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ORIGINAL RESEARCH

A rapid and highly sensitive method for the determination of glimepiride in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry: application to a pre-clinical pharmacokinetic study†

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ABSTRACT: A sensitive and specific liquid chromatography–positive electrospray ionization–tandem mass spectrometry method has been developed and validated for the determination of glimepiride (GPD) in human plasma. GPD and the internal standard (IS, glibenclamide) were extracted from a small aliquot of human plasma (200 μL) by a simple liquid–liquid extraction technique using ethyl acetate as extraction solvent. The compounds were separated on a YMC Propack, C_{18}, 4.6 × 50 mm column using a mixture of ammonium acetate buffer, acetonitrile and methanol (30:60:10, v/v) as mobile phase at 0.5 mL/min on an API 4000 Sciex mass spectrometer connected to an Agilent HPLC system. Method validation and pre-clinical sample analysis was performed as per FDA guidelines and the results met the acceptance criteria. GPD and IS were detected without any interference from human plasma matrix. The method was proved to be accurate and precise at linearity range of 0.02–100.00 ng/mL with a correlation coefficient of 0.999. The method was robust with a lower limit of quantitation of 0.02 ng/mL. Intra- and inter-day accuracies for GPD were 88.60–113.50 and 96.82–103.93%, respectively. The inter-day precision was better than 12.21%. This method enabled faster and reliable determination of GPD in a pre-clinical study. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: glimepiride; glibenclamide; LC-MS/MS; human plasma; method validation; rats; pharmacokinetics

INTRODUCTION

Glimepiride (GPD, Fig. 1, Amayrl®) is a potent oral sulfonylurea hypoglycemic agent with long duration of action. GPD is chemically, 3-ethyl-*N*,*N*-bis(3-ethyl-4-methyl-2-oxo-5H-pyrrol-2-yl)-4-methyl-2-oxo-5H-pyrrole-1-carboxamide. It is used in the management of type 2 diabetes (non-insulin dependent) by stimulating the body's natural insulin. GPD is extensively bound to plasma proteins (99.5%), metabolized by oxidative biotransformation and excreted mainly through urine (*Physician's Desk Reference*, 1998).

Several bioanalytical methods have been reported for estimation of GPD in biological samples; these include either high-performance liquid chromatography (HPLC) (Lehr and Damn, 1990; Malerczyk *et al.*, 1994; Pistos *et al.*, 2005a; Song *et al.*, 2004) or LC-MS/MS (Maurer *et al.*, 2002; Ho *et al.*, 2004; Kim *et al.*, 2004a,b; Salem *et al.*, 2004; Dotsikas *et al.*, 2005; Pistos *et al.*, 2005b).

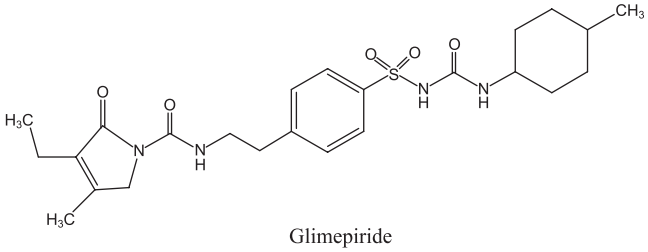

Figure 1. Structural representation of glimepiride and glibenclamide (IS).

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Abbreviations used: BSA, body surface area; DMSO, dimethylsulfoxide; GPD, glimepiride.

The reported HPLC methods are not ideal for pharmacokinetics because of high detection limits, time-consuming methods and long chromatographic run times. Although the reported LC-MS/MS methods are sensitive enough to characterize the human pharmacokinetic parameters following GPD oral administration (2 or 3 mg tablet), the reported lower limit of quantitation (LLOQ) for them was 0.1 ng/mL and hence was able to quantify the concentrations of GPD up to either 24 or 32 h only. Additionally, some of the reported LC-MS/MS methods require multi-step processing steps and/or need sophisticated instrumentation.

Herein we report a highly sensitive and robust LC-MS/MS method, which has overcome the drawbacks of the previously reported methods and enabled us to obtain a reproducible LLOQ of 0.02 ng/mL from 200 μL of human plasma. In order to accelerate the clinical sample analysis we optimized the extraction process to a simple and single-step procedure to enable faster sample processing. Attention was also given to reducing the run time by testing different analytical columns and mobile phase compositions, which helped us to achieve a total run time of 2.4 min with reproducible selectivity and specificity. The method developed by us offers a number of advantages over the existing methods, such as shorter analysis time, low plasma sample volume (200 μL), lack of extensive sample cleanup and the ability to analyze large numbers of samples.

