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DEVELOPMENT AND VALIDATION OF UPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF RIFAMPICIN, ISONIAZID AND PYRAZINAMIDE IN COMBINED PHARMACEUTICAL DOSAGE FORM

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ABSTRACT: As HPLC method is time consuming, very accurate and precise method was developed for simultaneous estimation of Rifampicin, Isoniazid and Pyrazinamide from Rifampicin, Isoniazid, Pyrazinamide and Ethambutol dosage form by UPLC. The experiment was carried out on Acquity UPLC @BEH C18, (100 mm x 2.1 mm), 1.7 µm column using the gradient composition of mixture of phosphate buffer pH 6.8 and acetonitrile in ratio of 96:4 v/v as mobile phase A and mixture of phosphate buffer pH 6.8 and acetonitrile in ratio of 45:55 v/v used as mobile phase B at flow rate 0.25 mL/min and detection wavelength 238 nm. The retention time of Rifampicin was about 3.982 min, Isoniazid was about 1.177 min and Pyrazinamide was about 1.409 min. (Figure 1). The detector response was linear in the range of 35.20 ppm to 240.00 ppm for Rifampicin, 35.20 ppm to 120.00 ppm for Isoniazide and 184.9 ppm to 645.00 ppm for Pyrazinamide.

KEYWORDS: Development and validation of UPLC method for Rifampicin, Isoniazid and Pyrazinamid.

INTRODUCTION

Rifampicin(7*S*,9*E*,11*S*,12*R*,13*S*,14*R*,15*R*,16*R*,17*S*,18*S*,19*E*,21*Z*)-2,15,17,27,29-pentahydroxy-11-methoxy 3,7,12,14,16,18,22-heptamethyl-26-{(E)-[(4-methylpiperazin-1-yl)imino]methyl}-6,23-dioxo-8,30-dioxa-24 azatetracyclo triaconta-1(28),2,4,9,19,21,25(29),26-octaen-13-yl acetate is a semisynthetic derivative of rifamycin, an antibiotic produced by Streptomyces mediterranei It is active in vitro against gram positive and gram negative cocci, some enteric bacteria, mycobacteria and chlamydia.

Isoniazide is pyridine-4-carbohydrazide is a chemo not antibiotic. Isoniazid is the most active drug for the treatment of tuberculosis caused by susceptible strains. Pyrazinamide is pyrazine-2-carboxamide is a relative of nicotinamide, stable and slightly soluble in water.[1] Few chromatographic method were reported along with other antituberculosis drugs like Clofazimine[2], Linezolid[3], Kynamycin[4] and Amikasin.[5]. Purpose of this study is to develop and Validate.[6] UPLC method for combination drug dosage form of Rifampicin, Isoniazid, Pyrazinamide and Ethambutol tablet.[7] The new proposed method was simple, accurate, precise, linear and rugged. Method was validated as per ICH guidelines[8.9.10] for simultaneous estimation of Rifampicin, Isoniazid, and Pyrazinamide in tablet dosage form.

MATERIALS AND METHODS

Chemical and Reagents

Rifampicin, Isoniazide and Pyrazinamide API, were gifted by Macleods Pharmaceutical Ltd. Mumbai, India, with purity 99.8%, 99.2% and 99.7%, respectively. The combination tablet dosage form of Rifampicin, Isoniazid, Pyrazinamide and Ethambutol contain 150, 75, 400 and 275mg strength tablets of Macleods Pharmaceutical Ltd. Mumbai, India were used. Water (Milli-Q), Anhydrous dibasic sodium phosphate, Methanol, Acetonitrile, Orthophosphoric acid, were purchased from reliable source and used as it is unless and until stated.

Instruments / Equipments

Waters Acquity UPLC with UV detector having Acquity UPLC@BEH C18, (100 mm x 2.1 mm) 1.7 µm, Analytical Balance (Sartorius), pH meter(Lab India), used for the study.

Method

The separation of drug was achieved with gradient method on a reverse phase Acquity UPLC @BEH C18, (100 mm x 2.1 mm) 1.7 µm column at wavelength 238nm, injection volume 1.7µl and column oven

temperature was 35°C and sample temperature was 10°C. The gradient program is of 6 minutes and is as follow.

