http://dx.doi.org/10.5012/bkcs.2013.34.11.3284

Pentafluorophenylprophyl Ligand-based Liquid Chromatography-Tandem Mass Spectrometric Method for Rapid and Reproducible Determination of Metformin in Human Plasma

Jeong Soo Yang, Hyeon Ju Oh,[†] Jin Ah Jung,[‡] Jung-Ryul Kim,[‡] Tae-Eun Kim,[‡] Jae-Wook Ko,[‡] Soo-Youn Lee,[§,*] and Wooseong Huh[#,*]

Clinical Trial Center, Clinical Research Institute, Samsung Medical Center, Seoul 135-710, Korea
[†]Department of Laboratory Medicine and Genetics, Samsung Medical Center, Seoul 135-710, Korea
[‡]Department of Clinical Pharmacology and Therapeutics, Samsung Medical Center, Seoul 135-710, Korea
[§]Department of Laboratory Medicine and Genetics and Department of Clinical Pharmacology and Therapeutics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea
[*]E-mail: sy117.lee@samsung.com
[#]Department of Medicine and Department of Clinical Pharmacology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea. [*]E-mail: wooseong.huh@samsung.com
Received June 22, 2013, Accepted August 20, 2013

This paper describes first development and validation of pentafluorophenylprophyl ligand-based liquid chromatography coupled to tandem mass spectrometry (PFPLC-MS/MS) method to determine metformin, a highly polar compound, in human plasma. Metformin and Phenformin (internal standard) were extracted from human plasma 50 μL with a single-step protein precipitation. The chromatographic separation was performed using a linear gradient elution of mobile phase involving 5.0 mM ammonium formate solution with 0.1% formic acid (A) and acetonitrile (B) over 3.0 min of run time on a Phenomenex Luna PFP column. The detection was performed using a triple-quadrupole tandem mass spectrometer (Waters Quattro micro) with electrospray ionization in the mode of positive ionization and multiple-reaction monitoring (MRM). The developed method was validated with 5.0 ng/mL of lower limit of quantification (LLOQ). The calibration curve was linear over 5-3000 ng/mL of the concentration range ($R^2 > 0.99$). The specificity, selectivity, carry-over effect, precision, accuracy and stability of the method met the acceptance criteria. The method developed in this study had had rapidness, simplicity and ruggedness. The reliable method was successfully applied to high throughput analysis of real samples for a practical purpose of a pharmacokinetic study.

Key Words : Metformin, Liquid chromatography-tandem mass spectrometry, Pentafluorophenylpropyl stationary phase, Pharmacokinetic study, Human plasma

Introduction

Metformin (Metformin HCl, *N,N*-dimethylimidodicarbonimidic diamide hydrochloride) is an oral anti-diabetic drug used for the treatment of type 2 diabetes.[1-3]

Metformin is a highly polar compound: log $P_{octanol/water}$ = –2.64 (4). Thus it had a poor retention and unfavorable peak shape (tailing and broadening) on a conventional reversed-phase liquid chromatographic technique, *e.g.*, C18 column, as shown in the literatures.[5,6] To compensate the defect, a hydrophilic interaction liquid chromatography (HILIC) method, well-known to be a powerful technique for the analysis of polar compounds, was employed to determine plasma metformin in human,[4] mouse[7] and rat.[8] In addition, another stationary phase with a nitrile-modified silica gel such as polar cyano (CN) column was also adopted to improve the retention of metformin in human plasma.[9,10] Recently a polar embedded-phase column (Phenomenex Synergi POLAR-RP 80A), an ether linked phenyl phase with proprietary hydrophilic end-capping designed to improve retention and selectivity for polar and aromatic analytes, was applied to chromatographic retention and separation of metformin from endogenous substances in plasma matrix.[11] Sharma *et al.*[12] used a monolithic column, *e.g.*, Chromolith RP-18e, to avoid ionization suppression of metformin by relatively rapid elution of matrix in rat plasma. A time dependent gradient of high flow rate (0.5 to 1.2 mL/min), not desirable for electrospray, was applied to decrease run time and band broadening, although the peak was still wide by almost 0.5 min.