EXPERIMENTAL

Chemicals and reagents. GPD with a purity of >99% was obtained from Unit II, Dr Reddy's Laboratories Limited (DRL), Hyderabad, India, and glibenclamide (EU standard, Fig. 1) was procured from the European Directorate for the Quality of Medicines (EDQM), Strasbourg. HPLC-grade acetonitrile and methanol were procured from Ranbaxy Fine Chemicals Ltd, New Delhi, India. Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Ammonium acetate was purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India. Ethyl acetate GR grade was purchased from Qualigens, Mumbai, India. All aqueous solutions including the buffer for the HPLC mobile phase were prepared with Milli Q (Millipore, USA) grade water. Control human plasma from healthy volunteers was obtained from Cauvery Diagnostics and Blood Bank, Secunderabad, India.

HPLC conditions. An Agilent high-performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany) 1200 series system equipped with degasser (G1322A) and pump (G1311A) along with auto sampler (G1367B) was used for the development and validation of the method. Chromatographic separation was carried out using a C_{18}, YMC Propack column, 5 μm, 4.6 × 50 mm (Waters, Milford, MA, USA). The mobile phase was composed of 0.01 M ammonium acetate buffer (not pH-adjusted):acetonitrile:methanol (30:60:10, v/v) and was used after degassing. Analysis was performed at ambient room temperature with a flow rate of 0.5 mL/min and a total run time of 2.4 min.

LC-MS/MS conditions. Tandem mass spectrometry (MS/MS) was performed with a triple quadrupole MDS Sciex (Foster City, CA, USA) API 4000 mass spectrometer equipped with a Turboionspray™ (ESI) source and the drug was monitored and quantified on Analyst 1.4.1 software. The source parameters, viz. curtain gas, nebulizer gas, auxiliary gas, collision gas (CAD), capillary temperature (TEM) and ion spray voltage, were set at 20, 40, 45, 5 and 500°C and 5.0 kV, respectively. The compound parameters, viz. declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP), for GPD and internal standard (IS) were 60, 20, 8, 10 and 60, 22, 8, 15, respectively. Detection of the ions was performed in multiple reaction monitoring (MRM) mode, monitoring the transition of the *m/z* 491.2 precursor ion to the *m/z* 352.2 product ion for GPD and *m/z* 494.2 precursor ion to the *m/z* 369.0 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution.

Preparation of stock solutions. Primary stock solutions of GPD were prepared from separate weightings. The primary stock solution was prepared in DMSO:methanol (2:3) and secondary stock solutions were prepared in 100% methanol and stored at −15°C. Working solutions of GPD were prepared in methanol, by appropriate dilution, at 0.2, 0.4, 1.0, 5.0, 10, 50, 100, 500 and 1000 ng/mL. The IS stock solution was prepared by dissolving 5.0 mg of glibenclamide in 5 mL methanol producing a concentration of 1 mg/mL. From this a working stock solution of 2 μg/mL was prepared to use as IS.

Calibration curves. Calibration curves were prepared daily by spiking 20 μL each of the corresponding standard solutions into 180 μL of blank plasma to produce calibration standards equivalent to 0.02, 0.04, 0.10, 0.50, 1.00, 5.00, 10.00, 50.00 and 100.00 ng/mL of GPD. Each sample also contained 100 ng/mL of IS. Zero plasma samples used in each run were prepared containing 100 ng/mL of IS only. In each run, a plasma blank, a zero standard (with IS) and a set of calibration standards were analyzed where the LLOQ and upper level of quantitation (ULOQ) were used in duplicate.

Quality control samples. Quality control (QC) samples were prepared at three levels, low level (three times the LLOQ), medium level (approximately 50% of the ULOQ) and a high level (80% of the ULOQ). QCs were prepared in bulk by spiking 20 μL of the corresponding standard solution in to 180 μL of blank plasma to produce a final concentration equivalent to 0.06, 40.00 and 80.00 ng/mL of GPD.

