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CHEMOMETRIC APPROACH FOR MULTIRESPONSE OPTIMIZATION OF ANTIRETROVIRAL DRUGS IN PHARMACEUTICAL FORMULATIONS AND HUMAN PLASMA SAMPLE BY RP-HPLC-PDA METHOD

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ABSTRACT

The simple, rapid, sensitive and robust high performance liquid chromatographic method has been developed, optimized and validated for the simultaneous estimation of antiretroviral drugs in pharmaceutical formulation and human plasma samples. First line prescribed nucleoside reverse transcriptase inhibitors used in the treatment against human immunodeficiency virus. The multi-combination therapy has to be proven for efficiently eradicate the HIV, such combinations they are lamividine, stavidine, nevirapine and zidovidine. The method was optimized with the aid of Chemometric tool and the optimized chromatographic condition was obtained Acetonitrile: Methanol: 0.25 % Triethylamine (pH 2.5 adjusted with Ortho phosphoric acid) (20: 20: 60 % v/v/v) at a flow rate of 0.8 ml min-1. The measurement was carried out PDA at 245 nm and the overall run time was less than 10 min and a 4-hydroxy cumarin used as the internal standard (IS). The plasma sample were extracted by simple protein precipitation method and extraction was carried out by using methanol as a co-solvent, then sample were evaporated by air-dry method then reconstituted with mobile phase. The optimized method can be applied for the synchronized quantitative determination of these drugs in formulation and pharmacokinetic, pharmacodynamic investigation of human plasma sample.

Key words: Chemometric, HPLC, zidovidine, lamividine, stavidine and nevirapine

INTRODUCTION

The fixed dose combination is a high efficacy in eradicating human immune deficiency virus (HIV), since single drug therapy rapidly ineffective for antiretroviral treatment because easily drug resistant will occur; and now a day's new paradigm is to combine more than two combinations only preferred for antiretroviral therapy. Lamivudine (LMV), Stavudine (STU), Nevirapine (NVP) and Zidovudine (AZT) are a nucleoside reverse transcriptase inhibitors (NRTI) were officially approved for the treating against antiretroviral therapy. AZT is the first officially USFDA approved for the treatment of HIV and LMV was approved for combination with other retroviral drugs and its special socioeconomic importance because of their widespread frequency in humans [1]. LMV is a synthetic dideoxy- nucleoside derivative that is active against HIV and hepatitis B virus (HBV) and the drug profile was given in. NVP is an inhibitor of DNA and RNA dependent DNA polymerase effectively inhibits the HIV-I. The STU is a thymidine analogue, the plasma half-life is one hour and eliminated via the kidney.

Lamivudine (LMV) is 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl] pyrimidin -2-one (Fig 1). There are various methods reported for the simultaneous estimation of LMV in formulation [2-4], in stability indicating assay [5], in the plasma samples [6-7] in simultaneous determination by LC-MS [8-9]. Simultaneous estimation of interacellural triphosphate metabolites of LMV in human peripheral blood mononuclear cells by combining with anion exchange solid phase extraction and LC–MS/MS [10] .

Stavudine (STU) is 1-[(2R, 5S)-5-(hydroxymethyl)-2, 5-dihydrofuran-2-yl]-5 -methyl pyrimidine -2, 4-dione (Fig 1). In the literature, many HPLC methods reported for the simultaneous estimation of STU in pharmaceutical formulation [11-13], in stability indicating assay [14-16], in biological matrices [17-19], HPTLC [20] .

Nevirapine (NVP) is 11-cyclopropyl -4-methyl-

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5H- dipyrido[2,3-e:2',3'-f] [1,4] diazepin-6-one (Fig 1). Various methods have been reported for the simultaneous estimation of NVP in formulation by HPLC [21-22], in stability and impurity identification [23-24]. In vitro protein adsorption studies nanosuspensions [25], in human plasma [26], in spectroscopic method [27], ion-pair chromatography [28] and pharmacokinetic studies in human serum using liquid–liquid extraction [29- 30], in human

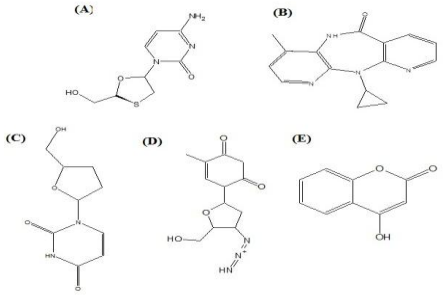

Figure 1 Chemical structure of (A) Lamivudine (B) Nevirapine (C) Stavudine (D) Zidovudine (E) 4-hydroxy cumarin (IS).

plasma sample by LC-MS-MS [31].

