DEVELOPMENT AND VALIDATION OF FORCED DEGRADATION STUDIES OF LOPINAVIR USING RP-HPLC AND CHARACTERIZATION OF DEGRADANTS BY LC-MS/MS

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ABSTRACT

Aim of the present work was to develop simple, shorter and effective HPLC method with UV detection (240 nm) and subsequent validation for forced degradation studies of lopinavir using RP-HPLC and characterization of degradants by LC-MS/MS. The method uses isocratic the mobile phase mixture of buffer and acetonitrile taken in the ratio in the ratio of 45:55 (v/v) on Hypersil BDS, C18, 100 x 4.6 mm, 5 μm column. The RSD for five injections was observed to 1.1 percentage and linearity range of 25-150 percentage of label claim established with 0.999 correlation. The observed result shows that the method was rapid, precise, accurate and simple. The method was validated as per ICH guidelines.

Key words: Lopinavir, Method development, LC-MS, Validation.

INTRODUCTION

Lopinavir (LPV) is a potent HIV protease inhibitor (PI) and a key ingredient of Highly Active Anti-Retroviral Therapy (HAART)[1]. LPV was developed by Abbott Laboratories to improve pharmacokinetics and to reduce HIV resistance of the company's earlier protease inhibitor, Ritonavir (RTV)[2]. LPV has low oral bioavailability when administered alone because of poor solubility, high first pass metabolism[3] and P-gp efflux[4]. RTV is co-administered with LPV orally in HAART in order to improve the bioavailability of LPV. RTV increases bioavailability of LPV due to its inhibitory effects on gut and liver

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Cytochrome (CYP) P450 enzymes and permeability glycoprotein (P-gp) efflux system[5]. LPV (Fig. 1) is chemically designated as (2*S*)-*N*-[(1*S*, 3*S*, 4*S*)-1-benzyl-4-{[(2,6-dimethyl-phenoxy)acetyl]amino}-3-hydroxy-5-phenylpentyl]-3-methyl-2-(2-oxotetrahydropyrimidin-1(2*H*)-yl)butanamide. Its molecular formula is $C_{37}H_{48}N_4O_5$ and its molecular weight is 628.80.[6]
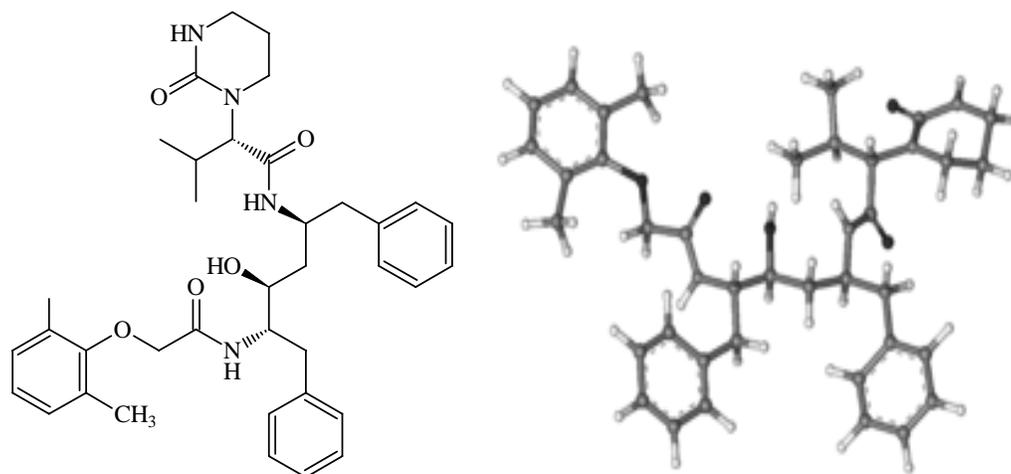

Fig. 1: Chemical structure of lopinavir

Literature survey revealed several analytical methods for the determination of ritoavir and lopinavir in tablets, capsules, and syrups which employ techniques such as high-performance liquid chromatography (HPLC)[7-9], ultra performance liquid chromatography (UPLC)[10] and high performance thin layer chromatography (HPTLC)[11]. In biological fluids, the active principles as well as their metabolites have been quantitatively determined by HPLC with UV detection, LC/MS/MS[12,13], spectroscopic method[14], micellar electro kinetic chromatography method[15] and Tandem mass spectrometry[16]. The present work describes a simple, isocratic method for the determination of lopinavir in tablets as for ICH guidelines[17-20].