The aim of this study was to develop a fast analytical method with reproducibility, selectivity and ruggedness, based on pentafluorophenylprophyl ligand-based liquid chromatography coupled to tandem mass spectrometric detection (PFPLC-MS/MS), for the high throughput assay of metformin in human plasma, and thus a successful application of the method to a pharmacokinetic study. To date, there has been no report on PFPLC-MS/MS method for the quantitative analysis of metformin in human plasma. The novel PFPLC-MS/MS achieved the following advantages: short

retention time (1.6 min) of metformin; rapid re-equilibration of the column to initial gradient condition resulting in short run time; reproducible measurement of metformin in plasma at 5-3000 ng/mL without noticeable interference affecting chromatography and ionization by endogenous components in plasma after one-step protein precipitation. We successfully applied the validated method to real sample analyses (n = 866) for a practical purpose, *i.e.*, a pharmacokinetic study of metformin administered in healthy Korean male subjects.[13]

Experimental

Materials and Reagents. Metformin hydrochloride (purity 97.0%, Lot No.STBB0847) and phenformin hydrochloride (internal standard, purity 98%, Lot No. 100M1940V) were purchased from Sigma-Aldrich (St. Louis, USA) and Fluka (St. Louis, USA) respectively. Methanol, acetonitrile and water used in the study were of HPLC grade and purchased from Burdick and Jackson (Muskegon, USA). All other chemicals including ammonium formate and formic acid were of analytical grade and purchased from Aldrich (St. Louis, USA).

Preparation of Standard Stock and Working Solutions. The stock solutions of metformin (1.0 mg/mL) and phenformin (1.0 mg/mL) were separately prepared by dissolving the reference standards in methanol–water (70:30, *v/v*) and stored at –70 °C. The stock solution of metformin was diluted with methanol–water (50:50, *v/v*) to prepare 100 μg/mL of a working solution. A working solution of internal standard (200 ng/mL) was prepared by diluting the internal standard stock solution with acetonitrile. The working solutions were stored at 4 °C.

Preparation of Calibration Curve and Quality Control Samples. A calibration curve sample at the highest concentration (upper limit of quantification, ULOQ) was prepared at 3000 ng/mL for metformin by spiking 0.2 mL of the working solution to 9.8 mL of blank human plasma. The ULOQ sample was diluted with blank plasma to yield calibration curve samples ranging between 5-3000 ng/mL. To prepare four quality control (QC) samples, *i.e.*, lower limit of quantification, low, medium and high quality control (LLOQ, LQC, MQC and HQC) samples, at the respective concentration levels of 5, 20, 1000 and 2000 ng/mL, a metformin working solution standard was independently prepared, and then spiked to blank plasma. The prepared bulk samples for calibration curve and quality control were divided into aliquots in micro-centrifuge tubes and stored in a freezer at –70 °C until analyses.

Sample Preparation. All frozen samples were thawed at room temperature and then vortex-homogenized. Protein precipitation was done to extract metformin from plasma. An internal standard working solution 200 μL was added to an aliquot of plasma 50 μL in an Eppendorf tube, which was vortex-mixed for 1.0 min and then centrifuged at 14000 rpm for 5.0 min. The supernatant was transferred into an autosampler vial prior to liquid chromatography–tandem mass spectrometric analysis.

Liquid Chromatography and Mass Spectrometry. An AcQuity UPLC system (Waters, Milford, MA, USA) with a cooling autosampler, column oven enabled to control temperature, a degasser and a binary pump was used to inject 3.0 μL of prepared samples onto a Phenomenex Luna PFP(2) analytical column (50 mm × 2.0 mm i.d., 3.0 μm particle size, 100 Å) which was maintained at 30 °C. Chromatographic separations of samples were done using linear gradient of mobile phases consisting of 5.0 mM ammonium formate with 0.1% formic acid (A) and acetonitrile (B) over 3.0 min of run time: 0 min (10% A); 1.0 min (80% A); 1.1 min (10% A) at 0.2 mL/min of flow rate. The strong and weak solutions used in autosampler were 10% acetonitrile with 0.1% formic acid and 80% acetonitrile, respectively.