Zidovudine (AZT) is 1-[(2R,4S,5S)-4-azido-5-(hydroxymethyl) oxolan-2-yl]-5-methyl pyrimidine-2,4-dione (Fig 1).There are several methods have been cited in the literature for the determination of AZT in pharmaceutical formulation [32-33] and in biological fluids [34-35], radioimmunoassay [36], determination of AZT and metabolites in cell extracts by LC with solid-phase extraction [37]. Thermal analysis in excipients used in solid dosage forms [38], and the pharmacokinetic properties of AZT and its metabolites by LC/ESI/MS-MS , micellar electrokinetic chromatography [39]. There are numerous method have been reported for the simultaneous analysis of LMV, STU, NVP and AZT in individually and combined with other drugs. There is no method available for the simultaneous estimation of these combinations with a single mobile phase.

The main objective of the present research was to develop and optimize the simultaneous quantification of RP-HPLC method for the determination of LMV, STU, NVP and AZT in pharmaceutical formulation and human plasma samples and the method was optimized with the help of Chemometric tool.

EXPERIMENTAL

2.1. Materials

2.1.1. Reference standards

Lamivudine (99.09%), stavidine (99.79%), nevirapine (99.86%) and zidovudine (99.66%), were provided as a gift samples by Heto drugs (Hyderabad, India).

2.1.2. Chemicals and reagents

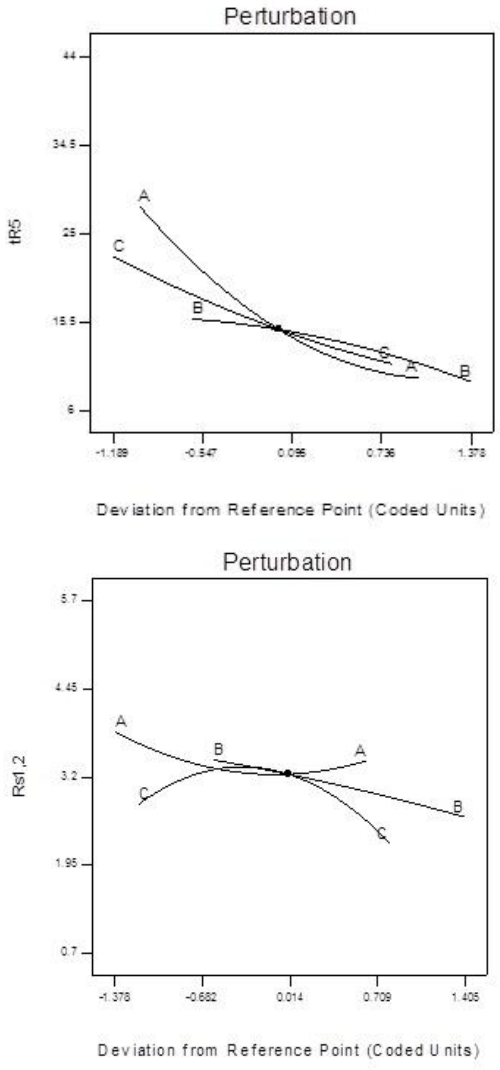

Figure 2 Petribution plot of a) Retention time of last eluted peak (tR_5) and (b) Resolution of critical peak of tR_1 and tR_2 ($Rs_{1,2}$).

Methanol and Acetonitrile were of HPLC grade and were purchased from Merck Inc. (USA). Triethylamine and ortho phosphoric acid were of AR grade purchased from Merck Inc. (USA). HPLC grade water was obtained from a Millipore Milli-Q Gradient water purification system (USA). The human plasma was gifted from Raja Muthiya Medical College and Hospital (RMMCH), Annamalai Nagar, India.

2.1.3. Software

Experimental design, data analysis and desirability function calculations were performed by using Design-Expert 8.0.0 (Stat-Ease Inc., Minneapolis) and Individual desirability function was estimated by JMP 9.0 (SAS NC, USA). The rest of the calculations were made by Microsoft excel 2007 software (Microsoft, USA).

