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# Development and Validation of a Reversed-Phase HPLC Method for Estimation of Glimepiride in Rabbit Serum and Its Application to Pharmacokinetic Studies

**Samhitha J[1*], Anilkumar KV[1], Nagaraju B[1]**
[1]Department of Pharmacology, Visveswarapura Institute of Pharmaceutical Sciences, BSK-2nd Stage, Bengaluru-560070

**Abstract:** A reversed-phase high-performance liquid chromatography (RP-HPLC) method reported for glimepiride was modified and standardized for its estimation in rabbit serum, using pioglitazone as an internal standard (IS), to support pharmacokinetic evaluation of the drug in a rabbit model. Chromatographic separation was achieved on an RP C18 column (250 × 4.6 mm, 5 µm) using acetonitrile and phosphate buffer (pH 4.3) in a 75:25 (v/v) ratio as the mobile phase at a flow rate of 1 mL/min, with UV detection at 258 nm. Glimepiride and the internal standard were extracted from rabbit serum by liquid–liquid extraction with dichloromethane, and the ratio of the peak area (AUC) of glimepiride to that of pioglitazone was used for quantification. The method was validated for linearity, system suitability, precision, and recovery/accuracy. The calibration curve was linear over 20–1000 ng/mL ($y = 0.0089x - 0.0078$, $r^2 = 0.9980$). The limit of detection (LOD) and limit of quantification (LOQ) were 18.55 and 20.00 ng/mL, respectively. The column gave 14,352 theoretical plates and a tailing factor of 1.08 for glimepiride, with retention times of 6.8–8.02 min for glimepiride and 4.2–5.05 min for the internal standard. Intra-day and inter-day precision (%RSD) were below 3% at all concentrations tested, and absolute recovery ranged from 98.85% to 99.82%. The validated RP-HPLC method is simple, sensitive, precise, and accurate, requires only 200 µL of serum, and is suitable for pharmacokinetic, bioavailability, and drug-interaction studies of glimepiride in rabbits.

**Keywords:** Glimepiride, Rabbit Serum, RP-HPLC, Pioglitazone, Internal Standard, Method Validation, Pharmacokinetics.



**Research Paper**

***Corresponding Author:**
*Samhitha J*
Department of Pharmacology, Visveswarapura Institute of Pharmaceutical Sciences, BSK-2nd Stage, Bengaluru-560070

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## INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by insulin resistance and progressive pancreatic beta-cell dysfunction, and it remains one of the most prevalent non-communicable diseases worldwide [1]. Oral hypoglycemic agents, particularly the sulfonylureas, continue to occupy an important place in its management, either as monotherapy or in combination with other antidiabetic agents such as metformin, thiazolidinediones, and alpha-glucosidase inhibitors [2].

Glimepiride (GLM), chemically 1-{(p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamide) ethyl] phenyl) sulfonyl}-3-(trans-4-methylcyclohexyl) urea, is a third-generation sulfonylurea that is widely prescribed for the treatment of T2DM. Like other members of this class, glimepiride acts as an insulin secretagogue: it binds to ATP-sensitive potassium channels on the pancreatic beta-cell membrane, causing membrane depolarization and triggering calcium influx that stimulates insulin secretion [3]. It also exerts extrapancreatic effects that improve the sensitivity of peripheral tissues to insulin. Owing to its once-daily dosing, favorable safety profile relative to older sulfonylureas, and relatively low incidence of severe hypoglycemia, glimepiride remains a widely used therapeutic option, often prescribed in combination regimens with metformin or insulin [4].

Accurate and sensitive bioanalytical methods for the quantification of glimepiride in biological matrices are essential, both for regulatory pharmacokinetic evaluation of formulations and for preclinical drug-interaction and bioavailability studies

[5]. A range of analytical techniques has previously been reported for the estimation of glimepiride in plasma, serum, and pharmaceutical formulations, including UV spectrophotometry, derivative spectroscopy, capillary electrophoresis, thin-layer chromatography, and various HPLC-based approaches coupled with UV, diode-array, or tandem mass-spectrometric detection [6]. While LC-MS/MS methods generally offer the highest sensitivity, they require expensive, specialized instrumentation that is not always accessible in routine laboratories, whereas many reported HPLC-UV methods involve long chromatographic run times, comparatively high limits of detection, or elaborate sample-preparation steps, which together limit their routine use in preclinical pharmacokinetic screening [7].

