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CHEMOMETRIC APPROACH FOR THE SIMULTANEOUS ESTIMATION OF ISONIAZID AND ETHAMBUTOL IN BULK AND FORMULATION BY RP-HPLC

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ABSTRACT

The intention was to develop chromatographic method for the determination of antitubercular drugs in combinations in bulk and formulation using a chemometric approach. A validated reverse phase HPLC method has been developed for the simultaneous estimation of isoniazid and ethambutol in bulks and Pharmaceutical formulations. The chromatographic separation was carried out on Inertsil C18 (4.6 x 250mm, 5µm) column at ambient temperature (25°C). The mobile phase containing 0.1 Molar ammonium-acetate buffer and acetonitrile in the ratio of 70: 30 at a flow rate of 1.0mL/min was used. The PDA detection was held at 255 nm (ISNZ) and 220nm (ETHAM). The retention time of isoniazid and ethambutol were found to be 2.272 and 3.285 minutes, accordingly. Method optimization was achieved using Box-Behnken design (DOE software), in which three key variables were examined for statistical analysis, namely, Flow rate, organic %, wavelength, pH. Retention times of respective drugs (R1 and R2) were taken as the response parameters. The actual and expected response values were very close. The developed HPLC method was validated by determining its sensitivity, selectivity, linearity, accuracy and precision. Isoniazid linearity was observed in the concentration range of 10-60µg/ml, while ethambutol linearity was identified in the range of 20-120µg/ml. The accuracy of the method was assessed by percentage recovery studies at three different levels at 50%, 100% and 150% of its working concentration. The mean recovery of Isoniazid and Ethambutol was found to be 100.07% and 100.09%, indicates the good accuracy of the method. This developed method can be used for the routine analysis for the estimation of isoniazid and ethambutol in pharmaceutical dosage forms.

KEYWORDS

Chemometry, Box Behnken, RP-HPLC and Antitubercular drugs.

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INTRODUCTION

Ethambutol is a tuberculosis treatment drug. It's generally taken with other tuberculosis medications including isoniazid, rifampicin and pyrazinamide. Isoniazid, also known as isonicotinylhydrazide (INH), is an antibiotic that is used as a first-line

medication for both latent and active tuberculosis prevention and treatment. It is powerful towards mycobacteria, mainly Mycobacterium tuberculosis. A review of the literature reveals that few analytical procedures, such as spectrophotometry, HPLC, and bioanalytical techniques, have been published for the determination of Ethambutol and Isoniazid in individual and combined dosage forms and for the simultaneous measurement of Ethambutol and Isoniazid in bulk and combination dosage form, no chemometric approach has been published. Hence the present attempt was made to develop a simple, precise, accurate, robust, and cost-effective RP-HPLC method for the simultaneous estimation of Isoniazid and Ethambutol by chemometric approach.

MATERIAL AND METHODS

The chromatographic method was developed by utilizing Waters -2695 equipped with PDA, 2998 detector using stationary phase of Inertsil C18 (4.6 x 250mm, 5µm) column. Empower 2 Software was used to integrate the system. All chemicals used were analytical grade and the solvents which are used in the mobile phase were HPLC grade.

Software

Design Expert® 13.0. (Stat-Ease Inc., Minneapolis) was employed to set an Experimental design, and to perform data analysis and desirability function for optimizing RP- HPLC method

Preparedness of mobile phase

A 0.1 Molar ammonium- acetate buffer was made ready by liquifying ammonium acetate (7.71g) in 800 millilitres of milliq water and the pH was altered to 4 using acetic acid and ammonium hydroxide. Then the capacity has been complete to one litre with H2O. Then A 0.45-micron membrane sieve was used to filter the aforesaid mixture. The mobile phase was produced by mixing 0.1 molar ammonium - acetate buffer fluid with ACN in a fraction of 70: 30 (volume/volume).

Diluent

HPLC grade water.