2.2. Equipment and apparatus

The study was performed by using Shimadzu (Japan) chromatography equipped with an LC-20 AD and LC-20 AD vp solvent-delivery module, an SPD-20A PDA detector, rheodyne model 7125 injector valve fitted with a 20µL sample loop. The system was controlled through a system controller (SCL-10A) and a personal computer using a Shimadzu chromatographic software (LC Solution, Release 1.11SP1) installed on it. The mobile phase was degassed using a sonicator (Branson Ultrasonics Corporation, USA). Absorbance spectra were recorded using a UV-Visible spectrophotometer (Model UV-1601PC, Japan) employing quartz cell of 1 cm path length. The chromatography analyses were done on a Phenomenex® analytical column Gemini C18 (150mm × 4.6mm I.D and 5µm particle size).

2.3. Standard solutions

Stock standard solutions of LMV, STU, NVP, AZT and IS *(4-hydroxy coumarin)* were prepared using mobile phase as a diluents and make them a 1.0 mg/ml of the concentration. Standard solutions employed for the optimization procedure constituted a mixture of LMV, STU, NVP and AZT at 10.0, 10.0, 10.0, 10.0, and 5.0 µgmL^{-1} were prepared respectively. For quantification of analytes in markets formulation samples, individual calibration curves (peak area ratios of LMV, STU, NVP, AZT to that of the IS versus drug concentrations) were established at five levels; 2.0-10 µg mL^{-1} for LMV, STU and 1.0- 5.0 for AZT and NVP in the presence of IS (5.0 µg mL^{-1}). In plasma assay method, the calibration curves were constructed at the same concentration levels.

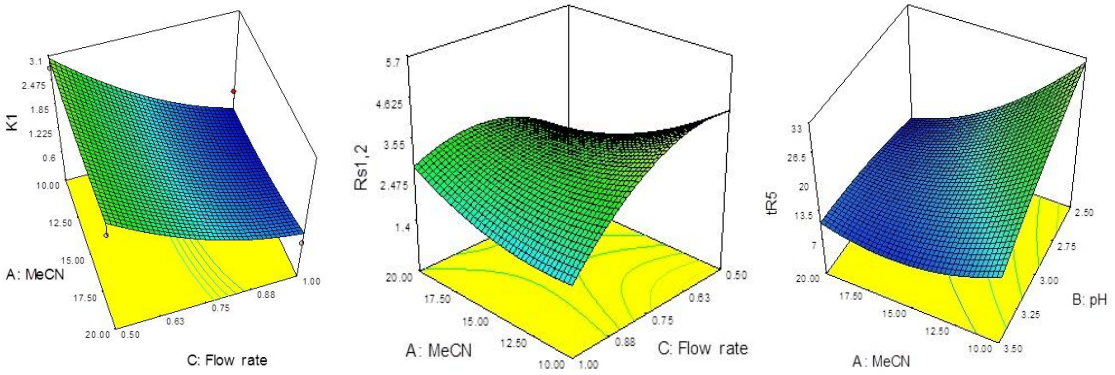

Figure 3 Surface response plots for the responses (A) Capacity factor (k_1) , (B) $Rs_{1,2}$, and (C) tR_5 MeCN concentration (A) is plotted against flow rate (C) for Capacity factor (k_1) , Resolution $Rs_{1,2}$ and MeCN concentration (A) is plotted against pH (B) for tR_5.

2.4. Formulation sample preparation

Twenty tablets were weighed and finely powdered. In the case of capsule dosage, the contents of the capsule were mixed thoroughly. An amount of pharmaceutical products powder equivalent to 50 mg of AZT, LMV, NVP, STU was accurately weighed and transferred in a 50 ml volumetric flask; and further IS (25 mg) was added followed by 25 ml of mobile phase. This mixture was sonicated for 15 min for complete extraction of drugs and the solution was made up to the mark with the mobile phase. Then further diluted, to obtain a concentration of 5.0 µg/ml for AZT, NVP and 10 µg/ml LMV, STU and IS as (5.0 µg/ml) and, respectively. The solution was centrifuged at 4500 RPM for 15 min; the clear supernatant was collected and filtered through a 0.2 µm membrane filter (Gelman Science, India) and 20 µl of this solution was injected into the HPLC system.

