

Rapid and Specific Approach for Direct Measurement of Glimepiride in Human Plasma by LC–ESI–MS–MS Employing Automated 96 Well Format: Application to a Bioequivalence Study

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A rapid liquid chromatographic method with electrospray ionization tandem mass spectrometric (LC–MS–MS) detection is developed and validated for quantification of glimepiride in heparinized human plasma. Plasma samples, without a drying and reconstitution step, are extracted by solid-phase extraction (SPE) and eluted with 0.9 mL of acetonitrile–methanol (1:1, v/v) containing 0.05% formic acid. The analyte and glimepiride d8 (internal standard, IS) are chromatographed on a C₁₈ column; the mobile phase is acetonitrile–2 mm ammonium formate (88:12, v/v), with the pH adjusted to 3.5 with formic acid, at a flow rate of 0.5 mL/min. The retention times of glimepiride and the IS are 0.93 min, and the runtime is 1.6 min per sample. Selected reaction monitoring of MH⁺ at *m/z* 491.20 and 499.26 result in stable fragment ions with *m/z* 351.80 and 359.96 for glimepiride and the IS, respectively. The response was a linear function of the concentration in the range of 2.0–650.0 ng/mL, with *r* ≥ 0.9994. The recovery of glimepiride and the IS ranged from 81.91 to 83.36%. The assay has excellent characteristics and has been successfully used for the analysis of glimepiride in healthy human subjects in a bioequivalence study. It was well suited to clinical studies of the drug involving large numbers of samples.

Introduction

Glimepiride is an oral blood-glucose-lowering drug of the sulfonylurea class. Glimepiride is 1-[[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrolin-1-carboxamido) ethyl] phenyl]-3-(trans-4-methylcyclohexyl) urea with an empirical formula of C₂₄H₃₄N₄O₅S, and a molecular weight of 490.6 (1, 2). Glimepiride lowers blood glucose by stimulating the release of insulin from pancreatic beta cells. Extraparacrine effects (increasing the sensitivity of the peripheral tissues to insulin) may also play a role in the activity of glimepiride, such as other sulfonylureas. After oral administration, it is completely absorbed from the gastrointestinal tract. Peak plasma concentration is reached 2–3 h after dosing. Its bioavailability changes somewhat with food. Approximately 99.5% of glimepiride is bound to plasma proteins. A volume of distribution is 8.8 L. Glimepiride is completely metabolized in the liver. The mean plasma elimination half-life of glimepiride is 5–8 h (2).

Several chromatographic methods including liquid chromatography–UV (LC–UV) (3–7), LC–diode array detection (DAD) (8–9), and LC–tandem mass spectrometry (MS–MS) (10–19) have been developed to measure glimepiride in

biological fluids. All these reported methods are inadequate because of insufficient sensitivity, a long chromatographic run time, more plasma volume require for sample processing, and a high injection volume. All reported methods require a laborious extraction procedure, such as liquid–liquid extraction (LLE), which requires time consuming and error prone solvent evaporation and reconstitution steps.

The aim of the present study was to develop and validate a simple, reproducible, and high throughput bioanalytical method based on mass spectrometry detection for the rapid quantitation of glimepiride in human plasma (0.2 mL). The method runtime was 1.6 min per sample, the lower limit of quantitation (LLOQ) was 2.0 ng/mL, the correlation coefficient (*r*) was better than 0.9994, and injection volume was 5.0 μL, which helps to increase the ESI-MS source life and to reduce the column backpressure during the analysis of large numbers of clinical samples. In this paper, a simple, rapid, and economical method for the determination of glimepiride is reported. The process of evaporation and reconstitution introduced in LLE considerably reduced labor, cost, and time for analysis. Also, the method was sufficiently sensitive to study the pharmacokinetics of 4 mg glimepiride formulation in healthy subjects.

