

# Performance Liquid Chromatographic Analysis of Glipizide: Application to In Vitro and In Vivo Studies

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## Abstract

This paper describes the validation of a sensitive, accurate, and reproducible method for the determination of a release profile of glipizide from controlled-release dosage forms. In this method, an in vitro dissolution profile of commercial controlled-release dosage forms is determined using a reversed-phase C<sub>18</sub> column, mobile phase (acetonitrile–buffer, 0.05M KH<sub>2</sub>PO<sub>4</sub> adjusted to pH 3.5 with orthophosphoric acid), and UV detection at a wavelength of 275 nm. The method is validated for linearity, accuracy, precision, and detection and quantitation limits. The same method can be exploited to determine the plasma concentration of glipizide. The peak area versus plasma concentration is linear over the range of 12.5–1000 ng/mL and the detection limit was 5 ng/mL in plasma. The average accuracy was 99.90% with a relative standard deviation (RSD) of not more than 3%. Repeatability and reproducibility were found to be good with an RSD of less than 3%.

## Introduction

Glipizide (1-cyclohexyl-3-[[p-[2-(5-methylpyrazinecarboxamido)ethyl]phenyl]sulfonyl]urea), a second generation sulfonylurea, is effective in controlling the blood glucose in patients with noninsulin-dependent diabetes mellitus (1). Glipizide is completely absorbed, and a peak plasma concentration varying between 380 and 611 ng/mL (0.85–1.35 nmol/mL) is attained usually 1–3 h after a single oral 5-mg dose (2). Plasma drug concentration declines to 12.0 ng/mL after 24 h. It has a half-life of approximately 2–4 h. It has been found that controlled-release preparations of glipizide have shown a better efficacy than immediate-release dosage forms (3,4). These dosage forms enable long-term management of diabetes by avoiding the problems associated with tight control of blood glucose concentrations such as hypoglycemia tolerance and seizures, while simultaneously avoiding the problems associated with conventional moderate control of blood glucose concentrations (pathological complications associated with hyperglycemia such as nephropathy, retinopathy, neuropathy, etc.) (5).

Over the past 10–15 years, it has been established that dissolu-

tion testing is probably the most important in vitro test that can be used to evaluate and control certain variables associated with formulation excipients, design, and manufacturing, which may alter the release characteristics of the active moiety from the formulation. The importance of dissolution testing has been well recognized by most of the official compendia, including the United States Pharmacopoeia (USP) (6). Over 500 monographs for pharmaceutical dosage forms in the USP prescribe dissolution testing. It is also an integral component of new drug applications to regulatory bodies worldwide (7).

A few bioassays for analysis of glipizide in plasma or serum have been reported. A thin-layer chromatography (TLC) determination coupled with fluorimetry of glipizide and its metabolites in serum after hydrolysis and densylation has been described by Huck (8). Hartvig et al. (9) determined glipizide and other sulphonyl urea in plasma by electron capture gas chromatography (GC) after extractive methylation. A perusal of the work done by research groups reveals that radioimmunoassay techniques, electrospray mass spectrometry (MS) (10), micellar electrokinetic chromatography (11), and ion-pair liquid chromatography (LC) (12,13) have also been used. However, high-performance liquid chromatography (HPLC) provides greater resolution than TLC and has proven to be an attractive alternative to GC analysis. Hence, HPLC techniques have been developed to quantitate glipizide in plasma or whole blood using different solvent systems for extraction, as well as mobile phase with different internal standards (14–19). Most of these HPLC methods use benzene (carcinogenic) as an extraction solvent because of one or the other problems encountered with the solvents, and they have large elution times (25–30 min).

Glipizide, normally administered in the doses of 2.5, 5, 10, and 20 mg, has an A<sub>1</sub> (absorbance 1%, 1 cm at wavelength 275 nm) of 237. It is difficult to ascertain the release profile of the glipizide formulations, particularly at lower doses, in the initial hours of dissolution using spectrophotometric analysis because of the interference by factors such as excipients in the formulations. Therefore, an analytical method was developed and validated for linearity, accuracy, precision (repeatability), and limit of detection (LOD) and quantitation (LOQ). The objective of the present study was to compare and evaluate the in vitro product performance of the commercially available sustained-release tablets and a quanti-

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tative determination of the drug in plasma samples. Also, this method requires no internal standard, as homogeneity of the peak was validated by ratio chromatograms of the samples at two detection wavelengths.

