ISSN 0975-6299 Vol 2/Issue 1/ Jan-Mar 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

ANALYTICAL CHEMISTRY

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE DETERMINATION OF GLIBENCLAMIDE IN RAT SERUM

Corresponding Author

ANIL KUMAR A

Drug Metabolism and Pharmacokinetics Lab, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India

Co Author

PRASHANTH S[1], PRADEEP KUMAR Y [1], MADHU B[2] AND [2]*

[1]Vaagdevi College of Pharmacy, Kakatiya University, Warangal, India
[2]Drug Metabolism and Pharmacokinetics Lab, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India

ABSTRACT

A rapid, sensitive, accurate and specific HPLC assay with UV-Visible detection (253nm) for the determination of glibenclamide in rat serum was developed and validated. Glipizide was used as an internal standard (IS). The serum proteins were precipitated by a single step liquid-liquid extraction using methanol. Chromatographic separation was achieved with a combination of acetonitrile and 25mM monobasic potassium dihydrogen orthophosphate (pH 3.5 adjusted with phosphoric acid) at 60:40 v/v ratios was run isocratically through a C18 (250mmX4.6mm, 5µm) reverse phase analytical column. Analytical run time was less than 10 min. Mean recovery was 97.12% for 0.1-10 µg/ml concentrations. The assay exhibited good linear relationship. Quantification limit was at 50ng/ml of glibenclamide and accuracy and precision were over the concentration range of 0.1-10 µg/ml. The method was validated with excellent sensitivity, accuracy, precision and recovery. The assay has been applied successfully to pharmacokinetic studies.

ISSN 0975-6299 Vol 2/Issue 1/ Jan-Mar 2011

KEY WORDS

Glibenclamide, HPLC, Methanol, Rat serum

INTRODUCTION

Glibenclamide (glyburide) is a potent, second generation oral sulfonylurea antidiabetic agent widely used to lower blood glucose levels in patients with type II non-insulin-dependent diabetes mellitus and as well as in gestational diabetes. It acts mainly by stimulating endogenous insulin release from beta cells of pancreas[1]. The pharmacokinetics of glibenclamide has been widely described in animals and man[2] and it is known to be metabolized extensively via oxidative pathways. Because of its relatively prolonged duration of action and the possibility of episodes of hypoglycemia, it is essential to monitor the serum concentrations of glibenclamide.

It occurs as a white or almost white, odorless crystalline powder. Solubility of the drug in water is approximately 4 µg/ml at pH 4 and 600 µg/ml at pH 9 and 3 mg/ml in alcohol. Glibenclamide is rapidly and completely absorbed from the gastrointestinal tract. As there is no significant first pass metabolism, 100% of the oral dose is bioavailable[3]. Glibenclamide concentration–time curves in serum exhibit biphasic elimination with a terminal elimination rate of 1.4–5 h[4].

Different HPLC methods coupled with UV detection[5–11], fluorescence detection[12] or mass spectrometry[13–15] has been developed for the determination of glibenclamide in biological fluids. However, some of these methods were not sufficiently specific and sensitive, some were not validated and some were time-consuming and expensive and not directly applicable for the determination of glibenclamide in rat serum.

The aim of the present study is to develop a simple and rapid RP-HPLC method with UV detection for the quantitative determination of glibenclamide in rat serum. The method uses HPLC-UV with glipizide as internal standard. This method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy. This analytical method can be used for the estimation of glibenclamide in biological samples and successfully to pharmacokinetic studies.

MATERIALS AND METHODS

Glibenclamide and glipizide obtained from Alka Pharmaceuticals, Hyderabad, India. Methanol (HPLC grade), ammonium acetate and Ortho-phosphoric acid were purchased from Merck. Double distilled Water for analytical purpose was obtained from milli-Q R-O system. Serum samples were obtained from healthy rats.

(1) Chromatographic conditions L

The HPLC system consisted of a Cyberlab LC-10 AD liquid chromatographic pump, Rheodyne injection port (Rheodyne, Cotati, CA,USA) with a 20 µl sample loop and SPD-10A UV-Visible spectrophotometer detector (Cyber lab corporation, USA). Data collection, integration and calibration were accomplished using Class VP chromatography Data system.

