



# Impact of sample storage conditions on gliclazide quantification in rat plasma by UHPLC/UV method: storage recommendation and pharmacokinetic application

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## ABSTRACT

The effect of different storage durations/temperatures on the stability of gliclazide (GLZ) in plasma was not studied before in the literature. A simple, reproducible ultra-high performance liquid chromatography coupled with ultraviolet detection (UHPLC/UV) was established for monitoring GLZ in rat plasma was established, with all stability parameters fully evaluated. The developed method provided simple solvent extraction for both GLZ and Glibenclamide as an internal standard (I.S.) with a short run time ( $\leq 7$  minutes). A linear calibration curve (0.1–40  $\mu\text{g/ml}$ ), with good intra- and inter-day precision and accuracy, was established based on 40- $\mu\text{l}$  plasma volume. Lower and upper limits of quantification allowed effective monitoring of the expected variations in GLZ absorption. The stock solution, freeze–thaw, short-term and bench stability results safeguard the rationale of the UHPLC/UV method. However, GLZ plasma samples were only stable for 1 week storage at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ . The present study emphasized the importance of proper storage conditions, with recommendations for direct analysis of GLZ in plasma after sample collection or maximum storage for 1 week at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  until analysis, to ensure accurate measurement of the drug in plasma. This method was effectively applied in a pharmacokinetic study of GLZ single oral dose from the market product Diamicon<sup>®</sup> MR 30 mg administered in six rats.

## INTRODUCTION

Analytical methods employed for drug and/or metabolites quantitation in biological fluids display a major role in the evaluation of pharmacokinetics, bioavailability (BA), and bioequivalence (BE). Thus, to ensure reproducible outcomes that can be interpreted, a well-characterized and fully validated bioanalytical method is a prerequisite to perform a successful BA/BE study. Validation of a bioanalytical method provides all steps needed to ensure precise quantification of analytes in biological matrices, which are reliable for the proposed use (FDA, 2018).

Gliclazide (GLZ), an oral hypoglycemic drug, is used for the treatment of (type II) diabetes. GLZ is readily absorbed from the gastrointestinal tract (GIT) with extensive metabolism in the liver by the processes of hydroxylation, N-oxidation, and oxidation. Nearly 60%–70% of drug dose is excreted in urine with excretion in an unchanged form limited to 5%. GLZ is highly protein-bound with plasma half-life of 6–14 hours (British Pharmacopoeia, 1998; Reynolds, 1993). GLZ enhances insulin secretion; also, it possesses beneficial extrapancreatic effects; thus, it is potentially useful in type I diabetes as well (Fu and Zhong, 2001; Holmes *et al.*, 1984). High variations in GLZ oral absorption were previously documented (Frey *et al.*, 2003; Palmer and Brogden, 1993). Such variations are reportedly related to the drug characteristic dissolution pattern along the GIT, with early dissolution in the stomach proceeding to variations in intestinal absorption with reported enterohepatic circulation in both human (Davis *et al.*, 2000; Hong *et al.*, 1998; Palmer and Brogden, 1993)

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and rat (Murthy and Mayuren, 2008; Reddy and Kumar, 2017; Satyanarayana *et al.*, 2007) models. Enterohepatic circulation is often associated with the existence of two peaks in GLZ plasma concentration-time profiles (Murthy and Mayuren, 2008; Reddy and Kumar, 2017; Satyanarayana *et al.*, 2007). Therefore, the development of a precise and sensitive analytical method which can monitor both low/high concentrations of GLZ in biological fluids and detect the existence of GLZ multiple peaks is presently considered.

