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Validation of a sensitive LC–MS assay for quantification of glyburide and its metabolite 4-transhydroxy glyburide in plasma and urine: An OPRU Network study

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Abstract

Glyburide (glibenclamide, INN), a second generation sulfonylurea is widely used in the treatment of gestational diabetes mellitus (GDM). None of the previously reported analytical methods provide adequate sensitivity for the expected sub-nanogram/mL maternal and umbilical cord plasma concentrations of glyburide during pregnancy. We developed and validated a sensitive and low sample volume liquid chromatographic–mass spectrometric (LC–MS) method for simultaneous determination of glyburide (GLY) and its metabolite, 4-transhydroxy glyburide (M1) in human plasma (0.5 mL) or urine (0.1 mL). The limits of quantitation (LOQ) for GLY and M1 in plasma were 0.25 and 0.40 ng/mL, respectively whereas it was 1.06 ng/mL for M1 in urine. As measured by quality control samples, precision (% coefficient of variation) of the assay was <15% whereas the accuracy (% deviation from expected) ranged from −10.1 to 14.3%. We found that the GLY metabolite, M1 is excreted in the urine as the glucuronide-conjugate.

Keywords: Glyburide; Metabolites; Liquid chromatography–mass spectrometry; β-Glucuronidase; Gestational diabetes mellitus; Plasma; Umbilical cord plasma; Urine

1. Introduction

Glyburide (glibenclamide, INN) (GLY), an oral sulfonylurea hypoglycemic agent, has similar outcomes as insulin when used in the treatment of gestational diabetes mellitus (GDM) [1]. GDM is typically diagnosed in the third trimester of pregnancy and affects up to 8% of all pregnancies [2]. It is a condition characterized by insulin resistance and an increased risk for progression to type 2 diabetes mellitus in later life. In pregnancy, GDM is associated with increased risk of adverse pregnancy outcomes including macrosomia, shoulder dystocia, cesarean delivery, preeclampsia, intrauterine fetal demise, and perinatal mortality [3–5]. GLY improves glycemic control by increasing the release of insulin from the β cells of the pancreas. When compared to treatment with exogenous insulin, GDM patients on GLY have higher compliance, similar maternal glucose control and similar infant outcomes such as macrosomia and neonatal hypoglycemia [1]. For these reasons, GLY is increasingly used as first-line therapy for GDM.

Even though oral hypoglycemic agents are titrated to response during pregnancy, there is a narrow window of opportunity to achieve glycemic control. A prolonged period of titration and dosage adjustments may expose the fetus to abnormally high glucose concentrations and associated complications including macrosomia. Since pregnancy can accelerate the clearance of drugs [6,7], GLY plasma concentrations may be much lower during pregnancy (sub-nanogram/mL) and therefore the duration of action shorter than achieved in non-pregnant individuals. To study the pharmacokinetics of GLY during pregnancy and its maternal-fetal transfer, a highly sensitive and specific assay is needed that is capable of measuring sub-nanogram/mL plasma concentration of the drug.

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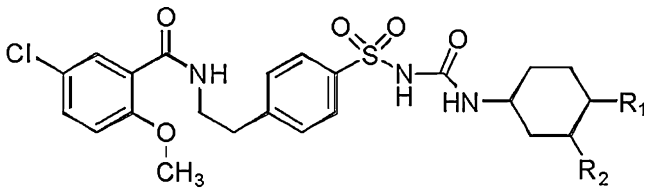
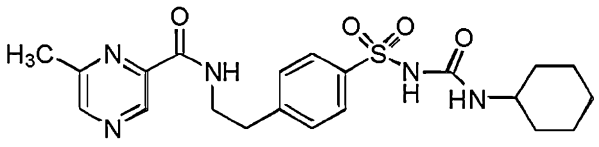


R1, R2=H → Glyburide, molecular weight: 494
R1=OH→ 4-transhydroxy glyburide, molecular weight: 510
R2=OH→ 3-Cishydroxy glyburide, molecular weight: 510



Glipizide, molecular weight: 446

Fig. 1. The chemical structure of GLY, M1 or M2 and the internal standard, GP (bottom).

The clearance of GLY is dominated by hepatic phase I metabolism to its hydroxylated metabolites. The two major metabolites of GLY in urine, which are also pharmacologically active [8,9], are 4-transhydroxy glyburide (M1) and 3-cishydroxy glyburide (M2) [10,11] (Fig. 1). There are conflicting data in the literature regarding the identity of the cytochrome P450s (CYPs) involved in the metabolism of GLY [12,13].

