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Research Article

Development and validation of a highly specific for quantification of irbesartan in human plasma and its application to a bioequivalence study

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ABSTRACT

A simple LC-MS/MS method was developed for the determination of irbesartan in human plasma with high sensitivity and specificity. The extraction process utilized a liquid-liquid extraction technique with a mixture of ethyl acetate and hexane (90:10, v/v), achieving highly efficient recovery of irbesartan. Chromatographic separation was performed on a Luna® HST C_{18} column (50 mm x 3 mm, 2.5 µm), using a mobile phase consisting of 0.1% aqueous formic acid and acetonitrile (33: 67, v/v) at flow rate of 0.2 mL/min, with a total runtime of 4.0 minutes. Irbesartan was detected by tandem mass spectrometer with positive ionization mode using the multiple reaction monitoring (MRM). The mass transition ion-pairs were m/z 428.95>206.96 and 428.95>195.01 amu for $[Irbesartan+H]^+$ and m/z 435.98>234.97 and 435.98>291 amu for $[Valsartan +H]^+$ (internal standard, IS), with, retention times of 1.44 and 2.24 minutes respectively. Key parameters of full validation were evaluated, and all consistently met the acceptance criteria. The limit of detection (LOD) and lower limit of quantification (LLOQ) for irbesartan were determined to be 60 pg/mL and 5.00 ng/mL, respectively. The method demonstrated linearity over concentration ranges of 5.00 - 6012.62 ng/mL with a correlation coefficient (r) consistently greater than 0.997(n = 3) using $1/X^2$ weighting. The within-run precision ranged from 2.43% to 7.61% and with accuracy from 92.42 to 106.20%. The between-run precision ranged from 4.73% to 8.66% with accuracy from 98.56%to 101.20%. Additionally, we investigated the effects of different plasma conditions, including hemolysed and hyperlipidemic plasma, on accuracy and precision. The results demonstrated that all measured values fell within acceptable tolerance limits. The relative recovery of irbesartan was determine to be 80.34%, 75.32%, and 74.26% for the LQC, MQC and HQC levels, respectively, while the IS demonstrated a relative recovery of 76.93%. The matrix effect exhibited no significant interference, as evidenced by the comparison of peak responses from six determinations at LQC and HQC levels, prepared in extracted drug-free human plasma obtained from six individual normal plasma sources, with those of neat standards at the corresponding concentrations. This ensures the reliability of the quantification. The calculated matrix factor values were 0.91 for LQC and 1.00 for HQC for irbesartan, while the matrix factor for valsartan was 0.92. Moreover, the IS-normalized matrix factor values were 0.99 for LQC and 1.09 for HQC for irbesartan. In addition, no significant matrix effect was observed when hemolysed and hyperlipidemic plasma samples were analyzed. Stability studies confirmed that irbesartan remained stable in human plasma under various conditions. It was stable for up to 29 days at -80˚C for long term storage, up to 24 hours at 4˚C and up to 6 hours at room temperature (25˚C). The new LC-MS/MS method exhibit high sensitivity, specificity, and a broad wide linearity range, (5 to 6000 ng/mL) with a short run time of 4.0 minutes using valsartan as an internal standard. The method was developed and validated using a single-step liquid – liquid extraction requiring only 100 µL of plasma. This positive ESI-LC-MS/MS method is simple, reproducible and robust, allowing high-throughput analysis with a large sample capacity per batch. It was successfully applied to the quantification of irbesartan in human plasma for bioequivalence studies of higher strength of irbesartan tablet (300 mg) in Thai volunteers.

Keyword: irbesartan, LC-MS/MS, Method development and validation, bioequivalence, liquid-liquid extraction

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1. INTRODUCTION

Irbesartan is a potent, long-acting and high selective angiotensin II receptor antagonist that specifically binds to the AT1 receptor subtype and widely indicated for the treatment of hypertension with safe and well tolerated[1]. It's chemical name is 2-Butyl-3-({4-[2-(2H-1,2,3,4-tetrazol-5-yl) phenyl]phenyl}methyl)-1,3-diazaspiro[4.4]non-1-en-4-one ($C_{22}H_{28}N_6O$, Figure 1) and molecular weight of 428.53 g/mol[1-2]. The chemical formula of irbesartan represent a five-membered hetero-cyclic ring with four nitrogen atoms, classifying it as a tetrazole derivative antihypertensive agents. It's a high permeability and low solubility antihypertensive agents with an absolute bioavailability of 60-80% and reaches peak plasma concentration within 2 h after oral adminis-tration. Irbesartan is also the first choice of anti-hypertensive agent for treatment of mild to moderate hypertension [3-4]. Irbesartan is important and interred target for the pharmaceutical industry of generic drug in Thailand.

In this present work, we aimed to develop a robust and efficient method for the determination of plasma irbesartan using high performance liquid chromatography–positive electrospray ionization mass spectrometry (LC-MS/MS). LC-MS/MS is a powerful analytical technique that combines the separation capability of liquid chromatography (LC) with the high specificity and sensitivity of mass spectrometry (MS). A triple-quadrupole mass spectrometer, tandem mass spectrometry to enable ion selection and fragmentation for enhanced analyte detection. The first quadrupole (Q1) scans a selects a specific precursor ion for analysis. The selected ion then undergoes collision-induced dissociation (CID) in the second quadrupole (Q2), which functions as a collision cell by introducing a collision gas (Argon) into the ion's flight path. The resulting fragment ions are subsequently analyzed by the third quadrupole (Q3), allowing for highly specific and sensitive quantification. The use of electrospray ionization (ESI) in positive mode enhances ionization efficiency while maintaining a relatively gentle ionization process, making it particularly suitable for low-level drug bioanalysis. Compared to conventional chromatographic methods, LC-MS/MS provides superior selectivity, lower detection limits, and the ability to analyze complex biological matrices with minimal interference[5]. Although analytical literatures have been previously reported for irbesartan determine methods including chromatography methods using liquid chromatography with ultraviolet detection[6-8], fluorescence detection[9] or mass spectrometry[10-12]. Several previously developed methods exhibited varying analytical run times, (2.5-35 minutes)[6-15] resulting in higher consumption of chemicals, reagents and time consuming[6,8]. More over some of the previously developed methods exhibited low sensitivity, with LLOQ ranging from 45 to 50 ng/mL[11, 13] that needs to usage more volume of sample[13, 15] and some of them had complex, multi-steps sample preparation procedures[13]. The linearity range and sensitivity remain a major challenge in developing methods for accurate bioanalysis[10,12,14]. Insufficient linearity, especially at maximum blood concentration (C_{max}), requires sample dilution and reanalysis, which adds complexity, extends the bioanalysis workflow and increases uncertainties in analytical results. To address these limitations, we developed and validated a highly sensitive and specific LC-MS/MS method with a broad linearity range (5–6000 ng/mL) and a short run time of 4.0 minutes, using valsartan (Figure 1) as an internal standard. Our method employs a single-step liquid–liquid extraction requiring only 100 µL of plasma, with a limit of detection (LOD) of 60 pg/mL and an LLOQ of 5 ng/mL. The method is simple, reproducible, and rugged, allowing for the high-throughput analysis of irbesartan in human plasma. Furthermore, plasma types were carefully selected to assess matrix effects on accuracy and precision. Hemolysed plasma, containing lysed red blood cells, may alter protein binding and ionization efficiency, while hyperlipidemic plasma, with high lipid content, can introduce matrix interferences. Evaluating these conditions ensured the method's robustness and accuracy across physiological variations. The results demonstrated that our LC-MS/MS method offers excellent selectivity, sensitivity, precision, and accuracy, making it suitable for bioequivalence studies involving higher-strength irbesartan formulations (300 mg tablets) in Thai volunteers.