Preparation of working standard

1ml of Isoniazid stock solution (100µg/mL) and 2ml of Ethambutol stockpile fluid (100µg/mL) were fetched to 10millilitres standard flagon and the final volume was made with HPLC ranked H2O to become the strength of 10µg/mL of ISNZ and 20µg/mL ETHAM.

Assortment of wavelength

Standard fluids (10µg/mL) of Isoniazid and Ethambutol have been skimmed in ultraviolet spectra array of 200 to 400 nanometres exclusively. Lambda max of the UV spectra was determined and fixed as detection wavelength.

Exploration of risk assessment

The studies objective remained to figure out what factors influence the Target Quality Profile (TQP). Before making a risk assessment, the essential analytical qualities were used to illustrate the right correlations between parameters and the goal quality profile. This research was utilised to classify potential sources of difficulty to figure out the cause of insufficiency, inconsistency, and let down (flaw). That is also used to figure out how important it is to extract critical factors to accomplish TQP. The components that affect the goal response are given low, medium, high notches. For streaming and collecting only a limited feature, multiple factors were employed and subsequently split into low, medium, and high-risk stages. Finally, utilising experiment designs, three factors were chosen to optimise answers. The target quality profile was identified using this study in the form of retention times (RT1 and RT2) derived from the independent factors[1-3].

Optimisation by RSM

The created approach has been optimised utilising the response- surface method, which included the usage Design-Expert and Box Behnken Design (BXBD). Flowing rate (parable A), organic phase (parable B), and pH (parable C) were chosen as independent factors for statistical analysis. Table No.1 shows the low level and top level of independent factors. As robustness parameters, response variables like retention durations of two chemicals (represented as R1 for isoniazid and R2

for ethambutol) were used. The statistical computations for variable screening and technique optimization were completed employing Design of Experiment software. Seventeen runs were performed using three sovereign parables which are portrayed.

Authentication of experimental strategy

For every parameter, way AOV was used to assess the importance of the impacts among parables. The sovereign variables flow rate, organic phase, and pH were elected and their effects on Retention time 1 (ISNZ) and Retention time 2 (ETHAM) were assessed. The retort coefficient for each dependent parable were computed in order to evaluate the impact of each retort. Each response coefficient was examined for arithmetical significance, and non-significant retort coefficients were eliminated, resulting in substantial polynomial retort equations for RT1 and RT2.

Method validation

The established innovative technique has been authenticated rendering to ICH criteria. The authentication was taking place by diverse strictures such like specificity, linearity, preciseness, accurateness, quantitation's limits, robustness and system suitability[4-7].

System suitability

It was resolved by injecting 10 microlitres each for 6 duplicate provisions of standard fluid containing 10µg/ml of isoniazid and 20µg/ml of ethambutol. Various method parameters as in resolution, plate count (N) and the tailing factor was measured in percent RSD.

Specificity

Specificity of the scheme was established by interjecting distinctly blank, standard and specimen solutions of isoniazid and ethambutol in triplicate. The peak purity analysis verified the findings. The particularity of the technique was evaluated to discover any hindrances in the segregation of the ISNZ and ETHAM in company of additives.

Linearity

The capacity of an analytical process to yield outcomes that have been right proportionate to the analytic potency, or by a mathematical transformation, within a certain range is known as linearity. Dissimilar capacities of standard fluids of medications have been interjected to attain a potency array of 10 to 60 microgram per mL for Isoniazid and 20 to 120 microgram per mL for ethambutol, in 6 duplicates. The monotonicity in relations of deliberate peak areas set against respective strength of medications was assessed by regular linear regressive study. The slope, intercept (with corresponding confidence intermissions) and correlative coefficients (r2) was considered[8].

