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Determination of glimepiride in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A sensitive and specific high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS–MS) method has been developed at our center for the determination of glimepiride in human plasma. After the addition of the internal standard, plasma samples were extracted by liquid–liquid extraction technique using diethyl ether. The compounds were separated on a prepacked C_{18} column using a mixture of acetonitrile, methanol and ammonium acetate buffer as mobile phase. A Finnigan LCQ^{DUO} ion trap mass spectrometer connected to an Alliance Waters HPLC was used to develop and validate the method. The analytical method was validated according to the FDA bioanalytical method validation guidance. The results were within the accepted criteria as stated in the aforementioned guidance. The method was proved to be sensitive and specific by testing six different plasma batches. Linearity was established for the range of concentrations 5.0–500.0 ng/ml with a coefficient of determination (r^2) of 0.9998. Accuracy for glimepiride ranged from 100.58 to 104.48% at low, mid and high levels. The intra-day precision was better than 12.24%. The lower limit of quantitation (LLOQ) was identifiable and reproducible at 5.0 ng/ml with a precision of 7.96%. The proposed method enables the unambiguous identification and quantitation of glimepiride for pharmacokinetic, bioavailability or bioequivalence studies.

Keyword: Glimepiride

1. Introduction

Glimepiride, 1-[[*p*-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido) ethyl]phenyl] sulfonyl]-3-(*trans*-4-methylcyclohexyl) urea, is a new oral sulfonylurea hypoglycemic agent. It contains a sulfonylurea nucleus and a cyclohexyl ring. Glimepiride is a white to yellowish white, crystalline, odorless to practically odorless powder, which is practically insoluble in water [1]. Glimepiride is used in the management of non-insulin dependent (type II) diabetes mellitus and is completely absorbed from the GI tract after oral administration [1,2].

Up to our best knowledge, there is only one published chromatographic technique on glimepiride determination in biological fluids [3]. The authors of this reference stated that high-performance liquid chromatography (HPLC) methods used usually to determine related sulphonylureas have been explored to quantify glimepiride in human biological fluids, however, the shortcoming of these methods seemed to be unreliability with regard to sensitivity and specificity, especially in the ng/ml range [3]. Their HPLC-UV method was used to quantify glimepiride in human serum after pre-column derivatization with 2,4-dinitrophenylamine [3]. Though, we consider this kind of methods requires special arrangements and is considered time-consuming due to the derivatization procedures.

It is well known that HPLC tandem MS (MS–MS) further enhances specificity and provides an improved signal-to noise ratio compared with single-stage MS [4]. Additionally, the ion trap mass spectrometer enables MS–MS at an affordable price compared with a triple-stage quadrupole MS system. The purpose of this work was to exploit the high selectivity and sensitivity of an ion trap detector operated in MS–MS mode with an ESI interface for the development and validation of a robust reversed-phase LC–MS–MS method for glimepiride determination in human plasma. It was essential to establish an assay capable of quantifying glimepiride at concentrations down to 5.0 ng/ml. At the same

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time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of glimepiride.

2. Experimental

2.1. Reagents

The following chemicals and reagents were used: Acetonitrile (HPLC grade, Panreac, Spain), methanol (HPLC grade, Acros, Belgium), ammonium acetate, potassium chloride, chloroform and diethyl ether (BDH Laboratories, UK), hydrochloric acid (Riedel-de Häen, Germany), dimethylsulphoxide (SDS, France), and glacial acetic acid (Scharlau, Spain). A Milli-Q® (Millipore, France) water purification system was used to obtain the purified water for the HPLC analysis. Glimepiride and glibenclamide were obtained from Julphar Co. (UAE). Lithium heparin plasma of healthy volunteers was obtained from Jordan University Hospital (Jordan).