The chromatographic separation of glibenclamide and internal standard (glipizide) were accomplished using 250x4.6mm phenomenex C18 5µm reverse phase analytical column. The mobile phase consisted of acetonitrile and 25mM monobasic potassium dihydrogen orthophosphate (pH 3.5 adjusted with phosphoric acid) at 60:40 v/v ratios was run isocratically. Before use, the mobile phase was filtered by passing it through a 0.45µm filter and the filtrate is degassed by using bath sonicator. The mobile phase was pumped at an isocratic flow of 1 ml/min at

room temperature. The peaks were determined using a UV detector set at a wavelength of 253 nm. All the procedures were performed at ambient temperature.

(II) Preparation of stock solution

Stock solution of glibenclamide was prepared in methanol at a concentration of 1mg/ml and was kept at -20^{0}c. This stock solution was diluted with mobile phase to obtain the concentrations required for preparation of standard working solutions. Glibenclamide working solutions were in the range of 0.1 µg/ml-10 µg/ml. The internal standard was prepared by dissolving 10µg of glipizide in 1ml of methanol. Samples for the determination of recovery, precision and accuracy were prepared by spiking quality control (QC) standard glibenclamide concentrations (0.4, 0.8, 1, 4, 8 µg/ml) and stored at -20^{0}c.

(III)Extraction procedure

In 2ml micro centrifuge tube, 100µl of serum was added along with 100µl internal standard solution (glipizide 10µg/ml). The serum proteins were precipitated by the addition of 500µl of methanol and then the tubes were vortexed for 30sec and centrifuged at 3000g/min for 15min. The supernatant was transferred to a clean and similarly labeled tube. The resulted solution was evaporated, reconstituted with 0.1ml of mobile phase and 20µl solution was injected into HPLC.

(IV) Assay validation

The RP-HPLC assay validation was done as per ICH Q2A and Q2B guidelines[16, 17].

(a)Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Standard calibration samples were prepared by making serial dilutions from the stock solution of glibenclamide (1mg/ml). Calibration curve of concentration versus peak area ratio was plotted at concentration range of 0.1 µg/ml-10 µg/ml.

The limit of detection (LOD) and the limit of quantification (LOQ) were measured according to the FDA's guidance for bioanalytical method validation in 2001[18]. The limit of detection was defined as the lowest concentration of glibenclamide resulting in a peak height greater or equal to three times from background noise (S/N ≥ 3). The quantification limit was established by assessing the signal-to-noise ratio level in proportion of 10:1 for each signal. The LOQ was investigated in extracted samples from five different days. For the determination of LOQ, the percentage deviation and % RSD are to be less than 20%.

(b)Precision and Accuracy

The precision and accuracy were determined by analyzing spiked standard and extracted samples at different concentrations ranging from 0.1µg/ml-10µg/ml. The precision of an HPLC method was determined as the coefficient of variation (%RSD) of intra- and inter-day. The intra-day precision was determined by analyzing the spiked standard and extracted samples prepared within a day. The inter-day precision was determined by analyzing the spiked standard and extracted samples analyzed on five different days. After concentrations were calculated by re-fitting peak area ratios obtained with different standard solutions into a derived regression equation from the set of these standard solutions, %R.S.D. was determined at each concentration of the standard solutions from their average value and S.D.

The accuracy of the HPLC method was demonstrated by percentage deviation. The calculated concentrations (or conc. found) were obtained by re-fitting peak area ratios from standard solutions of known concentrations (or conc. added) into a derived regression equation. The conc. found and conc. added was then used to

determine the absolute percentage deviation at each concentration of the standard solutions.

(c)Recovery

The absolute recovery was calculated by comparing the peak area ratio of extracted and unextracted samples containing glibenclamide and glipizide. Each measurement was made in triplicates.

Recovery (%) = Peak area ratio of extracted standard/Peak area ratio of unextracted standardX100

(d)System suitability

The purpose of system suitability to define a set of parameters that are measured prior to each experiment that will tell the analyst if the system is performing adequately or not. The suitability parameters that are evaluated for HPLC method includes peak area reproducibility and retention time.