Detection limits (LOD) and Quantification limits (LOQ)

The created method's LOD and LOQ were derived through using response's standard deviation and slant of the linearity curvature of medications by means of the formulary as per ICH guide,

Analyte detection's limit = 3.3 × sigma/S

Analyte quantization's limit = 10 × sigma/S

"S" is the slope of the standard's linearity plot, whereas sigma is the SDV of the y intercepts of regressive lines[9].

Accuracy (% Recovery study)

The accurateness of the technique was measured paying the standard's adding way, where specimens comprising an artificial blend of isoniazid and ethambutol have been barbed at 3 dissimilar potency stages of 50 percent, 100percent and 150percent. Retrieval study were achieved in triplicates by scheming the percent recovery and a couple of RSDs for each medication.

Precision

The preciseness of the established methodology was assessed by activity Intra-day preciseness and Inter-day preciseness schemes. within-day (Intra) preciseness acted upon by examining 3 duplicates of 3 dissimilar potencies (LQC, MQC, and HQC) on the similar day and the peak areas deliberated was stated in percentage form (%RSD). The amid days (Inter) preciseness investigation has been carried out in duplicate on 3 distinct days by means of the aforesaid medication strengths and the percent RSD was computed[10].

Ruggedness

The extent and intensity of the specimens that could be evaluated determine the method's ruggedness. Here, investigation of specimens from a standardized mix was achieved by investigating the specimens practical and ecological conditions and guaranteeing that the outlined parameters remained among the required confines. Using different analysts and equipment, a static dosage amalgamation assay was performed under varied investigational settings.

Robustness

As outlined through ICH, the strength of an investigative technique denotes to its competence to stay unpretentious by tiny and cautious discrepancies in methodology parameters. A natural action method's insusceptibility to tiny, deliberate variations associated with factors like the mobile segment configuration, flowing degree might be a life of the technique's forte. The sturdiness of the developed HPLC technique was analyzed by varying the system suitability parameters like flowing rate (±0.2 millilitre/minute). The results of robustness were evaluated in terms of % RSD[11-12].

Claim of the established technique to medicinal dose forms

Assay

Preparation of the standard

30 milligram of Isoniazid and 60 milligrams of Ethambutol were precisely balanced and shifted to a 100millilitre standard flagon, 30ml of diluent was adjoined, sonicated for 5 minutes. Then the ultimate capacity was finished with dilutant and branded as standards stock (300 microgram/ml of Isoniazid and 600 microgram/ml Ethambutol). 1 millilitre of afore said stock fluid has been tubed out and attuned to 10ml with diluent to become the final strength of 30ppm of ISNZ and 60ppm of ETHAM.

Preparation of sample

Ten tablets of ISNZ and ETHAM combination (Cadila pharmaceutics, the tag claim ISNZ 300milligram and ETHAM 600mg) were balanced; the mean mass of the tablet was calculated and crushed to a well residue. Accurately weighed residue specimen equal to 30 milligrams of ISNZ and 60 milligrams of ethambutol were put into a 100mL standard flagon and then it was liquified in 50 millilitre diluents by keeping the bottle in a sonication soak at room warmth for 15 min. Later, the resulting specimen was permitted to stance for five minutes and completed the closing capacity with the dissolvent. This solution was riddled. 1ml of above filtrate (300ppm of ISNZ and 600ppm of ETHAM) was shifted to 10ml standard flagon and capacity was completed to get the potency of 30ppm of Isoniazid and 60ppm Ethambutol. The above solution is injected into the HPLC. The mean assay percentage of the tablets and their % RSD was determined.

Stability studies

The steadiness of the investigated specimen was assessed for the isoniazid and ethambutol. The solution was stored at ambient heat deprived of the defencing from light and verified subsequently 0, 12 and 24 hrs.