2.2. Preparation of stock solutions

Primary stock solutions of glimepiride were prepared from separate weightings. The primary stock solutions were prepared in dimethylsulphoxide: chloroform (1:2, v/v) and stored at −20 °C. Two milliliters of this primary stock solution were diluted in methanol to produce a final concentration of 100 μg/ml. The internal standard stock solution was prepared by dissolving 5.0 mg of glibenclamide in 50 ml acetonitrile producing a concentration of 100 μg/ml. Working solutions of glimepiride were prepared in methanol, by appropriate dilution, at: 250, 500, 1250, 2500, 5000, 15,000 and 25,000 ng/ml.

2.3. Calibration curves

Calibration curves were prepared by spiking different samples of 1 ml plasma each with 20 μl of one of the above mentioned working solutions to produce the calibration curve points equivalent to 5.0, 10.0, 25.0, 50.0, 100.0, 300.0 and 500.0 ng/ml of glimepiride. Each sample contains also 200 ng/ml of internal standard. Zero plasma samples used in each run were prepared containing 200 ng/ml of internal standard only. In each run, a plasma blank sample (no IS) was also analyzed.

2.4. Quality control samples

Quality control (QC) samples were prepared at three levels, low level (three times the lower limit of quantitation, LLOQ), middle level and a high level (80% of the upper limit of quantitation limit, ULOQ). QCs were prepared daily by spiking different samples of 1 ml plasma each with 20 μl of the corresponding standard solution to produce a final concentration equivalent to 15.0, 250.0 and 400.0 ng/ml of glimepiride and 200 ng/ml of internal standard.

2.5. Extraction

QC, calibration curve and blank plasma samples were extracted using a liquid–liquid extraction technique. One milliliter of 0.05 M KCl (adjusted with HCl to pH 1.0) was added to each tube containing 1 ml of plasma, afterward the samples were extracted with 7 ml diethyl ether. The samples were then shaken for 20 min and centrifuged for 5 min at 2000 × *g* (Eppendorf 5810R, Germany). The resulting samples were frozen and the upper ethereal layer decanted into another tube where it was evaporated to complete dryness under nitrogen stream at 30 °C. Samples were reconstituted with 60 μl of methanol–water (50:50, v/v) then vortexed for 30 s ready for direct injection into the HPLC system.

2.6. HPLC conditions

Chromatography for separation and determination of the drug was carried out by applying the samples to a prepacked 5 μm (50 mm × 2.1 mm, i.d.) C_{18} XTerra column (Waters, Milford, MA, USA), using a 2690 Alliance high-performance liquid chromatograph (Waters, Milford, MA, USA). The analytical column was protected by a Phenomenex C_{18} guard column (4 mm × 2.0 mm, i.d.). The combination of the mobile phase, prepared by mixing ammonium acetate buffer (0.02 M, pH = 3.5): acetonitrile: methanol in the ratio of 40:35:25 (v/v), and a flow rate of 0.28 ml/min was found to be adequate for the samples analysis. Separations were performed at room temperature.

2.7. LC–MS–MS conditions

Drug monitoring and quantitation were done using a Finnigan LCQ^{DUO} quadrupole ion trap mass spectrometer (Finnigan ThermoQuest, USA) equipped with an ESI source (Finnigan) and run by XCALIBUR 1.2 software.

Operating conditions for the ESI source, used in the positive ionization mode, were optimized by constantly adding glimepiride in methanol (0.1 mg/ml) to the HPLC flow by a syringe pump via a T-connector in the infusion mode. The signal was optimized on the total ion current in MS mode, producing a transfer capillary temperature of 230 °C, a spray voltage of 4.5 kV, and a sheath gas flow of 56 units (units refer to arbitrary values set by the LCQ software). At the same time, the selection of ions and the collision voltages were optimized using LCQ software. In the MS–MS experiments, the protonated precursor molecular ions $[MH]^+$ of glimepiride (*m*/*z* 491) and the IS (*m*/*z* 494) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 35%. The mass spectra resulting from these fragmentations were acquired in the SRM mode at *m*/*z* 352 for glimepiride and *m*/*z* 369 for IS. These product

ions, *m*/*z* 352 for glimepiride and *m*/*z* 369 for the IS, were extracted for quantification.

