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A stability indicating LC method for lopinavir

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ABSTRACT

An isocratic reverse phase liquid chromatographic (RP-LC) assay method was developed for the quantitative determination of lopinavir in bulk drug and in pharmaceutical dosage form, used to treat antiviral. The developed method is also applicable for the related substances determination. The chromatographic separation was achieved on Agilent Zorbax SB- C18 250× 4.6mm, 5µm. The LC method employs solution A as mobile phase. The solution A contains a mixture of phosphate buffer pH 4.0: acetonitrile (55:45, v/v). The flow rate was 1.5 mL min^{-1} and the detection wavelength was 210 nm. In the developed HPLC method the resolution between lopinavir and its potential impurities namely Imp-1, Imp-2, Imp-3 and Imp-4was found to be greater than 10.0. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. Considerable degradation was found to occur in thermal, UV, acid medium and oxidative stress conditions. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 99.7%. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness.

KEYWORDS

Column liquid chromatography;
lopinavir;
Forced degradation;
Validation;
Stability indicating.

INTRODUCTION

lopinavir, (2*S*, 3S, 5S)-2-(2, 6-dimethylphenoxy acetyl) amino-3-hydroxy-5-[2*S*-(1-tetrahydropyrimid-2-onyl)-3-methylbutanoyl] amino-1, 6-diphenylhexane is known to have utility for the inhibition of antiviral. lopinavir prevents cleavage of the Gag-Pol polyprotein, resulting in the production of immature, non-infectious viral particles. lopinavir is particularly effective for the inhibition of antiviral with ritonavir. In *vitro* experiments with human hepatic microsomes indicate that lopinavir primarily undergoes oxidative metabolism. lopinavir is extensively metabolized by the hepatic cytochrome P450 system, almost exclusively by the CYP3A isozyme.

Few chromatographic methods have been appeared in the literature. for the determination of lopinavir, in human plasma[1-4]. One stability indicating LC method appeared in literature for the lopinavir sort gelatin capsules[5]. So far, to our present knowledge there is no stability indicating LC method for the related substance determination and quantitative estimation of lopinavir for bulk drug samples. This paper describes the assay and related substances method validation for accurate quantification of lopinavir and all 4 impurities in bulk samples and in pharmaceutical dosage forms also. (TABLE 7).

EXPERIMENTAL

Chemicals

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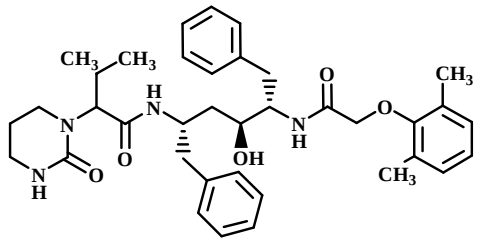
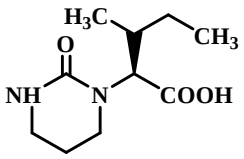


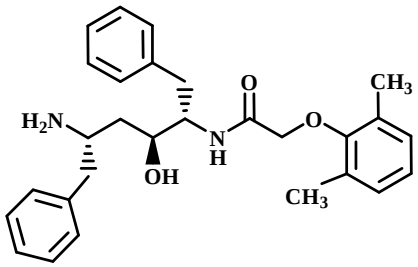
lopinavir

[1S-[1R*, (R*), 3R*, 4R*]]-N-[4-[[(2,6-dimethylphenoxy) acetyl]amino]-3-hydroxy-5-phenyl-1-(phenylmethyl) pentyl] tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2H)-pyrimidine acetamide



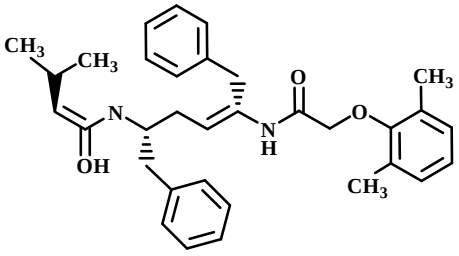
Imp-1

2S-(1-Tetrahydro-pyrimid-2-onyl)-3-methyl-butanoic acid



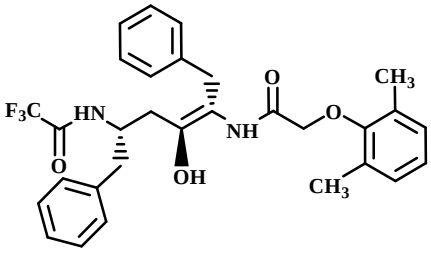
Imp-2

(2S,3S,5S)-2-(2,6-Dimethylphenoxyacetyl) amino-3-hydroxy-5-amino-1,6-diphenyl hexane



