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Research Article

DOE-BASED OPTIMIZATION STRATEGY IN MULTICOMPONENT HPLC ANALYSIS FOR DETERMINATION OF ANTI-VIRAL DRUGS IN COMBINED DOSAGE FORMS

Prafulla Kumar Sahu

Department of Pharmaceutical Analysis & Quality Assurance, Raghu College of Pharmacy, Dakamarri, Bheemunipatnam (M), Visakhapatnam, Andhra Pradesh 531 162.

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ABSTRACT

Owing to know the complexity in reverse phase high performance liquid chromatography (RP-HPLC); a deeper understanding of input method variability is highly essential for achieving the desired separation. During the method optimization, a mathematical relationship between the experimental variables and the chromatographic responses is to be established with the help of a suitable design of experiment (DoE). The present study demonstrated the use of a full factorial design (FFD) to identify the significant variables' effects influencing the separation of five anti viral drugs i.e.; Lamividine (LAM), Stavudine (STA), Atazanavir (ATA), Ritonavir (RIT), and Abacavir (ABA) in a RP-HPLC method. Influence variables such as mobile phase flow rate, buffer composition (ratio of acetic acid and triethylamine) and organic modifier concentration (acetonitrile), each at two levels were screened for the desirability of critical responses like $RS_{LAM\text{-}STA}$, $RS_{ATA\text{-}RIT}$, T_{LAM}, T_{STA}, T_{ATA} and T_{RIT}. A statistical program (Design Expert 12.0) was used to calculate and optimize the six responses simultaneously by means of a multiple response optimization algorithm. 50mM acetic acid and 50mM triethylamine (75:25 %v/v) buffer and acetonitrile (52: 58 v/v) at a flow rate of 0.25ml/min was found to be the optimal condition to produce desirable responses. The developed RP-HPLC method was further validated with respect to the current regulatory requirements. The optimized method gives rapid and efficient separation with complete resolution between the five peaks, and represents an improvement over the existing reported methods.

*** Corresponding author:**
Dr. Prafulla Kumar Sahu, Department of Pharmaceutical Analysis & Quality Assurance, Raghu College of Pharmacy, Dakamarri, Bheemunipatnam (M), Visakhapatnam, Andhra Pradesh-531 162. Email: kunasahu1@rediffmail.comMobile: +91-8121139575

INTRODUCTION

The use of antiretroviral agents in combination is common. To decrease the pill burden and improve patient compliance, combination antiretroviral therapies compile two or more active drugs. Nucleoside Reverse Transcriptase Inhibitors are frequently recommended as one of the first-line therapy for the treatment of HIV infections in combination with other class of antiretroviral drugs, i.e., protease inhibitors. Furthermore, generic preparations containing LAM dual combinations with STA, ATA and ABA are the more commonly prescribed antiretroviral medications to treat the HIV viral infections to lower the prescription costs (1, 2). ATA is frequently prescribed as dual combinations with LAM, ABA, and RIT. Co-administration of ATA with low-dose RIT has been an important innovation in the treatment of HIV-I infection owing to its several benefits including ease of dosing, favorable resistance profile, minimal toxicity, and lower effect on lipid and glucose metabolism (3). Hence, existence of a suitable analytical method for simultaneous determination of all the five drugs in bulk, pharmaceutical formulations and biological samples is highly desirous.

HPLC method development and optimization become more complicated when the sample contains mixture of multiple drugs. Due to structural difference, each chemical entity from the sample mixture possesses distinct affinity towards the stationary phase and the mobile phase, causing various constituents to travel at different velocities and separate. Thus desired separation may be expected through proper tuning of some of the intrinsic parameters like; polarity, pH, flow rate, composition of mobile phase; nature and type of stationary phase; and environmental factors like temperature and detector type and settings (4, 5). However, OFAT (changing **one-factor-at-a-time** while keeping others constant) based traditional approach for method optimization can certainly achieve the desired separation, but exaggerate time, money, and labor. Such an approach for HPLC method development is quite unpredictable, critical to fix errors, and often unsuccessful to promise true optimal conditions (5, 6). A large number of variables affecting the HPLC process need to be carefully identified, investigated and controlled to attain a true optimal system. DoE as a chemometric tool to solve the problems systematically can serve to enable us finding the global optimal condition thereby

obtaining information-rich results with a minimum effort, time and resources (5, 7-10).