From the literature survey, quantification of GLZ has been reported employing high performance liquid chromatography (HPLC)/UV (Al Mahmud *et al.*, 2015; Damanjeet and Lakshmi, 2009; Khan *et al.*, 2014; Ravi kumar *et al.*, 2013; Rouini *et al.*, 2003), electrochemical (Kuo and Wua, 2005), calorimetric (Awasthi and Kulkarni, 2014), or mass detection (Hu *et al.*, 2009; Mendes *et al.*, 2007; Wang *et al.*, 2008; Zhong *et al.*, 2005). HPLC continues to be the most widespread, cost-effective method for the analysis of drugs in biological samples. Analytical methods like liquid chromatography with tandem mass spectrometry are not readily affordable for routine analysis and product development due to their high equipment cost. Also, they are usually intended for human biological samples with a relatively large plasma volume. Rats are considered ideal applicants for preclinical pharmacokinetic evaluation of drugs due to their small size, cost-effective value, and ease in handling.

To date, quantification of GLZ in rat plasma is limited (Adhikari *et al.*, 2014; Resztak *et al.*, 2014; Talari *et al.*, 2011), with no published full validated reports in accordance with the international guidelines [United States-Food and Drug Administration (US-FDA), Europe, Middle East, and Africa (EMA), and World Health Organization (WHO)]. Hence, no evidence on their applicability for *in vivo* evaluation of different GLZ formulation in rat plasma was proved.

It is quite essential that therapeutic drug monitoring should provide a precise quantity of administered drugs for productive diagnosis in patients (Seeger *et al.*, 2009). Storage of biological samples for prolonged times at inappropriate temperatures may cause erroneous results for many bioanalyses.

Evaluation of sample stability of various drugs was previously reported (Gao *et al.*, 2007; Ingels *et al.*, 1995; Taylor and Sethi, 2001). Those studies emphasized the importance of optimizing storage conditions for exact measurement of tested drugs.

Assessment of GLZ plasma stability under different storage conditions was not reported before in literature; therefore, it is investigated for the first time in the present study.

The present work aimed to develop an accurate and reproducible UHPLC method for GLZ quantification in rat plasma with high sensitivity. The developed method will be validated in agreement with US-FDA regulations (FDA, 2018), through optimizing both chromatographic conditions and plasma sample preparation procedures using a small plasma volume (40  $\mu$ l). GLZ plasma stability under different storage conditions was investigated for the first time. The applicability of the developed method in a pharmacokinetic study of GLZ single oral dose from the market product (Diamicon<sup>®</sup> MR 30 mg) in six rats was carried out.

## MATERIALS AND METHODS

### Materials

GLZ pure powder and Glibenclamide as an internal standard (I.S.) were kindly provided from Sigma, Egypt. All chemicals employed were of analytical grades, as for methanol (MeOH), and acetonitrile (ACN); they were of HPLC grades acquired from Prolabo, France. Milli-Q deionized water (Millipore Corp., Burlington, MA) was used. Diamicon<sup>®</sup> MR tablets 30 mg (Batch number 25298, Servier, Egypt) was used as a market product.

### Apparatus

The UHPLC system consisted of Waters Acquity<sup>®</sup> Arc, with Quaternary Solvent Manager-R, Sample Manager (FTN-R), equipped with 2489 UV/Vis detector together and Empower<sup>®</sup> 3 computer software. Symmetry C18 analytical column was used [P.S. 5  $\mu$ m (length 3.9 cm; diameter 150 mm)], with a packed preguard column and Symmetry C18 inserts (5  $\mu$ m).

### Chromatographic settings

An isocratic elution scheme was initiated for GLZ chromatographic separation using mobile phase system of 55:45 (v/v) of ACN and deionized water, respectively, with pH adjusted to 3.8. A 1 ml/minute flow rate was used and UV detector  $\lambda$  was 230 nm.

### Standard solutions

GLZ and Glibenclamide stock solutions (100  $\mu$ g/ml, each) were prepared in MeOH and ACN, respectively. Dilution of the stocks was carried out with the equivalent mobile phase to provide standard working solutions of both analytes.

