VALIDATION OF LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRIC METHOD FOR SIMULTANEOUS DETERMINATION OF LOPINAVIR AND RITONAVIR IN HUMAN PLASMA, AND ITS APPLICATION IN A BIOEQUIVALENCE STUDY IN THAI VOLUNTEERS

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ABSTRACT:

Background: A reliable, sensitive and rugged LC-MS/MS method was developed and validated for simultaneous determination of lopinavir and ritonavir in human plasma in order to use in a bioequivalence study.

Methods: Extraction of both analytes and their deuterated internal standards (ISTDs) from plasma samples was performed by using a simple liquid-liquid extraction method. Chromatographic separation was performed on Shimadzu UPLC system using ZORBAX Eclipse XDB-C8 (4.6 x 150 mm, 5 µm) analytical column. The mobile phase consisted of 2 mM ammonium formate (pH 4.5) and organic mixture (10% methanol in acetonitrile) (25:75). The isocratic mode of mobile phase was pumped at a flow rate 0.7 mL/min. The run time for analysis of each sample was 4.3 minutes. Ionization and detection of analytes and ISTDs were performed by TSQ Quantum Ultra (USA) triple quadrupole mass spectrometer with electrospray ionization probe. All analytes were monitored using the multiple reaction monitoring (MRM) in positive ion mode. Calibration curves with a range of 9.946 to 4923.332 ng/mL for lopinavir and 1.013 to 302.643 ng/mL for ritonavir were established using 0.25 mL human plasma.

Results: The intra- and inter-day precision and accuracy was 1.1 to 8.3% for precision and 95.6-100.1 % for accuracy. The mean recovery for lopinavir, ritonavir, ISTD of lopinavir and ISTD of ritonavir were 61.9%, 56.3%, 65.7% and 59.8%, respectively. The method was applied to a bioequivalence study of lopinavir/ritonavir 100/25 mg tablets manufactured by the Government Pharmaceutical Organization (GPO), Thailand, in 50 healthy Thai male volunteers under fasting condition. The results showed the bioequivalence of lopinavir/ritonavir 100/25 mg tablets of GPO to the reference product Aluvia® 100/25 mg tablets.

Conclusions: According to the results of bioequivalence study, the generic product of lopinavir/ritonavir 100/25 mg tablets of GPO can be used interchangeably with the reference product Aluvia® 100/25 mg tablets.

Keywords: Lopinavir, Ritonavir, Validation, Bioequivalence, Liquid chromatography tandem mass spectrometry

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INTRODUCTION

Acquired immune deficiency syndrome (AIDS) was first recognized in 1981 [1]. Then, human immunodeficiency virus (HIV) was isolated and demonstrated as a cause of AIDS [2]. HIV attacks cells with CD4 receptors such as T-lymphocytes, macrophages and dendritic cells that causes depletion in those cells. Profound suppression of several immune cells renders patients susceptible to infection by opportunistic pathogens [1].

Lopinavir and ritonavir are members of

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protease inhibitors and their structures are shown in Figure 1. They were designed as transition-state peptidomimetics that bind to an active site of HIV protease. Ritonavir is combined to other PIs for pharmacokinetic purposes rather than pharmacodynamic reasons. For examples, small amount of ritonavir in the lopinavir/ritonavir formulation is expected to act as a booster, which increases plasma concentrations lopinavir through its CYP3A4 activity [3]. Co-admistration of co-formulated lopinavir and low dose ritonavir (400/100 mg or 400/200 mg twice daily) and NRTIs gives good therapeutic efficacy [4]. Additionally, administration of lopinavir/ritonavir (100/25 mg) tablets twice daily in accordance with FDA weight band-based dosing resulted in satisfactory exposure of lopinavir in HIV-infected children [5].

In Thailand, according to essentials of HIV/AIDS treatment and prevention 2014, boosted PIs e.g. lopinavir/ritonavir is indicated as the second-line anti-HIV agents for treatment of HIV-infected children who fail to achieve desirable outcomes with the first-line drugs. Therefore, the Government Pharmaceutical Organization (GPO) has developed a generic lopinavir/ritonavir 100/25 mg with lower price to enhance affordability of this medicine for HIV patients.

