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Stability indicating analytical method development and Validation for Isoniazide, Thiacetazone and Pyridoxine by RP-HPLC UV method

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ABSTRACT

A simple, rapid and precise liquid chromatographic method for simultaneous determination of isoniazid thiacetazone and pyridoxine, in a tablet dosage form has been developed. Chromatographic analysis was performed on a HYPERSIL ODS column (C_{18}) column (150*4.6 mm) of 5µ particle size, applying isocratic elution with a mobile phase. A mixture of 40 volumes of Ammonium Formate Buffer: 60 volumes of Acetonitrile were prepared. The mobile phase was sonicated for 10min to remove gases Wave length: 254nm Injection volume:20µl Column temperature: 25^{o} C. UV detection was performed at 254 nm. The total run time was 8 min. Retention times for isoniazid thiacetazone and pyridoxine are 2.7428, 3.6267, 8.217 mins, respectively. The method was validated with respect to linearity, accuracy, precision, specificity and sensitivity in accordance with ICH guidelines. The percent recovery for Isoniazide is 99.99%, Thiacetazone 99.14% and Pyridoxine 99.4%. The linearity was determined at five levels over the range of 50%-150% of standard concentration in diluents. The concentration range at which linearity was established is 200-600 □g/ml for Isoniazide Thiacetazone 100-300 and Pyridoxine 2-6g/ml. The coefficient of correlation (R^2 = 0.998) for Isoniazide, and Thiacetazone for (R^2 = 0.997) and (R^2= 0.996) for Pyridoxine. The plate count was found to be more than 2000, tailing for the same peak is not more than 2.0 and % RSD is not more than 2.0%. High recovery and low coefficients of variance confirmed the suitability of the method for the simultaneous analysis of the three considered drugs. Isoniazide, Thiacetazone and Pyridoxine was subjected to various stress conditions like hydrolytic degradation, thermal degradation, Peroxide degradation, photolytic degradation. The developed method was found to be stable in all the conditions. The developed method is stability indicating and can be used or routine analysis of Isoniazide, Thiacetazone and Pyridoxine free of interference from their degradation products in suspension formulation.

INTRODUCTION

Isoniazid (Laniazid, Nydrazid), also known as isonicotinylhydrazine (orINH), is an organic compound that is the first-line medication in prevention and treatment of tuberculosis. Isoniazid (INH) is a synthetic, bactericidal anti tubercular agent that is active against many mycobacterium's, primarily those that are actively dividing. Thioacetazone is used in the treatment of tuberculosis [1], it has only weak activity against *Mycobacterium tuberculosis* and is only useful in preventing resistance to more powerful drugs like isoniazid and rifampicin [2]. It is never used on its own to treat tuberculosis. Pyridoxine is one form of vitamin B_6. Its hydrochloride salt pyridoxine hydrochloride is used as vitamin B_6 dietary supplement [3].

EXPERIMENTAL WORK

Chemicals

Water	HPLC Grade
Methanol	HPLC Grade
Potassium Dihydrogen ortho Phosphate	AR Grade
Acetonitrile	HPLC Grade
Di potassium hydrogen ortho phosphate	AR Grade
Ammonium acetate	AR Grade
Ammonium formate	AR Grade

Instrument and chromatographic condition

All solvent used in this work are HPLC & AR grade. Instrument and chromatographic condition RP-HPLC Shimadzu LC20AT infinity series separation model equipped with UV Detector was employed in this method. The Lab solutions software was used for LC peak integration along data acquisition and data processing [4]. The column used for separation of analyte is Hypersil ODS (150×4.6mm) 5µ. Mobile phase consisting of phosphate buffer: Acetonitrile in the ratio of 60:40 % v/v at flow rate 1 ml/min [5]. it was filter through 0.45 µm nylon filter and sonicated for 5 min in ultrasonic bath. Sample was analyzed at 231 nm at an injection volume of 20µl [6].

Preparation of phosphate buffer

1.625 gm of Potassium Di Hydrogen ortho phosphate and 0.3 gms of Di Potassium Hydrogen ortho phosphate was weighed and dissolve in 100 ml of water and volume was made up to 550 ml with water. Adjust the P^H using ortho phosphoric acid. The buffer was filtered through 0.45µ filters to remove all fine particles and gases [7].

