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Short communication

Application of monolithic column in quantification of gliclazide in human plasma by liquid chromatography

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Abstract

A simple, rapid and sensitive isocratic reversed phase HPLC method with UV detection using a monolithic column has been developed and validated for the determination of gliclazide in human plasma. The assay enables the measurement of gliclazide for therapeutic drug monitoring with a minimum quantification limit of 10 ng ml^{-1}. The method involves simple, one-step extraction procedure and analytical recovery was complete. The separation was carried out in reversed-phase conditions using a Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column with an isocratic mobile phase consisting of 0.01 M disodium hydrogen phosphate buffer-acetonitrile (52:48, v/v) adjusted to pH 4.0. The wavelength was set at 230 nm. The calibration curve was linear over the concentration range 10–5000 ng ml^{-1}. The coefficients of variation for inter-day and intra-day assay were found to be less than 6.0%.

Keywords: Gliclazide; Plasma; HPLC; Monolithic column

1. Introduction

Gliclazide is a second-generation sulphonylurea oral hypoglycaemic agent used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM). It improves defective insulin secretion and may reverse insulin resistance observed in patients with NIDDM. These actions are reflected in a reduction in blood glucose levels, which is maintained during both short and long term administration, and is comparable with that achieved by other sulphonylurea agents. Gliclazide is readily absorbed from the gastro-intestinal tact with peak concentrations in plasma occurring about 2–4 h and it is highly protein bound [1–4]. Various high-performance liquid chromatography methods have been developed for the determination of gliclazide in biological fluids [5–11]. Most of the methods have various limitations, including time-consuming sample clean-up and laborious extraction steps, low sensitivity (30 ng ml^{-1} or more), complex derivatization techniques, use of large sample volumes (1 ml or more) or use of expensive solid phase extraction cartridges. Moreover, most of the aforementioned methods need long chromatographic elution time for analysis of gliclazide in plasma and were not suitable in all conditions. Park et al. [12] have reported a semi-micro HPLC for quantification of gliclazide in human plasma. The limit of quantification (LOQ) was reported as 100 ng ml^{-1} in this study and the total analysis time was 8 min. A sensitive LC–MS assay has been also presented by Maurer et al. [13] for the analyses of gliclazide in plasma. However, this method is not available for most laboratories because of its specialty requirement and financial reasons. Monolithic silica columns exhibit a tailor-made bimodal pore structure with both macropores or through pores and mesopores. The most unique feature of these columns is their high permeability, which is nearly twice as high as that of packed columns. Therefore, monolithic silica columns can be operated at high flow rates, thus allowing fast separations of various mixtures. They represent an approach that provides high rates of mass transfer at lower pressure drops as well as high efficiencies even at elevated flow rates. By this means, much faster separations are possible and the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns

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packed with porous particles. This enhances the speed of the separation process and reduces backpressure and unspecific binding without sacrificing resolution [14,15]. This paper describes the development and validation of a simple, rapid and sensitive isocratic reversed phase HPLC method for the determination of gliclazide in human plasma using a monolithic column with UV detection. Separation was performed on a reversed-phase monolithic column, which has lower separation impedance comparing to the particulate packings, and therefore it allows easy optimizing chromatographic conditions to obtain desirable resolution in a short time. The sample preparation only involves protein precipitation and no evaporation step is required. Also, the use of a smaller sample volume and shorter analysis time provide advantages as compared with some previous methods [9–10] that require large sample volume (1–2 ml) for and longer run time for analysis of gliclazide. We also demonstrate the applicability of this method for pharmacokinetic studies in humans.

2. Experimental

2.1. Chemicals

Gliclazide and glibenclamide were supplied by Kimidarou Pharmaceuticals (Tehran, Iran). Gliclazide is available as oral tablet containing 80 mg of gliclazide and other inactive ingredients. HPLC-grade acetonitrile and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation and purified additionally with a Milli-Q system.