2.8. Data treatment

The linearity of glimepiride method determination in human plasma was tested for the range of concentrations 5.0–500.0 ng/ml. Calibration curves were prepared by determining the best-fit of peak area ratios (peak area analyte/peak area internal standard) versus concentration, and fitted to the equation $y = bx + a$ by unweighted least-squares regression.

3. Results and discussion

3.1. Separation and specificity

Glimepiride and IS gave protonated precursor molecular ions $[\dot{M}H]^+$ in the MS mode. The major ions observed were *m*/*z* 491 for glimepiride (Fig. 1) and *m*/*z* 494 for the IS (Fig. 2). The most intense product ions observed in the MS–MS spectra were *m*/*z* 352 for glimepiride and *m*/*z* 369 for the IS. The corresponding SRM ion spectra of glimepiride and the IS are shown in Figs. 1 and 2, respectively.

The product ion chromatograms extracted from supplemented plasma are depicted in Fig. 3. As shown, the retention times of glimepiride and the IS were 1.45 and 0.52 min, respectively. The total HPLC–MS–MS analysis time was 2.5 min per sample. No interferences of the analytes were observed because of the high selectivity of the MS–MS technique. Fig. 3A shows also an HPLC chromatogram of a blank plasma sample indicating no endogenous peaks at the retention times (t_R) of glimepiride or internal standard (glibenclamide).

The product ion chromatograms obtained from an extracted plasma sample of a healthy volunteer who participated in a bioequivalence study conducted on 24 persons, is depicted in Fig. 4. Glimepiride was unambiguously identified and was quantified as 245 ng/ml.

3.2. Method validation

In our laboratory, samples analysis is always carried out in a GLP-compliant manner and therefore the LC–MS–MS methods need to be validated according to currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [5]. The following parameters were considered.

To test the specificity, six batches of human plasma were tested. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/spectroscopic conditions and compared with those obtained with an aqueous solution of the analyte at a concentration near to the LLOQ. No significant interference at the retention time of the drug or internal standard