Table 1: Gradient program

Time (minutes)	Mobile phase Ratio A:B (%)	Comment
0	95:05	Linear gradient
1.5	0:100	Linear gradient
3.5	0:100	Linear gradient
3.51	95:05	Linear gradient
6.00	95:05	Re-equilibration

Solution preparation
Buffer solution
Buffer was prepared by dissolving about 1.4 g of anhydrous dibasic sodium phosphate in 1000 mL of water, mixed. Adjusted the pH of the solution to 6.8 ± 0.05 with orthophosphoric acid, mixed. Filtered.

Diluent: Prepared a mixture of methanol and buffer solution (4: 96 v/v).
Mobile phase A: Prepared a mixture of buffer solution and acetonitrile (96: 4 v/v).
Mobile phase B: Prepare a mixture of buffer solution and acetonitrile (45: 55 v/v).

Standard Preparation
Standard preparation
16 mg Rifampicin standard, 8 mg of Isoniazid standard and 43 mg of Pyrazinamide standard was taken in to a 100 mL volumetric flask. Added 4 mL methanol sonicated for 2-3 min. Added about 50 mL of buffer solution and sonicated to dissolve. Allowed to equilibrate at room temperature and the volume was made with buffer and mixed.

Sample solution preparation
Weighed 20 tablets and crushed them to a fine powder. Transferred an accurately weighed quantity of crushed tablet powder, equivalent to 8 mg of Isoniazid to a 100 mL volumetric flask. Added 4 mL of methanol and sonicated for 2-3 minutes. Added 50 mL of buffer. Sonicated for 5 minutes. Allowed to equilibration at room temperature, diluted with buffer to volume. Filtered through PVDF syringe filter (Axiva or equivalent) by discarding the first few mL of the filtrate. The final concentration of solution was 160, 80 and 430 µg/mL of Rifampicin, Isoniazide and Pyrazinamide, respectively.

3 Method validation
3.1 Specificity
The blank, standard solution and sample solution were prepared as described in the methodology. The impurity solutions and spiked sample solution were prepared at working concentration and injected into the UPLC system. The retention time (RT) of all peaks observed in the resulting chromatograms is recorded. The percentage assay for sample solution and spiked sample solution was determined. The obtained results are presented in result Table 2.

3.2 Linearity and Range
To determine Linearity, a series of solutions were prepared by quantitative dilutions of the stock solution of Rifampicin, Isoniazide and Pyrazinamide standards to obtain solutions at 22%, 80%, 100%, 120% and 150% of the working concentration of 0.16 mg/mL i.e. 160 ppm for Rifampicin, 44%, 80%, 100%, 120% and 150% of the working concentration of 0.08 mg/mL 80 ppm and 43%, 80%, 100%, 120% and 150% of the working concentration of 0.43 mg/mL i.e. 430 ppm Pyrazinamide. This corresponded to a concentration range of 35.20 ppm to 240.00 ppm for Rifampicin, 35.20 ppm to 120.00 ppm for Isoniazide and 184.9 ppm to 645.00 ppm for Pyrazinamide.

Each solution was injected and the peak areas were recorded. The values of concentration, corrected concentration, mean peak area, Slope, intercept, correlation coefficient of the regression line and residual sum of squares were calculated and represented in the table 3 and with graphical representation in Figure 2 for Rifampicin, and Figure 3 for Isoniazide and Figure 4 for Pyrazinamide.

3.3 Precision
To check system precision Rifampicin, Isoniazide and Pyrazinamide standard solution was prepared as per methodology and peak response were measured in five replicates. The mean and relative standard deviations were calculated. The results are presented in the following result Table 4.

3.4 Repeatability
The assay was carried out as described in the methodology on six samples. The percentage assay of Rifampicin, Isoniazide and Pyrazinamide were calculated. The mean, relative standard deviation and 95 % confidence interval of the results were calculated. The results obtained for assay of Rifampicin, Isoniazide and Pyrazinamide are presented in Table 5.

