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Rapid Determination of Imatinib in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry: Application to a Pharmacokinetic Study

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A simple, fast and robust analytical method was developed to determine imatinib in human plasma using liquid chromatography-tandem mass spectrometry with electrospray ionization in the positive ion mode. Imatinib and labeled internal standard were extracted from plasma with a simple protein precipitation. The chromatographic separation was performed using an isocratic elution of mobile phase involving 5.0 mM ammonium formate in water–5.0 mM ammonium formate in methanol (30:70, v/v) over 3.0 min on reversed-stationary phase. The detection was performed using a triple-quadrupole tandem mass spectrometer in multiple-reaction monitoring mode. The developed method was validated with lower limit of quantification of 10 ng/mL. The calibration curve was linear over 10-2000 ng/mL ($R^2 > 0.99$). The method validation parameters met the acceptance criteria. The spiked samples and standard solutions were stable under conditions for storage and handling. The reliable method was successfully applied to real sample analyses and thus a pharmacokinetic study in 27 healthy Korean male volunteers.

Key Words : Imatinib, LC-MS/MS, Plasma, Validation, Pharmacokinetic

Introduction

Imatinib (Imatinib mesylate, 4-[(4-methyl-1-piperazinyl)-methyl]-*N*-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]phenyl]benzamide methanesulfonate), an inhibitor of the Bcr-Abl, Abl, c-KIT and PDGF-protein kinase, is a drug used to treat a rare form of cancer called gastrointestinal stromal tumor (GIST), philadelphia chromosome positive chronic myeloid leukemia (CML) and Ph+ chronic phase CML.[1,2]

Analytical methods based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been described to determine imatinib in plasma.[3-12] Several papers reported the employment of solid-phase extraction (SPE) procedure[6,11] or liquid-liquid extraction (LLE) technique[7,8] to develop more sensitive quantitative method by the selective extraction and enrichment of imatinib from plasma. On the other hand, protein precipitation has also been described for simple sample preparation.[3-5,9,10,12] In most case, reversed-phase liquid chromatography (RPLC) has been applied to separation of imatinib due to its non-polarity although hydrophilic interaction liquid chromatography (HILIC) served as an alternative technique to RPLC by Hsieh *et al.*[9]

The aim of this study was to develop an efficient LC-MS/MS method, which exhibited improved performance in terms of sensitivity, robustness, run time and sample preparation, to determine imatinib in human plasma. The LC-MS/MS methods, described by the previous studies,[3-12] had some limitations of time consumption, cost and method complexity due to high volume requirement of plasma sample by low dilution factor (4 to 11), relatively long run time (3 to 12 min) and complex extraction methods such as solid-phase extraction and liquid-liquid extraction. Unlike the previous studies, our method achieved protein precipitation for extraction, high dilution factor of plasma (14.4), retention time of 1.3 min and high-throughput analyses of real samples (n = 1101) that resulted in high sensitivity, simplicity of sample preparation, short run time and good robustness. No matrix interference effect by endogenous substances in plasma was found. The method validated in the study was successfully applied to real sample analyses for a practical purpose of a pharmacokinetic study of imatinib administered in healthy Korean male subjects.

Experimental

Materials and Reagents. Imatinib mesylate α-form (purity 99.29%, Batch No. IMαWS040511) and d_8-labeled internal standard of imatinib (purity 99.50%, Lot No. 1224-030A2) were purchased from Nosch (Hyderabad, India) and TLC

PharmaChem., Inc (Ontario, Canada), respectively. Methanol, acetonitrile and water used in the study were of HPLC grade and purchased from Burdick and Jackson (Muskegon, USA). 5 M ammonium formate solution was purchased from Agilent (Santa Clara, USA).