Of the GLY LC–MS assays published [14–20], none have the desired sensitivity to simultaneously determine sub-nanogram/mL plasma concentrations of GLY and its metabolites, 4-transhydroxy glyburide and 3-cishydroxy glyburide, even with the use of tandem mass spectrometry. We have developed and validated a highly sensitive assay for the determination of GLY and its metabolite, 4-transhydroxy glyburide requiring a small volume of plasma (0.5 mL) or urine (0.1 mL), using a commonly used single quadrupole LC–MS.

2. Experimental

2.1. Chemicals and reagents

Glyburide was purchased from Calbiochem (San Diego, CA, USA). M1 and M2 were synthesized [21]. Glipizide (GP), used as an internal standard, was obtained from Sigma Chemical Company (St Louis, MO, USA). Optima grade dichloromethane, acetonitrile, methanol and water were purchased from Fisher Scientific (Pittsburgh, PA). *n*-Hexane (HPLC grade) was obtained from Acros Organics (Morris Plains, NJ, USA). β-Glucuronidase (EC-number 3.2.1.31) from *H. pomatia* (glucuronidase activity of 99400 U/mL and sulfatase activity of 441.6 U/mL) was purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals used were of reagent grade. Outdated human plasma was procured from the Puget Sound Blood Center (Seattle, WA, USA).

2.2. Preparation of calibrators and quality control samples

2.2.1. Plasma

Stock solutions of GLY, M1 and M2 were prepared in acetonitrile at 0.25, 0.1 and 0.1 mg/mL, respectively. These stock solutions were diluted to 12.5, 1.25 and 0.125 μg/mL in methanol:water (1:1, v/v) and aliquots of these diluted solutions (≤400 μL) were further diluted in 50 mL of blank human plasma to yield the following calibrators for GLY: 0.26, 0.51, 1.03, 2.57, 5.14, 10.3, 25.7 and 51.4 ng/mL, M1: 0.40, 0.80, 2.00, 4.00, 8.00, 20.0 and 30.0 ng/mL and M2: 0.40, 0.80, 2.00, 4.00, 8.00, and 20.0 ng/mL. For the preparation of quality control (QC) samples, an independent stock solution was prepared as above to yield the following concentrations in plasma for GLY: 0.55, 5.54 and 41.6 ng/mL, M1: 0.40, 4.00 and 25.0 and M2: 0.40, 4.00 and 15.0 ng/mL. A solution of the internal standard, GP, was prepared in methanol (1.0 mg/mL) and further diluted with methanol:water (1:1, v/v) to achieve a final concentration of 50 ng/mL All the calibrators and QCs were aliquoted and stored at −80 °C along with clinical study samples.

2.2.2. Urine

Stock solutions and dilutions of M1 and M2 for urine calibration curve were made as described above, except that the matrix was blank human urine in place of plasma. The calibrators for M1 in urine were 1.06, 2.64, 5.28 10.6, 26.4 and 52.8 ng/mL whereas they were 0.45, 1.13, 2.26, 4.52, 11.3 and 22.6 ng/mL for M2. QC samples were prepared independently of the calibrators to yield the following concentrations for M1: 4.75, 14.3 and 35.6 ng/mL and M2: 2.27, 6.80 and 17.0 ng/mL of urine. All the calibrators and QCs were aliquoted and stored at −80 °C along with clinical study samples.

2.3. Instrumentation

LC–MS analysis was conducted using an Agilent (Agilent Technologies, Palo Alto, CA) 1100 series HPLC system consisting of a G1312A binary pump, a G1322A degasser, and an ALS G1329A autosampler interfaced to an Agilent 1100 series G1946D mass spectrometer equipped with an electrospray ionization (ESI) interface. Selected ion monitoring (SIM) chromatograms were acquired using Agilent Chemstation version 10.02.

2.4. Chromatographic and mass spectrometer conditions

An Agilent Zorbax XDB-C8 (2.1 × 50 mm, 5 μm) analytical column with a Phenomenex Security Guard C18 (2.0 × 4 mm) guard column were used with a gradient mobile phase system to chromatographically separate GLY, M1, M2 and GP. The mobile phase consisted of a mixture of methanol and water (pH 6), both containing 0.5 mM ammonium formate. Elution of analytes was carried out using a flow rate of 0.25 mL/min and the following gradient: from the initial conditions (20/80 organic/aqueous), the organic phase was increased linearly to 45% over 1 min, held for 3 min, increased linearly to 75% organic over 1.5 min, held for 1 min, then further increased linearly to 90% organic over 10 s,

held for 3 min and then returned to initial conditions of 20/80 organic/aqueous. To allow for column re-equilibration, the run time was set at 15 min.

For optimum sensitivity, the following parameters were set: fragmentor voltage of 50 V for GLY and GP, 250 V for M1 and M2, capillary voltage 5000 V, drying gas temperature 350 °C, drying gas flow 10 L/min and nebulizer pressure 25 psig (172.4 kPa). Mass spectrometric data were collected between 2.5 and 9.0 min. At all other times, column eluent was diverted to waste using post-column splitting.

