Determination of Glimepiride in Human Plasma by Liquid Chromatography–Electrospray Ionization Tandem Mass Spectrometry

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Received March 12, 2003

A sensitive method for quantitation of glimepiride in human plasma has been established using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). Glipizide was used as an internal standard. Glimepiride and internal standard in plasma sample was extracted using diethyl ether-ethyl acetate (1 : 1). A centrifuged upper layer was then evaporated and reconstituted with the mobile phase of acetonitrile-5 mM ammonium acetate (60:40, pH 3.0). The reconstituted samples were injected into a C_{18} reversed-phase column. Using MS/MS in the multiple reaction monitoring (MRM) mode, glimepiride and glipizide were detected without severe interference from human plasma matrix. Glimepiride produced a protonated precursor ion ($[M+H]^+$) at m/z 491 and a corresponding product ion at m/z 352. And the internal standard produced a protonated precursor ion ($[M+H]^+$) at m/z 446 and a corresponding product ion at m/z 321. Detection of glimepiride in human plasma by the LC-ESI/MS/MS method was accurate and precise with a quantitation limit of 0.1 ng/mL. The validation, reproducibility, stability, and recovery of the method were evaluated. The method has been successfully applied to pharmacokinetic studies of glimepiride in human plasma.

Key Words : Glimepiride, Glipizide, Liquid-liquid extraction, Liquid chromatography, Tandem mass spectrometry

Introduction

The sulfonylurea glimepiride (1-[[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl)urea) is widely used in the treatment of non-insulin-dependent Type II diabetes mellitus. Glimepiride is almost completely bioavailable from the gastrointestinal tract and achieves metabolic control with the lowest dose (1-8 mg daily). In addition, it maintains a better physiological regulation of insulin secretion than glibenclamide during physical exercise, suggesting that there may be a risk of hyperglycemia with glimepiride.[1-3] Figure 1 shows the structures of glimepiride and glipizide (internal standard).

Several different methods have been reported for qualitative and quantitative analysis of glimepiride in human plasma and biological samples; these include micellar electrokinetic capillary chromatography (MECC) with diode-array detection (DAD) or ultraviolet (UV) detection,[4,5] high performance liquid chromatography (HPLC) with DAD[6,7] and UV detection,[8] and derivative UV spectrophotometric detection.[9] However, these methods are not ideal for pharmacokinetics, because they have high detection limits and are time-consuming owing to the derivatization step, arduous sample preparation, and long chromatographic run times.

Recently, Magni *et al.*[10] reported the identification of four sulfonylureas (tolbutamide, chlorpropamide, glibenclamide, glipizide) in plasma by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS). Their LC-MS identification method has low specificity because this method uses only one ion per compound for selective ion monitoring (SIM) detection. In addition, the detection of glimepiride using LC-ESI/MS/MS has not been reported.

The present paper reports a novel quantification method for glimepiride in human plasma, using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) with liquid-liquid extraction (LLE). This method is not only more selective and reliable but also faster and simpler than other methods. The validation, reproducibility, stability, and recovery of the sample preparation method have been evaluated. Also, we applied this sample preparation method and LC-ESI/MS/MS to the pharmacokinetic study of glimepiride in human plasma.

Experimental Section

Reagents and solutions. Glimepiride and glipizide (internal standard) were obtained from CJ Corp. (Seoul, South Korea). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA), and diethyl ether, ethyl acetate, ammonium acetate and formic acid from Sigma-Aldrich Co. (St. Louis, MO, USA). A stock solution of glimepiride (1 mg/mL) and glipizide (1 mg/mL) were prepared in the mobile phase (5 mM ammonium acetate : acetonitrile = 40 : 60, pH 3.0 with formic acid). From these stock solutions, working standard solutions containing from

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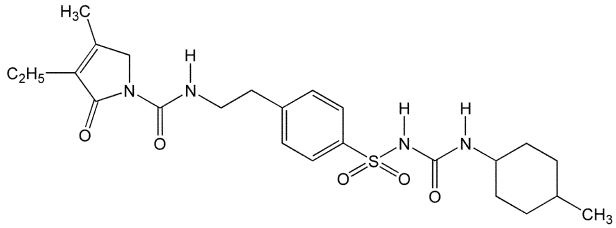

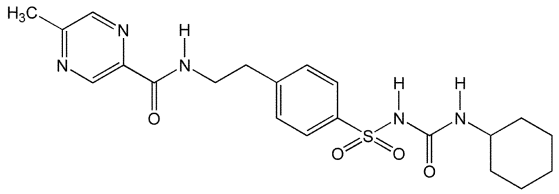

Figure 1. Structures of (a) glimepiride and (b) glipizide (the internal standard).

