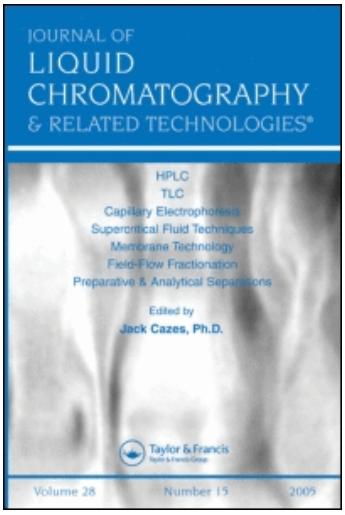

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Novel Stability-Indicating RP-LC Method for the Determination of Nelfinavir Mesylate and its Related Impurities in Drug Substance and Pharmaceutical Formulations

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Abstract: A simple, isocratic liquid chromatographic method was developed for determination of nelfinavir from its impurities, degradation products, and assay for the first time. This method involves the usage of a C_{18} (Hypersil BDS C_{18}, 250 mm × 4.6 mm, 5 μm) column. The method was validated over the range of LOQ to 120% of impurity specification limit and LOQ to 150% of analyte concentration for assay. The mobile phase consists of the mixture of 50 mM of sodium phosphate buffer, acetonitrile, and methanol in the ratio of 30:50:20. The flow rate was set at 1.0 mL/min with UV detection monitored at 220 nm. The drug substance was subjected to stress conditions of hydrolysis, oxidation, photolysis, and thermal degradation. The developed method was validated for linearity, range, precision, accuracy, and specificity. This method can be conveniently used in the

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quality control laboratory for routine analysis for both the assay and impurities of drug substances, drug products, and also for the evaluation of stability study samples.

Keywords: Column liquid chromatography, Forced degradation, Stability-indicating method, Nelfinavir, Impurities, Drug substance

INTRODUCTION

Nelfinavir mesylate is the fourth HIV-1 protease inhibitor (PI) to be marketed and is the first to be available in a pediatric formulation. Nelfinavir mesylate (Figure 1) is an inhibitor of the human immunodeficiency virus (HIV) protease and is administered in tablet form with the brand name of Viracept®. Tablets are available for oral administration as a light blue, capsule shaped tablet with a clear film coating in 250 mg strength (as nelfinavir free base), and as a white oval tablet with a clear film coating in 625 mg strength (as nelfinavir free base). The chemical name for nelfinavir mesylate is [3*S*-[2(2*S**, 3*S**), 3a,4ab,8ab]]-*N*-(1,1-dimethylethyl)decahydro-2-[2-hydroxy-3-[(3-hydroxy-2-

