Validation and Application of HPLC–ESI–MS/MS Method for the Determination of Irsogladine

N. H. Hoang[2], N. L. Huong[2], S.-Y. Hong[1] and Je Won Park[1*]

[1]*School of Biosystem and Biomedical Science, Korea University, Seoul 02841, Republic of Korea*
[2]*Department of BT Convergent Pharmaceutical Engineering, SunMoon University, Chungnam 31460, Republic of Korea*

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A highly sensitive analytical tool for the fast quantification of irsogladine in human plasma was developed. Cleanup using a solid-phase extraction technique is a simple method for extracting both irsogladine and lamotrigine (internal standard) spiked into human plasma. The resolvable separation of both analytes through reversed-phase high-performance liquid chromatography (HPLC) was carried out within 5 min. The HPLC–electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) method, which was operated in a selected reaction monitoring mode specific to the target analytes, was verified for use in the quantification of irsogladine. The inter- and intra-day precision (relative standard deviation, RSD) of irsogladine spiked into quality control samples were <7%, and their accuracies were between 96.6% and 102.1%. The calibration curve for irsogladine spiked into human plasma was linear over the range from 1.8 to 100 ng mL^{-1} with lower limit of quantification at 1.8 ng mL^{-1}. The established method was successfully applied for a bioequivalence study of irsogladine.

Keywords: Irsogladine, human plasma, HPLC–ESI–MS/MS, bioequivalence study

Introduction

As a selective phosphodiesterase inhibitor, irsogladine (6-[2,5-dichlorophenyl]-1,3,5-triazine-2,4-diamine maleate) elevates intracellular cyclic adenosine 3′,5′-monophosphate level via non-selective inhibition of phosphodiesterase isozymes and features gastric cytoprotection [1]. Since its development for the treatment of acute gastritis and peptic ulcer disease, irsogladine maleate has been used clinically as an anti-ulcer drug in Japan, Korea, and China [2]. This mucosal protective drug is now used frequently for the treatment of aphthous stomatitis and inflammatory bowel disease [1]. Recent reports suggest that irsogladine has remarkable effects against various gastrointestinal diseases, including nonsteroidal anti-inflammatory drug-induced gastric and small intestinal lesions, and gastric ulcer caused by *Helicobacter pylori* [3]. A couple of methods have been described, mainly by Chinese researchers, for the quality control of irsogladine in the final products such as pharmaceutical dosage form [4, 5]. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was the dominant analytical technique [5], and two kinds of the bioequivalence studies on irsogladine have been published by employing the abovementioned HPLC–UV methods [6, 7]; one was done for the phase 1 clinical trials of this anti-ulcer agent [6], whereas the other for different dosage forms [7]. However, there is no publication concerning the mass spectrometric (MS) determinations of irsogladine in biological matrices. Thus, the aim of this study was to develop and validate a fast, resolvable, and sensitive HPLC–electrospray ionization (ESI)–MS/MS analytical tool for the bioequivalence study of irsogladine maleate.

Experimental

Chemicals and Reagents. Methanol and water were purchased from J.T. Baker (Philipsburg, NJ, USA). Irsogladine maleate (98% purity) was obtained from Santa Cruz (Dallas, TX, USA), whereas formic acid and lamotrigine (≥98%), as the internal standard (IS), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Blank human plasma in ethylenediaminetetraacetic acid-tri-potassium anticoagulant (mixed gender) was from Biochemed Services (Winchester, VA, USA). An irsogladine stock solution (10 μg mL^{-1} in methanol) was diluted with blank human plasma to prepare seven-point non-zero calibration standards (1.8, 2, 10, 20, 40, 60, and 100 ng mL^{-1}). An irsogladine stock solution was serially diluted also with blank human plasma, resulting in quality control (QC) samples containing low (5 ng mL^{-1}, LQC), medium (50 ng mL^{-1}, MQC), and high (80 ng mL^{-1}, HQC) concentrations of irsogladine. A lamotrigine IS stock solution (20 μg mL^{-1} in methanol) was prepared. Each test (film-coated) and reference (sugar-coated) preparations of irsogladine maleate were kindly donated by Taejoon Pharm. Ltd. (Seoul, Republic of Korea) as 4 mg tablet dosage form, respectively.