Sample preparation. QC, calibration curve and blank plasma samples were extracted using a liquid–liquid extraction technique. For the determination of GPD in plasma, a 200 μL aliquot of plasma sample in RIA vial was spiked with 10 μL of 2 μg/mL of IS, vortexed for 10 s and extracted with 2 mL of ethyl acetate by vortex mixing for 4 min on a Vibramax 100 (Heidolph, Germany). The contents were centrifuged at 3200 rpm for 4 min using a Multifuge (Heraeus Instruments, Germany) at 22 ± 2°C and 1.8 mL of clear supernatant was

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separated and evaporated to dryness at 50°C under nitrogen gas. Resultant residue was reconstituted with 200 µL of mobile phase, vortexed for 30 s and 20 µL was injected onto the analytical column.

Selectivity and specificity. The lack of chromatographic interference at retention times of GPD and IS from endogenous plasma components was investigated. Six different lots of human blank plasma samples and set of blanks spiked with LLOQ concentration were evaluated for selectivity and specificity.

Calibration curve. Calibration curves were acquired by plotting the peak area ratio of GPD:IS against the nominal concentration of calibration standards. The concentrations used were 0.02, 0.04, 0.10, 0.50, 1.00, 5.0, 10.0, 50.00 and 100.00 ng/mL. The results were fitted to linear regression analysis using $1/X^2$ as weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except at LLOQ, which was set at 20% (Shah *et al.*, 2001; *United States Pharmacopeia*, 1995).

Precision and accuracy. Analyzing six replicates at four different QC levels, i.e. 0.02, 0.06, 40.00 and 80.00 ng/mL, estimated the intra-day assay precision and accuracy. The inter-assay precision was determined by analyzing six replicates at four different QC levels on five different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within ±15% coefficient of variation (CV), except for LLOQ, where it should not exceed ±20% of CV (Shah *et al.*, 2000; *United States Pharmacopeia*, 1982).

Recovery and matrix effect. The recovery of GPD and IS through LLE procedure was determined by comparing the peak area of the extracted plasma samples at three QC levels (n = 4) to the peak area of neat standard in post extracted samples (Hubert *et al.*, 1994). The matrix effect was determined at three concentration levels (low, medium and high) by comparing peak areas of neat standard in post-extracted samples with those of other neat solution standards (methanol). Recoveries and matrix effects were determined at low, medium and high concentrations, viz. 0.06, 40.00 and 80.00 ng/mL, whereas the recovery of the IS was determined at a single concentration of 100 ng/mL.

Stability experiments. The stability of GPD and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 12 h (in the autosampler at 4°C) after the initial injection. The peak areas of the GPD and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of GPD in the biomatrix during 8 h (bench-top) was determined at ambient temperature (22 ± 2°C). Analyzing the QC samples stored at –80°C for at least 110 days assessed freezer stability of GPD in human plasma. The stability of GPD in human plasma following repeated freeze–thaw cycles was assessed using QC samples spiked with GPD. The samples were stored at –80°C between freeze–thaw cycles. The stability of GPD was assessed after three freeze–thaw cycles. All the stability experiments were carried out at low and high QC concentrations in six replicates. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% DEV) and precision (i.e. ±15% CV).

Pharmacokinetic study. The pharmacokinetic study was carried out in male Wistar rats. The animals were fasted overnight (~14 h) and had free access to water throughout the experimental period. GPD was administered by oral gavage at a dose of 100 µg/kg [calculated from 1 mg dose of human based on body surface area (BSA) calculation] as a suspension in 0.25% sodium carboxymethylcellulose. Animals were provided with standard diet 3 h post dose. The rats were anesthetized in ether and blood samples (~0.50 mL, sparse sampling protocol) were collected from retro-orbital plexus into microfuge tube (containing 10 µL of saturated EDTA) at 0.5, 1, 2, 3, 5, 10, 24, 48 and 72 h post-dose. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 12,800 rpm for 5 min. Rat plasma (200 µL) samples were spiked with IS and processed as described above and data was accepted based on the performance of QCs prepared using rat blank plasma (two QCs each at three concentration levels). Plasma concentration–time data of GPD was analyzed by non-compartmental method.