2.5. Sample preparation

2.5.1. Plasma

A 0.5 mL aliquot of plasma was added to 50 μL of the working solution of internal standard (2.5 ng). The plasma was acidified by adding 100 μL of 2 M HCl followed by addition of 5.0 mL of *n*-hexane:methylene chloride (1:1, v/v). The sample was briefly vortex mixed then placed on a shaker plate for approximately 30 min. After centrifugation at 3000 × *g* for 10 min the upper organic layer was transferred to a disposable glass culture tube and evaporated to dryness under vacuum with centrifugation (RC 1010, Jouan Inc. Winchester, Virginia). The dried residue was reconstituted in 100 μL of the starting mobile phase. After vortex mixing, 15 μL aliquots of the samples were injected onto the LC–MS. Plasma clinical samples with expected concentrations outside the calibration range were diluted with the corresponding blank matrix.

2.5.2. Urine and β-glucuronidase treatment

To determine if M1 and M2 were excreted in the urine as glucuronide conjugates, we incubated a study subject's urine (0.1 mL) at 37 °C with 0, 500, 1000 or 2000 U/mL of β-glucuronidase for 2, 16 or 36 h. Incubation with 1000 U/mL for 16 h resulted in complete deconjugation of M1. M2 was found not to be conjugated. Therefore all the further analysis of urine samples included this deconjugation step.

To a 100 μL aliquot of urine sample, 1 mL of 100 mM sodium acetate buffer (pH 5, adjusted with glacial acetic acid) containing β-glucuronidase 1000 U/mL were added. Samples were incubated for 16 h at 37 °C. After incubation, the samples were brought to room temperature and the internal standard GP (7.5 ng) was added. The urine sample was acidified with 300 μL of 2 M HCl followed by addition of 5.0 mL of *n*-hexane:methylene chloride (1:1, v/v). The sample was then processed as described above for plasma.

As acids can potentially deconjugate metabolites, we determined the effect of 2 M HCl used in our extraction process on deconjugation of the metabolite conjugates. As only M1 is conjugated, to determine the concentration of unconjugated M1 metabolite in plasma, the above acid extraction process was compared with an acid free extraction process (acetonitrile-plasma protein precipitation) in five clinical study subjects at the M1 C_{max} concentration. Briefly, 100 μL of the subject's plasma, calibrators, and quality control samples were precipitated with 200 μL acetonitrile. The sample was vortexed for 1 min. After centrifugation, the supernatant was transferred to a disposable glass culture tube and processed as described above for plasma and compared to that extracted using 2 M HCl.

2.6. Validation

2.6.1. Specificity

Ten different blank human plasma samples and five different urine samples were processed with and without internal standard to identify the presence of interfering peaks.

2.6.2. Extraction efficiency and matrix effects

Extraction efficiency for GLY, M1 or M2 from the plasma matrix was determined by adding GLY, M1 or M2 at three different concentrations to blank plasma in triplicate. The absolute peak area of these samples at each concentration was compared to a reference sample. The reference sample for each concentration was prepared in triplicate by extracting blank human plasma, then adding the compounds to the extract and processing as described above. Percent extraction efficiency was calculated by the ratio of peak area of the analyte in the extraction efficiency sample to that in the reference sample. The matrix effects on the quantification of analytes in three different batches of plasma or urine in triplicate were evaluated by comparing the peak area of analytes resolved in blank sample post extraction (the final solution of blank plasma or urine after extraction and reconstitution) with that resolved in mobile phase. If the % difference is greater than ±15%, an exogenous matrix effect is implied.

2.6.3. Calibration curve and sample quantification

The calibration curve for GLY in plasma was divided into a lower calibration curve (0.26–2.57 ng/mL) and a higher calibration curve (2.57–51.4 ng/mL). For plasma, the calibration curves ranged from 0.40 to 30.0 ng/mL and 0.40 to 20.0 ng/mL for M1 and M2, respectively whereas for urine they ranged from 1.06 to 52.8 ng/mL and 0.45 to 22.6 ng/mL, respectively. The peak area ratios of GLY, M1 or M2 to that of GP were analyzed by linear regression to estimate the slope, intercept and correlation coefficient of the calibration curve. The concentration of the analytes in the quality controls and clinical samples were calculated by inverse regression.

2.6.4. Intra-day and Inter-day precision and accuracy

To determine intra-day precision and accuracy, low, mid and high concentrations of QC samples were analyzed in pentuplicate. Precision was determined as the %CV (coefficient of variation) of peak area ratios for the pentuplicate of each QC sample. Accuracy was evaluated by calculating the concentration of each QC sample, using the calibration curve that was run on the same day, and determining the percent deviation from expected concentration.

