

DEVELOPMENT AND VALIDATION OF A NOVEL RP-HPLC METHOD FOR PHARMACOKINETIC STUDIES OF GLICLAZIDE IN RAT

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Abstract

A sensitive, simple and accurate reverse phase HPLC method was developed to determine plasma levels of gliclazide (GL) in rat. The method uses liquid-liquid extraction that is not expensive and ibuprofen as internal standard, that is easily available. Mean recovery for GL and internal standard was 80% and 82% respectively. Relative standard errors and CV% ranged from 0.47 to 8.99 and from 0.72 to 12.54% for intraday and interday HPLC injections, respectively. The limit of quantitation (LOQ) and the limit of detection (LOD) for GL in serum were 0.12 µg/mL and 0.06 µg/mL respectively. The developed HPLC method is a suitable and rapid method to be adopted for pharmacokinetic studies in rat and human.

Rezumat

Articolul prezintă o metodă nouă HPLC pentru determinarea glicazidei din serul de șobolan. Aceasta are avantajele extracției lichid-lichid, o tehnică puțin costisitoare, care utilizează un standard intern (ibuprofen), ușor accesibil.

Metoda este rapidă și ușor de aplicat pentru determinări pe animal sau om, în cadrul studiilor farmacocinetice.

Keywords: reverse-phase HPLC, gliclazide, pharmacokinetics, rat

Introduction

In recent years, diabetes mellitus has become a common disease affecting human health seriously. About 90% of diabetic patients are found to suffer from type II (or non-insulin-dependent) diabetes mellitus. Gliclazide (GL) (Fig. 1), *N*-(4-methylbenzenesulfonyl)-*N*-(3-azabicyclo-[3.3.0] oct-3-yl) urea, is a second-generation sulfonylurea commonly used in the treatment of non-insulin dependent diabetes mellitus (NIDDM) [1]. Its mean half life is 10 h and has 85 to 95% protein binding in plasma [2].

The low water-solubility of GL leads to a low dissolution rate and variable bioavailability. There are few complications with the clinical use of GL [3] and that could be due to the practical insolubility of GL in water (55 mg/mL at 37°C). In this study we developed a rapid and accurate HPLC method to evaluate plasma levels of GL microcrystal and the substance *per se* after oral administration in rats.

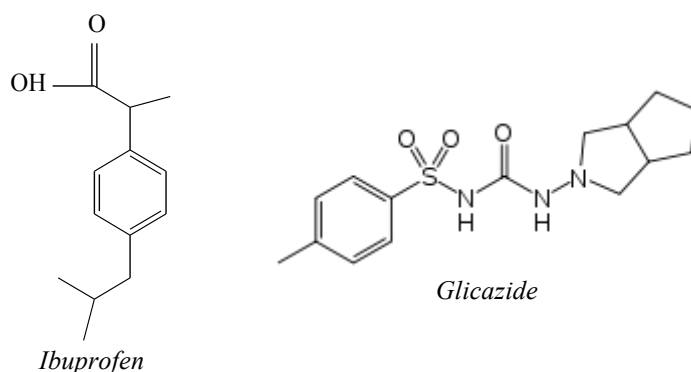


Figure 1

The chemical structure of ibuprofen and gliclazide.

Several HPLC and capillary electrophoresis (CE) methods were reported in the literature for the determination of GL in biological specimens [4-7]. Najib *et al* [4] used the liquid extraction and HPLC-UV for bioequivalence studies of two brands of GL. The limit of detection (LOD) was 0.6 μM in this study. Rouini *et al* [7] described a method using toluene extraction of GL in plasma, and the analysis was performed using HPLC-UV with a 90 nM LOD. However, none of these reported methodologies involved shorter retention time using a simple process of liquid-liquid extraction. We investigated the optimum conditions for the analysis of GL and we successfully established a HPLC method with simple sample preparation and high sensitivity for the determination of GL in rat plasma.