Pharmacokinetic analysis. Pharmacokinetic parameters were calculated by employing a non-compartmental analysis (Gibaldi and Perrier, 1982). The peak plasma concentration (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the plasma concentration–time curve up to the last quantifiable time point, $AUC_{(0-t)}$, was obtained by the linear and log–linear trapezoidal summation. The $AUC_{(0-t)}$ extrapolated to infinity [i.e. $AUC_{(0-\infty)}$] by adding the quotient of C_{last}/K_{el}, where C_{last} represents the last measurable time concentration and K_{el} represents the apparent terminal rate constant. K_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life ($t_{1/2}$) of the terminal elimination phase was obtained using the relationship $t_{1/2} = 0.693/K_{el}$.

RESULTS AND DISCUSSION

Specificity and selectivity

Figure 2 shows a typical chromatogram for the control human plasma (free of analyte and IS), human plasma spiked with GPD at LLOQ and IS and an *in vivo* plasma sample obtained 2 h after oral administration of GPD suspension to rats. No interfering peaks form endogenous compounds were observed at the retention times of analyte and IS. The retention time of GPD and IS were 1.82 and 1.70 min, respectively. The total chromatographic run time was 2.4 min, which was shorter than those reported in the literature (Maurer *et al.*, 2002; Ho *et al.*, 2004; Kim *et al.*, 2004a,b; Salem *et al.*, 2004; Dotsikas *et al.*, 2005; Pistos *et al.*, 2005).

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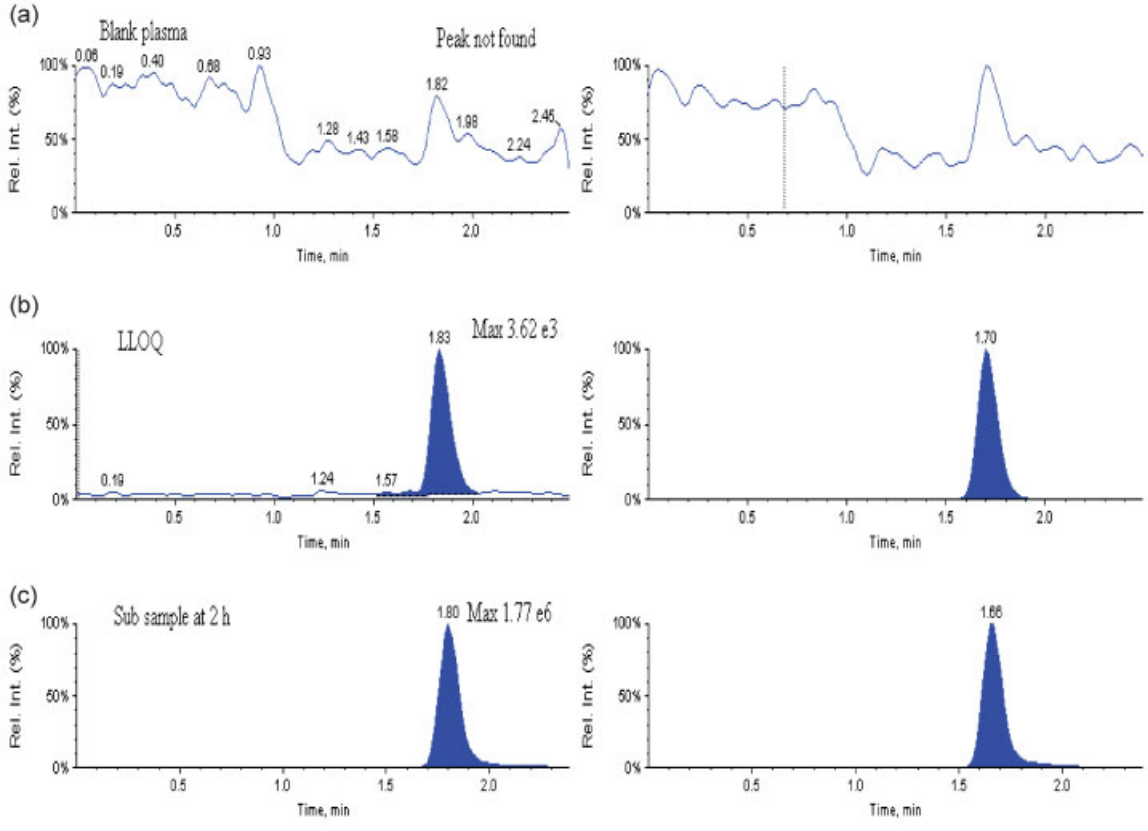

