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Short communication

Impurity profile study of lopinavir and validation of HPLC method for the determination of related substances in lopinavir drug substance

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ABSTRACT

Several related substances (RS4–RS10) were detected in lopinavir drug substance at levels ranging from 0.03% to 0.1% by employing gradient RP-HPLC. The related substances were identified by LC–MS analysis. These related substances were isolated and characterized by Mass, ^{1}H NMR and FT-IR spectral data. The separation was achieved on a YMC Pack ODS-AQ (250 mm × 4.6 mm, 5 μm) column thermostated at 45 °C using 0.02 M KH_2PO_4 (pH 2.5): acetonitrile as a mobile phase in gradient elution mode. A PDA detector set at 210 nm was used for detection. The investigated validation elements showed the method has acceptable specificity, accuracy, linearity, precision, robustness and high sensitivity with detection limits and quantitation limits ranging from 0.028 μg/ml to 0.063 μg/ml and 0.084 μg/ml to 0.192 μg/ml respectively. The method can be used for routine quality control analysis and stability testing of lopinavir drug substance.

1. Introduction

Lopinavir is a human immunodeficiency virus (HIV) protease inhibitor [1] and is chemically designated as (2*S*,3*S*,5*S*)-2-(2,6-dimethylphenoxyacetyl)amino-3-hydroxy-5-[2*S*-(1-tetrahydropyrimid-2-onyl)-3-methylbutanoyl]amino-1,6-diphenylhexane (Fig. 1). This class of drugs inhibits the HIV protease preventing cleavage of the gag-pol polyprotein, reducing the probability of viral particles reaching a mature infectious state. Administered alone, lopinavir has insufficient bioavailability. However, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of cytochrome P450 3A4 [2,3] and therefore lopinavir is co-administered with sub-therapeutic doses of ritonavir by oral route of administration.

Several HPLC methods have been described in the literature for the determination of lopinavir [4–20]. These are determination of lopinavir alone and simultaneously with other antiretrovirals in human plasma by RP-HPLC [4–18]. Donato et al. have reported a HPLC method for the assay of lopinavir and ritonavir in soft-gel capsules, which described an isocratic RP-HPLC method [19]. However, this paper was restricted to the determination of lopinavir and ritonavir only from its degradation products and the details of degradation impurities formed under the stress conditions employed were not discussed. More recently Seshachalam et al. have reported an isocratic RP-HPLC method for the lopinavir assay and determination of its process and degradation impurities in bulk drug and pharmaceutical formulation [20]. The impurities discussed in this paper [20] were Lopinavir alanine analog (*N*-(1-benzyl-4-[2-(2,6-dimethylphenoxy)-acetylamino]-3-hydroxy-5-phenylpentyl)-2-(2-oxo-tetrahydro-pyrimidin-1-yl)-propionamide), RS2, RS3 and RS7. Although, this paper has addressed those issues which were not dealt by Donato et al., it did not study for the comprehensive impurity profiling. During the study of synthetic process in our laboratory, the impurities RS4–RS10 were also observed. The two related substances, 4-hydroxy lopinavir (RS4) and 4-oxo lopinavir (RS5) were identified metabolites of lopinavir and had been reported by Kumar et al. [3].

As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product [21,22]. Further, lopinavir is not yet official in any of the pharmacopoeia.

Therefore, the present study aimed at the development of a simple RP HPLC method with gradient elution for the determination of process and degradation related substances in lopinavir bulk drugs and validation as per ICH guidance documents. The intermediate lopinavir amino alcohol (RS1) and starting material

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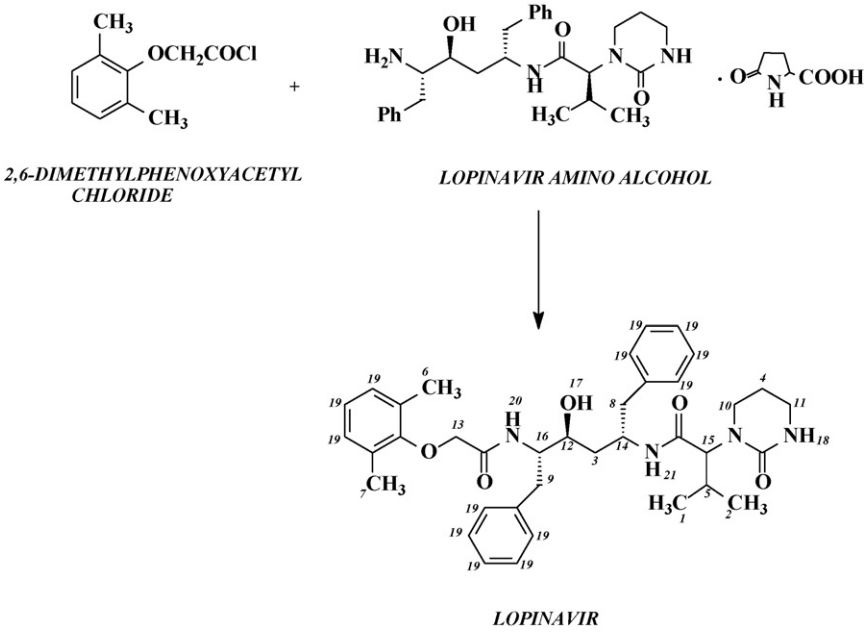


Fig. 1. Reaction scheme for the synthesis of lopinavir.

2,6-dimethylphenoxy acetic acid (RS2) were also present in the sample at levels below 0.1%. The molecular weights of related substances in lopinavir bulk drug were determined by LC–MS analysis and were isolated and characterized. The lopinavir acid hydrolysis degradation product (RS3) reported by Seshachalam et al. [20] was also isolated.

Correction factors (CF) of all these related substances against lopinavir were determined and used for their quantitative determination.

2. Experimental

2.1. Materials and reagents

HPLC grade acetonitrile and methanol procured from Merck (India) were used. Potassium dihydrogen orthophosphate, ammonium acetate, hydrochloric acid, sodium hydroxide and hydrogen peroxide were all of AR grade, procured from Merck (India). HPLC grade water obtained from Millipore system (Millipore Inc., USA) was used throughout the analysis. Orthophosphoric acid was obtained from Fluka Chemicals, Switzerland. The investigated sample, lopinavir was synthesized in APL Research Centre (A Division of Aurobindo Pharma Limited, Hyderabad, India). Related substances were isolated in the lab.