EXPERIMENTAL

Materials and methods

Chemicals

Qualified standards and samples of lopinavir were obtained from local laboratories and were used without any further purification. The chemicals like potassium dihydrogen orthophosphate, triethylamine and ortho phosphoric acid were purchased from Merck,

Mumbai. Millipore water generated from TK water system. The analytical column used was Hypersil BDS, C18, 100 x 4.6 mm, 5 μ.

Instruments

A Waters prominence HPLC system equipped with a quaternary UFLC LC-20AD pump, a DGU-20A$_5$ degasser, a SPD-M20A diode array detector, a SIL-20AC auto sampler, a CTO-20AC column oven and CBM-20A communications bus module was used for method development and validation studies.

Standard preparation

Accurately Weighed and transferred 10 mg of lopinavir working standards into a 10 mL clean dry volumetric flask, add 0.4 mL of methanol as diluent, sonicated for 10 mins and make up to the final volume with water.

Preparation of sample

5 Tablets were weighed and calculate the average weight of each tablet. Then the weight equivalent to 5 tablets were transferred into a 100 mL volumetric flask, 70 mL of methanol added and sonicated for 30 mins, further the volume made up with water and filtered. From the filtered solution 0.2 mL pipette out into a 10 mLvolumetric flask and made up to 10 mL with methanol.

Chromatographic conditions

The chromatographic column used was Hypersil BDS column with dimensions of 100 mm x 4.6 mm with 5 µm particle size. The column temperature was maintained at 30ºC and detection was monitored at a wavelength of 240 nm. Injection volume was 10 µL and the mobile phase flow was set at 1.0 mL/min. The water, acetonitrile in the ratio 30:70 v/v was used as diluents for preparation of solutions.

Method validation

The developed method for determination of lopinavir was validated for system suitability along with method selectivity, specificity, linearity, range, precision, accuracy, range, ruggedness, robustness according to the ICH guidelines.

Method validation parameters

The system suitability was conducted using standard preparation and evaluated by injecting five replicate injections. Specificity is the ability of analytical method to assess un equivocally the analyte in the presence of component that may be expected to be present.

Performed the specificity parameter of the method by injecting Diluent, placebo into the chromatographic system and evaluated by show any peak at the retention time of analyte. Performed the linearity with lopinavir in the range of 25 to 150% of specification limit. Recorded the area response for each level and calculated slope, intercept & correlation coefficient. Also performed precision at higher level by injecting six times into the chromatographic system.

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation of series of measurements. The system precision was conducted using lopinavir and evaluated by making six replicate injections. The accuracy of the method by recoveries of lopinavir sample solutions at different concentration levels ranging from 50 to 150%. The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Method development includes selection of appropriate chromatographic conditions/factors like detection wave length, selection and optimization of stationary and mobile phases. The wavelength of 240 nm was selected due to it produces less noise, which minimizes problems that may exhibit around the active ingredient when attempting to quantify lopinavir. Preliminary development trials were performed with various BDS columns of different types and dimensions from different manufacturers were tested for the peak shape and the number of theoretical plates for specification concentrations. Finally by switching to Hypersil BDS, C18, 100 x 4.6 mm, 5 μm column there a significant improvement in the peak shapes with 1.2 tailing factor.

System suitability

The RSD from six replicate injections of standard preparation was 1.1%. Tailing factor for lopinavir peak was 1.2.

Selectivity

Performed the specificity parameter of the method by injecting diluent, standard preparation sample preparation and placebo preparation into the chromatographic system and recorded the retention times. Specificity study of the method proved no peak observed at

retention time of lopinavir. Specificity results of lopinavir given in the below Table 1. The selectivity chromatograms shown in the Figs. 2-3.

