ORIGINAL ARTICLE

Simultaneous High-performance Liquid Chromatography Determination of Non-nucleoside Reverse Transcriptase Inhibitor and Protease Inhibitors: Global Optimization Technique

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Abstract

Introduction: An improved highly sensitive and robust reversed-phase high-performance liquid chromatography (HPLC) method for simultaneous determination of atazanavir (ATV), efavirenz (EFV), lopinavir (LPV), and ritonavir (RTV) along with carvedilol (IS) as an internal standard. **Materials and Methods:** Shimadzu HPLC system (Tokyo, Japan) containing a LC10AD and LC10 ADvp with UV detector (SPD-10A) and Thermo Hypersil C_{18} column was used in chromatography. The mobile phase comprising acetonitrile (ACN): 10 mM potassium di-hydrogen orthophosphate (55:45) delivered at a flow rate of 1 mL/min and monitored at a wavelength of 210 nm. The method developed was optimized using central composite design, a chemometric tool to optimize the factors (ACN, buffer concentration, and flow rate) effecting and interacting with the responses (k_1, $Rs_{2,1}$, $Rs_{3,2}$, and tR_5). **Results and Discussion:** The analysis time is 5.8 (<6 min). The method developed was validated according to ICH Q2 (R1) guidelines and confirmed the linearity, accuracy, precision, and specificity. The LOD and LOQ were found to be 1.065 and 3.227 ng/mL for ATV; 0.850 and 2.576 ng/mL for RTV; 0.744 and 2.255 ng/mL for LPV; and 0.315 and 0.955 ng/mL for EFV. **Conclusion:** The method developed is of highly sensitivity and aptness of the method was recognized by applying to three different combinations of commercially available formulations, confirmed applicability for routine analysis.

Key words: Atazanavir, carvedilol, central composite design, efavirenz, high-performance liquid chromatography, lopinavir, ritonavir

INTRODUCTION

The human immunodeficiency virus (HIV) build-ups and archives resistance easily which commands the standard therapy for HIV/AIDS. Highly active antiretroviral therapy (HAART) recommends prescribing three or more drugs from more than one class.

Atazanavir (ATV), ritonavir (RTV), and lopinavir (LPV) are protease inhibitors; efavirenz (EFV) is a HIV - I specific non-nucleoside reverse transcriptase inhibitor (NNRTI). ATV is chemically described as, 3,12-bis(1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-((4-(2- pyridinyl)phenyl) methyl)-, dimethyl ester,[1] RTV is chemically described as 1,3-thiazol-5-ylmethylN-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate.[2] LPV is chemically described as (2S) -N-[(2S, 4S, 5S) -5-[2- (2, 6-dimethylphenoxy) acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)

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butanamide1[3] EFV is chemically described as (S)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one,[4] their chemical structures were given in Figure 1.

Although several high-performance liquid chromatography (HPLC) methods for individual determination of ATV,[5] RTV,[6-8] LPV,[9] and EFV[10] and simultaneous determination were published,[11-19] literature review affirms no method was reported for the optimization combination of protease inhibitors and NNRTI (ATV, RTV, LPV, and EFV) by applying chemometrics-central composite design (CCD). The core objective of the current effort was to develop and validate a highly sensitive, accurate, and specific simultaneous HPLC method, for quantifying the above-mentioned drugs individually, and in combinations simultaneously both in formulations and plasma for further bio-availability, pharmacokinetic studies in a feasible and uncomplicated method using an internal standard (Carvedilol).

MATERIALS AND METHODS

Apparatus

Shimadzu HPLC system (Tokyo, Japan) contains a solvent delivery system (LC10AD and LC10 ADvp), a Rheodyne injector with a 20 µL loop (model 7125, USA), and SPD-10A UV detector. A personal computer with LC solution, release 1.11SP1 Shimadzu chromatographic software and SCL-10A (system controller) controls the entire system. Degassing of the mobile phase was done by a sonicator (Branson Ultrasonic Corporation, USA). A double beam UV-Visible spectrophotometer: 2202 Systronics and quartz cell of path length 1 cm were used for recording absorbance spectra.