3.5 Accuracy / Recovery
Recovery solutions were prepared by spiking Rifampicin, Isoniazide and Pyrazinamide stock solutions to placebo powder to obtain solutions in the range 50 % to 150 % (i.e. at 50 %, 100 % and 150 %) of the target concentration (0.16 mg/mL (160 ppm)) Rifampicin, (0.08 mg/mL (80 ppm)) Isoniazide and (0.43 mg/mL (430 ppm)) Pyrazinamide in triplicate.

The percentage recovery of Rifampicin, Isoniazide and Pyrazinamide were calculated for each of the recovery solution and the mean recovery was determined. The results are presented in the result Table 6.

3.6 Robustness

The Assay method was carried out as described in the methodology and by making the following alterations in the chromatographic conditions

- Changing Column oven temperature 35±5°C(30°C, 40°C)
- Changing the pH of mobile phase Buffer 6.8±0.2 (pH = 6.6, pH = 7.0)

The observed values were presented in table 7.

System suitability

To check the system suitability, Rifampicin, Isoniazide and Pyrazinamide standard solution was prepared as described in the methodology. The standard solution was injected into the UPLC system at the start of each validation parameter and peak responses were measured. The system suitability parameters of mean and relative standard deviation of areas, and the theoretical plates and tailing factor for the peaks for Rifampicin, Isoniazide and Pyrazinamide were measured.

3.7 Solution Stability

Considering the decomposition of analytes and standards over a time period the method development should investigate the stability of analytes and standards. It is measure of bias in assay result generated during preselected time interval.

3.8 Filter Compatibility

Sample solution was prepared as described in the methodology. The following variations were carried out at the sample filtration stage; the sample solution was centrifuged and diluted as per methodology. The sample solution were centrifuged and filtered through 0.2µm PVDF (25 mm) syringe filter. The obtained results using the 0.2 µm PVDF filter (25 mm) in sample are well within the acceptance criteria i.e. absolute difference of not more than 2.0. However, 0.2 µm PVDF filter was selected as the filter of choice

3.9 Filter Saturation

The saturation of 0.2µm PVDF (25 mm) syringe filter was optimized by filtering and discarding 1.0 mL, 3.0 mL and 5.0 mL sample solution using separate filters, followed by filtration of further 5 mL aliquots and collection of the filtrates in separate test tubes. From the results, it was concluded that the volume of 5.0 mL is sufficient to saturate the filters.

3.10 Forced degradation study

Stress testing.[11] (forced degradation study) help to identify the degradation products, stability of the molecules and also validate the stability and specificity of the analytical procedure. Study was performed for following factor.

- Thermal degradation with Heated the powder at 80°C for 14 hours.
- Photolytic degradation.[12], Powder covered with aluminum foil exposed. Also powder was directly exposed (without aluminum foil) in the photo stability chamber, as per ICH guidelines.
- Thermal and Humidity degradation with at 40°C/75%RH for 24 hours exposed.
- Acid degradation with acid media, powder was kept in 0.01M HCl for 1 hrs at 40°C on water bath.
- Base degradation with base media, powder was kept in 0.01M NaOH for 2 hrs. at room temperature.
- Oxidative degradation with Oxidative degradation media, powder was kept in 5 ml of 0.03% H_2O_2 for 2 hrs. at 40°C on water bath.

3.10.1 Summary of forced degradation results

The summary of degradation given with Table 7. The maximum degradation was observed in acid degradation (Liquid degradation) and thermal degradation (Solid degradation) chromatograms of same were shown in Figure 5 & 10.

RESULTS

4.1 Specificity

No peak observed due to blank solution, placebo solution and known impurity solution at the same retention time of the main peak as observed in the standard solution and sample solution

Table 2: Linearity results for Assay of Rifampicin, Isoniazid and Pyrazinamide.