2.2. Instruments and chromatographic conditions

The chromatographic apparatus consisted of a model Wellchrom K-1001 pump, a model Rheodyne 7125 injector and a model K 2501 UV detector connected to a model Eurochrom 2000 integrator, all from Knauer (Berlin, Germany).

The separation was performed on Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column from Merck (Darmstadt, Germany). The wavelength was set at 230 nm. The mobile phase was a mixture of 0.01 M disodium hydrogen phosphate buffer-acetonitrile (52:48, v/v) adjusted to pH 4.0 at a flow rate of 1.2 ml min^{-1}. The mobile phase was prepared daily and degassed by ultrasonication before use. The mobile phase was not allowed to recirculate during the analysis.

2.3. Standard solutions

Stock solutions (2 mg ml^{-1}) of gliclazide were prepared in methanol. Then 100, 1000, 2000, 3000, 4000 and 5000 ng ml^{-1} working standards were prepared in plasma from the stock solution and stored at +4 °C.

2.4. Sample preparation

To 450 μl of plasma in a glass-stoppered 15 ml centrifuge tube was added 50 μl of glibenclamide as internal standard (3 μg ml^{-1}) and 500 μl of acetonitrile. After mixing (30 s), the mixture centrifuged for 10 min at 4000 rpm. Then 30 μl of supernatant was injected into liquid chromatograph.

2.5. Biological samples

Twelve male healthy volunteers were included in this study. The study protocol was approved by the Ethics Committee of Shaheed Beheshti University of Medical Sciences and written informed consent was obtained from the volunteers. Gliclazide was administered in a single dose of 80 mg to the volunteers after over night fasting. Plasma samples were collected at several intervals after dosing and then frozen immediately at −20 °C until assayed.

2.6. Stability

The stability of gliclazide was assessed for spiked plasma samples stored at −20 °C for up to two months and at ambient temperature for at least 12 h. The stability of stock solutions stored at −20 °C was determined for up to one month by injecting appropriate dilutions of stocks in distilled water on day 1, 15 and 30 and comparing their peak areas with fresh stock prepared on the day of analysis. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

2.7. Plasma standard curve

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at −20 °C. After thawing, stock solution of gliclazide was added to yield final concentrations ranging from 10 to 5000 ng ml^{-1}. Internal standard solution was added to each of these samples to yield a concentration of 300 ng ml^{-1}. The samples were then prepared for analysis as described above.

2.8. Selectivity and specificity

Control human plasma, obtained from 12 healthy volunteers, was assessed by the procedure as described above and compared with respective plasma samples to evaluate selectivity of the method. Metformin and some beta blockers such as propranolol, atenolol and sotalol were also tested for potential interferences.

2.9. Precision and accuracy

The precision and accuracy of the method were examined by adding known amounts of gliclazide to pool plasma (quality control samples). For intra-day precision and accuracy five replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days.

2.10. Limit of quantification (LOQ) and recovery

For the concentration to be accepted as LOQ, the percent deviation from the nominal concentration (accuracy) and the rel-

Table 1
Relative recovery of gliclazide from plasma

Gliclazide spiked concentration (ng ml^{-1})	Gliclazide concentraion found ($n=6$)	Recovery (mean ± S.D.) (%)
250	232.5	93.0 ± 2.1
1500	1420.2	94.7 ± 1.5
3500	3390.8	96.9 ± 1.2

ative standard deviation (R.S.D.) must be ±10% and less than 10%, respectively, considering at least five-times the response compared to the blank response. The relative analytical recovery for plasma at three different concentrations of gliclazide (250, 1500 and 3500 ng ml^{-1}) was determined. Known amounts of gliclazide were added to drug-free plasma and the internal standard was then added. The relative recovery of gliclazide was calculated by comparing the peak areas for extracted gliclazide from spiked plasma and a standard solution of gliclazide in methanol containing internal standard with the same initial concentration (six samples for each concentration level) (Table 1).