2.5. Extraction procedure for plasma sample

1.0 ml of blank plasma and 1.0 ml of cold methanol was transferred into a 10 ml centrifuge glass stoppered tube spiked with the standard stock solutions of AZT, LMV, NVP, STU and IS get a final concentration of 5.0, 10.0, 10.0, 5.0 µg mL^{-1}. The mixture was gently shaken for 10 min and centrifuging on a laboratory centrifuge (Remi ®, R&C, Remi equipment, Mumbai, India) at 5000 RPM for 15.0 min. The supernatant organic layer was transferred to the petri-dish to allow to air dry

for 3.0 hours. The residue was reconstituted in 100 µL of mobile phase and vortex mixed for 45 seconds, then the aliquots of 20 µL were injected into the HPLC system. The same procedure was carried out for blank plasma samples to check the specificity of the extracts. To find the efficiency of the extraction procedure, the spiked plasma sample was extracted according to the above procedure, but the addition of IS after extraction. The percentage recovery was estimated by comparing the peak areas of each analyte spiked sample with that from the blank plasma sample to which the drug was added previous to the evaporation step (Equation-1).

$$\% R = \frac{D_{(spike)} / IS}{D_{(non\ spike)} / IS} X100 \quad (1.0)$$

Where, D (spike) is the area of the each analyte in spiked plasma sample; D (non spike) is the area

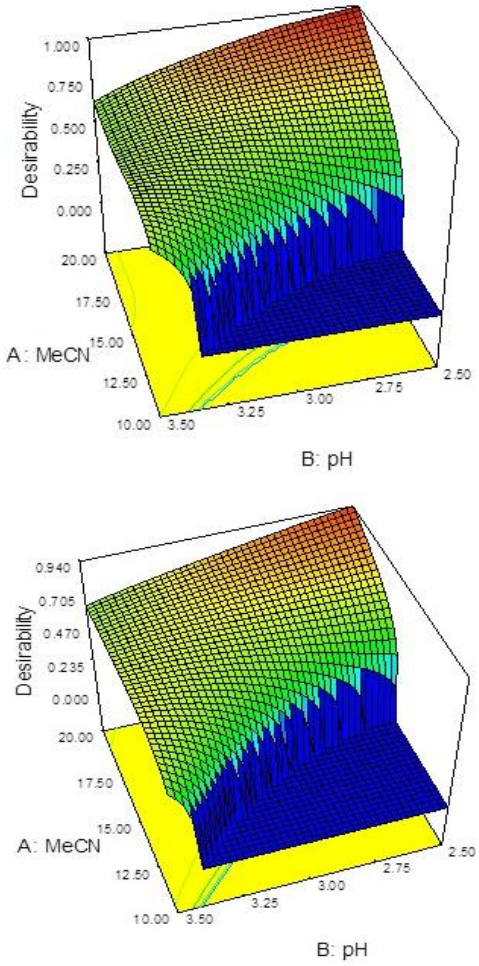

Figure 4 Graphical representation of global desirability (a) optimal formulation condition (D = 0.998) and (b) optimal plasma condition (D = 0.935).

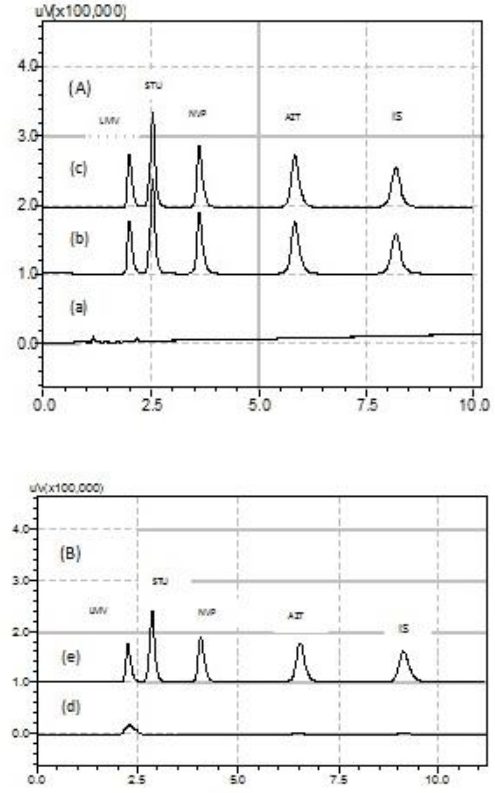

Figure 5 Corresponding chromatograms of LMV, STU, NVP, AZT and IS under optimal condition for (A) formulation, (a) a placebo solution (b) a synthetic mixture of LMV, STU, NVP, AZT and IS (c) real samples of Emtri and LAZID-N tablets. (B) spiked human plasma sample, (d) blank plasma (e) plasma spiked with drugs (LMV, STU, NVP, AZT and IS (4-hydroxy coumarin).

of each analyte obtained by the addition of the drug previous to the evaporation step, considering a recovery of 100%.