### Calibration samples

Calibration samples were set by spiking GLZ working solutions in a concentration range of 0.1–40  $\mu$ g/ml in blank rat plasma. Preparation of quality control (QC) samples was done similarly, at concentrations of 0.2  $\mu$ g/ml for low quality control (LQC), 1 and 5  $\mu$ g/ml for medium quality control (MQC), and 30  $\mu$ g/ml for high quality control (HQC). Spiked samples were stored at  $-20^{\circ}\text{C}$  till analysis.

### Sample extraction

To 40  $\mu$ l of rat plasma, 12.5  $\mu$ l of I.S. (4  $\mu$ g/ml) was added. Following brief vortex mixing, 1 ml of ethyl acetate was added followed by 2 minutes vortex and centrifugation (4,000 rpm) for 5 minutes at a temperature adjusted to  $4^{\circ}\text{C}$  (cooling centrifuge, Sigma 3-16KL, Germany). Separation of the upper organic layer was done, followed by evaporation at  $60^{\circ}\text{C}$  in a vacuum concentrator (miVac concentrator, DUC-23050-B00, USA) till dryness. The residue was reconstructed with 100  $\mu$ l of mobile phase and vortex mixed (2 minutes). Finally, aliquots of supernatant (50  $\mu$ l) were injected into the UHPLC system for analysis.

### UHPLC/UV method validation

The developed method was validated as instructed by US-FDA guidelines (FDA, 2018) in terms of the following parameters.

#### Selectivity

Selectivity was calculated by injecting pooled blank plasma samples ( $n = 6$ ) to confirm the absence of interference peaks around the expected retention time of both analytes.

#### Linearity

For determination of linearity, two calibration curves were plotted with two different ranges (i.e., 0.1–2 and 1–40  $\mu\text{g/ml}$ ). The construction of two calibration curves omits the necessity for sample dilution as well as repeated analysis needed for unknown samples. The ratio of peak area of drug to I.S. against drug concentrations was established and fitted by linear least-squares regression model.

#### Lower limit of quantification (LLOQ)

The lowest detectable sample concentration that is noticed from any background noise but still not quantifiable is termed as limit of detection (LOD). LLOQ signifies the lowest concentration in the calibration curve, where the ratio of signal to noise  $\geq 5$ , with precision  $< 20\%$ , and accuracy of about  $20\%$  (FDA, 2018).

#### Extraction recovery

GLZ extraction recovery from rat plasma was determined at different concentration levels ( $n = 6$  per concentration). Measurements of absolute recovery % were conducted by comparing the peak area of GLZ to I.S. with equivalent values in standard solutions.

#### Accuracy and precision

Intraday and interday precision and accuracy were considered within 1 day and 3 successive days, respectively, for different GLZ concentrations ( $n = 6$ ).

Coefficient of variation (CV %) was used for estimation of precision, while accuracy was evaluated as relative error (RE %). The acceptable values for accuracy and precision were RE% within  $\pm 15\%$  and CV%  $\leq 15\%$ , respectively, with RE% of  $\pm 20\%$  and CV  $\leq 20\%$ , for LLOQ.

#### Stability assessment

Pre-extraction stability parameters include stock solution, three freeze and thaw cycles, short- and long-term plasma. GLZ stock solution was evaluated at ( $-20^\circ\text{C}$ ) after 4 weeks. Freeze and thaw stability was measured after three complete cycles at ( $-20^\circ\text{C}$ ), where QC samples were processed and compared against fresh sets. Short-term plasma stability was measured after 24 hours storage at room temperature. Long-term plasma stability was evaluated after storage for 1 and 2 weeks at ( $-20^\circ\text{C}$ ) and ( $-80^\circ\text{C}$ ), where stored QC samples were compared with fresh ones.

The post-extraction (bench) stability was investigated by keeping processed samples at room temperature for 6 hours and reanalyzed. Also, dry extract of LQC samples (after sample treatment and before reconstitution) was stored for 4 weeks at ( $-20^\circ\text{C}$ ) and ( $-80^\circ\text{C}$ ) and then reanalyzed.