RESULTS AND DISCUSSION

The λ max of Isoniazid and ethambutol was found to be at 255nm and 220nm respectively. A Box Behnken design was employed to optimize the retention times for simple and precise simultaneous determination of isoniazid and ethambutol in bulk as well as formulations. Seventeen experiments were performed by using three independent variables such as flow rate, the organic phase, and pH to optimize the responses Retention times RT1 and RT2, the results were depicted in Table No.2. All the experiments were carried out in a randomized order for minimizing the effects of unrestrained variables, which may responsible for bias in the measurements. The statistical tools provide the numerical verification of variables and their effects on responses.

Effect of sovereign variables on retention time 1 (RT 1)

The effect of a sovereign variable on Retention time 1 (ISNZ) was expressed by the 3D response, contour plot, and polynomial equation. 2nd order quadratic polynomial for response R1 (Retention time 1) is given as follows.

R1 (Retention time 1) = +2.39 +0.1825 A +0.0537 B +0.0138 C -0.1025 AB -0.1625AC -0.1850BC +0.0850 A^2 -0.0125 B^2 +0.1375 C^2

In this polynomial equation, A, AB, AC, BC, C^2 are the significant model terms as they have P-values less than 0.05. The F-value of 12.44 for the model indicates that it is significant[13]. An F-value of this magnitude has a 0.16 percent chance of occurring due to noise. The F-value of 2.41 for the lack of fit indicates that it is not significant in comparison to the pure error. There's a 20.75 percent possibility that a big Lack of Fit F-value is caused by noise. It's acceptable if there's a Non-significant lack of fit. Seventeen experiments were performed to optimize the retention time of isoniazid and ethambutol. The RT1 results were varied from 2.12 min to 2.88 min. The regression coefficients of the independent variables such as flow rate, organic phase, and pH had a positive effect on retention time 1 and were found to be 0.1825, 0.0537, and 0.0138 respectively. Thus, it clearly indicates, retention time increases with an increase of A, B, and C. However, flow rate, organic phase, and pH together showed a negative influence on retention time. The negative regression coefficient for AB, AC, and BC was observed and it clearly indicates, decrease in retention time with the simultaneous increase in AB, AC, and BC.

Effect of sovereign variables on retention time 2 (RT 2)

The effect of a sovereign variable on Retention time 2(ETHAM) was expressed by the 3D response, contour plot, and polynomial equation. 2nd order quadratic polynomial for response R2 (Retention time 2) is given as follows.

R2 (Retention time 2) = +3.66 +0.1138A +0.2438B +0.0625C -0.2225AB -0.1800AC -0.1250BC +0.0447A^2 +0.0347 B^2 +0.0173C^2

In this polynomial equation, A, B, C, AB, AC, BC are significant model terms as they have P-values less than 0.05. The F-value of 22.49 for the model suggests that it is statistically significant. An F-value of this magnitude has a 0.02 percent chance of occurring due to noise. The F-value of 0.08 for the Lack of Fit indicates that it is not significant in comparison to the pure error. There's a 97.02 percent likelihood that a big Lack of Fit F-value is caused by noise. We want the model to fit, thus a non-significant lack of fit is better. Seventeen experiments were performed to optimize the retention time of isoniazid and ethambutol. The RT 2 results were varied from 3.15 min to 4.1 min. The regression coefficients of the independent variables such as flow rate, organic phase, and pH had a positive effect on retention time 2 and were found to be 0.1138, 0.2438, and 0.0625 respectively. Thus, it clearly indicates, retention time 2 increases with an increase of A, B, and C. However, flow rate, organic phase, and pH together showed a negative influence on retention time. The negative regression coefficient for AB, AC, and BC was observed and it clearly indicates, decrease in retention time 2 with the simultaneous increase in AB, AC, and BC.