0.01 μg/mL to 20 μg/mL glimepiride were prepared by sequential dilution with the mobile phase.

Sample preparation. Plasma specimens (1 mL) were pipetted into conical glass tubes and spiked with 0.1 mL of 2 μg/mL internal standard solution. After adding 6 mL of diethyl ether-ethyl acetate (1 : 1, v/v) to the glass tubes, the plasma samples were shaken 15 min. The two phases were separated by 5 min of centrifugation at 2000 g. The upper organic layer was transferred into another conical glass tube and completely evaporated at 30 °C under a stream of nitrogen. The dry residue was reconstituted with 120 μL mobile phase and then 20 μL of the reconstituted sample were injected into the LC-MS/MS system.

LC-MS/MS conditions. Tandem mass spectrometry (MS/MS) was performed with a Quattro micro triple quadruple mass spectrometer (Micromass Co., Manchester, UK) equipped with an electrospray ion source. The sample (20 μL) was delivered into the ESI source by LC (liquid chromatograph and autosampler, Model Waters HT 2795, Waters Co., Milford, MA, USA) with C_{18} Capcell Pak column (2.0 × 150 mm, 5.0 μm particle size). The mobile phase was composed of 5 mM ammonium acetate and acetonitrile (40 : 60, pH 3.0 with formic acid) and was used after degassing. The flow rate was 200 μL/min and the total run time was 6 min.

The electrospray interface was maintained at 300 °C. Nitrogen nebulization was performed with a nitrogen flow of 1100 L/h. Argon was used as collision gas. Glimepiride and the internal standard were detected by the MRM scan mode with positive ion detection; the parameter settings were: capillary voltage at 3.2 kV, cone voltage at 20 V, extractor at 2 V, RF lens at 0.1 V, source temperature at 120 °C, collision cell entrance potential at -1.0 V, collision energy at 14 eV, collision cell exit potential at 0 V, multiplier at 650 V, and dwell time of 0.50 s.

Mass calibration was performed by infusion of a 10^{-4} M polyethylene glycol 1000 (PEG 1000) solution into the ionspray source. The peak widths of precursor and product ions were maintained at ~0.7 mass unit at half-height in the MRM mode.

Validation procedures and calibration curves. To assess the intraday precision and accuracy of the method, five replicate analyses were performed on plasma standards containing six different concentrations (0.1, 0.5, 2, 10, 50 and 200 ng/mL) of glimepiride. Five replicate analyses of the same six samples were also performed to determine the initial interday precision and accuracy. The accuracy was expressed as [(mean observed concentration)-(spiked concentration)]/(spiked concentration) × 100%, with the precision expressed as relative standard deviation (RSD).

For the quality control (QC) samples the appropriate QC working solution (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 μg/mL; 400 μL) was added to 50 mL polypropylene tubes containing 39.6 mL human control plasma to yield QC concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL. The QC samples were used to construct the calibration curve. The calibration curves (y = mx + b) were generated by a weighted linear least-squares regression of the peak area ratios (y) of the analytes to their internal standards versus the concentrations (x) of the calibration standards. Concentrations of analytes in QC samples were calculated using the resulting peak area ratios and the regression equations of the calibration curves. The bulk QC plasma samples were then vortex mixed, and 1.5 mL aliquots were transferred to 2 mL microcentrifuge tubes and capped, and stored at -70 °C.

Pharmacokinetic assay. For the human assay, a single 2 mg dose of glimepiride was administered orally to 28 volunteers who were advised about the nature and purpose of the study. The volunteers were of good health and had not taken any medication for at least two weeks before the study. The group consisted of healthy males with a mean age of 24.3 ± 1.7, mean weight of 70.1 ± 8.2 kg, and mean height of 173.8 ± 6.0 cm. Blood samples of these assay were taken, using heparin vacutainer collection tubes, 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 hr after ingestion. Human plasma was obtained by centrifugation at 2000 g for 10 min. The plasma specimens were stored at -70 °C before analysis.