To determine inter-day precision and accuracy, the QC samples were analyzed on five separate days. Precision was determined as the %CV of the measured concentrations of each QC sample ran on five separate days. Accuracy was evaluated by calculating the % difference between the nominal concentration and the mean concentration of each QC (low, mid and high) sample on five separate days. Limits of acceptable intra-

and inter-day precision and accuracy were set at <15% CV and ±15% deviation from expected, respectively.

2.6.5. Limit of quantification and detection

The limit of detection (LOD) was set as the lowest concentration of the compounds that could be detected with a signal to noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration in the calibration curves that can be quantified with an acceptable level of precision of ≤15% and accuracy of ±15%. LOQ samples were analyzed in triplicate and evaluated as unknown samples on three different days.

2.7. Method application

This method was applied to samples obtained from a multi-center clinical trail investigating the steady-state pharmacokinetics of GLY in gestational diabetic subjects. After local Institutional Review Board approval and written informed consent, venous heparinized plasma and urine samples were collected over a dosing interval from a pregnant subject who received GLY (1.25 mg) every 12 h. In addition, at the time of delivery, maternal and umbilical cord plasma samples were obtained simultaneously.

3. Results

3.1. Mass spectral analysis and chromatography

The mass spectrum of GLY, GP (25 μg/mL), M1 and M2 (10 μg/mL) was determined by direct injection of neat solutions of each compound in 1:1 methanol: water into a mass spectrometer set in a positive ion mode. Protonated pseudomolecular $[M+H]^+$ ions of GLY and GP appeared at *m/z* 494.2 (Fig. 2) and 446.2, respectively. These pseudomolecular ions (494.2, 446.2) showed no matrix interferences with endogenous substances occurring at the same mass or retention time. The hydroxylated metabolites of GLY, M1 and M2 showed a prominent ion at *m/z* 532.2, which are sodium adducts of the metabolites (mol. wt. 510.2). The remaining small number of charged fragments were all of relatively insignificant intensities. The capillary voltage, drying gas temperature/flow and nebulizer pressure were selected to optimize the specificity and sensitivity of *m/z* 494.2, 446.2 and 532.2 ions.

Under the optimized chromatographic conditions, GLY, GP, M1 and M2 were eluted with retention times of 8.2, 6.5, 5.6 and 6.9 min, respectively. Fig. 3A shows the typical SIM chromatogram of a plasma calibrator containing 2.5 ng/mL of GLY (15 μL injection). In addition, analysis of ten different blank human plasma samples (e.g., Fig. 3B) and five different blank human urine samples (e.g., Fig. 3D) showed no interfering peaks at the retention times of the compounds of interest.

3.2. Linearity, precision and accuracy of calibrators and quality control samples

Calibration curves for GLY and its metabolites over the range described above were linear with a correlation coefficient greater than 0.990 when evaluated with uniformly weighted least square regression (weight = 1) (Table 1).

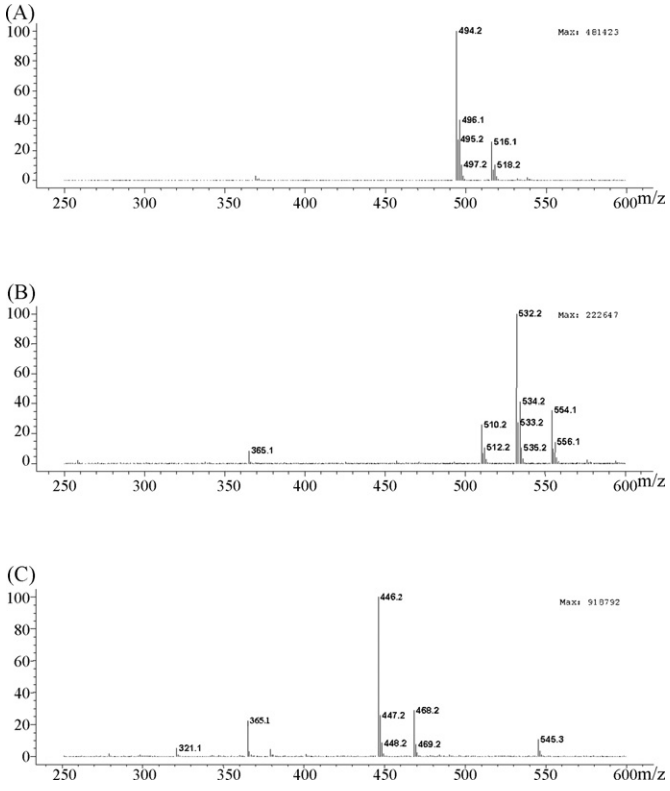


Fig. 2. Full mass spectrum of GLY in positive ionization mode with fragmentor voltage of 50 V using direct injection of GLY (25 μg/mL) in 1:1 methanol:water Positive ionization mode gave two times better sensitivity as compared to negative ionization mode (A). Full mass spectrum of M1 in positive ionization mode with fragmentor voltage of 250 V using direct injection of M1 (10 μg/mL) in 1:1 methanol:water. M2 showed similar mass spectrum as M1. Abundance of sodium adducts (*m/z* 532.2) for M1 or M2 was three-fold greater than that of the pseudomolecular ions (*m/z* 510.2) (B). Full mass spectrum of GP in positive ionization mode with fragmentor voltage of 50 V using direct injection of GP (25 μg/mL) in 1:1 methanol:water (C).