Table 1: Selectivity results of lopinavir

| S. No.	Sample	Retention time
1	Blank	-
2	Placebo	-
3	Standard	3.872
4	Sample	3.874
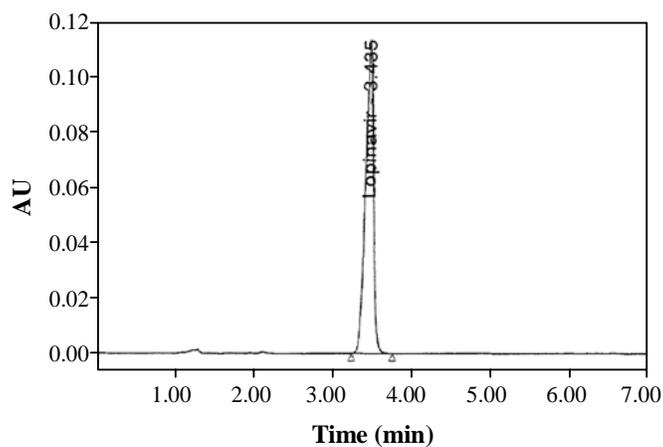
Fig. 2: Chromatogram of lopinavir standard
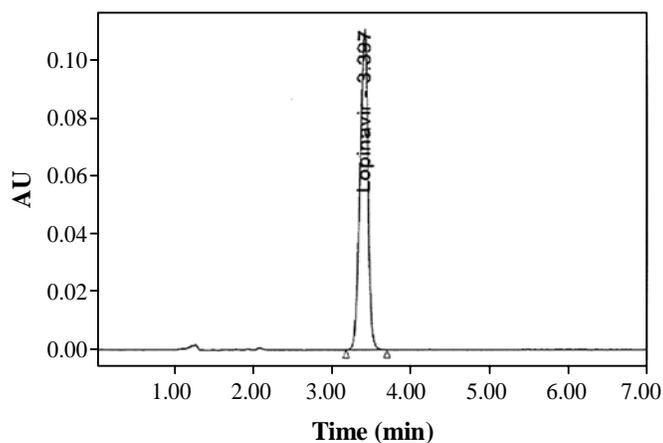
Fig. 3: Chromatogram of lopinavir sample

Linearity

To demonstrate the linearity with lopinavir standard in the range of 25 to 150% of specification limit**.** Correlation coefficient of lopinavir was 0.999. The linearity results shown in the below Table 2.

Table 2: Linearity results of lopinavir

S. No.	Concentration (ppm)	Area response
1.	25	162228
2.	50	332752
3.	75	512499
4.	100	687708
5.	125	859443
6.	150	1025060

Accuracy

Accuracy study found that the mean % of recovery was more than 97.0% and less than 103.0% at each level 50 to 150% of concentration levels, hence method is accurate. The accuracy results are given Table 3.

Table 3: Accuracy results

S. No.	Level (%)	Mean recovery (%)
1.	50	99.85563
2.	100	99.94564
3.	150	98.64364

Precision

The precision of test method was validated by assaying six samples prepared on lopinavir and calculate relative standard deviation of assay results. The precision results are given Table 4.

Table 4: Precision results

S. No.	Area of lopinavir
1	772761
2	780428
3	788569
4	767714
5	789584
6	781798
Average	780142
SD	8624.7
% RSD	1.1

LOD and LOQ

The limit of detection and limit of quantification of test method was validated based on signal to noise ratio method. The LOD and LOQ data given Table 5.

Table 5: LOD and LOQ establishment data

Parameter	Concentration (ppm)
LOD	0.285
LOQ	0.864

Degradation of lopinavir

The degradation behavior of LV under various stress conditions was investigated by LC. Typical chromatograms are shown in Fig. 2.

Oxidation

To 1 mL of stock solution of lopinavir, 1 mL of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60°C. For HPLC study, the resultant solution was diluted to obtain 100 µg/mL solutions and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid degradation

To 1 mL of stock solution lopinavir, 1 mL of 2 N hydrochloric acid was added and refluxed for 30 mins at 60°C. The resultant solution was diluted to obtain 100 µg/mL solution and 10 µL solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali degradation studies

To 1 mL of stock solution Lopinavir, 1 mL of 2 N sodium hydroxide was added and refluxed for 30 mins at 60°C. The resultant solution was diluted to obtain 100 µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral degradation studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6 hrs at a temperature of 60°C. For HPLC study, the resultant solution was diluted to 100 µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Thermal/dry heat degradation studies

The standard drug solution was placed in oven at 105°C for 6 hr to study dry heat degradation. For HPLC study, the resultant solution was diluted to 100 µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photostability studies