Comprehensive literature review evidenced that only limited number of HPLC methods (11-15) is available for simultaneous estimation of antiretroviral agents including the above drugs. All of these methods adopt OFAT strategy for method optimization and are impaired by inefficient or time-consuming procedures and lack of statistical evaluation of significant variables. However, no HPLC method has been developed for exclusive analysis of the five drugs so far. The present study particularly focuses on the DoE-based optimization of the newly developed HPLC procedure for simultaneous assay of the five analytes in tablet dosage forms. The method demonstrated to be reliable, robust and fit for the purpose. The strategy adopted was effective to achieve improved separation efficiency by minimizing trial and error without much useless experiments.

EXPERIMENTAL

Chemicals and reagents

All the materials were of analytical-reagent grade unless stated otherwise. HPLC-grade water (Qualigens, Mumbai, India), HPLC grade acetonitrile and methanol (FINAR, Ahmedabad), Curcumin (Molychem, Mumbai, India), Metanil yellow (Molychem, Mumbai, India), trifluoroacetic acid (FINAR, Mumbai, India), formic acid (EMPARTA, ACS, Mumbai, India) were procured from the local vendor, Visakhapatnam.

Apparatus used

A binary gradient HPLC system (Shimadzu, Kyoto, Japan) consisting two pumps LC 20AD, a photo diode array detector SPD-M20A, with a manual sample injector all from Shimadzu was used for the entire study. The output signal was monitored and integrated using LC solutions software (Shimadzu, Kyoto, Japan). Other apparatus used were Electronic balance (Shimadzu), Ultra sonicator (SONICA, Soltec), pH meter (Elico), microfilter paper (0.45µ, Millipore), filtration apparatus (Borosil) etc.

Mobile phase preparation

The mobile phase consists of solvent A and solvent B. Where, solvent A is various mixtures of 50mM acetic acid and 50mM triethylamine 25:75 v/v, 50:50 v/v and 75:25v/v; while solvent B is acetonitrile. Soon after solvent A and B were prepared, they were filtered using 0.45µm membrane filter (milipore) and subjected to sonication for one hour separately. A diluent comprising mixture of acetonitrile and water (50:50) was also made for further use in

standards and quality control solutions preparations.

Standards and quality control solutions

All the solutions and samples were prepared using the diluent comprising mixture of acetonitrile and water (50:50) unless stated otherwise. For the preparation of primary stock solutions (1mg/ml), accurately weighed LAM, STV, ATA, RIT and ABA were dissolved in small amount of acetonitrile prior to volume adjustment with the diluent. Working standards (100 µg/ml) were prepared from suitable dilution of the primary stock solutions individually. All the standard solutions were refrigerated until use. In the similar fashion, a mixture of all analytes was also prepared freshly before analysis, which was further diluted to get a series of calibration standards of appropriate concentrations. Calibration curves (five points) were constructed between the concentration range of 2 to 100 µg/ml. Quality control (QC) samples for precision and robustness testing were prepared by diluting working (mixture of all analytes) or calibration standards.

Method Optimization

FFD was used to identify the significant variables' effects influencing the separation of the five analytes in a RP-HPLC method. Influence variables such as mobile phase flow rate, buffer composition (ratio of acetic acid and triethylamine) and organic modifier concentration (acetonitrile), each at two levels were screened for the desirability of responses such as RS1 (resolution between lamivudine and stavudine), RS3 (resolution between atazanavir and ritonavir), T1 (tailing factor of lamivudine), T2 (tailing factor of stavudine), T3 (tailing factor of atazinavir) and T4 (tailing factor of ritonavir). A statistical program, Design Expert Version 12.0 (Stat-Ease Inc.) was used to calculate and optimize the six responses simultaneously by means of a multiple response optimization algorithm. The parameters and their levels selected for the optimization are given in Table 1.

Table 1: Operational parameters selected along with their levels for optimization of the method by FFD.