Dividing the calibration curve for GLY into lower and higher curves minimized the percent deviation from expected for the lower points on the curve (data not shown).

The intra- and inter-day precision and accuracy of quality control samples for GLY, M1 or M2 in plasma or urine which were run on five different days are shown in Table 2. All values were within the preset limits of ≤15% CV and ±15% deviation from expected (Table 2). These data are within the preset limits suggested by guidelines for bioanalytical method validation [22].

3.3. De-conjugation of phase II metabolites of glyburide metabolites

β-Glucuronidase treatment of GDM subject's urine revealed that M1 was conjugated while M2 was not. Complete deconjugation of M1 was achieved by incubating the urine samples at 37 °C with 1000 U/mL of β-glucuronidase for 16 h. This process increased the amount of M1 in the urine by 234%.

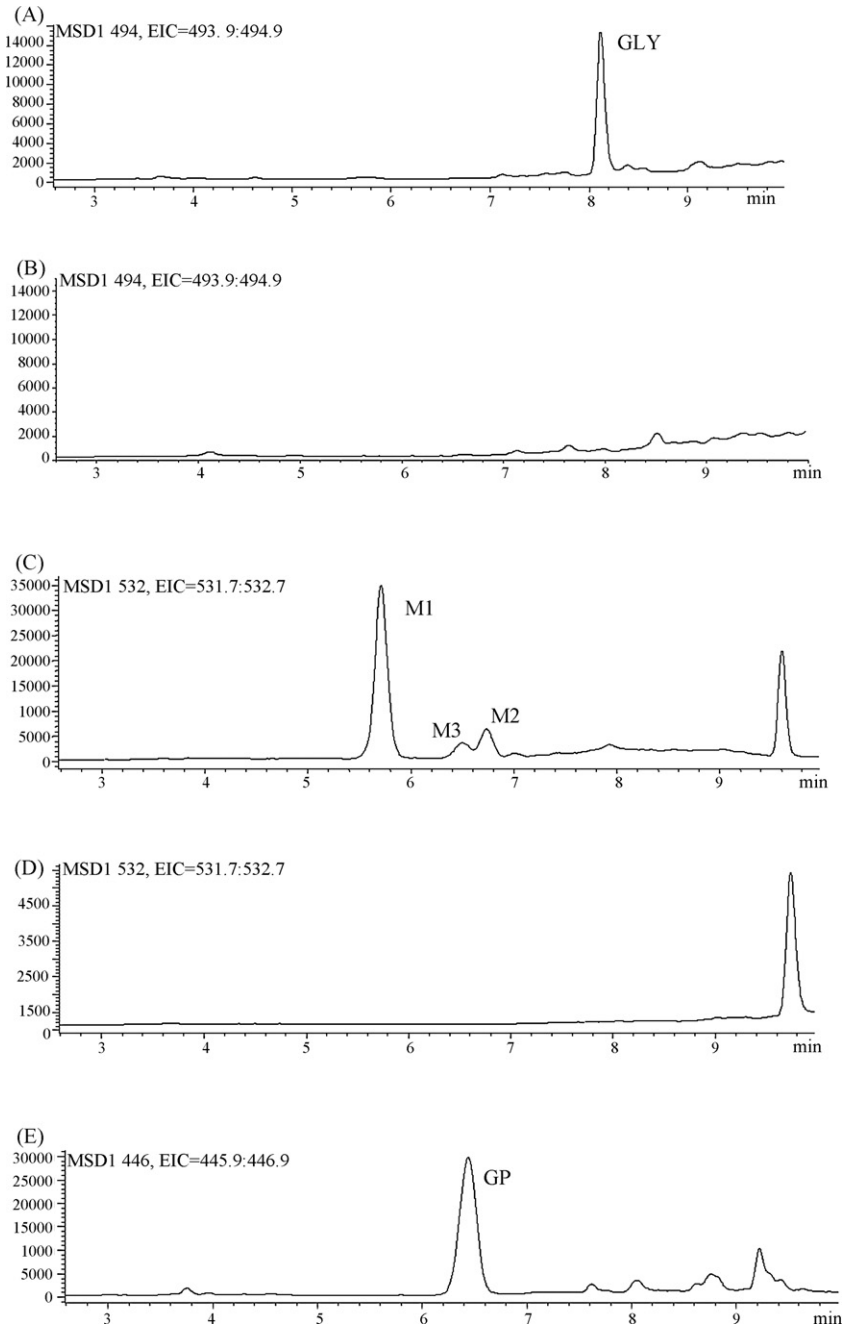


Fig. 3. Selected ion monitoring chromatogram for plasma-calibrator containing 2.5 ng/mL of GLY (*m*/*z* 494.2) (A). Selected ion monitoring chromatogram for blank plasma (*m*/*z* 494.2). The chromatogram shows no interference at the retention time of GLY (B). Selected ion monitoring chromatogram for clinical urine sample treated with 1000 U/mL of β-glucuronidase for 16 h, showing M1, M2 and M3 (*m*/*z* 532.2) (C). Selected ion monitoring chromatogram for blank urine (*m*/*z* 532.2). The chromatogram shows no interference at the retention time of M1, M2/M3 (D). Selected ion monitoring chromatogram for 50 ng/mL of GP (*m*/*z* 446.2) (E).

3.4. LOD, LOQ, extraction efficiency and matrix effects

The LOD was determined to be 0.13 ng/mL for GLY in plasma and 0.25 ng/mL for M1 and M2 in plasma or urine. The LOQ was 0.25, 0.40 and 0.40 ng/mL of plasma for GLY, M1 and M2, respectively, and 1.06 and 0.40 ng/mL of urine for M1 and M2. The mean percent extraction efficiency of GLY, M1 and M2 from plasma over the range described above was $78.3 \pm 4.5\%$, $40.0 \pm 3.8\%$ and $48.5 \pm 3.9\%$ (mean ± SD), respectively. The effect of matrix on the quantification of analytes (% difference in the ratio of peak area of analytes in blank plasma or urine to that in mobile phase) was less than 15% (Table 3).