Results and Discussion

We have developed a rapid and sensitive method for detecting glimepiride in human plasma, using LLE and LC-ESI/MS/MS for pharmacokinetic studies.

Under electrospray ionization condition, glimepiride and glipizide (internal standard) exhibit a fairly high sensitivity in positive ion detection mode rather than in negative ion detection mode. The analysis for compounds with basic sites (*e.g.*, amines), as for glimepiride and glipizide, should be performed at a low pH using positive ion detection.

Figure 2(a) shows the full scan first quadrupole positive ion spectrum of glimepiride, whereas Figure 2(b) shows that of the internal standard. These formed protonated precursor ion $[M+H]^+$ as major ion peaks. These spectrums were obtained from a working standard solution (1 μg/mL).

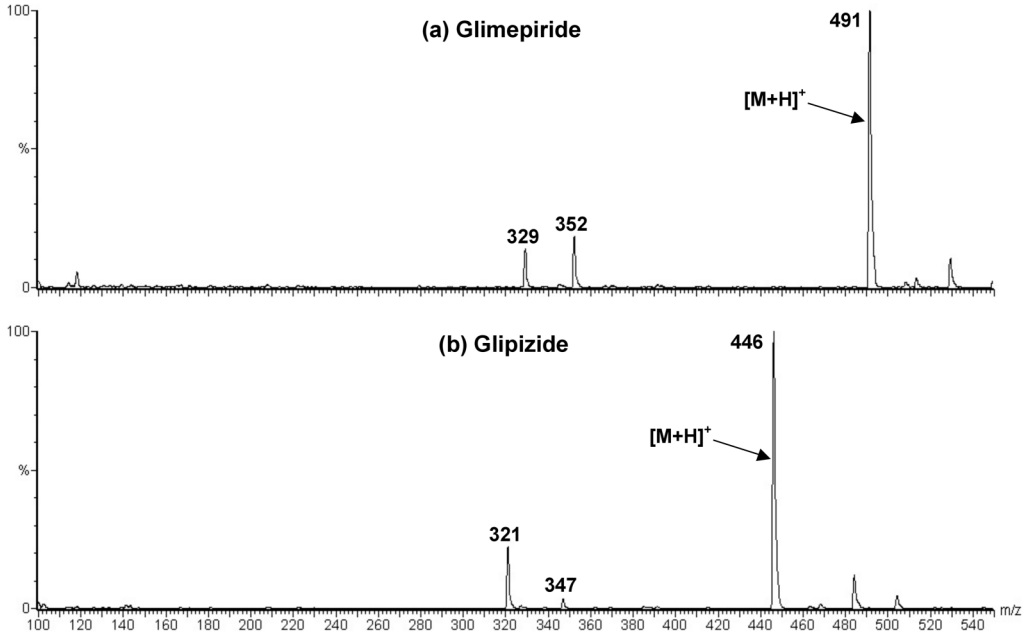

Figure 2. Full scan first quadrupole spectrum of (a) glimepiride and (b) glipizide (internal standard) working standard (1 μg/mL).

Glimepiride produced a protonated precursor ion ($[M+H]^+$) at m/z 491 with a major product ion at m/z 352. On the other hand, glipizide (internal standard) produced a protonated precursor ion ($[M+H]^+$) at m/z 446, with a major product ion at 321. Figure 3(a) and 3(b) show the product ion spectrum of glimepiride and the internal standard, respectively. The product ion mass spectrum and their postulated rationalization in terms of major fragmentation patterns of glimepiride and glipizide are illustrated in Figure 3. The most abundant product ions (m/z 352 for glimepiride and m/z 321 for glipizide) were selected for MRM analysis.

Multiple reaction monitoring (MRM) mode was used for quantitation and achieved very high sensitivity and selectivity. By using MRM mode in MS/MS, glimepiride and internal standard were detected without severe interference from the human plasma matrixes. Figure 4 shows the LC-MS/MS chromatogram of glimepiride in human blank plasma without internal standard. From Figure 4, no interference was observed in drug-free human plasma samples at the retention times of glimepiride and glipizide.