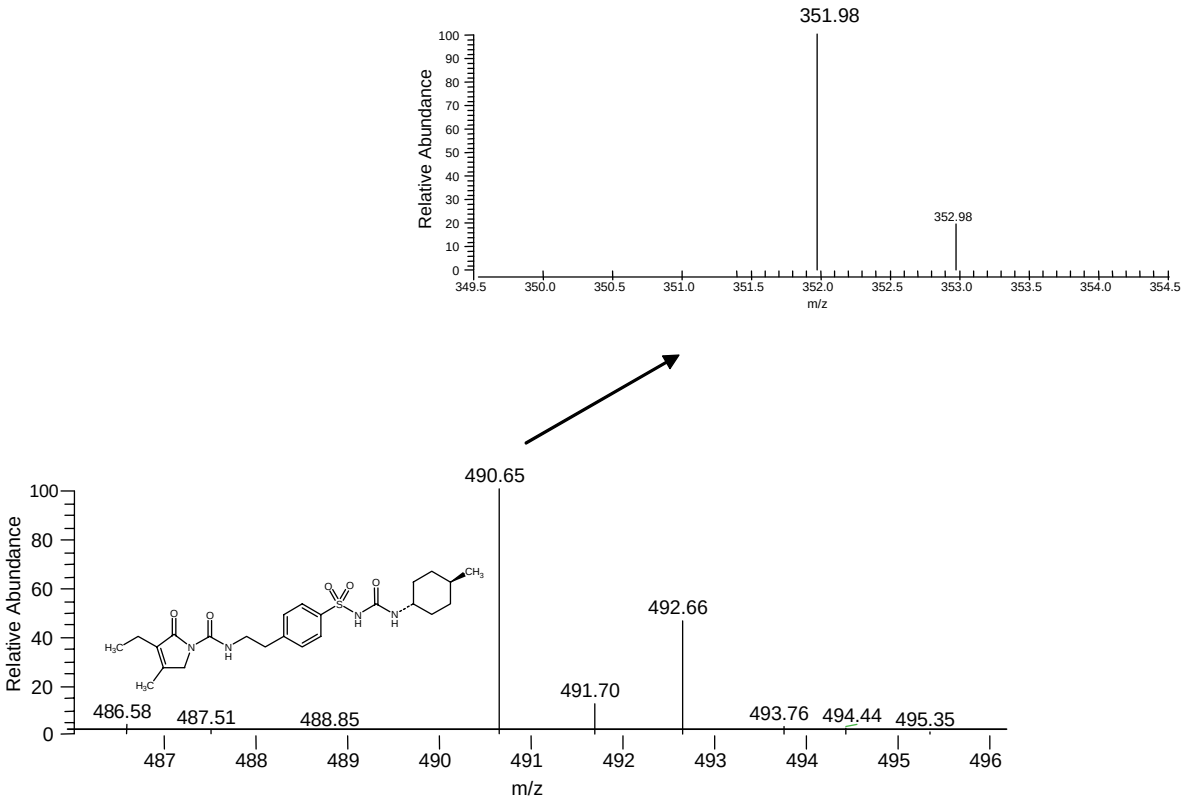

Fig. 1. Positive ion electrospray mass spectrum (bottom) and product ion mass spectrum (top) used in SRM for glimperide determination.

