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Determination of glimepiride in human plasma using semi-microbore high performance liquid chromatography with column-switching

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Abstract

A fully automated semi-microbore high performance liquid chromatographic (HPLC) method with column-switching using UV detection was developed for the determination of glimepiride from human plasma samples. Plasma sample (900 μl) was deproteinated and extracted with ethanol and acetonitrile. The extract (70 μl) was directly injected into a Capcell Pak MF Ph-1 pre-column where the primary separation occurred to remove proteins and retain drugs using a mixture of acetonitrile and 10 mM phosphate buffer (pH 2.18) (20:80, v/v). The analytes were transferred from the pre-column to an intermediate column using a switching valve and then subsequently separated on an analytical column and monitored with UV detection at 228 nm. Glimepiride was eluted with retention time 34.9 min without interference of endogenous substance from plasma. The limit of quantification (LOQ) was 10 ng/ml for glimepiride. The calibration curves were linear over the concentration range of 10–400 ng/ml (r^2 = 0.9997). Moreover, inter- and intra-day precisions of the method were less than 15% and accuracies were higher than 99%. The developed method was successfully applied for the quantification of glimepiride in human plasma and was used to support a human pharmacokinetic study following a single oral administration of 2 mg glimepiride.

Keywords: Glimepiride; Column-switching; Bioavailability

1. Introduction

Glimepiride, 1-[[*p*-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]-phenyl]-sulfonyl]-3-(trans-4-methylcyclohexyl)urea (Fig. 1a), is a new oral antidiabetic drug in the sulfonylurea class, with the advantage of being completely bioavailable [1], being effective at low doses in patients with non-insulin-dependent diabetes mellitus, showing linear pharmacokinetics [2] and having a prolonged effect [3]. As with the other sulphonylureas, glimepiride appears to lower blood glucose levels by stimulating insulin release from the pancreas [4]. In order to achieve appropriate control of blood glucose level, the treatment of non-insulin development Type II diabetes usually starts with diet and exercise. If this still results in insufficient metabolic control, oral hypoglycemic drugs or insulin are added to the non-pharmacological measures [5]. Glimepiride achieved metabolic control with the lowest dose (1–8 mg daily) of all the sulphonylureas. In addition, it maintains a more physiological regulation of insulin secretion than glibenclamide during physical exercise, suggesting that there may be less risk of hypoglycemia with glimepiride [6,7].

To the best of our knowledge, a few chromatographic techniques have been reported on glimepiride determination in biological fluids [8–10] and pharmaceutical dosage form [11,12]. High performance liquid chromatography (HPLC) methods to determine related sulphonylureas have been explored to quantify glimepiride in human biological fluids and

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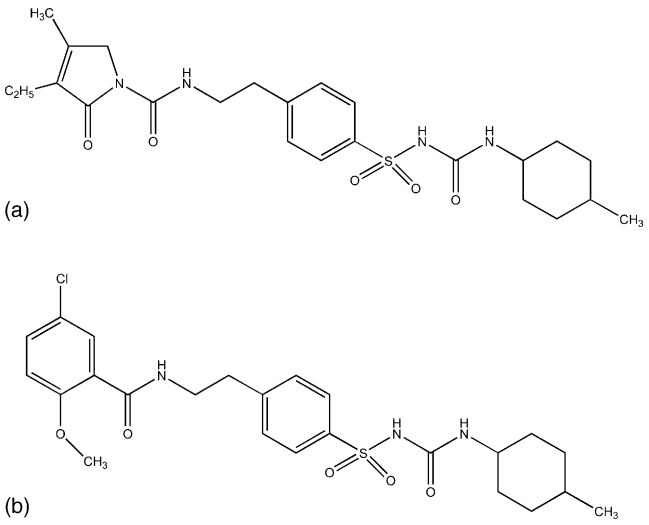

Fig. 1. Chemical structures of (a) glimepiride and (b) glibenclamide.