2.2. HPLC (analytical) instrumentation and operating conditions

A Waters HPLC (Milford, MA, USA) equipped with Alliance 2695 separations module and 2996 photodiode array detector was used. HPLC columns used in the present study were: (1) Targa C_{18}, 5 μm, 250 mm × 4.6 mm (Higgins Analytical Inc., CA, USA); (2) Inertsil ODS-3V, 5 μm, 250 mm × 4.6 mm (G.L. Sciences, Japan); (3) YMC Pack ODS-AQ, 250 mm × 4.6 mm, 5 μm (YMC Inc., USA) and (4) Symmetry C_{18}, 5 μm, 250 mm × 4.6 mm (Waters Corporation, USA). The column YMC Pack ODS-AQ (250 mm × 4.6 mm, 5 μm) thermostated at 45 °C was used for the separation. 0.02 M potassium dihydrogen orthophosphate buffer was prepared by dissolving 2.72 ± 0.10g potassium dihydrogen orthophosphate in 1000 ml of water and pH was adjusted to 2.5 ± 0.05 using orthophosphoric acid, filtered through 0.45 μm membrane filter (Millipore PVDF) and degassed in ultrasonic bath prior to use as mobile phase A. Acetonitrile was used as mobile phase B. The flow rate and injection volumes were 1.0 ml/min and 20 μl respectively. The analysis was carried out under gradient conditions as follows, time (min)/A (v/v):B (v/v); $T_{0.01}$/60:40, $T_{30.0}$/35:65, $T_{40.0}$/20:80, $T_{50.0}$/20:80, $T_{52.0}$/60:40 and $T_{60.0}$/60:40. The data were acquired at 210 nm for 50 min and processed by use of Empower Pro data handling system. For the analysis of forced degradation samples, the photodiode array detector was used in the scan mode from 200 nm to 400 nm. The peak homogeneity was expressed in terms of peak purity values.

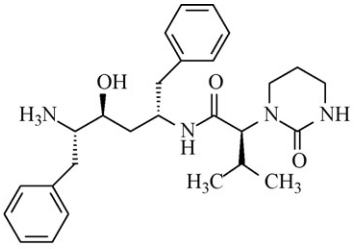
2.3. Analytical procedure

A mixture of water and acetonitrile in the ratio of 30:70 (v/v) was used as diluent in the preparation of analytical solutions. Lopinavir working reference standard solution (1000 μg/ml) spiked with each of RS5 and RS6 at a level of 0.3% (w/w) was used as system suitability solution. Lopinavir working reference standard at 3 μg/ml and lopinavir drug substance at 1000 μg/ml concentrations were used as diluted standard solution and sample solution respectively. Solutions of all the related substances (0.3 mg/ml) were prepared by dissolving known amounts of the substances initially in 3 ml of acetonitrile, made up to the mark with diluent. These solutions were diluted further adequately to study the validation attributes. The specification limits used for validation studies were 0.3% for the related substances RS3, RS4, RS5 and 0.15% for the remaining related substances. 20 μl of system suitability solution, blank, six replicate injections of diluted standard solution and sample solution were separately chromatographed. A resolution of not less than 3.0 between RS5 and RS6 was set as system suitability requirement. The R.S.D. of not more than 5.0% for lopinavir peak areas obtained from six replicate injections of diluted standard solution is used to verify the system precision. All the known and unknown related substances were determined against mean area obtained from

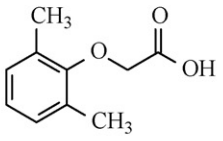
replicate injections of diluted standard solution and correction factors (CF). The CF for each related substance was calculated w.r.t. lopinavir, from the ratio of slope of lopinavir to slope of individual related substance obtained from the regression analysis.

2.4. Procedure for forced degradation study

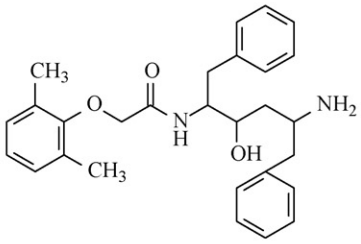
Forced degradation of lopinavir drug substance was carried out under acid/base hydrolytic, oxidative, thermolytic, photolytic and humid stress conditions. Solutions were prepared by dissolving drug substance in diluent and then treating with aqueous 1 M hydrochloric acid, aqueous 5 M sodium hydroxide and aqueous 30% hydrogen peroxide at 85 °C for 60 min, 120 min and 120 min respectively. After the degradation, these solutions were diluted with diluent and analyzed in the proposed method. For thermal stress, sample of drug substance was placed in a controlled temperature oven at 80 °C for 78 h. For photolytic stress, the sample was exposed to photolyte of 1.2×10^6 lx for 120 h and for degradation under humidity sample was exposed to 92% r.h. at 25 °C for 120 h. After the exposure to the above stress conditions, solutions of these samples were prepared by dissolving respective samples in diluent and diluted to the desired concentration and subjected for analysis using the proposed method.



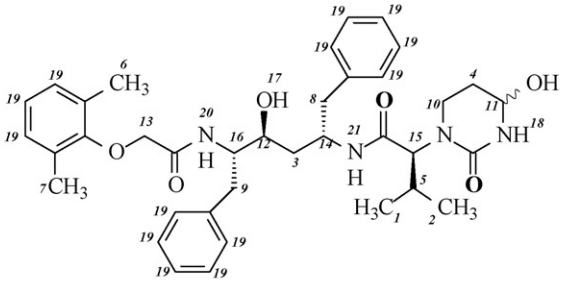
Lopinavir amino alcohol (RS1), RRT~0.15



2,6-Dimethylphenoxy acetic acid (RS2), RRT~0.33

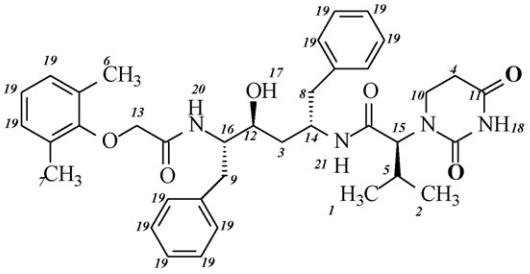


Lopinavir acid hydrolysis degradation product (RS3), RRT~0.39



4-Hydroxy lopinavir, (RS4), RRT~0.76

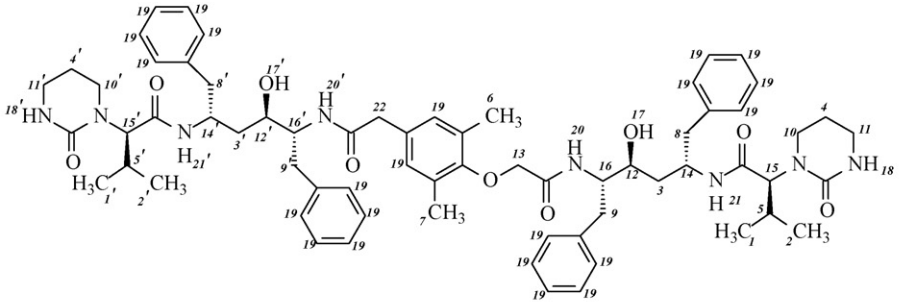
(2*S*,3*S*,5*S*)-2-(2,6-dimethylphenoxyacetyl)amino-3-hydroxy-5-[2*S*-(1-tetrahydropyrimid-4-hydroxy-2-onyl) -3-methyl butanoyl]amino-1,6-diphenylhexane)



4-Oxo lopinavir (RS5), RRT~0.88

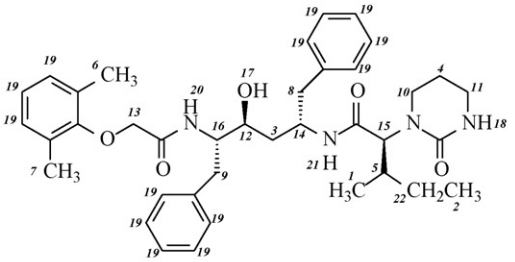
(2*S*,3*S*,5*S*)-2-(2,6-dimethylphenoxyacetyl)amino-3-hydroxy-5-[2*S*-(1-tetrahydropyrimid-2,4-dionyl)-3-methyl butanoyl] amino-1,6-diphenylhexane

Fig. 2. Chemical structures of lopinavir related substances, RS1–RS10.