Nowadays, liquid chromatography-tandem mass spectroscopy (LC-MS/MS) has been used extensively as an analytical tool to quantify drugs and/or related metabolites in biological matrices because of its high sensitivity and selectivity. Although there are several published methods for determination of lopinavir and ritonavir in human plasma using LC-MS/MS, the extraction processes are complicated [6-12].

The aims of this study were to develop and validate a simple and sensitive analytical method using LC-MS/MS to determine plasma concentration of lopinavir and ritonavir in bioequivalence study and to demonstrate the interchangeability between the generic lopinavir/ritonavir 100/25 mg tablets and the reference product.

EXPERIMENTAL

Chemicals and reagents

Lopinavirand ritonavir-d6 standards were purchased from TLC Pharmachem (Ontario, Canada). Ritonavir reference standard was obtained from USP (Maryland, USA). Lopinavir-d8 was procured from Toronto Research Chemicals Inc. (Ontario, Canada). Ultrapure water was produced in-house using a Milli-Q Advantage A10 ultrapure water purification system obtained from Merck Millipore (Bangkok, Thailand). HPLC grade acetonitrile was procured from Thomas Baker (Mumbai, India). Methanol HPLC grade and diethyl ether were purchased from Burdick & Jackson (Ulsan, Korea). Formic acid (98-100%) was obtained from Merck (Darmstadt, Germany). Ammonium formate was procured from LobaChemie (Mumbai, India). Sodium hydroxide was purchased from RCI Lab scan (Bangkok, Thailand). Blank K_2EDTA plasma was obtained from clinical research center, Department of Medical Sciences, Ministry of Public Health (Nonthaburi, Thailand) and stored at -22°C until use.

Standard stock, calibration standards and quality control sample preparation

Lopinavir, ritonavir and their internal standards (ISTD, lopinavir-d8 and ritonavir-d6, respectively) were dissolved individually in methanol to obtain stock solutions with a concentration of 2 mg/mL. Then stock solution of lopinavir and ritonavir were serially diluted with methanol: water(50:50) to obtain spiking solutions for calibration curve (CC). Stock solutions and spiking solutions for quality control (QC) samples were prepared separately from solutions for CC. Working solutions for ISTD were prepared from their stock solutions with methanolto the appropriate concentrations. All solutions were kept in a refrigerator at 2-8°C. The CC samples were prepared by spiking each spiking solution of lopinavir and ritonavir into screened blank plasma at concentration of 9.946-4923.332 ng/mL for lopinavir and 1.013-302.643 ng/mL for ritonavir. The QC samples used for each experiment in validation process were prepared in the same manner as CC samples.

Chromatographic conditions

Chromatography for separation of lopinavir and ritonavir was performed on Shimadzu UPLC system using ZORBAX Eclipse XDB-C8 (4.6 x 150 mm, 5 µm) analytical column. The column was maintained at 40°C in column oven. The mobile phase consisted of 2 mM ammonium formate (pH 4.5) and organic mixture (10% methanol in acetonitrile) (25:75). The isocratic mode of mobile phase was pumped at a flow rate 0.7 mL/min. The auto-sampler was set at 4°C and the injection volume of samples was 5µL. The run time for analysis of each sample was 4.3 minutes.

Mass spectrometric conditions

Ionization and detection of analytes (lopinavir and ritonavir) and ISTDs were performed by TSQ Quantum Ultra (USA) triple quadrupole mass spectrometer with electrospray ionization probe. All analytes were monitored in positive ion mode using

the multiple reaction monitoring (MRM) of m/z 629.370 → 155.120 for lopinavir, m/z 721.300 → 268.100 for ritonavir, m/z 637.390 → 163.160 for lopinavir-d8 and m/z 727.330 → 274.100 for ritonavir-d6 with scan width of 0.050 amu and scan times of 200 msec. The spray voltage, vaporization temperature, capillary temperature and collision pressure were set at 4000 V, 300°C, 350°C, 1.2 mTorr. The collision energy for lopinavir, ritonavir, lopinavir-d8 and ritonavir-d6 were 42, 27, 42 and 25 V, respectively.