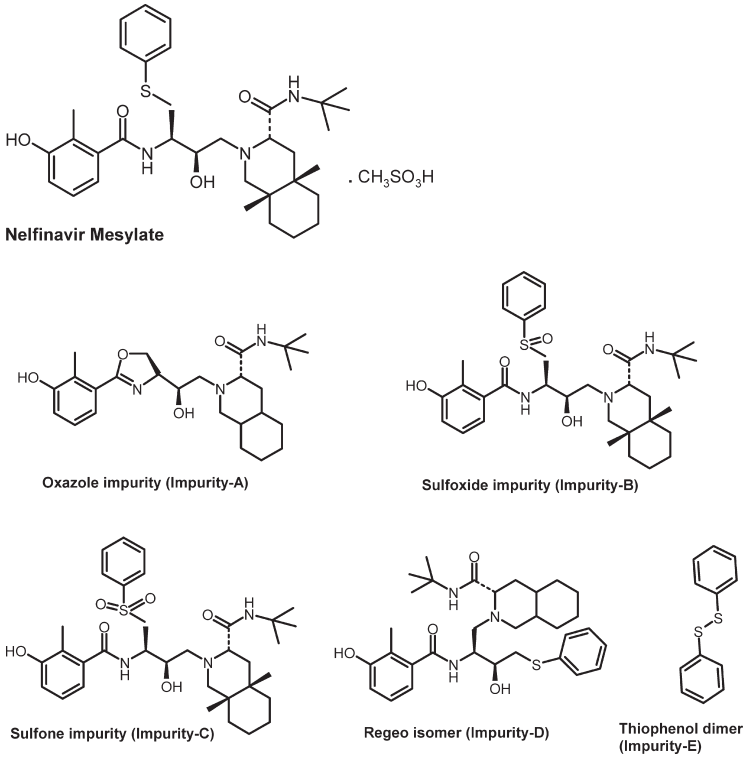

Figure 1. Structural formulae of nelfinavir mesylate and its impurities.

methylbenzoyl) amino]-4-(phenylthio)butyl]-3-isoquinoline carboxamide monomethanesulfonate (salt) and the molecular weight is 663.90. Nelfinavir mesylate was approved by United States Food and Drug Administration (FDA) for treatment of HIV patents in the year 1997.

Many analytical methods were available for the simultaneous determination of nelfinavir with other HIV protease inhibitors or non-nucleoside reverse transcriptase inhibitors employing the techniques of HPLC,[1–7] LC-MS-MS,[8] HPLC-MS,[9] and ion-pair HPLC.[10] However, all of these methods were related to the determination of nelfinavir including simultaneous determination with other drugs administered together to HIV patents, and to the determination of nelfinavir in biological samples. Few analytical methods were available for individual determination of nelfinavir mesylate through HPTLC.[11] Another method was reported in the literature for determination of nelfinavir in bulk drugs and dosage forms through stability-indicating HPLC[12] by Jing et al. However, the method proposed by Jing et al. involves the determination of nelfinavir with regard to the intermediates. Controls of intermediates are to be addressed through in-process quality control checks and are removed during the process of synthesis. Potential process related impurities, degradation products like sulfone and sulfoxide impurities, were not resolved in the method proposed by Jing et al.

The safety and efficacy of the drug substance is governed by the impurities, which are formed due to various reaction transformations during the manufacturing process of drug substance. These impurities are classified as organic impurities, inorganic impurities, residual solvents; and the level of impurity presence shall be justified in the specifications, if exceeding the identification threshold.[13] The available literature indicates the impurities namely, regeo isomer (impurity-D) and thiophenol dimer impurity (impurity-E), and are the potential impurities formed during the synthesis of nelfinavir.[14]

To our current knowledge, no liquid chromatographic method was reported for determination of process related impurities, namely oxazole impurity (impurity-A), regeo isomer (impurity-D), thiophenol dimmer impurity (impurity-E), and degradation products, namely sulphoxide impurity (impurity-B), and sulphone impurity (impurity-C) of nelfinavir drug substance. The aim of the present work was to develop a simple, selective, precise, robust, and accurate method for determination of nelfinavir in the presence of its impurities and degradation products. The method was developed and validated in accordance with the requirements of ICH guidelines.[15]

EXPERIMENTAL

Chemicals, Reagents, and Analytical Column

Qualified impurity standards and drug substances were obtained from the antiviral research laboratory of Matrix Laboratories Limited and were used

without any further purification. The water used for dilutions and making solutions was obtained through the Milli-Q water purification system available in the laboratory. The drug product was purchased from the market.

HPLC buffer grade sodium dihydrogen phosphate dihydrate was purchased from Qualigens Fine Chemicals, a division of Glaxo SmithKline Pharmaceuticals Limited. HPLC grade acetonitrile was purchased from Merck limited, India, a part of Merck group, Germany. HPLC grade methanol was purchased from M/s. J.T. Baker, USA. The analytical HPLC column, Hypersil-BDS C18 with dimensions of 250 mm × 4.6 mm, 5 μm particle size was purchased from Thermo-electron Corporation, USA.

