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A Validated Bioanalytical Method for Determination of Glimepiride in Human Plasma Using UPLC-MS/MS

Magda M. Ayad[1], Mervat M. Hosny[1], Heba M. El-Sayed[1*], Mohamed G. Elwan[2]

[1]Analytical Chemistry Department, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt, 44519,[2]International Center for Bioavailability, Pharmaceutical, and clinical Research (ICBR), El-Obour City, Egypt

***Abstract*:** *In the present work, a sensitive and rapid ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed and validated for the simultaneous determination of glimepiride (GPD) in human plasma using GPD-d5 as the internal standard (IS). The analyte was separated on an Acquity UPLC BEH C18 column with an isocratic elution system composed of acetonitrile and 0.1 % ammonium formate (70:30) at a flow rate of 0.30 mL/min. GPD was eluted at 1.00 min. Mass spectrometric detection was performed on a triple quadrupole (TQD) mass spectrometer operated in positive electrospray ionization (ESI) mode. The Multiple Reaction Monitoring (MRM) transitions were m/z 491.188→126.009 and m/z 496.188→ 131.009 for GPD and GPD-d5, respectively. The method was validated over a dynamic concentration range of (1– 100) ng/mL. The lower limit of quantification (LLOQ) of the developed method was 1 ng/mL. The intraday and interday precision (RSD %) were less than 10 % and accuracy (RE %) was within ± 5.40 %. Application of this method to the clinical samples after a single oral dose of 1 mg GPD in volunteers was successfully achieved.*

Keywords: *glimepiride, human plasma, UPLC–MS/MS.*

I. Introduction

Glimepiride **(**GPD) is an oral sulfonylurea hypoglycemic agent which is used in the treatment of type 2 diabetes. Chemically, it is 1-[[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl) urea, Fig. 1. The primary mechanism of action of GPD for lowering blood glucose is dependent on stimulating the release of insulin from functioning pancreatic cells. In addition, extra-pancreatic effects may also play a role[1,2]. GPD maintains a better physiological regulation of insulin secretion than other sulfonylurea during physical exercise [3]. Various liquid chromatography (LC) methods have been reported for determination of GPD in pharmaceutical formulations[4,5]or biological fluids [6–13].

UPLC is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 μm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis. Owing to its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceuticals and biomedical analysis.

In the present work, a bioanalytical validated UPLC-MS/MS method was applied for determination of GPD in human plasma offering several advantages and improvements in comparison with other UPLC methods [5, 12, 13] as it promotes the ability to separate and identify GPD with high sensitivity (1ng/ml) and marked reductions in the overall time of analysis (1 min). This article developed a quality system to ensure integrity, accuracy and precision of data generated during sampling, sample treatment and bioanalysis. The method was successfully applied to 34 volunteers after a single oral dose.

II. Experimental

2.1. Instrumentation and Reagents

GPD (purity 98.86%), obtained from EIPICO Company-Egypt and GPD-d5 (IS, purity 95.0%) were obtained from TRC (Toronto, Canada). Analytical grade ammonium formate was purchased from the Scharlau Company (Spain). Acetonitrile (HPLC grade) was procured from Sigma Company (Germany). HPLC grade water was obtained using a Milli Q system (Millipore, France). Blank samples of human plasma were obtained from Alshabrawishi blood bank (Cairo, Egypt).

Liquid chromatography was performed on an Acquity UPLC unit (Waters Corp., Milford, USA). Analytical method conditions are mentioned in table 1.

2.2.Method

2.2.1. Standard solutions, calibration standards and quality control (QC) sample

GPD stock standard solution was prepared by dissolving an equivalent amount of 50 mg GPD in 5.0 mL dimethyl sulfoxide then complete to 50.0 mL methanol to make up the concentration of 100µg/mL. GPD diluted stock solution was prepared by diluted 1.0 mL from stock solution with 50 mL methanol to make up stock solution containing 20µg/mL. The stock solutions were further diluted with methanol to obtain working solutions at several different concentration levels.

Calibration standards and QC samples in plasma were prepared by diluting the corresponding working solutions with blank human plasma. Final concentrations of the calibration standards were 1, 2, 4, 10, 20, 50, 75 and 100 ng/mLfor GPD in human plasma, respectively. Similarly, the concentrations of QC samples in plasma were 3, 40, and 80 ng/mL.