RESULTS

(i) Chromatography

Sensitive, rapid, specific and reproducible HPLC method has been developed and validated for quantitative determination glibenclamide in rat serum samples. After the pretreatment with a rapid single liquid–liquid extraction step, the rat serum containing glibenclamide was separated by reverse phase HPLC with UV detection at 253 nm. The representative chromatograms of glibenclamide standard concentration and blank are shown in Fig. 1 and Fig. 2. The retention time of glibenclamide and glipizide were 8.23 and 4.7 min respectively and the peaks were sharp. There was good baseline separation of glibenclamide.

(ii) Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

Peak area ratio of glibenclamide and glipizide were measured. A representative calibration graph of peak area versus concentration in the range of 0.1µg/ml-10µg/ml resulted in regression equation of the calibration curve was calculated as y = 2.445x +91.84 (correlation coefficient, r^2 = 0.9997), where *y* is the peak area ratio of glibenclamide and glipizide and *x* is the concentration of glibenclamide. These results demonstrated a good linearity between the peak area ratios versus concentrations. The limit of detection (LOD) and limit of quantitation (LOQ) was 15ng/ml (*S*/*N*≥3) and 50ng/ml.

(iii) Precision and accuracy

The precision of the assay method was validated by the determination of the intra- and inter-day coefficient of variation (%R.S.D.) and percentage deviation. The intra-day and inter-day precision has been done over the concentration range of 0.1µg/ml-10µg/ml. the average %RSD of intra-day and inter-day precision was 3.18% and 2.80% respectively. All %RSD are less than 5%. The accuracy of the method was verified by comparing the concentrations measured for glibenclamide spiked from extracted sample with actual added concentrations. The intra- and inter-day accuracy data expressed as percentage deviation of glibenclamide assay and the data was shown in Table.1

Table.1
Precision and accuracy of HPLC assay for glibenclamide

Spiked concentrations (µg/ml)	Calculated concentration (µg/ml, mean± S.D, n=5)	R.S.D (%)	Deviation (%)
Intra-day (n=5)			
0.4	0.39±0.01	2.56	7.49
0.8	0.78±0.03	3.84	2.12
1	1.11±0.05	4.50	0.07
4	3.82±0.12	3.14	0.03
8	7.89±0.15	1.90	0.05
Inter-day (n=5)			
0.4	0.4±0.03	0.75	1.78
0.8	0.81±0.03	3.7	2.63
1	1.05±0.05	4.76	0.57
4	3.95±0.10	2.53	0.13
8	7.92±0.18	2.27	0.1

(iv) Recovery

The recovery of glibenclamide after liquid-liquid extraction procedures was evaluated at five concentrations of 0.4, 0.8, 1, 4, 8 µg/ml. Absolute recovery was calculated by comparing the peak area ratios for direct injection of pure glibenclamide and glipizide in methanol with those obtained by methanol extracted serum samples containing same amount of glibenclamide and glipizide. Table.2 shows the recovery efficiency of glibenclamide from rat serum samples and the average extraction efficiency of were found to be 97.12%.

Table .2
Recovery of glibenclamide from rat serum sample

QC samples (µg/ml)	Concentration of QC samples after extraction	Recovery (%)
0.4	0.387	96.7
0.8	0.785	98.1
1	0.971	97.1
4	3.860	96.5
8	7.78	97.2

(v) System suitability

The %R.S.D. for area response for the drug was 1.65%, which is within the acceptance value ±2%. The %R.S.D. for retention time for the drug was 0.3% respectively, which is within the acceptance range of ±2%.

DISCUSSION

The present method for the determination of glibenclamide in rat serum samples is sensitive, rapid, specific, accurate and reproducible. The excellent separation is demonstrated in the chromatograms and no interfering peaks were observed. The calibration curve was linear and the method was suitable for the analysis of serum samples over the range of 0.1 to 10µg/ml.

The accuracy of the method was in compliance with the proposed limits and the precision of the method was satisfactory. The system suitability of the method shows that the performance of the chromatographic system is not significantly influenced by variations of the operational parameters inside an accepted domain. This method shows the system suitability parameters are within the limits only.