Operational parameters	Low	High
Flow rate (ml/min)	0.25	0.35
%B at start of gradient	52	58
Composition of buffer (acetic acid: triethylamine)	25:75	75:25

Developing the Experimental Design

Experimental design was applied with the aid of a FFD to obtain the optimum combined effect of as mobile phase flow rate, buffer composition (ratio of acetic acid and triethylamine) and organic modifier concentration (acetonitrile), each at two levels (low and high). The influence of the three independent variables on responses, i.e., RS1 (resolution between lamivudine and stavudine), RS3 (resolution between atazanavir and ritonavir), T1 (tailing factor of lamivudine), T2 (tailing factor of stavudine), T3 (tailing factor of atazinavir) and T4 (tailing factor of ritonavir) as dependent variables was investigated.

Various flow rate, buffer composition and organic modifier concentrations were inspected to identify the optimum chromatographic conditions, leading to the following observations:

Flow rate: A change in flow rate usually has a larger effect on the retention of the analytes and their elution. Depending on the mechanism, the change in the resolution because of change in mobile phase flow rate may sometimes be imperceptible or quite substantial and may lead to greater differences in selectivity. A critical change in flow rate may affect the peak area, retention time, and peak shape.

Buffer composition: A change in buffer composition usually has a larger effect on the pH value, which, in turn, can influence the degree of dissociation and thus the UV absorption of ionic analytes. The pH level affects the degree of dissociation of acidic/ basic compounds and of free (residual) silanol groups on the surface of the stationary phase. This means that the pH value could be used as a means of controlling the degree of interaction between a polar/ ionic analyte and the stationary phase. Thus, pH can be used to influence retention time and peak shape. In order to achieve good reproducibility of a separation, it is important that the buffers used have a sufficient buffer capacity. Inadequate buffering may lead to change the equilibriums between pKa values of solute with buffer pKa values and mobile phase pH; hence deterioration of peak shape.

Due to the aforementioned factors, a FFD with the three independent variables at three levels was used to determine the response pattern and optimum combination of variables. The second-order polynomial (regression) equation was used to represent the effects of the variables. Statistical comparison within variables was carried out by two-way analysis of variance (ANOVA).

A p-value of less than 0.05 was considered to be statistically significant.

Conducting the Experiments

According to the FFD, 12 experimental trials were conducted for the three variables each at two levels, and the translations of the coded levels are shown in the Table 2. The influence of the three independent variables on responses was observed.

Table 2: Full Factorial Design for optimization of the RP-HPLC method.

Run	Factors		
	Flow rate (mL/min)	%B at start of gradient	Acetic acid: Triethylamine (v/v)
1	0.35	58	1:3
2	0.35	52	1:3
3	0.25	52	1:3
4	0.25	58	3:1
5	0.25	52	1:3
6	0.25	52	3:1
7	0.35	52	3:1
8	0.35	52	3:1
9	0.25	58	1:3
10	0.25	58	3:1
11	0.35	58	3:1
12	0.35	58	1:3

Method validation

At the optimal condition, the proposed RP-HPLC method has been validated with respect to the following parameters outlined by ICH [ICH Q2 (R1), 2005]:

Linearity

Suitable dilutions from working standard solutions were prepared to yield a series of solutions in the concentrations range of 2-100 µg/ml for all the analytes. The resultant solutions were chronologically injected in duplicate into HPLC column. A calibration curve was constructed there by plotting the corresponding peak areas against the concentrations to obtain regression equation and correlation coefficient, which is used to indicate the linearity of the method

Precision

Intra-day precision of the method was determined using three quality control samples (30, 50 and 70 µg/ml) each nine injections on the same day and percentage relative standard deviation (% RSD) were calculated. Furthermore, these experiments were repeated on three consecutive days to assess inter-day precision.

Accuracy

Accuracy was determined by analyzing a known concentration of standard drug spiked with marketed formulation at 80%, 100% and 120% levels and then determining the percentage recovery.