## Experimental

### Drug samples

Glipizide and its reference standard were obtained ex-gratis from Jenburkt Pharmaceuticals (Gurjat Industrial Development Corp., Gurjat, India) (11,12). Glucotrol XL (Pfizer, New York, NY), Glynase (Maharashtra Industrial Development Corp., Maharashtra, India), and Glytop SR (Sidmak Laboratories, Gurjat, India) were obtained as a gift sample [the gift sample was taken from the control samples preserved by the quality control department of the company under the schedule M (good manufacturing practice) of the Drugs and Cosmetics Act, 1940; provision of schedule M inserted in 1986]. Plasma (single-donor plasma O-negative NO-601981) was obtained from the Post Graduate Institute of Medical Education and Research (Chandigarh, India).

### Reagents

Membrane filters (0.2  $\mu\text{m}$ , type CN, 47 mm and 0.45  $\mu\text{m}$ , type CN, 25 mm) were purchased from Advanced Microdevices Pvt. Ltd. (Ambala, India). HPLC-grade acetonitrile and methanol and potassium dihydrogen orthophosphate (guaranteed reagents, E. Merck, Mumbai, India) were used.

### Chromatographic conditions

A Shimadzu LC-10A series chromatograph equipped with two LC-10AS solvent delivery units, a C-R7A chromatopac data processor fitted with an additional channel board, and an SPD-10A dual wavelength UV-vis detector (Shimadzu, Kyoto, Japan) were used. The detector collected the data in dual wavelength mode by sequential scanning of grating (20). The samples were injected manually using a Rheodyne 7125 injector (Rheodyne, Cotati, CA) with a 20- $\mu\text{L}$  loop (for in vitro samples) and 50- $\mu\text{L}$  loop (for in vivo samples). The separation was achieved using a  $\text{C}_{18}$  column. The mobile phase was filtered through 0.45- $\mu\text{m}$  membrane and degassed at reduced pressure in an ultrasonic bath (3210E-DTH, Branson, Danburg, CT). HPLC with atmospheric pressure chemical ionization and mass spectrometric detection in the positive ion mode using a heated nebulizer surface was carried out on Finnigan Mat (Model LCQ, ThermoFinnigan, Bremen, Germany).

### Columns used

The columns used in this study were a Waters Spherisorb S5 ODS2 (4.6  $\times$  250 mm), Waters  $\mu$ Bondopak  $\text{C}_{18}$  (39  $\times$  300 mm), and Phenomenex Bondclone 5- $\mu\text{m}$  (300  $\times$  3.9 mm).

### Mobile phase

The mobile phase was acetonitrile-buffer (50:50 phosphate buffer, pH 3.5 adjusted with orthophosphoric acid). For LC-MS samples, 50mM ammonium acetate buffer with pH 3.5 was used.

### Flow rate

For in vitro samples, the flow rate was 1.5 mL/min, and for in vivo samples it was 1.2 mL/min.

### Stock solution

#### Standard stock solution

Five milligrams of glipizide was dissolved in 100 mL of methanol.

#### Working standards for in vitro samples

The calibration curve standards for in vitro samples were prepared by adding a known amount of glipizide from the stock solution to phosphate buffer (pH 7.5) dissolution medium and contained 5 ng–2  $\mu\text{g/mL}$  of glipizide.

#### Working standards for in vivo samples

For plasma samples, 20 mL of the stock solution was diluted to 100 mL with methanol to achieve a final concentration of 10,000 ng/mL. The calibration curve standards were prepared by adding known amounts of glipizide to plasma and contained 1–1000 ng/mL of glipizide.

### Sample preparation

#### In vitro

All the dissolution samples were filtered through 0.45- $\mu\text{m}$  membrane filter.