The chemicals hydrochloric acid, sodium hydroxide and hydrogen peroxide used for degradation studies were of Laboratory reagent grade, purchased from Ranbaxy Fine Chemicals Limited, India.

Instruments

The following instruments were used in the validation study: System-1: Waters HPLC system consisting of 2695 separation module, 2487 dual wavelength detector, thermo stated auto sampler, column heater, degasser, and was controlled by Waters Empower software. System-2: Agilent 1100 series pumping system with G1311A quaternary gradient, auto sampler, G1379A degasser, G1314A variable wavelength detector, G1316A column heater, and G1329A sample cooler, controlled by Waters Empower software. System-3: Waters HPLC system consisting of 2695 separation module, 2996 photodiode array detector, auto sampler, column heater, degasser, and sample cooler, and was controlled by Waters Empower software.

Chromatographic Conditions

The buffer was prepared by dissolving 7.8 g of sodium dihydrogen phosphate dihydrate in 1000 mL of Milli-Q water. The mobile phase consisted of acetonitrile, buffer, and methanol (50:30:20, v/v/v) and was pumped at a flow rate of 1 mL/min. The column temperature was maintained at ambient temperature, (25 ± 2°C) and detector wavelength was set at 220 nm. The injection volume for all analyses was set at 20 μL.

Preparation of Stock Solutions of Impurities and Drug Substance

The stock solutions of each impurity and drug substance were prepared with a concentration of 5 mg/mL in mobile phase. The solutions were further diluted to obtain the test concentrations at each stage of validation on an experimental need basis.

Preparation of Drug Product Samples

Each tablet of nelfinavir mesylate obtained from the market contains 292.3 mg of nelfinavir mesylate (250 mg of nelfinavir base). The tablet was crushed and dissolved in mobile phase. The contents were filtered to remove the excipients and the filtrate was collected in a 250 mL volumetric flask, diluted to obtain the sample concentration of 1.0 mg/mL solution.

Analytical Method Development

Initial developmental activities were carried out with stationary phases like C_8, cyano, and phenyl columns with the buffers consisting of acetate, formate, and phosphates using different compositions of buffer, acetonitrile, and methanol. The separations using the above conditions did not yield fruitful resolution between the impurities of interest and methane sulphonic acid (M.S.Acid).

The analytical conditions were optimized with mobile phase composition (acetonitrile, buffer, methanol, 50:30:20 v/v/v), using the HPLC column (4.6 mm × 250 mm, 5 μm) with C_{18} stationary phase. The Hypersil-BDS column was used in all validation studies.

Validation of Analytical Method for Impurities

Linearity of the analytical method was studied with six different concentrations of impurities ranging from the LOQ to 3 μg/mL (LOQ to 120% of specification limit (0.5%) with respect to analyte working concentration of 500 μg/mL) and LOQ to 750 μg/mL for nelfinavir drug substance (LOQ to 150% of analyte working concentration). Each solution was injected, in duplicate, into the chromatographic system and the mean value was taken into consideration.

Accuracy and precision for the impurities was evaluated using a 3 × 3 matrix by spiking three known concentrations of impurities, each, with triplicate injections. Thus, the known quantities of impurity standards at 50%, 100%, and 150% of the specification limit in the drug substance were spiked, and the recovery of spiked impurities at each concentration was studied.

Intermediate precision was evaluated by intra day analysis of six replicate injections of the drug substance spiked with the impurities of the specification limit. The concentrations were calculated using the area responses of each impurity from the mean values.

The detection and quantification limit of impurities and drug substance were achieved through a series of dilutions of stock solutions to obtain signal to noise ratio of about 3:1 and 10:1, respectively.

Specificity and selectivity of the analytical method was studied in view of resolution between the nearest eluting impurities and peak symmetry by spiking all known impurities in the drug substance.