Sample Preparation and HPLC–ESI–MS/MS. One-hundred microliters of each sample was combined with equal volume of methanol (containing 5 ng mL^{-1} of IS) and centrifuged. The supernatant (100 μL) was diluted with 900 μL of HPLC mobile phase. This mixture was loaded onto an Oasis mixed-mode cationic exchange (MCX) SPE cartridge (Waters, Milford, MA, USA), previously conditioned with methanol (3 mL) and then with HPLC mobile phase. The column was washed with 0.3% formic acid (6 mL) and then air dried completely. The absorbed irsogladine was eluted twice with 100 μL 0.5% methanolic ammonium hydroxide. The eluents were combined, evaporated to dryness by vacuum centrifugation at room temperature, and kept in a freezer prior to analysis. After that, the extracts were reconstituted in the HPLC mobile phase (50 μL), and a portion of this solution (10 μL) was subjected to HPLC–ESI–MS/MS analyses. The HPLC system consisted of a Spectra SYSTEM P1000XR quaternary pump, AS3000 auto-sampler, and an LTQ linear ion-trap MS from Thermo Finnigan (San Jose, CA, USA). Isocratic HPLC was performed using methanol–water–formic acid (65:35:0.2, *v*/*v*/*v*) at 130 μL min^{-1} flow rate. The HPLC

* Author for correspondence: jewonpark@korea.ac.kr

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column (Acquity BEH C_8, 1.7 μm, 2.1 × 100 mm, Waters) was operated at 35 °C for 5 min. Positive-ion MS spectra were acquired using an ESI source at atmospheric pressure. The optimized instrument settings were as follows: capillary temperature, 330 °C; capillary voltage, 37 V; spray voltage, 5 kV; a tube lens offset voltage, 70 V; auxiliary gas flow, 12; sheath gas flow, 20; relative collision energies (% RCE), 28.

Method Validation. According to the recent draft revision of US Food and Drug Administration guidance on bioanalytical method validation [8], the bio-analytical method validation described below was performed.

Recovery and Selectivity. QC samples with three different levels (n = 6) spiked with 5 ng mL^{-1} of IS were prepared, and the precision of irsogladine recovery at each level was evaluated. The recoveries were determined by comparing the concentrations obtained from the QC samples after Oasis MCX SPE with those from the irsogladine-free matrices with the spiked concentrations. The matrix effect was quantitatively examined by comparing the peak areas obtained from the extracts of two QC samples (LQC and MQC, n = 6) after SPE with those from the irsogladine standards (diluted in HPLC mobile phase) with the same concentrations.

Linearity and Sensitivity. Linearity of the calibration curve was examined as least-square liner regression by plotting the ratios of the peak area of irsogladine to IS acquired from seven-point calibration samples versus the nominal concentrations of irsogladine spiked. According to the comparison of the peak areas of analyte from the spiked samples (or analyte response) with that from the blank plasma sample (or blank response), the lower limit of quantification (LLOQ) was determined as the lowest concentration of irsogladine at a signal-to-noise ratio of 5:1 (n = 5).

Accuracy and Precision. The inter- and intra-day accuracy and their precision were presented as the percentage of the theoretical concentration and the percentage of relative standard deviations (RSD), respectively. The intra-day (within-run) precision and accuracy were investigated by analyzing five replicates of QC samples (n = 15) for a day, whereas the inter-day (between-run) precision and accuracy were evaluated by analyzing three batches of QC samples (n = 30) on two consecutive days.