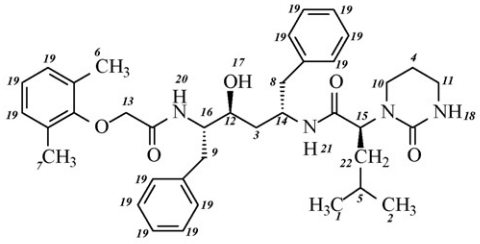
Carboxymethyl analog (RS6), RRT~0.94

(2*S*,3*S*,5*S*)-2-[2,6-dimethyl-4-[[(2*S*,3*S*,5*S*)-3-hydroxy-5-[(2*S*)-(1-tetrahydropyrimid-2-onyl)-3-methylbutanoyl]amino-1,6-diphenyl-2-hexyl] aminocarbonylmethyl]phenoxyacetyl]amino-3-hydroxy-5-[2*S*-(1-tetrahydropyrimid-2-onyl)-3-methylbutanoyl]amino-1,6-diphenylhexane



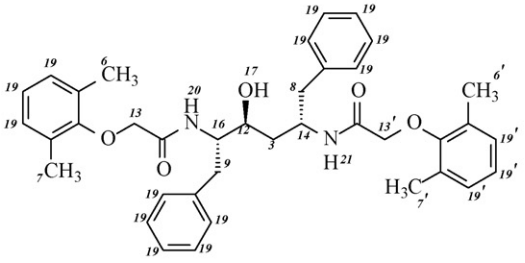
Lopinavir isoleucine analog (RS7), RRT~1.12

(2*S*,3*S*,5*S*)-2-(2,6-dimethyl phenoxyacetyl)amino-3-hydroxy-5-[2*S*-(1-tetrahydropyrimid-2-onyl)-3-methyl pentanoyl]amino-1,6-diphenylhexane



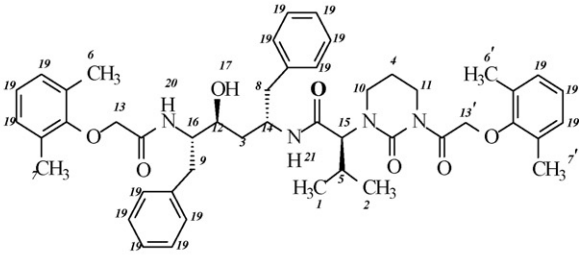
Lopinavir leucine analog (RS8), RRT~1.15

(2*S*,3*S*,5*S*)-2-(2,6-dimethylphenoxyacetyl) amino-3-hydroxy-5-[2*S*-(1-tetrahydropyrimid-2-onyl)-4-methyl-pentanoyl]amino-1,6-diphenylhexane



Lopinavir diamide (RS9), RRT~1.59

(2*S*,3*S*,5*S*)-2,5-*Bis*[(2,6-dimethylphenoxyacetyl)amino]-3-hydroxy-1,6-diphenylhexane



Diacylated lopinavir (RS10), RRT~1.68

(2*S*,3*S*,5*S*)-2-(2,6-dimethylphenoxyacetyl)amino-3-hydroxy-5-[2*S*-{1-tetrahydropyrimid-3-(2,6-dimethyl phenoxyacetyl)-2-onyl}-3-methyl butanoyl]amino-1,6-diphenylhexane

Fig. 2. (*Continued*).

2.5. Preparative HPLC instrumentation and operating conditions

A Shimadzu LC-8A Preparative Liquid Chromatograph equipped with SPD-10A VP, UV–vis detector [Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan] was used. Peerless Basic, C_{18} (500 mm × 30 mm, particle size 10 μm,) column was employed for isolation of impurities. Mobile phase consisted of water and acetonitrile. Flow rate was kept at 25 ml/min and detection was carried out at 210 nm. The gradient program was as follows, time (min)/A (v/v):B (v/v); $T_{0.01}$/100:0, $T_{20.0}$/40:60, $T_{70.0}$/20:80, $T_{80.0}$/50:50.

2.6. LC–MS/MS analysis

LC–MS/MS analysis was carried out using PerkinElmer triple quadrupole mass spectrometer (API 2000, PE SCIEX) coupled with a Shimadzu HPLC equipped with SPD 10A VP UV–vis detector and LC AT VP pumps [Foster city, CA, USA]. Analyst software was used

for data acquisition and data processing. The turbo ion spray voltage was maintained at 5.5 kV and temperature was set at 375 °C. High pure nitrogen gas was used as auxiliary gas and curtain gas. Zero air is used as nebulizer gas. LC–MS spectra were acquired from m/z 100 to 1200 in 0.1 amu steps with 2.0 s dwell time. Lopinavir sample was subjected to LC–MS/MS analysis. The analysis was carried out using a reversed phase column YMC Pack ODS-AQ, 250 mm × 4.6 mm, 5 μm. Mobile Phase A used was 0.1% aqueous formic acid and Mobile Phase B was acetonitrile. Detection was carried out at 210 nm and flow rate was kept at 1.0 ml/min. Water and acetonitrile mixture in the ratio of 50:50 (v/v) was used as diluent. Data acquisition time was 60 min. The gradient program was as follows: time (min)/A (v/v):B (v/v); $T_{0.01}$/70:30, $T_{30.0}$/30:70, $T_{40.0}$/20:80, $T_{50.0}$/20:80, $T_{52.0}$/70:30, $T_{60.0}$/70:30. Seven related substances were detected in the laboratory batch sample. The masses of detected peaks were 645.6[$(MH)^+$], 643.6[$(MH)^+$], 1136.1[$(MH)^+$], 643.4[$(MH)^+$], 643.4[$(MH)^+$], 609.2[$(MH)^+$] and 791.4[$(MH)^+$]. Based on these mass values, the structures given in Fig. 2 were suggested.

2.7. ^{1}H NMR spectroscopy

The ^{1}H NMR spectra were recorded on Bruker 300 MHz spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard with sixteen number of scans over a spectral width (sw) of 20 ppm, time domain 32 K (td), relaxation delay (d1) 2.0 s and 01P, 5.0 ppm.

2.8. Mass spectrometry

Mass spectra were recorded on PerkinElmer PE SCIEX-API 2000 mass spectrometer equipped with a Turbo ion spray interface at 375 °C. Detection of the ions was performed in electron spray ionization positive ion mode. Spectra were acquired from m/z 100 to 1200 in 0.1 amu steps with 2.0 s dwell time with 10 number of scans.