Preparation of Standard Stock and Working Solutions. The stock solutions of imatinib (2.0 mg/mL) and d_8-imatinib (0.1 mg/mL) were separately prepared by dissolving the reference standards in methanol-water (50:50, v/v) and stored at 5 °C for three months. An aliquot of stock solution of imatinib was diluted with methanol-water (50:50, v/v) to yield eight working solutions for calibration curve at 1-200 μg/mL. Another aliquot of stock solution of imatinib was independently diluted with methanol-water (50:50, v/v) to prepare four working solutions for quality control at 1, 3, 25 and 150 μg/mL. A working solution of internal standard (5 μg/mL) was prepared by diluting the internal standard stock solution with 50% methanol in water. All working solutions were stored at 5 °C for three months.

Preparation of Calibration Curve and Quality Control Samples. The calibration curve samples were prepared at eight concentrations of 10-2000 ng/mL by spiking 10 μL of each working solution for calibration curve to 990 μL of blank human plasma. 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 10, 30, 250 and 1500 ng/mL, 10 μL of each quality control working solution was spiked to 990 μL of blank human plasma. The samples prepared 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 imatinib from human plasma. An internal standard working solution 10 μL and acetonitrile 300 μL was added to an aliquot of 100 μL plasma in an Eppendorf tube, which was vortex-mixed for 1.0 min and then centrifuged at 14000 rpm for 5.0 min. The supernatant 200 μL was vortex-mixed with 5 mM ammonium formate solution 500 μL in a clean tube, and then transferred into an autosampler vial prior to liquid chromatography-tandem mass spectrometry 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 10 μL of prepared samples onto a Waters XBridge shield RP18 (100 mm × 2.1 mm i.d., 3.5 μm particle size), which was maintained at 40 °C. Chromatographic separations of samples were done using isocratic elution of mobile phases involving 5 mM ammonium formate in water–5 mM ammonium formate in methanol (30:70, v/v) at 0.3 mL/min of flow rate over 3.0 min of run time. The strong and weak solutions used in autosampler were 80% methanol and 5 mM ammonium formate in 70% methanol, respectively.

A Quattro Micro API triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI interface was used to detect imatinib and internal standard in the column effluents. 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 Admini-stration (FDA) guideline requirements to confirm reliability and reproducibility of the method for the determination of imatinib in human plasma.[13] Briefly, the method was evaluated in term of system suitability, specificity, selectivity, carry-over effect, linearity, matrix effect, extraction efficiency, intra-/inter-day precision and intra-/inter-day accuracy. The stabilities of samples spiked with imatinib under storage and handling conditions was assessed in terms of freeze/thaw, short-term and long-term and post-extracted (processed) samples. In addition, we have evaluated the short- and long-term stabilities of imatinib and internal standard in prepared standard solutions that were stored and handled during the method validation and the period of real sample analyses, respectively.

Pharmacokinetic Study. The study was conducted in compliance with the Declaration of Helsinki and in accordance with Good Clinical Practice.[14] Imatinib mesylate 200 mg was administered to 27 healthy volunteers in the fasted state. The serial blood samples were obtained for the determination of plasma imatinib. The pharmacokinetic parameters were calculated using non-compartmental analysis with Phoenix WinNonlin 6.1 software.

Results and Discussion

Conditions for LC-MS/MS and Sample Preparation. The chromatographic conditions have been developed to obtain peaks of imatinib and internal standard with high intensity, good shape and stable response that were not interfered with endogenous substances derived from plasma matrices. We have applied conventional reversed-phase liquid chromatography such as C18 column on which imatinib was well retained and separated due to its relatively non-polar property. Under the diverse mobile phase conditions, *i.e.*, methanol and acetonitrile in water with acidic and ammonium buffers, several C18 columns have been tested: core-shell-typed columns such as Phenomenex Kinetex C18 (50 mm × 2.1 mm i.d., 2.6 μm) and Agilent Poroshell 120 EC-C18 (50 mm × 3.0 mm i.d., 2.7 μm), and porous-typed columns such as Imtakt Cadenza CD-C18 (50 mm × 2.1 mm i.d., 3.0 μm), Cadenza 5CD-C18 (100 mm × 2.1 mm i.d., 5.0 μm) and Waters XBridge shield RP18 (100 mm × 2.1 mm i.d., 3.5 μm). Finally, XBridge shield RP18 column was chosen for liquid chromatographic separation of imatinib and internal standard considering their peak shapes (tailing and symmetry). Various contents of mobile phase modifiers such as formic acid and ammonium buffers under isocratic elution of methanol in water were investigated to optimize mobile phase conditions that produced the reproducibility in peak shape and response of analyte and internal standard on