PREPARATION OF SOLUTIONS

Preparation of Mixed Standard Solution: (400 µg/ml, 200 µg/ml & 4µg/ml)

Weigh accurately 37.5mg of Thiacetazone and 75mg of Isoniazide and 0.75mg of Pyridoxine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase From above stock solution 200µg/ml of Thiacetazone and 400µg/ml of Isoniazide and 4µg/ml of Pyridoxine is prepared by diluting 5.3ml to 10ml with mobile phase. This solution is used for recording chromatogram [8].

Preparation of Sample Solution

10 tablets (each tablet contains 37.5mg of Thiacetazone and 75mg of Isoniazide and 0.75mg of Pyridoxine) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed [9]. Tablet stock solutions of Thiacetazone (200µg/ml) and Isoniazide (400µg/ml) and Pyridoxine (4µg/ml) were prepared by dissolving weight equivalent to 37.5mg of Thiacetazone and 75mg of Isoniazide and 0.75mg of Pyridoxine and dissolved in sufficient mobile phase [10]. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 200µg/ml of Thiacetazone and 400µg/ml of Isoniazide and 4µg/ml of Pyridoxine was made by adding 5.3ml of stock solution to 10 ml of mobile phase [11-12].

Table 1: Summary of Chromatographic conditions

Column	ODS Hypersil C-18 (150*4.6mm) 5µ
Pump mode	Isocratic
Flow rate	1.0 ml/min
Wave length	254 nm
Injection volume	20µl
Column temperature	25^o C
Mobile phase ratio	Ammonium formate: Acetonitrile (40:60)

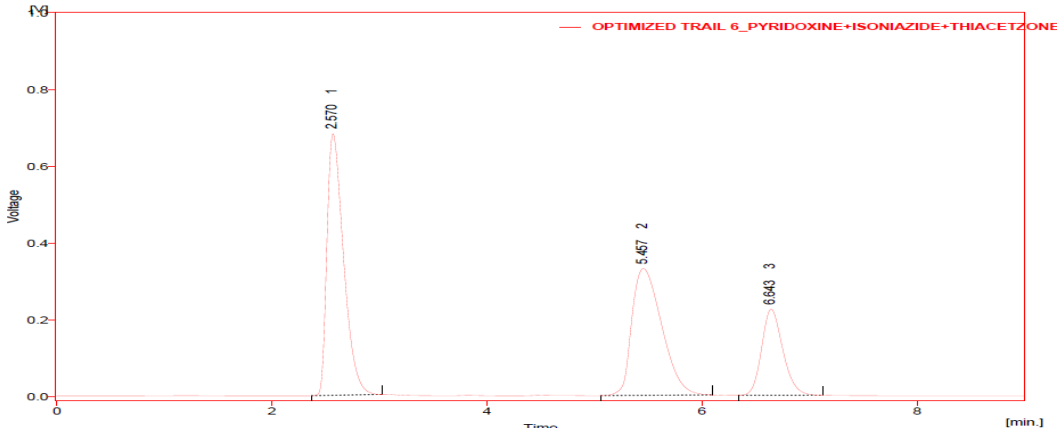

Fig: 3 Typical chromatogram of Thiacetazone, Isoniazide & Pyridoxine

Method validation

The validation of method was carried out as per ICH guideline. The parameters assessed were specificity, linearity, precision, accuracy, stability, LOD and LOQ. Specificity Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences including degradation product and related substances.

Accuracy

The accuracy was determined by calculating % recoveries of Thiacetazone, Isoniazide & Pyridoxine. It was carried out by adding known amount of each analyte corresponding to three conc. Levels (80, 100, 120) of the label claims to the excipients. At each level, six determinations were performed and the accuracy results were expressed as percent analyte recovered by proposed method.

Precision

Method precision

Precision of an analytical method is usually expressed as the standard deviation. Method precision was demonstrated by preparing six samples as per test method representing single batch and were chromatographed. The precision of the method was evaluated by computing the %RSD of the results. The individual values and the low % RSD observed on the values are within acceptance criteria and indicates that method is precise.

Linearity

The purpose of the test for linearity is to demonstrate that the entire analytical system (including detector and data acquisition) exhibits linear responses and directly proportional over the relevant conc. Range for the target conc. of the analyte. The linear regression data for the calibration plot is the indicative of a good linear relationship between peak and concentration over wide range. The correlation coefficient was indicative of high significance.