2.11. Pharmacokinetic analysis

Gliclazide pharmacokinetic parameters were determined by non-compartmental methods. Elimination rate constant (K) was estimated by the least-square regression of plasma concentration–time data points in the terminal log-linear region of the curves. Half-life was calculated as 0.693 divided by K. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration at time t (AUC_{0-t}) was calculated using the linear trapezoidal rule. The area was extrapolated to infinity ($AUC_{0-\infty}$) by addition of C_t/K to AUC_{0-t}, where C_t is the last detectable drug concentration. Peak plasma concentration (C_{max}) and time to peak concentration (T_{max}) were obtained directly from the individual plasma concentration versus time curves.

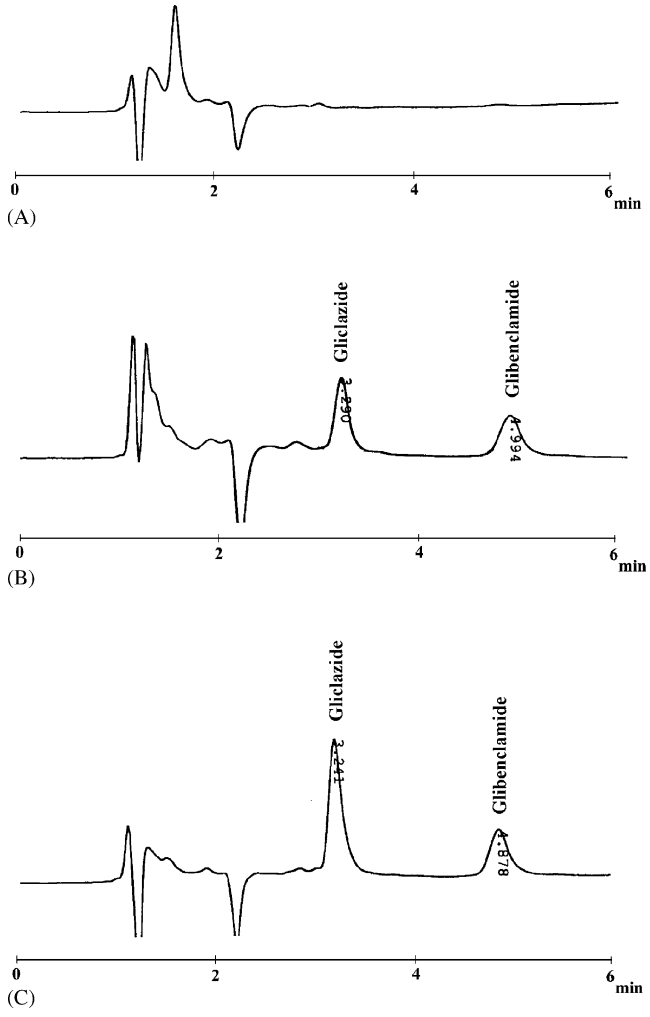

Fig. 1. Chromatograms of (A) blank plasma; (B) blank plasma spiked with 400 ng ml^{-1} gliclazide and 300 ng ml^{-1} glibenclamide (internal standard); and (C) plasma sample from a healthy volunteer 2 h after oral administration 80 mg of gliclazide.