Liquid chromatography-tandem mass spectrometry is still limited to conditions that are suitable for mass spectrometry

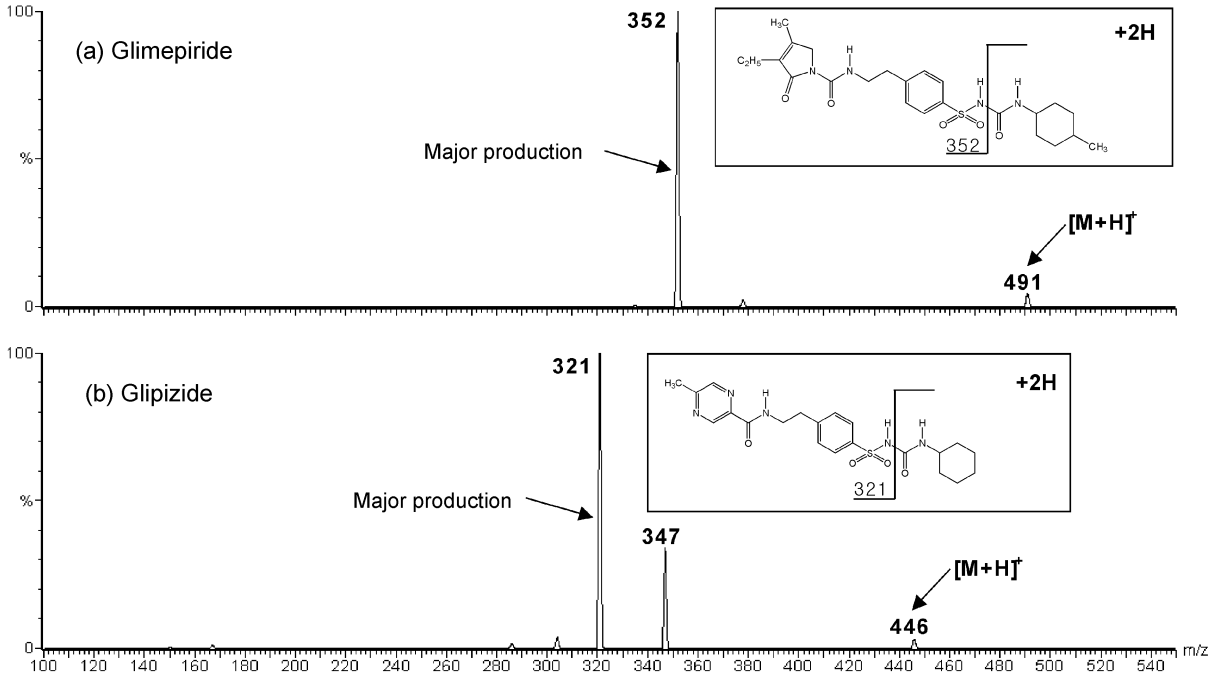

Figure 3. Product ion spectrum of (a) glimepiride and (b) glipizide (internal standard) working standard (1 μg/mL).