Robustness

Robustness of method was investigated under a variety of conditions including changes of composition of buffer in the mobile phase, flow rate and temperature. This deliberate change in the method has no effect on the peak tailing, peak area and theoretical plates finally the method was found to be robust.

Ruggedness

The ruggednss of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts. % RSD Assay values between two analysts not greater than 2.0%, hence the method was rugged.

Limit of Detection and Limit of Quantitation:

The LOD can be defined as the smallest level of analyte that gives a measurable responses and LOQ was determined as the lowest amount of the analyte that was reproducibly quantified. These two parameters were calculated using formula based on standard deviation of the response and slope. LOD and LOQ were calculated by equation, LOD=3.3 x σ/s and LOQ= 10 x σ/s, where s = standard deviation, S = slope of calibration curve.

Assay of Thiacetazone, Isoniazide & Pyridoxine in pharmaceutical dosage form

Assay of marketed product was carried out by using the developed method. Sample solutions were prepared and injected into RP –HPLC system. The sample solution was scanned at 254 nm. The percentage drug estimated was found to be 99.91% , 99.84% and 99.39% respectively as Thiacetazone, Isoniazide & Pyridoxine. The chromatogram showed two single peaks of Thiacetazone, Isoniazide & Pyridoxine was observed with retention time 2.570, 5.457 & 6.643 min respectively.

Forced degradation studies

Stress studies are performed according to ICH guidelines under following conditions.

Acid degradation

To 5 ml of sample solution add 1ml of 0.1N HCL and sonicate place it aside for 3hrs, then neutralize the solution with 1 ml of base and then transfer above solution into10 ml volumetric flask dilute with mobile phase and record the chromatogram .

Alkaline degradation

To 5 ml of sample solution add 1ml of 0.1N NaOH and sonicate place it aside for 3hrs, then neutralize the solution with 1 ml of acid and then transfer above solution into 10 ml volumetric flask dilute with mobile phase and record the chromatogram.

Peroxide degradation

To 5 ml of sample solution add 1ml of 3% H2 O 2 and sonicate place it aside for 3hrs, then transfer the above solution into 10 ml volumetric flask dilute with mobile phase and record the chromatogram.

Photolytic degradation

Expose about 100 mg of sample in UV light chamber at 365 nm for 3hrs. Weigh accurately this powder equivalent to 10 mg of Ornidazole and 15 mg of Diloxanidefuroate into a 100ml volumetric flask and make up the volume and sonicate for 30 minutes with intermittent shaking and volume is made up to the mark with mobile phase and record the chromatogram.

Thermal degradation

Expose about 100 mg of sample in to dry heat to 80˚ C for 3hrs. Weigh accurately this powder equivalent to 10 mg of Ornidazole and 15 mg of Diloxanide furoate into a 100ml volumetric flask and make up the volume and sonicate for 30 minutes with intermittent shaking and volume is made up to the mark with mobile phase and record the chromatogram. Record the peak area of stressed samples then compare it with peak area of unstressed sample to determine the % degradation.

$$\% \text{ degradation} = \frac{\text{Response of unstressed sample} - \text{response of stressed sample}}{\text{Response of unstressed sample}} \times 100$$

RESULTS AND DISCUSSION

The HPLC separation and quantification were achieved on thermohypersil BDS (4.6 x 150mm, 5μ). The mobile phase was prepared by mixing ammoniumformate buffer and acetonitrile in the ratio of (40: 60)v/v that run isocratic ally at the flow rate of 1.0ml/min. The wavelength at which detector was set is 254nm. The optimized chromatographic conditions and chromatogram is shown in table 4.1.8 and figure 4.1.9 respectively. Calibration curve at five levels over the range of 50%-150% of standard concentration in a diluents whose concentration was. The calibration curves were obtained by plotting the peak area Vs concentration. The calibration curves are shown in figure 4.1.2.14 & 4.15 and table 4.17.