Figure 2. Typical MRM chromatograms of GPD (left panel) and IS (right panel) in (a) human blank plasma, (b) human plasma spiked with GPD at LLOQ (0.02 ng/mL) and IS, (c) a 2.0 h *in vivo* plasma sample showing GPD along with IS peak obtained following oral dosing of GPD to rats. This figure is available in colour online at www.interscience.wiley.com/journal/bmc

Calibration curve

The plasma calibration curve was constructed using eight calibrators, viz. 0.02–100 ng/mL. The standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to $y = mx + c$ using a weighting factor ($1/X^2$). The average regression ($n = 5$) was found to be ≥0.999. The lowest concentration with the CV <20% was taken as the LLOQ and was found to be 0.02 ng/mL. The percentage accuracy observed for the mean of back-calculated concentration for five linearities was within 94.08–110.25, while the precision (% CV) values ranged from 1.06 to 9.01.

Accuracy and precision

Table 1 summarizes the accuracy and precision data for intra and inter-day plasma test samples for GPD.

Recovery and matrix effect

The results of the comparison of extracted plasma samples to the neat standard in post extracted samples were estimated for GPD at 0.06, 40.00 and 80.00 ng/mL and the absolute mean recoveries were 87.32, 83.71 and 84.02%, respectively, across the concentrations. The absolute recovery of IS at 100 ng/mL was 98.91%. The matrix effect was calculated by comparing peak areas of neat standard in post-extracted samples with those of other neat solution standards (methanol). Accuracy values in the matrix effect experiment were 109.16, 108.89 and 111.60% for QCs at concentration levels of 0.06, 40.00 and 80.00 ng/mL for GPD, and 112.33% for IS at 100.00 ng/mL, indicating no impact of the matrix effect on the validated analytical method.

Stability

Auto-sampler and bench top stability. Over a period of 12 h injection time in the auto sampler at 4°C temperature and over the bench-top for an 8 h period, the predicted concentrations for GPD at 0.06 and 80.00 ng/mL, samples deviated within the acceptable limits from the nominal concentrations (Table 2).

Freeze–thaw stability. Table 2 also shows the results of the analyses of the QC samples following three repeated freeze–thaw cycles. GPD was shown to be stable in the frozen plasma at −80°C for at least three freeze–thaw cycles.

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Table 1. Intra- and inter-day precisions data of GPD quality controls in human plasma

Theoretical concentration (ng/mL)	Run	Measured concentration (ng/mL) Mean	SD	%CV	Accuracy (%)[a]
Intra-day variation (6 replicates at each concentration)					
0.02	1	0.022	0.002	7.51	109.02
	2	0.019	0.003	14.05	97.53
	3	0.018	0.001	7.25	88.60
	4	0.021	0.003	12.83	104.15
	5	0.021	0.003	12.07	103.60
0.06	1	0.068	0.000	0.47	113.50
	2	0.054	0.002	3.63	94.62
	3	0.054	0.002	4.26	89.75
	4	0.067	0.002	2.29	112.20
	5	0.064	0.004	6.81	106.22
40.0	1	39.883	2.199	5.52	99.82
	2	37.820	3.370	8.91	94.64
	3	38.000	1.328	3.50	94.97
	4	36.633	1.331	3.63	91.60
	5	41.917	1.199	2.86	105.00
80.0	1	80.333	3.643	4.54	100.63
	2	74.267	3.907	5.26	92.82
	3	73.967	1.808	2.45	92.45
	4	74.960	4.862	6.49	93.66
	5	85.217	2.827	3.32	106.50
Inter-day variation (30 replicates at each concentration)					
0.02		0.020	0.002	12.21	101.10
0.06		0.062	0.007	10.86	103.93
40.0		38.886	2.648	6.811	97.22
80.0		77.430	5.917	7.641	96.82

%CV = (SD/mean × 100).
[a] (Mean assayed concentration/theoretical concentration) × 100.