2.6. Chromatographic conditions

Chromatographic separations of LMV, STU, NVP and AZT were carried out C18 as a stationary phase, with the ternary mobile phase consisted of a MeCN; MeOH and 0.25% of TEA (pH 2.50) pH was adjusted with 10 % ortho phosphoric acid and detection was carried out in PDA detector at 245 nm.

RESULTS AND DISCUSSION

3.1. Preliminary experiments

Table 1.0 Experimental responses and rotatable central composite design arrangements[a]

Point Type	Factors			Response		
	MeCN (A)	pH (B)	Flow Rate (C)	k_1	$Rs_{1,2}$	tR_5
Fact	20.00	2.50	0.50	2.562	2.837	13.221
Center	15.00	3.00	0.75	1.478	3.148	14.372
Center	15.00	3.00	0.75	1.478	3.148	14.372
Fact	20.00	2.50	1.00	0.778	2.306	6.627
Axial	15.00	3.00	1.17	0.612	1.455	8.798
Axial	23.41	3.00	0.75	2.525	3.971	13.151
Fact	20.00	2.50	1.00	0.778	2.306	6.627
Axial	15.00	3.00	0.33	4.623	1.57	31.144
Fact	10.00	3.50	1.00	0.896	0.729	12.455
Axial	15.00	2.16	0.75	1.47	4.519	17.898
Fact	10.00	2.50	1.00	1.053	1.274	24.198
Fact	10.00	3.50	0.50	2.837	2.91	12.455
Center	15.00	3.00	0.75	1.482	3.149	14.359
Center	15.00	3.00	0.75	1.48	3.16	14.347
Fact	20.00	3.50	0.50	1.503	1.352	16.579
Fact	20.00	3.50	1.00	0.865	2.283	6.477
Center	15.00	3.00	0.75	1.477	3.171	14.327
Axial	6.59	3.00	0.75	1.625	5.606	43.597
Fact	10.00	2.50	0.50	2.792	5.128	40.365
Axial	15.00	3.84	0.75	1.53	2.68	9.861
Center	15.00	3.00	0.75	1.479	3.188	14.353

Initial studies were carried out from the literature by trial and error method to identify the basic requirements of liquid chromatographic method developments such as (i) type of stationary phase (C18, C8 and C6), (ii) range of pH, (iii) flow rate (iv) type of mobile phase additives (Diethylamine, Tryethylamine), based on the studies we estimated independent factors that have influenced on dependent responses. To obtain an acceptable analytical retention time, good quality of separation (resolution, capacity factor), there is need to optimize the chromatographic separation. For the optimization purpose we utilized rotatable central composite Design and derringer desirability function.

(i) Selection of stationary phase:

There are different types of stationary phase available for the reverse phase HPLC and we tried phenyl ,C18, C8 and C6 columns. Well resolved peak separation and excesses of asymmetric factor, less peak resolution were observed on C8 and C6 columns. Moreover phenyl columns are not suitable for this analyte. Among these C18 gave good peak separation and satisfactory retention time, resolution and capacity factor.

(ii) Selection of mobile phase:

Initially acetonitrile was selected as the organic phase and HPLC water was selected as an aqueous phase, then various ranges of pH (pH was adjusted with 10% orthophosphoric acid) were tried. In the above combination of mobile phase were tested in different proportion (50:50, 40:60, 60:40, 70:30) and at 50: 50 (MeCN: water (pH 3.5) ratio only we observed valuable retention time but poor resolution, capacity factor and poor peak separation, then introduced methanol to overcome this problem.

(iii) Selection of Additives:

Table 2.0 Response models[a] and statistical parameters obtained from ANOVA and CCD (after backward elimination)

Responses	Regression model	Adjusted R^2	Model P Value	% CV	Adequate precision
k_1	+1.49- 0.026 A-0.072B-0.94 C+0.16AC	0.8580	< 0.0001	8.29	9.716
$Rs_{1,2}$	+3.19 -0.29A-0.54B-0.43C+0.16AB+0.80AC+0.37A^2	0.8305	< 0.0001	7.35	10.423
$/tR_5$	+14.50-7.16A-3.66B-5.16C+5.36AB+1.58BC+3.99A^2	0.9044	< 0.0001	14.35	12.600

[a] Only significant coefficients with $p < 0.05$ are included. Factors are in coded levels.