Software

Design - Expert® trial version 7.0.0. (Stat-Ease Inc., Minneapolis) was employed for the optimization (data analysis and desirability function calculations). The remaining calculations for the analysis were accomplished using 2007 Micro soft Excel (Microsoft, USA).

Chemicals and reagents

Active pharmaceuticals/references standards of ATV, EFV, LPV, and RTV were gifted by Cipla Pharma, Mumbai, India. HPLC grade acetonitrile (ACN), procured from SD Fine Chemicals, Mumbai, India. Milli-Q Academic (Millipore, Bengaluru, India) was used to prepare HPLC grade water. The pharmaceuticals: Efavir (EFV 600 mg) Lopimune (LPV 200 mg and RTV 50 mg) and Synthivan (ATV 300 mg and

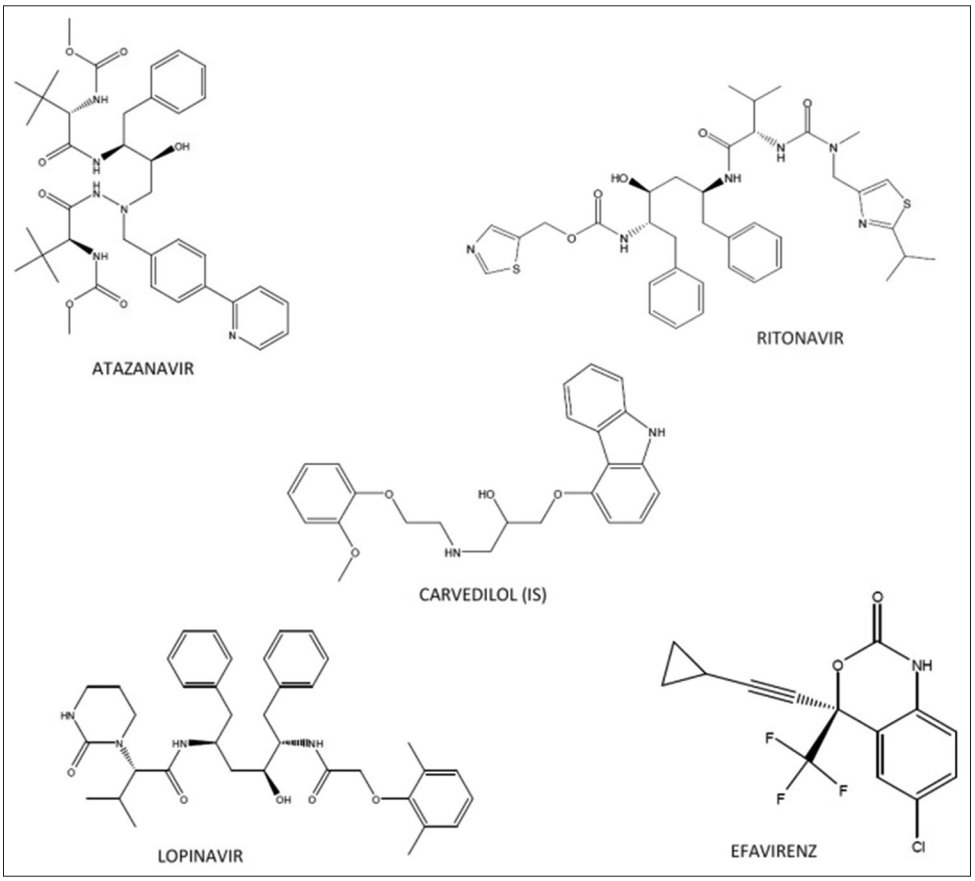

Figure 1: The chemical structures of atazanavir, ritonavir, carvedilol, lopinavir, and efavirenz

RTV 100 mg) tablets (Cipla Pharma, Mumbai, India) were procured from retail pharmacy.

Stock and working standard solutions

Prepared standard stock solutions of EFV, LPV, ATV, RTV, and IS at 1000 µg/mL, individually with a mixture of ACN and KH_2PO_4 in the ratio of 55:45 v/v and preserved and protected at 4°C away from light. Further serial dilutions were diluted with the mobile phase to give a series of concentration 1–15 µg/mL. The optimization was done using solution comprising of EFV, LPV, ATV, and RTV 10 µg/mL while IS concentration was fixed as 5 µg/mL.