Materials and Methods

Materials

GL (Unites State Pharmacopoeia reference standard) from Kimidaru Co. (Iran), ibuprofen (IB) working standard (used as internal standard, I.S.) was purchased from Sekhasari Co., India. Brij 35, nonionic polyoxyethelene surfactant (Fluka, US), acetonitrile and methanol were purchased from Merck Chemical Company (Darmstadt, Germany). All other chemicals and reagents were of analytical grade.

Microcrystal preparation

A pH-change from 11 to 5 under stirring and in the presence of Brij 35 as stabilizer was used to prepare micronized GL crystals [8]. In our pharmacokinetic studies, we used both the substance *per se* and the microcrystals.

HPLC system

A Waters model 515 pump, 2487 Dual UV absorbance detector and a Waters system solvent module iIEEE 488 pump were used. Isocratic separation was achieved at ambient temperature using Waters C₁₈ (3.9×150 mm). The mobile phase was a mixture of phosphate buffer pH 3.4 and acetonitrile in a ratio of 45/55 % and it was filtered (Millipore, HVLP, 0.45 µm) under vacuum for degassing before using. Each run lasted less than 5 min at a flow-rate of 1 mL/min.

Reagents and standard solutions

Stock solutions of GL and IB (50 µg/mL in acetonitrile) were prepared and stored in fridge during the experiments. A 10 µg/mL solution of IB in deionised water was prepared freshly every day as working standard solution. HPLC grade acetonitrile, methanol and double distilled water were used throughout the analysis.

Male Wistar rats were obtained from the animal house of Isfahan University of Medical Sciences. All the animals were cared according to the rules and regulations of the Institutional Animal Ethics Committee (IAEC) guidelines of the Health Ministry, Iran.

Extraction procedure

50 µL of the IS (ibuprofen as internal standard) and GL working solution, 100 µL of 0.07M phosphate buffer (pH=4.4) were added to 100 µL of serum. After vortex mixing for 10 sec, 1 mL of toluene was added and the mixture was shaken vigorously for 1 min. The mixture was then centrifuged for 15 min at 10000 rpm (8500 g) (Eppendorf 5415C, Germany). An 800 µL aliquot of the upper organic layer containing GL and I.S. was transferred to a clean glass tube and evaporated under air stream to dryness at 50°C. The residue was re-dissolved in 100 µL of mobile phase and a 50 µL aliquot was injected onto the HPLC column.

Calibration procedure

100 μ L of GL standard solutions at concentrations of 0.12, 1, 2, 3, 4, 8, 10, 20, 36, 50 μ g/mL, 50 μ L of I.S. working solution and 100 μ L of 0.07M phosphate buffer (pH 4.4) were added to 100 μ L of rat plasma. After the extraction process the residue was re-dissolved in 100 μ L of mobile phase and a 50 μ L aliquot was injected onto the HPLC column.

Method validation parameters

The inter- and intra-day variations of the method were determined by repeating the experiment in 3 days and 3 times in a day, respectively using the concentrations of calibration curve range. The LOD (limit of detection) and LOQ (limit of quantification) were determined as the concentration that produced a signal to noise ratio of 3 and 5 respectively. The LOQ is the lowest concentration that is quantifiable with an acceptable CV (%) and Error (%). The recovery percent of GL and IB were obtained by comparing the area under the peaks after extraction of different concentrations with the same concentrations in double distilled water.

In vivo application of the developed analytical method

Male Wistar rats weighing 180–220 g housed in a light–dark cycle and temperature-controlled environment were used for *in vivo* studies. Food was withheld during the experiment and the night before.

Diabetes was induced in rats by an intraperitoneal injection of streptozotocin solution (16 mg/mL) (Zanosar[®], 45 mg/kg) in citrate buffer pH 4.5. The animals were diabetic 72 h after injection. Rats were considered diabetic and included in the study when fasted glycemia (by Glucose-SL Kit, Zeistchem, Iran) was higher than 300 mg/dL.