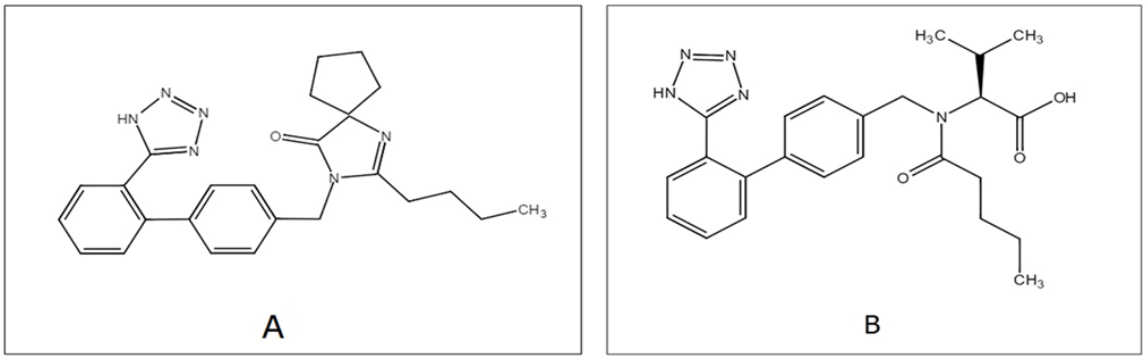

Figure 1 Chemical structures of irbesartan (A) and valsartan (B)

2. MATERIALS AND METHODS

2.1 Instrumentation

The liquid chromatography system was performed on **s**eparation module of a Acquity Ultra Performance LC™, (Waters, Co., Ltd. USA) equipped with a Quattro Premier XE mass spectrometer, (Micromass Technologies, UK). Data acquisition and analysis were performed using Masslynx 4.1 SCN627 software (Micromass Technologies, UK). Chromatographic separation was achieved using a Luna® HST C_{18} column (50 mm x 3 mm, 2.5 µm) (Phenomenex Inc.,Torrance, CA) maintained at 30±5°C.

2.2 Chemicals and reagents

Reference substance of irbesartan, (on the as is basis; 99.6%), valsartan (on the anhydrous basis, 99.8%), Acetaminophen, (on the as is basis; 99.8%), and Chlorpheniramine Maleate, (on the as is basis; 99.8%) were purchased from The United States Pharmacopeial Convention, Inc. (USA). Type I water was prepared by a Milli Q system (Millipore Corporation, Massachusetts, USA). HPLC-grade acetonitrile and propan-2-ol, analytical reagent grade ethyl acetate and hexane were purchased from Scharlau, (Barcelona, Spain). HPLC-grade methanol was purchased from Fisher Scientific (Loughborough, United Kingdom). Analytical reagent grade formic acid was purchased from Merck (Darmstadt, Germany). Six individual sources of normal drug-free human plasma anticoagulated with dipotassium ethylenediaminetetraacetic acid (K_2EDTA), one source of hyperlipidaemia and one source of haemolysed plasma were obtained from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. These plasma sources were used for all experimental purpose related to method validation parameters.

2.3 Standard solution preparation and calibration curve

Irbesartan primary stock standard solutions were accurately prepared in methanol for two separate sets for the calibration standards (CS) and quality control (QC) with a final concentration of 1.82 and 1.60 µg/mL, respectively. Working standard solutions were prepared by diluting stock standard solution with 50% methanol achieve a final concentration of the linearity range of analytical detection was 5.00 to 6012.62 ng/mL. The final concentrations in 100 µL of 8 different concentration levels of the CS samples were detailed as 5.00, 10.00, 100.20, 751.50, 1,503.00, 3,000.99, 4,809.60, 6,012.00 ng/mL and four different concentration levels of the QC sample at 5.01, 15.02, 2502.75 and 4503.95 ng/mL as lower limit of quantification (LLOQ), validated low quality control sample (LQC), validated medium quality control sample (MQC) and validated high quality control sample (HQC) concentration of Irbesartan, respectively. All of standard solutions and sample preparation were prepared under protected from light condition and stored at -70 ± 10 °C upon use.

2.4 LC-MS/MS and chromatographic conditions

Chromatographic separation was performed using a Luna® HST C_{18} column (50x3 mm, 2.5µm, Phenomenex, USA) thermostated at 30±5°C. The mobile phase was Milli Q water containing 0.10% (v/v) formic acid (solvent A) and acetonitrile (solvent B). (33:67, v/v (%)). The separation was performed under isocratic conditions with a constant flow rate of 0.2 mL/min. LC–MS/MS experimental conditions utilized the multiple reaction monitoring (MRM), detection of irbesartan, and internal standard (valsartan) was performed in the positive ESI mode for their respective $[M-H]^+$ ions. at the following transitions: m/z 428.95>206.96 and 428.95>195.01 amu and 435.98>234.97 and 435.98>291 amu for the quantification and confirmation of [Irbesartan $+H]^+$,and [Valsartan $+H]^+$ respectively. Instrument settings of the MS/MS were optimized as follows: source temperature 120°C; desolvation temperature 350°C; cone gas flow 30 L/Hr and desolvation gas flow 650 L/Hr. The injection volume was 2 µL and the autosampler temperature was 10±5°C.

2.5 Sample preparation

Sample extraction was performed using a liquid-liquid extraction (LLE) technique, followed by chromatographic separation of the irbesartan and its internal standard (valsartan) using an LC-MS/MS system. All procedures were carried out under light-protected conditions. The developed extraction method was applied to calibration standards, quality control (QC) samples and clinical samples. Briefly, 100 µL of plasma was mixed with 20 µL of the internal standard solution (valsartan), followed by the addition of 30 µL of 1 M formic acid to acidify the sample. Subsequently, 1 mL of extraction solvent (ethyl acetate: hexane, 9:1, v/v) was added, and the mixture was thoroughly vortexed. After phase separation, 800 µL of the organic layer was carefully transferred and evaporated to dryness under a gentle stream of nitrogen at 30 °C. The dried residue was reconstituted in 600 µL of a mixture of acetonitrile and 0.1% formic acid (1:1, v/v), and a 10 µL aliquot was injected into the LC–MS/MS system for analysis.