A Quattro Micro API triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI (eletrospray ionization) interface was used to detect metformin and phenformin (IS) in the column effluents. The optimum mass spectrometric conditions in multiple-reaction monitoring (MRM) mode are summarized in Table S1. Data acquisition and analysis were performed using the MassLynx^{TM} NT 4.0 software with the Quan-Lynx^{TM} program.

Method Validation. We have validated the method in concordance with the United State of Food and Drug Administration (FDA) guideline requirements to confirm reliability and reproducibility of the developed method.[14] The details are described in supplementary. Briefly, the method was evaluated in terms of specificity, selectivity, carry-over effect, linearity, sensitivity (LLOQ), intra-/inter-day precision and intra-/inter-day accuracy. The stabilities of samples spiked with metformin under storage and handling conditions was assessed in terms of freeze/thaw, short-term and long-term and post-extracted (processed) samples.

Results and Discussion

Conditions for LC and Tandem Mass Spectrometry. The major issue of this study was to develop a fast liquid chromatographic separation (coupled to mass spectrometry) that gave a sufficient retention of a highly polar compound, *e.g.*, metformin, on a stationary phase to decrease matrix effect. Several columns have been tested: Waters dC18, Luna HILIC, Luna CN and Luna PFP. A polar reversed-phase stationary phase, *e.g.*, Waters dC18 column, did not show a satisfactory retention of metformin on the polar stationary phase. Moreover suppression of ionization by matrix effect in plasma brought about decrease in the sensitivity of metformin. The other columns, *e.g.*, Luna HILIC, Luna CN and Luna PFP, showed enhanced interactions between metformin and each stationary phase. However a cyano stationary phase (Luna CN) had somewhat poor reproducibility in response of metformin, and a hydrophilic interaction liquid chromatography (Luna HILIC) required additionally long run time for stable re-equilibration of the stationary phase after complete gradient elution of mobile phases. Finally, a pentafluorophenylpropyl stationary phase

(Phenomenex Luna PFP) was chosen because the column exhibited a sufficient retention of metformin, and achieved the best performance in terms of speed (run time 3 min) and reproducibility in retention and response of metformin peak with its tailing and broadening minimized. Various contents of mobile phase modifiers such as formic acid and ammonium buffers under gradient combinations of water in acetonitrile were investigated to optimize mobile phase composition that produced the best sensitivity and peak shape for analyte and internal standard. An excellent response in positive ion electrospray for analyte and internal standard was obtained by using formic acid as an acidic modifier in the mobile phase. The use of ammonium formate buffer improved the peak shapes and increases also the sensitivity. A higher percentage of the aqueous portion in the mobile phase achieved shorter retention time of the analyte in liquid chromatography as well as improvement in peak shape. Consequently, high sensitivity and good separation of metformin from the endogenous substances in plasma for relatively short run time (retention time 1.60 min) were obtained by introduction of the prepared samples into a pentafluorophenylpropyl stationary phase with a gradient mobile phases consisting of 5 mM ammonium formate with 0.1% formic acid and acetonitrile. The developed PFPLC separation overcame the limitations of liquid chromatographic conditions reported in the previous literatures: long run time[9-11] and high flow rate over 0.6 mL/min[4,7,10-12] unsuitable for the mass spectrometer (Waters Quattro Micro API triple quadrupole).