Experimental

Chemicals, reagents, standards

Pharmaceutical grade glimepiride was supplied by Vardha Biotech (Mumbai, Maharashtra, India) and was certified to contain 99.56% glimepiride. Glimepiride-d8 was supplied by BDG Synthesis (Wellington, New Zealand) and was certified to contain 96.9% glimepiride-d₈. Both standards were used without further purification. The organic solvents used were of gradient grade and were obtained from Ranbaxy (Delhi, India). Water was obtained from a Milli-Q Gradient water purification system (Millipore, Bedford, MA). Formic acid was of suprapur grade and obtained from Merck (Darmstadt, Germany). Ortho-Phosphoric acid was suprapur and obtained from Merck (Darmstadt, Germany). Ammonium formate, used for mobile phase preparation, was of molecular biology-tested grade from Sigma-Aldrich (Steinheim, Germany). HLB cartridges were obtained from Waters (Milford, MA). Control human plasma was obtained from a Blue Cross laboratory (Ahmadabad, Gujarat, India) and was stored below –70°C.

Main stock solutions (1.00 mg/mL) of glimepiride and IS were prepared in methanol. The main stock solution of

glimepiride was further diluted with methanol–water 50:50 (v/v) to an intermediate concentration of 20 µg/mL. Working solutions of glimepiride of different concentrations required for preparation of spiking plasma calibration standard (CS) solutions and quality control (QC) samples were subsequently prepared using intermediate stock solution. The main stock solution of the IS was further diluted with water to furnish a concentration of 500.0 ng/mL working solution. All main stock, intermediate stock, and working stock solutions were stored at 2–8°C. Drug-free plasma (i.e., control plasma) was stored below –70°C in a deep freezer and left to thaw completely for nearly 1 h before use. A blank plasma was spiked with 5% working stock solution of glimepiride to achieve the desired concentration of glimepiride for CS and QC samples. The spiked QC samples were stored below –70°C for studies of freeze-thaw and long-term stability.

CSs were prepared immediately before extraction by spiking 190 µL blank human plasma with glimepiride to furnish concentrations of 2.0, 4.0, 20.0, 60.0, 120.0, 240.0, 360.0, 480.0, and 650.0 ng/mL. QC samples were prepared at concentrations of 6.0, 180.0, and 450.0 ng/mL.

Sample preparation by an automated SPE method (Freedom EVO)

Spiked plasma stability samples of glimepiride were removed from the deep freezer and maintained below –70°C and left at room temperature to thaw. The samples were vortexed, mixed adequately, and centrifuged before pipetting. As soon as the stability samples (0.2 mL) were thawed, these samples and freshly prepared CS and QC samples were spiked with 25.0 µL of IS (500.0 ng/mL). Ortho-phosphoric acid (2.5%) in water (700 µL) was then added, and the sample was mixed and loaded onto HLB Oasis 96 well cartridges previously conditioned with 0.9 mL methanol, followed by 0.9 mL Milli Q water. The sample was then mixed and loaded into HLB Oasis 96 well cartridges. The cartridges were washed sequentially with 1.8 mL water and 0.9 mL 5% methanol in water and eluted with 0.9 mL of acetonitrile–methanol (1:1, v/v), containing 0.05% of formic acid. The eluate was collected in labeled LC vials, and 5.0 µL was injected for analysis.

Eight Channel Robotic Arm Liquid Handling Systems (LHS, Tecan, Switzerland) were used to extract the plasma samples. All extraction steps, from the conditioning of the cartridges to the elution step, were performed by using fully automated Freedom EVOware software. A total of 96 samples were processed within 1 h.

During the pre-method validation, a manual SPE for glimepiride was tested with an automated SPE technique. An automated SPE procedure unit gives better accuracy, precision, and linearity than a manual SPE technique. As a result, an automated SPE method was implemented for method validation and clinical study sample analysis.

LC–MS–MS

LC was performed with a Prominence pump, autosampler, autoinjector, and a column oven from Shimadzu (Kyoto, Japan). MS was performed with a TSQ Quantum triple-quadrupole MS manufactured by Thermo Finnigan (Thermo Electron, San Jose,

CA). All LC and MS–MS conditions were controlled by LCquan software, version 2.5.6.