For pharmacokinetic studies in small-animal models, where only limited serum volumes can be collected at each time point, a bioanalytical method must combine adequate sensitivity with a simple, low-volume extraction procedure and a short chromatographic run time. Rabbit models are frequently used for preliminary pharmacokinetic, bioavailability, and drug-interaction evaluation of oral antidiabetic agents prior to more resource-intensive studies in larger species, making a validated, serum-based assay for glimepiride in this species particularly useful for early-stage drug development and academic research alike [8].

In the present work, a reversed-phase HPLC method previously reported for the estimation of glimepiride was modified and standardized for application to rabbit serum [9], using pioglitazone as an internal standard to normalize peak-area response and correct for injection variability. The method was designed to require only a small serum volume (200 µL), to use a straightforward liquid-liquid extraction with dichloromethane, and to achieve sensitivity adequate for quantification at low nanogram-per-milliliter concentrations. The developed method was fully validated for linearity, system suitability, precision, and recovery/accuracy, in accordance with standard bioanalytical validation practice, with the aim of providing a simple, economical, and reliable tool for pharmacokinetic, bioavailability, and drug-interaction studies of glimepiride in the rabbit model.

## Experimental

### *Materials*

To ensure a high degree of analytical precision, all reagents and chemicals used were of HPLC or analytical reagent (AR) grade. The glimepiride reference standard was provided as a gift sample by Dr. Reddy's Laboratories (Hyderabad, India). High-purity acetonitrile was procured from Qualigens Chemicals (Mumbai, India), while dichloromethane and orthophosphoric acid were obtained from SD Fine Chemicals (Mumbai, India). In-house triple-distilled water, further purified by 0.2 µm membrane filtration to prevent column contamination, was used throughout for mobile phase and solution preparation. Pioglitazone, also a gift sample from Dr. Reddy's Laboratories, was used as the internal standard (IS) to normalize peak-area response and ensure injection reproducibility during serum analysis.

### *Instrumentation and Chromatographic Conditions*

Quantitative analysis was performed using a Shimadzu Class-VP series HPLC system (Shimadzu, Japan) configured for high-sensitivity bioanalytical work. The system comprised dual LC-10AT VP pumps for precise flow control, an SCL-10A VP system controller, and an SPD-10A VP variable-wavelength UV/VIS detector. A CTO-10AS VP column oven was used to maintain thermal stability and reproducible retention times. Chromatographic separation was achieved on an RP C18 column (250 mm × 4.6 mm i.d., 5 µm particle size; YMC Inc., USA). Data acquisition, integration, and processing were performed using Empower software (version 2.30).

The mobile phase consisted of acetonitrile and phosphate buffer (pH 4.3) in the ratio of 75:25 (v/v). The phosphate buffer was filtered through a 0.22 µm membrane filter before use. The mobile phase was pumped at a flow rate of 1 mL/min, and the column effluent was monitored at 258 nm. The ratio of the peak area of glimepiride to that of the internal standard, pioglitazone, was used for quantification of glimepiride in serum samples.