Imp-3

N-[1-Benzyl-4-[2-(2,6-dimethylphenoxy)-acetylamino]-5-phenyl-pent-3-enylidine]-3-me4hyl-but-1-enimidic acid



Imp-4

(2S,3S,5S)-2-(2,6-Dimethylphenoxyacetyl)amino-3-hydroxy-5-trifluromethylcarbonylamino-1,6-diphenylhexane

Figure1: Chemical structures and labels of lopinavir and its impurities

Samples of lopinavir its related impurities and 200mg lopinavir tablets were received from Hetero group India (Figure 1), HPLC grade acetonitrile was purchased from Merck, Darmstadt, Germany. Analytical reagent grade potassium dihydrogen phosphate monohydrate and orthophosphoric acid were purchased from Merck, Darmstadt, Germany. High pure water was prepared by using Millipore Milliq plus water purification system.

Equipment

The LC System, used for method development, forced degradation studies and method validation was Waters 2695 binary pump plus auto sampler and a 2996 photo diode array detector. The output signal was monitored and processed using empower software on Pentium computer (Digital equipment Co).

Chromatographic conditions

The chromatographic column used was Agilent Zorbax SB- C18 250×4.6mm, 5µm particles. Mobile phase contains a mixture of phosphate buffer pH 4.0: acetonitrile (55:45, v/v).The flow rate of the mobile phase was 1.5mL min^{-1}. The column temperature was maintained at 25°C and the detection was monitored at a wavelength of 210 nm. The injection volume was 20µL. mobile phase was used as diluent.

Preparation of solutions

1. Preparation of standard solutions

A stock solution of lopinavir (2.0 mg mL^{-1}) was prepared by dissolving appropriate amount in the diluent. Working solutions of 1000 and 100µg mL^{-1} were prepared from above stock solution for related substances determination and assay determination, respectively. A stock solution of impurity (mixture of Imp-1, Imp-2, Imp-3 and Imp-4) at 0.0015 mg mL^{-1} was also prepared in diluent.

2. Preparation of sample solution

Twenty tablets were weighed and the content transferred into a clean and dry mortar, grinded well. Then equivalent to 200 mg of drug was transferred to 100mL volumetric flask, 70 mL of diluent added and kept on rotatory shaker for 10min to disperse the material com-

pletely and sonicated for 10 min and diluted to 100mL (500 μg mL^{-1}). The resulting solution was centrifuged at 3,000 rpm for 5 min. Supernant solution was taken 10 mL and diluted to 100mL with diluent (100μgmL^{-1}). This was filtered using 0.45μnylon 66-membrane filter.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities[6]. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used.

The specificity of the developed LC method for lopinavir was determined in the presence of its impurities namely Imp-1, Imp-2, Imp-3, Imp-4 and degradation products. Forced degradation studies were also performed on lopinavir to provide an indication of the stability indicating property and specificity of the proposed method[8,9]. The stress conditions employed for degradation study includes light (carried out as per ICH Q1B), UV light 254 nm, heat (105°C), acid hydrolysis (1.0M HCl), base hydrolysis (1.0M NaOH), water hydrolysis, oxidation (30% H_2O_2). For the above studies, study period was 48 hours. Peak purity of stressed samples of lopinavir was checked by using 2996 Photo diode array detector of Waters (PDA). The purity angle is within the purity threshold limit obtained in all stressed samples demonstrates the analyte peak homogeneity. All stressed samples of lopinavir[UV light 254 nm, heat (105°C), acid hydrolysis (1.0M HCl), base hydrolysis (1.0M NaOH), water hydrolysis, oxidation (30% H_2O_2)] were studied for extended run time of 100 min (with 90% acetonitrile in mobile phase) to check the late eluting degradants

Assay studies were carried out for stress samples against qualified reference standard and the mass balance (% assay+% of impurities + % of degradation products) was calculated. Assay was also calculated for bulk samples and drug product by spiking all four impurities (Imp-1, Imp-2, Imp-3 and Imp-4) at the specification level (i.e. 0.15% of analyte concentration which is 1000μg mL^{-1}).