Preparation of the sample solution

Twenty tablets of Efavir (EFV 600 mg), Lopimune (LPV 200 mg and RTV 50 mg), and Synthivan (ATV 300 mg and RTV 100 mg) tablets were weighed and analyzed separately. Accurately weighed and transferred amounts equivalent to 60 mg of EFV; 100 mg of LPV and 25 mg of RTV; 90 mg of ATV and 30 mg of RTV; into three different 50 mL volumetric flasks each containing suitable quantity of IS. Added 25 mL of mobile phase and the mixtures were sonicated for 10 min to complete the extraction of drugs, added mobile phase further to make up the volume up to the mark. From these stock solutions, serial dilutions were done to acquire final concentrations of 6 µg/mL of EFV; 20 µg/mL of LPV and 5 µg/mL of RTV; 18 µg/mL of ATV; and 6 µg/mL of RTV; and the IS concentration was fixed as 5 µg/mL. The prepared solutions were centrifuged for 15 min at 4000 rpm; the supernatant clear solution was collected, filtered using 0.2 µm membrane filter (Gelman Science, India), 20 µL from these solutions were injected for analysis.

Chromatographic procedure

The chromatography was done in an air-conditioned laboratory (25 ± 2°C), by injecting 20 µL of sample solutions using Thermo Hypersil C_{18} – column (15 cm × 4.6 mm i.d., 5 µm) with a flow rate of 1 mL/min at 210 nm. Mobile phase comprising ACN: 10 mM KH_2PO_4 of pH3.8 (±0.02) using 0.1N o-phosphoric acid (55:45) was vacuum filtered through 0.45 membrane filter, Gelman Science, India, and degassed using an ultrasonic bath for 15 min.

RESULTS AND DISCUSSION

Preliminary screening

Preliminary experiments were performed with water, ACN and MeOH (methanol), peaks were not eluted and hence included the KH_2PO_4. Mobile-phase additives comprise a key role in the separation of analytes consisting of basic or acidic functional groups.[20] All the analytes were weak acids; acidic buffer pH mobile phase will suppress the dissociation providing sufficient retention on the stationary phase while increased content of ACN speeds up the elution of analytes. Potassium dihydrogen orthophosphate and ammonium acetate selected for the first series of trials and KH_2PO_4 resulted early elution and good symmetry of peaks. Furthermore, another series of trials were performed for obtaining the best responses by altering the ratios of the components of mobile phase and selected the lower and upper levels of ACN, buffer along with flow rate and optimized using CCD.

Optimization by central composite design analysis

Initially, 2^k Factorial design [Table 1] was applied, investigated and found the model is significant for Rs 2,1 response, since $P < 0.05$, implies to consider the quadratic equation.[21] Due to the flexibility and applicability for HPLC method optimizations by understanding the individual effects and interaction effects of the factors,[22,23] central composite design (CCD) is employed to obtain a second-order predictive model. The quadratic mathematical model with three independent factors is given in Equation (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

Modeled response and regression coefficient were denoted by Y and β, and the factors A(ACN), B (buffer strength), and C (flow rate) denoted by X_1, X_2, and X_3, respectively. Statistical results acquired from ANOVA for the models are shown in Table 2. Backward elimination was done to eliminate the insignificant terms ($P > 0.05$) to obtain a reasonable model.[24]

Based on the preliminary experiments, the low level and high level of selected factors were fixed as ACN (A: 50–55 mL), buffer strength (B: 10–20 mM), and flow rate (C: 0.5–1.0 mL/min) whereas buffer volume was not included as a factor and the proportion taken is the remaining volume to make 100 mL. To assess the eminence of the work under various investigational conditions, the responses defined were: (1) Capacity factor of

Table 1: Fractional factorial design of experiments and obtained responses[a]

STD	RUN	A	B	C	K1	Rs. 2.1	Rs 3.2	tR_5
8	1	50	10	0.5	1.587	7.57	4.349	17.815
5	2	50	20	0.5	1.511	10.077	4.509	18.208
7	3	50	10	1	1.388	6.186	3.573	8.93
4	4	50	20	1	1.235	8.373	3.755	9.327
3	5	55	10	0.5	1.474	4.488	2.923	12.759
6	6	55	20	0.5	1.29	6.85	3.025	12.817
1	7	55	10	1	1.22	3.857	2.493	6.422
2	8	55	20	1	1.084	5.634	2.514	6.44