Adjusted R1 and R2 were found to be within acceptable bounds (R2> 0.9), indicating that the experimental model fits polynomial equations well. The model is significant for the quantification process since the appropriate precision value of all responses was determined to be larger than 4, indicating a sufficient signal. The coefficient of variation (C.V.) represents the model's repeatability for all responses within the limit (percent C. V< 10). The 3D plots in Figure No.1 demonstrated the effects of variables on distinct responses R1 and R2. The graphical interpretation of the interactions can be done using three-dimensional (3D) plots of the model. As a result, the 3D graphs of the model were accustomed assess the outcomes. The responses were mapped against two experimental factors while the other factors are held constant at their central level. The response surface plot was obtained for the maximum desirability function (D = 0.922), which clearly indicates that obtained mathematical model is excellent The ANOVA findings are tabulated in Table No.3 and Table No.4. The maximum desirability value was obtained at flow rate 1ml/min, ACN 30%, and pH 4. Table No.5 displays the actual and expected response values. As can be seen, the expected and observed experimental values were very similar. The

Optimized Chromatographic Conditions of ISNZ and ETHAM are displayed in Table No.6.

Validation

Method validation featured various ICH parameters including system suitability, specificity, linearity, LOD, LOQ, accuracy, precision, ruggedness, robustness, and stability. The system suitability parameters for Isoniazid and Ethambutol such as theoretical plates and tailing factor were found to be 7095, 1.11, and 9722, 1.16. The specificity of the method was found out through non-interference of excipients in identical conditions of the assay. This demonstrates the uniqueness of the developed method. Isoniazid linearity was observed in the concentration range of 10-60µg/ml, while ethambutol linearity was identified in the range of 20-120µg/ml (Table No.7). The calibration curve of isoniazid was linear with slope, y-intercept, and the correlation coefficient was found to be 5159, 3192, and 0.998 respectively and the calibration curve of Ethambutol was linear with slope, y-intercept, and correlation coefficient were found to be 5737, 1133, and 0.999 respectively (FigureNo.2). The conventional addition method was used to test accuracy at three concentration levels of 50%, 100%, and 150 percent. The mean recovery of Isoniazid and Ethambutol was found to be 100.07% and 100.09%. The preciseness of the developed technique was evaluated by exploiting intra-day and inter-day precision studies. The mean % RSD values of isoniazid for the intraday and inter-day precisions were found to be 0.8362 and 0.8293 respectively.

The mean % RSD values of Ethambutol for the intraday and inter-day precisions were found to be 0.6867 and 0.705 respectively. The approach was found to be reliable, with percent RSD values of less than 2% for replicate measurements. LOD and LOQ of Isoniazid and Ethambutol were 3.15, 9.55, and 4.03 and 12.2µg/mL respectively.

Ruggedness was assessed utilizing two analysts and two HPLC tools. Variation of system suitability parameters such as flow rate (0.2mL/min) was used to test the robustness of the devised HPLC technique. The resultant % RSD values of Isoniazid and Ethambutol were found to be within the acceptable limit (NMT 2%) hence the developed method was found to be rugged and robust. The percentage purity of Isoniazid and Ethambutol was found to be 99.86 % and 100.31% respectively. Medication samples have been examined at room temperature for as much as 24 hours to decide the stableness of the drug solution. Based on preceding research, the garage length becomes chosen. The result showed that no significant change in the concentration of Isoniazid and Ethambutol and thus found to be stable. This method is potentially useful for the analysis of commercial formulations and laboratory preparations[14-17].

Table No.1: Investigational features and stages used in the BXBD

S.No	Factor	Name	Level		
			Low (-)	Medium (0)	High (+)
1	A	Flow rate (ml/min)	0.8	1	1.2
2	B	ACN (%)	30	40	50
3	C	pH	4	4.5	5