Method validation

1. Precision

The precision of the related substance method was checked by injecting six individual preparations of (1000μg mL^{-1}) lopinavir spiked with 0.15% each Imp-1, Imp-2, Imp-3 and Imp-4. The % RSD of area for each Imp-1, Imp-2, Imp-3 and Imp-4 was calculated.

The intermediate precision (ruggedness) of the method was also evaluated using different analyst, different column and a different instrument in the same laboratory.

Assay method precision was evaluated by carrying out six independent assays of test sample of lopinavir against qualified reference standard. The % RSD of six assay values obtained was calculated.

2. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for Imp-1, Imp-2, Imp-3 and Imp-4were estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration[7]. The precision study was also carried out at the LOQ level by injecting six individual preparations of Imp-1, Imp-2, Imp-3 and Imp-4 and calculated the % RSD for the areas of each impurity.

3. Linearity

Linearity test solutions for assay method were prepared from stock solution at seven concentration levels from 25 to 200% of assay analyte concentration (25, 50, 75, 100, 125, 150 and 200μg mL^{-1}). The peak area versus concentration data was performed by least-squares linear regression analysis.

Linearity test solutions for related substance method were prepared by diluting the impurity stock solution (2.4) to the required concentrations. The solutions were prepared at six concentration levels. From LOQ to 200% of the permitted maximum level of the impurity (i.e. the LOQ , 0.015%,0.0375%, 0.075%, 0.15%, 0.225% and 0.3% for an analyte concentration of 1000μg mL^{-1}). The correlation coefficient, slope and Y-intercept of the calibration curve were reported.

4. Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels, i.e. 50,100

and 150μg mL^{-1} in bulk sample and drug product. The percentages of recoveries were calculated.

The bulk sample does not show the presence of Imp-1 and Imp-2, it shows 0.03% of Imp-3, 0.05% of imp-4. The study was carried out in triplicate at 0.075%, 0.15% and 0.225% of the analyte concentration (1000μg mL^{-1}). The percentage of recoveries for Imp-1, Imp-2, Imp-3 and Imp-4were calculated.

5. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately changed and the resolution between lopinavir, Imp-1, Imp-2, Imp-3 and Imp-4was evaluated. The flow rate of the mobile phase was 1.5 mL min^{-1}. To study the effect of flow rate on the resolution, 0.2 units changed it from 1.3 to 1.7 mL min^{-1}. The effect of pH on solution of impurities was studied by varying ± 0.1 pH units (at 3.9 and 4.1 buffer pH). The effect of column temperature on resolution was studied at 20^0C and 30^0C instead of 25°C. In the all above varied conditions, the components of the mobile phase were held constant as stated in Section 2.3.

6. Solution stability and mobile phase stability

The solution stability of lopinavir in the assay method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 48h. The same sample solutions were assayed for 6 h interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h interval up to 48 h. Mobile phase prepared was kept constant during the study period. The % RSD of assay of lopinavir was calculated for the study period during mobile phase and solution stability experiments.

The solution stability of lopinavir and its impurities in the related substance method was carried out by leaving spiked sample solution in tightly capped volumetric flask at room temperature for 48 h. Content of Imp-1, Imp-2, Imp-3 and Imp-4were determined for every 6 h interval up to the study period.

Mobile phase stability was also carried out for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. Content of Imp-1, Imp-2, Imp-3 and Imp-4were checked in the test solutions. Mobile phase prepared was kept constant during the study period.

RESULTS AND DISCUSSION

Method development and optimization

The target of the chromatographic method is to get the separation of impurities, namely Imp-1, Imp-2, Imp-3 and Imp-4 from lopinavir and each other. The tailing factor of the lopinavir is high, by using different stationary phases like C18, and C8 and different mobile phases containing buffers like phosphate, sulphate and acetate with different pH (2-8) and using organic modifiers like acetonitrile, methanol and ethanol in the mobile phase. pH of the buffer to 4.0 has played a significant role in achieving the separation between impurities and the symmetry of lopinavir peak.