[a]Randomized

Table 2: Response models and statistical parameters obtained from ANOVA for CCD

Responses	Regression model	Adjusted R^2	Model *P* value	% C.V.	Adequate precision
K1	+1.16–0.069*A-0.075*B-0.17*C+0.054* B^2+0.14 *C^2	0.9188	<0.0001	4.75	23.608
$Rs_{2,1}$	+6.63–1.41*A+1.17*B– 0.65*C–0.23*B^2	0.9624	<0.0001	5.05	39.576
$Rs_{3,2}$	+3.28–0.65*A-0.33*C+0.074A*C+0.056*C^2	0.9774	<0.0001	2.83	46.671
tR_5	+9.93–1.85*A-4.22*C+0.63*A*C+1.59*C^2	0.9844	<0.0001	4.75	59.013

the first peak carvedilol (k_1); (2) resolution between ATV and carvedilol (IS) ($Rs_{2,1}$); (3) resolution between RTV and ATV ($Rs_{3,2}$); and (4) retention time of last peak (EFV) (tR_5).

A total of 20 experimental runs acquired from the design were processed and obtained response variables [Table 3]. All experiments were conducted in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Two replicates were performed for each investigational run to recognize the error variance in the experiment and to assess the prognostic validity of the said model. The obtained results were entered, analyzed and found significance of the model for all the three factors since $P < 0.05$, therefore, proceeded for optimization and selected the optimal condition having Derringers desirability value 0.949, mobile phase comprises ACN: 10 mM KH_2PO_4 (55:45) delivered with 1 mL/min flow rate and monitored at a wavelength of 210 nm. Simultaneously, the condition suitable for plasma was also selected having Derringers desirability value 0.754, mobile phase comprises ACN: 10 mM KH_2PO_4 (51.2:48.8) 1 mL/min flow rate and results were tabulated in Table 4.

The investigational data prove that the adjusted $R^2 \geq 0.80$ has good agreement with the second-order polynomial equations.[25] For the models, *P* value (< 0.05) shows that the model is of immense value, and for adequate precision, the assessment value more than 4 indicates that the model is desirable,[26] and the ratio obtained for this model is 23.6–59.01. Hence, in the separation process, the model was found to be significant.

Among the fitted models, tR_5 contains the interaction term with bigger absolute coefficient: *AC* (+ 0.63) [Table 2], which is statistically important for tR_5 and illustrates an increase in ACN concentration causes sharp decrease in tR_5.

In Figure 2 perturbation plot: Study indicates the effect of a single factor on a particular response, with all other factors, kept stable at a reference point.[27] The effect of individual factors on the resolution $Rs_{(2,1)}$ response to considerate the experimental procedure and Factor A has more effect on resolution, compare to B and C. Three dimensional response plots for k_1, $Rs_{2,1}$, $Rs_{3,2}$, and tR_5 are shown in Figure 3.

Global optimization

Global optimization was done with the identified targets: (a) Resolution between the $Rs_{2,1}$; $Rs_{3,2}$ critical pair peaks, (b) first peak for capacity factor, and (c) last peak for total elution time. Chemometric technique: Desirability function was employed with various targets to optimize the above responses.[28] The desirability function is shown in equation (2):

Table 3: Central composite rotatable design arrangement and responses[a]