2.6 Method validation

This LC-MS/MS method was validated in terms of selectivity, selectivity in presence of concomitance drug, carry over, recovery of extraction, matrix effect, linearity, LLOQ, intra-day and inter-day precision and accuracy, stability(short-term, long term, freeze and thaw stability, post-preparative stability, stock solution stability, re-injection reproducibility), dilution integrity, robustness, effect of hemolysed and hyperlipidaemic plasma on accuracy and precision, and accuracy and precision of QC samples in an analytical batch run. Each calibration curve included 2 blank samples (plasma without internal standard and plasma with internal standard), and 8 concentrations of CS samples. Set of 6 replicate QC samples was included 5.01, 15.02, 2502.75 and 4503.95 ng/mL for LLOQ, LQC, MQC and HQC, respectively were used in general term of validation runs. Set of 4 replicate QC samples was included 15.02, and 4503.95 ng/mL for, LQC, and HQC, respectively were used in stability studies terms of validation runs. The CS and QC sample was performed by spiked irbesartan standard solution into pooled plasma from six sources of normal drug-free human K_2EDTA plasma for general validation. Specific studies performed by using hemolysed drug-free human K_2EDTA plasma and hyperlipidaemic drug-free human K_2EDTA plasma.

2.7 Matrix effect

The matrix effect is defined as the direct or indirect alteration in analyte response caused by co-eluting, undetected compounds present in the sample matrix. To evaluate potential ionization suppression or enhancement caused by human plasma, the matrix effect was assessed at two concentration levels: low quality control (LQC; 15.02 ng/mL) and high quality control (HQC; 4503.95 ng/mL). For each level, four replicates of drug-free human plasma extracts from six different individual sources were post-spiked with irbesartan and the internal standard (valsartan) after extraction. The peak responses were compared to those of neat standard solutions at equivalent concentrations. For each analyte and internal standard (IS), the matrix factor (MF) is calculated as:

MF = (Peak response in presence of matrix ions)/(Peak response in absence of matrix ions

- Peak response in presence of matrix ions: Peak area obtained from a blank matrix spiked with the analyte after extraction.
- Peak response in absence of matrix ions: Peak area obtained from a neat solution of the analyte in solvent.

For IS-normalized matrix factor assessment (IS-normalized MF), the MF of irbesartan was divided by the MF of the internal standard. The IS-normalized MF was calculated as:

$$\text{IS-normalized MF} = MF_{analyte} / MF_{IS}$$

- $MF_{Analyte}$: Matrix factor of the analyte.
- MF_{IS}: Matrix factor of the internal standard (IS).

Matrix factor between 0.85 and 1.15 was considered acceptable, indicating no significant matrix effect. A value below 0.85 indicated ion suppression, whereas a value above 1.15 indicated ion enhancement. The coefficient of variation (%CV) of matrix factors and IS-normalized MF across the six plasma sources was required to be less than 15%.

2.8 Carry over

Carry-over refers to the presence of residual analyte signal in a blank sample following the injection of a sample with a high analyte concentration. To evaluate this effect, a blank plasma sample was injected immediately after the upper limit of quantification (ULOQ) sample. This assessment aimed to determine whether residual analyte or internal standard remained adsorbed onto or retained within the LC–MS/MS system components (e.g., injector, tubing, or analytical column). Carry-over was considered acceptable if the peak area of any interfering signal in the blank sample did not exceed 20% of the analyte response at the lower limit of quantification (LLOQ) for irbesartan, and 5% of the LLOQ response for the internal standard, valsartan.

2.9 System suitability

System suitability testing was performed to verify the proper functioning of the LC–MS/MS system prior to the analysis of each analytical batch. A reference standard solution containing irbesartan and the internal standard (valsartan) was injected at the start of each run to assess instrument sensitivity, chromatographic retention, and peak shape. The acceptance criteria required that the retention times remain within ±2% of the established method setpoint and that the signal intensity be adequate, with a signal-to-noise (S/N) ratio greater than 10 at the LLOQ level. These parameters ensured consistent and reliable system performance prior to sample analysis.

2.10 Robustness

Robustness was assessed by introducing minor but intentional variations in critical analytical parameters

to evaluate the method's reliability under routine laboratory conditions. Two conditions were tested: (1) replacing the analytical column with another of the same type and specification (Luna® HST C18, 50 × 3.0 mm, 2.5 µm), and (2) preparing key reagents (extraction solvent, reconstitution solvent, and mobile phase) by a different analyst. Six replicate QC samples at four concentration levels (LLOQ, LQC, MQC, HQC) were analyzed under each condition. Method performance was evaluated based on accuracy, precision (%CV), and retention time reproducibility, with a %CV of ≤2% accepted for chromatographic stability.

2.11 Bioequivalence study design

An open-label, single-dose, randomized, two-treatment, two-period, two-sequence crossover study was conducted in 24 healthy Thai volunteers at the Siriraj Clinical Research Center. Subjects received a 300 mg dose of irbesartan from each formulation with