The chromatographic separation was achieved on Zorbax SB-C18 (250×4.6) mm with 5μm particles. The mobile phase contains a mixture of pH 4.0 phosphate buffer and acetonitrile in the ration of 55:45(v/v) Buffer consist of 0.01mM potassium dihydrogen orthophosphate, pH adjusted to 4.0 ± 0.05 using ortho phosphoric acid.

The flow rate of the mobile phase was 1.5mLmin^{-1}. The column temperature was maintained at 25°C and the detection was monitored at a wavelength of 210 nm. The injection volume was 20μL. mobile phase was used as diluent. There was no interference of blank with impurities (Imp-1, Imp-2, Imp-3 and Imp-4) and lopinavir. The interference of excipients (Lactose, micro crystalline cellulose and magnesium stearate) was also checked by injecting sample solutions of excipients. There was no interference of excipients with Impurities (Imp-1, Imp-2, Imp-3 and Imp-4) and lopinavir peak. In the optimized conditions lopinavir, Imp-1, Imp-2, Imp-3 and Imp-4 were well separated with a resolution of greater than 10 and the typical retention times of Imp-1, Imp-2, Imp-3, lopinavir and Imp-4 were about 1.2, 7.1, 15.3,23.1and 64.8 min respectively. The system suitability results are given in TABLE 1. The developed LC method was found to be specific for lopinavir and its four impurities namely Imp-1, Imp-

TABLE 1: System suitability report

Compound	USP resolution (R_S)	USP tailing factor
Imp-1	--	1.3
Imp-2	25.08	1.2
Imp-3	20.76	0.9
Lopinavir	10.40	1.0
Imp-4	30.84	1.1

TABLE 2: Summary of forced degradation results

Stress condition	Time	% Assay of active substance	Mass balance (%Assay + % impurities +% Degradation products)	Remarks (Major degradant)
Acid hydrolysis (1.0M HCl, reflux)	2 h	98.0	99.7	Imp-2, Imp-3, Imp-4
Base hydrolysis (1.0MNaOH, Reflux)	2 h	99.6	99.8	Impurity-4
Oxidation (30% H_20_2)	2 h	99.1	99.8	Unknown impurities
Water hydrolysis (Reflux at 60 deg)	2 h	99.7%	99.8	No degradation products formed
Thermal (105°C)	48 hrs	99.6	99.9	Unknown impurities
UV light	254 nm	98.7	99.8	Impurity-2, Impurity-4
Light (photolytic degradation)	1200 KLUX	99.4	100.0	Impurity-2

TABLE 3: Results of accuracy study for drug substance

Added (µg)(n=3)	Recovered (µg)	% Recovery
50	49.7	99.4
100	99.5	99.5
150	148.2	98.8

n =3, Number of determinations

TABLE 4: Results of accuracy study for drug product

Added (µg)(n= 3)	Recovered (µg)	% Recovery
50	49.9	99.8
100	100.6	100.6
150	147.6	98.4

n =3, Number of determinations

2, Imp-3 and Imp-4 (TABLE 2).

Analysis was performed for different batches of bulk drug samples (n=3) and for pharmaceutical dosage forms (n=3). Results were given in TABLE 7. Stability study results as per ICH Q1A (R2) for lopinavir[6] were given in TABLE 8 and TABLE 9.

Method validation

TABLE 5: Results of robustness study

S.no	Parameter	Variation	Resolution(R_s) between Imp1 and Imp-2	Resolution(R_s) between Imp 3 and Lopinavir
1	Temperature (± 5^{0}C of set temperature)	(a) At 20°C	24.0	19.1
		(b) At 30°C	23.2	19.1
2	Flow rate (± 0.2mL of the set flow)	(a) At 1.3 ml/min	25.0	19.3
		(b) At 1.7 ml/min	23.2	19.1
3	pH(±0.1unit of set pH)	(a) At 3.9	25.0	18.1
		(b) At 4.1	25.0	18.1

1. Precision

The %RSD of assay of lopinavir during assay method precision study was within 1.0% and the %RSD of area of Imp-1, Imp-2, Imp-3 and Imp-4in related substance method precision study were within 4.5 %. Confirming the good precision of the method.