### Pharmacokinetic application

Six healthy male Wistar rats were provided from the animal house of the National Research Centre and quarantined for 1 week. The selected weight range was 200–250 g. The study protocol was accepted by the Medical Ethical Committee of the National Research Centre (registration approval number 16–058). Evaluation of the developed UHPLC method was carried out in rat plasma by giving a single dose (4 mg/kg) of the market product via an oral feeding tube. Rats were given a standardized type of food and allowed to drink water throughout the study. Rats were injected with 2 ml of glucose (5% solution for injection) via intraperitoneal route at specific time intervals.

Blood sampling was taken under general anesthesia via the retro-orbital plexus as described earlier (Parasuraman *et al.*, 2010). Blood samples were collected in heparinized vacutainer tubes before dosing (0 hour) and at specified time intervals after dosing, followed by centrifugation at 4,000 rpm for 10 minute, and the obtained plasma was then separated promptly and frozen at  $-20^\circ\text{C}$  until assayed.

### Pharmacokinetic analysis

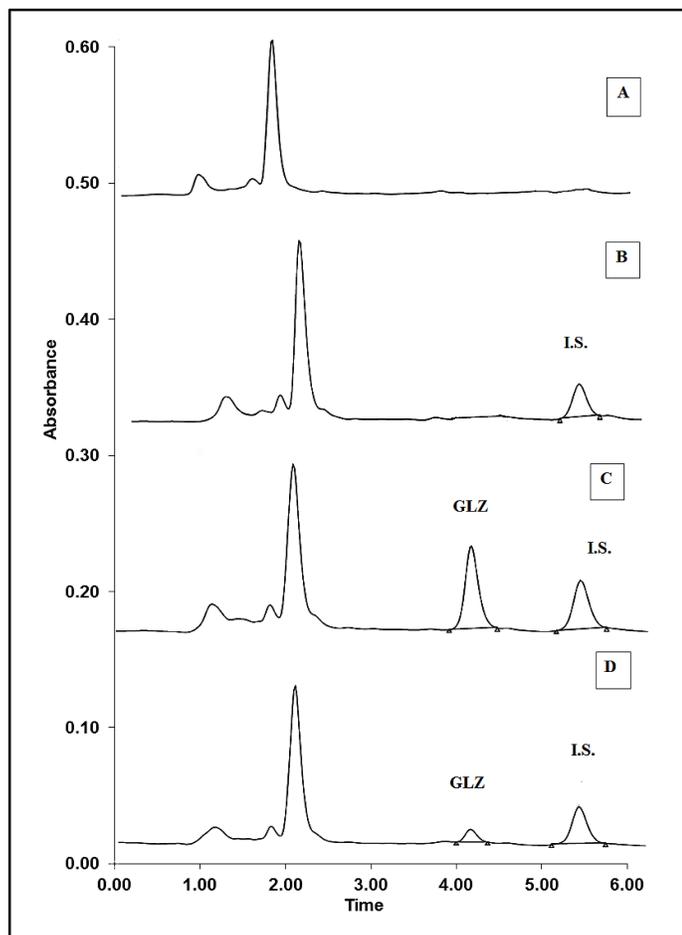
The parameters of pharmacokinetics were evaluated from the plasma concentration versus time data using a noncompartmental model employing the WinNonLin, professional 2.1 computer program (Pharsight, Sunnyvale, CA). The following parameters were evaluated:  $C_{\text{max}}$ ,  $T_{\text{max}}$ ,  $\text{AUC}_{0-24}$ ,  $\text{AUC}_{0-\infty}$ ,  $K_{\text{el}}$ , and  $T_{1/2}$ .