Stock solution of the internal standard was prepared by dissolving 0.94 mg IS in 0.1 mL dimethyl sulfoxide then complete to 10.0 mL methanol. This solution was further diluted with acetonitrile to make up stock standard solution containing 0.014 µg/mL IS.

All stock solutions, working solutions, calibration standards and QCs were immediately stored at −80 ˚C.

2.2.2. Sample preparation

Plasma samples, calibration standard and QCs samples were thawed to room temperature. Place an appropriate number of disposable Eppendorf tubes in a rack. The tubes should be properly labeled. Dispense 250.0 µL of the samples (blank, zero, standards, QC_L, QC_M, QC_H or volunteer's human plasma samples) into appropriate tubes. Add 750 µl of 0.014 µg /mL IS in acetonitrile. Vortex samples for 30 s then centrifuge for 20.0 min at 4000 rpm. Separate 250.0 µl from supernatant in another appropriate number of disposable tubes in a rack, the tubes should be properly labeled. Evaporate in concentrator for 30 minutes at 60 °c. Reconstitute the samples with 250 µl water and vortex for 30 s before injection of 10.0µL from supernatant to UPLC-MS/MS system.

2.2.3. Bioanalytical method validation

This method was fully validated according to the United States Food and Drug Administration (US FDA) guidelines for the validation of a bioanalytical method [14].

Selectivity

Selectivity is assessed to show that the intended analytes are measured and that their quantitation is not affected by the presence of the biological matrix, known metabolites, degradation products, or co-administered drugs. Selectivity was evaluated by comparing six different sources of blank plasma samples collected under controlled conditions to simulate the plasma matrix harvested from participants. Samples were extracted and analyzed after ensuring that selectivity requirements were met and the interferences from the extracted components in the signals of both GPD and IS were evaluated.

Carryover

Carry-over should be assessed during validation by injecting blank samples after a high concentration sample or calibration standard at the upper limit of quantification

Linearity

Eight concentrations of GPD (1–100ng/mL) were separately extracted and assayed on three separate days. A calibration graph was constructed by plotting the peak area ratios against concentrations. The LLOQ was defined as the lowest concentration on the calibration curves.

Precision and accuracy

Different concentrations of QC samples were used for the assessment of the precision and accuracy of the method on three separate days. Extraction recovery experiments were determined by comparing the analytical result of QC_L, QC_M and QC_H of GPD. Recovery of IS was determined in the same way. Matrix effect percentage (ME %) was calculated using the following equation; ME (%) = Ai/Ar × 100 where Ai/Ar is the ratio between the peak areas of analytes obtained from plasma samples spiked with analytes after extraction (Ai) to those of the pure standard solutions (Ar) at the same concentrations. The matrix effect of IS was evaluated similarly.

Stability

The reliability of the results was ensured using a stability assay comprising freeze-thaw stability, auto-sampler stability, short-term and long-term stability for matrix based samples. Sample stability was tested by

determination of QC samples after short-term (24 h) storage at room temperature and on storage at −80°C for 39 days. In addition, the effect of three freezes (−80 °C)–thaw (room temperature) cycles was also studied.

III. Results and Discussion

3.1. Method development

Optimization of the chromatographic conditions was done to achieve a high sensitivity, good peak shape and a short run time for GPD and IS, table 1.

3.2. Selectivity

Comparing UPLC–MS/MS chromatogram of GPD in human plasma samples with those of blank blood sample shows that there was no interference of other components. The retention time of GPD, and IS was 1.0 min, Fig. 2.

3.3 Carry over

Carry over in the blank sample following the high concentration standard was not greater than 20% of the lower limit of quantification (LLOQ) and 5% for the internal standard.

3.4. Linearity and sensitivity

A good linearity in the concentration range of 1–100 ng/mL for GPD was obtained. The lower limit of quantification (LLOQ) was 1 ng/mL for GPD. The % RSD was 12.12 at LLOQ level, which was within the accepted limits, table 2.