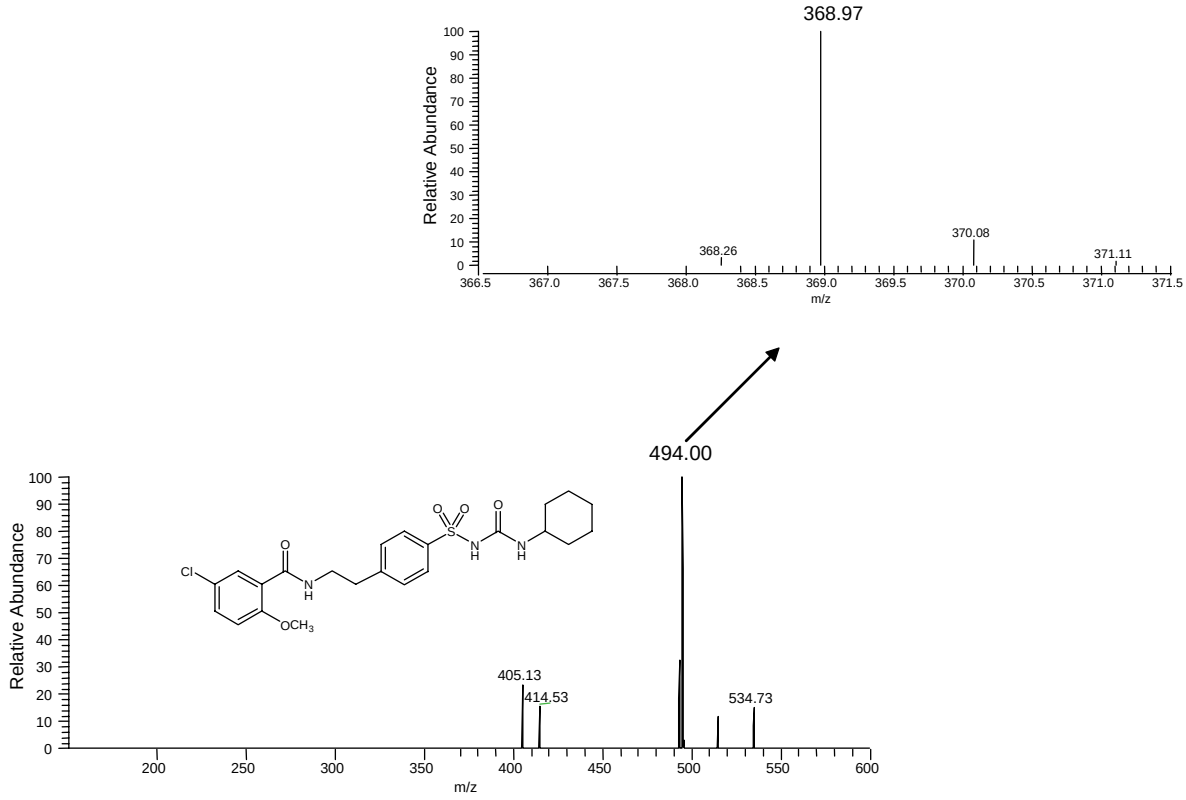

Fig. 2. Positive ion electrospray mass spectrum (bottom) and product ion mass spectrum (top) used in SRM for glibenclamide (internal standard) determination.

were found, as showed in the chromatograms presented in Fig. 1.

Linearity was tested for the range of concentrations 5.0–500.0 ng/ml, employing standard calibration curves of at least seven points (non-zero standards). In addition, a blank and zero plasma samples were also analyzed to confirm absence of interferences, these two samples were not used to construct the calibration function. The method exhibited a reliable linear response for the range of concentrations from 5.0 to 500.0 ng/ml. Results of six representative calibration curves for glimepiride HPLC determination are given in Table 1. The table also shows the back calculated

Table 1
Back-calculated concentrations of glimepiride calibration standards and statistics for precision and accuracy from six representative calibration curves

Calibration curves		Concentration of standards (ng/ml)									
		5.00	10.00	25.00	50.00	100.00	300.00	500.00	*a*	*b*	*R*
First	Calculated concentration	5.82	11.08	23.19	47.55	103.49	297.47	500.48	−2.64E−02	1.33E−02	9.9993E−01
	Accuracy (%)	116.39	110.82	92.75	95.10	103.49	99.16	100.10			
Second	Calculated concentration	5.26	11.05	23.98	47.67	102.93	296.92	499.92	−1.90E−02	1.33E−02	9.9995E−01
	Accuracy (%)	105.26	110.53	95.94	95.34	102.93	98.97	99.98			
Third	Calculated concentration	5.92	11.08	25.84	49.91	99.91	295.22	504.59	−1.88E−02	1.28E−02	9.9989E−01
	Accuracy (%)	118.44	110.78	103.38	99.81	99.91	98.41	100.92			
Fourth	Calculated concentration	5.76	9.82	23.26	47.48	100.29	309.66	496.38	−2.37E−02	1.28E−02	9.9974E−01
	Accuracy (%)	115.16	98.20	93.03	94.95	100.29	103.22	99.28			
Fifth	Calculated concentration	5.77	10.54	24.13	48.12	100.23	302.57	498.66	−2.29E−02	1.28E−02	9.9997E−01
	Accuracy (%)	115.47	105.39	96.53	96.23	100.23	100.86	99.73			
Sixth	Calculated concentration	5.80	11.27	25.09	48.69	100.17	295.48	500.95	−2.22E−02	1.28E−02	9.9995E−01
	Accuracy (%)	115.94	112.66	100.38	97.34	100.17	98.49	100.19			
	Mean	5.72	10.81	24.25	48.23	101.17	299.55	500.17	−2.22E−02	1.30E−02	9.9990E−01
	CV%	4.08	5.01	4.30	1.94	1.58	1.88	0.54	13.1	1.99	0.0085