Three groups of normal and diabetics rats (n=6) were selected for HPLC analysis studies. Different formulations were intragastrically administered to each group (n=6) as suspended in normal saline by a feeding tube. Normal saline was preferred to any other vehicle because it did not make any change on blood glucose level. We administered:

1. Normal saline to diabetic and normal control group of rats.
 2. GL substance *per se* (40 mg/kg body weight) to diabetic and normal rats.
 3. GL Microcrystals (40 mg/kg body weight) to diabetic and normal rats.
- 300 μ L plasma samples were collected using retro-orbital blood sampling [9]. The plasma collected before GL administration was used as a blank. All blood samples were centrifuged immediately; the plasma was separated and stored at -20°C until analysis.

Table I
Reproducibility and accuracy of the HPLC assay method (n=6)

Concentration ($\mu\text{g} / \text{mL}$)	Between- days			Within-day		
	Mean Concentration ($\mu\text{g} / \text{mL}$)	CV (%)	Error (%)	Mean Concentration ($\mu\text{g} / \text{mL}$)	CV (%)	Error (%)
2	1.94	0.72	2.97	1.94	0.78	2.76
3	2.80	2.22	6.60	2.85	2.28	4.87
4	4.35	8.66	8.64	4.35	8.66	8.64
8	7.82	7.53	2.25	7.28	8.13	8.99
10	10.14	5.08	1.45	9.75	12.54	2.48
20	21.22	5.57	6.09	21.26	5.57	6.09
36	35.83	1.02	0.47	36.02	4.57	0.04
50	51.36	3.17	2.73	49.64	7.31	0.71

CV=coefficient of variation

Results and Discussion

Specificity and selectivity

Figure 2 compares the chromatograms of plasma sample containing GL (retention time =2.8 min) and internal standard (IB) (retention time =4 min) 90 min after administration of microcrystals containing 40 mg/kg GL in diabetic rats and blank plasma spiked with internal standard (IB).

Recovery

Extraction efficiency was determined by comparison of the peak areas of the spiked plasma samples with those of un-extracted GL solution. The recoveries were not concentration dependent, which resulted in a good linearity of the calibration curve. The mean recoveries were 80% for GL and 82% for IB.

Accuracy and precision

The results of intra- and inter-day reproducibility of the method are shown in Table I. Coefficient of variation (CV %) and error % are less than 12.54 and 8.99, respectively. Results of CV% and error% which are below 20% indicate that the method is reproducible within day and between days.

Linearity, limit of detection and limit of quantification

The standard curves were linear over the concentration ranges of 2-50 $\mu\text{g}/\text{mL}$ using line-fit plot in regression analysis with a correlation coefficient of 0.999. The detection limit (LOD) for GL was approximately 0.06 $\mu\text{g}/\text{mL}$ at a signal to noise ratio of 3:1 and the limit of quantitation (LOQ) was 0.12 $\mu\text{g}/\text{mL}$. GL was measurable at the first sampling time in plasma of rats (0.25 h) and after 24 h of sampling.

In vivo application of the developed analytical method

This method has been used for determination of GL in rat plasma following a single oral administration of 40 mg/kg of microcrystals and GL substance *per se* in 6 streptozotocin-induced diabetic rats. Figure 3 shows the typical plasma concentration-time profile for both microcrystals and GL substance *per se*.

In the present study we described a reproducible, sensitive, simple and easy method for analysis of GL in rat plasma that can be used for *in vivo* pharmacokinetic studies. Ibuprofen used as internal standard is more available than 4-hydroxybenzoate [10] or nadoxolol [11] which were reported before. The LOQ of 0.5 $\mu\text{g/mL}$ that was reported in previous study is not a suitable sensitivity for the pharmacokinetic investigations [12]. Another advantage of the present method is the need of very low plasma sample which is particularly important in animal studies. The method used by Poirier *et al* [11] required 250 μL of plasma samples while in the present developed method 100 μL plasma was enough. The solid phase extraction procedure reported by Yu *et al* [13] for sample preparation is an expensive sample clean-up method, while the method used in the present study was based on a liquid-liquid extraction that is less expensive and has a shorter run time.

Figure 2 presents the chromatograms of GL and IB, 90 min after the administration of microcrystals containing 40 mg/kg body weight GL and IB in diabetic rats.