## RESULTS AND DISCUSSION

### Chromatographic and extraction procedures

Initial trials were conducted to select suitable entity and type of column, strength of solvent and optimum flow rate, for the achievement of good separation, better peaks shape, and shorter run times for both analytes. A mixture of ACN and water (55:45, v/v) with pH of 3.8 at 1 ml/minute flow rate allowed elution of both GLZ and I.S. with better resolution using a Symmetry C18 column. Our proposed method proceeded without the need for buffers; with retention times for GLZ and I.S. equal to 4.2 and 5.4 min, respectively and a total run time less than 7 minutes (Fig. 1). On the other hand, Resztak *et al.* (2014), separated GLZ and Glibenclamide from rat plasma at retention times of 9.4 and 14.7 minutes, respectively, using a buffer system of 0.04 M potassium dihydrogenphosphate (pH 3.8)/ACN.

A protein precipitation method was proposed during the early stage of sample treatment using ACN and MeOH. However, the extraction efficiency was poor, with tailed peaks and many endogenous compounds extracted simultaneously. Thus, the liquid-liquid extraction method was adopted using ethyl acetate as



**Figure 1.** Representative UHPLC chromatograms in rat plasma: (A) blank sample; (B) sample spiked with 2 µg/ml Glibenclamide (I.S.); (C) sample spiked with 0.5 µg/ml GLZ and 2 µg/ml I.S.; (D) sample evaluated 3 hours after single oral administration of 4 mg/kg GLZ from Diamicon®, MR 30 mg.

an extracting solvent, which proved to provide high recovery for GLZ with no significant interferences.

## Method validation

### Selectivity

Figure 1 showed the following chromatograms of rat plasma: blank sample with no spiking (Fig. 1A), sample spiked with 2 µg/ml I.S. (Fig. 1B), sample spiked with 0.5 µg/ml GLZ and 2 µg/ml I.S. (Fig. 1C), and sample collected from rat 3.0 hours following a single oral administration of market product (Fig. 1D)

### Linearity, LOD, and LLOQ

In this study, the detection of GLZ concentration was done using two standard calibration curves covering the range of 0.1–40 µg/ml (Table 1). The determination coefficients ( $R^2$ ) values were equal to 0.9913 and 0.9922 for the low (0.1–2 µg/ml) and high (1–40 µg/ml) calibration curves, respectively. The

LOD and LLOQ for GLZ were 0.05 and 0.1 µg/ml, respectively (based on 40 µl plasma). Detailed results of regression equations with slopes and intercepts for both calibration curves were presented in Table 1. It must be noted that the developed method provided such a wide calibration range (0.1–40 µg/ml) to monitor expected variations in GLZ plasma concentrations. Adhikari *et al.* (2014) provided a narrow calibration range of 0.25–5.00 µg/ml (based on 100 µl plasma sample), which might not be sufficient to properly detect varying concentrations of the drug in rat plasma.

### Extraction recovery

GLZ % extraction recoveries were within the range of  $85\% \pm 3.08\%$  ( $n = 6$ ) at all studied concentrations with relative standard deviation values below 7.3%, demonstrating good consistency.

### Accuracy and precision

The results are summarized in Table 2, where intra- and interday precisions values (CV %) were less than 15% with intra- and interday accuracy ranging from 91.37 to 105.85 and 93.92% to 105.65% in rat plasma, respectively. All precision and accuracy parameters were in compliance with the acceptance criteria stated in international guidelines, indicating the proper reproducibility, accuracy, and low intra- and interday variations of the developed UHPLC method. Our study complied with the recommended international regulations by conducting the tests on six concentration levels (i.e., 0.2, 0.5, 1, 5, 10, and 30 µg/ml).

### Stability

The stability of GLZ in rat plasma during sample handling, analysis, and storage was evaluated for the first time and results were summarized in Table 3.

#### Pre-extraction stability

1. Stock solution stability. GLZ stock solution in ACN was stable for 4 weeks of storage at  $-20^\circ\text{C}$  with percentage recoveries ranging from 87.93% to 104.88% for different QC samples (Table 3).

2. Freeze and thaw stability. GLZ stability in rat plasma was not affected over three freezing and thawing cycles, where percentage recoveries ranged from 89.92% to 112.59% for the tested QC samples (Table 3).