From the selected above mobile phase we added 0.05 to 1.0 % diethylamine and there are no significant changes in resolution, and the peak overlapping. Then we tried acetic acid (0.1- 0.5 %) in aqoues phase small variation in resolution, so atlast we tried with 0.05 - 1.0 % of triethylamine, it produced significant improvements in resolution and good peak shape.

3.2 Central composite design and analysis

A rotatable central composite design (R-CCD) was employed for the simultaneous separation and optimization of LMV, STV, NVP and AZT in pharmaceutical formulation and human plasma sample. From the preliminary experiments, C18 as a stationary phase and a ternary mobile phase consisted of MeCN; MeOH and 0.25% of TEA were employed as the factors, in which concentration of MeCN, flow rate, pH of 0.25% TEA were varied and MeOH concentration was fixed at 20.0 % v/v in the mobile phase because there is a very small significant difference in responses. ***(Table 1.0)*** shows the levels of each factor studied for finding out the optimal values and responses. As can be seen in this table, the ranges of each factor used were: MeCN concentration (10-20% v/v), pH of 0.25% TEA (2.5- 3.5) and flow rate (0.5-1.5 ml/min). The dependent variable responses, they are capacity factor of the first eluted peak LMV (k_1), resolution of the critical separated peak LMV, NVP and retention time of last eluted peak (tR_5) were selected. The order of drug elution was determined by individually injected and conformed. The design matrix, the experimental results and replicates ($n_c = 6$) of the central points were performed to identify the experimental error and all the experiments were performed in a randomized order ***(Table 1)***. The quadratic mathematical model for three independent factors is given in Equation (2.0).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (2.0)$$

Where Y is the responses (k, Rs or tR_5) to be modeled, β is the regression coefficient and X_1, X_2 and X_3 represents factors A, B and C respectively. Statistical parameters obtained from ANOVA from the reduced models are given in ***(Table 2)***, and the insignificant terms ($P > 0.05$) were eliminated to the model from backward elimination process to obtain a simple and realistic model. The adjusted R^2 were well within the acceptable limits of $R^2 \geq 0.80$, which revealed that the experimental data shows a good fit with the second-order polynomial equations. For all the reduced models, P value of < 0.05 was obtained, implying these models were significant. In this study, the adequate precision value is a measure of the signal (response) to noise (deviation) ratio was found to be in the range of 9.716 –12.60, which indicates an adequate signal and therefore the model is significant for the separation process. The coefficient of variation (C.V.) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10%. In this model CV was found to be less than 10 %, excepting tR_5 (14.35%). Hence, the examination of diagnostic plots such as (a) normal probability plot of residuals, (b) plot of residuals vs. predicted values were analyzed for response tR_5. The normal probability plot provides the residuals follow a normal distribution, in which case the points will follow a straight line. The points on this plot falls on rather close to the straight line, so the seems appropriate. The plot of residual vs predicted values is a measurable number of standard deviations the actual value deviates from the predicted value. This plot will indicate it is possible to conclude that they were randomly distributed around zero and there is no evidence of outliers (no point falls away from the mean more than three times the standard deviation). Since, the assumptions of normality and constant variance of the residuals were found to be satisfied; the fitted model for the tR_5 was accepted. As can be seen in (***Table 2)***, the interaction term with the largest

Table 3.0 Criteria for the optimization of the individual responses for the analysis of pharmaceutical formulation (Criteria I) and plasma sample (Criteria II)

Responses	Lower Limit	Upper Limit	Criteria I		Criteria II	
			Goal	R I	Goal	R I
k_1	0.612	2.0	Maximize	4	Target=2.0	5
tR_5	7.00	2.5	Minimize	3	Minimize	3
$Rs_{1,2}$	0.729	2.0	Maximize	4	Maximize	4

RI- Relative importance

absolute coefficients between the fitted models is *AB* (+ 5.36) of tR_5 model. The positive interaction between *A* and B is statistically significant (P<0.0001) for tR_5 model. The study reveals that changing the fraction of MeCN from low (–1) to high (+1) results in a rapid decline in tR_5 both at the low (–1) and high level (+1) of pH. Further, at low level of factor A, an increase in the pH results in a marginal decrease in the retention, capacity factor and resolution. Therefore, when the MeCN concentration is set at its lowest level, the pH has to be at its highest level to shorten the analysis time. Especially this interaction is synergistic, as it led to a decrease in analysis time. The existence of such interactions emphasizes the necessity to carry out active multifactor experiments for optimization of the chromatographic separation.