Stability. The stability of irsogladine was examined by analyzing three replicates of two QC samples (LQC and HQC) subjected to specific conditions for different time intervals, and the percentage deviations were calculated by comparing the mean concentrations of irsogladine obtained from the QC samples with those from freshly prepared ones. In order to investigate the bench-top stability of irsogladine in human plasma, the above-described QC samples were stored at room temperature for three different time intervals (3, 6, and 9 h) prior to sample preparation. The freeze–thaw stability of the analyte was determined after three freeze–thaw cycles on consecutive days. The stability of irsogladine stock solutions spiked with IS (5 ng mL^{-1} of lamotrigine) was performed at a storage temperature of 4 °C for a week. Besides that, unlike other stability studies using two QC samples, the long-term stability of irsogladine in human plasma was evaluated only by analyses of LQC samples (n = 3) kept at −20 °C for 0, 6, 12, 24 h, and a month.

Bioavailability Study. The above method was employed to quantify the irsogladine concentration in a randomized and single oral dose bioequivalence study in 20 healthy volunteers. The clinical study was approved by the ethics committee, and all subjects signed an informed consent form. The volunteers were administered a single oral dose of 4 mg irsogladine maleate as reference or test preparation. Blood samples were collected at pre-dose and then at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 168, 336, and 504 h post-dose. The plasma was separated by refrigerated centrifugation at 3000 rpm for 5 min and then kept at −80 °C prior to analysis. The procedures described before (including methanol precipitation coupled with MCX SPE cleanup) were also applied to the above plasma samples prior to HPLC–ESI–MS/MS analysis. Pharmacokinetic parameters were determined from the plasma irsogladine concentration–time data by non-compartmental analysis using WinNonlin Professional software (version 5.1; Pharsight Co., Mountain View, CA, USA). The pharmacokinetic parameter measured from the data was area under the plasma concentration–time curve from 0 h to time (AUC_{0-t}), whereas the maximum plasma concentration (C_{max}) and the time (T_{max}) required to reach C_{max} were determined from the individual plasma concentration–time curve [9].

Results and Discussion

ESI–MS/MS Fragmentation Studies. The chemical structures of irsogladine and lamotrigine, as the IS, are shown in Figure 1. In addition, there was no matrix effect (i.e., retention time shift and non-symmetric peak) on the detection of irsogladine spiked into the QC samples. The MS/MS fragmentation patterns of authentic irsogladine and IS were examined to identify characteristic product ions and fragmentation processes. A $[M + H]^+$ molecular ion at m/z 256.1 for both irsogladine and its structural analog IS was used as the precursor ion for MS/MS experiment. MS/MS of the irsogladine precursor ion yielded a characteristic fragment ion at m/z 109.1, similar to IS (Figure 1). HPLC–ESI–MS/MS tracing was done in the selected reaction monitoring mode by choosing the mass pair specific to both analytes to detect the following transition: 256.1 > 109.1 for both irsogladine and IS. In the recent publication on the HPLC–ESI–MS/MS determination of