3. Short-term plasma stability. GLZ plasma samples were stable at room temperature for 24 h, as shown in Table 3.

4. Long-term plasma stability. GLZ plasma stability was evaluated at  $-20^\circ\text{C}$  or  $-80^\circ\text{C}$  for 1-2 weeks to ensure samples stability during the whole analysis period. No difference was observed between fresh samples and the ones kept at  $-20^\circ\text{C}$  or  $-80^\circ\text{C}$  for 1 week, with percent recoveries values of 102.76%–113.34% and 98.76%–112.04%, respectively, for all QC samples (Table 3).

However, GLZ plasma samples stored at  $-20^\circ\text{C}$  or  $-80^\circ\text{C}$  for 2 weeks were not stable, with only 30%–40% of GLZ detected for all tested QC samples [the obtained percentage recoveries were out of the acceptable limits stated by FDA (2018)]

**Table 1.** Linear regression data for GLZ in rat plasma by UHPLC/UV method.

Calibration curve range (ug/ml) <sup>a,b</sup>	Slope (± SD)	Intercept (± SD)	R <sup>2c</sup>	LOD <sup>d</sup>	LLOQ <sup>e</sup>
0.1–2	15.522	−0.519	0.9913	0.05	0.1
1–40	0.3517	0.125	0.9922		

<sup>a</sup>Peak area ratio of GLZ/I.S. versus concentration.

<sup>b</sup>Data expressed as mean ± SD (*n* = 6).

<sup>c</sup>R<sup>2</sup> = determination coefficient.

<sup>d</sup>LOD = limit of detection.

<sup>e</sup>LLOQ = lower limit of quantification.

**Table 2.** Results of precision and accuracy of GLZ in rat plasma.

Intra-day				
Added concentration (ug/ml)	Mean determined concentration <sup>a</sup> (ug/ml) ± SD	Precision (%) <sup>b</sup>	Accuracy (%) <sup>c</sup>	
0.2	0.2117 ± 0.0045	2.13	105.85	
0.5	0.5113 ± 0.0565	11.05	102.26	
1	0.9137 ± 0.0076	0.83	91.37	
5	4.6097 ± 0.2122	4.60	92.19	
10	9.9403 ± 0.2613	2.63	99.40	
30	30.3490 ± 2.1340	7.03	101.16	
Inter-day				
0.2	0.2113 ± 0.0181	8.57	105.65	
0.5	0.5113 ± 0.0513	10.03	102.26	
1	1.0188 ± 0.0511	5.02	101.88	
5	4.6959 ± 0.0457	0.97	93.92	
10	9.9272 ± 0.2610	2.63	99.27	
30	28.8628 ± 0.9310	3.23	96.21	

<sup>a</sup>Data expressed as mean ± SD (*n* = 6).

<sup>b</sup>Presented as % CV.

<sup>c</sup>Calculated as % RE.

**Table 3.** Stability results of GLZ in rat plasma.

Stability conditions	Percentage of GLZ recovery from initial concentration (%) <sup>a</sup>			
	LQC samples (ug/ml)	MQC samples (ug/ml)		HQC samples (ug/ml)
	0.2	1	5	30
Bench (25°C, 6 hours)	87.93%(± 3.91)	88.82%(± 4.13)	91.98%(± 15.15)	90.93%(± 5.94)
Stock solution (−20°C, 4 weeks)	90.76%(± 0.31)	88.89%(± 3.91)	87.93%(± 4.31)	104.88%(± 0.31)
Short-term plasma (25°C, 24 hours)	88.12%(± 0.33)	89.93%(± 0.11)	95.44%(± 0.21)	100.76%(± 0.45)
Freeze and thaw (−20°C, 3 cycles)	89.92%(± 3.17)	104.59%(± 5.91)	112.59%(± 2.11)	93.44%(± 4.27)
Long-term plasma (−20°C)				
1 week	102.76%(± 1.98)	110.91%(± 1.18)	113.34%(± 1.00)	112.67%(± 2.23)
2 weeks	All QC samples were ≤ 60%(± 4.33)			
Long-term plasma (−80°C)				
1 week	98.76%(± 2.36)	103.45%(± 6.22)	112.04%(± 1.89)	108.76%(± 2.36)
2 weeks	All QC samples were ≤ 53.23%(± 6.69)			

LQC: low quality control; MQC: medium quality control; HQC: high quality control.