Robustness of the method was studied by small but deliberate variations in mobile phase composition, columns of different lots, and flow rate of mobile phase.

Forced Degradation Studies

The drug substance, nelfinavir mesylate at the concentration level of 1 mg/mL was used for all degradation studies to evaluate the method for its stability indicating nature and to identify the degradation products formed under different stress conditions. The details of the degradation studies performed were as follows.

Acid degradation studies were carried out by heating the solution of drug substance in 0.1 N hydrochloric acid at 80°C for 12 h. The solution was then cooled to room temperature and neutralized with dilute sodium hydroxide prior to HPLC analysis. The alkaline degradation studies were performed by heating the solution of the drug substance in 0.1 N sodium hydroxide to 80°C for 12 h. The heated solution was cooled to room temperature, neutralized with dilute acid prior to HPLC analysis. Oxidative degradation studies were performed using 10% hydrogen peroxide and was kept at room temperature (25 ± 2°C) for 5 days and then analyzed by HPLC.

Photo stability studies were performed by spreading the solid sample of the drug substance as a thin layer in a petri dish, placing in photo stability chambers equipped with ultraviolet light, with overall illumination of >1.2 million lux hours, with an energy of not less than 200 watt hours/meter. Another set of samples was kept in the dark for the same period.

Thermal degradation was carried out by exposing the solid sample of drug substance at 50°C for 30 days. The degradation under neutral conditions was carried out in water at 80°C for 30 days.

Analytical Method Validation for Assay

The accuracy of the method was studied by spiking with three different concentrations of the drug substance at 50%, 75%, and 100% of analyte concentration in the stressed sample. The recovery of the drug substance was calculated using area responses at each concentration level.

Precision of the method was studied by repeatability and intermediate precision studies. Repeatability studies were performed by analyses of three different concentrations of the drug substance, with six injections at each concentration, on the same day. Intermediate precision was evaluated by repetition of the same concentrations at different days.

The specificity of the method was studied by determination of peak purity of the drug substance in the stressed sample by a PDA detector and, also, with the resolution between the adjacent peaks.

RESULTS AND DISCUSSION

The data presented in Table 1 indicates that the analytical method was linear over the range of LOQ to 120% for impurities and LOQ to 150% for the drug substance. The correlation coefficient values presented in the table (>0.999) shows good agreement with respect to linearity of the method over the range of study. The response factors calculated using the slope values of each impurity. These response factors of each impurity were found to be close to the drug substance.

The recovery data of impurities presented in Table 2 for different concentrations shows that the average recovery of impurities was found in the range of 98.3% to 100.0%. This indicates that the method was sufficiently accurate and precise. The intermediate precision data presented in Table 3 indicates that the relative standard deviation values of the samples analyzed at different days with different analysts range from 0.70% to 2.99%, indicating that the method was sufficiently precise to determine impurities of the drug substance.

Limit of detection (LOD) and limit of quantitation (LOQ) were evaluated through a series of dilutions of all impurities. The samples were prepared in duplicate and the signal to noise ratio of each impurity was determined. The LOD concentration was that of the concentration, where the ratio of signal to noise yielded about 3.0. The LOQ concentration was that of the concentration, where the signal to noise ratio was determined to be about 10.0. The results of LOD and LOQ of each impurity and drug product were presented in Table 4.

The results pertain to the robustness study presented in Table 5. The data presented in Table 5 indicates that the deliberate changes to the chromatographic conditions, did not significantly affect the system suitability

Table 1. Linearity details

Compound name	Slope	y-Intercept correlation	Coefficient
Impurity-A	13358.47	2506.67	0.99999
Impurity-B	13719.37	8141.23	0.99995
Impurity-C	13419.06	15214.9	0.99992
Impurity-D	13991.47	3914.14	0.99999
Impurity-E	14322.52	9848.09	0.99998
Nelfinavir	13473.69	51019.82	0.99988