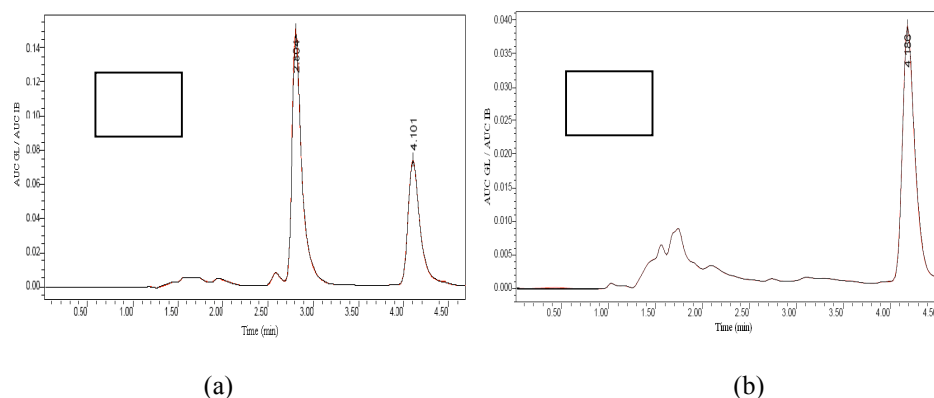


Figure 2

HPLC Chromatograms of a) drug (GL) (2.8 min) and internal standard (IB) (4 min) 90 min after administration of microcrystals containing 40 mg/kg body weight GL in diabetic rats and b) blank plasma spiked with internal standard (IB) ($n=6$).

The retention times of GL and IB are 2.8 and 4.1 min, respectively. Any peak tailing and interference between the peak of the drug and the internal standard was resolved using different pHs of mobile phase and different ratios of the buffer: acetonitrile mixture. The optimum flow rate of the mobile phase was determined as 1 mL/min to detect the drug and internal standard with suitable retention time. This retention time is far less than similar studies for HPLC analysis of GL [7, 14]. There was a good linear regression plotting the peak area ratios of GL to IB vs. GL plasma concentrations. The equation for this regression was $Y = 302 X - 0.3377$ ($r^2 = 0.999$).

The applicability of the method was evaluated by analyzing the samples after administration of 40 mg/kg body weight GL as microcrystal and substance *per se* to diabetic and normal rats. As it is indicated in Figure 3, C_{max} and t_{max} showed significant differences between microcrystals and substance *per se* using the same dose ($p < 0.05$) after administration of 40 mg/kg body weight of microcrystals and substance *per se* to diabetic rats.

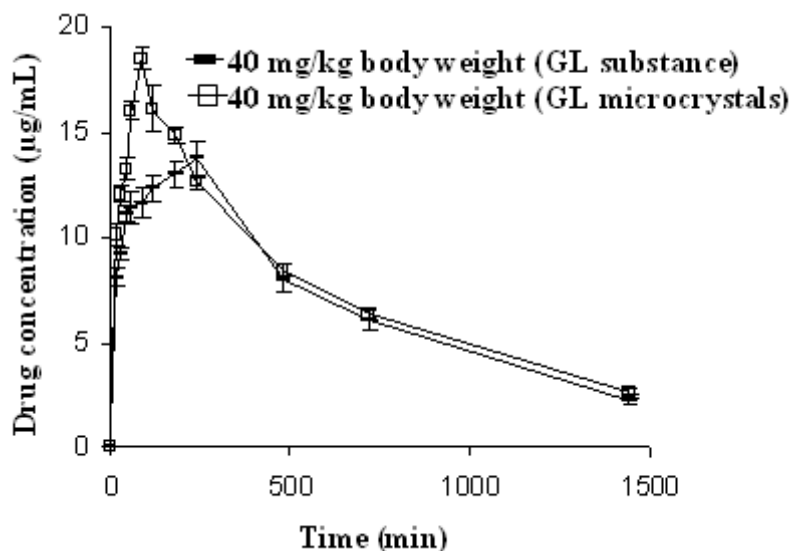


Figure 3

Plasma level concentrations of GL after oral administration of 40 mg/kg body weight of GL substance *per se* and GL microcrystals in diabetic rats ($n=6$).