2.9. FT-IR spectroscopy

FT-IR spectra were recorded for lopinavir and seven related substances, RS4 to RS10 on PerkinElmer model-spectrum one instrument using KBr pellet method.

2.10. Isolation of related substances by preparative HPLC

Impurities were present in the crude samples at about 0.13–0.3% level by area normalization. All seven related substances were isolated by preparative HPLC as per the conditions described in Section 2.5. Fractions collected were analyzed by analytical HPLC as per the conditions mentioned in Section 2.2. Fractions of >95% were pooled together, concentrated in vacuo to remove acetonitrile. The aqueous solution was lyophilized using freeze drier (Virtis advantage 2XL). Related substances RS4 to RS8 were obtained as white powders and RS9 and RS10 were obtained as an off-white powders. The chromatographic purities had been determined by the HPLC method described in Section 2.2 and the purities were 94.4%, 94.8%, 90.9%, 97.0%, 93.5%, 98.4% and 91.0% respectively.

2.11. Synthesis of lopinavir

The reaction scheme for the synthesis of lopinavir is shown in Fig. 1 [23].

3. Results and discussion

3.1. Detection of related substances

Laboratory batches of lopinavir were analyzed for their related substances identification using the HPLC method described in Section 2.2. These samples were subjected to LC–MS/MS analysis using the method described in Section 2.6. The isolated related substances were co-injected with lopinavir to confirm the retention times. All the related substances were well resolved from lopinavir peak and the representative resolution mixture chromatogram was shown in Fig. 3. Relative retention times of the related substances with respect to lopinavir are shown in Fig. 2.

The related substance RS1 is an intermediate, RS2 is a starting material of the process and RS3 is lopinavir acid hydrolysis degradation product. The presence of these substances was confirmed by co-injection with lopinavir in HPLC.

3.2. Structural elucidation

Based on the mass values obtained from the LC–MS/MS spectral data and ^{1}H NMR chemical shift data, the structures of the related substances were confirmed as given in Fig. 2. The ^{1}H NMR chemical shift values of lopinavir and all related substances are presented in Table 1 and FT-IR spectral data are presented in Table 2.

Based on the spectral data, the structures elucidated for RS3 and RS7 matched with the lopinavir acid hydrolysis degradation product and lopinavir isoleucine analog reported by Seshachalam et al. [20]; RS4 and RS5 matched with the metabolites M-3 and M-1 reported by Kumar et al. [3] respectively.

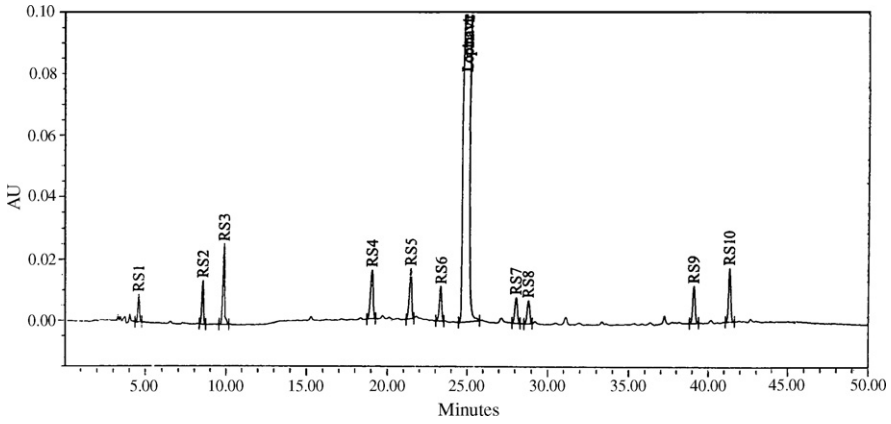


Fig. 3. Typical HPLC chromatogram of lopinavir drug substance spiked with related substances at specification level.

Table 1
Comparative ^{1}H NMR assignments for lopinavir and related substances.

Position[a]	Lopinavir δ (ppm), multiplicity	RS4 δ (ppm), multiplicity	RS5 δ (ppm), multiplicity	RS7 δ (ppm), multiplicity	RS8 δ (ppm), multiplicity	Position[a]	RS6 δ (ppm), multiplicity	Position[a]	RS9 δ (ppm), multiplicity	Position[a]	RS10 δ (ppm), multiplicity
1	0.73–0.77 (2d, 6H)	0.79 (d, 6H)	0.77 (m, 6H)	0.71 (d, 3H)	0.83–0.88 (m, 6H)	1, 1′	0.71–0.76 (m, 12H)	1	–	1	0.72–0.76 (m, 6H)
2				0.78–0.86 (m, 3H)		2, 2′		2	–	2	
3	1.50 (m, 4H)	1.50 (m, 4H)	1.50–1.95 (m, 2H)	1.50 (m, 4H)	1.35–1.60 (m, 5H)	3, 3′	1.40–1.55 (m, 8H)	3	1.65 (m, 2H)	3	1.40–1.65 (m, 4H)
4						4, 4′		4	–	4	
5	2.05 (m, 1H)	2.05 (m, 1H)	2.27 (m, 1H)	1.85 (m, 1H)		5, 5′	2.05 (m, 2H)	5	–	5	2.00 (m, 1H)
6	2.15 (s, 6H)	2.17 (s, 6H)	2.16 (s, 6H)	2.15 (s, 6H)	2.14 (s, 6H)	6	2.06 (s, 6H)	6, 6′	2.14–2.16 (2s, 12H)	6	2.15 and 2.24 (2s, 12H)
7						7		7, 7′	–	7	
8	2.50–2.82 (2m, 4H)	2.55–2.80 (2m, 4H)	2.55–2.84 (m, 4H)	2.50–2.80 (m, 4H)	2.65 and 2.80 (m, 4H)	8, 8′	2.50–2.81 (m, 8H)	8	2.78 and 2.85 (2d, 4H)	8	2.70 (m, 4H)
9						9, 9′		9		9	
10	2.90 (t, 2H)	–	3.00 (m, 2H)	2.93–3.02 (m, 4H)	2.81 and 3.04 (2m, 4H)	10, 10′	–	10	–	10	2.81 and 3.55 (m, 4H)
11	3.02 (t, 2H)	4.75 (m, 1H)	–			11, 11′	2.90–3.25 (m, 8H)	11	–	11	
12	3.60 (m, 1H)	3.60 (m, 1H)	3.64 (m, 1H)	3.60 (m, 1H)	3.55 (m, 1H)	12, 12′	3.60 (m, 2H)	12	3.70 (m, 1H)	12	3.62 (m, 1H)
13	4.06 and 4.09 (ABq, 2H)	4.06 and 4.10 (ABq, 2H)	4.09 and 4.12 (ABq, 2H)	4.07 and 4.09 (ABq, 2H)	4.07 and 4.08 (ABq, 2H)	13	3.99 (ABq, 2H)	13, 13′	3.95 and 4.05 (ABq, 2H)	13, 13′	4.09 and 4.12 (ABq, 2H)
14	4.15 (m, 1H)	4.15 (m, 1H)	4.22 and 4.26 (2m, 2H)	4.20 (m, 1H)	4.20 (m, 2H)	14, 14′	4.20 (m, 2H)	14	4.35 (m, 2H)	14	4.20–4.40 (m, 3H)
15	4.28–4.32 (m, 2H)	4.34 (m, 2H)		4.30 (m, 1H)		15, 15′	4.28–4.32 (m, 4H)	15		15	
16			4.33 (m, 1H)	4.45 (m, 1H)	4.77 (m, 1H)	16, 16′		16		16	
17	5.00 (d, 1H)	5.05 (d, 1H)	5.05 (brs, 1H)	5.00 (d, 1H)	5.00 (d, 1H)	17, 17′	4.80 and 5.05 (2d, 2H)	17	5.08 (d, 1H)	17	5.00 (d, 1H)
18	6.28 (s, 1H)	–	10.15 (s, 1H)	6.30 (s, 1H)	6.28 (s, 1H)	18, 18′	6.29 (m, 2H)	18	–	18	–
19	6.93–7.26 (m, 13H)	6.98–7.28 (m, 11H)	6.94–7.25 (m, 13H)	6.93–7.26 (m, 13H)	6.94–7.26 (m, 13H)	19, 19′	6.78 (s, 2H) and 7.04–7.23 (m, 20H)	19	6.93–7.27 (m, 6H)	19	6.93–7.26 (m, 16H)
20	7.45 (d, 1H)	7.47 (d, 1H)	7.51 (d, 1H)	7.45 and 7.55 (2d, 2H)	7.39–7.47 (m, 2H)	20, 20′	7.51 and 7.70 (2d, 2H)	20	7.52–7.86 (2d, 2H)	20	7.49–7.73 (2d, 2H)
21	7.52 (d, 2H)	7.67 (d, 1H)	7.95 (d, 1H)			21, 21′	7.41 (d, 2H)	21		21	
22	–	5.44 (d, 1H)	–	0.90 and 1.35 (m, 2H)	1.51 (m, 2H)	22	3.25 (s, 2H)	22	–	22	–