The compounds were separated on a 100 mm × 3 mm, 3.0-µm particle, Betasil C₁₈ reversed-phase column (Thermo). The column temperature was 45°C, and the autosampler and tray temperatures were 10°C. The mobile phase was acetonitrile–2 mM ammonium formate (88:12, v/v), and the pH was adjusted to 3.5 with formic acid at a flow rate of 0.5 mL/min. The MS was equipped with an electrospray ionization (ESI) ion source and was used in positive-ionization SRM mode. The ion-spray conditions for glimepiride and the IS were: collision gas pressure, 1.5 mTorr; sheath gas, 40.0 (arb. units); auxiliary gas, 20.0 (arb. units); capillary temperature, 380.0°C; and ion-spray potential (IS), 3500.0 V. The tube lens offset and collision energy (CE) applied for glimepiride and IS was 80 V, 13 V, 78 V, and 16 V, respectively. The MS–MS transition selected to monitor glimepiride was *m/z* 491.20 to the product ion at *m/z* 351.80 (see Figure 1). The IS was monitored using the transition from 499.26 to the product ion at *m/z* 359.96 (see Figure 2). The protonated molecules were fragmented using argon as the CID gas. Because of the high specificity of tandem MS detection, no peaks other than the analyte and the IS peaks were seen in the analyte runs. The TIC of the chromatograms for glimepiride is presented in Figure 3.

The LCquan software provided a standard method for calculations for quantitative analysis. Peak areas for all the SRMs were automatically integrated, and the glimepiride-to-IS peak-area ratios of the calibrators were used in a linear regression analysis with a weighting factor of 1/x². The response curve was used to calculate the concentration of the calibrators, the QC, and the stability samples.

Results and Discussion

Method development

The objective of this method was to develop and validate (20) a simple, rapid, and sensitive method for the extraction and quantification of glimepiride that would be suitable for the determination of the pharmacokinetic of the compound in clinical studies. During the method development, different detections, chromatographic, and sample/extraction conditions were evaluated to achieve the maximum response and good peak shape.

Initially, tuning the MS conditions in both positive and negative modes was performed for glimepiride and the IS, and the response was found to be much higher in a positive-ionization mode. With a short 3.0-mm i.d. and 3.0-µm particle size Betasil C₁₈ column, the retention times of glimepiride and the IS were low enough. In several initial trials with ammonium formate and methanol for the mobile phase optimization, the response was low when pH of the ammonium formate was 3.5. The p*K*_a value of glimepiride was ~6.2. When 2 mM ammonium formate of pH 3.5 was used with acetonitrile, a good peak shape and an improved signal were obtained with a low background noise, resulting in a higher specificity. The maximum backpressure during analysis was ~55 bars. The optimum column oven temperature was 45°C, which resulted in symmetrical peaks. The signal obtained by use of the optimized chromatographic and detection condition enabled elimination of the laborious steps of evaporation and reconstitution. The SPE

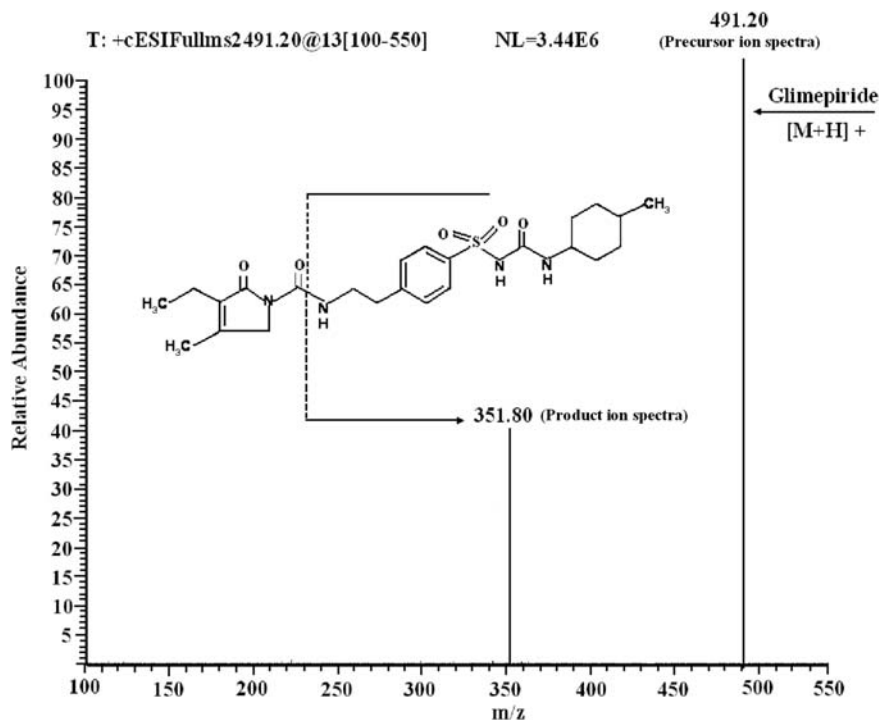


Figure 1. Full scan and product ion spectra of glimepiride.