Accuracy is determined at three different levels 50%, 100%, 150%. The percent recovery for Isoniazide, Thiacetazone and Pyridoxine is 99.84%, 99.91% and 99.39% respectively, which is presented in the table 4.1.29,4.1.30 and 4.1.30. The precision of each method was ascertained separately from the peak area obtained by actual determination of six replicates of a fixed amount of drug i.e. (37.5μg/ml of isoniazid, 75μg/ml of thiacetazone and 0.75μg/ml of pyridoxine). The % RSD were calculated for Isoniazide, Thiacetazone and Pyridoxine and presented in the table 4.1.42.

Robustness of the method was studied by deliberate variations of the analytical parameters such as flow rate (± 0.2ml/min) & wavelength (± 2^{o}C). The

chromatograms and results of robustness is given in figure 4.24 and table 4.29 & 4.30 respectively.

In the forced degradation studies it was found that Isoniazide showed no degradation in acid (0.1M HCl), base (0.1M NaOH), peroxide, heat and UV. In case of Thiacetazone showed showed no degradation in acid (0.1M HCl), base (0.1M NaOH), peroxide, heat and sunlight. Pyridoxine is unstable in acidic degradation and in UV degradation but overall the net degradation was within the limits.

Results of linearity

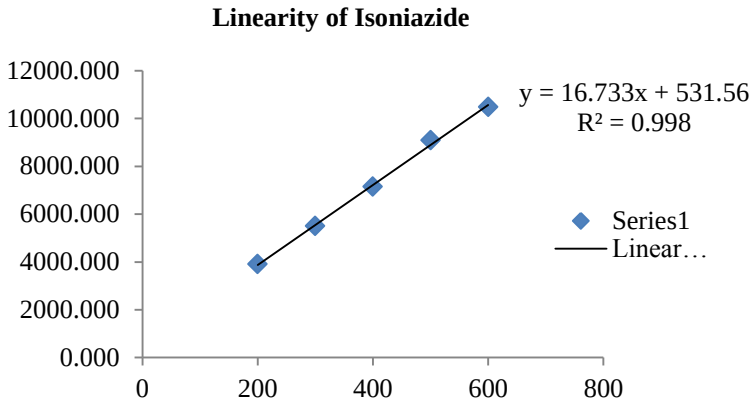

Fig: 4 Linearity curve of Isoniazide

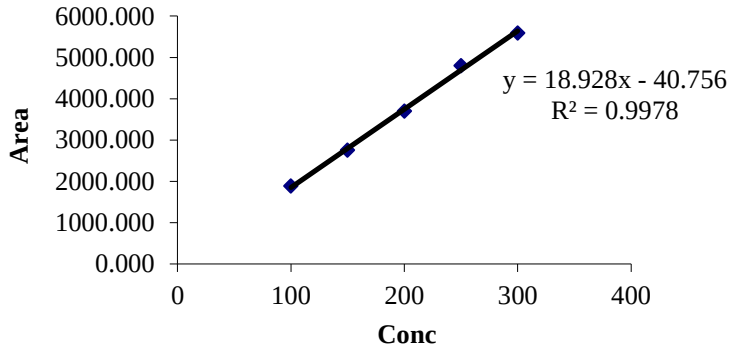

Fig: 5 Linearity curve of Thiacetazone

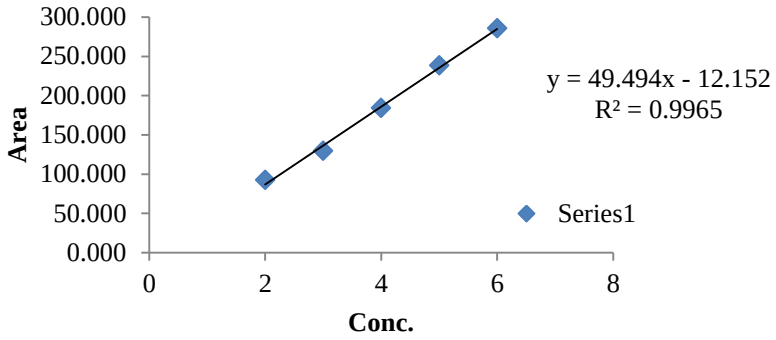

Fig: 6 Linearity curve of Pyridoxine

S.No.	Conc(µg/ml)	Area	S.No	Conc(µg/ml)	Area	S.No	Conc(µg/ml)	Area
1	200	3906.797	1	100	1883.716	1	2	92.174
2	300	5503.068	2	150	2752.347	2	3	129.514
3	400	7144.041	3	200	3695.500	3	4	183.990
4	500	9089.993	4	250	4801.689	4	5	238.072
5	600	10479.812	5	300	5591.017	5	6	285.364

Table: 3 Precision method of proposed RP- HPLC method.