Name	Retention Time (min)
Blank	No interference
Placebo	No interference
Isoniazid	1.19
Pyrazinamide	1.44
Ethambutol	Not Detected
Rifampicin	4.16
Rifampicin N-Oxide	3.43
Pyrazine-2-Carboxylic acid	0.91
3-Formyl Rifamycin	4.56
Rifampicin Quinone	4.68
3-Isonicotinoylhydrazinomethyl Rifamycin	3.84
Standard	Rifampicin: 4.15, Isoniazid:1.18, Pyrazinamide:1.45

Sample	Rifampicin: 4.15, Isoniazid: 1.18, Pyrazinamide:1.44
Spiked Sample	Rifampicin: 4.15, Isoniazid:1.18, Pyrazinamide:1.44

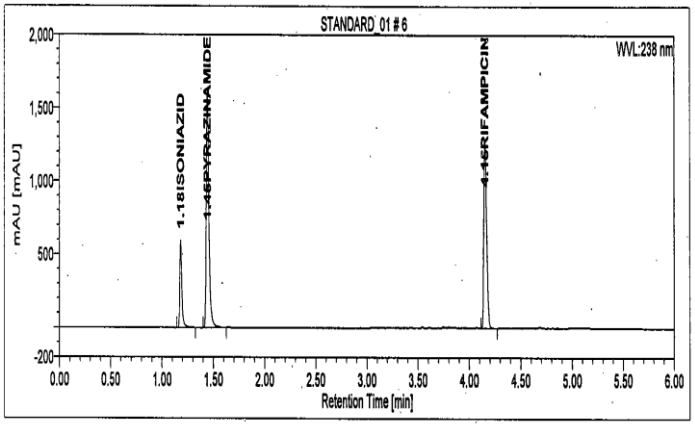

Figure 1: Typical elution pattern for Assay of Rifampicin, Isoniazide and Pyrazinamide

4.2 Linearity and Range

The plot of peak area of each sample against respective concentration was found to be linear in the range of 35.20 – 240, 35.20 - 120, and 184.9 – 645.00 ppm with correlation coefficient of 0.99999, 0.99966 and 0.99983 and linear regression equation Being Y=15.67125x+8.64102, Y=10.33968x+12.39258, and Y=6.63679x+8.64102 for Rifampicin, Isoniazide and Pyrazinamide respectively. Linear regression least square fit, slope (m), intercept (b), standard deviation, residual sum of squares and correlation coefficient data obtained from the measurements.

Table 3: Linearity results for Assay of Rifampicin, Isoniazid and Pyrazinamide.

Level	Rifampicin	Isoniazid	Pyrazinamide
22 % Rifampicin, 44 % Isoniazid, 43 % Pyrazinamide	559.257	381.089	517294
80 %	2019.268	662.670	829110
100 %	2526.000	828.226	1029061
120 %	3032.427	992.707	1229083
150 %	3793.363	1254.666	1545436
SLOPE	15.67125	10.33968	6.63679
INTERCEPT	8.64102	12.39258	32.08680
CORRELATION COEFFICIENT	0.99999	0.99966	0.99983
RESIDUAL SUM OF SQUARES	75.28489	294.65015	1739.20655
RANGE:	22 % to 150 % of target concentration	44 % to 150 % of target concentration	43 % to 150 % of target concentration