Table No.2: Optimization method parameters for Box Behnken experimental design

S.No	Std	Run	Factor 1 A: Flow rate ml/min	Factor 2 B: ACN %	Factor 3 C: pH	Response 1 R1 (Retention time 1) min	Response 2 R2 (Retention time 2) min
1	16	1	1	40	4.5	2.5	3.5
2	7	2	0.8	40	5	2.67	3.86
3	2	3	1.2	30	4.5	2.7	3.82
4	17	4	1	40	4.5	2.37	3.7
5	9	5	1	30	4	2.32	3.3
6	15	6	1	40	4.5	2.35	3.66
7	6	7	1.2	40	4	2.88	3.94
8	1	8	0.8	30	4.5	2.12	3.15
9	5	9	0.8	40	4	2.2	3.35
10	10	10	1	50	4	2.8	4.02
11	12	11	1	50	5	2.34	3.87
12	3	12	0.8	50	4.5	2.43	4.1
13	11	13	1	30	5	2.6	3.65
14	4	14	1.2	50	4.5	2.6	3.88
15	13	15	1	40	4.5	2.37	3.72
16	14	16	1	40	4.5	2.36	3.71
17	8	17	1.2	40	5	2.7	3.73

Table No.3: ANOVA results for response 1 (Retention time 1)

S.No	Source	Sum of Squares	df	Mean Square	F-value	p-value	-
1	Model	0.6912	9	0.0768	12.44	0.0016	Significant
2	A-Flow rate	0.2664	1	0.2664	43.15	0.0003	
3	B-ACN	0.0231	1	0.0231	3.74	0.0943	
4	C-pH	0.0015	1	0.0015	0.2449	0.6358	
5	AB	0.0420	1	0.0420	6.81	0.0350	
6	AC	0.1056	1	0.1056	17.11	0.0044	
7	BC	0.1369	1	0.1369	22.17	0.0022	
8	A^2	0.0304	1	0.0304	4.93	0.0619	
9	B^2	0.0007	1	0.0007	0.1065	0.7537	
10	C^2	0.0796	1	0.0796	12.89	0.0089	
11	Residual	0.0432	7	0.0062			
12	Lack of Fit	0.0278	3	0.0093	2.41	0.2075	not significant
13	Pure Error	0.0154	4	0.0038			
14	Cor Total	0.7344	16				

Table No.4: ANOVA results for response 2 (Retention time 2)

S.No	Source	Sum of Squares	df	Mean Square	F-value	p-value	-
1	Model	1.02	9	0.1129	22.49	0.0002	Significant
2	A-Flow rate	0.1035	1	0.1035	20.61	0.0027	
3	B-ACN	0.4753	1	0.4753	94.64	< 0.0001	
4	C-pH	0.0313	1	0.0313	6.22	0.0413	
5	AB	0.1980	1	0.1980	39.43	0.0004	
6	AC	0.1296	1	0.1296	25.81	0.0014	
7	BC	0.0625	1	0.0625	12.44	0.0096	
8	A^2	0.0084	1	0.0084	1.68	0.2362	
9	B^2	0.0051	1	0.0051	1.01	0.3478	
10	C^2	0.0013	1	0.0013	0.2495	0.6328	
11	Residual	0.0352	7	0.0050			
12	Lack of Fit	0.0019	3	0.0006	0.0751	0.9702	Not significant
13	Pure Error	0.0333	4	0.0083			
14	Cor Total	1.05	16				

Table No.5: The projected and experimental retort for the augmented chromatography settings

S.No	Factor	Optimized Level	
1	A: Flow rate	1 ml/min	
2	B: ACN	30 %	
3	C: pH	4.00	
4	Response	**Predicted**	**Observed**
5	X1: Retention time 1	2.263	2.272
6	X2: Retention time 2	3.279	3.285
7	Desirability	0.922	

Table No.6: Optimized chromatographic conditions

Instrument	Waters 2695 separations module, 2998 PDA detector
Column	Inertsil C18 (4.6 x 250mm, 5µm)
Wave length	255nm (ISO), 220nm (ETHAM)
Temperature	Ambient temperature (25^{o}C)
Flow rate	1.0 ml/min
Injection volume	10µl
Mobile phase	Ammonium Buffer: ACN(Isocratic)
Diluents	HPLC grade water
Run time	7 mins