The photochemical stability of the drug was also studied by exposing the 100 µg/mL solution to UV light by keeping the beaker in UV chamber for 7 days or 200 Watt hours/m^2 in photostability chamber For HPLC study, the resultant solution was diluted to obtain 100 µg/mL and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Mass spectral fragmentation

The analysis of the degradation products was carried by LC and LC-MS. Lopinavir was subjected to LC-MS/MS with atmospheric pressure chemical ionization (APCI) to know

the fragmentation pattern of drug. The MS^2 analysis of the precursor ion (m/z 704) of the drug given below with molecular structure and molecular weight.
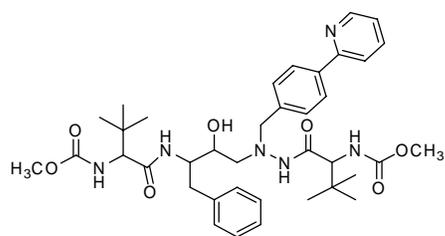
$C_{38}H_{52}N_6O_7$
Mol. Wt.: 704.86
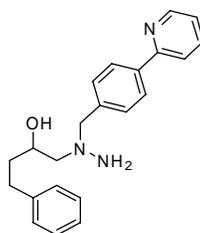
$C_{22}H_{25}N_3O$
Mol. Wt.: 347.45
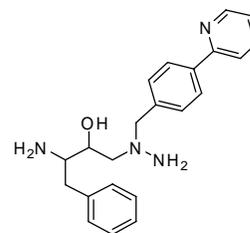
$C_{22}H_{26}N_4O$
Mol. Wt.: 362.47
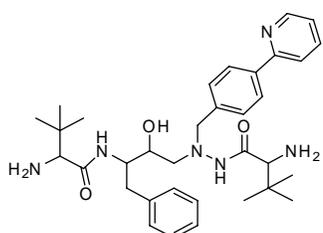
$C_{34}H_{48}N_6O_3$
Mol. Wt.: 588.78
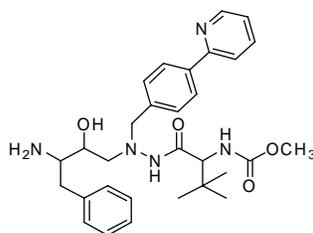
$C_{30}H_{39}N_5O_4$
Mol. Wt.: 533.66
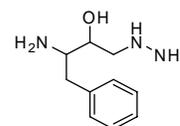
$C_{10}H_{17}N_3O$
Mol. Wt.: 195.26
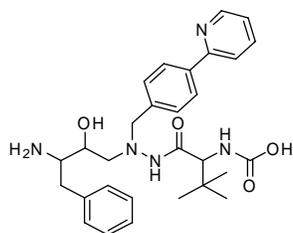
$C_{29}H_{37}N_5O_4$
Mol. Wt.: 519.64
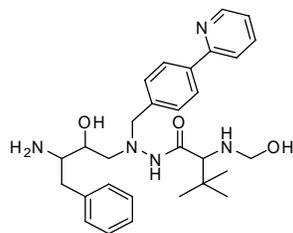
$C_{29}H_{39}N_5O_3$
Mol. Wt.: 505.65
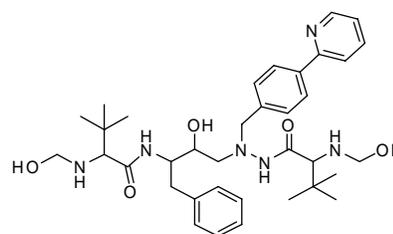
$C_{36}H_{52}N_6O_5$
Mol. Wt.: 648.84
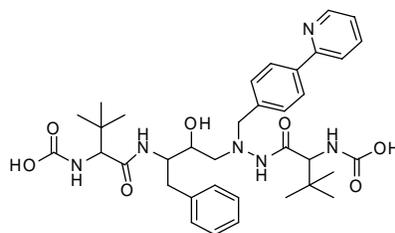
$C_{36}H_{48}N_6O_7$
Mol. Wt.: 676.8

CONCLUSION

A validated stability indicating assay LC-PDA method was developed to study the degradation behavior of Lopinavir under hydrolysis (acid, base and neutral), oxidation, thermal and UV conditions. LC-MS/MS characterization of degradation products was carried out and pathways of decomposition were proposed. The drug was found to be degraded extensively in all conditions except oxidation due to presence of carbamate and urea linkage, which were susceptible to hydrolysis

ACKNOWLEDGEMENT

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