We have optimized the mass spectrometric conditions (capillary voltage, cone voltage, collision energy, *etc*) for electrospray ionization and multiple-reaction monitoring of analyte and internal standard. The positive electrospray ionization mode was selected for the detection of analyte and internal standard, which were more sensitive in positive mode than negative mode. In the positive ion full scan, the protonated molecule ions $[M+H]^+$ were abundantly generated at *m/z* 129.8 for metformin and *m/z* 205.9 for internal

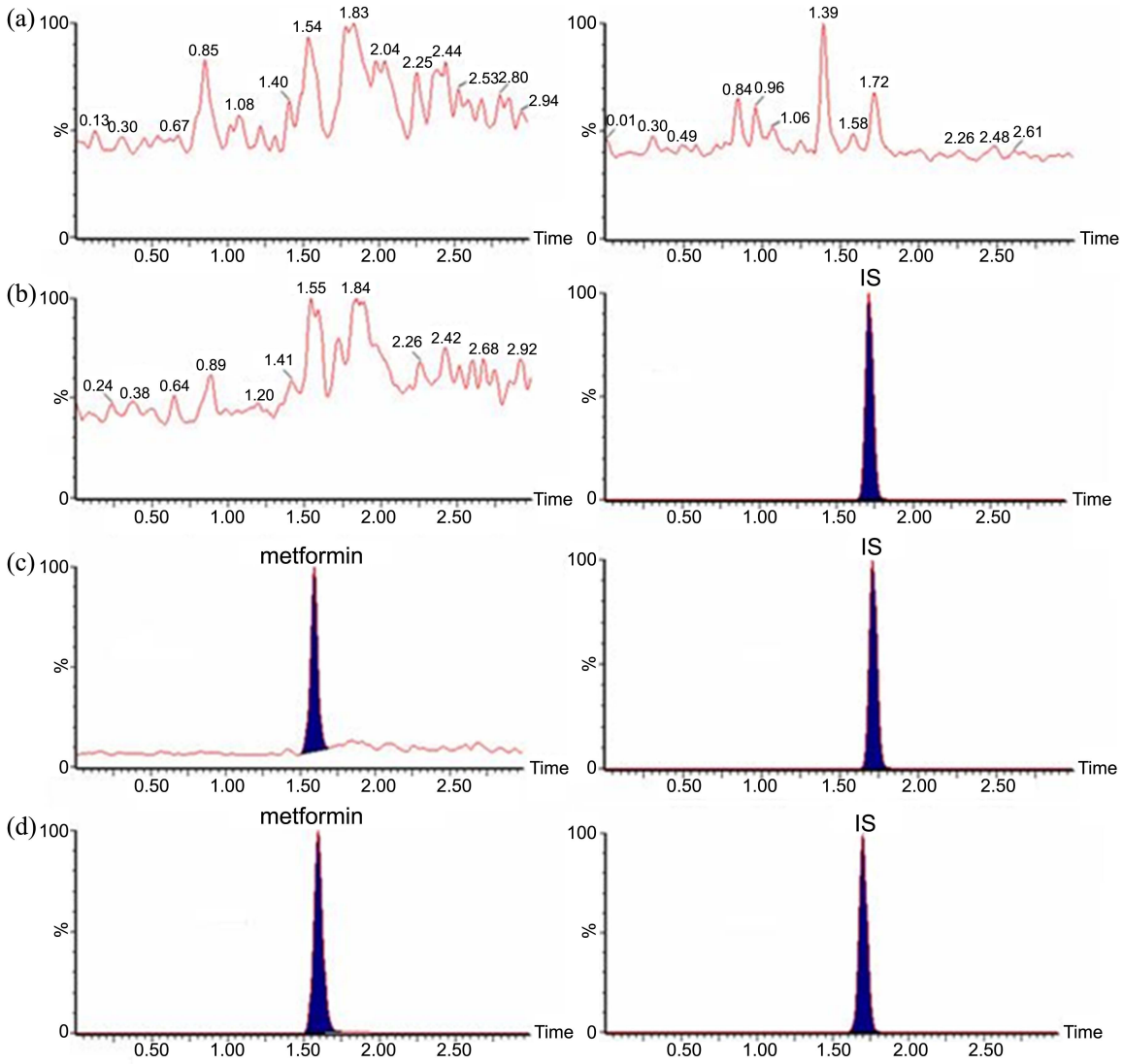

Figure 1. Representative multiple reaction monitoring (MRM) chromatograms of metformin (left panel) and internal standard (right panel) in (a) double blank plasma, (b) blank plasma spiked with internal standard, (c) blank plasma spiked with metformin and internal standard at LLOQ concentration (5 ng/mL), and (d) real plasma sample spiked with internal standard.

standard. More fragments were generated with increasing collision energy. The prominent stable product ion for metformin was obtained at *m/z* 70.7 with the collision energy of 20 eV. The internal standard was monitored by the most abundant product ion at *m/z* 104.8 with the collision energy of 25 eV.