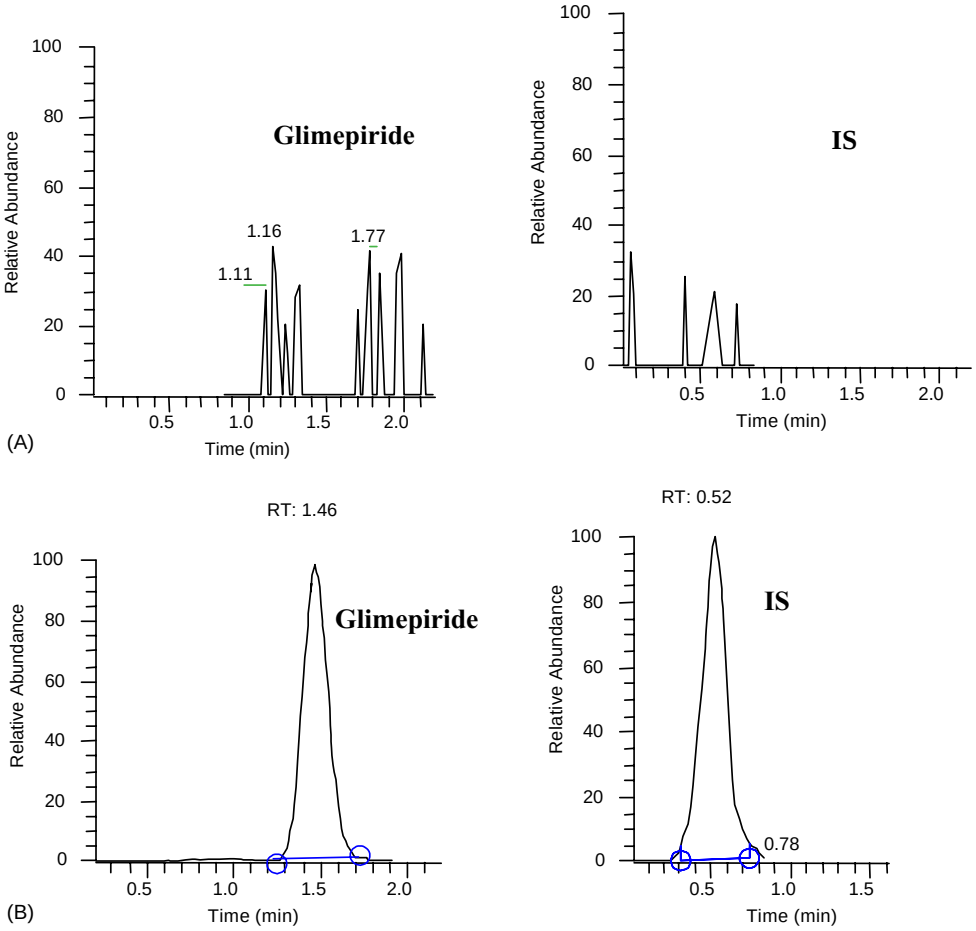

Fig. 3. LC–MS–MS chromatograms showing (A) a blank human plasma sample, (B) human plasma sample spiked with 5.0 ng/ml glimepiride and 200.0 ng/ml glibenclamide (internal standard).

concentration (apparent recovery) and the accuracy and precision for each point. The obtained results were within the acceptance criteria of no more than 20% deviation at LLOQ and no more than 15% deviation for standards above this point (LLOQ). The acceptance criteria for correlation coefficient was 0.998 or more, otherwise the calibration curve should be rejected.

The intra-day precision and accuracy of the assay was measured by analyzing five spiked samples of glimepiride at each QC level (15.0, 250.0 and 400.0 ng/ml). Intra-day