Sample preparation

A 250 µL aliquot of spiked plasma sample or study sample was pipetted to a pre-labeledtube. 50 µL of IS working solution mixture of lopinavir-d8 and ritonavir-d6 was added into aliquoted sample. The analytes and ISTDs were extracted using 0.1 M sodium hydroxide solution and diethyl ether. The samples were vortexed and centrifuged at rcf 3400 ± 100 for 5 minutes at 10°C, then the plasma layer was flash frozen and the organic layer was transferred into pre-labeled tube. The organic layer samples were evaporated at 40°C under vacuum to dryness and reconstituted with the mobile phase. The processed samples were transferred into appropriate auto-sampler vials for analysis.

Validation

The bioanalytical method validation of lopinavir and ritonavir in human was conducted as per U.S. FDA Guidance for Industry Bioanalytical Method Validation 2001 [13] and EMA guideline on bioanalytical method validation 2011 [14]. The method was validated for selectivity, linearity, precision, accuracy, recovery, dilution integrity, matrix effect, robustness and ruggedness, and stability. The calibration curve consisted of standard blank, standard zero (no analytes but contained ISTDs), eight concentration levels including the upper limit and the lower limit of quantification (ULOQ and LLOQ, respectively).

Selectivity of the method towards matrix components was assessed in six batches of normal blank plasma, one batch of each hemolyzed and lipemic blank. The selectivity towards expected co-administered medications (acetaminophen, amoxicillin, azithromycin, chlorpheniramine, domperidone, ibuprofen, omeprazole, and piroxicam) was also examined. Auto sample rcarryover was determined by sequential injection of aqueous ULOQ and reconstitution solution. Sensitivity of the method was considered from the lowest concentration of both analytes that could be measured with acceptable precision and accuracy (e.g. LLOQ). The response of analytes at the lowest concentration should be at least 5 times the response compared to blank response. Three generated CCs in acceptable precision and accuracy experiments were used to evaluate the linearity of the method. To determine intra-day precision and accuracy, six replicate analyses of each QC level (HQC, MQC, LQC and LOQ QC) were performed on the same day. Three precision and accuracy batches on different days were used to assess inter-day precision and accuracy. Recovery of the extraction method was determined by comparing average response of each analyte and ISTD in extracted LQC, MQC and HQC to aqueous QC samples. Matrix effect of each blank plasma batch was evaluated in order to demonstrate the effect of constituents in plasma to the analysis of analytes. Each blank plasma was extracted as mentioned in sample preparation and then reconstituted with aqueous samples of HQC and LQC that contained lopinavir, ritonavir and their ISTDs. Dilution integrity was also performed to ensure that dilution of samples did not affect the precision and accuracy. Blank plasma was spiked with spiking solution of lopinavir and ritonavir to obtain the spiked sample with concentration greater than ULOQ for 1.4 to 1.6 times. Robustness and ruggedness were performed to evaluate the performance of the instrument over the period run of long sequence. Short term and long term stability of analytes and ISTDs in solution, plasma and processed samples were investigated. Short term stability at room temperature and long term stability of stock solutions and spiking solutions kept at 2-8°C were determined by comparing the average response of analytes and ISTDs stability samples to samples prepared from fresh stock solutions. For stability of analytes and ISTDs in plasma, extracted QC samples were analyzed along with freshly spiked CC and fresh processed QC samples. Autosampler (or wet extract) stability, bench-top stability, wet extract bench-top stability, dry extract stability, dry extract bench-top stability and freeze-thaw stability were evaluated using six replicate of LQC and HQC levels. Long term stability of analytes in human plasma was determined at -65°C using LQC, MQC and HQC with six replicates of each.