STD	RUN	A	B	C	K1	Rs.2.1	Rs.3.2	tR_5
1	9	50	10	0.5	1.587	7.57	4.349	17.815
2	4	55	10	0.5	1.474	4.488	2.923	12.759
3	5	50	20	0.5	1.511	10.077	4.509	18.208
4	20	55	20	0.5	1.29	6.85	3.025	12.817
5	7	50	10	1	1.388	6.186	3.573	8.93
6	11	55	10	1	1.22	3.857	2.493	6.422
7	2	50	20	1	1.235	8.373	3.755	9.327
8	6	55	20	1	1.084	5.634	2.514	6.44
9	14	48.29	15	0.75	1.255	8.479	4.212	12.377
10	17	56.70	15	0.75	1.084	3.805	2.064	6.772
11	13	52.5	6.59	0.75	1.465	3.541	3.208	9.889
12	8	52.5	23.40	0.75	1.186	7.791	3.276	10.052
13	16	52.5	15	0.32	1.966	7.884	3.977	22.411
14	10	52.5	15	1.17	1.182	5.508	2.757	6.248
15	19	52.5	15	0.75	1.158	6.545	3.319	10
16	3	52.5	15	0.75	1.161	6.693	3.313	9.937
17	15	52.5	15	0.75	1.163	6.61	3.289	9.99
18	18	52.5	15	0.75	1.158	6.503	3.245	9.901
19	1	52.5	15	0.75	1.161	6.544	3.318	10
20	12	52.5	15	0.75	1.163	6.543	3.317	10

[a]Randomized

$$D = [d_1^{p^1} \text{x} d_2^{p^2} \text{x} d_3^{p^3} \text{x} ... \text{x} d_n^{pn}]^{\frac{1}{n}} \quad (2)$$

Weight of response (*pi*) values was rest at 1 for all the four responses. Value of *D* close to 1 shows that the recipe of the various criteria is coordinated in a global optimum.[29,30] Table 5 shows each response for the optimized criteria. The bar graph achieved for the desirability function is depicted in Figure 4. The forecast effectiveness of the model was established by performing the analytical experiment under the exact optimal condition, and the resultant chromatogram.

Table 4: The comparison of observed and predictive values of different objective functions under optimal conditions

Optimum conditions	ACN (mL)	Buffer (mM)	Flow (ml/min)	K1	Rs.2.1	Rs.3.2	tR_5
I	Desirability value (D) =0.949						
	54.909	10	0.9999				
	Experimental value			1.269	3.333	2.503	5.860
	Predicted value			1.199	3.220	2.455	6.122
	Percentage error			5.4	3.49	1.94	4.28
II	Desirability value (D) =0.754						
	51.182	10	0.9999				
	Experimental value			1.335	5.214	3.129	8.155
	Predicted value			1.302	5.320	3.310	7.939
	Percentage error			2.53	1.99	5.494	2.72

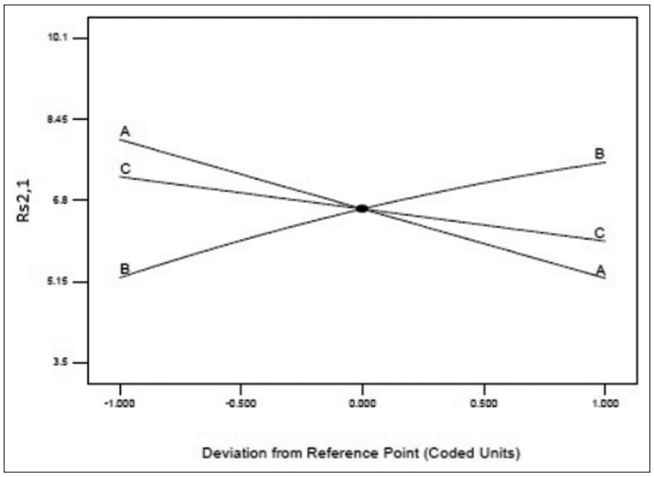

Figure 2: Perturbation plots showing the effect of each of the independent variables on $Rs_{2,1}$. Where A is the concentration of can (mL), B the buffer concentration (mM), and C the mobile phase flow rate (mL/min)

Predicted error = Experimental – predicted/predicted ×100 (4)

Method validation

Developed and optimized method was validated for accuracy, detection limit, linearity, specificity, precision, and quantitation limit by following the ICH guidelines.[31]

Linearity

Linearity was established for the concentration range of approximately 20–200% of the nominal range of ATV (3–15 µg), EFV (2–6 µg), LPV (2–10 µg), and RTV (1–5 µg). Calibration curves were plotted for the analytes peak areas (Y) versus their respective concentrations (x). Performed linear regression analysis for the resultant calibration curves and correlation coefficients for all the analytes (R^2) were determined as >0.999. The representative linear regression equations were: y = 0.065x + 0.001 for ATV; y = 0.156x + 0.001 for EFV; y = 0.085x + 0.002 for LPV; and y = 0.062x + 0.001 for RTV. In view of the fact that, the correlation coefficients barely are not fine markers of linearity studies,[32] executed one-way ANOVA too[33] for all the analytes, and found that the F_{calc} is less than the F_{crit} at 5% significance level, representing absence of significant variation among the replicate estimations of individual concentration level.