Analytical sensitivities (γ) , which accounts for the variation in the standard deviation of the analytical signal measured for different concentration levels of GPD are listed in table 3.

3.5. Precision and accuracy

Table 4 shows that the intraday and interday precision were within 9.65%, and accuracy of the method ranged from -5.40% to 0.70%.

3.6. Recovery and matrix effect

The results of the recovery in plasma ranged from 51.20 % to 63.44%. The recovery of IS in plasma was 80.03%. In addition, matrix effect in human plasma ranged from 1.0310 to 0.9864 at different QCs concentration.

3.7 Stability

Stability tests were carried out under different storage conditions using the low and high QC samples with three determinations for each. RSD of the mean test responses was within 15% in all stability tests, table 5. There was no significant effect on the determination of GPD in plasma samples kept at room temperature for 40 h. No degradation was observed through three freeze (−80 °C) –thaw (room temperature) cycles. As a result, GPD in samples were stable at −80 °C for 39 days.

IV. Conclusions

In the present work, a validated UPLC–MS/MS method was developed for the simultaneous quantitation of GPD in human plasma. The proposed method affords the sensitivity, very short elution time, accuracy and precision needed for the efficient determination of GPD in human plasma.

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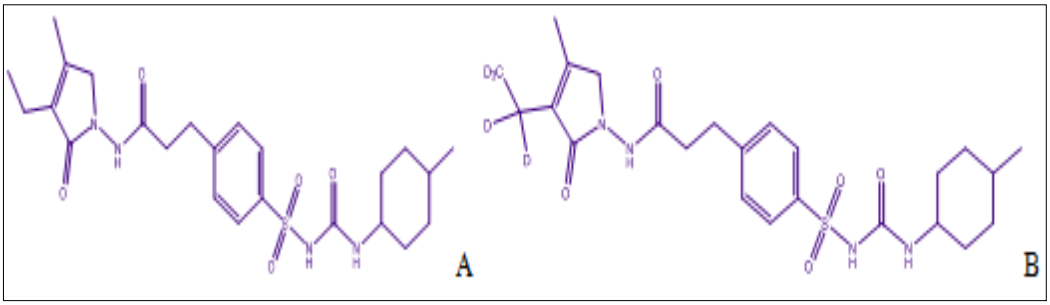

Figure 1: The chemical structures of GPD and IS in the present study: (A) GPD and (B) IS