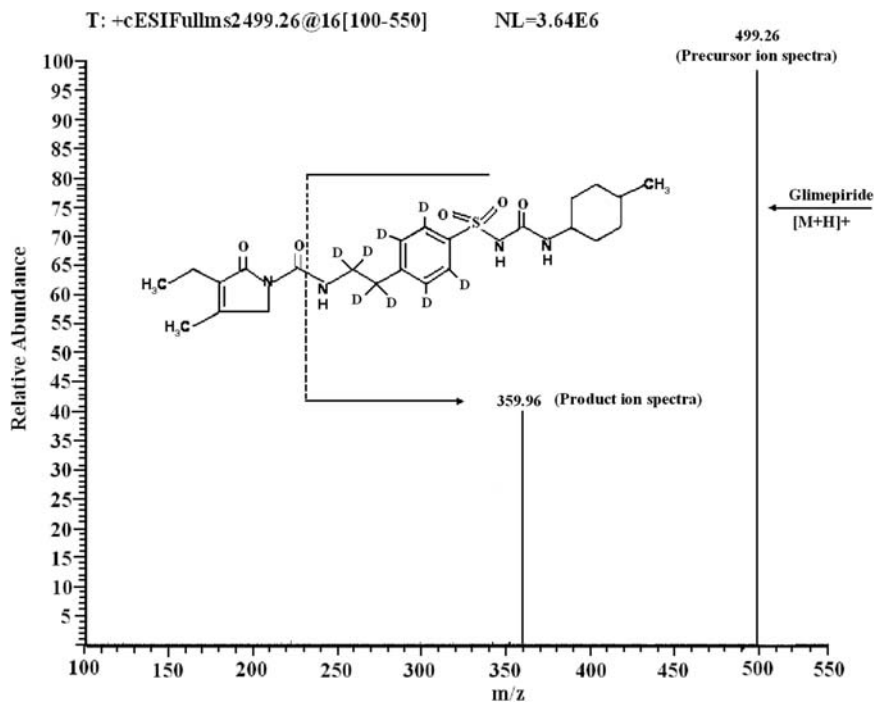


Figure 2. Full scan and product ion spectra of glimepiride-d8.

could be used without compromising the method, and further reduced the sample-processing time. The sample volume used for processing was 0.2 mL, and the method of extraction was

sufficiently rugged for routine analysis. The sample injected (5.0 μ L) avoided column backpressure and ESI source contamination during sample analysis in the clinical studies.

Specificity

The specificity of the method was investigated by comparing chromatograms obtained from six different sources of plasma. The area observed at the retention time of glimepiride was much less than 20% of the LLOQ area (2.0 ng/mL). The representative chromatograms, shown in Figure 4, indicate that there was no interference with the analyte and IS from endogenous substances in the plasma.

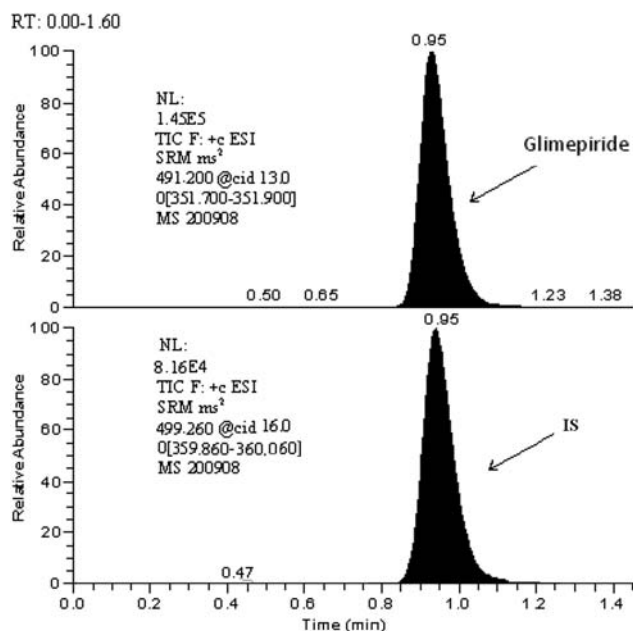


Figure 3. The representative TIC chromatograms of glimepiride and IS.