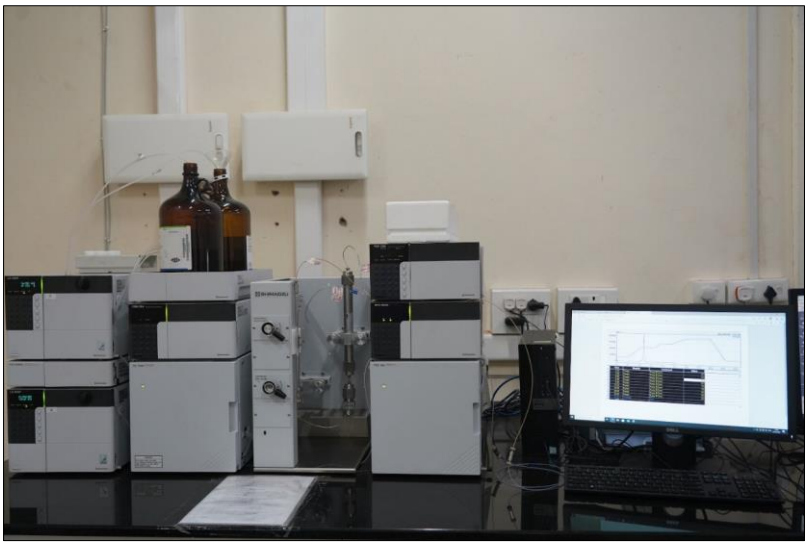


**Fig. 1: High-performance liquid chromatography (HPLC) system used for the analysis**

***Standard Solutions***

A primary stock solution of glimepiride (1 mg/mL) was prepared in methanol and stored at 4 °C. Appropriate dilutions were made in mobile phase to give working concentrations of 1 µg/mL and 500, 200, 100, 50, and 20 ng/mL, which were used to spike serum for construction of the calibration curve. Calibration samples were prepared by spiking 200 µL aliquots of blank serum with the appropriate amount of drug on the day of analysis. Samples for determination of recovery, precision, and accuracy were prepared by spiking bulk control rabbit serum at 20, 100, 500, and 1000 ng/mL and were stored at 4 °C.

A standard solution of pioglitazone (10 µg/mL) was prepared separately to serve as the internal standard. The IS was used to normalize peak-area variation and to ensure assay robustness against variation in injection volume.

For construction of the calibration curve, stock solutions of glimepiride and pioglitazone were each prepared by dissolving 10 mg of the compound in 10 mL of methanol. From the glimepiride stock, aliquots were diluted with methanol to prepare 100 µg/mL, 10 µg/mL, and 1.0 µg/mL working solutions; from the pioglitazone stock, an aliquot was diluted with methanol to prepare a 10 µg/mL working solution.

***Preparation of Calibration Standards and Extraction Procedure***

Blood collected from the rabbit was centrifuged at 2500 rpm for 30 min to separate the serum. Working calibration standards were prepared by taking 200 µL of serum in a 10 mL graduated test tube, to which 0.8 mL of 0.1 N HCl and appropriate aliquots of glimepiride solution (equivalent to 0.1, 0.3, 0.5, 1.0, and 5.0 µg of glimepiride) and of pioglitazone solution (equivalent to 0.5 µg) were added and mixed well. Dichloromethane (4 mL) was then added and the mixture was mixed for approximately 5 min, centrifuged at 2500 rpm for 15 min, and allowed to stand for 5 min. A 3 mL aliquot of the organic layer was transferred to a clean, dry test tube and evaporated to dryness on a water bath at 45 °C. The residue was reconstituted in 1.0 mL of mobile phase, and 20 µL was injected into the HPLC system. The ratio of the AUC of glimepiride to that of pioglitazone was calculated for each standard, and the calibration curve was constructed by plotting this ratio (y) against the corresponding glimepiride concentration (x), following the regression equation $y = mx + c$.

For the analysis of study serum samples, an analogous liquid–liquid extraction was used: to 200 µL of serum, 100 µL of pioglitazone (IS) working solution and 1 mL of 0.07 M phosphate buffer (pH 4.5) were added. After vortex-mixing for 10 s, 4 mL of dichloromethane was added and the mixture was shaken vigorously for 1 min, then centrifuged for 5 min at 8000 rpm. A 3 mL aliquot of the lower organic layer containing glimepiride was transferred to a clean tube and evaporated to dryness at 50 °C. The residue was reconstituted in 100 µL of mobile phase, and a 25 µL aliquot was injected onto the column, with the eluent monitored at 258 nm.

***Method Validation***

System suitability parameters (theoretical plates, tailing factor, retention time, and resolution), linearity, limit of detection (LOD), limit of quantification (LOQ), intra- and inter-day precision, and recovery/accuracy were evaluated as described in the Results and Discussion section below.