[a] Refer to Fig. 1 for numbering of lopinavir and Fig. 2 for numbering of RS4–RS 10, s, singlet; d, doublet; t, triplet; m, multiplet; brs, broad singlet; q, quartet; ABq, AB quartet.

Table 2
FT-IR spectral data of lopinavir and related substances.

Serial number	Component	IR (KBr) absorption bands (cm^{-1})
1	Lopinavir	3411, 3062, 3028, 2962, 2931, 2871, 1651, 1600, 1515, 1477, 1453, 1379, 840, 768, 701
2	RS4	3405, 3063, 3029, 2963, 2924, 2874, 1652, 1600, 1529, 1471, 1455, 1378, 841, 770, 701
3	RS5	3410, 3063, 3029, 2963, 2927, 2874, 1652, 1600, 1533, 1471, 1445, 1376, 840, 771, 701
4	RS6	3410, 3062, 3029, 2963, 2931, 2871, 1644, 1600, 1514, 1453, 1376, 765, 749, 701
5	RS7	3411, 3062, 3028, 2962, 2930, 2873, 1647, 1513, 1477, 1453, 1378, 840, 769, 753, 701
6	RS8	3411, 3061, 3028, 2954, 2868, 1651, 1600, 1516, 1477, 1455, 1370, 840, 768, 753, 701
7	RS9	3403, 3060, 3029, 2923, 2856, 1653, 1604, 1604, 1476, 1455, 1377, 842, 766 740, 698
8	RS10	3410, 3063, 3028, 2963, 2925, 2873, 1662, 1600, 1529, 1455, 1379, 769, 751, 701

3.3. Formation of impurities

RS4 and RS5 are formed via two pathways. In the first pathway, the components 4-hydroxy lopinavir amino alcohol and 4-oxo lopinavir amino alcohol present as impurities in the intermediate lopinavir amino alcohol condense with 2,6-dimethylphenoxyacetyl chloride, during the preparation of lopinavir. Also these two impurities originate from the oxidation of lopinavir on extended storage at higher temperature. RS6 originates from the condensation of lopinavir amino alcohol with 2,6-dimethylphenoxyacetyl chloride in the preparation of lopinavir, in which 4-carboxymethoxy-3,5-dimethylphenyl acetic acid present as an impurity in the raw material 2,6-dimethylphenoxyacetyl chloride gets condensed at both the carboxyl groups. RS7 and RS8 are formed via the impurities L-isoleucine and L-leucine present in L-valine, which is used in the preparation of penultimate steps of lopinavir amino alcohol undergo the reaction sequence employed to prepare lopinavir. RS9 originates from the synthesis of lopinavir by condensation of

Table 3
Selectivity of C_{18} columns of different manufactures in the optimized chromatographic conditions.

Column	Component	k	α	Rs	T
YMC ODS-AQ, 5 μm, 250 mm × 4.6 mm	RS1	0.4	5.00	–	0.9
	RS2	2.0	1.30	17.6	1.0
	RS3	2.6	2.27	8.2	1.0
	RS4	5.9	1.17	37.7	1.0
	RS5	6.9	1.09	8.9	1.1
	RS6	7.5	1.08	5.3	0.9
	Lopinavir	8.1	1.12	4.8	1.0
	RS7	9.1	1.04	8.8	0.9
	RS8	9.5	1.41	2.8	1.0
	RS9	13.4	1.06	38.3	1.0
	RS910	14.2	–	8.6	1.0
Targa C_{18}, 5 μm, 250 mm × 4.6 mm	RS1	0.8	3.25	–	1.2
	RS2	2.6	1.08	17.6	1.0
	RS3	2.8	2.71	7.9	0.9
	RS4	7.6	1.17	37.1	1.0
	RS5	8.9	1.04	8.1	0.9
	RS6	9.3	1.09	2.6	0.9
	Lopinavir	10.1	1.14	5.39	0.9
	RS7	11.5	1.03	7.9	0.9
	RS8	11.9	1.42	2.4	0.8
	RS9	16.9	1.06	32.4	0.9
	RS910	17.9	–	8.0	0.9
Inertsil ODS-3V, 5 μm, 250 mm × 4.6 mm	RS1	0.4	9.25	–	0.8
	RS2	3.7	1.08	31.0	1.1
	RS3	4.0	2.45	7.6	1.0
	RS4	9.8	1.16	35.1	0.9
	RS5	11.4	1.05	8.1	1.1
	RS6	12.0	1.09	3.4	1.0
	Lopinavir	13.1	1.14	5.5	1.0
	RS7	14.9	1.03	8.5	1.0
	RS8	15.3	1.39	2.5	0.9
	RS9	21.2	1.06	35.0	1.0
	RS10	22.5	–	8.4	1.0
Symmetry C_{18}, 5 μm, 250 mm × 4.6 mm	RS1	0.3	10.00	–	0.8
	RS2	3.0	1.07	27.7	1.1
	RS3	3.2	2.59	7.5	1.0
	RS4	8.3	1.17	37.5	0.9
	RS5	9.7	1.02	9.0	0.9
	RS6	9.9	1.12	1.3	1.1
	Lopinavir	11.1	1.14	7.3	0.9
	RS7	12.7	1.04	9.0	1.0
	RS8	13.2	1.44	2.7	1.0
	RS9	19.0	1.07	38.6	0.9
	RS10	20.3	–	9.5	1.0

k, Capacity factor; α, selectivity; Rs, resolution; T, tailing factor

Table 4
Forced degradation studies data.