Table 2. Stability data of GPD quality controls in human plasma

QC (spiked) concentration (ng/mL)	Stability	Mean ± SD[a] (ng/mL), $n = 6$	Accuracy (%)[b]	Precision (%CV)
0.06	0 h (for all)	0.068 ± 0.000		
	three freeze–thaw cycles	0.068 ± 0.002	100.00	2.62
	8 h (bench top)	0.065 ± 0.003	95.58	4.82
	12 h (in-injector)	0.067 ± 0.002	98.53	6.03
	110 days at –80°C	0.061 ± 0.004	89.71	6.48
80.0	0 h (for all)	80.333 ± 3.643		
	three freeze–thaw cycles	84.117 ± 4.250	104.71	5.05
	8 h (bench top)	75.050 ± 2.215	93.42	2.95
	12 h (in-injector)	83.567 ± 3.782	104.03	4.53
	110 days at –80°C	78.127 ± 4.586	97.25	5.87

QC, quality control; %CV, coefficient of variation.
[a] Back-calculated plasma concentrations.
[b] (Mean assayed concentration/mean assayed concentration at 0 h) × 100.

Plasma long-term stability. GPD was found to be stable when stored at –80°C for at least 110 days. Both accuracy and precision of QC samples in this evaluation were within the assay variability limits (Table 2).

Application of the method. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of GPD in rats at a dose of 100 μg/kg. Profiles of the mean plasma concentration versus time were shown in Fig. 3. The QCs also met the acceptance criteria as shown in Table 3. Maximum concentration in plasma (C_{max} 50.45 ± 13.32 ng/mL) was achieved at 1.75 ± 0.96 h (T_{max}). The half-life ($t_{1/2}$) of GPD was 9.12 ± 1.36 h, while the $AUC_{(0-\infty)}$ was 271.44 ± 92.12 ng h/mL. The current method could quantify the plasma concentrations of GPD in rats at 100 μg/kg dose up to 72 h post-dose, whereas all the reported methods (Maurer *et al.*, 2002; Ho *et al.*, 2004; Kim *et al.*, 2004a,b; Salem *et al.*, 2004; Dotsikas *et al.*, 2005; Pistos *et al.*, 2005b) could quantify concentrations

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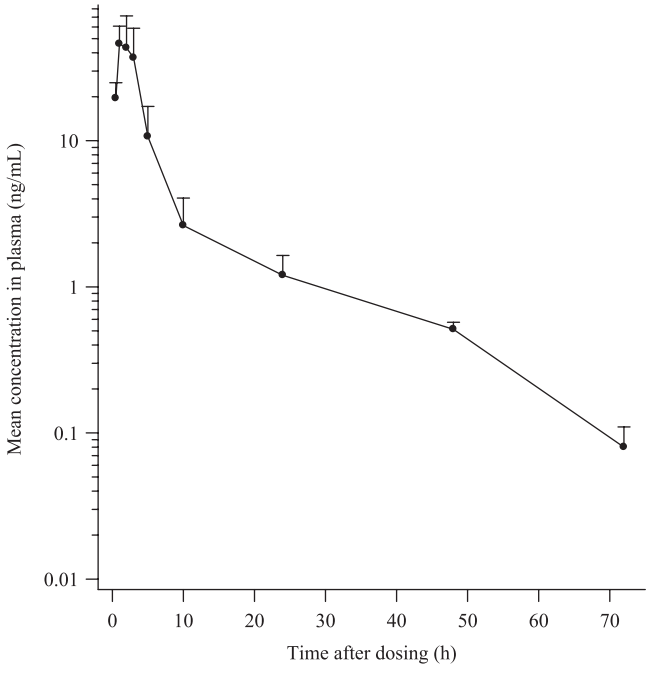

Figure 3. Mean plasma concentration–time profile of GPD in rats following an oral dose of 100 μg/kg.

Table 3. GPD quality controls in rat plasma

Theoretical concentration (ng/mL)	Calculated concentration (ng/mL)	Percentage accuracy
0.06	0.07	113
	0.06	92.8
40.0	43.9	110
	32.1	88.3
80.0	82.5	103
	82.4	102

of GPD up to either 24 or 32 h following 2 or 3 mg tablet oral administration to humans.

CONCLUSIONS

In summary, we have developed and validated a highly sensitive, specific, reproducible and high-throughput LC-MS/MS assay to quantify GPD using commercially available IS from small volumes of human plasma (200 μL). To the best of our knowledge, the present method offers the highest sensitivity (0.02 ng/mL) compared with other methods described in the literature. This developed LC-MS/MS method can be successfully used for pharmacokinetic, bioequivalence or bioavailability and drug–drug interaction studies.

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