Method Validation

Figure 1(a), (b) and (c) show the representative chromatograms of double-blank plasma (free of analyte and internal standard), blank plasma spiked with internal standard and blank plasma spiked with analyte and internal standard at LLOQ concentration, respectively. No obvious interference, which affected significantly LLOQ response, around each retention time of analyte and internal standard in double-blank plasma samples from six different humans demonstrates the absence of endogenous substances for the determination of metformin. The retention times were 1.60 and 1.74 min for metformin and IS, respectively. When plasma samples spiked with metformin were analyzed to investigate the potentially chromatographic interferences for internal standard, no signal affecting significantly response of internal standard was observed on chromatograms (data not shown).

For carry-over effect experiment, blank plasma samples were analyzed right after highest concentration (3000 ng/mL) calibration standards six times repeatedly. No carry-over has influenced the quantification of metformin because the area of peak observed at retention time of metformin in blank sample was less than 20% in comparison with that in LLOQ (5.0 ng/mL) sample (results not shown).

A linear calibration curve was constructed by preparing and analyzing a double blank sample, a blank sample spiked with internal standard and eight calibration curve samples over the concentration range between 5-3000 ng/mL and then by plotting peak area ratio of the analyte to internal standard *vs*. analyte concentration in plasma sample. The calibration data were analyzed by weighted ($1/x$) least-squares linear regression. All calibration curves exhibited good linearity due to the correlation coefficients (r^2) above 0.99, and met the acceptance criteria because each back-calculated standard concentration from a calibration equation was within ± 15% deviation from the nominal value. The precisions of correlation coefficient and the curve slope were 0.11 and 4.4%, respectively on five consecutive days. In addition, the precision and accuracy of mean concentrations in the calibration samples at 5-3000 ng/mL exhibited relative standard deviations of less than 6.2% and relative error values of –3.7 to 1.9%, respectively (Table S2). It was, therefore, demonstrated that the linearity of calibration standard curves had a reliable reproducibility across the calibration range. The lower limit of quantification (LLOQ) was determined as a 5.0 ng/mL because the intra- and inter-day precision and intra- and inter-day accuracy of calculated concentration did not exceed acceptance limits, 20% and ± 20%, respectively.

The intra- and inter-day precision and accuracy of the method were determined by analyzing four QC samples at 5, 20, 1000 and 2000 ng/mL of metformin in five replicates along with one standard curve on each of five runs (5 days). The results are presented in Table 1. The intra- and inter-day precision ranged from 0.9 to 9.3% and 2.2 to 9.8%, respectively, at all QC levels. The intra- and inter-day accuracy was between –6.8 to 2.1% and from –2.3 to 2.3%, respectively. The results indicate that the method was accurate and precise due to the values of relative standard deviations and

Table 1. Intra- and inter-day precision and accuracy of the method for the determination of metformin in human plasma (5 runs, five replicates per run)

Nominal concentration (ng/mL)	Intra-day (1 day, n = 5)			Inter-day (5 days, n = 25)		
	Mean ± SD (ng/mL)	RSD (%)	RE (%)	Mean ± SD (ng/mL)	RSD (%)	RE (%)
5	5.1 ± 0.5	9.3	2.1	5.1 ± 0.3	6.4	2.3
20	18.6 ± 0.7	3.6	-6.8	19.6 ± 1.9	9.8	-1.9
1000	964.7 ± 13.4	1.4	-3.5	978.8 ± 22.0	2.2	-2.1
2000	1886.4 ± 16.2	0.9	-5.7	1955.0 ± 56.3	2.9	-2.3