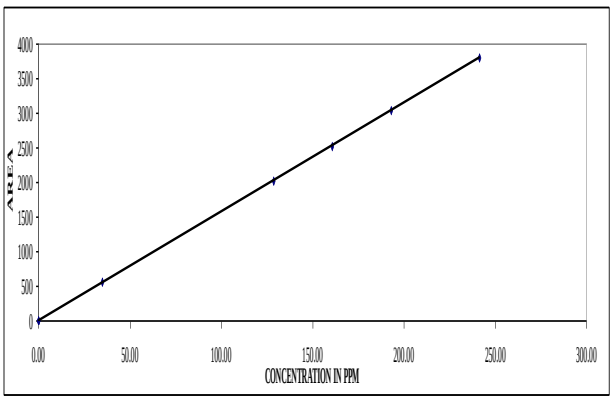

Figure 2: **Linearity graph for Rifampicin**

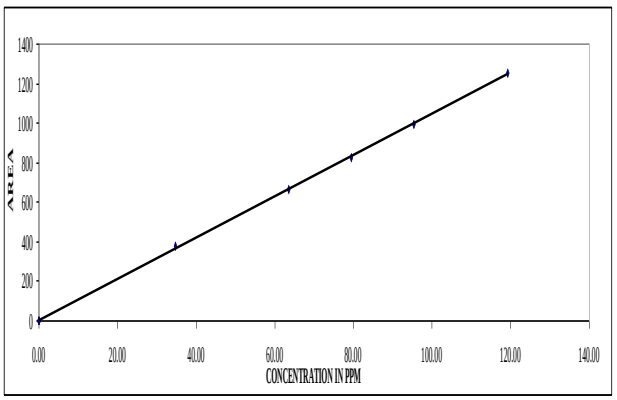

Figure 3: **Linearity graph for Isoniazid**

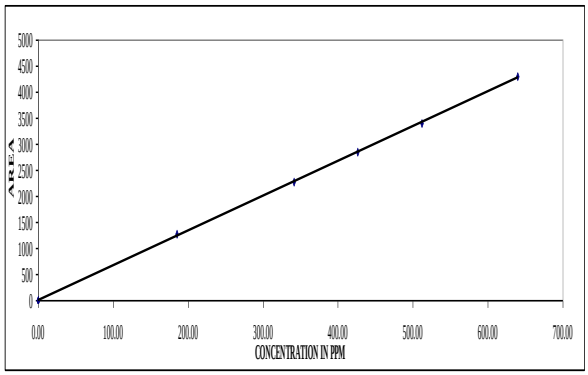

Figure 4: **Linearity graph for Pyrazinamide**

The correlation co-efficient was found to be 0.99999 for Rifampicin, 0.99966 for Isoniazide and 0.99983 for Pyrazinamide, which are well within the acceptance criteria of not less than 0.999. Hence it had been concluded that the method was linear in the range of 35.20 ppm to 240.00 ppm for Rifampicin, 35.20 ppm to 120.00 ppm for Isoniazide and 184.90 ppm to 645.00 ppm for Pyrazinamide.

4.3 Precision

Table 4: System presicion for Assay of Rifampicin, Isoniazide and Pyrazinamide

Injection No.	Peak Area (Rifampicin)	Peak Area (Isoniazide)	Peak Area (Pyrazinamide)
1	2507.607	829.080	2880.530
2	2507.600	827.930	2871.840
3	2517.690	833.280	2891.910
4	2482.730	821.490	2849.820
5	2485.250	821.910	2852.410
Mean	2500.170	826.740	2869.300
% RSD	0.61	0.61	0.63

The relative standard deviations for areas of peaks due to Rifampicin, Isoniazide and Pyrazinamide in five replicate injections of standard solution are 0.61 %, 0.61 % and 0.63 % respectively, which are well within the acceptance criteria of not more than 2.0 %.

4.4 Repeatability

Table 5: Repeatability results for Assay of Rifampicin, Isoniazide and Pyrazinamide

Sample No	Spl. wt (mg)	% Assay		
		Rifampicin	Isoniazid	Pyrazinamide
1	111.80	101.6	102.4	99.9
2	107.99	98.7	99.1	102.2
3	111.48	101.7	102.4	99.8
4	107.43	98.4	99.3	102.5
5	111.48	102.2	102.2	99.6
6	107.22	100.1	100.1	103.3
MEAN		100.5	100.9	101.2
% RSD		1.63	1.57	1.61
95 % CONFIDENCE INTERVAL		1.31	1.27	1.31

The relative standard deviation of the assay results for six individual sample preparations in repeatability for Rifampicin was 1.31 %, Isoniazide was 1.27 % and Pyrazinamide was 1.31 % for Rifampicin, Isoniazide, Pyrazinamide and Ethambutol Hydrochloride tablets tablets (150 mg / 75 mg / 400 mg/ 275mg) which was well within the acceptance criteria of not more than 2.0 %.