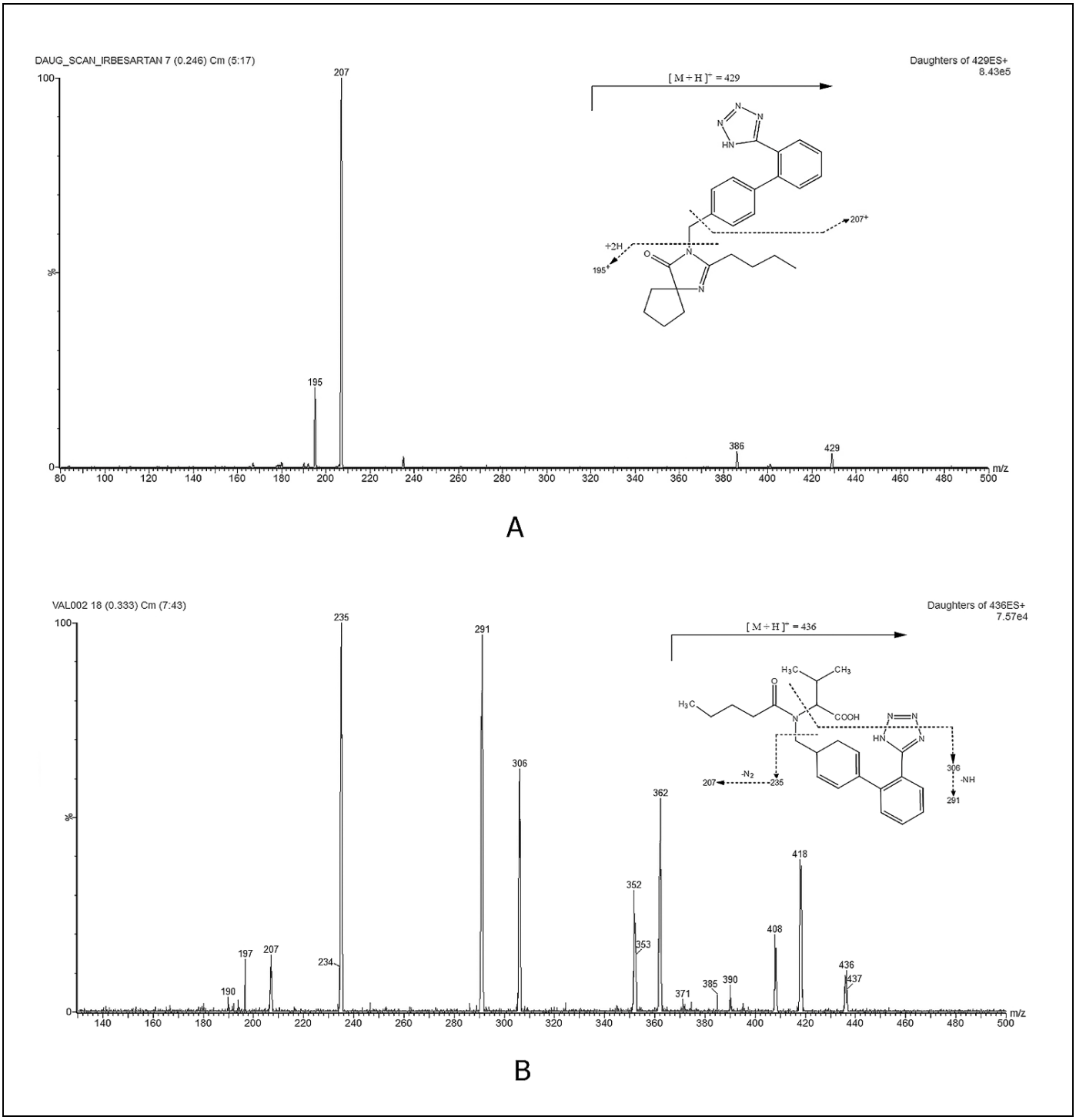

Figure 2 The product ions scan mass spectra and chemical structure of (A) Irbesartan and (B) Valsartan, (IS).

at least 14-day washout period. Blood samples (15 time points: pre-dose and up to 72 h post-dose) were collected in K_2EDTA tubes and immediately centrifuged. Plasma was separated, stored at −70°C, and analyzed using the validated LC-MS/MS method. The study protocol was approved by the Siriraj Institutional Review Board (Approval No. SI 220/2015) and conducted under GCP guidelines.

3. RESULTS AND DISCUSION

3.1 LC-MS/MS Method development for irbesartan bioanalysis

The LC-MS/MS system has been complete optimization process for the best specific experimental condition. The tandem mass spectrometer was infused a solution containing standard of irbesartan or valsartan (I.S.) directly into the electrospray ionization source. The manually fine tuning of the mass spectrometer by using adding of 5 µL/min flow of 500 ng/mL irbesartan standard solution through a T-connector between the LC system and the mass spectrometer with a mobile phase was consisting of Milli Q water containing 0.10% (v/v) formic acid (solvent A) and acetonitrile (solvent B). The isocratic elution mode of mobile phase was performed by 33%A and 67%B. The irbesartan molecule ion can be identified and improve the detection specificity. The following parameters of mass spectrometer were applied during measurements as source temperature,120°C; desolvation temperature,350°C; cone gas flow,30 L/Hr; desolvation gas flow,650L/Hr. Compound dependent parameters were set as followers: voltage of the source with positive electrospray ionization (ES+), capillary voltage (3.2 kV), cone voltage (30.0 V) and optimized the collision energy for the best abundant and specific daughter ions. Our observation revealed that the abundant of $[Irbesartan+H]^+$ and $[Valsartan +H]^+$,(I.S.) in ESI positive ion mode higher than in negative ion mode for 5-10 fold. The full scan spectra shown prominent and stable product ions fragmentation and no adduct ions of all compounds was found. (Figure 2) The present LC-MS/MS analyses were conduct using multiple reaction monitoring (MRM) detection mode, which provided a high selectivity for the quantification of irbesartan. The mass transition ion pairs were employed to monitor specific precursor-to-product ion transitions at m/z 428.95>206.96 and 428.95>195.01 amu for the quantification and confirmation of $[Irbesartan +H]^+$, respectively. Similarly, the transitions at m/z 435.98>234.97 and 435.98>291 amu were utilized for the quantification and confirmation of $[Valsartan +H]^+$ (internal standard, IS), respectively. This analytical approach ensured the accuracy, precision and highly specific selectivity of the method for irbesartan (*m/z* 428.95), while effectively eliminating the possibility of false-positive findings. The chromatographically separation was using reversed-phase high performance liquid chromatography with isocratic elution. Irbesartan and its internal standard were separated on a Luna HST C18 column (50x3 mm, 2.5µm, Phenomenex, USA) with column temperature at 30±5°C. The sample loop for injection mode was full loop with injection volume of 2 µL. All samples were placed in a sample organizer at 10±5°C. The syringe cleaning system were operated before and after sample injection using 200 µL of a weak wash solvent as Milli Q water containing 80% (v/v) acetonitrile and followed with 600 µL of a strong wash solvent as 5% propan-2-ol in acetonitrile containing 0.1% (v/v) formic acid. A mobile phase was consisting of Milli Q water containing 0.10% (v/v) formic acid (solvent A) and acetonitrile (solvent B). The isocratic elution mode of mobile phase was performed by 33%A and 67%B and delivered with a flow rate of 0.200 mL/min with 4.0 minute of total run time. The retention time for irbesartan and valsartan (IS) were 1.44 and 2.24 minutes respectively. The chromatographic separation developed in this present work revealed that enables efficient analysis with highly sensitivity and reproducibility, providing a typical peak shape, consistent retention time and signal to noise ratio within a short LC run time. The chromatographic conditions were using a reversed phase isocratic liquid chromatography method. In this study, the use of non-volatile salts was avoided to prevent the ion suppression in the LC-MS/MS system.