pharmaceutical dosage form. However, the shortcoming of these methods seemed to be unreliable with regard to sensitivity and specificity, especially in the ng/ml range. Moreover, the HPLC-UV method was used to quantify glimepiride in human serum after pre-column derivatization with 2,4-dinitrophenylamine [8], which method is time-consuming due to the derivatization procedures. An APCI-LC–MS procedure has advantages of fast screening, reliable identification and fully calibrated quantification of glimepiride [9]. However, this method has low sensitivity to detect glimepiride in human plasma after an oral administration. HPLC tandem MS (MS–MS) procedure further enhances specificity and provides an improved signal-to-noise ratio compared with single-stage MS. Additionally, though the ion trap mass spectrometer enables MS–MS at an affordable price compared with a triple-stage quadrupole MS system [10], this method is more sophisticated and complicated compared with HPLC-UV method which is commonly used, and the apparatuses are much more expensive than HPLC-UV systems.

The purpose of this study was to develop a sensitive, convenient and accurate semi-microbore HPLC method using column-switching for the determination of glimepiride in human plasma. The utility of this methodology was demonstrated by its application to a human pharmacokinetic study of glimepiride.

2. Experimental

2.1. Materials

Glimepiride and glibenclamide (internal standard, I.S.) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were analytical grade and used without further purification.

2.2. Preparation of standards

Stock solutions of glimepiride (200 μg/ml) were made by dissolving in a mixture of acetonitrile and water (80:20, v/v) and stored at −20 °C. Standard solutions of glimepiride in human plasma were prepared by spiking the appropriate volume (less than 10 μl per ml) of various diluted stock solutions giving trial concentrations of 10, 50, 200 and 400 ng/ml. Internal standard, glibenclamide (Fig. 1b), a structurally related analog of glimepiride, was dissolved in acetonitrile to make a stock solution at a final concentration of 100 ng/ml.

2.3. Preparation of samples

To remove plasma protein, 2 ml of ethanol was added to each 0.9 ml of plasma samples in a glass tube and the mixture was vortexed for 30 s. Then, 2 ml of acetonitrile containing 100 ng/ml of glibenclamide as internal standard was added and vortexed for 10 min. The tubes were centrifuged at 1000 × *g* for 20 min and clear supernatants were transferred into another glass tubes after filtering through a 0.2 μm reg cellulose syringe filter (National Science, Seoul, Korea). The supernatants were evaporated under nitrogen at 40–45 °C and the residues were reconstituted in 150 μl of mobile phase. Then, the samples were filtered through a 0.2 μm reg cellulose syringe filter (National Science). Seventy μl of filtered aliquot was injected into the HPLC system for analysis with an autosampler.

2.4. Column-switching system and HPLC condition

The configuration of the column-switching system (Fig. 2) using three columns consisted of the Nanospace SI-1 se-

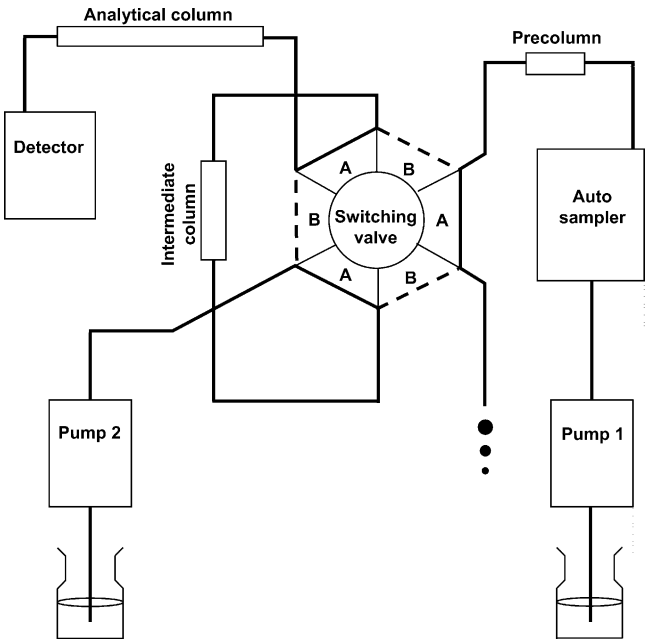

Fig. 2. Schematic diagram of a column-switching system. Solid line, position A; dashed line, position B.

ries (Shiseido, Tokyo, Japan), i.e. two 2001 pumps, a 2002 UV–vis detector, a 2003 autosampler, a 2004 column oven, a 2012 high pressure switching valve and a 2009 degassing unit. The signals were processed by dsChrom98 (Donam, Seoul, Korea).