<sup>a</sup>Average of six determinations (*n* = 6)

(Table 3). Hence, for accurate measurements of GLZ in plasma, it is highly recommended that samples must be measured either immediately after collection or stored at −20°C or −80°C for not more than 1 week until analysis.

#### Post-extraction Stability

1. Bench stability. As can be seen in Table 3, extracted GLZ samples can endure bench stability for 6 h at 25°C, with GLZ percentage recoveries equal to 87.93%, 88.82%, 91.98%, and

90.93% for concentrations 0.2, 1, 5, and 30  $\mu\text{g/ml}$ , respectively. On the other hand, when the dry extract (after sample treatment, before reconstitution) was 4 weeks stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  and then reanalyzed, several interfering peaks were detected and GLZ characteristic peak was clearly reduced.

For routine clinical practice, the measurement of plasma concentration levels often requires several days, especially in hospitals with high patient capacity. Biological samples are usually stored under refrigerated conditions or in freezers; hence by identifying different factors affecting the stability of drugs, clinicians might optimize sample storage and provide a better scope of how to manage accurate dosing in patients.

This is the first study that provided a fully validated bioanalytical report for estimating GLZ in rat plasma, including the impact of long-term storage at different temperatures on the

stability of GLZ in plasma. No study has evaluated the stability of GLZ or its storage conditions in detail.

### Pharmacokinetic application in rat plasma

The validated UHPLC/UV method was further applied in a pharmacokinetic study in a rat model. GLZ single dose (4 mg/kg) from the market product (Diamicon<sup>®</sup> MR 30 mg) was given to six rats using an oral feeding tube. The individual plasma concentration versus time profiles of GLZ in six rats was presented in Figure 2 with corresponding pharmacokinetic parameters summarized in Table 4.

The data showed that absorption of GLZ from the innovator product gave  $C_{\text{max}}$  values ranging from 2.88 to 19.65  $\mu\text{g/ml}$ . Also,  $T_{\text{max}}$  values ranged from 1.5 to 5 hours, while  $\text{AUC}_{0-24}$  and  $\text{AUC}_{0-\infty}$  values attained values from 15.67 and 19.59 to 178.04 and 192.09  $\mu\text{g}\cdot\text{hours/ml}$ , respectively. As expected, the results showed high variations in GLZ plasma concentrations among the six rats (Table 4). However, the developed UHPLC method provided LLOQ and Upper limit of quantification (ULOQ) sufficient enough to detect such variations. The phenomenon of high variations in GLZ, BA was previously reported by other researchers (Frey *et al.*, 2003; Palmer and Brogden, 1993).

Figure 2 also showed multiple peak behavior of GLZ which was detected in plasma concentration-time profiles of five rats. As previously reported, the existence of two peaks usually occurs due to enterohepatic circulation in rats and humans (Davis *et al.*, 2000; Murthy and Mayuren, 2008; Satyanarayana *et al.*, 2007), with the first and second peaks appearing at 2 and 8 hours after dosing, in rats, respectively (Davis *et al.*, 2000; Murthy and Mayuren, 2008; Satyanarayana *et al.*, 2007). Multiple peak phenomenon is an ongoing crucial issue when managing  $C_{\text{max}}$  in BA/BE studies, with relevant examples related to this issue in the literature (Emara *et al.*, 2014; Ezzet *et al.*, 2001; Marzo, 2007). Nevertheless, most of the pharmacokinetic research articles dealing with oral administration of GLZ in rats were carried out without discussing the above-mentioned phenomenon (Adhikari *et al.*, 2014; Resztak *et al.*, 2014; Talari *et al.*, 2011).