3.5. Application to the clinical study samples

This assay was designed to analyze the plasma and urine samples obtained to study the steady-state pharmacokinetics of GLY in gestational diabetes mellitus subjects. The plasma concentration–time profile of GLY and its metabolite, M1 for a representative gestational diabetic clinical study subject who

Table 1
Inter-day variability of the slope and correlation coefficient of the calibration curves of GLY, M1 and M2 in human plasma and M1 and M2 in urine (run on five different days)

Analyte	Slope	Correlation coefficient	*y*- intercept
	Mean (% CV)	Range	Mean
Plasma			
GLY lower calibration curve (0.25–2.50 ng/mL)	0.0807 (9)	0.992–0.998	0.0013
GLY higher calibration curve (2.50–50.0 ng/mL)	0.0723 (11)	0.994–0.998	0.0608
M1 (0.40–30.0 ng/mL)	0.0062 (7)	0.997–0.999	−0.0000
M2 (0.40–20.0 ng/mL)	0.0065 (16)	0.993–0.998	0.0002
Urine			
M1 (1.06–52.8 ng/mL)	0.0021 (17)	0.994–0.999	−0.0064
M2 (0.45–22.6 ng/mL)	0.0026 (16)	0.993–1.000	−0.0022

Table 2
Intra-day and inter-day precision (% CV) and accuracy (% deviation from expected) of quality control samples of GLY, M1 and M2 in human plasma or M1 and M2 in urine

	Intra-day ($n = 5$ replicates)			Inter-day ($n = 5$ days)		
Plasma GLY (ng/mL)	0.55	5.54	41.6	0.55	5.54	41.6
Mean ± SD	0.60 ± 0.02	5.86 ± 0.52	42.7 ± 3.1	0.56 ± 0.04	5.38 ± 0.4	42.19 ± 2.7
CV (%)	3.6	8.8	7.3	7.2	7.8	6.5.
Deviation from expected (%)	7.5	5.9	2.8	1.6	−2.8	1.6
Plasma M1 (ng/mL)	0.40	4.00	25.0	0.40	4.00	25.0
Mean ± SD	0.42 ± 0.03	4.15 ± 0.23	25.3 ± 0.9	0.40 ± 0.02	3.99 ± 0.3	25.7 ± 1.2
CV (%)	7.9	5.6	3.7	3.8	6.8	4.8
Deviation from expected (%)	4.6	3.6	1.0	1.0	−0.3	2.6
Plasma M2 (ng/mL)	0.40	4.00	15.0	0.40	4.00	15.0
Mean ± SD	0.40 ± 0.02	4.21 ± 0.25	15.0 ± 0.7	0.40 ± 0.03	3.91 ± 0.2	14.9 ± 0.4
CV (%)	3.9	5.9	4.8	8.6	5.6	2.6
Deviation from expected (%)	1.1	5.2	−0.3	−0.3	−2.3	−0.9
Urine M1 (ng/mL)	4.75	14.3	35.6	4.75	14.3	35.6
Mean ± SD	4.85 ± 0.09	13.7 ± 1.8	37.6 ± 2.3	4.82 ± 0.34	14.4 ± 0.4	36.03 ± 1.3
CV (%)	1.8	13.2	6.2	7.1	2.9	3.6
Deviation from expected (%)	2.0	−3.7	5.5	1.5	1.1	1.1
Urine M2 (ng/mL)	2.27	6.80	17.0	2.27	6.80	17.0
Mean ± SD	2.06 ± 0.11	5.83 ± 0.58	15.4 ± 1.3	2.08 ± 0.07	6.11 ± 0.6	16.1 ± 1.9
CV (%)	5.5	10.0	8.1	3.5	9.8	11.4
Deviation from expected (%)	9.2	14.3	9.3	−8.5	−10.1	−5.1