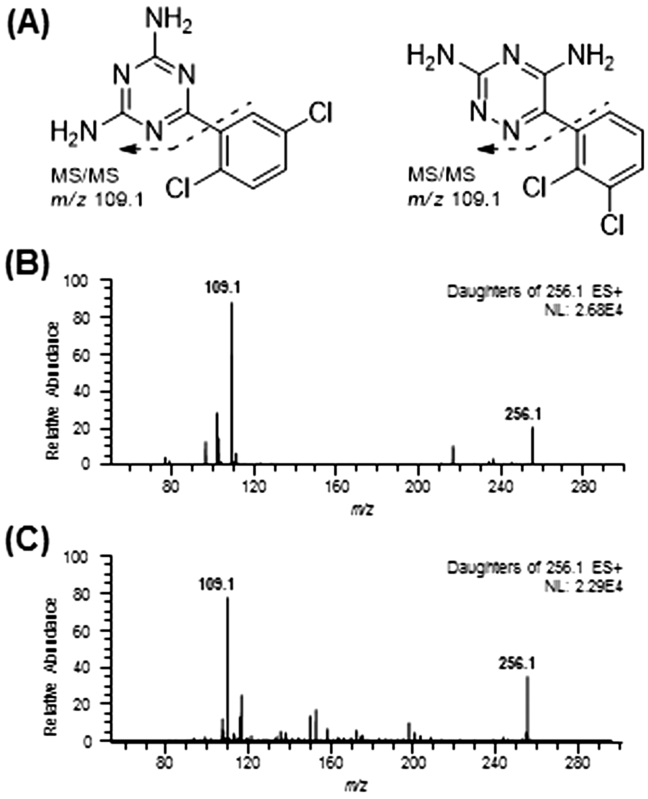

Figure 1. Chemical structures of (A) irsogladine (left) and lamotrigine internal standard (right) and representative fragmentation patterns of (B) the electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) spectra of the protonated molecular ion of irsogladine at m/z 256.1, (C) the ESI–MS/MS spectra of the protonated molecular ion of lamotrigine internal standard at the same m/z value

lamotrigine in human serum, the authors used a very similar mass transition (256.1 > 109.0), acquired for IS in this study [10].

Validation of HPLC–ESI–MS/MS Method. The retention times of lamotrigine IS and irsogladine, which were spiked into the QC samples, which in turn underwent optimized SPE cleanup procedures using the Oasis MCX cartridge, were 1.8 and 3.1 min, respectively (Figure 2). The representative chromatograms obtained from both the blank plasma sample and the spiked plasma at the LLOQ level (1.8 ng mL^{-1}) were also depicted in Figure 2, indicating the absence of any interfering peaks derived from the human plasma or IS. In addition, the matrix effects of human plasma on the irsogladine determination were estimated as 95.3 ± 4.6% and 93.2 ± 6.9% for LQC and MQC samples (Table 1), respectively, ensuring that the developed method herein is selective enough to be unaffected by the variations (i.e., endogenous components and metabolites) in the sample matrices.

Good linear correlations were obtained for the quantification of irsogladine (1.8–100 ng mL^{-1}) in the seven-point calibration curves as follows: $y = 0.1067x + 0.6965$, $r^2 = 0.9993$, where x represents the concentration of irsogladine spiked into the human plasma samples and y does the ratio of peak area of irsogladine to IS acquired by HPLC–ESI–MS/MS analysis. The lower limit of detection (LLOD) of irsogladine was determined to be 16 pg per injection (10 μL), whereas the LLOQ was approximately 1.8 ng mL^{-1}. The most recent publication employing HPLC with UV detection for irsogladine in tablet dosage forms reported that a linearity was within 10 to 80 μg mL^{-1}, as well as the LLOD obtained was 10 ng [5], demonstrating that the tandem MS/MS-aided analytical tool developed herein appears to be superior. Analyses of the human plasma samples spiked with irsogladine at LLOQ level (1.8 ng mL^{-1}) revealed that intra- and inter-day accuracy were 98.1 and 98.3% with %RSD values of 9.0 and 10.4%, respectively (Table 1). In addition, when we further examined the accuracy and precision of the developed method onto the QC samples with three different levels, the precision (%RSD) values were all below 7%, and the intra-day and inter-day accuracy ranged from 96.6% to 101.8% and 98.2% to 102.1%, respectively (Table 1). These results thus confirmed that the method herein appears to be reproducible, accurate, and precise. Recoveries of irsogladine spiked at 5, 50, and 80 ng mL^{-1} in QC samples were determined to be 92.7 ± 5.1%, 91.4 ± 4.8% and 89.6 ± 8.7%, whereas those of IS was averaged at 93.0 ± 4.9%.