In order to remove proteins and concentrate glimepiride and glibenclamide from plasma samples, a large volume of plasma was pre-separated on Capcell Pak MF Ph-1 column (10 mm × 4.0 mm i.d., Shiseido) using a washing solvent at a flow-rate of 0.5 ml/min. A washing solvent was used as a mixture of acetonitrile and 10 mM potassium phosphate buffer containing 0.04% of triethylamine (20:80, v/v, pH 2.18). The drug molecule fractions from primary separation were transferred to an intermediate column (Capcell Pak C_{18} UG 120 U, 35 mm × 2.0 mm i.d., Shiseido) and the final separation was performed on Capcell Pak MG C_{18} column (5 μm, 250 mm × 1.5 mm i.d., Shiseido) using a mobile phase at a flow-rate of 0.1 ml/min. A mixture of acetonitrile and 10 mM potassium phosphate buffer containing 0.04% of triethylamine (52:48, v/v, pH 2.18) was used as a mobile phase. The column was maintained at 30 °C and the eluent was monitored at 228 nm.

2.5. Column-switching procedure

Step 1 (0.0–7.1 min, valve position A): Plasma sample (70 μl) was introduced onto a Capcell Pak MF Ph-1 column where plasma proteins, glimepiride and glibenclamide were separated using the washing solvent at a flow-rate of 0.5 ml/min. The intermediate column and analytical column were equilibrated using the mobile phase.

Step 2 (7.1–13.1 min, valve position B): When the valve status was changed to B, target drug-containing zone separated in Capcell Pak MF Ph-1 pre-column was focused onto the top of an intermediate C_{18} column using the washing solvent at a flow-rate of 0.5 ml/min. The analytical column was equilibrated using the mobile phase.

Step 3 (13.1–40.0 min, valve position A): The analytes trapped in the intermediate C_{18} column were transferred to a Capcell Pak MG C_{18} column and separated by using 0.1 ml/min of mobile phase when the valve status was switched back to the A position. In the meanwhile, the MF Ph-1 column was equilibrated with the washing solvent.

2.6. Validation of the method

Evaluation of the developed HPLC method with column-switching was based on proportionality (linearity assay), precision and accuracy.

2.6.1. Specificity

Drug-free blank human serum was tested for interference using the proposed HPLC column-switching method, and the result was compared with those obtained from glimepiride and the internal standard.

2.6.2. Linearity

The calibration curve consisted of the four concentrations; 10, 50, 200 and 400 ng/ml for glimepiride. The calibration curves were obtained by linear regression; the ratio of glimepiride peak area to internal standard peak area was plotted versus glimepiride concentration in ng/ml

2.6.3. Precision and accuracy

The intra- and inter-day precision (coefficients of variation, CV%) and inter-day accuracy (bias%) of the assay procedure were determined by the analysis of five samples at each concentration level in the same day and one sample at each concentrations in five different days, respectively.

2.6.4. Sensitivity

The limit of quantification (LOQ) was defined as the lowest concentration at which the precision expressed by CV% was lower than 20%, the accauracy expressed by bias% was within 80–120% and ratio of signal to noise was better than 10.

2.6.5. Robustness

The robustness of a method is its ability to remain unaffected by small deliberate variations in the method parameters [13]. The robustness of this HPLC method with column-switching was determined by analysis of samples under various conditions such as the column length of the pre-column (10–150 mm) and analytical column (150 and 250 mm), pH (2.0–2.3) of the washing solvent and mobile phase, the percentage of acetonitrile in the washing solvent (5–25%) and mobile phase (40–80%) and the injection volume (50–100 μl). The effects on retention time and peak parameters were investigated.