3.2 The sample extraction procedure

Sample preparation for LC-MS/MS analysis is critical steps in bio-analysis because it's significantly impacted MS result by enhance or suppress ionization of the interesting substance. In this work, proper sample extraction workflow was studied. Sample extraction was performed by liquid-liquid extraction technique (LLE) and then was followed by chromatographic separation of the irbesartan and its internal standard (valsartan) on an LC-MS/MS system. From the chemical formula of irbesartan represent a tetrazole agent. It's containing acid groups with pKa value as 4.24 and a partition coefficient (octanol/water) of 10.1 at pH of 7.4. The several of non-polar organic solvents were tried for the best extraction solvent. The result shown that a mixture of ethyl acetate: hexane, 90:10 (v/v) has shown suitable with highly recovery result. The acidic modifier was required to achieve better peak area and shape. Briefly, 20 µL of valsartan as an internal standard (IS) working solution (1,000.17 ng/mL) was added in 100 µL of irbesartan standard spiked plasma sample and then mixed by a Vortex Genie2 G5605 (Scientific Industries, USA). All samples were adjusted to acidic pH and mixed with 1,000 µL of an extract

solvent; ethyl acetate: hexane, 90:10 (v/v) for 10 minutes then centrifuged at 10,000 rpm, 4°C for 15 minutes by a refrigerated centrifuge Legend RT (Sorvall, Germany). The organic layers were transferred into a new conical polypropylene tube and were evaporated under a nitrogen stream until the sample was dried by a Turbo Vap LV evaporator (Caliper LifeSciences, USA). The residues were reconstituted and were injected into the LC-MS/MS system. The samples were prepared under the protected from light condition. This present work aimed to develop a simple assay with a single step of sample extraction, achieving a highly consistent recovery coefficient ranging from of 80.34 at the low-quality control (LQC) to 74.26 at the high-quality control (HQC). The matrix effect on the ionization of irbesatan and the internal standard (IS) from plasma matrix was thoroughly investigated. It had no effect in the electrospray ionization source, with values ranging from 0.91 to 1.00 for LQC and HQC level for irbesartan and 0.92 for valsartan. The sample preparation procedures described in this study were applied to standard spiked samples calibration standard and quality control sample, as well as clinical samples.

3.3 Assay performance and validation

Our present quantitative LC-MS/MS method has been developed. It is importance to evaluate the performance of this developed method that suitable for quantitative analysis. Method validation was performed followed by the requirement set by USFDA[16]/EMEA[17] guidelines. These two standard guidelines are the most accepted in the pharmaceutical industry. All of validation terms proposed by the standard guidelines have to consider for bioanalytical method and all validation results met the acceptable limit of standard guidelines. The validation results are shown in Table 1-3.

Table 1 Precision and accuracy of irbesartan calibrations standards (CS) in human plasma obtained on the developed LC-MS/MS method.

CS Level (ng/mL)	1	2	3	4	5	6	7	8
Nominal Concentration	5.00	10.00	100.21	751.58	1503.16	3001.30	4800.08	6012.62
Measured Concentration	5.05	9.79	101.07	774.26	1528.22	3025.42	4721.79	5796.02
% CV	3.73	7.32	3.79	2.12	1.34	3.14	4.34	1.26
% Accuracy	100.98	97.91	100.86	103.02	101.67	100.80	98.37	96.40

[a]Mean, n = 3 sets of calibration curve

Table 2 Calibration curve equation of Irbesartan[b]

Linearity	Calibration curve equation	r^2
Linearity 1	0.101068 * x + (-0.015999)	0.997075
Linearity 2	0.101447 * x + (-0.0129237)	0.998940
Linearity 3	0.101695 * x + (-0.0318977)	0.998444

[b] The calibration curve with a $1/x^2$ weighing factors.

Table 3 Within-run and between-run precision and accuracy study for irbesartan quality control sample (QC).

	Nominal concentration (ng/mL)		Measured value (ng/mL) Mean ± SD	Accuracy		Precision	
				Within-run[c]	Between-run[d]	Within-run[c]	Between-run[d]
LLOQ	Day 1		4.63 ± 0.19	92.42		4.21	
	Day 2	5.01	5.32 ± 0.36	106.20	98.56	6.80	8.66
	Day 3		4.81 ± 0.37	96.03		7.61	
LQC	Day 1		14.02 ± 0.67	93.35		4.77	
	Day 2	15.02	15.66 ± 0.75	104.23	98.56	4.77	6.27
	Day 3		14.74 ± 0.56	98.11		3.82	
MQC	Day 1		2484.98 ± 0.67	99.29		3.54	
	Day 2	2502.75	2626.78 ± 96.27	104.96	101.20	3.66	4.73
	Day 3		2486.80 ± 126.20	99.36		5.07	
HQC	Day 1		4261.07 ± 141.63	94.61		3.32	
	Day 2	4503.95	4700.91 ± 114.38	104.37	100.47	2.43	5.30
	Day 3		4612.78 ± 179.25	102.42		3.89	

[c] Six replicates (n=6) at each concentration of QC level for within-run analysis.
[d] Three runs (n=18) at each concentration of QC level for between-run analysis.