3. Results and discussion

Under the chromatographic conditions described, gliclazide and the internal standard peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Fig. 1 shows typical chromatograms of blank plasma in comparison to spiked samples analyzed for a pharmacokinetic study. The average retention times of gliclazide and glibenclamide were 3.2 and 4.7 min, respectively. None of the drugs mentioned above interfered with analytes peaks as well. The calibration curve for the determination of gliclazide in plasma was linear over the range 10–5000 ng ml^{-1}. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (r) for calibration curves were equal to or better than 0.999. The relative standard deviation values of the slope were equal to or better than 6%. For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves. The relative analytical recovery for plasma at three different concentrations of gliclazide was determined. Known amounts of gliclazide were added to drug-free plasma in concentrations ranging from 250 to 3500 ng ml^{-1}. The internal standard was added and the relative recovery of gliclazide was calculated by comparing the peak areas for extracted gliclazide from spiked plasma and a standard solution of gliclazide in methanol containing internal standard with the same initial concentration. The average recovery was 94.86 ± 1.96% ($n=6$). The limit of quantification, as previously defined, was 10 ng ml^{-1} for gliclazide. This is sensitive enough for drug monitoring and other purposes such as pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of gliclazide. As shown in Table 2, coefficients of variation were less than 6%, which is acceptable for the routine measurement of gliclazide. Stability was determined for spiked plasma samples under the conditions as previously described. The results showed that the samples were stable during the mentioned conditions. The aim of our study was to develop a rapid and sensitive method for the routine analysis of biological samples in pharmacokinetic gliclazide research. This method is well suited for routine application in

Table 2
Reproducibility of the analysis of gliclazide in human plasma ($n = 5$)

Concentration added (ng mL^{-1})	Concentration measured (mean ± S.D.)	
	Intra-day	Inter-day
250	218.4 ± 12.6 (5.8)	215.8 ± 12.8 (5.9)
1500	1542.4 ± 53.7 (3.5)	1534.1 ± 53.2 (3.5)
3500	3671.3 ± 80.4 (2.2)	3664.7 ± 83.2 (2.3)

Values in parentheses are coefficients of variation (%).

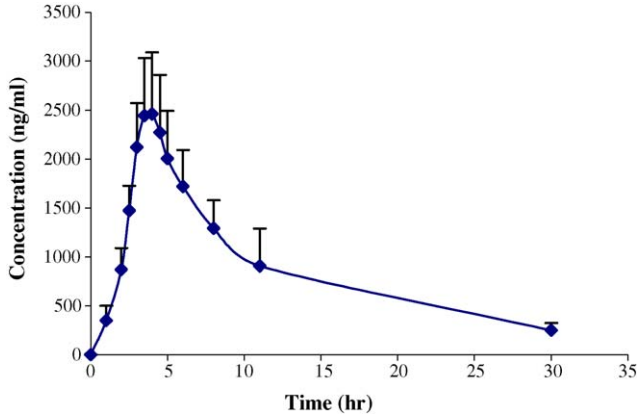

Fig. 2. Mean plasma concentration–time profile of gliclazide in healthy volunteers ($n = 12$) after a single 80 mg gliclazide.

the clinical laboratory because of the speed of analysis and simple extraction procedure. Owing to the use of the monolithic column, which has lower separation impedance compared with the particulate packings, much faster separations are possible. The productivity of chromatographic processes can be increased by at least one order of magnitude as compared with traditional chromatographic columns packed with porous particles. Accordingly, the chromatographic elution step is undertaken in a short time (less than 5 min) with high resolution. The sample preparation only involves protein precipitation and no evaporation step is required. Also, the use of a smaller sample volume provides an advantage as compared with some previous methods that require 1–2 ml of plasma for analysis of gliclazide. Over 500 plasma samples were analyzed by this method without any significant loss of resolution. No change in the column efficiency and backpressure was also observed over the entire study time, thus proving its suitability. In this study, plasma concentrations were determined in 12 healthy volunteers, who received 80 mg of gliclazide each. Fig. 2 shows the mean plasma concentration–time profile of gliclazide. The derived pharmacokinetic parameters of 12 healthy volunteers are summarized in Table 3. These pharmacokinetic parameters are in good agreement with that found previously [16].

Table 3
Pharmacokinetic parameters of gliclazide in healthy volunteers following a single oral dose of 80 mg of gliclazide

Parameter	Result (mean ± S.D.)
T_{max} (h)	3.5 ± 0.4
C_{max} (ng ml^{-1})	2686.2 ± 461.2
AUC_{0-t} (ng h ml^{-1})	26984.1 ± 1765.4
K_{el} (h^{-1})	0.078 ± 0.002
$T_{1/2}$ (h)	8.9 ± 0.4

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