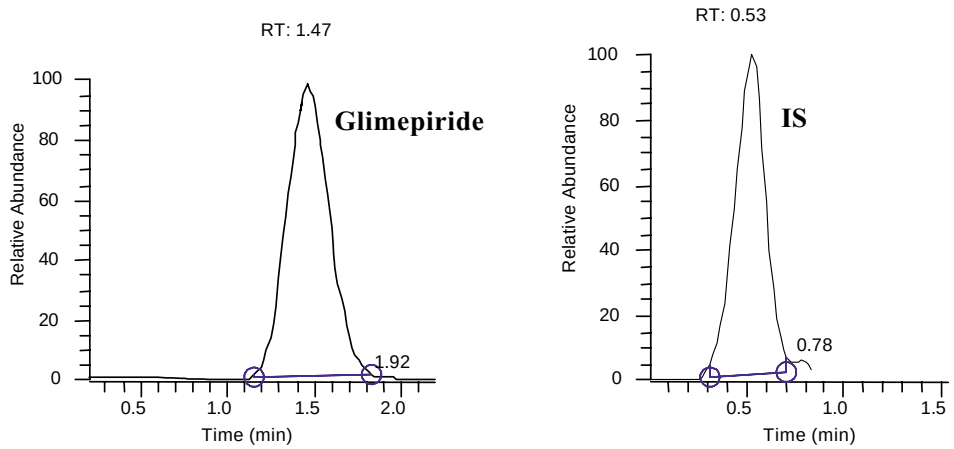

Fig. 4. LC–MS–MS chromatograms showing volunteer's plasma sample after the administration of an oral single dose of 3 mg tablets of glimepiride. The sample's concentration was 245 ng/ml.

Table 2
Inter-day accuracy, precision and relative error for glimepiride determination in human plasma samples

Analyzed on day	Glimepiride concentrations in human plasma		
	Low QC (15.0 ng/ml)	Medium QC (250.0 ng/ml)	High QC (400.0 ng/ml)
1	16.991	281.815	408.665
	12.974	238.324	374.735
	15.473	285.380	399.614
	16.616	215.058	454.547
	15.104	236.659	452.012
2	15.369	260.772	451.741
	15.104	242.820	449.664
	16.100	212.718	454.401
	15.969	287.019	407.555
	13.968	252.011	399.963
3	14.653	281.321	453.231
	15.265	245.365	450.111
	14.369	255.555	424.123
	15.111	256.963	399.969
	15.136	280.147	398.122
Mean	15.213	255.462	425.23
S.D.	1.008	24.311	27.949
Precision as CV (%)	6.626	9.516	6.573
Accuracy (%)	101.42	102.18	106.31
R.E. (%)	1.42	2.18	6.31

accuracy of the method for glimepiride ranged from 100.58 to 104.48%, while the intra-day precision ranged from 8.28 to 12.24% at the concentrations of 15.0, 250.0 and 400.0 ng/ml.

The inter-day precision and accuracy was determined over 3 days by analyzing 45 QC samples. Data for the inter-day precision and accuracy are presented in Table 2. These results were within the acceptance criteria for precision and accuracy which establish the deviation values should be within 15% of the actual values.

The absolute recoveries were evaluated for both glimepiride and IS by comparing peak areas of the extracted samples with the unextracted pure authentic standard solutions peak areas at three QC levels (15.0, 250.0 and 400.0 ng/ml). The absolute recovery determined for glimepiride was shown to be consistent, precise and reproducible. Results ranged from 86.36 (6.81) to 91.99% (2.83) at the three QC levels (15.0, 250.0 and 400.0 ng/ml). Absolute analytical recovery of internal standard (glibenclamide) was 85.47% (0.78).

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantitation. The lower limit of quantitation for glimepiride was proved to be 5.0 ng/ml, with 109.72% accuracy and 7.96% precision. Fig. 3B shows the chromatogram of an extracted plasma sample spiked with 5.0 ng/ml of glimepiride (LLOQ). The LLOQ met the following criteria: LLOQ response was more than five times the response of the blank and the LLOQ response was identifiable, discrete and reproducible with precision of 20% and accuracy of 80–120%.

Table 3
Data showing the stability glimepiride in human plasma during storage and samples handling

Stability	Low QC (15 ng/ml)	Medium QC (250 ng/ml)	High QC (400 ng/ml)
Short-term	99.97 (6.38)	104.26 (14.55)	103.4 (5.54)
Freeze and thaw	103.56 (10.28)	113.59 (2.58)	96.75 (7.02)
Long-term	93.72 (4.75)	103.29 (5.52)	94.65 (3.64)

Mean recovery percentage (CV%), $n = 5$.