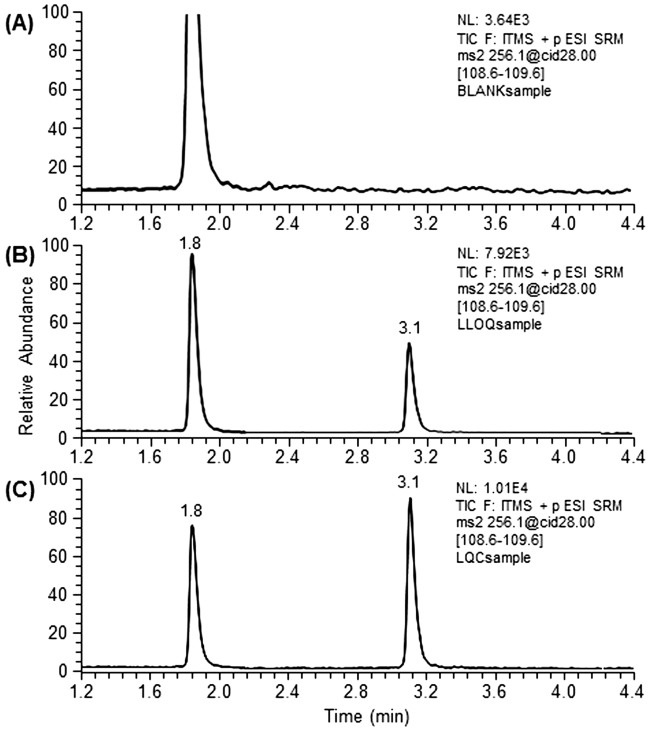

Figure 2. Representative chromatograms of (A) irsogladine-free blank human plasma, (B) irsogladine LLOQ sample, and (C) the extracts of a low-level QC sample supplemented with lamotrigine internal standard (retention at 1.8 min) and irsogladine (3.1 min), both at 5 ng mL^{-1}. Tracing was done by HPLC–ESI–MS/MS in the selective reaction monitoring mode for both analytes: 256.1 > 109.1

In case of short-term stability studies, the noticeable changes of irsogladine content were not observed; the deviations were found to be in the range of 93.6% to 99.8% and 96.9% to 100.6% for the bench-top stability (kept at room temperature for 9 h) and the freeze–thaw (3 cycles) stability studies, respectively. Furthermore, the long-term stability of QC samples ($n = 3$) kept at −20 °C for a month also exhibited 93.2% accuracy with 5.7% RSD (Table 2). Therefore, these stability results clearly indicated that irsogladine spiked in human plasma sample was stable up to 9 h at 25 °C, as well as the repeated freeze–thawing cycle did not influence the stability of the analyte. In case of irsogladine stock solutions, these were stable up to 7 days at 4 °C. This is the first report on the quantitative determination of the plasma irsogladine contents at the ppb (part per billion) level.

Applicability of the Validated Method to Bioequivalence Study. The validated analytical protocol was then used to evaluate the bioequivalence of reference and test preparations (4 mg irsogladine maleate) in 20 healthy volunteers. Typical time profiles vs. plasma concentration are exhibited in Figure 3, and the pharmacokinetic parameters are summarized in Table 3. The bioequivalence of two preparations was determined based on C_{max} and AUC_{0-504} by analysis of variance (ANOVA) and T_{max} by a Wilcoxon test. The times for reaching the maximum plasma concentration of irsogladine reference (sugar-coated) and test (film-coated) tablet were averaged at 1.4 and 1.3 h, respectively, whereas the peak plasma levels were 83.6 ± 10.6 and 84.0 ± 9.4 ng mL^{-1}, respectively. The elimination half-lives of irsogladine from the plasma were averaged within the range of 126.7 to 131.1, which were the same as those from the other bioequivalence study of irsogladine [7]. However, the elimination patterns found in this study were not in accordance with those (152 h) determined from the phase 1 study [6], and this inconsistency might be caused by the different dosage forms administrated. With respect to the abovementioned parameters, the results demonstrate that there was no significant difference between the two preparations (film- vs. sugar-coated tablet), thus concluding that the two preparations were almost bioequivalent.