Stress condition	Degradation (%)	Observation	Peak purity	
			PA	PT
Undegraded	–	–	0.323	0.603
Acid (1 M HCl/85 °C/60 min)	15.1	Increase in levels of RS1 (1.3%) and RS3 (13.8%)	0.236	0.443
Base (5 M NaOH/85 °C/120 min)	Below 0.5	No increase in levels of known related substances except an additional peak at ~1.07 RRT (0.2%)	0.243	0.533
Oxidation (30% H_2O_2/85 °C/120 min)	Below 1	Increase in levels of RS3 (0.2%), RS6 (0.1%), RS10 (0.1%), and additional peak at RRT ~0.84 (0.3%)	0.295	0.662
Thermal (80 °C/78 h)	Nil	–	0.226	0.506
Photolytic (10K lx/120 h)	Below 0.7	Increase in levels of RS2 (0.1%), RS4 (0.1%), RS5 (0.1%), RS6 (0.1%), RS9 (0.1%) and RS10 (0.1%)	0.236	0.494
Humidity 92% r.h./25 °C/120 h	Nil	Increase in levels of RS5 (0.1%) and RS10 (0.1%)	0.252	0.535

PA, Purity angle; PT, purity threshold.

2,6-dimethylphenoxyacetyl chloride on both the amino groups of 2,5-diamino-3-hydroxy-1,6-diphenyl hexane. RS10 originates from the condensation of lopinavir with 2,6-dimethylphenoxyacetyl chloride at NH of tetrahydropyrimidine ring of lopinavir during the synthesis.

3.4. Optimization of chromatographic conditions

In the preliminary experiments lopinavir and all the related substances were subjected to separation using RP-HPLC method described by Seshachalam et al. [20]. The retention factors (k) of all these related substances were found to be in between 0.3 and 24. Because of high dependence on mobile phase composition; the attempts to improve selectivity, peak shapes and to reduce the retention times by altering buffer pH, acetonitrile and methanol composition were not successful.

Therefore, gradient mode of separation was chosen using 0.02 M KH_2PO_4 buffer as mobile phase A, acetonitrile as mobile phase B, and YMC Pack ODS-AQ column of 250 mm × 4.6 mm i.d., particle size 5 μm. Separation of all the related substances was achieved by optimizing gradient program, pH of the buffer and column temperature. Finally, a good separation of the related substances was achieved with sharp elution of peaks and good selectivity with variable mixtures of mobile phase A (0.02 M KH_2PO_4, pH 2.5) and mobile phase B (acetonitrile) under the gradient conditions, time (min)/A (v/v):B (v/v); $T_{0.01}$/60:40, $T_{30.0}$/35:65, $T_{40.0}$/20:80, $T_{50.0}$/20:80, $T_{52.0}$/60:40 and $T_{60.0}$/60:40, column temperature maintained at 45 °C with PDA detector set at 215 nm. The higher column temperature in the procedure evidenced the related substances and lopinavir were stable throughout the column separation process. A typical chromatogram showing the separation of the related substances spiked at specification level was given in Fig. 3. The column selectivity [24] of the method is further investigated using, Inertsil ODS-3V, Targa C_{18} and Symmetry C_{18} of similar dimensions and the data on the column efficiency parameters were given in Table 3. The results of these studies indicated that the peak separation pattern obtained using Targa C_{18} column is much similar to that of YMC Pack. This could be due to similar percentage of carbon load (16%), surface area and pore size of the column packing as seen from the column catalogues. Where as peak separation obtained with Inertsil (15% carbon load) showed the decrease in resolution of RS4 and RS6 in comparison with the previous two columns data, while the change in the retention and resolution of other peaks is relatively less. However, with Symmetry C_{18} (20% carbon load) columns, the resolution between RS5 and RS6 decreased to below 1.5. As the hydrophobicity of the Symmetry column is higher out of these four columns, this column did not separate these two peaks. The data presented in Table 3 suggested, Targa C_{18} column may be used as an alternative column. The optimized method was validated as per ICH guidelines [25].

4. Validation

4.1. Specificity (selectivity)

The data on degradation studies revealed that the degradation products were well separated from the lopinavir and known related substances and the peak purity data (purity angle is less than purity

Table 5
Linearity data for lopinavir and related substances.

Component	Calibration range (μg/ml)	Regression equation	SES[a]	SEI[b]	CC[c] (r)	δ[d]	Correction factor[e]	LOD (μg/ml) (%R.S.D.)	LOQ (μg/ml) (%R.S.D.)
Lopinavir	0.030–4.523	$y = 58524x - 156$	2667 (±6150)	6090 (±14044)	0.9999	749	1.00	0.042 (12.7)	0.128 (1.2)
RS1	0.015–2.254	$y = 36729x - 191$	1667 (±3844)	1895 (±4370)	0.9997	706	1.60	0.063 (12.0)	0.192 (0.9)
RS2	0.015–2.306	$y = 62956x + 711$	2974 (±6858)	3459 (±7976)	0.9999	527	0.93	0.028 (12.2)	0.084 (1.5)
RS3	0.030–4.525	$y = 60076x + 54$	2710 (±6249)	6192 (±14279)	0.9999	755	0.97	0.041 (13.6)	0.126 (2.2)
RS4	0.030–4.512	$y = 53402x + 766$	238 (±549)	543 (±1252)	0.9999	740	1.10	0.046 (18.8)	0.139 (5.2)
RS5	0.030–4.493	$y = 51523x + 88$	2339 (±5394)	5311 (±12247)	0.9999	889	1.14	0.058 (13.7)	0.174 (4.5)
RS6	0.015–2.256	$y = 51191x + 80$	2295 (±5292)	2612 (±6023)	0.9999	520	1.14	0.034 (11.8)	0.102 (3.3)
RS7	0.015–2.275	$y = 55882x - 259$	2444 (±5636)	2810 (±6480)	0.9999	499	1.05	0.029 (13.1)	0.089 (2.8)
RS8	0.015–2.249	$y = 53869x - 118$	708 (±1633)	804 (±1854)	0.9999	518	1.09	0.032 (13.1)	0.096 (2.8)
RS9	0.015–2.260	$y = 73250x + 575$	3329 (±7677)	3815(±8797)	0.9998	1056	0.80	0.048 (11.5)	0.144 (5.6)
RS10	0.015–2.260	$y = 72996x - 545$	3054 (±7043)	3500(±8071)	0.9998	1051	0.80	0.048 (12.4)	0.144 (3.2)

[a] SES: Standard error of slope and the values given in parentheses are 95% confidence limits.
[b] SEI: Standard error of intercept and the values given in parentheses are 95% confidence limits.
[c] CC: Correlation coefficient.
[d] δ: Standard deviation of peak areas (residual standard deviation of y on x).
[e] CF: Correction factor.