A rapid single-step liquid-liquid extraction with methanol shows good recovery. The method described here is appropriate for a clinical study. In conclusion, the HPLC method described here successfully applied for pharmacokinetic study of glibenclamide.

Figure 1

Typical chromatogram of glibenclamide and glipizide (IS) 10µg/ml in rat serum

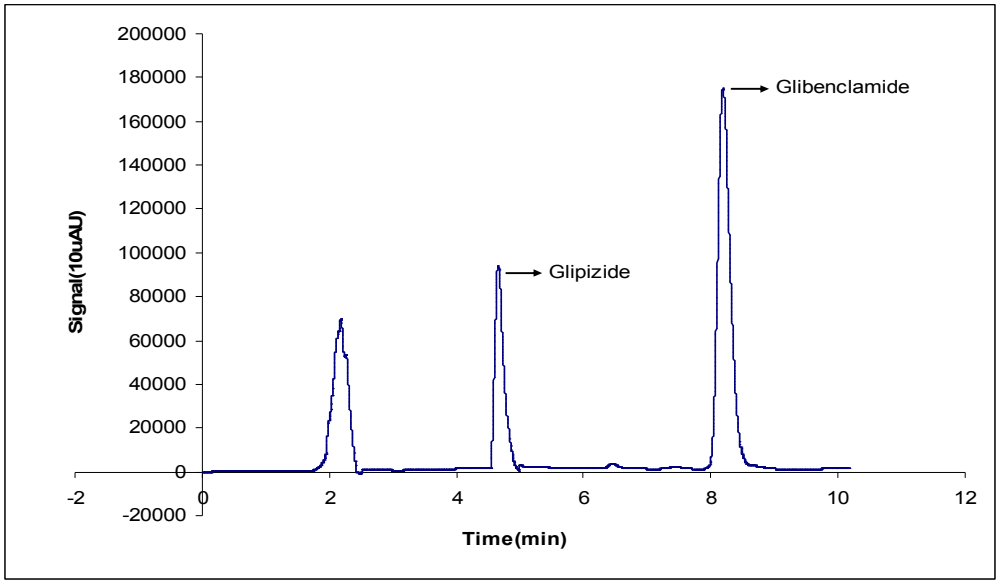
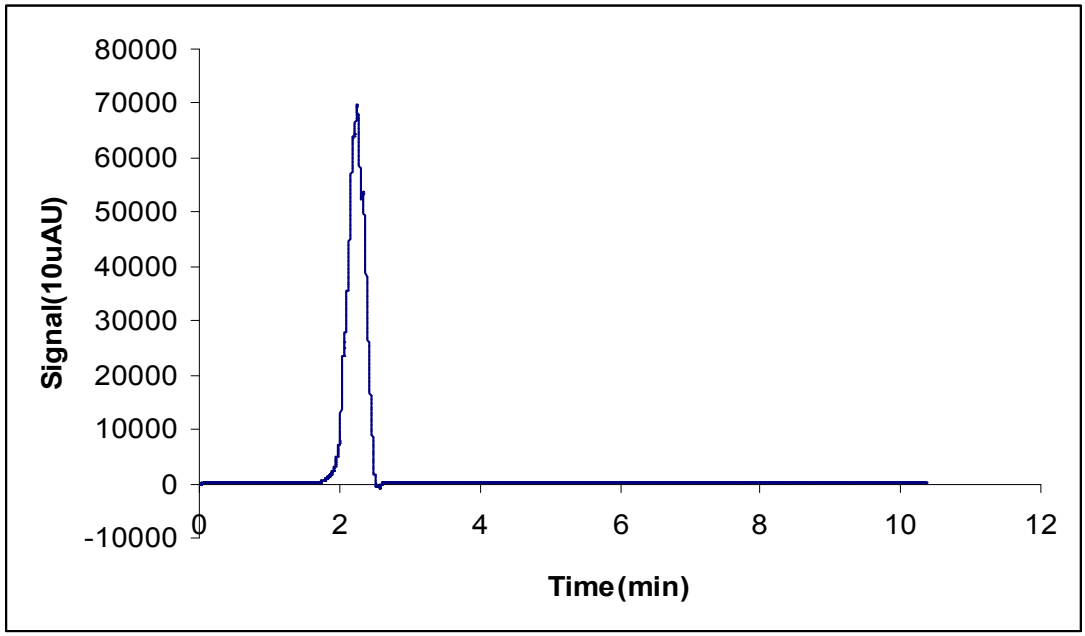