4.5 Accuracy / Recovery

Table 6: Recovery study for Assay test of Rifampicin, Isoniazide and Pyrazinamide

Level	% Recovery		
	Rifampicin	Isoniazid	Pyrazinamide
50 %	101.4	99.2	100.1
	100.5	98.9	99.8
	100.0	97.7	98.9
100 %	100.8	101.0	100.0
	101.2	100.5	99.6

	100.5	100.1	99.2
150 %	101.5	100.7	99.3
	101.1	100.7	99.2
	100.4	100.0	98.6
Mean % Recovery	100.9	99.5	99.4
% RSD	0.60	1.07	0.51

The % recovery for Rifampicin, Isoniazide and Pyrazinamide at 50 %, 100 % and 150 % of target concentration (0.16 mg/mL (160 ppm)) Rifampicin, (0.08 mg/mL (80 ppm)) Isoniazide and (0.43 mg/mL (430 ppm)) Pyrazinamide ranged from 100.0 % to 101.5 % for Rifampicin, 99.8 % to 101.2 % for Isoniazide and 97.7 % to 101.0 % for Pyrazinamide respectively, which are well within the acceptance criteria of 98.6% to 100.1 %.

The mean recoveries are 101.2 % for Rifampicin, 100.9 % for Isoniazide and 100.7 % for Pyrazinamide, which were also within the acceptance criteria of 99.4 % to 102.0 %.

Based on the above obtained recovery results, it was concluded that method for assay of Rifampicin, Isoniazide and Pyrazinamide in Rifampicin, Isoniazide, Pyrazinamide and Ethambutol Hydrochloride tablets (150 mg / 75 mg / 400 mg/ 275mg) was accurate

4.6 Forced degradation study

Table 7: Forced degradation summary result for Assay method of Rifampicin, Isoniazide and Pyrazinamide

Force Degradation condition	**% Degradation**			**Peak Purity**		
	Rifa	**Iso**	**Pyra**	**Rifa**	**Iso**	**Pyra**
Initial	----	----	----	Peak Pure	Peak Pure	Peak Pure
Heat at 80°C in oven for 14 hours (Thermal Degradation)	X	13.6	0.8	Peak Pure	Peak Pure	Peak Pure
Photolytic degradation, as per ICH guidelines (Control)	----	----	----	Peak Pure	Peak Pure	Peak Pure
Photolytic degradation, as per ICH guidelines (Exposed)	3.8	X	X	Peak Pure	Peak Pure	Peak Pure
Thermal and Humidity at 40°C/75% RH for 24 hours	3.4	4.9	X	Peak Pure	Peak Pure	Peak Pure
5 mL of 0.01 M HCl heated at 40°C for 1 hr on water bath.	0.8	8.7	4.5	Peak Pure	Peak Pure	Peak Pure
5 mL of 0.01 M NaOH kept at room temperature.	5.7	X	2.6	Peak Pure	Peak Pure	Peak Pure
5 mL of 0.03 % H_2O_2 heated at 40°C for 2 hours on water bath	X	3.5	0.5	Peak Pure	Peak Pure	Peak Pure

Note: X indicates No degradation, Rifa indicates Rifampicin; Iso indicates Isoniazide and Pyra indicates Pyrazinamide.

The peaks due to Rifampicin, Isoniazide and Pyrazinamide were found to the spectrally pure in all the degradation conditions, indicating that there was no co-elution with main peaks. Based on the above results it was concluded that the method for assay of Rifampicin, Isoniazide and Pyrazinamide estimation in Rifampicin, Isoniazide, Pyrazinamide and Ethambutol Hydrochloride tablets (150 mg / 75 mg / 400 mg/ 275mg) tablets was specific and stability indicating.