2.6.6. Matrix effect

To show signal suppression caused by matrix components, matrix effects were determined by comparing the peak area of the analytes at the concentrations of 10, 200 and 400 ng/ml in the samples after protein precipitation to that of each analyte obtained in neat solutions.

2.6.7. Stability

Stability of glimepiride and internal standard in human plasma was assessed by placing standard solutions containing 10, 200 and 400 ng/ml concentration of glimepiride under ambient conditions for 24 h. The freeze-thaw stability of glimepiride and internal standard was also assessed by analyzing standard solutions containing 10, 200 and 400 ng/ml concentration of glimepiride undergoing three freeze (−70 °C)-thaw (room temperature) cycles versus regularly treated standard solutions. The stability of glimepiride and internal standard in protein-precipitated standard solutions containing 10, 200 and 400 ng/ml concentration of glimepiride was analyzed by storing processed samples in the autosampler of the chromatographic system at 20 °C for a period of up to 14 h.

2.7. Bioavailability study of glimepiride in human

The validated method was applied to evaluate the bioavailability of glimepiride. Seven healthy male volunteers were selected for this study according to medical history, physical examination and standard laboratory test results (blood cell count, biochemical profile and urinalysis). The demographic data of these volunteers were mean age 24 years, mean height 176.5 cm and mean weight 67.9 kg.

After an overnight fast, a catheter was introduced in a forearm vein and a pre-dosing blood sample was collected. Each volunteer was then orally administered one tablet of glimepiride 2 mg, namely Amaryl® (Handok-Aventis, Seoul, Korea) with 240 ml of water. The volunteers continued to fast for 4 h and a standard lunch was served. Heparinized venous blood samples (8 ml) were withdrawn from the forearm vein before administration and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 10 h postdosing, transferred to vacutainer tubes and centrifuged at 2000 × *g* for 20 min. After centrifugation, plasma samples were stored at −70 °C prior to analysis. The pharmacokinetic parameters were calculated by bioavailability analytical program, BA Calc 2002 provided by College of Pharmacy in Seoul National University [14].

3. Results and discussion

3.1. Column-switching procedure

In the present study, semi-microbore HPLC was used for the determination of glimepiride in plasma samples. The method has many advantages such as increased sensitivity, high resolution, economic and environmental aspects. To establish a column-switching system for the simultaneous determination of glimepiride and internal standard (glibenclamide) in plasma, the pre-column packing material, washing solvent and valve-switching time must be chosen in order to obtain complete recovery and remove interference components [15–17].

The Capcell pak MF column is one of the restricted access materials (RAM). It is not an internal-surface reversed-phase (ISRP) column, but a mixed functional phase column [18]. Capcell Pak MF Ph-1 column, used as the pre-column possesses long hydrophilic polyoxyethylene chains and hydrophobic phenyl groups on the surface of 80 Å silica in order to limit the access of large molecules such as proteins and retain glimepiride and glibenclamide longer. Therefore, the column minimizes undesirable secondary interactions of plasma proteins with the silica surface [19–24]. The separation of glimepiride and glibenclamide in plasma on the pre-column was evaluated using washing solvent to obtain the good recovery and to determine the appropriate time for column-switching. Glimepiride and glibenclamide were retained in the pre-column during the exclusion of the plasma proteins which was seen at the position of void volume. The peak of glimepiride and glibenclamide appeared from 7.1 to 9.3 min and 10.3 to 13.1 min, respectively. Therefore, the eluate of pre-column from 7.1 to 13.1 min after injection of plasma sample was transferred to the intermediate C_{18} column by switching the valve to B position.

The analytes isolated from MF Ph-1 by the valve-switching step to B position were transferred to the top of intermediate C_{18} column (35 mm × 2.0 mm i.d., Shiseido) to obtain sharp peaks in the final separation, protect a main column from high pressure and save the analysis time. Without an intermediate column, it might take more time to transfer glimepiride and internal standard fraction from the pre-column to analytical column at a flow-rate of 0.1 ml/min [19–23]. Since the analytical column's diameter was 1.5 mm, the flow-rate was reduced to 0.1 ml/min during analysis [21].