The %RSD of assay results obtained in intermediate precision study was within 1.0% and the %RSD of area of Imp-1, Imp-2, Imp-3 and Imp-4 were well within 5.0 %, confirming the ruggedness of the method. (TABLE 6)

2. Limit of detection and limit of quantification

The limit of detection of Imp-1, Imp-2, Imp-3 and Imp-4 were 0.006, 0.007, 0.007 and 0.008% (of analyte concentration, i.e.1000µg mL^{-1}) respectively for 20 µL injection volume. The limit of quantification of Imp-1, Imp-2, Imp-3 and Imp-4 were 0.02, 0.018, 0.023, and 0.022% (of analyte concentration, i.e. 1000µg mL^{-1}) respectively for 20µL injection volume. The precision at LOQ concentration for Imp-1, Imp-2, Imp-3 and Imp-4were below 2%.

3. Linearity

Linear calibration plot for assay method was obtained over the calibration ranges tested, i.e. 25-200µg mL^{-1}and the correlation coefficient obtained was greater than 0.999. The results show that an excellent correlation existed between the peak area and concentration of the analyte. The slope and Y-intercept of the calibration curve were 79 and 145 respectively.

Linear calibration plot for related substance method was obtained over the calibration ranges tested, i.e. LOQ to 0.3% for Imp-1, Imp-2, Imp-3 and Imp-4. The correlation coefficient obtained was greater than

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TABLE 6: Results of intermediate precision

S.no	Parameter	Variation	%RSD for assay	%RSD for related substances	Resolution between Imp-1 and Imp-2	Resolution between Imp 3 and lopinavir
1	Different system	(a) Waters 2695 Alliance system	0.1%	< 5.0%	23.8	19.0
		(b) Agilent 1100 series VWD system	0.2%	< 5.0%	23.5	20.2
2	Different column	(a) B.No: USCL 019544	0.2%	< 5.0%	23.7	19.2
		(b)B.No: US CL 019484	0.2%	< 5.0%	23.2	20.2
3	Different analyst	(a) Analyst-1	0.2%	< 5.0%	23.8	19.4
		(b) Analyst-2	0.2%	< 5.0%	23.6	20.3

TABLE 7: Batch analysis

Batch No: Bulk B.No:	Imp-1	Imp-2	Imp-3	Imp-4	Purity by HPLC	Assay by HPLC
Batch#1	ND	ND	0.08%	0.05%	99.80%	99.9%
Batch#2	0.04	ND	0.08%	0.04%	99.82%	99.8%
Batch#3	ND	ND	0.06%	0.02%	99.88%	100.2%
Drug product B.No#						
Batch#1	ND	ND	0.06%	0.06%	99.80%	99.8%
Batch#2	0.01	ND	0.04%	0.04%	99.72%	99.8%
Batch#3	0.02	ND	0.06%	0.05%	99.78%	99.7%