#### In vivo

Glipizide is a weak acid with a pKa of 5.94. Because it was the aim to keep the drug undissociated to the largest possible degree, a pH of 3.0–3.5 seemed appropriate for extraction. One milliliter of plasma spiked with the drug solution was taken and dilute acid (hydrochloric) was added to it resulting in a pH of approximately 3. It was then extracted with 5 mL of toluene on a vortex mixer for 15 min. The organic layer was then separated and evaporated to dryness. Because toluene has a boiling point of 111°C, it was evaporated in a specially designed assembly consisting of a long glass tube (27.5 cm) with one side attached to a blower pump (Perfit high vacuum pump, Gupta Scientific Industries, Ambala, India) through plastic tubing. The tube had further joints (4.5 cm) attached to it, to which teflon tubing (9.5 cm) was attached so that the air could be easily blown into the tubes. The air blowing from the pump increased the surface area of the solvent being exposed to the high temperature (40°C) inside the glass tubes. The stand containing the test tubes was placed in a water bath (Haake circulators, GmbH U Co., Kalsruhe, Germany) maintained at 40°C. The residue was reconstituted with 500  $\mu\text{L}$  of mobile phase with sonication.

### Dissolution testing

The in vitro dissolution tests were performed using the USP apparatus I (basket method) dissolution apparatus (PTWS3C, Pharma Test, Hainberg, Germany) with six replicates. The dissolution medium was 900 mL of phosphate buffer (7.5 pH) maintained at 37°C  $\pm$  0.5°C (21). The basket rotation speed was held at 100 rpm. In all experiments, 5 mL of dissolution sample was withdrawn at 1–6, 8, 10, 12, 18, and 24 h and replaced with an equal volume of the fresh medium to maintain a constant total volume.

Samples were passed through a membrane filter (0.45  $\mu\text{m}$ ) and assayed by HPLC at 275 nm. Cumulative percentages of the drug released from the dosage forms were calculated.

## Results and Discussion

### Validation of peak homogeneity

A test of homogeneity was applied to the separated drug peak to confirm its single component character. Among the techniques available for the purpose, which included ratio chromatography (22,23), derivative spectroscopy (24), photodiode array detection (25), LC-MS (26), etc., the first and last were employed in our studies. For ratio chromatography, the two detection wavelengths were 254 and 275 nm. The chromatogram in Figure 1 is the ratio chromatogram of two wavelengths obtained when the detector was employed in dual wavelength mode, and the data processor had the provision for two-channel recording. The ratio chromatogram shows clear separation of drug peaks from other resolved peaks. The ratio chromatograms accrue from the relative absorbance variations in  $A_{\lambda_1(t)}$  and  $A_{\lambda_2(t)}$  at the two wavelengths  $\lambda_1$  and  $\lambda_2$  as per the following equations (20):

$$R(t) = \frac{A_{\lambda_1(t)}}{A_{\lambda_2(t)}} \quad \text{-- (when } A_{\lambda_1(t)} > A_{\lambda_2(t)} \text{)} \quad \text{Eq. 1}$$

$$R(t) = \frac{A_{\lambda_1(t)}}{A_{\lambda_2(t)}} \quad \text{-- (when } A_{\lambda_1(t)} < A_{\lambda_2(t)} \text{)} \quad \text{Eq. 2}$$

where  $R(t)$  is the ratio chromatograms signal. Because baseline drifts  $D_{\lambda_1}$  and  $D_{\lambda_2}$  may be contained at time in  $A_{\lambda_1}$  and  $A_{\lambda_2}$ , the ratio chromatogram is actually obtained from the following equation:

$$R(t) = \frac{A_{\lambda_1(t)} + D_{\lambda_1}^-}{A_{\lambda_2(t)} + D_{\lambda_2}^-} - 1 \quad \text{Eq. 3}$$