Thiacetazone			**Isoniazide**		
S.No.	**Rt**	**Area**	**S.No.**	**Rt**	**Area**
1	3.650	3715.615	1	2.750	7094.150
2	3.620	3796.979	2	2.747	7278.146
3	3.650	3681.836	3	2.750	7137.760
4	3.587	3832.778	4	2.723	7297.014
5	3.603	3810.629	5	2.737	7240.482
6	3.650	3712.663	6	2.750	7124.908
avg	3.6267	3791.750	**avg**	2.7428	7195.410
stdev	0.0276	66.684	**stdev**	0.0109	86.888
%RSD	**0.76**	**1.76**	**%RSD**	**0.40**	**1.21**

Pyridoxine		
S.No.	**Rt**	**Area**
1	6.010	173.685
2	6.060	175.481
3	6.010	178.947
4	6.030	178.698
5	6.057	171.136
6	6.010	173.232
avg	6.030	175.197
stdev	0.0238	3.132
%RSD	**0.39**	**1.79**

Table: 4 Recovery data for Isoniazide

Recovery level	Accuracy Isoniazide					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
50%	200	3919.240	**3919.240**	**199.98**	**99.99**	
	200	3919.240				
	200	3919.240				
100%	400	7144.041	**7141.947**	**399.75**	**99.94**	
	400	7144.041				99.91%
	400	7137.760				
150%	600	10451.725	**10459.405**	**598.90**	**99.82**	
	600	10457.861				
	600	10468.629				

Table: 5 Recovery data for Pyridoxine

Recovery level	Accuracy Pyridoxine					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
50%	2	97.276	**98.012**	**1.99**	**99.41**	
	2	98.479				
	2	98.281				
100%	4	173.990	**174.336**	**3.99**	**99.66**	
	4	174.509				99.39%
	4	174.509				
150%	6	283.199	**278.872**	**5.95**	**99.12**	
	6	276.093				
	6	277.324				

Table: 6 Recovery data for Thiacetazone

Recovery level	Accuracy Thiacetazone					Average % Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	% Recovery	
50%	100	1902.289	**1914.006**	**99.14**	**99.14**	
	100	1915.232				
	100	1924.498				
100%	200	3695.500	**3666.904**	**198.99**	**99.50**	
	200	3658.506				99.84 %
	200	3646.706				
150%	300	5597.148	**5588.994**	**302.69**	**100.90**	
	300	5577.529				
	300	5592.306				

Table: 7 Robustness data

Parameter	Thiacetazone		Isoniazide		Pyridoxine	
	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor
Flow						
0.8ml/min	4.463	1.863	3.387	1.950	7.503	1.459
1.0 ml/min	3.650	1.775	2.750	1.857	6.010	1.426
1.2ml/min	3.00	1.788	2.777	1.833	5.040	1.341
Wavelength						
252nm	3.620	1.825	2.747	1.914	6.060	1.313
254nm	3.650	1.775	2.750	1.857	6.010	1.426
256nm	3.620	1.805	2.747	1.943	6.060	1.429

Table: 8 Result of Ruggedness

Thiacetazone	%Assay	Isoniazide	%Assay	Pyridoxine	%Assay
Analyst 01	99.89	**Analyst 01**	99.87	**Analyst 01**	99.49
Analyst 02	100.12	**Analyst 02**	98.43	**Analyst 02**	100.45
%RSD	0.16	**%RSD**	1	**%RSD**	0.67

Table: 9 Results of Forced Degradation studies.