Capcell Pak MF Ph-1 pre-column, intermediate C_{18} column and MG C_{18} column showed no decrease in efficiency after more than 300 injections of plasma samples (equivalent to about 20 ml plasma).

3.2. Method validation

3.2.1. Specificity

Drug-free human plasma was screened and no endogenous interference was detected at the retention time of glimepiride (34.9 min) and internal standard (32.2 min). Fig. 3 shows a chromatogram of blank human plasma (a) and a representative chromatogram of a processed plasma sample containing 200 ng/ml of glimepiride and 100 ng/ml of internal strandard (b).

3.2.2. Linearity

The calibration curves were linear in the validated range. The mean equation of the calibration curve including four points was $y = 0.0054 \pm 0.0005x - 0.0351 \pm 0.0075$ with correlation coefficient as $r^2 = 0.9997 \pm 0.0005$, where y represents the ratio of glimepiride peak area and the internal standard one and x represents the glimepiride concentration in ng/ml.

3.2.3. Precision and accuracy

The intra- and inter-day precision and accuracy results are shown in Table 1. The values obtained were lower than the limits required for biological samples; the precision and accuracy of LOQ (10 ng/ml) were less than 20% and those of the other concentrations were less than 15%.

Table 1
Reproducibility of glimepiride determination in human plasma (n = 5)

Glimepiride concentration (ng/ml)	Precision (CV%)		Accuracy
	Intra-day	Inter-day	
10 (LOQ)	12.9	7.9	109.5
50	12.0	6.1	100.0
200	10.4	10.5	99.1
400	9.3	8.7	99.9

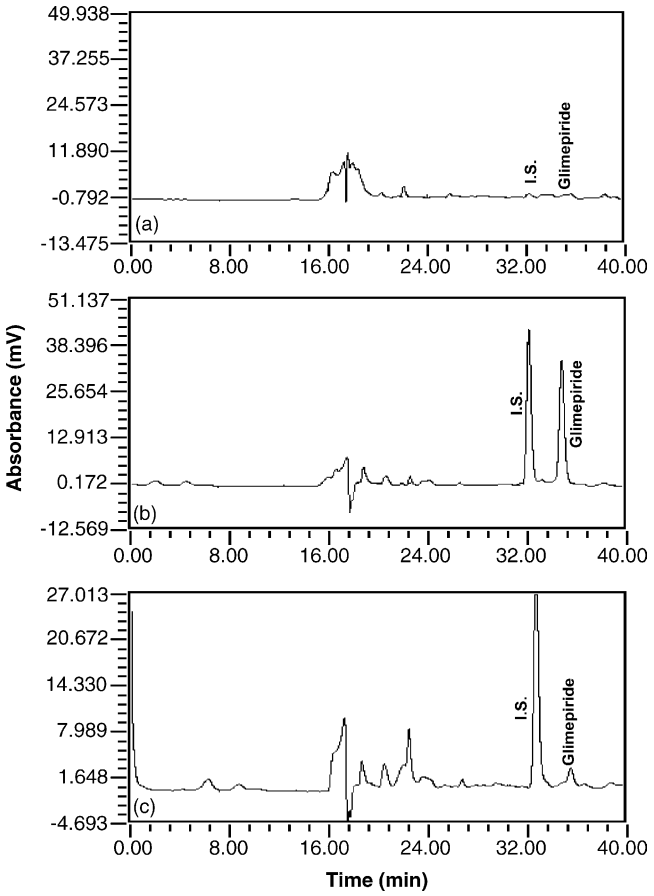

Fig. 3. Chromatograms of (a) blank plasma, (b) blank plasma spiked with glimepiride (200 ng/ml) and glibenclamide as internal standard (I.S.) (100 ng/ml) and (c) blank plasma spiked with glimepiride (10 ng/ml) and glibenclamide as internal standard (I.S.) (100 ng/ml).