Table 2. Recovery studies of impurities of nelfinavir

Name of the impurity	Measured concentration, ±S.D (n = 3)	RSD (%)	Recovery (%)
At 50% Level (1.25 μg/mL)			
Impurity-A	1.25, 0.01	0.80	100.00
Impurity-B	1.23, 0.01	0.89	98.64
Impurity-C	1.24, 0.02	1.61	99.20
Impurity-D	1.23, 0.008	0.62	98.56
Impurity-E	1.24, 0.004	0.34	99.12
At 100% Level (2.5 μg/mL)			
Impurity-A	2.47, 0.044	1.76	98.80
Impurity-B	2.47, 0.074	2.99	98.68
Impurity-C	2.47, 0.044	1.76	98.80
Impurity-D	2.47, 0.032	1.30	98.92
Impurity-E	2.46, 0.064	2.61	98.52
At 150% Level (3.75 μg/mL)			
Impurity-A	3.70, 0.065	1.76	98.75
Impurity-B	3.69, 0.081	2.19	98.32
Impurity-C	3.71, 0.095	2.56	98.85
Impurity-D	3.72, 0.052	1.40	99.20
Impurity-E	3.74, 0.015	0.41	99.65

Table 3. Intermediate precision.

Name of the impurity (n = 6)	Measured concentration (μg/mL), standard deviation (±SD), RSD (%)			
	Analyst-1, system-1	Analyst-1, system-2	Analysts-2, system-1	Analyst-2, system-2
	Day-1	Day-1	Day-2	Day-2
Impurity-A	2.47, 0.044, 1.76	2.49, 0.06, 2.41	2.49, 0.067, 2.67	2.46, 0.047, 1.92
Impurity-B	2.47, 0.074, 2.99	2.46, 0.059, 2.38	2.49, 0.057, 2.29	2.47, 0.035, 1.42
Impurity-C	2.47, 0.044, 1.76	2.48, 0.017, 0.70	2.44, 0.021, 0.85	2.47, 0.07, 2.83
Impurity-D	2.47, 0.032, 1.30	2.47, 0.02, 0.81	2.49, 0.032, 1.29	2.46, 0.061, 2.49
Impurity-E	2.46, 0.064, 2.61	2.47, 0.055, 2.23	2.5, 0.021, 0.83	2.47, 0.045, 1.83

Table 4. LOD and LOQ values of nelfinavir and its impurities

Impurity details	LOD	S/N ratio	LOQ	S/N ratio
Impurity-A	0.04 μg/mL	3.0:1	0.13 μg/mL	9.9:1
Impurity-B	0.04 μg/mL	2.9:1	0.12 μg/mL	10.2:1
Impurity-C	0.03 μg/mL	3.0:1	0.11 μg/mL	10.0:1
Impurity-D	0.04 μg/mL	3.1:1	0.14 μg/mL	9.9:1
Impurity-E	0.03 μg/mL	2.9:1	0.10 μg/mL	10.1:1
Nelfinavir	0.03 μg/mL	3.2:1	0.12 μg/mL	10.6:1

parameters. The observed responses to the parameters changed were as expected. The increase in flow shows the decrease in retention and decrease in flow increase in retention of components without any significant effect in the relative retention time of impurities and their elution pattern. Similarly, the increase in composition of acetonitrile decreases the retention times of components and decrease in composition shows the increase in retention times without any significant effect.

The specificity and selectivity of the method was studied by resolution of impurities with each other and no interference either with principal peak or with neighboring eluted peak. The data presented in Table 6 indicates that the minimum resolution between the closest eluting peaks was 7.06.

A typical chromatogram spiked with all impurities presented in Figure 2 indicates that the method was selective for determination of nelfinavir from its impurities.