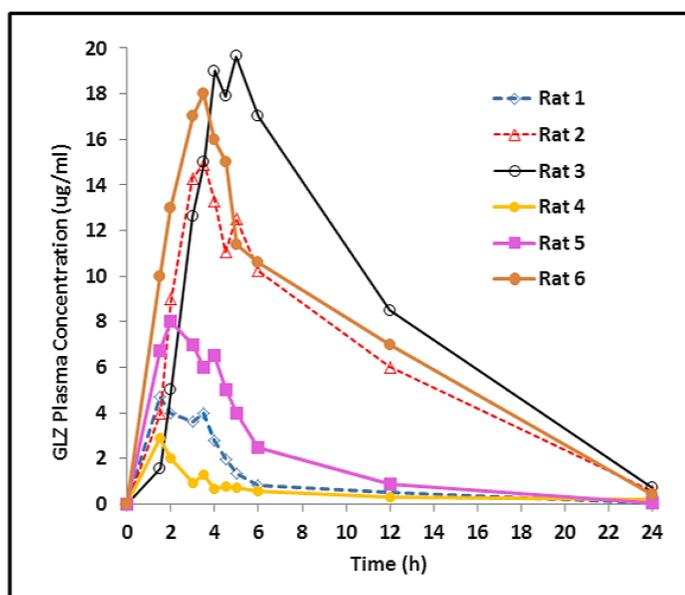


Figure 2. Individual plasma concentration versus time profiles, following oral administration of 4 mg/kg GLZ from Diamicon<sup>®</sup>, MR 30 mg in six rats.

Table 4. Pharmacokinetic results following single oral administration of 4 mg/kg GLZ from Diamicon<sup>®</sup> MR 30 mg in six male Wistar rats.

Pharmacokinetic parameters	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6
$C_{\text{max}}$ ( $\mu\text{g/ml}$ )	4.71	14.92	19.65	2.88	8.41	17.56
$T_{\text{max}}$ (hour)	1.50	3.50	5.00	1.50	2.00	3.50
$\text{AUC}_{0-24}$ ( $\mu\text{g}\cdot\text{hour/ml}$ )	26.82	145.11	178.04	15.67	30.56	156.78
$\text{AUC}_{0-\infty}$ ( $\mu\text{g}\cdot\text{hour/ml}$ )	28.28	151.49	192.09	19.59	33.99	169.54
$T_{1/2}$ (hour)	5.05	7.85	10.05	12.79	6.55	8.99
$K_{\text{el}}$ ( $\text{hour}^{-1}$ )	0.14	0.09	0.07	0.05	0.09	0.08

## CONCLUSION

The present study investigated the stability of pre- and postextracted GLZ plasma samples stored for several weeks at  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$  for the first time. The authors drew attention to the importance of proper storage conditions, with recommendations for either direct analysis of GLZ in plasma after sample collection or storage at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  for not more than 1 week until analysis. The proposed UHPLC/UV method offered the following additional advantages: a full validated bioanalytical report, a small plasma sample volume of  $40\ \mu\text{l}$ , a wide calibration concentration range, and a short run time. The LLOQ and ULOQ limits were safely applied for *in vivo* evaluation of therapeutic concentrations of GLZ in rat plasma as well as monitoring expected variations in GLZ plasma concentrations.

## ABBREVIATIONS

GLZ	Gliclazide
I.S.	Internal standard
ACN	Acetonitrile (Methanol)
QC	Quality control
LQC	Low quality control
MQC	Medium quality control
HQC	High quality control
LOD	Limit of detection
LLOQ	Lower limit of quantification
ULOQ	Upper limit of quantification
BA	Bioavailability
BE	Bioequivalence

## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

The study protocol was accepted by the Medical Ethical Committee of the National Research Centre (registration approval number 16–058).

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