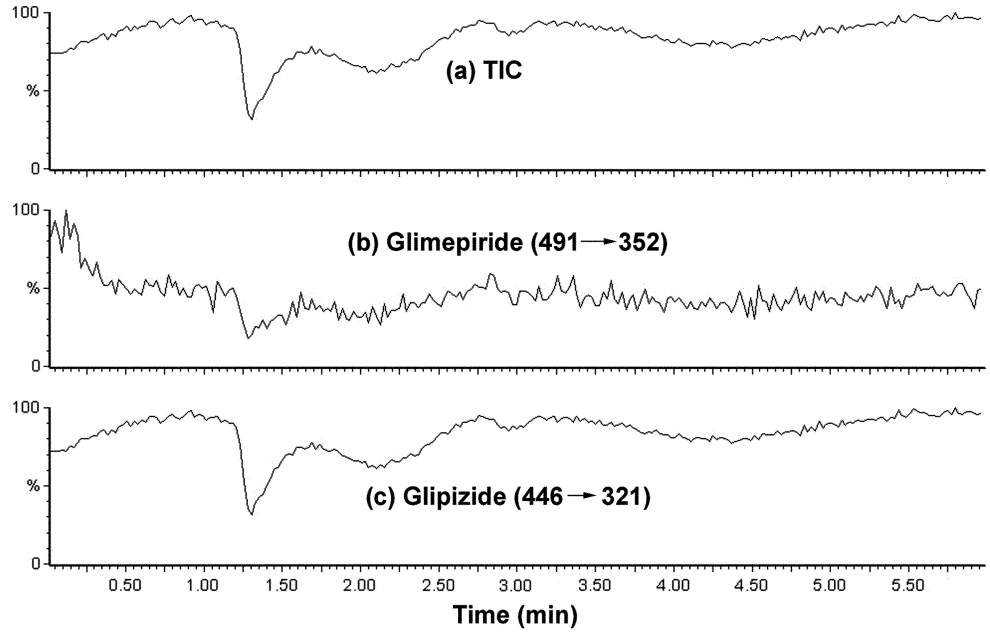

Figure 4. LC-MS/MS chromatograms of glimepiride in human blank plasma without internal standard.

operations. There are restrictions on pH, solvent choice, solvent additives, and flow rates for LC to achieve optimal ESI-MS/MS sensitivity. For the chromatographic analysis and electrospray ionization of glimepiride and glipizide we initially attempted to develop a reversed phase chromatographic method with methanol or acetonitrile as mobile phase. Acetonitrile was used instead of methanol, because acetonitrile affords better sensitivity and resolution in the analysis of glimepiride and internal standard. Ammonium acetate buffer was used because it was easily miscible with organic solvents and led to improved peak symmetry and ionization. When we used ammonium acetate buffer it was found that much higher ion intensities were achieved in the presence of ammonium acetate. The amount of acetonitrile in mobile phase was optimized at 60%. Likewise, the pH of the mobile phase was optimized at 3.0 by use of formic acid. Under these conditions, the sensitivity of glimepiride and internal standard was very high (Figure 5). Figure 5(a) show the total ion chromatogram (TIC) of quantitation limit of glimepiride in human plasma, and Figure 5(b) and 5(c) show the extracted ion chromatogram (XIC) of glimepiride and glipizide, respectively.

The effect of the matrix was also evaluated. Chromatography might result in co-elution of glimepiride and internal standard with endogenous interferences, which might not be detected by MS/MS but which might affect the ionization

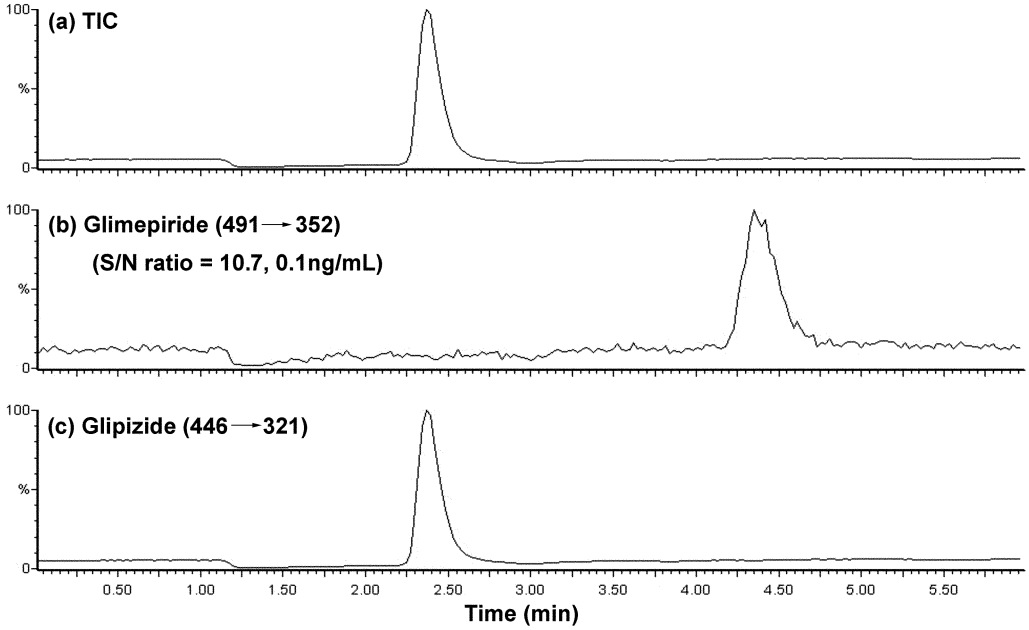

Figure 5. LC-MS/MS chromatograms of (a) TIC, (b) glimepiride and (c) glipizide (internal standard) in human plasma.