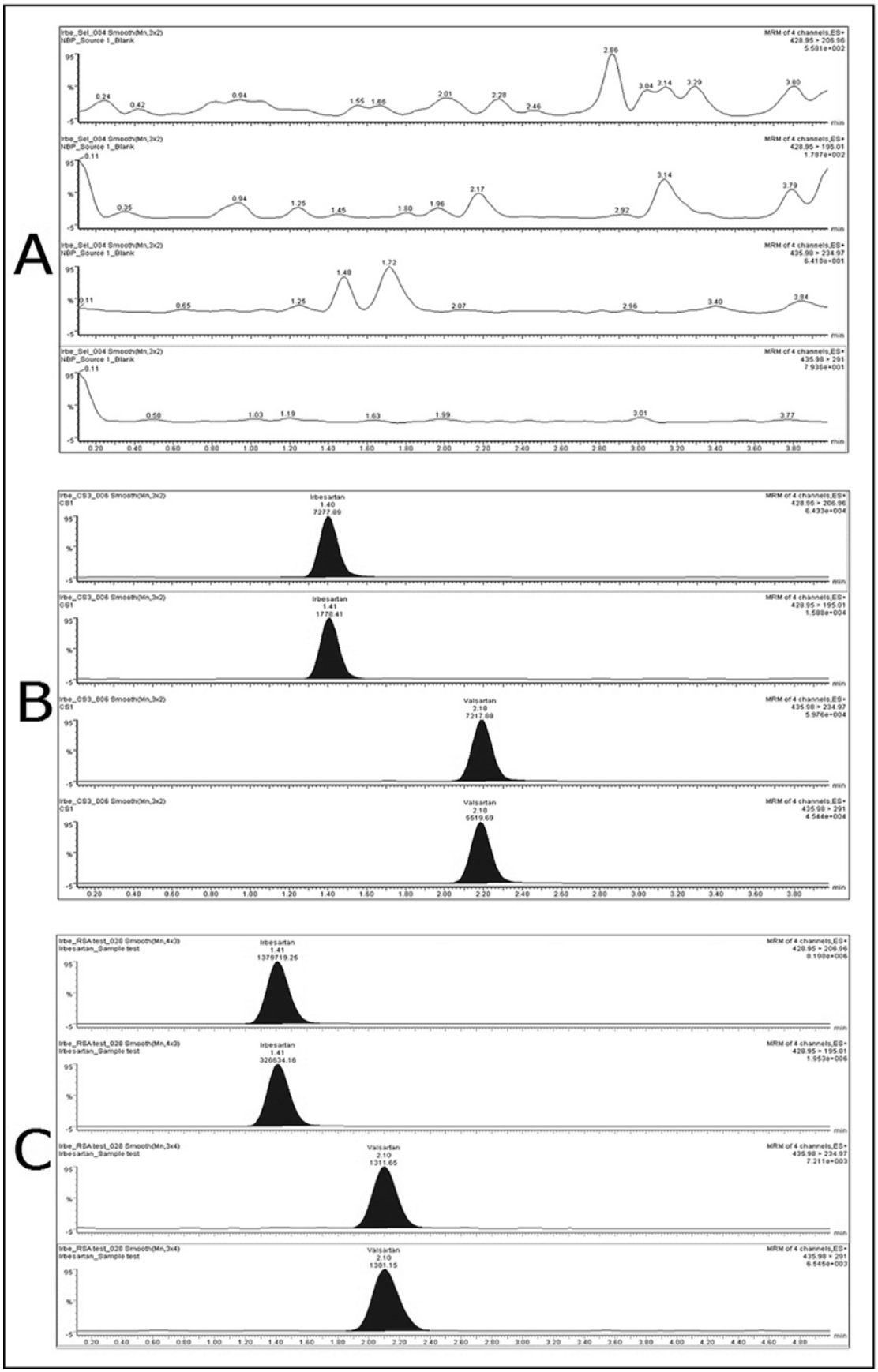

Figure 3 The representative LC-MS/MS chromatograms obtained from (A) blank human plasma sample, (B) LLOQ plasma sample at 5 ng/mL (C) clinical plasma sample at 1 h after an oral administration of 300 mg irbesartan in a fasting state. All of samples were processed by the developed extraction procedure and LC-MS/MS conditions.

3.3.1 Specificity/selectivity

The chromatograms of selectivity (Figure 3) were free from co-eluting peaks and no cross-irbesartan/internal standard interference was observed. No interference peaks from the endogenous plasma matrix, were detected at the retention time of irbesartan. Irbesartan and the IS had a good peak shapes and good separation. The specificity/selectivity test was compared with those obtained in absence and presence of concomitant medication were no interference in terms of retention time and peak area of the irbesartan at a concentration of the LLOQ (5 ng/Ml) and the IS. The specificity/selectivity result proved this analytical method, which appropriate with highly sensitive and specific for determination of irbesartan and valsartan (IS) in human plasma.

3.3.2 Sensitivity

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can be reliably distinguished from background noise using the chromatogram generated by the developed analytical method. The LOD is typically determined from non-extracted samples, prepared by diluting the analyte in an appropriate solvent such as the working standard solution, reconstitution solvent, or mobile phase. An S/N (signal-to-noise) ratio of at least 3 is generally required to confirm detection. The lower limit of quantification (LLOQ) is defined as the lowest concentration of an analyte that can be quantitatively determined with acceptable accuracy and precision. The LLOQ is established based on the analysis of extracted samples using the validated method, and the analyte response at this level should be at least five times the response observed in a blank sample, corresponding to an S/N ratio of ≥5. For irbesartan, the LOD was determined to be 60 pg/mL, while the LLOQ was established at 5.01 ng/mL. Representative chromatogram of irbesartan and the internal standard (valsartan) at the LLOQ level in human plasma is presented in Figure 3(B). The precision and accuracy of measurements at the LLOQ concentration are summarized in Table III. Within-run precision ranged from 4.21% to 7.61%, with corresponding accuracy values between 92.42% and 106.20%. Between-run precision and accuracy were 8.66% and 98.56%, respectively.

The potential impact of hemolyzed and hyperlipidemic plasma on assay performance at the LLOQ concentration was also evaluated. Precision in hemolyzed and hyperlipidemic samples was 6.62% and 7.21%, respectively, while accuracy was 105.93% and 103.00%, respectively. These results indicate that neither hemolysis nor hyperlipidemia had a significant effect on the accuracy or precision of the assay at the LLOQ level.

3.3.3 Carry-over

The carry-over of chromatographic system was tested in order to evaluate the possibility carry-over effect of previously injected sample which absorbed on or trapped within the LC-MS/MS system. No significant carry-over effect at the retention time was observed. The chromatographic system was clear from interfering peak when neat blank samples were sequentially injected followed by the highest standard sample into the LC-MS/MS system. The acceptance criteria were limit the peak response area of interfering peak in last neat blank sample was less than 20% and 5%, for Irbesartan and Valsartan (IS) respectively compared with LLOQ.

3.3.4 Linearity and weighting factor determination

Linearity of the assay was demonstrated by extracted and analyzed of 3 sets of calibration standards consisted of eight non-zero standards of irbesartan. The calibration curves were found to be linear over the concentration range of 5 to 6012 ng/mL. The results showed that the response of all concentrations was less than 20% CV of the LLOQ from nominal concentration and 15% CV of standards other than LLOQ from nominal concentration (Table 1). The calibration model was selected based on the analysis of the data by linear regression with or without weighing factors (none, 1/x and $1/x^2$). The best linear fit and least square residuals for the calibration curve with a $1/x^2$ weighing factors, giving a representative mean linear regression equation for the calibration of irbesartan (Table 2). The coefficient of determination (r^2) was 0.997, 0.999, 0.998 for three sets of calibration curve, respectively.

3.3.5 Recovery

The effectiveness of the extraction procedure was evaluated. The relative recovery of irbesartan were 80.34%, 75.32%, and 74.26% for LQC, MQC and HQC levels. The relative recovery of valsartan (IS.) was 76.93 %. The results indicated that the extraction procedure of this study had good efficiency and reproducibility.

3.3.6 Matrix effect

The presence of endogenous matrix components in plasma samples can potentially affect the ionization efficiency of irbesartan in LC–MS/MS analysis. Therefore, evaluation of the matrix effect is a critical aspect of method validation. In this study, the mean matrix factor (MF) values for irbesartan were 0.91 at the LQC level and 1.00 at the HQC level, while the MF for the internal standard (valsartan) was 0.92. The IS-normalized matrix factors for irbesartan were 0.99 (LQC) and 1.09 (HQC), with corresponding coefficients of

variation (%CV) of 4.04% and 8.26%, respectively. These values fall within the generally accepted range of 0.85–1.15 and %CV below 15%, indicating that no significant matrix effect was observed under the tested conditions.