***Data and Statistical Analysis***

Data are presented as mean ± SEM. Statistical significance was assessed using Student's paired t-test (SAS version 9.2). Pharmacokinetic parameters for

glimepiride were computed using a non-compartmental model in R (version 3.1.0; package "bear", version 2.6.3; GNU General Public License).

The pharmacokinetic parameters estimated were: Cmax (ng/mL), Kel ($h^{-1}$), t1/2 (h), AUC0–24 (ng·h/mL), AUC0–∞ (ng·h/mL), MRT0–24 (h), MRT0–∞ (h), Tmax (h), Vd (L), CL (h/L), AUMC0–24 (ng·$h^2$/mL), and AUMC0–∞ (ng·$h^2$/mL). Concentration values below the LOQ were set to zero for all pharmacokinetic and statistical calculations.

# Results and Discussion

### *Chromatography*

Representative chromatograms of individual blank rabbit serum and of a serum sample containing glimepiride, metformin, and the internal standard (pioglitazone) are shown in Fig. 2 and Fig. 3, respectively. No endogenous interfering peaks were observed in blank serum at the retention time of glimepiride, confirming the specificity of the method. The retention time of glimepiride was found to be 6.7–7.52 min.

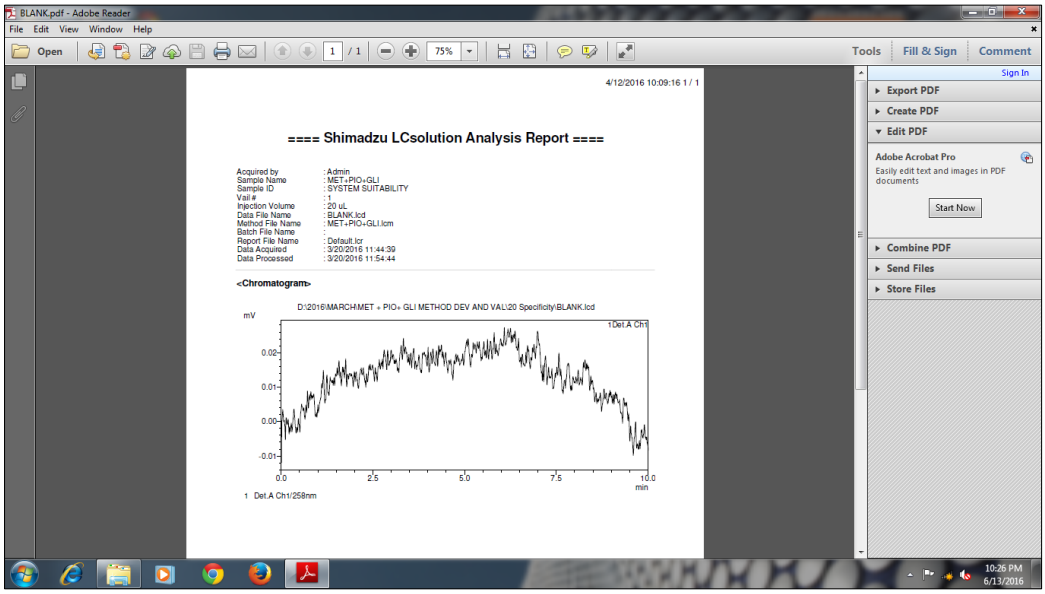
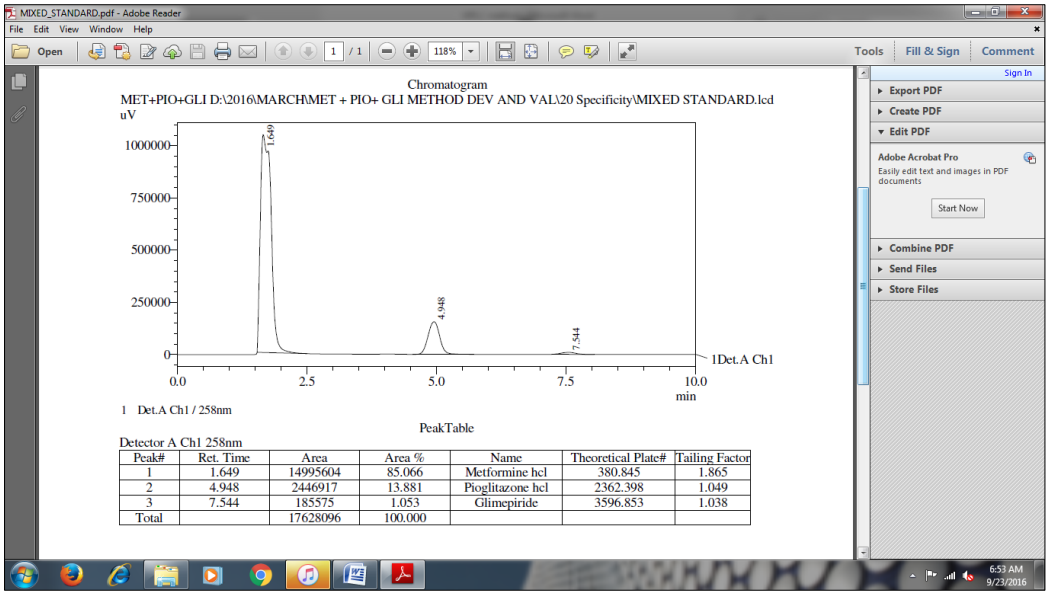