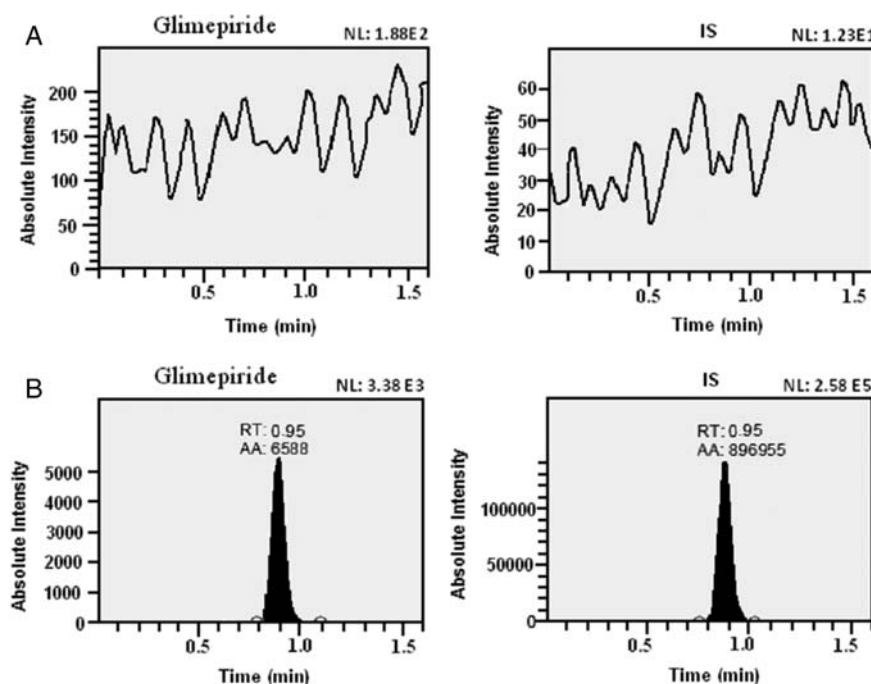


Figure 4. Representative chromatograms for glimepiride: (A) the extracted blank plasma; (B) the extracted 2.0 ng/mL of glimepiride.

Calibration curves

The linearity of the method was determined by a weighted least-square linear regression analysis of standard plots associated with a nine-point standard calibration curves. Best-fit calibration curves of peak-area ratio against the concentration were drawn. The concentration of glimepiride was calculated from the simple linear equation using a regression analysis of the spiked plasma CSs with a reciprocal of the square of the drug concentration, $1/x^2$, as a weighting factor. The calibration plots were linear from 2.0 ng/mL to 650.00 ng/mL with $r \geq 0.9994$.

Precision and accuracy

Intra-assay precision and accuracy were calculated at LLOQ (2.0 ng/mL), low quality-control (LQC, 6.0 ng/mL), middle quality-control (MQC, 180.0 ng/mL), and high quality-control (HQC, 450.0 ng/mL) levels for the six replicates, each of the same analytical run. Inter-assay precision and accuracy were calculated after the replicates in five different analytical runs. The results are given in Table I.

Recovery

The recovery of glimepiride was calculated by comparing the peak area of the analyte from the extracted plasma standard with that obtained from an un-extracted standard at the same concentration for the QC samples containing 3.0, 180.0, and 450.0 ng/mL. IS recovery was tested at 500.0 ng/mL by comparing six extracted and un-extracted samples at each concentration. The percent mean recovery for glimepiride was observed as 81.91. The mean recovery of IS was 83.36% at a concentration 500.0 ng/mL. The results are given in Table IIA and Table IIB.