Application of the method in bioequivalence study

The validated method was used for analysis of lopinavir and ritonavir in plasma from fifty healthy Thai male volunteers. The study design was a comparative randomized, single dose, two-way crossover, open-label study to determine the bioequivalence between lopinavir/ritonavir 100/25 mg tablets manufactured by the Government Pharmaceutical Organization (GPO, Thailand, batch

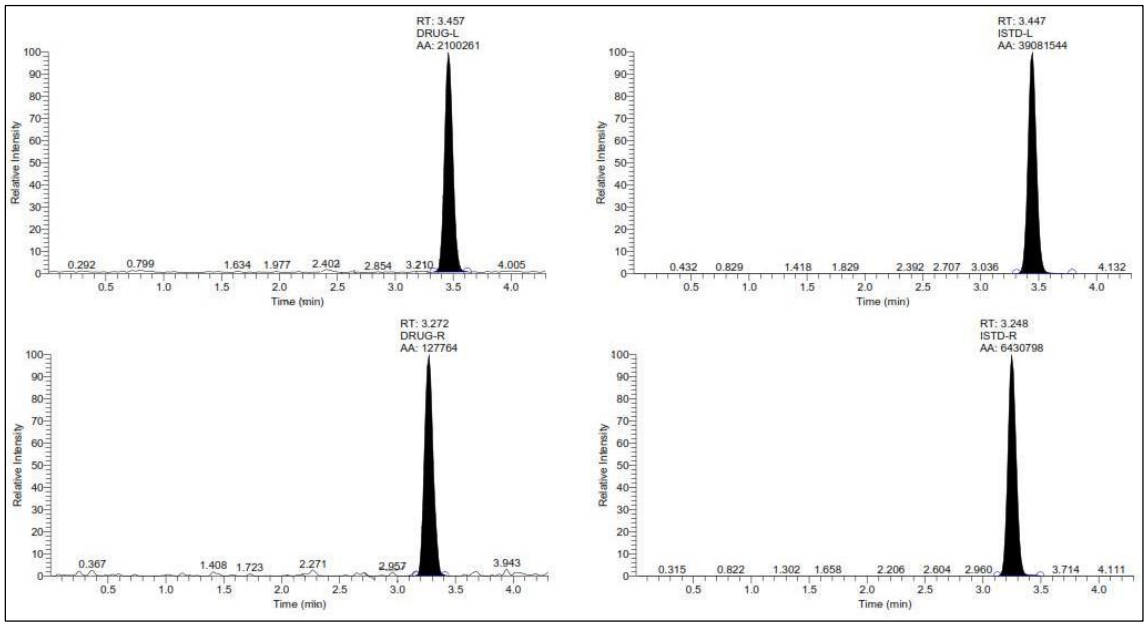

Figure 1 The representative chromatograms of lopinavir (DRUG-L) and ritonavir (DRUG-R) of LLOQ

