Rapid and Sensitive Method for Quantitative Determination of Lopinavir and Ritonavir in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: A rapid and sensitive liquid chromatography-mass spectrometric (LC-MS-MS) method for the simultaneous determination of lopinavir and ritonavir in human plasma using abacavir as internal standard has been developed and validated. Sample preparation of plasma involved solid phase extraction. Detection was performed using an Applied Biosystems Sciex API 2000 Mass spectrometer. The assay of lopinavir and ritonavir was linear over the range of 50 ng mL⁻¹ to 20000 ng mL⁻¹ and 20 ng mL⁻¹ to 3000 ng mL⁻¹ respectively with a precision of < 15% and accuracy in the range of 85-115%. The limit of quantification in plasma for lopinavir and ritonavir was 50 ng mL⁻¹ and 20 ng mL⁻¹ respectively. The described method has the advantage of being rapid and easy and it could be applied in therapeutic monitoring of these drugs in human plasma.

Keywords: Human immunodeficiency virus (HIV)-1, Lopinavir, Ritonavir, LC-MS-MS

Introduction

Lopinavir is an inhibitor of the HIV protease. Ritonavir inhibits the CYP3A-mediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir¹⁻⁶.

Lopinavir is chemically designated as [1S-[1R*, (R*), 3R*, 4R*]]-N- [4-[(2,6-dimethylphenoxy) acetyl] amino]-3-hydroxy-5-phenyl-1- (phenylmethyl) pentyl] tetra

hydro-alpha- (1-methylethyl)-2-oxo-1 (2*H*)-pyrimidineacetamide. Its molecular formula is $C_{37}H_{48}N_4O_5$, and its molecular weight is 628.80¹. Ritonavir is chemically designated as 10-Hydroxy-2-methyl-5-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5*S*-(5*R**,8*R**,10*R**,11*R**)]. Its molecular formula is $C_{37}H_{48}N_6O_5S_2$ and its molecular weight^{1,3} is 720.95. The present paper deals with a biological assay of lopinavir and ritonavir simultaneously. Benefits of this method are improved sensitivity and recovery, which are mainly due to instrumental advances. The present LC-MS-MS method is well suited for studies generating large number of samples. In addition, much improved sensitivity was achieved with lower limit of quantification (LLOQ) of 50.0 ng mL⁻¹ for lopinavir and 20.0 ng mL⁻¹ for ritonavir. Recoveries were about 80-90% for lopinavir and ritonavir.

Experimental

Chemicals and reagents

Working standard of lopinavir and ritonavir and abacavir were obtained from Cipla. Methanol and acetonitrile of HPLC grade and all other chemicals used were of analytical grade.

Working standard preparation

Stock solutions (1000 µg mL⁻¹) of lopinavir, ritonavir and abacavir were prepared in methanol. Working solutions with different concentrations were prepared by dilution of stock with diluent (methanol: water 50: 50). Working solution for internal standard was also prepared in same diluent.

Sample extraction

Plasma samples to be processed were thawed at room temperature. Strata-X 33 µm Polymeric Reversed Phase (30 mg/1mL) cartridges were conditioned by passing through them 1 mL of methanol followed by 1 mL of water. To prepare samples for loading, 200 µL plasma was mixed with 50 µL of internal standard (approximately 2.000 µg mL⁻¹). Sample was vortexed to mix, centrifuged for 5 min at 15000 rpm and loaded on cartridge. Samples were washed with 2 mL water. Analyte and internal standard were eluted from the cartridges using 2000 µL of mobile phase. 2 µL of sample was injected onto LC-MS/MS.

Instrumentation and chromatographic conditions

The HPLC system (shimadzu) consist of an isocratic pump, an auto sampler and a Hypersil Hypurity C₁₈ column (50 x 4.6 mm, *id* 5 µ). The mobile phase was prepared by mixing 2% formic acid with acetonitrile in the ratio of 20:80(v/v). Chromatography was performed at ambient temperature with a flow rate of 0.4 mL min⁻¹. Detection was performed by an Applied Biosystems Sciex API 2000 Mass Spectrometer using atmospheric turbo ion spray for ion production. API was performed in the positive ion mode. The LC-MS-MS detector was operated at unit resolution in the multiple reaction-monitoring (MRM) mode. The transitions of the protonated molecular ions of lopinavir at *m/z* 629.5 → 183.3, ritonavir at *m/z* 721.4 → 296.3 and abacavir at *m/z* 287.2 → 191.2. Product ion mass spectra of lopinavir and ritonavir are shown in Figure 1 and 2 respectively.

Figure 1. Mass spectra of lopinavir.
Molecular mass: 629.5 m/z and fragment mass: 183.3 m/z

Figure 2. Mass spectra of ritonavir.
Molecular mass: 721.4 m/z and fragment mass: 296.3 m/z

Results and Discussion

Validation procedures

Selectivity

Ten different sources of blank plasma samples were extracted and analysed for the assessment of potential interference with endogenous substances. The apparent response at the retention time of lopinavir and ritonavir was compared to the lower limit of quantification. Figure 3, 4, 5 and 6 shows representative chromatograms of selectivity for lopinavir and ritonavir.

Figure 3. Representative chromatogram of plasma blank for lopinavir.

Figure 4. Representative chromatogram of plasma spiked with lopinavir at the lower limit of quantification (50.0 ng mL⁻¹).