**Fig. 2: Typical chromatogram of blank rabbit serum**




| Peak# | Ret. Time | Area | Area % | Name | Theoretical Plate# | Tailing Factor |
|---|---|---|---|---|---|---|
| 1 | 1.649 | 14995604 | 85.066 | Metformine hcl | 380.845 | 1.865 |
| 2 | 4.948 | 2446917 | 13.881 | Pioglitazone hcl | 2362.398 | 1.049 |
| 3 | 7.544 | 185575 | 1.053 | Glimepiride | 3596.853 | 1.038 |
| Total | | 17628096 | 100.000 | | | |

**Fig. 3: Typical chromatogram of glimepiride, metformin, and internal standard (pioglitazone)**

### *Standard Graph and Linearity*

The calibration curve, constructed from the ratio of the AUC of glimepiride to that of pioglitazone against glimepiride concentration, was linear over the range 20–800 ng/mL. The regression equation obtained was $y = 0.0089x - 0.0078$ ($r^2 = 0.9980$) (Table 1, Fig. 4).

**Table 1: Construction of the standard graph of glimepiride (ratio of AUC of glimepiride to pioglitazone, internal standard)**

| Concentration of glimepiride (ng/mL) | SET-I | SET-II | SET-III | Average ± SEM |
|---|---|---|---|---|
| 20 | 0.195 | 0.175 | 0.184 | 0.185 ± 0.006 |
| 50 | 0.537 | 0.533 | 0.537 | 0.536 ± 0.001 |
| 100 | 0.981 | 1.009 | 0.999 | 0.996 ± 0.008 |
| 200 | 1.699 | 1.671 | 1.678 | 1.683 ± 0.008 |
| 500 | 4.185 | 4.187 | 4.194 | 4.189 ± 0.003 |
| 1000 | 9.102 | 9.321 | 8.691 | 9.038 ± 0.185 |