Sensitivity

Sensitivity of the method was studied by establishing the limit of detection (LOD) and limit of quantitation (LOQ).

$$LOD = 3.3 (\sigma/S)$$

$$LOQ = 10 (\sigma/S)$$

Where, σ is the standard deviation of the response and S is the slope of the calibration curve.

Robustness

Robustness of the proposed method was evaluated by deliberately varying the method parameters: flow rate (± 0.2mL/min) and % organic modifier (±2%). The resultant responses to the variations were statistically compared with the proposed method.

RESULTS AND DISCUSSION

Optimization of the Method with the Aid of Full Factorial Experimental Design

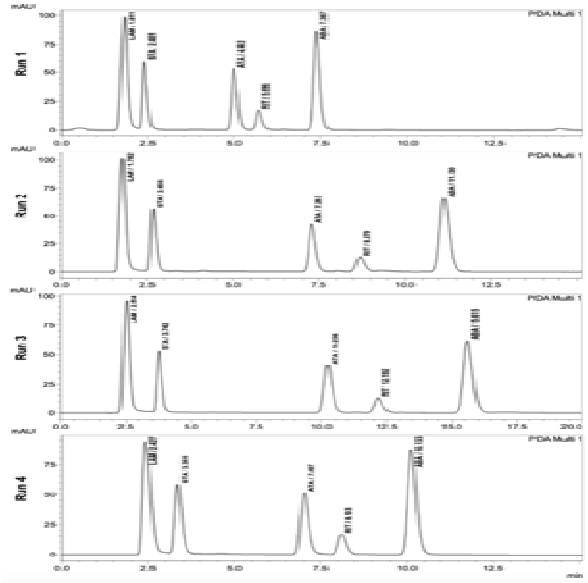
As per FFD, 12 experimental trials were conducted to investigate the influence of the three independent variables on responses, i.e., RS1 (resolution between lamivudine and stavudine), RS3 (resolution between atazanavir and ritonavir), T1 (tailing factor of lamivudine), T2 (tailing factor of stavudine), T3 (tailing factor of atazinavir) and T4 (tailing factor of ritonavir) (Table 3).

The resulted chromatograms from the experimental trials were given in Fig 1.

Table 3: Full Factorial Design and responses obtained for optimization of the RP-HPLC method.

Run	Responses						
	A:T	RS1	RS3	T1	T2	T3	T4
1	1:3	2.057	2.171	1.182	1.413	1.308	1.306
2	1:3	3.021	3.317	1.229	1.428	1.267	1.326
3	1:3	3.026	3.358	1.214	1.382	1.269	1.243
4	3:1	2.426	2.486	1.544	1.366	1.319	1.253
5	1:3	3.023	3.361	1.212	1.379	1.267	1.235
6	3:1	3.333	3.949	1.373	1.385	1.278	1.25
7	3:1	3.394	3.96	1.419	1.41	1.274	1.274
8	3:1	3.441	4.043	1.403	1.386	1.27	1.225
9	1:3	2.074	2.149	1.178	1.386	1.301	1.287
10	3:1	2.423	2.522	1.572	1.373	1.3	1.268
11	3:1	2.459	2.549	1.612	1.388	1.306	1.274
12	1:3	2.068	2.178	1.189	1.422	1.309	1.303

Where, A:T is ratio of acetic acid and triethylamine, RS1 (resolution between lamivudine and stavudine), RS3 (resolution between atazanavir and ritonavir), T1 (tailing factor of lamivudine), T2 (tailing factor of stavudine), T3 (tailing factor of atazinavir) and T4 (tailing factor of ritonavir)