In order to addition a better understanding of the results, the predicted models are presented in (***Fig. 2)*** as perturbation plots and response surface plots (three dimension) ***(Fig. 3.0)***. Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models, were chosen for the axes of the response surface plots. Therefore, factor A and C were nominated for the response surface plots of k_1, $Rs_{1,2}$ with the factor B held constant at a low level of pH 2.50 except tR_5 there is factor A and B were nominated for the response surface plot. All the 3D plots were beneficial to get an overall clarefecation of the MeCN concentration and pH influence the analysis time (tR_5). Perturbation plot provides outline views of the response surface plots, where it showed the response alters the each factor moves from a preferred reference point, with another factor held constant as the reference value. A steepest slope or curvature shows sensitiveness of the response to a specific factor, and it's indicates factor A (MeCN) mostly influence the retention time (tR_5) followed by factor B then factor C. The rest of factors (pH and flow rate) had a significant effect on capacity factor (k_1) and resolution ($Rs_{1,2}$).

3.3 Multi Response optimization

Derringer's desirability function was utilized for the global optimization of three responses and to selected multi-task optimal conditions for the analysis of the formulation and human plasma samples.

3.3.1. Optimum condition for formulation assay

The optimized conditions of each individual response are shown in ***(Table 3).*** In criteria I have been proposed for selecting an optimum run condition for analyzing marketed formulation samples. AS shown the criteria I, the responses tR_5 was minimized, to reduce the analysis time. At the same time $Rs_{1,2}$ was fitted in the range of 1.50 – 2.50 to allow baseline separation of LMV and STV, to avoid the initial noise and solvent front of the first eluted peak k_1 was to be maximized. The relative importance for k_1, $Rs_{1,2}$ and tR_5 was assigned in 5, 4 and 3 respectively. The above restrictions and conditions to be considered in the optimization process were carried out. The 3D response surface gained for the global desirability function is presented in ***(Fig 4).*** From the figure it can be concluded that there was a set of coordinates, producing high desirability value (D= 0.998) were concentrations of MeCN is 20.0 %, flow rate is 0.80 ml min^{-1} and pH of 2.50. The optimized condition for the analyzing marketed formulation assay condition were C18 as a stationary phase and mobile phase is MeCN: MeOH: 0.25 % TEA (pH 2.50 adjusted with ortho phosphoric acid) in the ratio of 20:20:60 % v/v, 0.80 ml min^{-1} as a flow rate and PDA detection at 245 nm. The predicted response values corresponding to the latter value of D were: k_1 = 1.50, $Rs_{1,2}$ = 2.77 and tR_5 = 8.86.

3.3.2. Optimum condition for plasma assay

Table 4.0 Comparison of predictive and experimental values of different objective functions under optimal conditions

Optimum Conditions	MeCN (%)	pH	Flow (ml/min)	k_1	$Rs_{1,2}$	tR_5
I	Desirability Value (D) = 0.998					
	20.0	2.5	0.80			
	Predicted value			1.50	2.772	8.860
	Experimental value		1.56	2.800	8.891	
	% Error		**4.0**	**1.01**	**0.34**	
II	Desirability Value (D) = 0.935					
	20.0	2.5	0.72			
	Predicted value			1.99	3.531	9.387
	Experimental value		2.03	3.602	9.619	
	% Error		**2.01**	**2.01**	**2.47**	

For the assay of plasma sample were used criteria II and was established by varying the response goals and their importance values shown in (**Table 3).** For instance, high k_1 value has to be selected for the separation of first eluted peak (LMV) to stay away from the initial disturbances of plasma components. Therefore k_1 was maximized up to 1.80 and high importance value of 5 was chosen. To meet the above response goals and the optimization procedure was carried out. The desirability function was maximized at an overall desirability of about D = 0.935**.** The coordinates, producing the maximum value were concentrations of MeCN was 20% v/v, pH is 2.5 and flow rate of 0.72 mL min^{-1}. The optimized plasma assay condition were performed a C18 as a stationary phase and mobile phase was MeCN-MeOH-0.25 % of TEA (pH was adjusted with 10 % ortho phosphoric acid) at a flow rate of 0.67 mL min^{-1} and PDA detection at 245 nm. The relationship between the experimental and predicted responses to both the predicted responses for both the predicted optimums I and II are shown in (**Table 4).** The percentage of prediction error (P.E) was calculated by the equation (3.0) [40], the difference should be 0.0- 6.0 % .