Specificity

Specificity defines the method's aptitude to differentiate the analyte and interfering substances and calculated by inspecting the separation and resolution of the drug peaks from formulation placebo (organized in agreement with the qualitative and quantitative composition of tablet formulation). Figure 5 shows no excipient peaks were coeluted with the analyte peaks which reveal the developed method is selective and specific.

Limit of detection (LOD) and quantitation (LOQ)

The LOD and LOQ for ATV, EFV, LPV, and RTV were determined as per the ICH guidelines.[31] LOD and LOQ were calculated by substituting σ (regression lines y-intercept's standard deviation) and S (calibration curve slope) in 3.3 σ/S and 10 σ/S, respectively. Calibration curve was specially constructed in a low region of 0.05–1.0% of the target analyte concentration.[34,35] The LOD and LOQ values were found to be 1.065 and 3.227 ng/mL for ATV; 0.850 and 2.576 ng/mL for RTV; 0.744 and 2.255 ng/mL for LPV; and 0.315 and 0.955 ng/mL for EFV.

Accuracy and recovery

Recovery studies were performed for 80%, 100%, and 120% concentration from the label claim of ATV, RTV, LPV, and

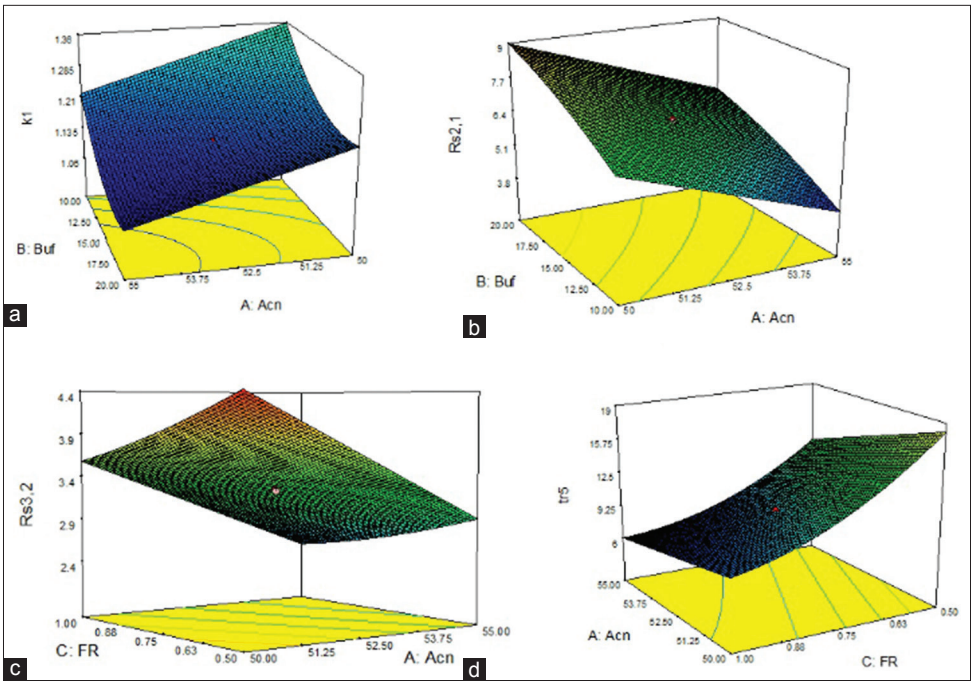

Figure 3: Response surfaces functions of acetonitrile (ACN%) and buffer concentration: (a) Capacity factor of the first peak (*k*1), (b) resolution of the critical pair ($Rs_{2,1}$); response surfaces functions of ACN% and flow rate: (c) Resolution of the critical pair ($Rs_{3,2}$) and (d) retention time of the last peak (tR_5)