Table 2. Stability of metformin in human plasma at various conditions (n = 3)

Conditions	Nominal concentration (ng/mL)	Mean ± SD	RSD (%)	RE (%)
Three Freeze-thaw cycles	20	19.8 ± 1.4	7.2	-0.8
	2000	1949.0 ± 57.2	2.9	-2.6
Room temperature, 12 h	20	20.0 ± 0.9	4.6	0.2
	2000	1877.8 ± 42.5	2.3	-6.1
–70 °C, 4 months	20	21.1 ± 2.3	10.9	5.7
	2000	1929.1 ± 63.9	3.3	-3.6
Post-extracted samples, 4 °C, 24 h	20	18.1 ± 0.3	1.4	-9.3
	2000	2078.3 ± 44.1	2.1	3.9

relative error values within the acceptance criteria (15 and ± 15%, respectively).

The stability results are summarized in Table 2. As the precisions and accuracies of the quality control samples at low and high concentration levels exhibited relative standard deviations and relative error values of within acceptance limits (15 and ± 15%, respectively), it was indicated that metformin in plasma samples was considered as stable without any noticeable degradation during storage and handling of samples under the conditions: three freezing and thawing cycles, 12 hrs at room temperature, 4 months at 70 °C, and 24 h in autosampler at 4 °C before liquid chromatography-tandem mass spectrometric analysis.

Application of the Method to Analysis of Real Samples. The representative chromatograms of real sample are shown in Figure 1(d). Like spiked samples, no chromatographic interference caused by endogenous substances in real samples was found around each retention time of metformin and internal standard.

In each batch run, we have prepared two sets of three QC samples (LQC, MQC and HQC), between which real samples were analyzed, to check the performance of the method. The accuracy of metformin concentration in the QC samples was evaluated for the integrity and validity of the analytical results of the real samples: no concentration exceeded ± 15% in comparison with each nominal one (results not shown). We have also evaluated reproducibility of calibration and QC samples throughout the analysis of real samples. As indicated in Table S3, the precision and accuracy of metformin concentrations in calibration and QC samples exhibited relative standard deviations of less than 6.8% and relative error values of –3.1 to 6.9% respectively, except for LLOQ in which the accuracy was –14.4%. In addition, the constancy in peak areas of internal standard over the whole analyses of all samples including calibration, QC and real plasma was assessed with the precision of 20.0% (n = 866). It was, therefore, demonstrated that the reliable method was practically applied to high-throughput analysis of real plasma samples as well as spiked blank samples. The quantitative results were successfully used to perform a pharmacokinetic study of metformin administered in Korean male subjects.[13] The measured concentration range of plasma metformin were within the calibration curves, and were in agreement with those reported in the literatures.[6,15] Metformin was absorbed with maximum plasma concentration of 1230 μg/L at 1.5 h. Thereafter, the plasma concentrations were declined biphasically with half-lives of 5.5 h. Area under the plasma concentration time curve from 0 to the last measurable concentration (AUC_{last}) and from 0 h extrapolated to infinity (AUC_{inf}) were 7719 μg·h/L and 7820 μg·h/L, respectively. For all subjects, the ratio AUC_{last}/AUC_{inf} was higher than 80%, as recommended by EMA guidelines.[16] The pharmacokinetic parameters in this study were similar to those in Caucasian patients after the multiple administrations with the same dose.[17]

Conclusion

We have developed a simple, reproducible, fast and robust analytical method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) for the quantification of metformin in human plasma. The one-step protein precipitation for simple sample preparation and pentafluorophenylpropyl (PFP) stationary phase chromatography for sufficient and fast retention of metformin gave acceptable validation results without any significant interference effect. The validated method was successfully employed throughout the whole analytical procedure of real samples to accomplish a pharmacokinetic study.

Acknowledgments. This study was supported by JW Pharmaceutical Corp (Seoul 137-864, Korea).

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