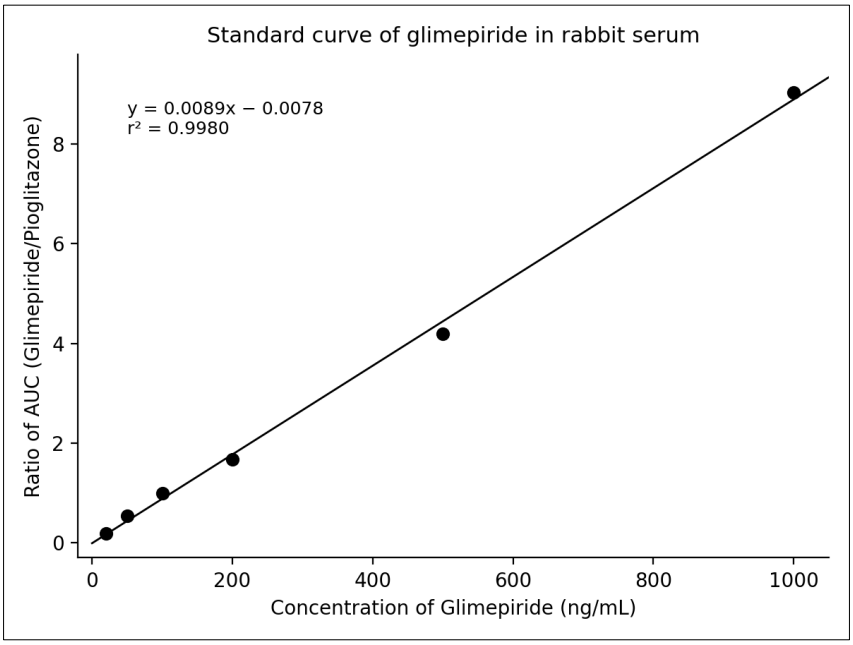



**Fig. 4: Standard curve of glimepiride in rabbit serum**

***Quantification***

The peak area of glimepiride relative to the internal standard was used for quantification in serum samples; the calibration curves were linear over the concentration range 20–800 ng/mL. The limit of quantification (LOQ), established from spiked calibration standards with %RSD below 20% and accuracy of 80–120%, was found to be 20 ng/mL. Sensitivity could be further increased, if required, by increasing the serum/injection volume.

***System Suitability***

System suitability parameters-theoretical plates, tailing factor, retention times, resolution, LOQ, and LOD-were determined and found to be within acceptable limits (Table 2).

**Table 2: System suitability parameters**

| Parameter | Value |
|---|---|
| LOD (ng/mL) | 18.55 |
| LOQ (ng/mL) | 20.00 |
| Theoretical plates | 14,352 |
| Tailing factor | 1.08 |
| Retention time of glimepiride (min) | 6.8–8.02 |
| Retention time of internal standard (min) | 4.2–5.05 |
| Resolution between drug and IS peaks | 1.75 |

***Precision***

Intra-day precision was determined by analysing three spiked serum samples at each concentration on the same day; inter-day precision was assessed over four different days. Inter-day %RSD ranged from 0.54 to 1.83, 0.23 to 0.79, 0.17 to 1.22, and 0.16 to 0.96 for the 20, 100, 500, and 1000 ng/mL concentrations, respectively (Table 3), well within the acceptance limit of <15% for inter- and intra-day precision.

**Table 3: Intra-day and inter-day precision of glimepiride in rabbit serum**

| Concentration (ng/mL) | Day | Mean | SD | %RSD |
|---|---|---|---|---|
| Intra-day (n = 3) | | | | |
| 20 | — | 19.96 | 0.09 | 0.43 |
| 100 | — | 99.55 | 2.72 | 2.73 |
| 500 | — | 489.52 | 9.17 | 1.87 |
| 1000 | — | 992.37 | 6.24 | 0.63 |
| Inter-day (n = 3) | | | | |
| 20 | 0 | 20.05 | 0.13 | 0.64 |
| 20 | 3 | 19.94 | 0.12 | 0.54 |
| 20 | 6 | 19.82 | 0.36 | 1.82 |
| 20 | 9 | 20.02 | 0.25 | 1.25 |
| 100 | 0 | 100.06 | 0.80 | 0.79 |
| 100 | 3 | 99.85 | 0.30 | 0.30 |
| 100 | 6 | 99.82 | 0.62 | 0.62 |
| 100 | 9 | 100.14 | 0.23 | 0.23 |
| 500 | 0 | 499.26 | 0.87 | 0.17 |
| 500 | 3 | 499.62 | 0.88 | 0.18 |
| 500 | 6 | 500.14 | 2.80 | 0.56 |
| 500 | 9 | 496.67 | 6.08 | 1.22 |
| 1000 | 0 | 987.70 | 6.57 | 0.66 |
| 1000 | 3 | 999.25 | 1.58 | 0.16 |
| 1000 | 6 | 992.75 | 4.30 | 0.43 |
| 1000 | 9 | 989.88 | 9.44 | 0.96 |