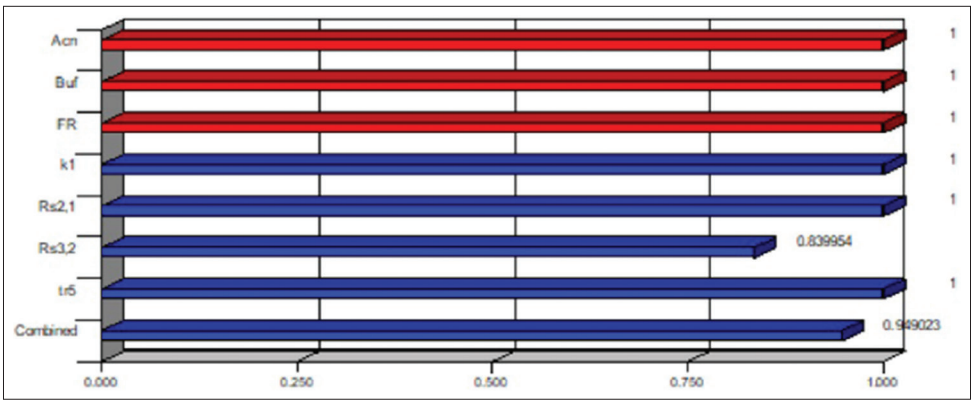

Figure 4: Graphical representation of the overall desirability function for Criteria I *D* (D = 0.949)

Table 5: Criteria for the optimization of the individual responses

Responses	Lower limit	Upper limit	Criteria I		Criteria II	
			Goal	Importance	Goal	Importance
K1	1.084	1.966	Target=1.2	3	Target=1.5	5
Rs.2.1	3.541	10.077	Minimize	1	Target=5.2	5
Rs.3.2	2.064	4.509	Minimize	3	In range	3
tR_5	6.248	22.411	Minimize	3	Target=8.1	4

EFV in Synthivan, Lopimune, and Efavir formulations and the results for ATV, RTV, LPV, and EFV were found to be 99.82, 99.97, 99.85 and 99.84 respectively, indicates within the acceptable range of 100±2%. Percentage recovery of each concentration level (n = 3) and mean percentage recovery (n = 9) were determined and tabulated in Table 6.

Precision

Three different concentrations of six replicates were injected on the same day and calculated; the percentage relative standard deviation was ≤2 (intraday precision). Repeated the analysis for 6 consecutive days and calculated, the percentage

relative standard deviation was ≤3 (interday precision), confirms the method's precision.[35] Precision studies results were tabulated in Table 7.

Robustness

Minute changes in the flow rate and mobile phase composition altered the responses capacity factor of the first peak, the resolution between second and first peak, the resolution between third and second peak, and retention time of the last peak slightly, not more than 2% which confirms the robustness of the method.

Application of the method

Three commercially available formulations: Efavir (EFV 600 mg), Lopimune (LPV 200 mg and RTV 50 mg), and Synthivan (ATV 300 mg and RTV 100 mg) were analyzed by applying the projected HPLC method, and corresponding chromatograms are presented in Figure 5. The results achieved when analyzing Efavir (EFV 600 mg); Lopimune (LPV 200 mg and RTV 50 mg); and Synthivan (ATV 300 mg and 100 mg RTV) tablets were 599.1 (0.08) mg of EFV; 199.046 (0.278) mg of LPV; and 49.696 (0.8) mg of RTV; and 299.926 (0.012) mg of ATV; and 99.703 (0.012) mg of RTV, respectively, the values inside the parentheses represent the percentage CV of the six replicates. The assay results confirm good harmony with the label claim of the above-mentioned formulations. <2% CV for all the three formulations represents, the method is precise.

Summary

Accurate, precise, and robust HPLC method was developed and validated for the optimum resolution and estimation of ATV, EFV, LPV, and RTV in pharmaceuticals. The current developed method is significant and worthwhile since in India most of the doctors are widely prescribing combinations of these anti-retroviral drugs of different classes according to HAART guidelines. The said HPLC method is most cost-effective, since it decreases overall quantification time and consumption of organic solvents. The method furnishes crucial data relating to the sensitivity and interaction effects of various chromatographic factors and their separation attributes. Retention time, separation, and capacity factor of the chromatographs were optimized employing chemometrics technique: Global optimization. The validation protocol assisted to assure the final optimum assay condition was highly sensitive, specific, precise, robust, and linear. Hence, the final optimum assay procedure can be applied in the quality control department for usual analysis of ATV, EFV, LPV, and RTV both in pharmaceuticals and biological matrices.