Where ND = Not Detected

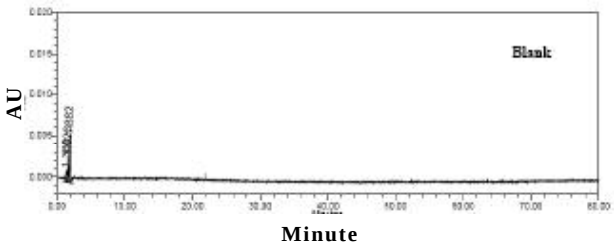

Figure2: Typical chromatogram of lopinavir spiked with impurities at 0.15% level

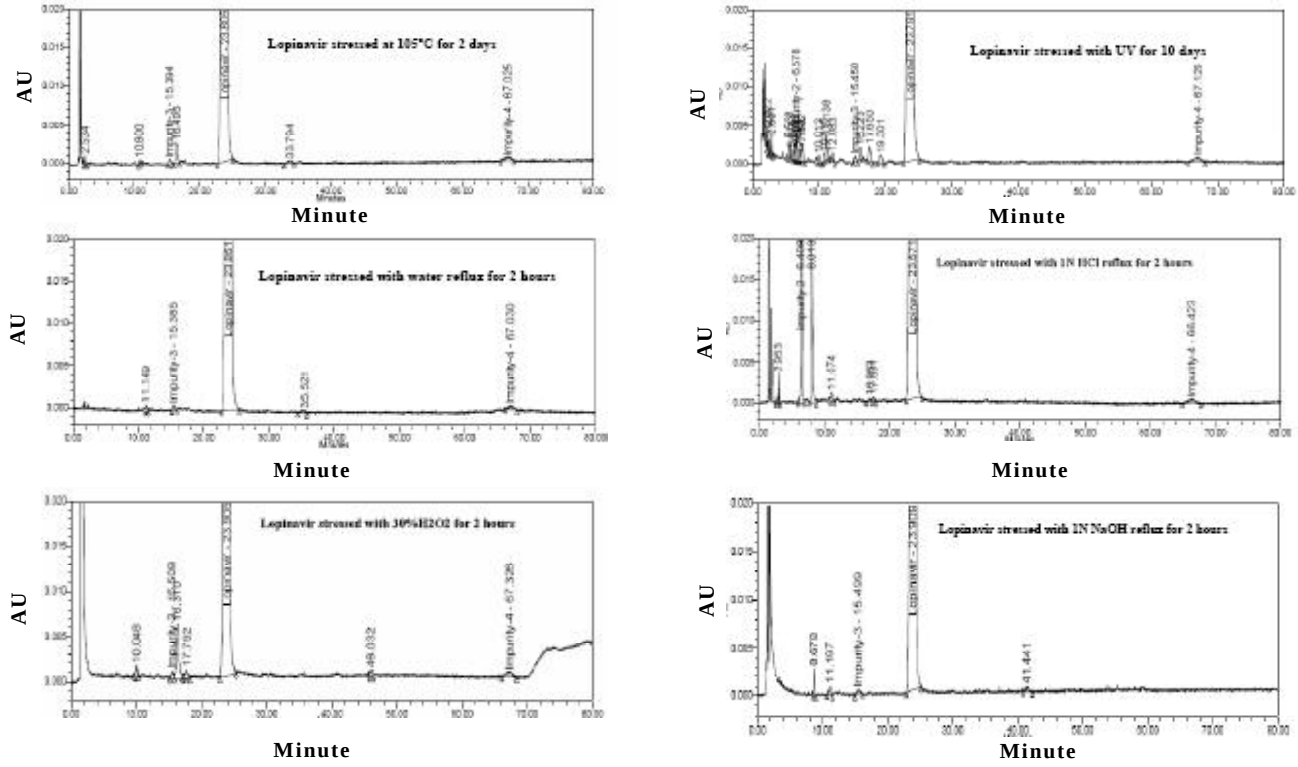

Figure 3: Typical chromatogram of lopinavir sample and stressed lopinavir

0.999. The results show that an excellent correlation existed between the peak area and concentration of Imp-1, Imp-2, Imp-3 and Imp-4.

4. Accuracy

The percentage recovery of lopinavir in bulk drug samples ranged from 98.8 to 99.5 (TABLE 3) and in pharmaceutical dosage forms ranged from 98.4 to 100.6 % (TABLE 4). The percentage recovery of Imp-1, Imp-2, Imp-3 and Imp-4 in bulk drugs samples ranged from 93.5 to 106.2. HPLC chromatogram of spiked sample at 0.15% level of all four impurities in

TABLE 8: Accelerated stability data (storage conditions: 40°±2°C / 75± 5% RH)