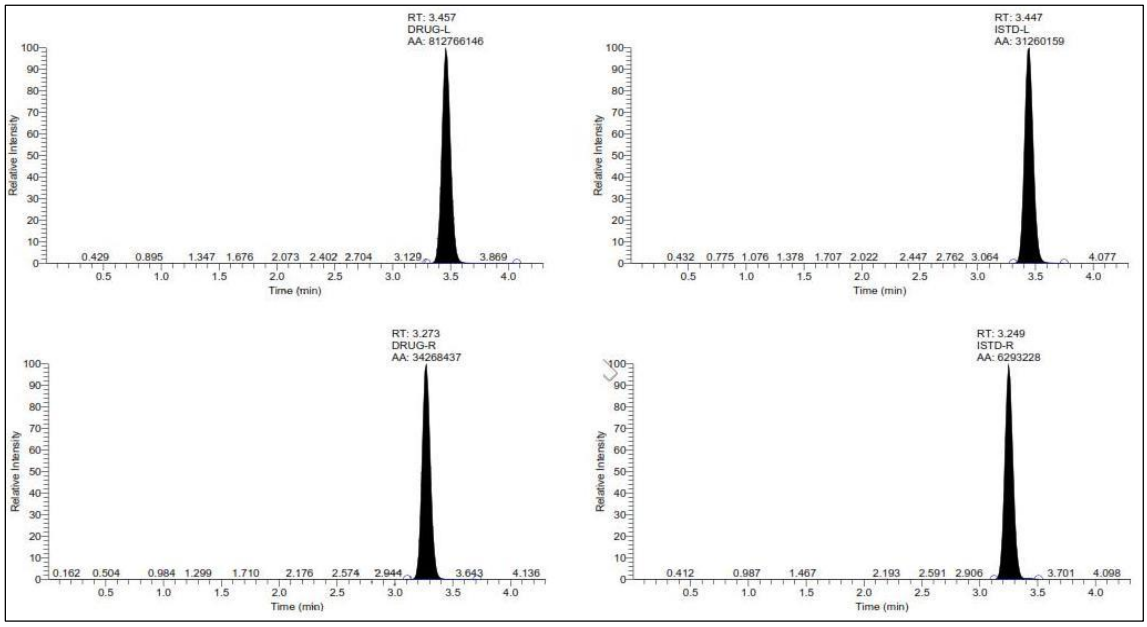

Figure 2 The representative chromatograms of lopinavir (DRUG-L) and ritonavir (DRUG-R) of ULOQ

Table 1 Precision and accuracy of back-calculated concentration of lopinavir and ritonavir in human plasma

Lopinavir				Ritonavir			
Nominal concentration (ng/mL)	**Mean back-calculated concentration (ng/mL)**	**%CV**	**%Nominal**	**Nominal concentration (ng/mL)**	**Mean back-calculated concentration (ng/mL)**	**%CV**	**%Nominal**
4923.332	4871.386	3.0	99.8	302.643	298.515	1.8	98.6
4430.999	4429.973	2.2	100.0	272.379	268.288	1.3	98.5
3691.022	3683.451	1.1	99.5	226.892	231.286	2.0	101.9
2458.221	2479.291	1.4	99.9	151.337	152.178	1.3	100.6
737.466	742.498	2.7	100.5	45.401	45.938	1.5	101.2
245.576	246.598	1.1	100.4	15.119	15.240	0.6	100.8
19.892	19.635	2.0	99.9	2.026	1.964	6.4	96.9
9.946	10.008	1.0	100.0	1.013	1.027	3.0	101.4

Table 2 Precision and accuracy of the method to quantify lopinavir and ritonavir in human plasma

QC level	Nominal concentration (ng/mL)	Intra-day (First P&A batch)				Inter-day			
		n	Mean back-calculated concentration (ng/mL)	%CV	% Nominal	n	Mean back-calculated concentration (ng/mL)	%CV	% Nominal
Lopinavir									
HQC	3720.582	6	3620.045	1.9	97.3	18	3567.459	2.1	95.9
MQC	2418.378	6	2373.190	1.9	98.1	18	2346.249	1.9	97.0
LQC	29.021	6	29.038	2.5	100.1	18	28.424	3.0	97.9
LOQ QC	9.983	6	9.874	1.1	98.9	18	9.559	4.6	95.8
Ritonavir									
HQC	229.317	6	227.698	2.1	99.3	18	227.531	1.8	99.2
MQC	149.056	6	147.891	1.8	99.2	18	146.999	1.6	98.6
LQC	2.758	6	2.691	4.5	97.6	18	2.665	4.5	96.6
LOQ QC	1.020	6	1.000	8.3	98.1	18	0.975	7.5	95.6

no.S550081, Mfg. date 12 Feb 2012, Exp. date 12 Feb 2014) and Aluvia®100/25 mg tablets manufactured by Abbott GmbH & Co.KG (Germany, batch no.275298D, Mfg. date 12 Dec 2012, Exp. date 30 Nov 2015) under fasting condition. The study protocol was approved by the ethic committee of the faculty of tropical medicine, Mahidol University. All volunteers were informed about risks and benefits of the study and signed informed consent before participating into the study.