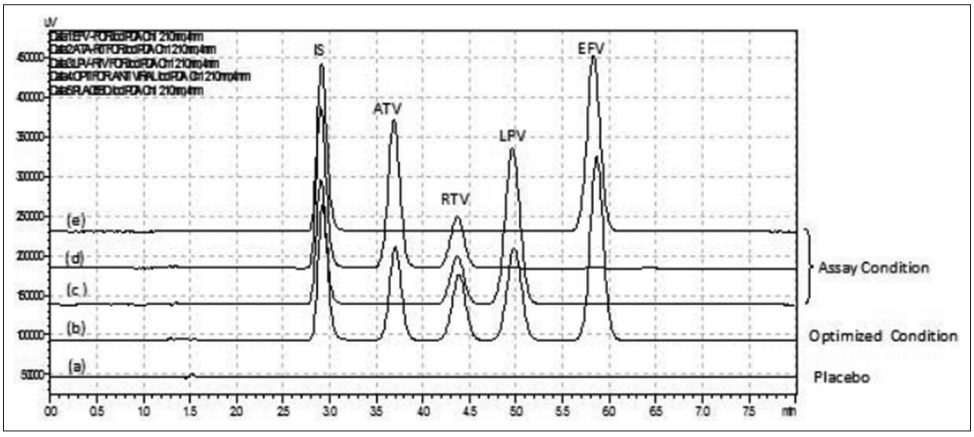

Figure 5x: Chromatograms corresponding to (a) a placebo solution; (b) a synthetic mixture of IS (5 µg/mL) atazanavir (), efavirenz (EFV), lopinavir (LPV), and ritonavir (RTV) (10 µg/mL); (c) a real sample of Lopimune tablets containing IS (5 µg/mL); RTV (5 µg/mL) and LPV (20 µg/mL); (d) a real sample of Synthivan tablets containing IS (5 µg/mL); ATV (18 µg/mL) and RTV (6 µg/mL); (e) a real sample of Efavir tablets containing IS (5 µg/mL); and EFV (6 µg/mL) under optimum assay conditions I for formulation

Table 6: Accuracy indicating assay method for the determination of atazanavir, ritonavir, lopinavir, and efavirenz

Accuracy (mean % recovery) (*n*=3)	Atazanavir	Efavirenz	Lopinavir	Ritonavir
80%W/W	99.96	99.80	99.74	99.94
100%W/W	100.00	99.85	99.89	99.99
120% W/W	99.99	99.44	99.92	99.97
(Mean % recovery, %CV) (*n*=9)	99.82,0.02	99.84, 0.24	99.85,0.21	99.97,0.04

Table 7: Precision indicating assay method for the determination of atazanavir, ritonavir, lopinavir, and efavirenz

Precision (%CV) (*n*=6)	Atazanavir		Efavirenz		Lopinavir		Ritonavir	
	Conc μgmL^{-1}	Results	Conc μgmL^{-1}	Results	Conc μgmL^{-1}	Results	Conc μgmL^{-1}	Results
Intraday	3	0.171	2	0.582	2	0.582	0.5	1.123
	9	0.249	4	0.951	6	0.717	1.5	1.025
	15	0.226	6	0.606	10	0.433	2.5	0.606
Interday	3	0.77	2	1.623	2	1.977	0.5	2.193
	9	0.24	4	1.316	6	1.299	1.5	1.316
	15	0.16	6	1.118	10	2.03	2.5	1.118

CONCLUSION

The newly developed and chemometrics assisted optimized HPLC method is highly sensitive than the other existing methods. It is cost minimizing, simple and fast (<6 min) method with good linearity and precision. The results found to confirm that the developed protocol can be employed in day to day analysis of all the four drugs (ATV, EFV, LPV, and RTV) individually and in combinations. Further, bioavailability and pharmacokinetic studies can be done employing plasma condition, Criteria II.

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