Table 5. Study results on method robustness

		Relative retention times of impurities, tailing factor				
Parameter altered	Variation	A	B	C	D	E
Flow rate	0.8 mL	0.14, 1.12	0.36, 1.01	0.47, 0.97	0.70, 0.93	1.31,0.92
	1.0 mL	0.14, 1.12	0.37, 1.00	0.48, 0.98	0.69, 0.93	1.31, 0.91
	1.2 mL	0.14, 1.11	0.37, 1.00	0.48, 0.97	0.69, 0.94	1.30,0.92
ACN ratio	45	0.13, 0.98	0.36, 0.98	0.48, 0.97	0.69, 0.94	1.31, 0.93
	50	0.14, 1.12	0.37, 1.00	0.48, 0.98	0.69, 0.93	1.31, 0.91
	55	0.14, 1.07	0.37, 1.00	0.48, 0.96	0.71, 0.95	1.31, 0.91
Column make	Hypersil	0.14, 1.12	0.37, 1.00	0.48, 0.98	0.69, 0.93	1.31, 0.91
	Wakosil (SGE)	0.15, 1.11	0.38, 1.02	0.47, 0.96	0.70, 0.91	1.33,0.94

ACN: Acetonitrile.

Table 6. System suitability parameters

Compound name	USP resolution	Tailing factor (as per USP)	No. of theoretical plates (N)
Impurity-A	—	1.11	9407
M.S.Acid	10.15	1.06	10712
Impurity-B	13.50	0.99	12319
Impurity-C	7.06	0.96	12742
Impurity-D	10.50	0.93	14463
Nelfinavir	10.02	0.92	19544
Impurity-E	8.17	0.90	11198

M.S.Acid: Methane sulfonic acid.

Results of Forced Degradation Studies

Two impurities of about 8% and one impurity of negligible degradation (thiophenol dimer impurity) were formed during the oxidative degradation studies (Figure 3).

Two impurities of about 5% were formed during alkaline hydrolysis conducted with sodium hydroxide (Figure 4). These two impurities were eluting at relative retention times of 0.36 and 0.48, respectively, and were identified as sulfoxide impurity and sulfone impurity, respectively. These impurities were further confirmed by spiking the impurities into a degraded sample as co-injection and determination of its peak purity.

No degradation was observed under the forced degradation studies conducted with acid hydrolysis. The product was also found stable under the conditions of thermal degradation, photolytic degradation, and neutral conditions.

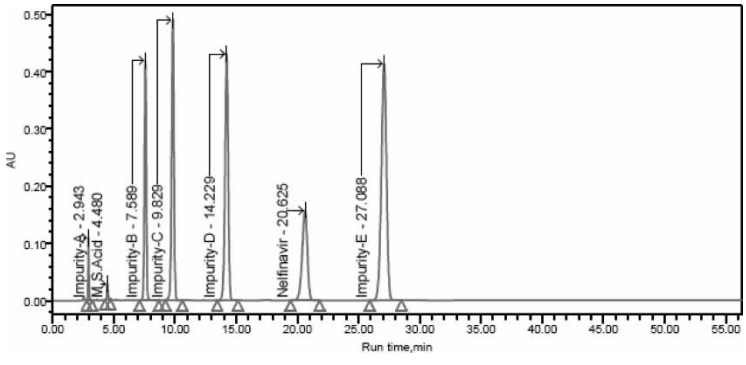

Figure 2. Typical system suitability test chromatogram of nelfinavir.

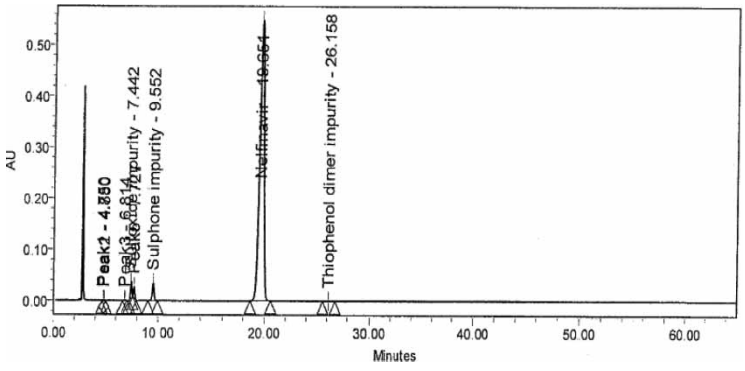

Figure 3. HPLC chromatogram of nelfinavir stressed with peroxide.