Table 6
Precision results for lopinavir related substances (Set I: repeatability and Set II: intermediate precision).

Component	Repeatability results (n = 6) (Mean result ± SD, %RSD)	Intermediate precision results (n = 6) (Mean result ± SD, %RSD)	Overall results (n = 12) (Mean result ± SD, %RSD)
RS1	0.171 ± 0.001, 0.6	0.179 ± 0.001, 0.6	0.175 ± 0.004, 2.3
RS2	0.142 ± 0.001, 0.7	0.147 ± 0.001, 0.7	0.144 ± 0.003, 2.1
RS3	0.291 ± 0.001, 0.3	0.299 ± 0.001, 0.3	0.295 ± 0.004, 1.4
RS4	0.291 ± 0.001, 0.3	0.302 ± 0.001, 0.3	0.297 ± 0.006, 2.0
RS5	0.354 ± 0.001, 0.3	0.366 ± 0.002, 0.5	0.360 ± 0.006, 1.7
RS6	0.171 ± 0.001, 0.6	0.177 ± 0.001, 0.6	0.174 ± 0.003, 1.7
RS7	0.150 ± 0.001, 0.7	0.153 ± 0.001, 0.7	0.151 ± 0.002, 1.3
RS8	0.143 ± 0.002, 1.4	0.149 ± 0.001, 0.7	0.146 ± 0.003, 2.1
RS9	0.147 ± 0.001, 0.7	0.153 ± 0.001, 0.7	0.150 ± 0.003, 2.0
RS10	0.195 ± 0.001, 0.5	0.202 ± 0.001, 0.5	0.198 ± 0.004, 2.0

threshold) of lopinavir indicated that is was spectrally pure. The data on forced degradation studies is given in Table 4.

4.2. Linearity

The linearity of peak areas versus different concentrations was evaluated for lopinavir and all the related substances using 10 levels ranging from 0.015 μg/ml to 4.5 μg/ml equivalent to 0.0015 (%w/w) to 0.45 (%w/w) with respect to sample concentration. The linear regression data for all the components tested were presented in Table 5. The UV CFs for each related substance were calculated against lopinavir, from the ratio of slope of lopinavir to slope of individual related substance obtained from the regression line. The data shown in Table 5 is confirmed the detector response at 210 nm were linear over the ranges studied for all components. These CFs are used while estimating the levels of known related substances and all other unknown impurities are estimated using the diluted standard solution.

4.3. Limits of detection and quantitation (LOD and LOQ)

The limit of detection and limit of quantitation were determined for lopinavir and for each of the related substances as per ICH guideline [25] from the standard deviation of the peak areas and slope of linearity data. The values of LOD and LOQ for lopinavir were 0.042 μg/ml, 0.128 μg/ml and they were for related substances, in the ranges; 0.028–0.063 μg/ml and 0.084–0.192 μg/ml respectively. The calculated LOD and LOQ concentrations of all the components were verified for precision. R.S.D. was in the range of 11.5–18.8% for LOD and 0.9–5.6% for LOQ respectively. The results were depicted in Table 5.

4.4. Precision

System precision was verified using diluted standard solution, which was analyzed for six times and R.S.D. of lopinavir peak areas was evaluated and found to be 1.0%. Precision of the method was studied for repeatability and intermediate precision. Repeatability was demonstrated by analyzing six separate lopinavir sample solutions that were prepared by spiking the related substances at specification level. The R.S.D. (0.3–1.4%, $n = 6$) for each related substance was evaluated.

The intermediate precision of the method was determined on six separate sample solutions prepared from same lot by spiking the related substances at specification level by a different analyst using different mobile phase and diluent preparations and instrument on a different day with different lot of same brand column. The overall R.S.D. was evaluated and found to be in the range of 1.3–2.3% for all related substances, which were within the acceptance criterion of NMT 10.0% R.S.D. The results were presented in Table 6.

4.5. Accuracy (recovery)

Accuracy of the method for all the related substances was determined by analyzing lopinavir sample solutions spiked with all the related substances at three different concentration levels of 50, 100 and 150% of each in triplicate at the specified limit. The recovery of all these related substances were found to be in-between the predefined acceptance criterion of 90.0–110.0% and the data is given in Table 7.

4.6. Stability of analytical solution

To determine the stability of sample solution, the sample solutions of lopinavir spiked with related substances at specified level were prepared and analyzed immediately after preparation and after different time intervals up to 15 h, while maintaining the sample cooler temperature at about 25 °C and at about 6 °C. The results from these studies indicated, the sample solution was unstable at room temperature and stable for at least 15 h at 6 °C temperature.

Table 7
Accuracy data of related substances.

Component	50% of specification level; amount (%w/w)			100% of specification level[a]; amount (%w/w)			150% of specification level; amount (%w/w)		
	Added[b]	Found[b]	%Recovery[c]	Added[b]	Found[b]	%Recovery[c]	Added[b]	Found[b]	%Recovery[c]
RS1	0.075	0.074	99.1 ± 0.8	0.151	0.150	99.3 ± 0.7	0.225	0.230	102.2 ± 0.4
RS2	0.075	0.075	100.4 ± 0.7	0.151	0.151	100.0 ± 0.7	0.226	0.223	98.8 ± 0.2
RS3	0.148	0.150	100.7 ± 0.6	0.298	0.296	99.3 ± 0.4	0.445	0.443	99.5 ± 0.8
RS4	0.150	0.145	97.1 ± 0.4	0.300	0.288	96.0 ± 0.0	0.449	0.426	94.9 ± 0.2
RS5	0.152	0.154	101.5 ± 0.4	0.304	0.317	104.5 ± 0.2	0.455	0.473	103.9 ± 0.4
RS6	0.077	0.079	102.6 ± 0.0	0.155	0.157	101.3 ± 0.0	0.232	0.235	101.2 ± 02
RS7	0.076	0.078	102.2 ± 0.7	0.152	0.156	102.8 ± 0.4	0.227	0.223	102.8 ± 0.3
RS8	0.074	0.071	95.9 ± 0.6	0.149	0.151	101.3 ± 0.0	0.223	0.228	102.2 ± 0.0
RS9	0.076	0.074	97.0 ± 0.8	0.152	0.154	101.5 ± 0.4	0.232	0.232	102.2 ± 0.0
RS10	0.076	0.078	101.7 ± 0.7	0.153	0.157	102.6 ± 0.0	0.230	0.230	100.6 ± 0.3

[a] 0.3% of RS3, RS4, RS5 and 0.15% of remaining related substances. [b] $n = 3$, average of three determinations. [c] Average ± R.S.D.

Table 8
Robustness data of related substances.