3.3.7 Precision and accuracy

Precision and accuracy results are summarized in table III. The ranged of within-run precision (2.43 to 7.61%) and accuracy (92.42 to 106.20%), respectively while the between-run precision (4.73 to 8.66%) and accuracy (98.56 to 101.20%), respectively. Effect of hemolysed and hyperlipidaemic on accuracy and precision was evaluated. The precision evaluated in hemolysed and hyperlipidaemic samples were 4.10 - 6.69 % and 4.21 – 7.21%, respectively. The accuracy evaluated in hemolysed and hyperlipidaemic samples were 99.88 – 105.93 % and 97.20 – 103.00%, respectively. The results indicating that both hemolysed and hyperlipidaemic have no significant effect on accuracy and precision of the assay.

3.3.8 Stability

The stability study of irbesatan is summarized in table 4 are the mean estimates obtained from 2 levels of QC sample (LQC and HQC, n = 4). All of the results showed that irbesartan was stable under the conditions in which the stability assessment. Stock stability of irbesartan and valsartan were established for 30 days at -70 ± 10°C were found to be within ±7% of their observed peak area at 30 days storage stock solution by respective peak area of freshly preparing stock solution. Irbesartan was proved to be stable in human plasma at specified storage conditions for post-preparative stability, re-injection reproducibility, bench top stability, freeze-thaw stability (three cycle), long-term stability (29 days) with percentage difference and precision (%CV) were within an acceptable range of ±15%. Accuracy of the observed mean concentration was within 85-115% of their respective nominal concentration.

3.3.9 Robustness

To evaluated when the change of method parameter using different column (same type) on the same instrument and using solutions (extraction solvents, reconstitution solvents and mobile phase) prepared by different analyst. The within-run precision (2.43 to 7.61%) and accuracy (92.42 to 106.20%), respectively while the between-run precision (4.73 to 8.66%) and accuracy (98.56 to 101.20%), respectively. All of values were within ±20% for LLOQ and ±15% for LQC, MQC and HQC of the actual values (85-115%), which were in an acceptable range.

3.3.10 Application of the proposed method.

The developed and validated LC-MS/MS method was successfully applied to quantify irbesartan plasma concentrations in a bioequivalence study. This

Table 4 Stability of irbesartan in different storage condition (n = 4)

Stability	Level	ng/mL	% CV	% Accuracy	% Change
Stock solution stability [e]					
Irbesartan					
15 h, 25 ± 2 °C	HQC	6,012.62	0.57	100.23	0.23
30 days, -70 ± 10°C	HQC	6,012.62	0.69	98.24	-1.76
Valsartan (IS)					
15 h, 25 ± 2 °C	IS	1,000.17	2.66	105.53	5.53
30 days, -70 ± 10°C	IS	1,000.17	1.24	98.63	-1.37
Post-preparative stability [f]					
48 hrs, 10 ± 5°C	LQC	15.02	4.52	103.75	4.43
(re-constitution samples)	HQC	4503.95	6.18	98.74	-0.11
5 days –70 ± 10°C	LQC	15.02	5.28	106.53	7.24
(dry samples)	HQC	4503.95	4.23	107.04	8.29
Re-injection reproducibility [f]	LQC	15.02	5.02	102.53	-0.17
24 hrs, 10 ± 5°C	HQC	4503.95	3.8	101.71	2.28
Bench top stability [f]	LQC	15.02	3.17	97.57	-1.78
5 h, 25 ± 2°C	HQC	4503.95	3.9	99.04	0.19
Freeze-thaw stability [f] (three cycle)	LQC	15.02	3.89	108.00	8.72
24 h, –70 ± 10°C	HQC	4503.95	2.77	101.74	2.92
Long-term stability [f]	LQC	15.02	3.94	104.09	4.78
29 days, –70 ± 10°C	HQC	4503.95	1.15	102.72	3.91

[e]Neat standard solution; [f]QC samples

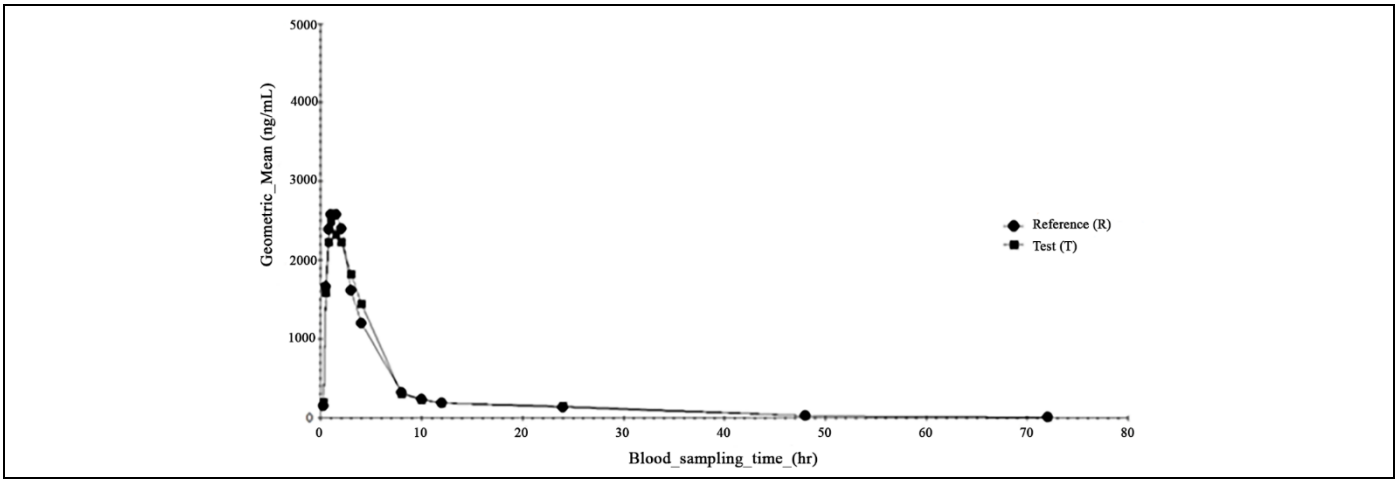

Figure 4 Representative data showing geometric mean of plasma concentration-time profiles of 24 healthy subjects after the administration of oral single dose of 300 mg of Irbesartan.