Table 1. Intraday precision and accuracy of measurement of glimepiride in human plasma

Glimepiride Nominal Concentration (ng/mL)	Glimepiride Calculated Concentration (ng/mL) [Mean ± SD][a]	Accuray (%)[b]	Precision (% RSD)
0.1	0.108 ± 0.006	8.0	5.6
0.5	0.520 ± 0.030	4.0	5.8
2	2.00 ± 0.04	0.0	2.0
10	10.1 ± 0.3	1.0	3.0
50	49.8 ± 1.5	-0.4	3.0
200	200 ± 1	0.0	0.5

[a]Averaged for five measurements at each concentration level (n = 5). [b]Accuracy = [(mean observed concentration)-(spiked concentration)]/(spiked concentration) × 100%.

Table 2. Interday precision and accuracy of measurement of glimepiride in human plasma

Glimepiride Nominal Concentration (ng/mL)	Glimepiride Calculated Concentration (ng/mL) [Mean ± SD][a]	Accuray (%)[b]	Precision (% RSD)
0.1	0.107 ± 0.008	7.0	7.5
0.5	0.510 ± 0.040	2.0	7.8
2	1.98 ± 0.07	-1.0	3.5
10	10.1 ± 0.3	1.0	3.0
50	50.2 ± 1.5	0.4	3.0
200	200 ± 2	0.0	1.0

[a]Averaged for five measurements at each concentration level (n = 5). [b]Accuracy = [(mean observed concentration)-(spiked concentration)]/(spiked concentration) × 100%.

efficiency of the analytes. This effect can lead to decreased reproducibility and accuracy for an assay and failure to reach the desired limit of quantitation. It is reported that the extent of ionization suppression seen is much more severe with electrospray ionization than with atmospheric pressure chemical ionization.[11] Therefore, analysts need to use a post-extraction spiked matrix blank and compare the results with an analytical standard in solution to determine the influence of the matrix on the analysis. A matrix blank is a representative biological sample that is free of the target analytes. A spiked matrix blank is a control sample that has been fortified with the target analytes at a defined, relevant level.[12] (matrix effect = [response of post-extracted spike/response of unextracted sample]). The absence of a matrix effect is indicated by a response ratio of 1.0. If responses are different a matrix effect is present. The present study was unable to detect a matrix effect.

The intraday precision, expressed as RSD (%), was 0.50%-5.8% for 0.10, 0.50, 2.00, 10.00, 50.00, and 200.00 ng/mL standard concentrations, based on five replicate analyses at each concentration level. The intraday accuracy, expressed as a percentage of nominal values was measured as (–)0.4%-8.0% for six standard concentrations, based on five replicate analyses at each concentration level. Table 1 shows the measured intraday precision and accuracy of glimepiride in human plasma. The interday precision was measured as 1.0%-7.8% for six standard concentrations, based on five replicate analyses at each concentration level. The interday accuracy was measured as (–)1.0%-7.0% for six standard concentrations, based on five replicate analyses at each concentration level. Table 2 shows the measured interday precision and accuracy of glimepiride in human plasma.

Standard calibration curves (reproducibility) were constructed on different working days (three days) using the human plasma. The response was linear throughout the concentration range of the study, with the coefficient of determination (r^2) always greater than 0.9997. The correlation equations was y = 0.01071x + 0.0038 (± 0.0021) in human plasma (Figure 6).

Analyte recovery from a sample matrix (also called extraction efficiency) is a comparison of the analytical response from an amount of analyte added to and extracted from the sample matrix (pre-extraction spike) with that from a post-extraction spike. (% recovery = (response of extracted spike)/(response of post-extracted spike) × 100). The % recovery of LLE was measured as 71.2%-79.8% for 0.1, 0.5, 2, 10, 50 and 200 ng/mL standard concentrations, with five