In both the cases of degradation, i.e., oxidative degradation and degradation under alkaline conditions, the fall in the response of the drug peak was in the corresponding rise in the peak responses of degradation products.

Stability-Indicating Nature of the Method

The data presented in Table 7 corresponds to the precision study by repeatability and intermediate precision. The relative standard deviation of repeatability experiments and intermediate precision were obtained in the range of 0.24% to 0.67% and 0.28% to 0.67%, respectively. The RSD values of $<1.0\%$ in both cases suggests that the method was sufficiently precise in the stated conditions.

The data corresponding to the accuracy of the method obtained by recovery study through three different concentrations of drug substance is

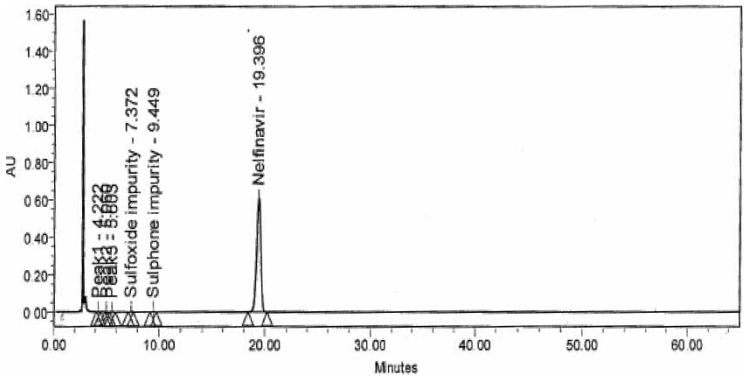

Figure 4. HPLC Chromatogram of nelfinavir stressed with base.

Table 7. Precision studies on nelfinavair stressed sample

Spiked concentration (μg/mL)	Measured concentration, ± S.D, RSD (%)	
	Repeatability (n = 6)	Intermediate precision (n = 6)
250	250.58 ± 1.68, 0.67	250.10 ± 1.66, 0.67
500	499.02 ± 2.32, 0.47	501.65 ± 1.77, 0.35
750	748.92 ± 1.81, 0.24	750.33 ± 2.06, 0.28

Table 8. Recovery studies of nelfinavir on stress samples

Recovery (μg/mL) (n = 6)	Actual concentration	Calculated concentration	Standard deviation (%)	RSD (%)
250	249.65	1.36	0.55	99.86
375	374.08	2.02	0.54	99.76
500	500.5	1.22	0.24	100.1

presented in Table 8. The data suggests that the percentage of recovery at all concentrations (50%, 75%, and 100% of analyte concentration) obtained at more than 99.0% indicates that good recoveries were obtained at the targeted concentrations.

The details of the data on stress studies presented in Table 9, indicates that the peak purity of the sample in all degraded conditions found at more than 0.999 indicates that the method is considerably precise and specific.

Figure 5 shows the peak purity of the stressed sample. The peak purity of the drug substance was found to be more than 0.999, indicating that the method was specific and there was no interference with degradation products. This suggests that the method was sufficiently selective for determination of the assay.