Figure 5. Representative chromatogram of plasma blank for ritonavir.

Figure 6. Representative chromatogram of plasma spiked with ritonavir at the lower limit of quantification (20.0 ng mL⁻¹).

Linearity

Linearity was studied over concentration of 50.000 ng mL⁻¹ to 20000.000 ng mL⁻¹ for lopinavir and 20.000 ng mL⁻¹ to 3000.000 ng mL⁻¹ for ritonavir. The calculation was done by linear weighted regression (1/X²).

Accuracy and precision

To validate the method for lopinavir and ritonavir, accuracy and precision were evaluated by analysis of three different concentration levels LQC (*i.e.* 150.0 ng mL⁻¹ of lopinavir and 60.0 ng mL⁻¹ of ritonavir), MQC (*i.e.* 8000.0 ng mL⁻¹ of lopinavir and 800.0 ng mL⁻¹ of ritonavir) and HQC (*i.e.* 18000.0 ng mL⁻¹ of lopinavir and 2700.0 ng mL⁻¹ of ritonavir). The results obtained for precision and accuracy of lopinavir and ritonavir are listed in Table 2.

Table 1. Data of precision and accuracy.

Nominal Concentration in ng mL ⁻¹	For lopinavir			For ritonavir		
	QC-1 (150.000)	QC-2 (8000.000)	QC-3 (18000.000)	QC-1 (60.000)	QC-2 (800.000)	QC-3 (2700.000)
	Observed conc.	Observed conc.	Observed conc.	Observed conc.	Observed conc.	Observed conc.
1	168.237	7563.268	17988.243	65.813	766.547	2701.386
2	138.184	7199.263	17869.587	59.996	729.562	2728.063
3	154.859	8027.006	17165.230	60.028	825.382	2630.722
4	159.090	8192.263	17172.471	70.583	807.584	2663.173
5	158.252	8246.126	17126.408	60.311	813.105	2585.505
6	145.301	8080.509	16656.402	53.271	784.695	2609.615
Mean	153.987	7884.739	17329.724	61.667	787.813	2653.077
SD	10.707	413.995	504.299	5.908	35.488	54.796
% CV(Precision)	6.95	5.25	2.91	9.58	4.50	2.07
% Accuracy	102.66	98.56	96.28	102.78	98.48	98.26

Recovery

For the recovery experiment, plasma extracted samples were prepared by spiking both lopinavir and ritonavir at three different concentrations levels (LQC, MQC, HQC) and extracted using extraction procedure. Recovery was carried out by comparing the area obtained from an extracted sample with standard (unextracted) sample. The results obtained for drug and internal standard recovery are listed in Table 2

Stability

The stock solution stability at room temperature for 24.0 h was compared with freshly prepared stock solution of lopinavir and ritonavir. Freeze thaw stability was done for LQC and HQC after three cycles at -20⁰ C. Room temperature stability in plasma was done for 24 h at two concentrations LQC and HQC. Auto sampler stability was done for 24 h at two concentrations LQC and HQC.

Analyte and internal standard responded best to positive ionization using atmospheric turbo ion spray for ion production. In order to get higher response hypersil hypurity C18 (4.6 X 50 mm) column was used. A number of different columns (Nucleosil, Nova pack, hypersil and Symmetry shield) were evaluated and hypersil hypurity C18 was found to give best chromatography with minimal matrix effects.

Table 2. Extraction recovery of drug and internal standard.

Nominal concentration in ng mL ⁻¹	For lopinavir			For ritonavir			Internal standard
	QC-1 (150.0)	QC-2 (8000.0)	QC-3 (18000.0)	QC-1 (60.0)	QC-2 (800.0)	QC-3 (2700.0)	5.0, µg mL ⁻¹
1	84.71	89.14	94.36	87.67	91.02	97.45	96.43
2	85.14	94.34	92.80	87.38	98.62	94.89	103.01
3	94.60	93.43	93.17	102.00	98.19	94.00	104.37
4	85.59	96.88	96.57	84.08	94.81	100.10	105.23
5	89.73	90.38	91.17	93.52	93.16	90.03	100.73
6	88.15	92.08	86.90	88.16	91.88	86.10	100.13
Mean	87.99	92.71	92.49	90.47	94.61	93.76	96.43
SD	3.780	2.801	3.278	6.417	3.205	5.055	103.01
RSD	4.30	3.02	3.54	7.09	3.39	5.39	104.37

The assay was found to be linear in the concentration range 50.0 to 20000.0 ng mL⁻¹ for lopinavir and 20.0 to 3000.0 ng mL⁻¹ for ritonavir ($r > 0.99$). Precision and accuracy were satisfactory at the three concentrations. Mean % recovery of the lopinavir and ritonavir was 91.06 and 92.95% respectively. Stability of analyte and internal standard stock solution in methanol was verified on storage for one month at 2-8°C.

Application of method

The method can be successfully applied to determine concentration of drug in a bioequivalence study of lopinavir and ritonavir.

Conclusion

Rapid and sensitive LC-MS-MS method is reported for the simultaneous determination of lopinavir and ritonavir in human plasma. The Assay can be successfully applied to determine concentration of the drug in a bioequivalence study of lopinavir and ritonavir. The method allows high samples throughput due to short run time and relatively simple sample preparation procedure.

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