CV, coefficient of variation; M1—4-transhydroxy glyburide; M2—3-cishydroxy glyburide.

Table 3
Matrix effects for GLY, M1 and GP in three different batches of human plasma and M1, M2 and GP in three different batches of human urine

Analyte	Peak area (Mean ± SD)		% Difference
	Post extraction ($n = 9$)	Mobile phase ($n = 6$)	
Plasma GLY	406635 ± 50727	456750 ± 12435	−11.0
Plasma GLY/GP	0.819 ± 0.133	0.858 ± 0.025	−4.6
Plasma M1	39279 ± 3049	45456 ± 1104	−13.6
Plasma M1/GP	0.079 ± 0.007	0.085 ± 0.002	−7.4
Plasma GP	496449 ± 61942	532198 ± 3152	−6.7
Urine M1	104047 ± 11581	117133 ± 5555	−11.2
Urine M1/GP	0.221 ± 0.038	0.223 ± 0.010	−1.04
Urine M2	41348 ± 3234	48361 ± 1364	−14.5
Urine M2/GP	0.088 ± 0.012	0.092 ± 0.002	−4.7
Urine GP	470634 ± 70229	524320 ± 3656	−10.2

% Difference = {[Mean peak area or area ratio (post extraction)/mean peak area or area ratio (mobile phase)] × 100} − 100.

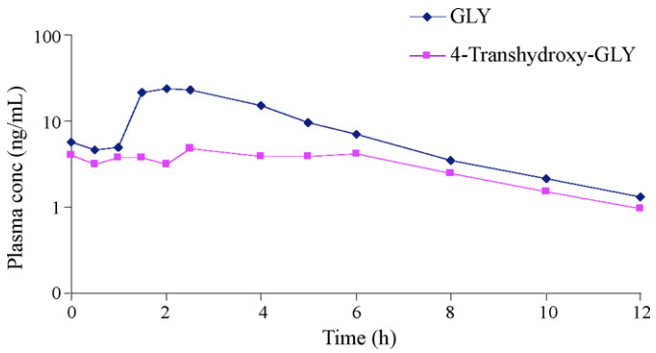


Fig. 4. Plasma concentration-time profile of glyburide and its metabolite M1 over a dosing interval in a gestational diabetes subject who received 1.25 mg oral dose of glyburide every 12 h. The amount of M1 excreted in the 12 h-urine was 0.47 mg.

received 1.25 mg of GLY every 12 h is shown in Fig. 4. Analysis of metabolites in plasma or urine from this subject showed that M2 partially overlapped with another GLY metabolite (M3). This metabolite, based on the elution time, is most likely 4-cishydroxy glyburide [25] (Fig. 3C). Since M2 could not be separated from M3 and a pure standard for M3 was not available to us we did not report the concentrations of M2 (see Section 4).

The amount of M1 excreted in the urine over a dosing interval (12 h), after deconjugation, was 37% of the dose. The maternal and cord venous GLY concentrations, simultaneously drawn at 4 h after drug administration on the day of delivery, were 1.73 and 0.57 ng/mL, respectively.

In 5 clinical study subjects at their C_{max} concentrations, the mean ± SD plasma concentration of M1 (pharmacologically active) using the acid extraction process were not significantly different from those obtained with acetonitrile-plasma protein precipitation (8.0 ± 5.1 vs. 8.6 ± 5.6 ng/mL; $p < 0.05$, paired t-test).

4. Discussion

There are several methods for quantification of GLY [14–20], but none have the required sensitivity to quantify the low maternal and umbilical cord plasma concentrations (<1 ng/mL) of GLY expected during pregnancy to determine the pharmacokinetics and the maternal-fetal transfer of the drug.