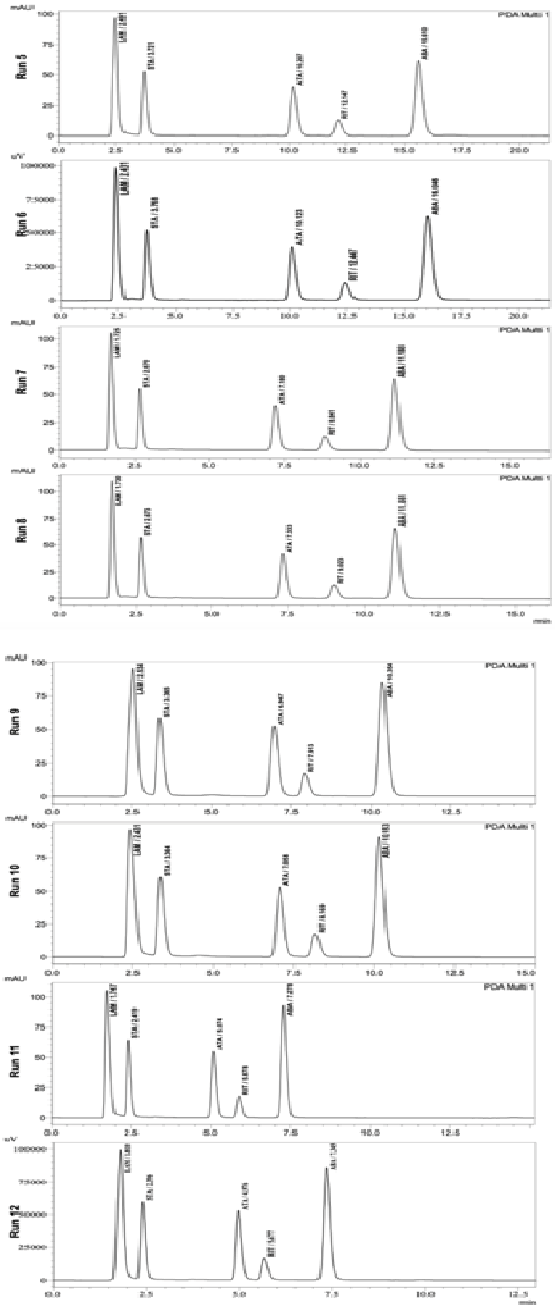


Fig1: Chromatograms resulted from all 12 FFD experimental trials

Data Analysis

ANOVA Statistical analysis (ANOVA) was performed so as to identify significant effects of the variables. The derived outcome indicates that the assumed regression models were significant and valid for each of the responses when the independent variables have a p-value <0.05 (Table 4)

Table 4: Table for ANOVA results

	RS1		RS3		T1		T2		T3		T4	
Source	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Model	1125.85	<0.0001	279.09	<0.0001	18.66	0.0006	10.35	0.0040	39.19	<0.0001	39.19	<0.0001
A-FLOW	2.76	0.1352	1.80	0.2168	0.0621	0.8096	22.97	0.0014	0.0000	1.0000	0.0000	1.0000
D-B CONC	2919.63	<0.0001	732.45	<0.0001	3.25	0.1091	0.3589	0.5657	115.91	<0.0001	115.91	<0.0001
G-A:T	455.15	<0.0001	103.01	<0.0001	52.68	<0.0001	7.72	0.0240	1.65	0.2351	1.65	0.2351

6.3 Optimization

From the ANOVA analysis it was concluded that a compromise among various responses and the combination of variable levels is crucial to reach the optimum conditions of separation. In this study, the optimization was performed for all the six responses based on the following criteria (Table 5): (i) maximum resolution between the peaks and (ii) good tailing factor. 50Mm acetic acid and 50Mm triethylamine (75:25) buffer and acetonitrile (52: 58) at a flow rate of 0.25ml/min was identified as the optimum condition. The chromatogram obtained from the above condition for separation of five anti-viral drugs is shown in Fig 2. Table 6 depicts the chromatographic characteristics of Anti-Viral drugs and its stress related

degradation products in the selected optimal condition.

Table 5: Criteria for optimization:

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:FLOW	is in range	0.25	0.35	1	1	3
D:B CONC	is in range	52	58	1	1	3
G:A:T	is in range	1:3	3:1	1	1	3
RS1	maximize	2.057	3.441	1	1	3
RS3	maximize	2.149	4.043	1	1	3
T1	minimize	1.178	1.612	1	1	3
T2	minimize	1.366	1.428	1	1	3
T3	minimize	1.267	1.319	1	1	3
T4	minimize	1.225	1.326	1	1	3