4.7 Robustness

Table 8: Robustness study system suitability results for Assay of Rifampicin, Isoniazid & Pyrazinamide

Altered condition	**Tailing Factor**			**Theoretical plates**			**% RSD (Five replicate Injections)**		
	Rifa	Iso	Pyra	Rifa	Iso	Pyra	Rifa	Iso	Pyra
Unaltered Repeatability Standard	1.68	1.35	1.23	74446	17156	14822	0.61	0.61	0.63
Column temperature 30°c	1.53	1.49	1.47	77485	15533	14017	0.61	0.54	0.52
Column temperature 40°c	1.65	1.47	1.46	79909	18208	14059	0.48	0.49	0.56
buffer pH 6.6	1.63	1.50	1.48	77799	17267	14080	0.58	0.65	0.66
buffer pH 7.0	1.61	1.49	1.48	78315	16724	14024	0.32	0.43	0.49

By making above alteration no significant change in result observed hence method seen roubst for routine analysis.

4.8 System suitability

During the complete validation the system suitability parameter i.e. theoretical plate, %RSD of replicate standard solution and tailing factor established and based on the overall validation parameter results , the following system suitability acceptance criteria recommended for consistent and reproducible result

- The column efficiency should not be less than 30000, 5000 and 50000 theoretical plates for Isoniazide, Pyrazinamide and Rifampicin peaks respectively.

- Relative standard deviation for area due to Rifampicin, Isoniazide and Pyrazinamide peak in five replicate inj
- ections of standard solution should be not more than 2.0 %.
- Tailing factor for Rifampicin, Isoniazide and Pyrazinamide peak should be not more than 2.0.

5.0 CONCLUSION

The method for the determination of assay of Rifampicin, Isoniazide and Pyrazinamide in Rifampicin, Isoniazide, Pyrazinamide and Ethambutol Hydrochloride tablets (150 mg / 75 mg / 400 mg/ 275mg) was validated. The method was evaluated for its specificity, precision, solution stability, accuracy, linearity and range, and robustness. The method meets all the acceptance criteria Hence it can be concluded that the method has been suitable for its intended use, i.e. to determine the assay of Rifampicin, Isoniazide and Pyrazinamide in Rifampicin, Isoniazide, Pyrazinamide and Ethambutol Hydrochloride tablets (150 mg / 75 mg / 400 mg/ 275mg).