Blood samples were collected for 22 sampling times (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10, 12, 16, 24 and 36 hours). The blood samples were centrifuged at 3000±100 rcf for 5 minutes below 10°C to separate plasma. All plasma samples were transferred to pre-labeled polypropylene tubes and stored upright frozen at -65±10°C until analysis.

RESULTS AND DISCUSSION

Selectivity

Eight batches of human blank plasma (six normal, one hemolyzed and one lipemic) containing K_2EDTA as an anticoagulant were evaluated for selectivity of the method. The results showed that there was no interference observed at the retention time and transition of lopinavir, ritonavir, lopinavir-d8 (ISTD-L) and ritonavir-d6 (ISTD-R) in any of the human blank plasma batches. In addition, the presence of co-administered drugs did not show any interference at the retention time and transition of analytes and their ISTDs. Based on the result, it can be concluded that the quantification method is selective for the analysis of lopinavir, ritonavir, ISTD-L and ISTD-R.

Autosampler carry-over

In autosampler carryover experiment, there was no any response found in reconstitution solution at the retention times of analytes and ISTDS after sequential injection of aqueous ULOQ. Furthermore, in other experiments e.g. precision and accuracy experiment, injection of extracted blank QC after extracted HQC did not show response at the retention times of analytes and ISTDs.

Sensitivity and linearity

The lower limits of quantification (LLOQ) were 9.946 ng/mL and 1.013 ng/mL for lopinavirand ritonavir, respectively. The representative chromatograms of lopinavir and ritonavir of LLOQ and ULOQ were shown in Figure 1 and 2. A linear equation was judged to produce the best fit for the concentration versus area response relationship. The regression type was 1/concentration² and peak area ratio for an eight point calibration curve was found linear from 9.946 to 4923.332 ng/mL for lopinavir and 1.013 to 302.643 ng/mL for ritonavir. The goodness of fit (r^2) was consistently greater than 0.99 during the course of method validation. Data of calibration curve were summarized in Table 1.

Precision and accuracy

Precision and accuracy of lopinavir and ritonavir were determined for limit of quantification (LOQ), low (L), medium (M) and high (H) concentrations of quality control samples in the human plasma. The intra-day and inter-day precision, which was expressed as %CV, were ≤ 15% for all quality control samples, except for LOQ QC, which was ≤ 20%. Accuracy, which was represented as %nominal, for intra-day and inter-day were within 85-115% of the nominal value for all quality control samples except for LOQ QC, which was within 80-120%. The results for intra-day and inter-day precision and accuracy of lopinavir and ritonavir in QC samples were summarized in Table 2.

Recovery, matrix effect and dilution integrity

The recovery was determined by comparing the

Table 3 Precision and accuracy for dilution integrity experiment

Dilution	Nominal concentration (ng/mL)	n	Mean back-calculated concentration multiplied with dilution factor (ng/mL)	%CV	% Nominal
Lopinavir					
1/2	7410.031	6	6832.905	1.8	92.2
1/10	7410.031	6	7310.782	7.0	98.7
Ritonavir					
1/2	443.877	6	430.140	2.3	96.9
1/10	443.877	6	464.639	8.6	104.7

Table 4 Stability of lopinavir under various conditions (n=6)

Storage condition	Comparison sample concentration (mean;ng/mL)	Stability sample concentration (mean;ng/mL)	% Mean ratio
Autosampler/Wet extract stability, 157 h at 2-8°C			
HQC	3351.193	3276.431	97.8
LQC	26.420	25.948	98.2
Bench-top stability, 17 h at room temperature			
HQC	3351.193	3326.122	99.3
LQC	26.420	26.356	99.8
Wet extract bench-top stability, 2 h at room temperature			
HQC	3351.193	3317.004	99.0
LQC	26.420	26.186	99.1
Dry extract stability, 50 h at -22°C			
HQC	3351.193	3367.170	105.8
LQC	26.420	27.025	105.4
Dry extract bench-top stability, 2 h at room temperature			
HQC	3351.193	3352.892	100.1
LQC	26.420	26.493	100.3
Freeze-thaw stability, 4 cycles			
HQC	3351.193	3302.184	98.5
LQC	26.420	26.220	99.2