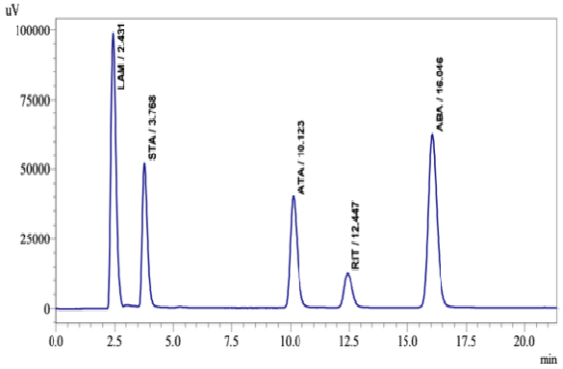


Fig 2: Chromatogram for the separation of five antiviral drugs obtained at the optimum condition

Table 6: Chromatographic Characteristics of the analytes at the Optimum Condition

Analyte	Peak	Retention time	Peak area	Resolution	Tailing factor
Lamivudine	1	2.431	1475143	0.000	1.373
Stavudine	2	3.768	782799	3.333	1.385
Atazanavir	3	10.123	831635	13.296	1.278
Ritonavir	4	12.447	294525	3.949	1.250
Abacavir	5	16.046	1590669	5.534	1.206

Method validation

Method validation

The developed method was further validated for various parameters to demonstrate its compliance to the current regulatory criterion. The method was found to be linear (r^2> 0.99) within the concentration of 2-100 µg/ml for all the analytes. The method was precise with %RSD < 0.143. During accuracy study, the percent recovery of the spiked sample was excellent (% recovery > 99.91) for all the analytes.The sensitivity of the method was established by the measured LODs of 0.371, 2.489, 1.599, 2.43, and 2.89 µg/mL and LOQs of 1.239, 4.297, 5.33, 8.101, and 8.386 µg/mL for LAM, STA, ATA, RIT, and ABA respectively. The validation summary has been depicted in Table 7. The method was found to be robust enough when influence of variations in the method parameters studied to be under control (Table 8).

Table 7: Method validation summary

Parameters	Lamivudine	Stavudine	Atazanavir	Ritonavir	Abacavir
System suitability[a]					
RT (min)	2.84	3.768	10.123	12.447	16.046
A_s	1.373	1.385	1.278	1.250	1.206
N	98530	10613.17	10613.17	10613.17	10613.17
Linearity					
Range (µg/ml)	2-100	2-100	2-100	2-100	2-100
r^2	0.999	0.998	0.998	0.999	0.997
Mean Slope	122496	68401	72217	24143	131350
Mean Intercept	38934	34796	30395	5445	146048
Sensitivity[b]					
LOD (µg/ml)	0.371	2.489	1.599	2.43	2.89
LOQ (µg/ml)	1.239	4.297	5.330	8.101	8.386
Precision (%RSD)[a]	0.03	0.03	0.143	0.12	0.033
Accuracy (% recovery)					
At 80 % level[b]	99.99	99.97	99.97	99.94	99.99
At 100 % level[b]	99.99	99.98	100.02	99.91	99.99
At 120 % level[b]	99.99	99.96	99.98	100.18	99.98

Where; RT - retention time, A_s - tailing factor, N - number of the theoretical plates, r^2- correlationcoefficient.

[a] - Average of six determinations.

[b] - Average of the three determinations.