Table 1. Intra- and inter-day accuracy, precision, recovery, and matrix effect of irsogladine supplemented into the blank human plasma

Batches	Intra-day ($n = 12$)			Inter-day ($n = 24$)			Recovery ($n = 9$, %, mean ± SD)	Matrix effect ($n = 6$, %)
Concentration added (ng mL^{-1})	Concentration found (ng mL^{-1}, mean ± SD)	Precision (%RSD)	Accuracy (%)	Concentration found (ng mL^{-1}, mean ± SD)	Precision (%RSD)	Accuracy (%)		
1.8	1.76 ± 0.2	9.0	98.1	1.77 ± 0.2	10.4	98.3		
5.0	4.83 ± 0.3	6.1	96.6	4.91 ± 0.3	6.8	98.2	92.7 ± 5.1	95.3 ± 4.6
50	50.2 ± 2.2	4.3	100.3	50.3 ± 2.3	4.7	100.6	91.4 ± 4.8	93.2 ± 6.9
80	81.4 ± 3.4	4.2	101.8	81.7 ± 3.1	3.9	102.1	89.6 ± 8.7	

Table 2. Stabilities of irsogladine in human plasma

Stability	Concentration added (ng mL^{-1}, n = 15)	Concentration found (ng mL^{-1}, mean ± SD)	Precision (%RSD)	Accuracy (%)
Bench top (up to 9 h)	5.0	4.7 ± 0.3	5.3	93.6
	80	79.8 ± 2.2	2.7	99.8
Freeze–thaw (3 cycles)	5.0	4.8 ± 0.4	8.9	96.9
	80	80.5 ± 3.5	4.4	100.6
Long term (a month)	5.0	4.7 ± 0.3	5.7	93.2

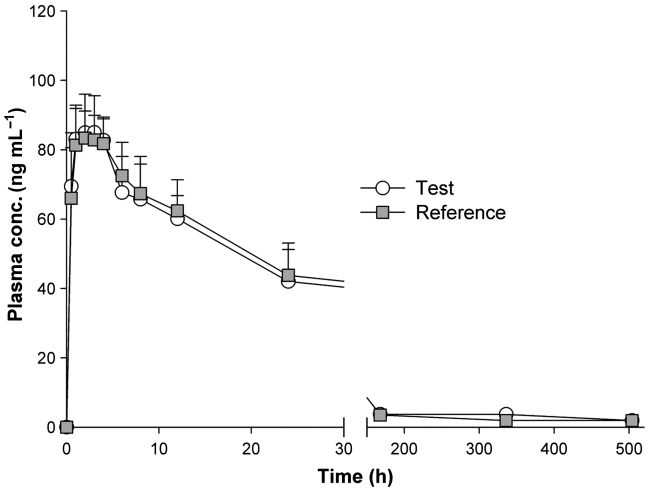

Figure 3. Mean plasma concentration–time profiles of irsogladine maleate after single oral dose of test and reference preparations to twenty healthy volunteers

Table 3. Pharmacokinetic parameters of irsogladine in healthy volunteers (mean ± SD, n = 20)

Parameters	Test tablet	Reference tablet
C_{max} (ng mL^{-1})	84.0 ± 9.4	83.6 ± 10.6
T_{max} (h)	1.3 ± 0.7	1.4 ± 0.6
$T_{1/2}$ (h)	126.7 ± 12.9	131.1 ± 16.2
$AUC_{0–504}$ (ng h mL^{-1})	11,005.4 ± 2028.6	11,442.6 ± 2127.2

Conclusions

The described method combining cationic-exchanging SPE cleanup, HPLC chromatographic separation, and ESI–MS/MS quantification provided a selective, sensitive, fast, and reproducible measurement of irsogladine in the human plasma, guarantying its successful application to a bioequivalence study. Indeed, as described earlier in Results and Discussion section, the validation parameters (including LLOD and linearity) obtained from this study were superior to those from the most recent publication [5], indicating that the HPLC–ESI–MS/MS method developed is the most sensitive tool available for the irsogladine bio-analysis.

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