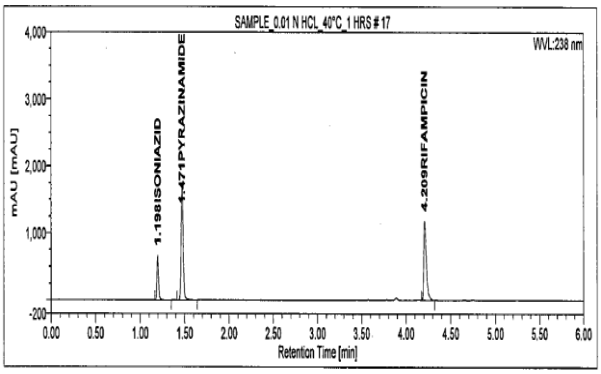

Figure 5: Chrmatograph of Acid Degradation

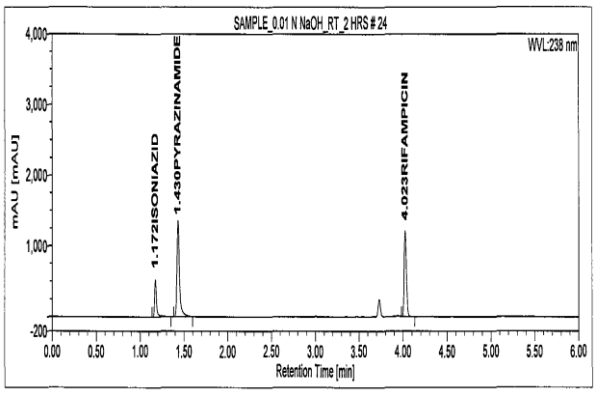

Figure 6: Chrmatograph of Base Degradation

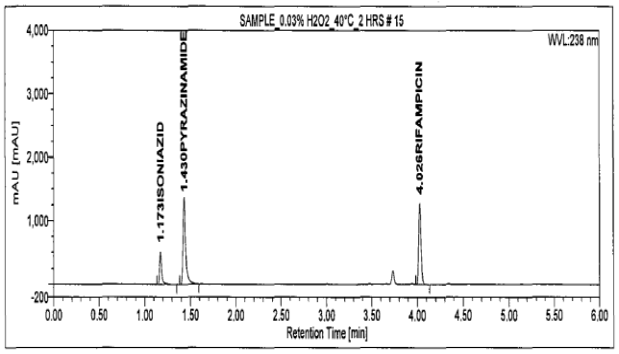

Figure 7: Chrmatograph of Peroxide Degradation

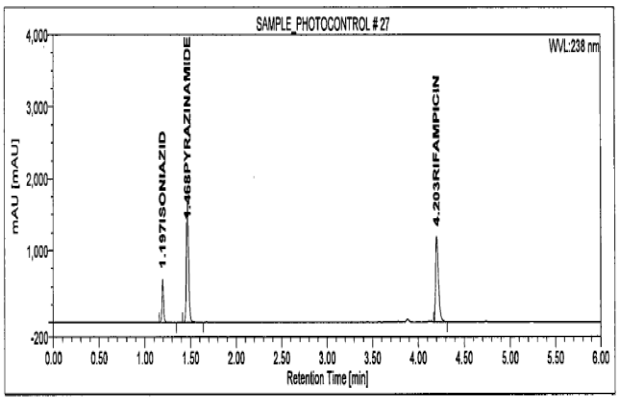

Figure 8: Chrmatograph of Photocontrol Degradation

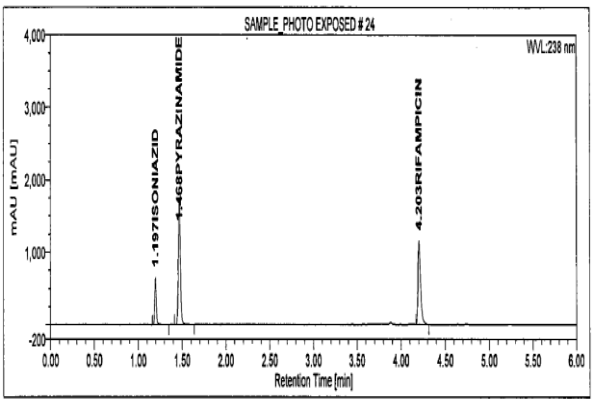

Figure 9: Chrmatograph of Photo Degradation

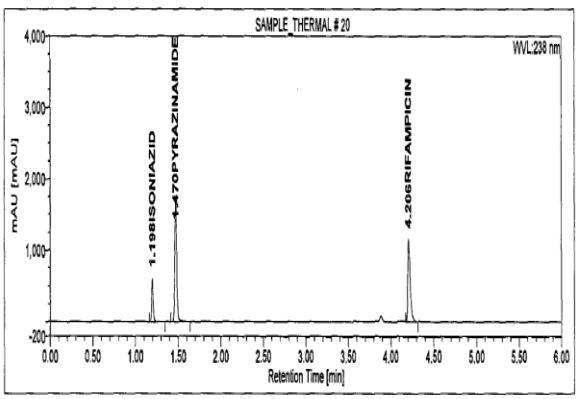

Figure 10: Chrmatograph of Thermal Degradation

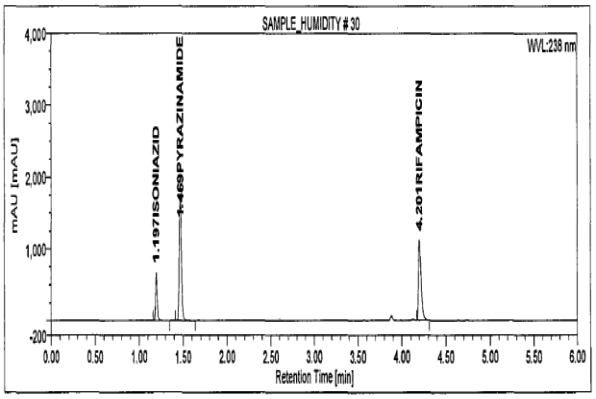

Figure 11: Chrmatograph of Thermal & Humidity Degradation

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