Table No.7: Linearity data of isoniazid and ethambutol

S.No	Isoniazid		Ethambutol	
	Conc (µg/mL)	**Peak Area**	**Conc (µg/mL)**	**Peak Area**
1	10	53268	20	112665
2	20	108254	40	235783
3	30	159319	60	346172
4	40	218130	80	466281
5	50	259386	100	562729
6	60	307542	120	694182

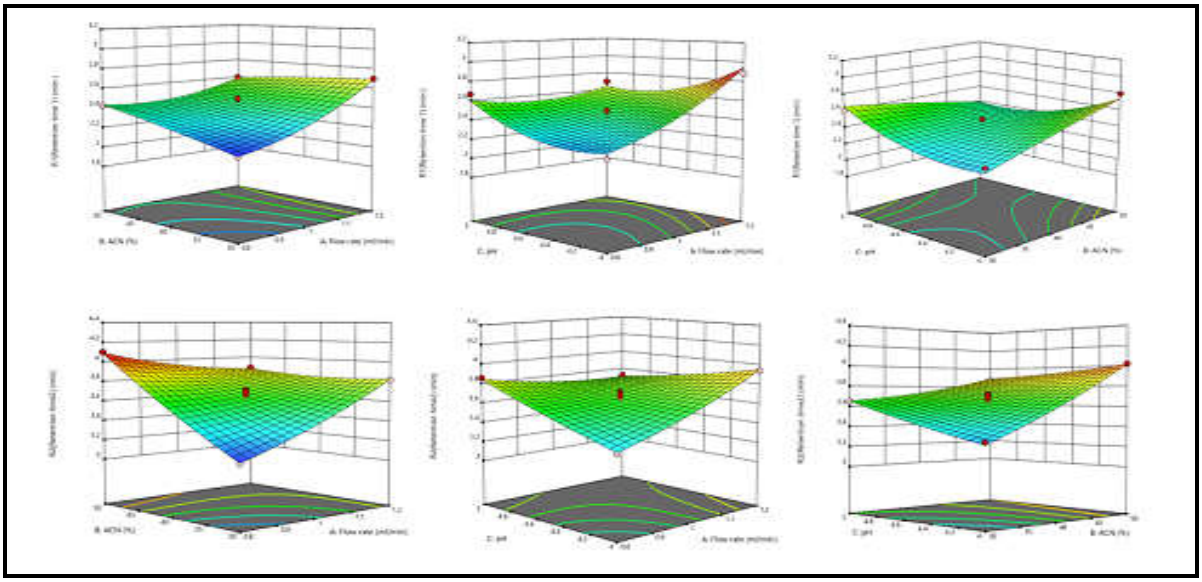

Figure No.1: 3D design plots screening the impact of sovereign variables on RT 1 and RT2