3.2.4. Sensitivity

The LOQ of glimepiride was estimated to be 10 ng/ml as shown in Table 1. The LOQ was a same value obtained by HPLC method after pre-column derivatization with a simple process [8]. This method was sufficiently sensitive, with a quantification limit lower than the minimum concentration recommended for plasma samples obtained after the administration of 2 mg glimepiride. The sensitivity of glimepiride is shown in Fig. 3c.

3.2.5. Robustness

The method was found to be robust when the column and the mobile phase were varied. The separation of all the compounds was possible within washing solvent and mobile phase at a pH range of 2.05–2.2. A pre-column packed with the same stationary phase but with different length of 10–150 mm (Capcell Pak MF Ph-1 column, 4.0 mm i.d., Shiseido) was investigated at a flow-rate 0.5 ml/min. The retention times for glimepiride and internal standard were shortened by reducing pre-column length and increasing the percentage of acetonitrile in the washing solvent. However, using more than 25% of acetonitrile in the washing solvent, the analytes could not remain in the intermediate column during the column-switching time. As shown in Table 2, variation of the percentage of acetonitrile in the mobile phase at a range of 50–80% significantly affects, as expected, the elution behavior. Increasing the proportion of organic solvent in the mobile phase resulted in a decrease of retention for all analytes. Adjusting the percentage of acetonitrile to 52% and pH to 2.08 was associated by enhancement of the peaks symmetry, improvement of the resolution of the analytes and shortening of the retention time

Table 2
Influence of the percentage of acetonitrile in the mobile phase on the retention times of the analytes

Acetonitrile (%)	Retention time (min)	
	Glimepiride	I.S.
50	42.3	38.3
52	34.9	32.2
55	30.2	28.5
60	26.6	25.4
80	17.5	17.3

3.2.6. Matrix effect

As shown in Table 3, little matrix effect was observed when samples were deproteinized by this method.

3.2.7. Stability

Freezing and thawing did not reveal any detrimental effect on the absolute concentrations of analyte spiked to human plasma and investigated at 10, 200 and 400 ng/ml. After completion of three freezing and thawing cycles, the measured concentrations of glimepiride still ranged between 89.8 and 96.3%.

Stability was also confirmed by storing processed plasma samples in the autosampler of the chromatographic system for a period of up to 14 h at approximately 20 °C. Over the entire timeframe observed concentrations of glimepiride ranged between 86.1 and 101.8% without any obvious trend of concentration changes over time.

Thus, these stability data clearly suggest that human plasma samples containing glimepiride could be handled under normal laboratory conditions without significant loss.

3.3. Application to the bioavailability of glimepiride

The present method was applied to determine glimepiride in human plasma after a single oral administration of Amaryl® tablet (2 mg, glimepiride). Plasma chromatograms

Table 3
Assessment of matrix effect (n = 3)

Glimepiride (ng/ml)	Peak area ratio (%) (protein precipitation/neat × 100)
10	95.1 ± 16.6
200	96.5 ± 12.4
400	100.1 ± 6.8