Table 5 Stability of ritonavir under various conditions (n=6)

Storage condition	Comparison sample concentration (mean;ng/mL)	Stability sample concentration (mean;ng/mL)	% Mean ratio
Autosampler/Wet extract stability, 157 h at 2-8°C			
HQC	215.872	210.334	97.4
LQC	2.484	2.457	98.9
Bench-top stability, 17 h at room temperature			
HQC	215.872	215.164	99.7
LQC	2.484	2.631	105.9
Wet extract bench-top stability, 2 h at room temperature			
HQC	215.872	214.391	99.3
LQC	2.484	2.475	99.7
Dry extract stability, 50 h at -22°C			
HQC	215.872	217.955	105.0
LQC	2.484	2.595	101.3
Dry extract bench-top stability, 2 h at room temperature			
HQC	215.872	214.619	99.4
LQC	2.484	2.571	103.5
Freeze-thaw stability, 4 cycles			
HQC	215.872	213.969	99.1
LQC	2.484	2.554	102.8

Table 6 90% Confident intervals of the ln-transformed primary pharmacokinetic parameters of lopinavir and ritonavir

Parameter	Lopinavir		Ritonavir	
	Ratio	90% CI	Ratio	90% CI
$AUC_{0\text{-tlast}}$	100.3	87.4 - 115.2	101.9	91.1 - 113.9
$AUC_{0\text{-}\infty}$	100.2	87.4 - 114.9	101.0	90.6 - 112.4
C_{max}	104.2	92.4 - 117.4	106.7	93.8- 121.3

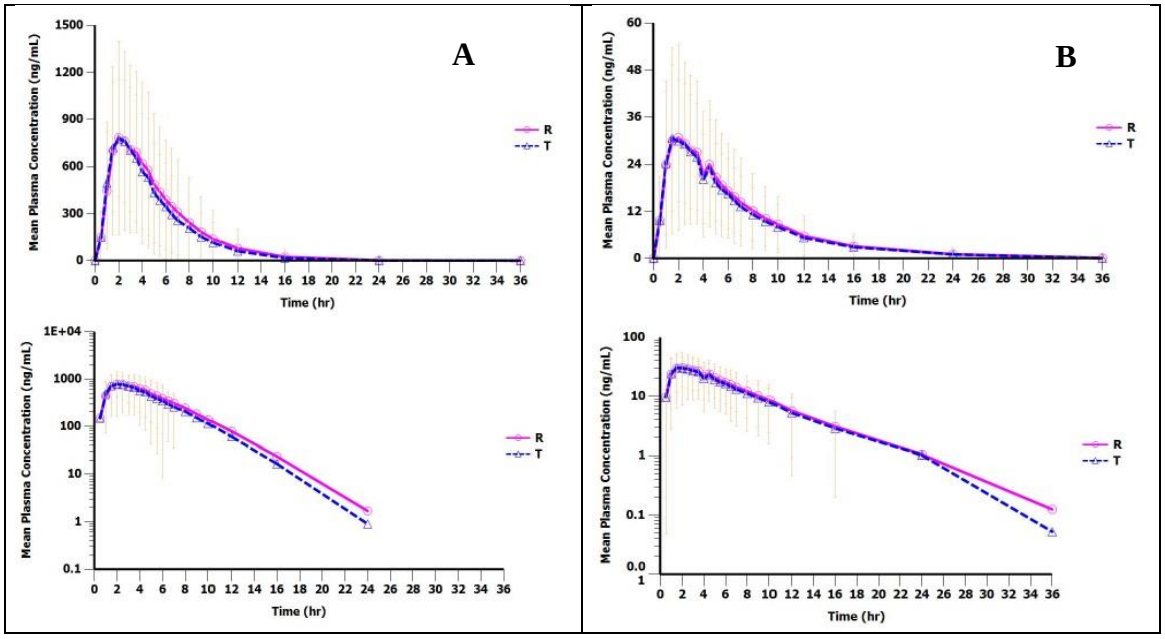

Figure 3A The linear and semi-logarithmic plots of mean (±SD) plasma concentration of lopinavir versus time curves of test (T) and reference (R) products. (N=45)