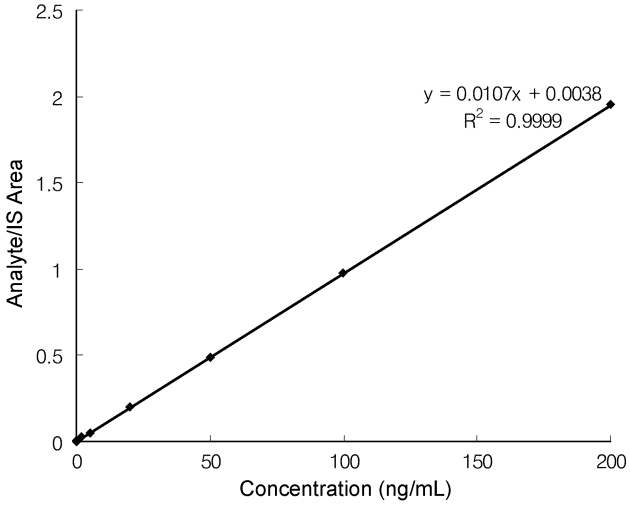

Figure 6. A calibration curve.

Table 3. The percent recovery of measurement of glimepiride in human plasma

Glimepiride Nominal Concentration (ng/mL)	% Recovery[a,b]
0.1	79.8
0.5	73.4
2	71.2
10	75.3
50	76.8
200	77.1

[a]Averaged for five measurements at each concentration level (n = 5). [b]% recovery = (response of extracted spike)/(response of post-extracted spike) × 100.

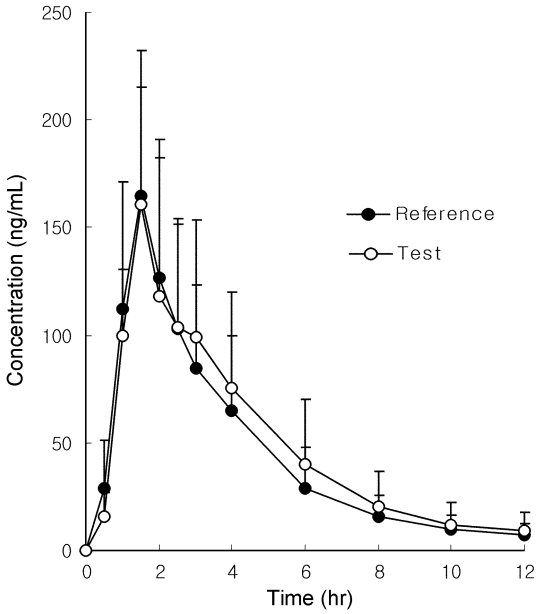

Figure 7. Plasma concentration of glimepiride in human plasma-time curve.

replicates at each concentration level.

The stability of glimepiride and internal standard was evaluated in the dissolution solvent and in human plasma. It was found that glimepiride and internal standard were stable for the duration of the experiment.

On the basis of a signal-to-noise ratio (S/N) of 10, the limit of quantitation (LOQ) for glimepiride was found to be 0.1 ng/mL on injection of 20 μL of sample into the LC-MS/MS system, and the lower limit of quantitation (LLOQ) for glimepiride was also found to be 0.1 ng/mL on the same system. The LLOQ is defined as the lowest concentration of the analyte that can be measured with a coefficient of variation and accuracy both less than 20%. These LLOQ values were sufficient for pharmacokinetic studies.

Determining the concentration of glimepiride in human plasma has been applied to pharmacokinetic studies by use of LLE with LC-MS/MS. Figure 7 shows the concentration of glimepiride in human plasma time curve. Figure 7 indicates that the proposed method is suitable for pharmacokinetic studies to determine the concentration of glimepiride in human plasma.

The method of Magni *et al.*[10] did not achieve glimepiride analysis. We infer from the fact that the retention time of glipizide is 7.7 minutes in his method that, if analyzed, glimepiride might be eluted later than glipizide. Therefore, this method has a longer chromatographic run time compared with our method. The LC-MS identification method has low specificity because this method used only one ion per compound for selective ion monitoring (SIM) detection. In addition, the LC-MS chromatogram of this method shows several interferences.

Conclusion

A highly sensitive and specific LC-MS/MS method for the determination of glimepiride in human plasma has been developed and validated, with a lower quantitation limit of 0.1 ng/mL, which is better than that attained by HPLC-UV[8] and LC-MS.[10] Validation experiments have shown that the assay has good precision and accuracy over a wide concentration range (0.1-200 ng/mL), and no interference caused by endogenous compounds was observed. This simple, rapid and robust assay enables the complete processing of large samples (about 1000 samples) for pharmacokinetic studies of glimepiride in human plasma.

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