Conditions	ISONIAZIDE Sample weight(mg)	Peak Area	% claim	% Degradation
Sample Control	142.897	7094.113	**100.83**	-
Alkali Degradation	143.895	7063.413	98.67	2.16
Acid Degradation	142.36	7019.616	99.12	1.71
Thermal Degradation	144.89	7242.156	100.48	0.35
Per Oxide Degradation	143.89	7101.615	99.21	1.62
UV Degradation	143.56	7077.686	99.10	1.73

Conditions	THIACETAZONE Sample weight(mg)	Peak Area	% claim	% Degradation
Sample Control	142.897	3715.615	**101.20**	-
Alkali Degradation	143.897	3655.350	97.49	3.71%
Acid Degradation	141.235	3633.349	98.73	2.47
Thermal Degradation	144.56	3704.680	98.36	2.84

Per Oxide Degradation	140.23	3632.527	99.42	1.78
UV Degradation	141.31	3645.933	99.02	2.18

Conditions	**PYRIDOXINE Sample weight(mg)**	**Peak Area**	**% claim**	**% Degradation**
Sample Control	142.897	173.685	**100.98**	-
Alkali Degradation	140.23	143.296	95.12	5.86
Acid Degradation	144.36	159.423	92.52	8.46
Thermal Degradation	139.98	151.135	94.26	6.72
Per Oxide Degradation	140.63	172.775	100.87	0.11
UV Degradation	143.25	154.546	92.23	8.75

CONCLUSION

In order to achieve simple, precise, accurate method development simultaneous elution of three components initial trials were performed with an objective to select adequate and optimum chromatographic conditions. Parameters such as ideal mobile phase and their proportions, detection wavelength, optimum pH, different columns and concentration of the standard solutions were carefully studied. Solvent and buffer (ammonium formate-Acetonitrile) were tested by using different proportions, such as ammonium formate - acetonitrile in the ratio of (50:50v/v), (30:70 v/v), (60:40 v/v) . Finally, a mixture of ammonium formate and acetonitrile in the proportion of 40:60 v/v was selected as the optimum mobile phase and a flow rate of 0.1ml/min. The separation was achieved on thermohypersil BDS (4.6 x 150mm, 5μ). Under these conditions, the analyte peaks were well resolved and free from tailing. The tailing factor was < 1.5 for both the analytes. The retention time obtained for Isoniazide was 2.742 min for thiacetazone it was 3.626 and for Pyridoxine it was 8.217. The developed method was validated by evaluating system suitability, recovery, linearity, precision, robustness, specificity. System suitability was evaluated by injecting 10 μl of standard solution six times, the chromatogram were recorded. System suitability parameter like, plate count and tailing factor were also recorded. The plate count was found to be more than 2000, tailing for the same peak is not more than 2.0 and % RSD is not more than 2.0%. The linearity was determined at five levels over the range of 50%-150% of standard concentration in a diluents. The concentration range at which linearity was established is 200-600 μg/ml for Isoniazide Thiacetazone 100-300 and Pyridoxine 2-6μg/ml. The coefficient of correlation (R^2 = 0.998) for Isoniazide, and Thiacetazone for (R^2 = 0.997) and (R^2= 0.996) for Pyridoxine.

Accuracy is determined at three different levels 50%, 100%, 150%. The recovery experiment was carried out by standard addition method. The percent recovery for Isoniazide is 99.99%, Thiacetazone 99.14% and Pyridoxine 99.4%. The precision of each method was ascertained separately from the peak area obtained by actual determination of six replicates of a fixed amount of drug i.e. (37.5 μg/ml of Isoniazide, 75μg/ml Thiacetazone and 0.75μg/ml of Pyridoxine). The RSD of Isoniazide, Thiacetazone and Pyridoxine is found to be 0.40 and 0.76 and 0.29 respectively. Robustness of the method was studied by deliberate variations of the analytical parameters such as flow rate (± 0.2ml/min) & wavelength (± 2nm). it was found that even if the most critical parameters like flow rate and temperature is changed, all system suitability parameters were within the acceptance criteria. Isoniazide, Thiacetazone and Pyridoxine was subjected to various stress conditions like hydrolytic degradation, thermal degradation, Peroxide degradation, photolytic degradation. The developed method was found to be stable in all the conditions. The developed method is stability indicating and can be used or routine analysis of Isoniazide, Thiacetazone and Pyridoxine free of interference from their degradation products in suspension formulation. All these factors make this method suitable for quantification of Isoniazide, Thiacetazone and Pyridoxine in pharmaceutical dosage form. It can therefore be concluded that use of the method can

save much time and money and it can be used in small laboratories with high accuracy and wide linear range. The method can be successfully used for routine analysis of Isoniazide, Thiacetazone and Pyridoxine in marketed formulation without interference.

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