Table I

Intra and Inter-Accuracy and Precision for Glimepiride

Quality control samples	Glimepiride intra assay precision and accuracy					Glimepiride inter assay precision and accuracy				
	Conc. added (ng/mL) [‡]	Mean conc. found (ng/mL)*	SD	Precision % CV	Accuracy (%)	Mean conc. found (ng/mL) [‡]	SD	Precision % CV	Accuracy (%)	
LLOQ	2.0	2.119	0.082	3.85	105.96	2.094	0.120	5.73	104.68	
LQC	6.0	6.309	0.229	3.64	105.16	6.17	0.375	6.08	102.84	
MQC	180.0	178.531	8.845	4.95	99.18	178.703	5.364	3.00	99.28	
HQC	450.0	442.802	14.052	3.17	98.40	460.667	18.664	4.05	102.37	

* Mean of six replicates.

† Mean of thirty replicates.

‡ Concentration.

Table IIA

Recovery of Glimepiride

QC Levels	Recovery (%)
LQC	79.54
MQC	83.65
HQC	82.54
Mean	81.91
SD	2.126
CV (%)	2.60

Table IIB

Recovery of IS (500.000 ng/mL)

Sr. No.	IS serial No.	Area of IS Unextracted	QC serial No.	Extracted
1	IS-1	773125	IS-1	638257
2	IS-2	762587	IS-2	635284
3	IS-3	764874	IS-3	635287
4	IS-4	756240	IS-4	632451
5	IS-5	747891	IS-5	635247
6	IS-6	768961	IS-6	635934
		6		6
Mean		762280		635410
SD		9084.028		1854.359
CV (%)		1.19		0.29
Recovery (%)		83.36		

Matrix effect

The matrix effects were investigated for six different samples of plasma, comprising four lots of normal control heparinized plasma, one lot of lipemic plasma, and one lot of haemolyzed plasma. Three samples each at the LQC and HQC levels were prepared from different lots of plasma (i.e., a total of 36 QC samples) and checked for accuracy to see whether the matrix affected the back-calculated value of the nominal concentrations for these different plasma samples. The results obtained were well within the acceptable limit of $\pm 15\%$, which clearly proves that elution of endogenous matrix peaks in the dead volume time does not affect the pattern of elution of glimepiride and IS, respectively.

Stability

Exhaustive experiments were performed to assess the stability of glimepiride in stock solution and in plasma samples under different conditions, simulating the conditions occurring during the analysis of study samples: room-temperature stability, extracted sample stability (process stability), freeze-thaw

stability, and the long-term stability of plasma samples. The results obtained were well within acceptable limits. The IS stock solution was also found to be stable.

Stock solutions of glimepiride and the IS were stable at room temperature for 88 h and at $2-8^{\circ}\text{C}$ for 10 days. Glimepiride in control human plasma was stable for 20 h at room temperature. Glimepiride in the final extract after SPE was found to be stable in an autosampler at 10°C for up to 78.0 h (process stability). Glimepiride was found to be stable through at least four freeze-thaw cycles. Glimepiride spiked plasma samples stored at -70°C to test long-term stability were stable for at least 26 days. Percentage changes of concentration in these stability experiments are listed in Table III.

Bioequivalence study

The design of this study comprised of an open randomized, two period, two sequence, replicate, crossover, comparative evaluation of the relative bioavailability of the test formulation of glimepiride with reference (4 mg amaryl, tablets) in 20 healthy adult human subjects under fasting conditions. All the subjects were informed of the aim and risk involved in the study and written consent was obtained. An ethics committee approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by the International Conference on Harmonization and the US Food and Drug Administration (FDA) (21). A health checkup for all subjects was done by a general physical examination, ECG, and laboratory tests like hematology, biochemistry, and urine examination. All subjects were negative for HIV, HBSAg, and HCV tests. They were orally administered a single dose of the test compound and reference formulation after a recommended washout period with 240 mL of water. Drinking water was not allowed and a supine position was restricted 2 h post dose. Standardized meals were provided as per schedule. Blood samples were collected in vacutainers containing heparin before collection of each time point's administration of drug. Blood samples were centrifuged at 3500 rpm for 10 min and plasma was separated and stored at -70°C until use.

