0.940	115	4.4	0.830	102	7.2	0.880	108	12.8
	164	146	89	7.3	142	86	7.6	171	104	0.4
M2a	0.872	0.970	111	2.0	0.850	97	8.7	1.00	115	8.1
	174	147	85	6.1	148	85	3.3	182	105	0.8
M2b	0.806	0.830	103	4.6	0.700	87	2.3	0.920	114	11.1
	161	136	85	9.1	136	85	14.4	184	114	4.8
M3	0.800	0.840	105	3.9	0.690	86	6.9	0.820	103	8.5
	160	136	85	5.1	137	86	12.7	166	104	2.7
M4	0.900	0.970	108	5.6	0.820	91	5.0	0.810	90	12.2
	180	172	95	9.1	168	93	6.9	169	94	0.8
MET	14.9	16.8	113	14.3	16.3	109	8.3	14.2	95	14.2
	2.97×10^3	3.20×10^3	108	5.1	3.07×10^3	103	2.4	2.81×10^3	95	3.1

^a GLB, glyburide; M1, 4-trans-hydroxycyclohexyl glyburide; M2a, 4-cis-hydroxycyclohexyl glyburide; M2b, 3-cis-hydroxycyclohexyl glyburide; M3, 3-trans-hydroxycyclohexyl glyburide; M4, 2-trans-hydroxycyclohexyl glyburide; MET, metformin.

^b accuracy = (obtained concentration/added concentration) $\times$ 100%.