Different mobile phase compositions were assessed to increase the baseline separation and sensitivity for detection of GLY and its metabolites. The column chosen produced sharp symmetrical peaks with the described mobile phase. The pH of the mobile phase had a marked effect on the chromatography of GLY (pK_a of GLY is 5.3) and its metabolites. A pH of above 8 resulted in GLY not being retained on the column whereas below pH 4, the elution times of GLY were >15 min. Ammonium formate (0.5 mM, pH 6) used in this assay facilitated the separation of GLY, its metabolites and internal standard, GP, within 10 min. Better resolution of M1 and M2 was achieved by holding the mobile phase conditions at 45% organic for 3 min in the initial phase of the gradient. The total run-time for compound separation and column equilibration was 15 min which is much shorter than in previously reported methods of 22–55 min [23–25]. This shorter run time increased the throughput of the clinical study samples.

The full mass spectra (ionization pattern) of the analytes were obtained by injecting neat solutions of each in 50:50 methanol:water. For GLY, mass spectra were collected both in positive and negative electrospray ionization modes. Positive ionization mode gave two times better sensitivity as compared to the negative ionization mode. GLY and GP were identified/quantified using protonated pseudomolecular ions $[M+H]^+$ as their abundance was much greater compared to the fragment ions. Also, these pseudomolecular ions showed no interferences with the endogenous compounds in the plasma. For GLY metabolites, M1 or M2, abundance of sodium adducts (532.2) was three-fold greater than that of the pseudomolecular ions (510.2) (Fig. 2B). The 50:50 methanol: water composition used to obtain the mass spectra of M1 or M2 is approximately similar to the composition of mobile phase at their elution times through the chromatographic gradient, which is, 55:45 methanol:water. These similar mobile phase conditions for consistent ionization is important especially for metabolites which were quantified using sodium adducts.

Validation of our method consisted of intra-day and inter-day reproducibility of QCs at three concentrations in plasma or urine. These concentrations were selected cover the entire range of the calibration curve. The precision (% CV) and accuracy (% deviation from expected) for plasma and urine at all concentrations were ≤15% and ±13%, respectively. Extraction efficiency of GLY, M1 and M2 measured over the calibration range was 78, 40 and 49%, respectively. The lower extraction efficiency of M1 and M2 was not a problem as the concentrations of these metabolites in the urine were sufficient to detect with confidence. There was no significant matrix effects observed on the quantification of analytes in plasma or urine (Table 3).

With clinical urine or plasma samples, we observed a peak that partially overlapped with the M2 metabolite. This closely eluting peak is likely 4-cishydroxy glyburide, as the elution time is consistent with that observed in the recent *in vitro* study with human liver microsomes [25]. Several unsuccessful attempts were made with different gradient profiles to separate these metabolites. As these closely eluting peaks could not be separated, the concentrations of M2 are not reported. The concentration of M1 in the plasma was that of the unconjugated metabolite as the acid extraction process did not affect this concentration.

In the urine, two other hydroxy metabolites apart from M1, M2 and M3, were detected when a larger volume (250 μL) of the urine sample was extracted. These metabolites could be 3-transhydroxy glyburide and 2-transhydroxy glyburide as the elution times of these metabolites are similar to those observed in a recent *in vitro* study [25]. Previous *in vivo* studies have not reported the presence of these metabolites, perhaps due to lack of sensitivity or further metabolism to other metabolites. As pure standards for these metabolites were not available to us, their concentrations in the urine could not be estimated.

Consistent with previous data [26,27], glyburide was not excreted unchanged in the urine. Previous studies [26,27] have not addressed the presence of phase II metabolites (glucuronide

or sulfate conjugates) of GLY metabolites in urine. Complete deconjugation of metabolite M1 in urine is necessary to determine the metabolic clearance of GLY to M1. We addressed this issue by determining the amount of β-glucuronidase and duration of incubation necessary to ensure complete hydrolysis of the glucuronides. We found that M1 in the urine was conjugated, while M2 was not. We also found that the acidic conditions of our extraction process were capable of partially deconjugating the M1 conjugate. Such deconjugation was not observed in the plasma, perhaps due to the presence of multiple conjugates of M1, with the acid-susceptible conjugates being present in the urine at a higher concentration. Alternately, the difference might be due to the higher concentration of acid used in extracting the urine samples. The quantification of M1 in this assay was 10-fold lower than previously reported [26].

5. Conclusion

We have developed and validated a reproducible, accurate, sensitive and selective LC-MS assay for quantification of GLY and its metabolite 4-transhydroxy glyburide in plasma or urine which requires small sample volume (0.5 mL plasma or 0.1 mL urine). This assay provides a 20-fold lower LOQ compared with the other single quadrupole methods [18] and a four-fold lower LOQ compared with tandem instrument methods [20]. This assay was capable of quantifying GLY plasma concentrations <1 ng/mL (Fig. 4) and therefore can be utilized to determine the GLY pharmacokinetics

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