The stability of the analytes in human plasma under different temperature and timing conditions, as well as the stability of the analytes in stock solution, was evaluated as follows.

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeded that expected to be encountered during the routine sample preparation (around 6 h). Samples were extracted and analyzed as above mentioned. Results are given below in Table 3. Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs.

The post-preparative stability of QC samples kept in the autosampler for 24 h, was also assessed. The mean recoveries of the low, mid and high QC levels were 97.06, 97.44 and 96.36%, respectively, whereas the precision (CV%) were 0.84, 3.21 and 8.50, respectively. The results indicate that glimperidie and internal standard can remain at the autosampler temperature for at least 24 h, without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The data that represent the stability of glimepiride plasma samples at three QC levels over three cycles of freeze and thawing are given in Table 3. The performed tests indicate that the analyte is stable in human plasma for three cycles of freeze and thaw, when stored at −20 °C and thawed to room temperature.

Table 3 summarizes also the long-term stability data of glimepiride in plasma samples stored for a period of eight weeks at −20 °C. The stability study of glimepiride in human plasma showed reliable stability behavior as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicated that storage of glimepiride's plasma samples at −20 °C is adequate, and no stability-related problems would be expected during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies.

The stability of stock solutions was tested and established at room temperature for 6 h. The recoveries for glimepiride and glibenclamide were 99.73 (CV 0.23%) and 97.40 (CV 1.88%) respectively. The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

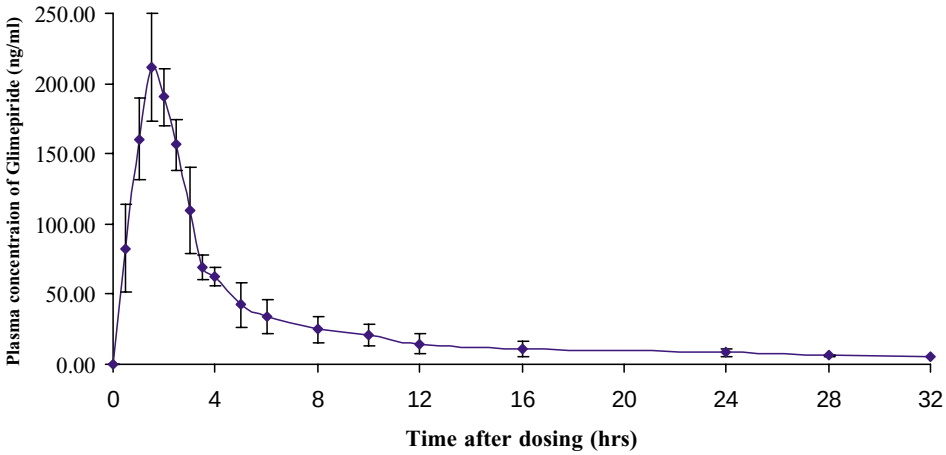

Fig. 5. Representative data showing mean plasma concentration–time profiles of three healthy volunteers after the administration of an oral single dose of 3 mg tablet of glimepiride. The error bars represents ± standard deviation.

4. Application

The method was applied to analyze plasma samples obtained from healthy volunteers after the administration of a single dose of 3.0 mg glimepiride tablet. The analyses were accomplished in accordance with the FDA bioanalytical method validation guidance [5]. The mean plasma concentration-time profile of three volunteers is represented in Fig. 5.

5. Conclusions

The combination of HPLC (under the isocratic conditions described) with ESI–MS–MS leads to very short retention times and yields both high selectivity and sensitivity. ESI is a "gentle" ionization technique that produces high mass-to-charge $[MH]^+$ precursor ions with minimal fragmentation of the analyte. No interference of the analyte was observed because acquisition was in the precursor ion selection mode followed by fragmentation.

It was shown that this method is specific and sensitive for the determination of glimepiride in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies.

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