Parameter altered	Variation	RS1		RS2		RS3		RS4		RS5	
		Mean[a] (R.S.D.)	%Difference[b]	Mean (R.S.D.)	%Difference	Mean (R.S.D.)	%Difference	Mean (R.S.D.)	%Difference	Mean (R.S.D.)	%Difference
Flow	−10%	0.175 (5.1)	2.3	0.149 (0.7)	4.9	0.301 (1.0)	6.5	0.313 (0.3)	7.6	0.355 (0.3)	0.3
	+10%	0.161 (0.6)	5.8	0.135 (0.0)	4.9	0.2977 (0.8)	5.4	0.287 (0.3)	1.4	0.328 (0.3)	7.3
Wavelength	−5 nm	0.172 (0.6)	0.6	0.147 (1.2)	3.6	0.311 (2.1)	3.2	0.316 (1.1)	8.6	0.346 (0.8)	2.3
	+5 nm	0.156 (0.5)	8.8	0.152 (1.5)	7.0	0.297 (4.1)	2.3	0.213 (0.4)	7.6	0.326 (0.6)	7.9
%Organic in mobile phase	−2% absolute	0.164 (4.3)	4.1	0.135 (0.7)	4.9	0.291 (0.3)	5.2	0.292 (0.7)	5.2	0.336 (1.5)	5.1
	+2% absolute	0.181 (1.3)	5.8	0.131 (0.8)	7.7	0.284 (0.7)	3.3	0.306 (5.2)	5.2	0.346 (5.4)	2.3
Column oven temperature	−5 °C	0.162 (3.1)	5.3	0.140 (0.7)	1.4	0.303 (0.3)	2.1	0.298 (1.3)	2.4	0.332 (2.2)	6.2
	+5 °C	0.162 (1.9)	5.3	0.137 (1.5)	3.5	0.301 (0.7)	1.2	0.306 (1.0)	5.2	0.331 (1.7)	6.5
pH	−0.2 units	0.185 (7.3)	8.2	0.138 (0.7)	2.8	0.306 (1.6)	1.7	0.301 (2.0)	3.4	0.333 (1.7)	5.9
	+0.2 units	0.160 (2.5)	6.4	0.137 (0.7)	3.5	0.300 (1.7)	3.4	0.286 (2.4)	1.7	0.325 (8.9)	8.2

Parameter altered	Variation	RS6		RS7		RS8		RS9		RS10	
		Mean (R.S.D.)	%Difference	Mean (R.S.D.)	%Difference	Mean (R.S.D.)	%Difference	Mean (R.S.D.)	%Difference	Mean (R.S.D.)	%Difference
Flow	−10%	0.183 (0.5)	7.0	0.161 (0.6)	7.3	0.152 (0.6)	6.3	0.152 (0.6)	6.1	0.186 (0.5)	4.6
	+10%	0.328 (0.9)	2.9	0.161 (0.6)	7.3	0.148 (0.0)	3.5	0.154 (0.6)	4.8	0.183 (4.9)	6.2
Wavelength	−5 nm	0.185 (2.4)	8.2	0.161 (1.3)	7.3	0.152 (3.1)	6.3	0.158 (0.6)	7.5	0.203 (0.6)	4.1
	+5 nm	0.156 (2.3)	8.8	0.144 (2.3)	4.0	0.132 (1.1)	7.7	0.144 (2.4)	2.0	0.186 (0.7)	4.6
%Organic in mobile phase	−2% absolute	0.182 (1.0)	6.4	0.157 (1.3)	4.7	0.153 (0.6)	7.0	0.151 (0.7)	2.7	0.186 (0.6)	4.6
	+2% absolute	0.175 (2.3)	2.3	0.151 (1.3)	0.7	0.150 (0.6)	5.6	0.148 (0.7)	0.7	0.182 (1.1)	6.7
Column oven temperature	−5 °C	0.181 (1.7)	5.8	0.162 (1.9)	8.0	0.146 (2.4)	2.0	0.154 (0.6)	4.8	0.192 (1.0)	1.5
	+5 °C	0.182 (3.6)	6.4	0.162 (1.9)	8.0	0.154 (2.4)	7.7	0.159 (1.3)	8.2	0.180 (0.6)	7.7
pH	−0.2 units	0.185 (1.5)	8.5	0.161 (2.4)	7.3	0.149 (2.4)	4.2	0.158 (1.9)	7.5	0.194 (2.6)	0.5
	+0.2 units	0.175 (2.5)	2.3	0.161 (2.4)	7.3	0.156 (3.6)	9.1	0.157 (1.9)	6.8	0.191 (1.0)	2.1

[a] $n = 6$, mean of six determinations.
[b] %Difference between the mean result of repeatability and mean result obtained from each of the variation for each individual related substance.

Table 9
Analysis of Lopinavir drug substance for related substances.

Sample	Related substances, mean[a]/%R.S.D. (%w/w)									
	RS1	RS2	RS3	RS4	RS5	RS6	RS7	RS8	RS9	RS10
Sample-1	ND	ND	ND	0.034 (6.3)	0.063 (4.3)	0.031 5.5)	0.031 (4.5)	ND	0.018 (5.9)	ND
Sample-2	ND	ND	ND	0.014 (7.4)	0.081 (3.8)	0.012 (5.2)	0.030 (4.1)	ND	0.032 (4.6)	ND
Sample-3	ND	ND	ND	ND	0.062 (3.3)	BLQ	0.013 (7.8)	ND	0.013 (6.5)	ND

ND: Not detected. BLQ: Below limit of qunatitation.
[a] Mean of three determinations.

4.7. Robustness

To evaluate the robustness of the method, the influence of small and premeditated alteration of analytical parameters on the quantification of the related substances and selectivity was studied. The parameters selected were mobile phase composition (±2% of gradient composition), pH of the mobile phase (±0.2 units), flow rate (±10%), wavelength (±5 nm) and column temperature (±5 °C). Only one parameter was changed while the others were kept unaltered. The mean and R.S.D. for each related substance were evaluated. The difference between the mean values (for all the related substances from each of the robustness parameters) from the repeatability mean results is found to be below 10.0%. The results were presented in Table 8. The studies indicated no effect on the determination of related substances and the selectivity. Therefore the test method is robust for the quantification of related substances.

5. Application of the method

Three batches of lopinavir drug substance, each in triplicate are analyzed using the proposed method. The results showed the presence of RS4, RS5, RS6, RS7 and RS9 in the range of 0.02–0.07%. R.S.D. ($n = 3$) for the related substances present in the samples was below 10.0%. The results were presented in Table 9.

6. Conclusion

A new, accurate and selective gradient RP-HPLC method was proposed for the determination of lopinavir related substances in lopinavir drug substance and validated as per the ICH guidelines. The method has higher sensitivity towards the determination of related substances than previously reported method. The method was found to be simple, selective, precise, accurate and robust. Four unknown related substances, which were not reported earlier, were identified by this method, isolated and characterized using spectral data. Therefore, this method can be used for routine testing as well as stability analysis of lopinavir drug substance. All statistical results (percentage, mean, R.S.D., percentage difference and recovery%) were within the acceptance criteria.

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