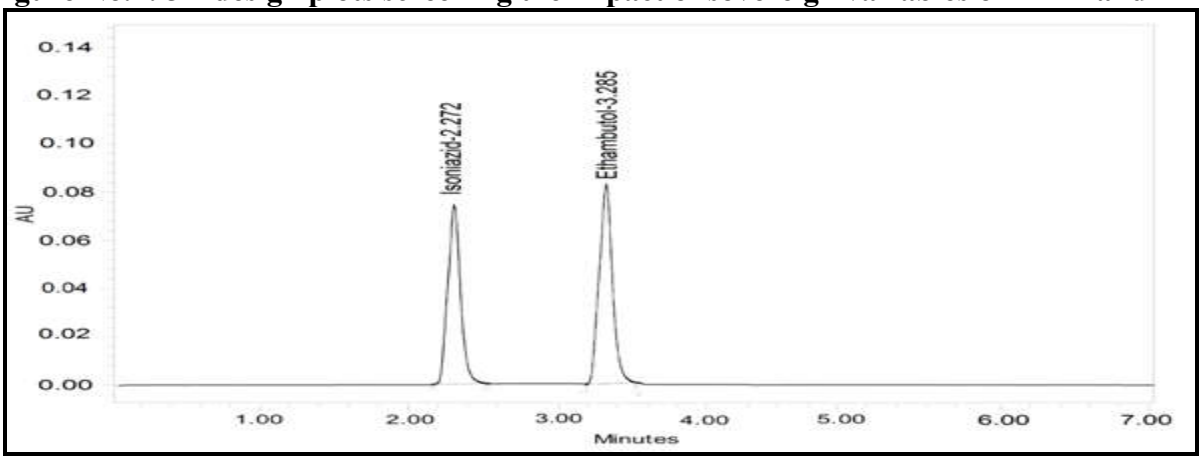

Figure No.2: Optimized chromatogram of ISNZ and ETHAM

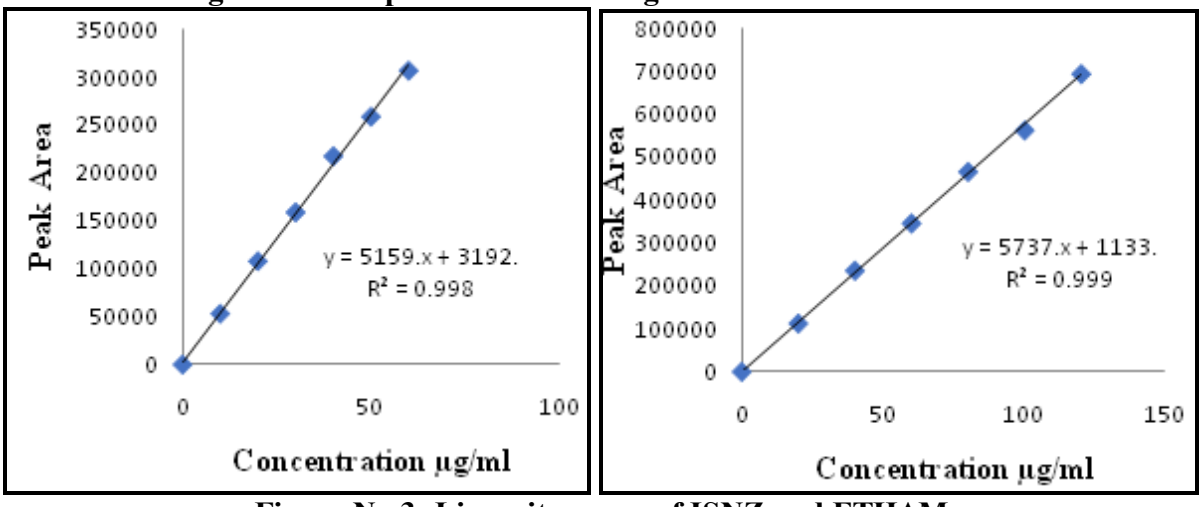

Figure No.3: Linearity curve of ISNZ and ETHAM

CONCLUSION

For the simultaneous estimation of antitubercular medicines, new RP-HPLC methods were devised and validated. Chemometry (Analytical Quality by Design) technique was successfully applied for the optimization of chromatographic method response parameters. The uses of analytical Quality-by-Design tools lead to deeper understanding on how critical variables influence method performance and resulting in more robust and reliable methods. For optimizing the procedure and examining the interaction of multiple variables, the Box-Behnken design was used. The developed methods shall considerably minimize the analysis time and organic solvent required for routine analysis. The reduction of organic solvent indicates that the developed methods are safe for environment as well as more robust technique, no need of frequent validation.

The HPLC procedures developed were found to be simple, accurate, precise and robust. All of the validation parameters yielded positive findings. The study proved that chemometrics can be effectively coupled with chromatography to enhance the separation process. Hence, the validated RP-HPLC method could be readily adapted for the simultaneous estimation of ISNZ and ETHAM in bulk and formulations.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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