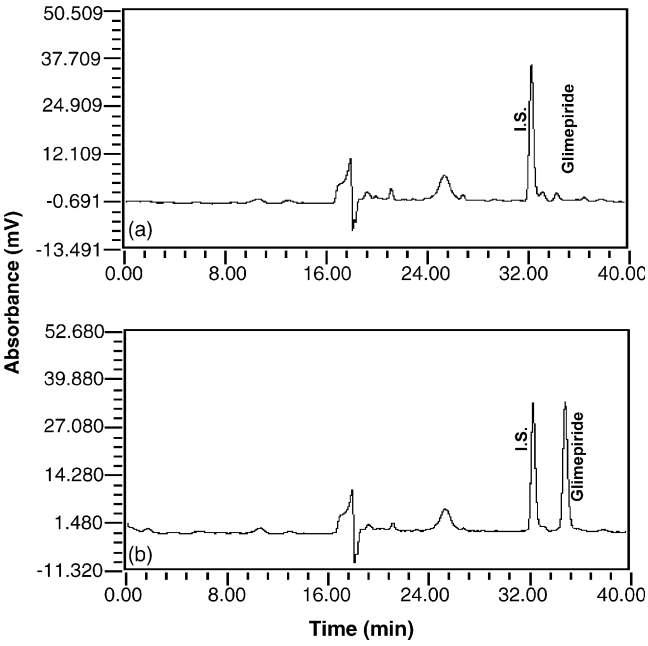

Fig. 4. Chromatograms of (a) human blank plasma and (b) plasma sample from a human subject at 2.5 h after an oral administration of 2 mg glimepiride.

of a volunteer administered glimepiride are shown in Fig. 4, which shows typical chromatograms of glimepiride in plasma at 0 h (a) and 2.5 h (b) after oral administration of 2 mg glimepiride to seven healthy volunteers. The plasma concentration of glimepiride at 2.5 h was 219.0 ng/ml (Fig. 4b).

Fig. 5 shows the mean plasma concentration–time profiles of glimepiride following an oral administration (2 mg)

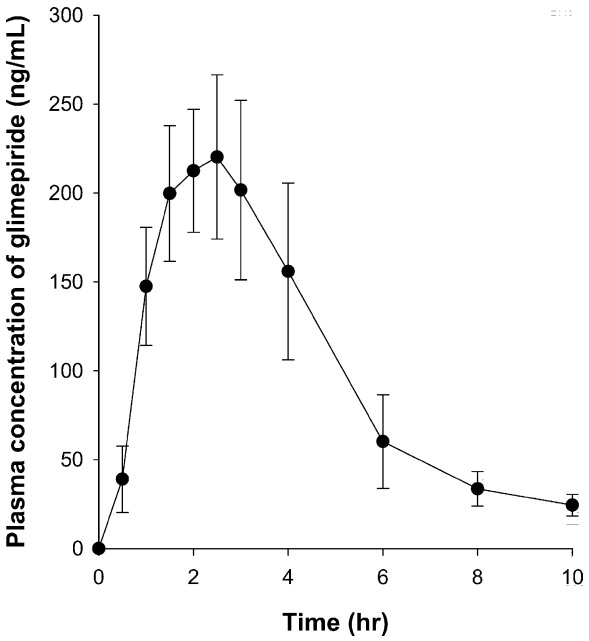

Fig. 5. Mean plasma concentration of glimepiride in human subjects after oral administration of 2 mg glimepiride. The results represent the mean ± S.D. (n = 7).

Table 4
Pharmacokinetic parameters of glimepiride in plasma of seven healthy subjects after an oral administration of 2 mg glimepiride

Parameter	Mean ± S.D.
C_{max} (ng/ml)	232.8 ± 28.4
T_{max} (h)	2.14 ± 0.38
$AUC_{0–10\,h}$ (ng·h/ml)	985.7 ± 217.5
CL (ml/min)	32.8 ± 7.9
$T_{1/2}$ (h)	2.16 ± 0.21

to human subjects. The C_{max} of glimepiride was reached at 2.14 ± 0.38 h as 232.8 ± 28.4 ng/ml after administration. The area under the curve from 0 to 10 h ($AUC_{0–10\,h}$) was 985.7 ± 217.5 ng·h/ml. The pharmacokinetic parameters of glimepiride are shown in Table 4. These values are comparable to the corresponding parameters obtained by single oral dose of 2 mg glimepiride in the previous reports [25,26].

From the results, it is suggested that the present column-switching semi-microbore HPLC analysis can be applied to the routine determination of glimepiride in biological fluid.

4. Conclusion

We have established a sensitive and specific automated analytical method for the determination of glimepiride in human plasma using semi-microbore HPLC technique with column-switching. The method shows satisfactory reproducibility, specificity and stability. Practical testing of the developed assay on samples from subjects also showed adequate sensitivity (10 ng/ml) for clinical studies compared with previous reports. This analytical method has been successfully used to evaluate the bioavailability of glimepiride in healthy volunteers.

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