Bulk drug B.No:Batch#1	Temperature 40 ± 2°C, Relative Humidity 75± 5%						
Duration	**Imp-1**	**Imp-2**	**Imp-3**	**Imp-4**	**Total impurities**	**Assay by HPLC**	**Remarks**
Initial	ND	ND	0.03%	0.05%	0.18%	99.8%	No significant change observed
1st month	ND	ND	0.03%	0.05%	0.30%	99.8%	No significant change observed
2nd Month	ND	ND	0.03%	0.06%	0.36%	99.8%	No significant change observed
3rd Month	ND	ND	0.03%	0.06%	0.12%	99.9%	No significant change observed
Drug Product, B.No: Batch#1							
Duration	**Imp-1**	**Imp-2**	**Imp-3**	**Imp-4**	**Total impurities**	**Assay by HPLC**	**Remarks**
Initial	ND	ND	0.06%	0.06%	0.20%	99.80%	No significant change observed
1st month	ND	ND	0.05%	0.05%	0.24 %	99.85%	No significant change observed
2nd Month	ND	ND	0.05%	0.07%	0.22%	99.78%	No significant change observed
3rd Month	ND	ND	0.04%	0.07%	0.25%	99.75%	No significant change observed

Where ND = Not Detected

TABLE 9: Long stability data (storage conditions: 25° ±2°C/60 ± 5% RH)

Bulk Drug B.No:Batch#1	Temperature 25±2°C, Relative Humidity 60± 5%						
Duration	**Imp-1**	**Imp-2**	**Imp-3**	**Imp-4**	**Total impurities**	**Assay by HPLC**	**Remarks**
Initial	ND	ND	0.03	0.06%	0.12%	99.9%	No significant change observed
1st month	ND	ND	0.04%	0.05%	0.14 %	99.8%	No significant change observed
2nd Month	ND	ND	0.04%	0.07%	0.22%	99.8%	No significant change observed
3rd Month	ND	ND	0.04%	0.07%	0.25%	99.7%	No significant change observed
Drug Product, B.No: Batch#1							
Duration	**Imp-1**	**Imp-2**	**Imp-3**	**Imp-4**	**Total impurities**	**Assay by HPLC**	**Remarks**
Initial	ND	ND	0.06%	0.06%	0.20%	99.80%	No significant change observed
1st month	ND	ND	0.04%	0.05%	0.18%	99.78%	No significant change observed
2nd Month	ND	ND	0.04%	0.05%	0.18%	99.72%	No significant change observed
3rd Month	ND	ND	0.06%	0.05%	0.22%	99.69%	No significant change observed

Where ND = Not Detected

lopinavir bulk drug sample are shown in figure 2.

5. Robustness

In all the deliberate varied chromatographic conditions carried out as described earlier (Flow rate, pH and Column temperature), the resolution between impurities, namely Imp-1 and Imp-2, Imp-3 and lopinavir was greater than 10.0, illustrating the robustness of the method (TABLE 5).

Solution stability and mobile phase stability

The %RSD of assay of lopinavir during solution stability and mobile phase stability experiments was within1%. No significant changes were observed in the content of Imp-1, Imp-2, Imp-3 and Imp-4 during solution stability and mobile phase experiments when performed using related substances method. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during assay and related substance determination were stable up to 48 h.

Results of forced degradation studies

Degradation was not observed in lopinavir stressed samples that were subjected to water and base hydrolysis. The degradation of drug substance was observed under light, heat, acid hydrolysis and oxidative conditions (Figure 3). lopinavir is highly sensitive towards acid and light. Considerable degradation of the drug substance was observed under Thermal and oxidative conditions leads to the formation of some unknown degradation peaks. Peak purity test results derived from PDA detector, confirmed that the lopinavir peak is homogeneous and pure in all the analyzed stress samples. Peak purity results for degraded peaks from PDA detector confirm that lopinavir peak and all-

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degradant peaks are homogeneous and pure in all analyzed stress samples. No degradants were observed after 30 min in the extended runtime of 100 min for all the lopinavir samples[UV light 254 nm, heat (105°C), acid hydrolysis (1.0M HCl), base hydrolysis (1.0M NaOH), water hydrolysis, oxidation (30% H_2O_2) with 90% acetonitrile as mobile phase.

The mass balance of stressed samples was close to 99.7%(TABLE 2). The assay of lopinavir is unaffected in the presence of Imp-1, Imp-2, Imp-3 and Imp-4 and its degradation products confirm the stability indicating power of the developed method. (TABLE 8) (TABLE 9).

CONCLUSIONS

The isocratic RP-LC method developed for quantitative and related substance determination of lopinavir in both bulk drug and pharmaceutical dosage form is precise, accurate and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of samples of lopinavir.

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