Conclusions

The developed method is suitable and valid for the determination of GL in rat plasma. The method uses liquid-liquid extraction that is not expensive and ibuprofen as internal standard that is easily available. The

retention time is very short in the developed HPLC method. This easy and rapid method can be used in rat and human studies in future. The validity, LOQ and linearity range of the method makes it acceptable for pharmacological studies in rats. The method was successfully used in determining the blood level of GL in diabetic and normal rats.

Acknowledgments

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References

1. K.J. Palmer, R.N. Brogden, Gliclazide an update of its pharmacological properties and therapeutic efficacy in non-insulin-dependent diabetes mellitus. *Drugs* 1993, 46, 92-125.
2. S.N. Davis, Insulin oral hypoglycemic agents, and the pharmacology of the endocrine pancreas. In: L.L. Brunton, J.S. Lazo, K.L. Parker, eds. Goodman and Gillman's: The Pharmacological Basis of Therapeutics, 11th Ed., New York: Mc Graw-Hill, 2006, 1634.
3. C.S. Winters, L. Shields, P. Timmins, P. York, Solid-state properties and crystal structure of gliclazide. *Journal of Pharmaceutical Sciences* 1994, 33, 300-304.
4. N. Najib, N. Idkaidek, M. Beshtawi, M. Bader, I. Admour, S.M. Alam, Q. Zaman, R. Dham, Bioequivalence evaluation of two brands of gliclazide 80 mg tablets (Glyzide & Diamicon)- in healthy human volunteers. *Biopharmracy Drug Disposition* 2002, 23: 197-202.
5. H. Noguchi, N. Tomita, S. Naruto, Determination of gliclazide in serum by high-performance liquid chromatography using solid-phase extraction. *Journal of Chromatography* 1992, 583, 266-9.
6. R. Paroni, B. Comuzzi, C. Arcelloni, S. Brocco, S. Kreutzenberg, A. Tiengo, A. Ciucci, P. Beck-Peccoz, S. Genovese, Comparison of capillary electrophoresis with HPLC for diagnosis of factitious hypoglycemia. *Clinical Chemistry* 2000, 46: 1773-1780.
7. M.R. Rouini, A. Mohajer, M.H. Tahami, A simple and sensitive HPLC method for determination of gliclazide in human serum. *Journal of Chromatographia B* 2003, 785, 383-386.
8. J. Varshosaz, R. Talari, A. Nokhodchi, Dissolution enhancement of gliclazide using pH change approach in presence of twelve stabilizers. *Journal of Pharmacy and Pharmaceutical Sciences* 2009, 12, 250-265.
9. J. Weiss, G.R. Taylor, F. Zimmermann, K. Nebendahl, Collection of body fluids. In: G.J. Krinke, ed. The handbook of experimental animals, The laboratory rat. San Diego: Academic Press, 2000, 488-489.
10. C.Y. Kuo, S.M. Wu, High-performance liquid chromatography with electrochemical detection for analysis of gliclazide in plasma. *Journal of Chromatographia A* 2005, 1088, 131-135.
11. J.M. Poirier, M. Perez, G. Cheymol, High-performance liquid chromatographic determination of gliclazide in human plasma. *Journal of Chromatography* 1987, 421, 223-226.
12. B.G. Charles, P.J. Ravenscroft, Measurement of gliclazide in plasma by radial compression reversed- phase liquid chromatography. *Clinical Chemistry* 1984, 30, 1789-1791.
13. Yu NH, Ho ENM, Tang FPW, Wan TSM, Wong ASY. Comprehensive screening of acidic and neutral drugs in equine plasma by liquid chromatography-tandem mass spectrometry. *J Chromatog A* 2008, 1189, 426-434.
14. AbuRuz S, Millership J, McElnay J. The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimepiride in plasma. *J Chromatog B* 2005, 817, 277-286.

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