The ratio chromatogram for a pure compound, as per equations 1 and 2, is output as a flat rectangular peak. The flat behavior appears because of the ratio,  $R(t)$ , at two wavelengths remains constant. The flatness is an ideal situation and requires zero baseline drifts during elution. When the baseline drifts are

contained in the measured absorbances, the peak containing no impurities shows symmetrical distortions, in accordance with equation 3. When a peak contains an impurity, the ratio  $R(t)$  varies, and the shape of the ratio chromatogram is distorted asymmetrically. The shape of the ratio chromatogram in this manner is a manifestation of the homogeneity of the resolved peak (27,28). The flat shape of ratio plots in Figure 1 (plasma samples) is indicative of the homogeneity of the drug peak. In order to confirm it, LC-MS of pure as well as plasma samples was performed, and the mass of glipizide in pure as well as test samples was determined. The mass was found to be the same (445.7 for glipizide peak) in both the samples. On the basis of mass analysis by LC-MS, the peak homogeneity of the drug was confirmed.

### Linearity

#### *In vitro* samples

Linearity was validated by measuring area responses at the concentration range of 0.00787–12  $\mu\text{g/mL}$ . Two separate stock solutions were prepared, the same serial dilutions were made, and each sample was injected in duplicate. A linear regression analysis was performed, and the calibration curve was prepared.

A typical chromatogram for a dissolution sample of Glucotrol XL is shown in Figure 2. The retention time of glipizide was approximately 3.9 min. Similarly, dissolution samples of Glytop SR and glipizide controlled-release formulation (29) showed well-separated peaks of glipizide. The calibration curve was found to be linear in the range tested (0.00787–11  $\mu\text{g/mL}$ ,  $r^2$  value 0.99983 and equation of the line,  $\text{conc.} = 0.05569619 \times \text{area} - 48.718897$ ).

#### *Plasma samples*

The concentration range of the samples was 1–1000 ng. A typical chromatogram for plasma samples of glipizide is shown in Figure 3. The retention time of glipizide was approximately 4.6 min. The calibration curve was found to be linear in the range tested (0.0125–1  $\mu\text{g/mL}$ ,  $r^2 = 0.99924$  and equation of line,  $\text{conc.} = 0.0006340519 \times \text{area} + 13.06175$ ).

### Accuracy

#### *In vitro* samples

Aliquots of dissolution medium were spiked with glipizide at six different concentrations (500, 2000, 4000, 6000, 8000, and 10,000 ng/mL). Each concentration was injected in duplicate, and area responses were compared with those of the same concentrations of reference standard. The average accuracy and the standard

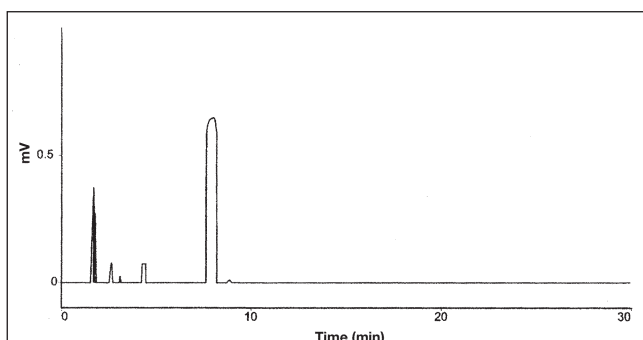


Figure 1. Validation of homogeneity for plasma samples. Dual wavelength ratio chromatogram.

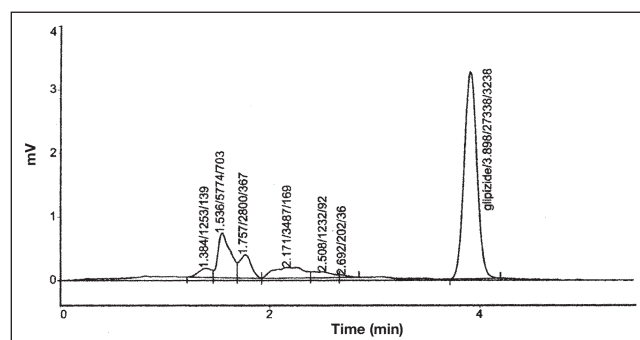


Figure 2. Representative chromatogram for a 5-mg in vitro dissolution sample of Glucotrol XL in 7.5 pH phosphate buffer.

deviations (SDs) were calculated. The average accuracy was found to be 99.62% with a relative standard deviation (RSD) of 1.04%, indicating very good accuracy.