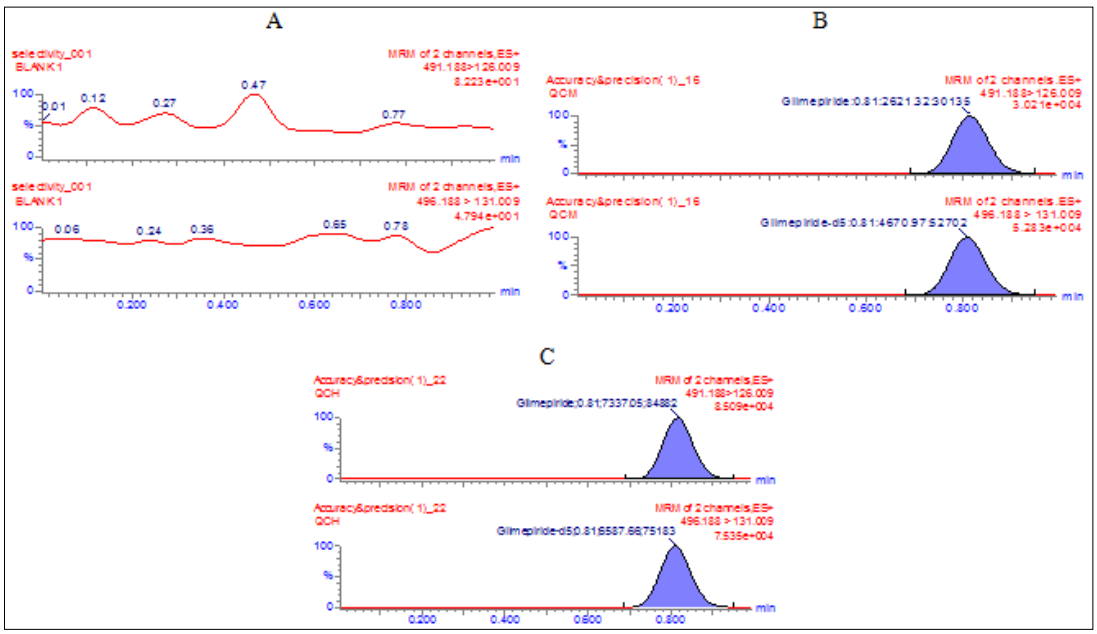

Figure 2: Representative chromatograms of GPD and IS in human plasma samples. (A) A blank plasma sample (B) spiked plasma sample at LLOQ (1 ng/mL) with the IS (C) spiked plasma sample at QC_H (80 ng/mL) with the IS

Table1: Summary of the analytical method parameters

Parameters	Description
Detection method	MS/MS 491.188>126.009(Glimepiride), 496.188>131.009(Glimepiride-d5). Capillary(Kv):3.5

	Cone(V):22 Collision energy(eV):36 Source Temp:150 °C Desolvation Gas(L/H):800 Desolvation temp: 250°C Cone Gas(L/H):50 Collision Gas(mL/min):0.10
Analytical column	BEH C_{18} 1.7µm (2.1*50 mm Column).
Auto-sampler temperature	10.0 ºC
Column temperature	30.0 ºC
Mobile phase	A: 30% (Ammonium formate 0.1 %) B:70% Acetonitrile
Flow Rate	0.3 mL/min
Injection volume	10.0 µL
Run time	1.0 min

Table 2: Summary of statistical evaluation of the measured area ratios

Sample	Nominal values (ng/mL)	Average Area ratio	SD	RSD (%)
1	1.000	0.0165	0.0020	12.12
2	2.000	0.0290	0.0041	14.14
3	4.000	0.0618	0.0032	5.18
4	10.000	0.1399	0.0097	6.93
5	20.000	0.2849	0.0129	4.53
6	50.000	0.6920	0.0539	7.79
7	75.000	0.9973	0.0310	3.11
8	100.000	1.2402	0.0338	2.73

Table 3: Analytical sensitivities (γ) for three GPD concentrations

Nominal values (ng/mL)	SD	Analytical sensitivity (γ) (Slope /SD)
3.000	0.146	0.0927
40.000	1.339	0.01011
80.000	1.940	0.00698

Table 4: Precision and accuracy of method for the determination of GPD in human plasma

Nominal concentration (ng/mL)	Intraday (n= 6 single day)			Interday (n= 8 from each day)		
	Mean conc. found (ng/mL)	% RSD	Relative Error (%)	Mean conc. found (ng/mL)	% RSD	Relative Error (%)
QC_L (3)	2.838	9.65	-5.40	3.021	2.910	0.70
QC_M (40)	38.918	2.98	-2.85	38.034	3.010	-5.06
QC_H (80)	78.605	2.20	-1.89	77.292	3.550	-3.53

Table 5: Stability Studies of GPD under different conditions

Stability		QC_L[a]				QC_H[b]			
Auto-sampler stability		0 h	20 h	40 h		0 h	20 h	40 h	
	Average	3.038	3.058	3.125		81.294	81.747	81.132	
	%stability	101.27	101.93	104.17		101.47	102.03	101.26	
Freeze and thaw cycle stability	FTC[c]	zero[d]	one	two	three	zero[d]	one	two	three
	Average	3.266	3.061	3.059	2.860	83.283	78.156	79.068	75.655
	% stability	108.87	102.03	101.97	95.33	103.95	97.55	98.69	94.43
Short term matrix-based stability		0 h		24 h		0 h		24 h	
	Average	3.086		3.125		81.326		82.291	
	% stability	102.87		104.17		101.51		102.71	
Long term matrix-based stability		0 d	17 d	39 d		0 d	17 d	39 d	
	Average	2.847	2.941	3.033		73.48	73.88	69.959	
	% stability	94.90	98.03	101.10		91.7	92.22	87.3	

[a] QC_L = 3 ng/mL
[b] QC_H = 80 ng/mL
[c]FTC : freeze and thaw cycle
[d]zero cycle is a freshly spiked and prepared sample