***Recovery and Accuracy***

Recovery of glimepiride from serum was estimated at 20, 100, 500, and 1000 ng/mL. Serum samples (in triplicate) containing glimepiride were extracted with dichloromethane and analysed; samples containing equivalent concentrations of glimepiride in methanol were injected directly, and the peak areas compared. Absolute recovery, calculated by comparing peak areas from direct injection with those from extracted, spiked serum, ranged from 98.85% to 99.82% (Table 4). Accuracy was confirmed by comparing measured concentrations in extracted serum with the nominal spiked concentrations.

**Table 4: Recovery of glimepiride from rabbit serum**

| Amount of glimepiride added (ng) | Mean amount found (ng, n = 3) | SD | Mean % recovery (n = 3) |
|---|---|---|---|
| 20.00 | 19.87 | 0.42 | 98.88 |
| 100.00 | 98.96 | 0.56 | 98.85 |
| 500.00 | 499.08 | 0.42 | 99.82 |
| 1000.00 | 992.97 | 6.28 | 99.45 |

***Pharmacokinetic parameters***

The pharmacokinetic parameters computed using the non-compartmental model are defined in Table 5.

**Table 5: Definitions of pharmacokinetic parameters computed for glimepiride**

| Parameter | Definition |
|---|---|
| Cmax | Maximum measured plasma concentration following treatment. |
| AUC0–24 | Area under the plasma concentration–time curve from time zero to the last measurable concentration, calculated by the linear trapezoidal method. |
| Tmax | Time to reach the maximum measured plasma concentration. |
| AUC0–∞ | Area under the plasma concentration–time curve from time zero to infinity, where AUC0–∞ = AUC0–t + Ct/λz (Ct = last measurable concentration; λz = terminal elimination rate constant). |
| λz (Kel) | First-order rate constant associated with the terminal (log-linear) portion of the curve, estimated by linear regression of time vs. log concentration using at least the last three non-zero plasma concentrations. |
| t1/2 (HL λz) | Elimination half-life, calculated as 0.693/λz. |
| MRT | Mean residence time — the average time drug molecules remain in the body after dosing; MRT = AUMC/AUC. |
| AUMC | Area under the first-moment curve, calculated by the trapezoidal rule. |

| Parameter | Definition |
|---|---|
| Vd | Volume of distribution — the theoretical volume the administered dose would need to occupy (if uniformly distributed) to produce the observed plasma concentration; Vd = total amount of drug in body / plasma drug concentration. |
| CL | Clearance — the volume of blood from which drug is completely removed per unit time; CL = 0.693 × Vd / t1/2. |

# DISCUSSION

The developed RP-HPLC method for glimepiride estimation in rabbit serum was specific, sensitive, and reproducible. No endogenous interference was observed at the retention times of glimepiride or the internal standard, confirming method specificity. The assay showed excellent linearity over 20–1000 ng/mL ($r^2$ = 0.9980) with an LOQ of 20 ng/mL, making it suitable for low-level serum quantification. Precision was excellent, with intra- and inter-day %RSD values below 3%, while recovery ranged from 98.88% to 99.82%, indicating efficient extraction and analytical reliability. Chromatographic performance was further supported by a tailing factor of 1.08 and 14,352 theoretical plates, demonstrating robust system suitability. The method is therefore appropriate for pharmacokinetic and bioavailability studies of glimepiride in rabbits.

# CONCLUSION

The validated RP-HPLC method described here is simple, accurate, precise, and highly sensitive for the estimation of glimepiride in rabbit serum. Its wide linear range, excellent recovery, low LOQ, and requirement of only 200 µL of serum make it well suited for pharmacokinetic, drug-interaction, and therapeutic-monitoring studies of glimepiride in rabbit models.

**Competing Interests**: The authors declare that they have no competing interests.

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The authors gratefully acknowledge the receipt of pure glimepiride and pioglitazone gift samples from Dr. Reddy's Laboratories, Hyderabad, India.

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