the column. The use of formic acid as an acidic modifier in the mobile phase decreased consistency in peak areas of imatinib and internal standard, which were injected onto chromatography repeatedly. In addition, ammonium formate in mobile phase improved the reproducibility of imatinib concentration at LLOQ rather than ammonium acetate. As the ratio of ammonium buffer in mobile phase was increased to 5 mM, the peak shapes was improved with tailing minimized. The use of acetonitrile as an organic mobile phase did not improved the peak shapes and sensitivities of imatinib and internal standard in comparison with methanol. Moreover, methanol was more miscible with ammonium buffer than acetonitrile. A higher percentage of the organic portion in the mobile phase achieved shorter retention time of the analyte in liquid chromatography as well as improvement in peak shape. An isocratic elution of mobile phase was used because it produced more stable response and shorter run time than gradient elution. Finally, a chromatographic separation of imatinib with reproducibility in peak shape and response was achieved by introduction of the

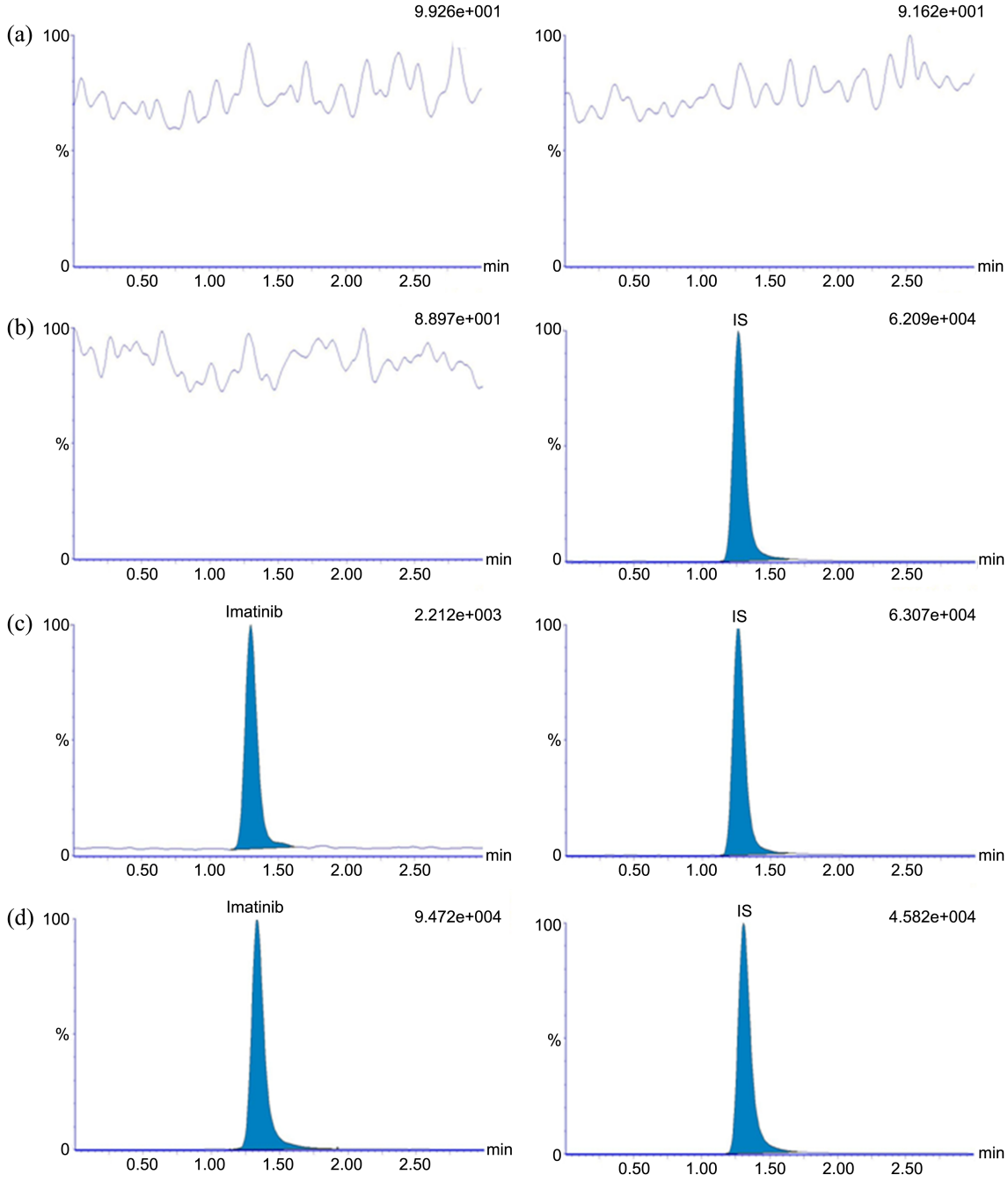

Figure 1. Representative multiple reaction monitoring (MRM) chromatograms of imatinib (left panel) and internal standard (right panel) in (a) double blank plasma, (b) blank plasma spiked with internal standard, (c) blank plasma spiked with imatinib and internal standard at LLOQ concentration, and (d) real plasma sample spiked with internal standard: concentrations of imatinib spiked to blank sample and estimated in real sample were 10 and 629.3 ng/mL, respectively.

prepared samples into a porous/reversed-stationary phase column (XBridge shield RP18) with an isocratic mobile phase consisting of 5 mM ammonium formate in water–5 mM ammonium formate in acetonitrile (30:70, v/v). The elution condition provided fast separation of analyte and internal standard from interferences by endogenous substances in plasma and sufficient ruggedness to analyze high-throughput real samples.

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 optimum conditions in multiple-reaction monitoring (MRM) mode are summarized in Table S1. 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* 494.4 for imatinib and *m/z* 502.3 for internal standard. More fragments were generated with increasing collision energy. The prominent stable product ion for imatinib was obtained at *m/z* 394.2 with the collision energy of 20 eV. The internal standard was monitored by the most abundant product ion at *m/z* 394.4 with the collision energy of 25 eV.

The optimization of sensitive LC-MS/MS conditions allowed of a simple and rapid sample preparation, for which protein precipitation was done to extract analyte and internal standard from human plasma. Two organic solvents, acetonitrile and methanol, were tested and compared. Acetonitrile was chosen as an extraction solvent because no difference of peak area between two solvents was observed and the extraction by methanol gave rise to a chromatographic interference with imatinib resulting in incorrect concentration at lower limit of quantification.