Table 9. Data on degradation studies

Degradation condition/media/duration	Degradation (%)	No. of impurities purity	Peak
Acidic/0.1 N HCl/80°C/12 h	0	0	0.99987
Alkaline/0.1 N NaOH/80°C/12 h	5%	2	0.99992
Neutral/H_2O/80°C/30d	0	0	0.99967
Oxidative/10%H_2O_2/5d	8%	3	0.99991
Photolytic/200 Wh/1.2 m lux h	0	0	0.99972
Thermal/50°C/30d	0	0	0.99988

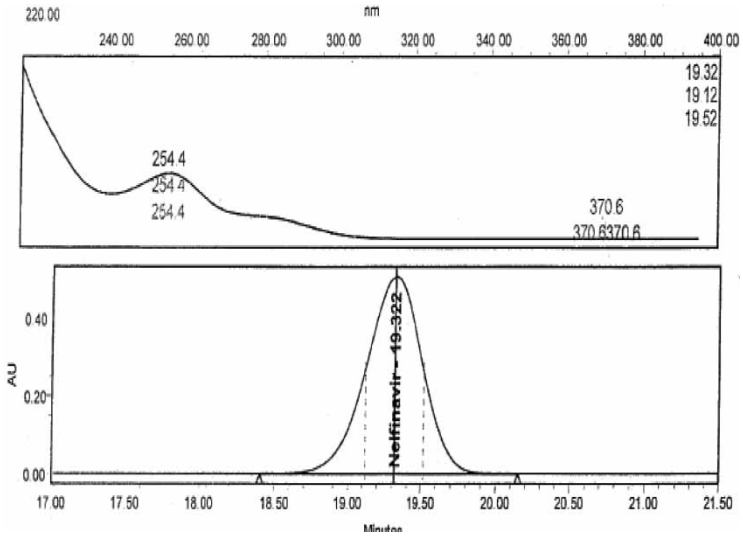

Figure 5. Peak purity spectra of nelfinavir obtained with stressed sample.

Analysis of Drug Product Samples

The drug product was analyzed using an external standard of a reference substance. The drug product of 10 samples of the same batch were analyzed and the potency was calculated using the following equation.

$$\text{Potency} = [(A_T/A_S) \times (W_S/W_T)] \times P$$

where A_T & W_T are peak area response and weight of test sample, i.e., drug

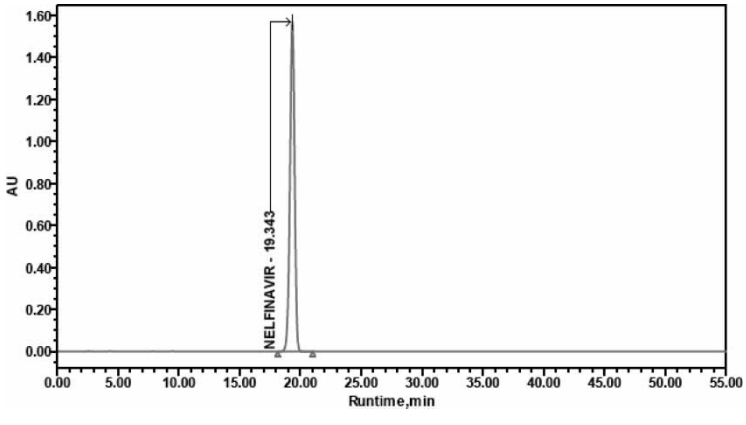

Figure 6. Typical chromatogram of nelfinavir drug product.

product, A_S, W_S, & P are the peak area response and weight and potency of standard, respectively.

The analyses of the drug product samples confirm the label claim of 250 mg nelfinavir base. The typical HPLC chromatogram of the drug product is presented in Figure 6.

CONCLUSIONS

The proposed HPLC method perfectly resolves the process impurities and degradation products with considerable resolution. Forced degradation studies indicate that the drug substance was found to degrade considerably under alkaline hydrolysis and oxidative degradation conditions. The drug substance was found stable under acid hydrolysis, thermal degradation, and photolytic degradation conditions.

The method is simple, linear, precise, and robust for determination of process related impurities and is also stability indicating. The method is recommended for routine analysis of product release in quality laboratories and for analysis of stability samples.

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