Table 8: Robustness test results

	Condition	Level	RT (min): mean±SD; %RSD	Peak area: mean±SD; %RSD	A_s: mean±SD;%RSD	N: mean±SD; %RSD
Lamivudine	Optimal	-	2.070±0.002; 0.04	1386982±116.51; 0.07	1.254±0.004; 0.29	35519±11.56; 0.69
	Flow rate(±0.1ml/min)	Decrease	2.065±0.004; 0.14	1375896±136.71; 0.08	1.213±0.004; 0.33	35173±34.18; 1.75
		Increase	2.062±0.001; 0.02	1369658±13.34; 0.03	1.273±0.022; 1.75	36711±13.13; 1.88
	Buffer pH (±0.2)	Decrease	2.072±0.001; 0.01	1389084±86.89; 0.065	1.219±0.007; 0.69	32524±54.79; 1.65
		Increase	2.079±0.01; 0.40	1381906±32.657; 0.57	1.223±0.007; 0.51	35130±24.006; 1.43
Stavudine	Optimal	-	2.979±0.013; 0.39	685993±31.527; 0.01	1.39±0.037; 0.57	15725±11.56; 0.37
	Flow rate (±0.1ml/min)	Decrease	2.982±0.04; 0.09	629531±54.51; 0.12	1.37±0.015; 0.97	16521±48.65; 1.19
		Increase	2.938±0.05;0.15	654069±25.56; 0.48	1.33±0.015; 0.79	15451±5.584; 0.46
	Buffer (pH) (±0.2)	Decrease	2.968±0.04; 0.12	634620±1393.02; 0.01	1.38±0.011; 0.59	16742±35.521; 1.56
		Increase	2.924±0.05; 0.16	605697±44.71; 0.54	1.36±0.020; 1.06	14699±25.040 0.65
Atazanavir	Optimal	-	7.153±0.008; 0.18	729008±19.09; 0.02	1.53±0.025; 1.04	11271±105.5; 0.67
	Flow rate (±0.1ml/min)	Decrease	7.157±0.003; 0.17	725982±35.59; 0.00	1.57±0.015; 0.97	10154±11.17; 1.87
		Increase	7.086±0.005; 0.15	724216±52.44; 0.00	1.83±0.015;0.83	13568±14.12; 1.23
	Buffer (pH) (±0.2)	Decrease	7.144±0.004; 0.09	727465±50.95; 0.01	1.78±0.01; 0.56	09865±52.564; 0.57
		Increase	7.107±0.005; 0.12	721705±11.53; 0.02	1.66±0.02; 1.35	10422±14.742; 0.24
Ritonavir	Optimal	-	8.359±0.008; 0.18	242025±19.09; 0.02	1.73±0.025; 1.04	5548±101.7; 0.76
	Flow rate (±0.1ml/min)	Decrease	8.287±0.003; 0.17	241917±35.59; 0.00	1.77±0.015; 0.97	5865±40.52; 0.35
		Increase	8.365±0.005; 0.15	223911±52.44; 0.00	1.73±0.015;0.83	5129±48.65; 0.84
	Buffer (pH) (±0.2)	Decrease	8.305±0.004; 0.09	213988±50.95; 0.01	1.78±0.01; 0.56	5135±51.39; 0.57
		Increase	8.350±0.005; 0.12	225725±11.53; 0.02	1.76±0.02; 1.35	5869±19.12; 0.65
Abacavir	Optimal	-	10.630±0.26; 0.46	1318526±10.05; 0.02	1.8±0.14; 1.04	25040±0.251; 1.64
	Flow rate (±0.1ml/min)	Decrease	10.690±0.11; 0.59	1319839±47.16; 0.04	1.86±0.97; 0.38	21699±0.15; 0.35
		Increase	10.618±0.15; 0.04	1312025±16.11; 0.13	1.82±0.02;0.017	23185±14.12; 1.23
	Buffer (pH) (±0.2)	Decrease	10.638±0.81; 0.02	1316938±55.19; 0.04	1.81±0.83; 0.18	24242±52.56; 0.24
		Increase	10.646±0.11; 0.02	1314742±25.04; 0.02	1.78±0.56; 0.15	21725±11.53; 0.12

Where, RT: retention time (min) of the peak; As: asymmetric factor of the peak; and N: number of theoretical plates.

* All the experiments were conducted in triplicate.

CONCLUSIONS

A DoE based strategy was exercised in the optimisation of a RP-HPLC method for simultaneous quantification of five antiretroviral agents. With the aid of risk analysis, characterisation, and multivariate statistical analysis, the approach allowed a significantly greater understanding of the method parameters and separation. Further, this improved the separation efficiency, reliability and robustness of the method there by reduced criticalities of the variables with limited experimental trials. The study demonstrated that all three variables assessed were significant to exhibit variability on the chromatographic responses. The design spaces established for the developed LC method was flexible and fit for use and can be used as a tool for the routine quality control and therapeutic drug monitoring of the drugs in various sample matrices.

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