Method Validation. The intra- and inter-day precisions (RSD) of peak area ratio (imatinib/d_8-imatinib in standard mixture solution) for system suitability throughout the method validation were 1.0-3.7% (n = 5) and 2.6% (n = 25), respectively, with reproducible peak shapes in terms of symmetry and width. 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 as shown in Figure 1(a), (b) and (c). In addition, no signal that affected significantly response of internal standard was observed in multiple-reaction monitoring chromatogram of blank plasma samples spiked with imatinib. The quantification of imatinib was not influenced by carry-over because no peak in double blank sample, analyzed right after highest concentration (2000 ng/mL) calibration standards, showed responses over 20 and 5% for imatinib and internal standard, respectively, in comparison with those in LLOQ (10 ng/mL) sample. All calibration curves by weighted ($1/x^2$) least-squares linear regression exhibited good linearity due to each correlation coefficient (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 linearity of calibration standard curves had a reliable reproducibility across the calibration range due to precise mean values of 0.15 and 4.27% for correlation coefficient and curve slope, respectively. In addition, the precision and accuracy of mean concentrations in the calibration samples between 10-2000 ng/mL exhibited relative standard deviations of less than 3.74% and relative error of –2.36 to 6.10%, respectively (Table S2). The lower limit of quantification (LLOQ) was determined as 10 ng/mL because the intra- and inter-day precision and accuracy of the calculated concentration did not exceed acceptance limits, 20% and ± 20%, respectively. As presented in Table 1, the method was accurate and precise due to the values of relative standard deviation and relative error within the acceptance criteria: intra-/inter-day precision 1.42 to 6.47% and 2.82 to 6.89%, respectively; intra-/inter-day accuracy –0.20 to 3.79% and –1.80 to 6.67%, respectively.

The plasma matrix did not influence the quantification of imatinib due to the standard deviation values below 2.3% between six different batches of blank plasma spiked at

Table 2. Stability of human plasma spiked with imatinib (n = 5)

Conditions	Nominal concentration (ng/mL)	Mean ± SD	RSD (%)	RE (%)
Three Freeze-thaw cycles	30	28.84 ± 1.15	4.00	-3.87
	1500	1604.16 ± 6.60	0.41	6.94
Room temperature, 24 h	30	28.48 ± 1.93	6.78	-5.07
	1500	1582.16 ± 34.43	2.18	5.48
70 °C, 166 days	30	28.70 ± 0.64	2.22	-4.33
	1500	1539.68 ± 34.75	2.26	2.65
Post-extracted samples, 10 °C, 24 h	10	9.54 ± 0.51	5.38	-4.60
	30	29.24 ± 0.69	2.38	-2.53
	250	250.32 ± 6.30	2.52	0.13
	1500	1608.64 ± 35.9	2.23	7.24

Table 1. Intra- and inter-day precision and accuracy of the method for the determination of imatinib 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 (%)
10	9.98 ± 0.65	6.47	-0.20	9.82 ± 0.68	6.89	-1.80
30	30.00 ± 0.58	1.93	0.00	29.71 ± 0.90	3.04	-0.97
250	252.42 ± 3.58	1.42	0.97	248.48 ± 7.00	2.82	-0.61
1500	1556.80 ± 36.01	2.31	3.79	1600.08 ± 53.72	3.36	6.67

LLOQ level after extraction. The extractions of imatinib and internal standard were consistent and reproducible due to the relative standard deviation values of 0.9-3.9% and 1.6-4.0%, respectively, with extraction efficiencies of 101.9-105.7% and 98.7-103.1%, respectively. The relative standard deviations and relative error values of within acceptance limits in Table 2 indicated that plasma samples spiked with imatinib was stable without any noticeable degradation during storage and handling of the samples under the conditions: three freezing and thawing cycles, 24 h at room temperature, 166 days at 70 °C, and 24 h in autosampler at 10 °C before liquid chromatography-tandem mass spectrometric analysis. The standard solutions of imatinib and internal standard (each 500 ng/mL) were stable under the conditions during the method validation because relative errors of each peak area in imatinib and internal standard solutions stored and handled at 5 °C for 5 days from that in freshly prepared solutions were –2.9 and –1.5% respectively, and standard solutions stored at room temperature for 6 h gave relative error of –0.3% for both imatinib and internal standard with relative standard deviations between 0.54.1%. The standard solutions, which were stored and used at 5 °C for 23 weeks throughout real sample analyses, were also stable due to relative error values of 1.5 and –0.2% for imatinib and internal standard, respectively with relative standard deviations from 1.1 to 2.0%.