Application of the method

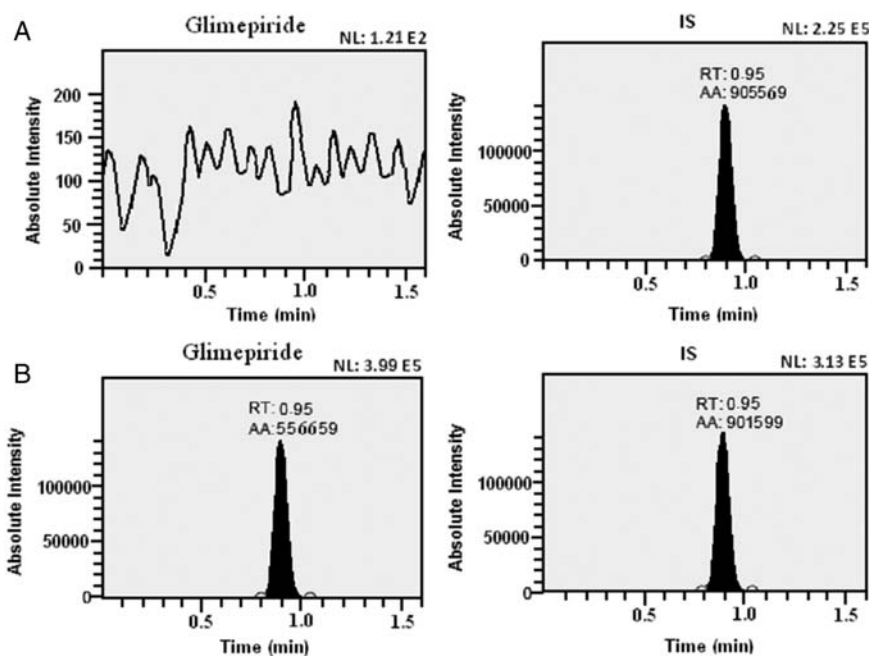
An automated SPE method was successfully applied for the assay of glimepiride in plasma samples from ongoing development of an immediate-release formulation. Plasma samples

Table III

Stability Results of Glimperiride

Stability experiments	Storage conditions	Mean comparison sample conc. found (ng/mL)	Mean stability sample conc. found (ng/mL)	% Mean change at quality control level
Bench top	Room temp. (20 h)	6.024	5.784	LQC -3.98
Process (extracted sample)	Auto sampler (78 h)	457.111	457.874	HQC 0.17
		6.654	6.005	LQC -9.75
Freeze and thaw stability in plasma	After 4th FT cycle at -70°C	435.125	454.258	HQC 4.40
		6.698	6.451	LQC -3.69
Long term stability in plasma	For 26 days at -70°C	467.234	458.154	HQC -1.94
		6.229	6.354	LQC 2.01
		178.964	177.367	MQC -0.89
		478.998	489.113	HQC 2.11

LQC = 2.300 ng/mL; MQC = 180.000 ng/mL; HQC = 450.000 ng/mL

**Figure 5.** Representative chromatograms for glimepiride: (A) the extracted pre-dose sample from the subject; (B) the extracted patient sample after 5.0 h of dosing.

were collected at: 0.00, 0.50, 1.00, 1.50, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 8.00, 10.0, 12.0, 15.0, 16.0, 18.0, 24.0, and 36.00 h after administration of a single oral dose of a 4-mg tablet to 20 male volunteers in each phase. A total of 1390 plasma samples from 20 volunteers were analyzed along with CSs and QC samples. A total 550 samples were analyzed per day. No interfering peak was found in pre-dose samples from all volunteers (see Figure 5). The concentration of glimepiride in the patient's samples reached a maximum at 5.0 h after dosing. It was well suited to pharmacokinetic and pharmacodynamic clinical studies of the drug involving a large numbers of samples.

Conclusion

The objective of this work was to develop a simple, rugged, automated, and a high throughput method for the estimation

of glimepiride in human plasma, especially in the absorption and elimination phase after the oral administration of 4 mg formulation. The advantage of the current work is the reduction in the labor commonly associated with an LLE technique on account of the evaporation and reconstitution part. This technique, introduced here for the first time, minimizes the chance for human errors, saves considerable time, and provides an automated sample preparation methodology. The run time per sample analysis of 1.6 min suggests the high throughput of the proposed method. Moreover, the limit of quantitation is low enough to monitor at least five half-life samples of glimepiride concentration with good intra-assay reproducibility (%CV) for the QC samples. From the results of all the validation parameters, the method can be useful for the therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for the clinical trials samples with desire precision and accuracy.

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