Percent recovery of glipizide from plasma samples was determined in duplicate by comparing the glipizide peak area after the injection of a standard drug solution (50, 100, 150, 250, 375, 450, 600, 800, and 1000 ng/mL) and extraction of plasma samples at the same concentration. Glipizide was quantitatively extracted from plasma, percent recovery being independent of the amount of drug present in the sample. The average recovery was found to be 99.90% with an RSD of 2.23%.

### Precision

A more comprehensive definition proposed by the International Conference on Harmonization (30) divides precision into three types: (a) repeatability, (b) intermediate precision, and (c) reproducibility.

### Repeatability

Repeatability was validated by measuring response factors at six concentration levels (1000, 3000, 5000, 7000, 9000, and 11,000 ng/mL). Each sample was injected in duplicate. The response factor was calculated by dividing the concentration of the sample with the area of the drug peak obtained in the chromatogram. Precision was expressed by the SD and RSD of measured response factor. An RSD value of 1.14 was obtained for the dissolution sam-

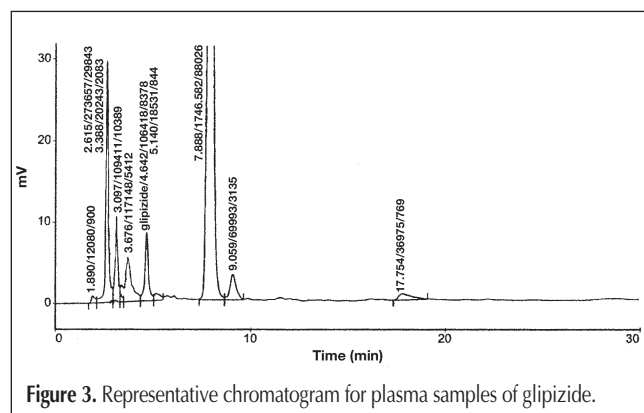


Figure 3. Representative chromatogram for plasma samples of glipizide.

Table I. Repeatability and Response Factors for Glipizide in Plasma Samples

Plasma concentration of glipizide (ng/mL)	Amount of glipizide injected ( $\mu$ g)	Area		Response factor ( $\mu$ g/area)	
		A <sub>1</sub>	A <sub>2</sub>	1	2
25	0.00125	38945	37452	3.20965E-08	3.33761E-08
100	0.005	144427	149867	3.46196E-08	3.33629E-08
150	0.0075	212537	220314	3.5288E-08	3.40423E-08
375	0.01875	556489	576891	3.36934E-08	3.25018E-08
450	0.0225	691593	689789	3.25336E-08	3.26187E-08
600	0.03	942391	895476	3.18339E-08	3.35017E-08
800	0.04	1220814	1207896	3.2765E-08	3.31154E-08
			<b>Average</b>	3.3323E-08	
			<b>RSD (%)</b>	2.94528943	

ples. Similarly for plasma samples, an RSD value of 2.94 (Table I) was obtained, thus indicating a high degree of repeatability.

### Intermediate precision

The intermediate precision was evaluated by examining the effect of different columns employed in the study on multiple days on chromatographic performance. The parameters such as tailing factor and theoretical plate number for glipizide were determined to evaluate precision. The values of tailing factors for Waters spherisorb, Waters  $\mu$ Bondapak, and Phenomenex Bondclone columns were found to be 1.02, 1.04, and 1.01, respectively. The plate numbers for the abovementioned columns were found to be 10,000, 9874, and 8765, respectively.

### Reproducibility

Reproducibility was validated by measuring the area at different concentrations (1000, 3000, 5000, 7000, and 9000 ng/mL for in vitro samples and 200, 400, and 500 ng/mL for in vivo samples). Each sample was injected ten times. RSD values for in vitro and in vivo samples were found to be 0.90 and 1.60, respectively.