Figure 2
Chromatogram of Blank serum



REFERENCES

1. Nanovskaya T, Nekhayeva I, Hankins G, Ahmed M, Effect of human serum albumin on transplacental transfer of glyburide. BioChem Pharmacol, 72: 632–639, (2006).
2. Greene MF, Oral hypoglycemic drugs for gestational diabetes, N Engl J Med 343: 1178–1179, (2000).
3. G. Neugebauer, G. Betzien, V. Hrstka, B. Kaufmann, E. von-Mollendorff, U. Abshagen, Absolute bioavailability and bioequivalence of glibenclamide (Semi-Euglucon N). Int. J. Clin. Pharmacol. Ther. Toxicol. 23: 453-460 (1985).
4. P. Marchetti, R. Giannarelli, A. di-Carlo, R. Navalesi, Pharmacokinetic Optimisation of Oral Hypoglycaemic Therapy, Clin. Pharmacokinet, 21: 308-317, (1991).
5. W.J. Adams, D.S. Krueger, Specific and sensitive high-performance liquid chromatographic determination of glyburide, J. Pharm. Sci. 68: 1138-1140, (1979).
6. M. Uihlein, N. Sistovaris, High-performance liquid column and thin-layer chromatographic determination of human serum glibenclamide at therapeutic levels, J. Chromatogr. 227: 93-101, (1982).
7. L. Zecca, S. Trivulzio, A. Pinelli, R. Colombo, O. Tofanetti, Determination of glibenclamide, chlorpropamide and tolbutamide in plasma by high-performance liquid chromatography with ultraviolet detection, J. Chromatogr. 339: 203-209, (1985).
8. H. Emilsson, S. Sjoberg, M. Svedner, I. Christenson, High-performance liquid chromatographic determination of glibenclamide in human plasma and urine, J. Chromatogr. 383: 93-102, (1986).
9. S. Othman, O. Shaheen, I. Jalal, A. Awidi, W. Al-Turk, Liquid chromatographic determination of glibenclamide in human plasma, J. Assoc. Off. Anal. Chem. 71: 942-944, (1988).

10. M.E. Abdel-Hamid, M.S. Suleiman, Y.M. el-Sayed, N.M. Najib, M.M. Hasan, A rapid high-performance liquid chromatography assay of glibenclamide in serum, J. Clin. Pharm. Ther. 14: 181-188, (1989).
11. T. Rydberg, E. Wahlin-Boll, A. Melander, Determination of glibenclamide and its two major metabolites in human serum and urine by column liquid chromatography, J. Chromatogr. 564: 223-233, (1991).
12. J.R. Valdes Santurio, E. Gonzalez Porto, Determination of glibenclamide in human plasma by solid-phase extraction and high-performance liquid chromatography, J. Chromatogr. B Biomed. Appl. 682: 364-370, (1996).
13. W.J. Adams, G.S. Skinner, P.A. Bombart, M. Courtney, J.E. Brewer, Determination of glyburide in human serum by liquid chromatography with fluorescence detection, Anal. Chem. 54: 1287-1291, (1982).
14. L. Ramos, R. Bakhtiar, F. Tse, Rapid Commun. Mass Spectrom. 13: 2439, (1999).
15. H. Zhang, J. Henion, Y. Yang, N. Spooner, Application of Atmospheric Pressure Ionization Time-of-Flight Mass Spectrometry Coupled with Liquid Chromatography for the Characterization of in Vitro Drug Metabolites, Anal. Chem. 72: 3342-3348, (2000).
16. ICH Topic Q2A, Validation of Analytical Methods: Definitions and Terminology, Step 5, CPMP/ICH/381/95.
17. ICH Topic Q2B, Validation of Analytical Procedures: Methodology, Step 4, CPMP/ICH/281/95.
18. Guidance for industry-Bioanalytical method validation, Internet at http://www.fda.gov/cder/guidance/index.htm