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

We have prepared two sets of three QC samples (LQC, MQC and HQC), between which real samples were analyzed in each batch run, to check the performance of the method. The accuracy of imatinib concentration in the QC samples was evaluated for the integrity and validity of the analytical results of 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 mean concentrations in calibration and QC samples exhibited relative standard deviations of less than 4.74% and relative error values of –3.27 to 4.16% respectively. The calibration curves applied to real sample analyses had good linearity and precision due to 0.9978 of mean correlation coefficient with 0.22% of relative standard deviation (n = 19). The response ratio of imatinib to internal standard over all calibration curves was reproducible because the precision value of curve slopes was 3.90% of relative standard deviation (n = 19). Moreover, the mean of curve slopes for real sample analyses was deviated by 0.98% from that of curve slopes used during the method validation. The consistency 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 18.5% (n = 1101).

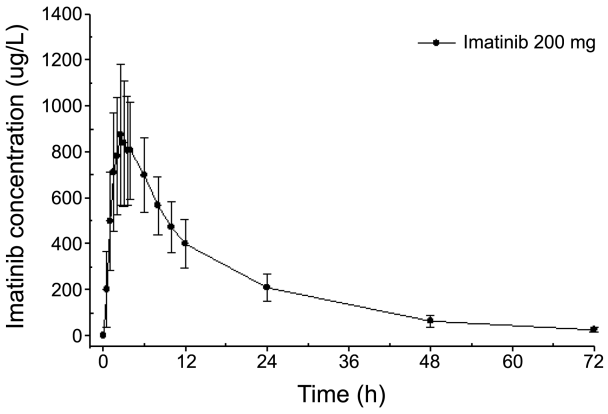

Figure 2. Mean plasma concentration *vs.* time profile of imatinib after oral administration of 200 mg in 27 healthy subjects

Table 3. Pharmacokinetic parameters of imatinib after oral administration of 200 mg (mean ± SD, n = 27)

PK parameter	Imatinib mesylate 200 mg (oral dose)	
	Arithmetic (mean ± SD)	Geometric (mean (CV))
C_{max} (μg/L)	986.3 ± 266.0	959.7 (27.0)
t_{max} (h)	2.9 ± 0.7	2.82 (23.84)
$t_{1/2}$ (h)	14.3 ± 2.3	14.1 (15.9)
AUC_{last} (h·μg/L)	14131.7 ± 3826.2	13659.9 (27.1)
AUC_{inf} (h·μg/L)	14938.9 ± 3523.7	14493.9 (23.6)
V_z/F (L)	289.8 ± 84.2	279.6 (29.1)
CL/F (L/h)	14.2 ± 3.4	13.8 (24.2)

It was, therefore, demonstrated that the method was reliable for high-throughput analysis of real plasma samples as well as spiked blank samples.

Pharmacokinetic Study. Figure 2 illustrates the mean plasma concentration *vs*. time profile of imatinib. Pharmacokinetic parameters, calculated by non-compartmental analysis, are summarized in Table 3. Imatinib was absorbed after single oral administration, with a mean t_{max} of 2.9 h. The terminal half-life of imatinib in plasma was approximately 14.3 h, and CL/F was 13.8 L/h at 200 mg. The parameters of imatinib in the study were in agreement with those reported in the literatures.[15,16] Pharmacokinetic parameters of imatinib in Korean healthy subjects in this study were similar to those in Europeans after the single administration with same dose. Likewise, population analysis of imatinib pharmacokinetics showed that ethnic effect was not significant.[17] Since imatinib exhibits dose proportionality from 25 to 1000 mg and it is reported that there is no difference in pharmacokinetics between healthy adults and patients, the results from the present study can be extrapolated to the targeted patients.[18]

Conclusions

We have developed a simple, reproducible, fast and robust high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) method for the quanti-

fication of imatinib in human plasma. The simple protein precipitation for sample preparation and fast reversed-phase chromatography for separation of analyte from endogenous substances in human plasma gave acceptable validation results without any significant interference effect. The developed and validated method was successfully applied throughout the whole analytical procedure of real samples to accomplish a pharmacokinetic study of imatinib.

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