$$\text{Predicted Error} = \frac{\text{Experimental - Predicted}}{\text{Predicted}} \times 100 \quad (3.0)$$

3.4 Validation of formulation and plasma assay method and its application

3.4.1. Method validation

An excellent linearity was established at five levels in the range of 2.0-10.0 μgmL^{-1} for LMV and STU, 1.0-5.0 μgmL^{-1} for NVP & AZT with more than 0.999 R^2 were observed the all analytes. Slope and intercept of the calibration curve were y= 0.198x+ 0.014 for LMV, y= 0.196x+0.012 for STU, y= 0.513x+0.001 for NVP and y= 0.197x+0.011 for AZT respectively. Since the correlation coefficient is not good indicators of linearity performance of an analytical procedure a one way ANOVA was conducted. For all the each analytes, the calculated F-Value (F*calc*) was obtained less than the theoretical F*crit* (F-Value) at a 5.0 % significance level, its indicating there was no significance difference between replicate determinations for each concentration level. The LOD and LOQ were estimated at 4.66 ng mL^{-1} and 14.14 ng mL^{-1} for LMV, 4.46 ng mL^{-1} and 14.70 ng mL^{-1} for STU, 1.69 ng mL^{-1} and 5.13 ng mL^{-1} for NVP, 2.91 ng mL^{-1} and 8.83 ng mL^{-1} for AZT, respectively. The optimized method is specifically in relation to the placebo used in the formulation and blank plasma used for in this study, but there are no excipients and matrix effect is observed and corresponding chromatogram presented in **Fig 5**. Accuracy, assessed by spike recovery and in which the % recovery of each level (*n* = 3) and mean % recovery (*n* = 9) were estimated at each level were found to be within the acceptable criteria of bias, ± 2.0 %. The mean % recovery (*n* = 9) for each analytes was also tested for significance by using the Student t – test. Since the t_{Calc} is less than the theoretical *t* value (t_{Crit} = 2.306), at 5% significance level, the null hypothesis (the recovery is unity or 100 %) were accepted. These results specify that the method is accurate and therefore the absence of interference from placebo excipients and blank plasma used in this study. The intra and inter-assay

precision (n = 6) was confirmed since, the % C.V. were well within the target criterion of ≤ 2.0 and ≤ 3.0, respectively. Robustness study reveals that small changes did not alter the retention times, retention factor and resolutions more than 2.0 % and therefore it would be concluded that the method conditions are robust.

3.4.2. Application of the method

This optimized method has to be utilized for the simultaneous quantitative analysis of AZT, LMV, NVP, and STV in pharmaceutical formulation and biological matrix. The method can be applied for the marketed (commercial) formulation samples such as Emtri tab containing (LMV= 150 mg, STV= 40 mg and NVP= 200 mg) and LAZID-N tab containing (AZT= 300 mg, LMV= 150 mg, NVP= 200 mg). The mean, % SD recoveries values achieved were within the parenthesis being the % C.V. of the six replicates and the % C.V. of the assay results were <2, indicating the precision of the analytical methodology.

The mean recoveries for each analyte were also tested for significance to realize whether the recovery means are different from the label claim of the tablet by employing student t-test. The values of t_{Calc} for AZT, LMV, NVP, and STV were obtained less than the t_{Crit} = 2.620 at 5% significance level, suggested that there was no significant difference within the mean recoveries of the analytes and the label claim of the analyzed commercial formulation. The optimized criteria II applied for the drug concentration in human plasama samples, it is support for pharmacokinetic and pharmacodynamics studies in clinical and preclinical.

CONCLUSION

A rapid, simple, robust and efficient isocratic reversed-phase high-performance liquid chromatography method was developed, optimized and validated for the simultaneous determination of the LMV, STU, NVP and AZT, in pharmaceutical formulations and human plasma sample with the help of chemometric tool. The optimized condition obtained in support of chemometric approaches enables baseline separation such as capacity factor, resolution of the LMV, STU, NVP and AZT in a reasonable analysis time. Chromatographic techniques when coupled with Chemometrics tools can provide a holistic view of the separation process, converting this combined technique into a powerful and convenient analytical tool.

The analytical results obtained lead to the conclusion that the developed method performs well with regard to precision, accuracy, rapidity, sensitivity and robustness, with single mobile phase allows to detect LMV, STU, NVP and AZT. Therefore, it could be successfully employed for the analysis of these antiretroviral drugs in formulations and plasma samples.

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