study evaluated the bioequivalence of 300 mg irbesartan tablets in 24 healthy Thai volunteers using a randomized, two-period crossover design at least 14-day washout period. The study aimed to compare the rate and extent of absorption of a generic irbesartan tablet formulation with that of the reference formulation, both administered at equivalent labeled doses. Each subject received either the test or reference formulation under fasting conditions. Blood samples were collected at 15 time points per period, processed, and stored at -70 °C until analysis. The chromatograms of plasma irbesartan and the internal standard are presented in Figure 3. The geometric mean of plasma concentration-time profiles of 24 healthy subjects after the administration of oral single dose of 300 mg of Irbesartan are presented in Figure 4. Point estimates and the 90% confidence intervals for the log-transformed ratios (Test/Reference) for the C_{max}, AUC_{0-72}, and $AUC_{0-\infty(obs)}$ should be within the acceptable range of 80.00% - 125.00% with the power more than 80%. The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, under approval number SI 220/2015

3.3.11 Advantage of the proposed method.

The developed LC-MS/MS method offers several advantages compared to previously reported methods, as summarized in Table V. It utilizes a simple and cost-effective one-step liquid-liquid extraction (LLE) with a mixture of ethyl acetate and hexane (90:10, v/v). LLE is an economical and rapid technique, especially for large sample sizes in bioanalysis. Moreover, this technique provides a highly pure extracted sample from the plasma matrix, minimizing potential effects on the analytical column and mass spectrometer. Preventing ion suppression and matrix effects in the LC-MS/MS system is crucial for ensuring reliable results. Some studies use solid-phase extraction (SPE)[12], which is expensive, involves multiple steps in the extraction process, and is time-consuming. Additionally, SPE requires a significant amount of chemicals, which is not ideal for waste management. However, the advantage of SPE is that it provides a very pure extracted sample. The protein precipitation technique is widely used in the sample preparation process due to its minimal steps[5,9,11,13]. However, the extracted sample often appears impure, as the plasma matrix can contaminate it. In analyses with large numbers of samples, it is essential to ensure that the extracted sample is clean enough to prevent any impact, such as accumulation in the column, which can induce ion suppression and matrix effects in the LC-MS/MS system. This method uses only 100 µL of plasma, which demonstrates the sensitivity of the method. The advantage of using a small plasma volume is that it ensures a better robustness, allowing a larger number of samples to be analyzed per batch. Literature search has shown that the plasma volume used can vary from 50 to 500 µL[13,14]. It has only 4 minutes of total runtime with a flow rate of 0.2 mL/min, requiring a small amount of chemicals and saving time. A literature review has shown that the total runtime for UPLC and LC systems ranges from 2.0 to 5.0 minutes[9,13], with flow rates varying from 0.4 to 1.0 mL/min[9,12-14]. The method has a wide linearity range of 5–6012.62 ng/mL and provides a high sensitivity with a limit of detection (LOD) of 60 pg/mL. There is no need to dilute samples and repeat the analysis. This makes the method suitable for handling a large number of samples in the study while ensuring the validity of the results. A literature review is summarized in Table 5. In addition, the present LC-MS/MS analysis was performed using multiple reaction monitoring (MRM) detection mode, which provided excellent selectivity for the quantification of irbesartan. The method employed specific precursor-to-product ion transitions at m/z 428.95 > 206.96 and 428.95 > 195.01

Table 5 Comparative summary of validated method results: literature review and present study

Year	Author	Method	Sample preparation technique	Plasma sample volume (µL)	Liquid Chromatography part			Irbesartan ion transition (m/z)		Linearity Range (ng/mL)	Recovery (%)	
					Flow rate (mL/mim)	Inject volume (µL)	Total Run time (minutes)	Quantification	Confirmation		IRB	IS
2015	Vargas M.et al.[6]	HPLC-UV	*PP	-	1.0	-	9.4	-	-	500-7000	-	-
2010	Rao RN.et al.[9]	HPLC-FL	**LLE	****DBS	1.0	20	25.0	-	-	6.0 – 2000	98.68	-
2015	Wani TA et al.[10]	UPLC-MS/MS	PP	200	0.4	5	2.0	427.2>193.08	-	2-500	82.94	84.62
2014	Ganesan M.et al.[11]	LC-MS/MS	LLE	100	0.5	5	4.0	429.79>207.12	-	45.8-10052.54	54.62-70.76	90
2014	Qiu X.et al.[12]	UPLC-MS/MS	PP	100	0.45	10	2.5	427.2>206.9	-	5-3000	90.7-97.5	-
2015	Nazareth C et al.[13]	LC-MS/MS	***SPE	300	1.0	10	2.5	427.200>193.100	-	50.197-6038.206	78.83-84.06	-
2023	Bhargavi PD et al.[14]	LC-MS/MS	PP	50	1.0	-	5.0	429.2>206.9	-	5.17-1025.15	84.6-87.3	-
2016	Patel CD et al.[15]	LC-MS/MS	LLE	500	1.0	5	3.5	427.1>193.0	-	10-5000	59.2-67.5	64.4
2025	this present work	LC-MS/MS	LLE	100	0.2	2	4.0	428.95>206.96	428.95>195.01	5-6012	74.26-80.34	76.93

Note: *PP = Protein precipitation, **LLE = Liquid-Liquid Extraction, ***SPE = Solid Phase Extraction, ****DBS = Dry blood spot

amu for the quantification and confirmation of [Irbesartan +H]$^+$, respectively. This analytical strategy ensured high accuracy, precision, and highly specific selectivity for irbesartan, with no evidence of false-positive findings.

4. CONCLUSIONS

In this present work, a reverse-phase chromatography method was successfully developed for the quantitative determination of irbesartan in human plasma using valsartan as an internal standard. The chromatographic conditions were optimized to ensure efficient separation, with no carryover effects observed thoughout the experiments. A highly sensitive and very specific LC-MS/MS method had a detection limit at picogram levels (60 pg/mL) and had a limit of quantification (LLOQ) of 5 ng/mL. We were optimized a sample preparation procedure for more specific for irbesartan, by the absence of interference by endogenous substance from biological matrix. All validated results were within the acceptance limit according to guidelines set by US FDA[16] and EMEA[17] for bioanalytical method validation. The better wide dynamic range of concentrations (5.00 - 6012.62 ng/mL) with the correlation coefficient were more than 0.997 (n=3) with a single step of liquid – liquid extraction for only small amount of 100 μL of plasma sample. Our proposed LC-MS/MS method showed highly precise, accurate and robust performance for quantification of irbesartan in human plasma used in bioequivalence study after oral administration of 300 mg tablet in Thai volunteers.

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Author contribution

Conceptualization: SC, SK, PP.
Methodology: SC, WC, PP.
Data curation: SC, WC, PJ, PP.
Writing – original draft preparation: PP.
Writing – review & editing: All authors.
All Authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare no conflict of interest.

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Ethics approval

The Institutional Review Boards of the Committee on Ethics, Faculty of Medicine Siriraj Hospital, Mahidol University approved the study protocol. (COA no. Si220/2015)

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