### LOD

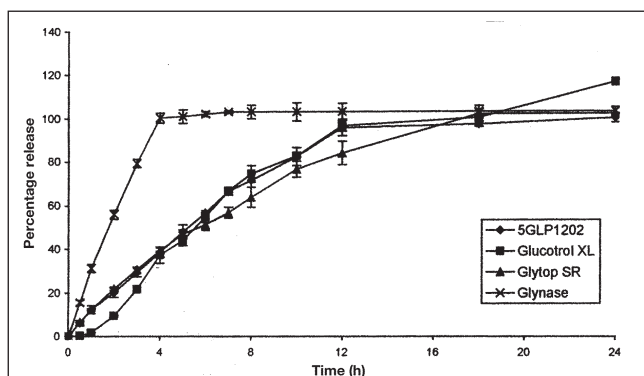
A standard solution of glipizide (25 ng/mL for in vitro samples and 40 ng/mL for in vivo samples), which resulted in a signal-to-noise ratio (S/N) of at least 30, was selected; the sample was subsequently diluted and measured using the procedure described previously. The dilutions were continued to obtain a concentration until the S/N was approximately 3. The minimum detectable concentrations of glipizide in dissolution and plasma samples were found to be 2 and 5 ng/mL, respectively.

### LOQ

A standard solution of glipizide (25 ng/mL for in vitro samples and 40 ng/mL for in vivo samples), which resulted in S/N of at least 30, was selected; the sample was subsequently diluted and measured at least six times using the procedure described previously. The dilutions were continued to obtain a concentration until the S/N was approximately 10. The LOQs for in vitro and in vivo samples were found to be 7.87 and 12.5 ng/mL, respectively.

### Dissolution testing

The dissolution profiles of Glucotrol XL, Glynase, Glytop SR, and glipizide controlled-release tablets (5GLP1202) are given in Figure 4. Glipizide was quantitated in dissolution samples by the previously mentioned, validated method. The same samples, when analyzed with a UV spectrophotometer, gave erroneous results because a percentage release of the drug was found to increase or decrease variably with time, which is not feasible. It was observed that the immediate-release dosage form (Glynase) released all of the drug in approximately 4 h, whereas the release of glipizide was retarded from sustained-release formulations (5GLP1202, Glucotrol XL, and Glytop SR). In the case of Glucotrol XL, the amount of the drug released in the first hour was very low because it works on the principle of osmotic



**Figure 4.** Release profile of glipizide controlled-release tablets (code 5GLP1202), Glucotrol XL, glyname, and Glytop SR in phosphate buffer (pH 7.5).

**Table II. Dissolution Parameters for Different Formulations**

Formulation	$t_{50}$ (h)	$t_{60}$ (h)	$t_{70}$ (h)	$t_{80}$ (h)	Release rate $\pm$ SD (mg/h)
Glipizide CR tablets (5GLP1202)	5.24	6.38	7.72	9.61	0.417 $\pm$ 0.11
Glucotrol XL	5.57	6.44	7.39	9.41	0.425 $\pm$ 0.24
Glytop SR	5.73	7.41	8.89	10.66	0.356 $\pm$ 0.14
Glyname	1.75	2.17	2.59	3.03	1.31 $\pm$ 0.11

pump. This system absorbs water and develops osmotic pressure inside by which the drug is released. The values of  $t_{50}$ ,  $t_{60}$ ,  $t_{70}$ , and  $t_{80}$  for all the formulations are given in Table II.  $t_{50}$ ,  $t_{60}$ ,  $t_{70}$ , and  $t_{80}$  values can be defined as the time (h) in which 50%, 60%, 70%, and 80% of the drug is released. The release rates of 5GLP1202 and Glucotrol XL were found to be comparable.

## Conclusion

An HPLC method for the quantitation of glipizide in dissolution and plasma samples was developed and validated for peak homogeneity, accuracy, precision, LOD, and LOQ. A release profile of glipizide from Glucotrol XL, Glytop SR, and glipizide controlled-release tablets was determined using this method.

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