Figure 3B The linear and semi-logarithmic plots of mean (±SD) plasma concentration of ritonavir versus time curves of test (T) and reference (R) products. (N=45)

detector response of lopinavir and ritonavir at three distinct levels of low, medium and high quality control samples with detector response obtained from the un-extracted (aqueous) quality control samples. The mean recovery for lopinavir, ritonavir, ISTD-L and ISTD-R were 61.9%, 56.3%, 65.7% and 59.8% respectively.

Matrix effect was evaluated through matrix factor, which was calculated by comparing area response in presence of matrix ions with mean area response in absence of matrix ions. Matrix factor was the quantitative measurement of the matrix effect due to suppression or enhancement of ionization in mass spectrometric detector. Then, matrix factors of both lopinavir and ritonavir were normalized with matrix factors of their ISTDs. The ISTD normalized matrix factors for lopinavir and ritonavir were about 1.0 with %CV of 0.8-2.0%. Based on the obtained results it could be concluded that, no significant ion suppression or enhancement was observed during ionization in mass spectrometric detector.

For dilution integrity, sample of lopinavir having concentration of 7410.031 ng/mL and ritonavir having concentration of 443.877 ng/mL were prepared in human plasma and were diluted with human blank plasma to 1/2 and 1/10 of the original concentration. The results for dilution integrity were shown in Table 3.

Robustness and ruggedness

Long batch was processed and performed with the CC standards and 35 sets of QC samples of each level (LQC, MQC, HQC and blank QC). This batch was processed by different analyst and analyzed using different column of same make. The precision and accuracy were ranged from 1.7-2.2 % and 93.5-95.8% for lopinavir. For ritonavir, the precision was 1.5-3.3% and the accuracy was 98.0-100.6%. The method was robust and rugged up to 150 injections.

Stability

Stock solutions of lopinavir, ritonavir and their ISTDs including spiking solutions of both drugs were stable at room temperature for 25 hours.For temperature range of 2-8°C,stock solutions of lopinavir, ritonavir and their ISTDs were stable for 28 days while spiking solution of the drugs were stable for 27 days. The results of short term stability of analytes in human plasma in several conditions were given in Table 4 for lopinavir and Table 5 for ritonavir. Moreover, the long term stability of lopinavir and ritonavir were established for 138 days at -65°C.

Application of the method in bioequivalence study

Forty-five subjects were included in pharmacokinetics and statistical analysis. The pharmacokinetic parameters were calculated by non-compartmental model using Phoenix

WinNonlin software version 6.3 (Pharsight Corporation, USA) and the statistical analyses was carried out using SAS® version 9.3 (SAS Institute Inc., USA). Figure 3A and 3B showed the plasma concentration versus time profiles after oral administration of the two lopinavir/ritonavir 100/25 mg formulations. The 90% parametric confidence intervals were calculated for the ln-transformed primary pharmacokinetic parameters of lopinavir and ritonavir and presented in Table 6.

CONCLUSION

A reliable, sensitive and rugged LC-MS/MS method with the simple liquid-liquid extraction was developed and validated for simultaneous determination of lopinavir and ritonavir in human plasma in accordance with U.S. FDA Guidance for Industry Bioanalytical Method Validation 2001 and EMA guideline on bioanalytical method validation 2011. The calibration curve ranges for both lopinavir and ritonavir were set based on the expected C_{max} of both drugs after oral administration of lopinavir/ritonavir 100/25 mg tablets. The method can be used to analyze samples up to 150 injections with precision and accuracy. Therefore, this method has proved to be suitable for bioequivalence study or pharmacokinetics of lopinavir/ritonavir 100/25 mg. Furthermore, according to the results of bioequivalence study, the generic product of lopinavir/ritonavir 100/25 mg tablets manufactured by GPO can be used interchangeably with the reference product Aluvia®100/25 mg tablets.

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