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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ISONIAZID, THIACETAZONE AND PYRIDOXINE HCl IN TABLET DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT

A simple, rapid, sensitive, precise, accurate and economical reverse phase liquid chromatographic method was developed and validated for the simultaneous estimation of Isoniazid, Thiacetazone and Pyridoxine HCl in tablet dosage form. The stationary phase used wasInertsil ODS Zodiac C18 column (250 X 4.6 mm,5µm) and Ammonium Acetate: Acetonitrile (30:70 v/v) mobile phase at a flow rate of 1.0ml/min and 10µl injection volume. UV detector was used for detection at a wavelength 254nm. The retention times of Isoniazid, Thiacetazone and PyridoxineHCl were found to be 2.742, 3.720 and 6.030 ± 0.01 mins respectively.The method was validated according to the ICH guidelines and results for specificity, accuracy (% recovery 98.66-100%), linearity (r^2=0.999), precision, LOD, LOQ, robustness (%RSD>2) and system suitability were found to be within limits. The developed method is simple and economical and can be applied for the routine quality control analysis of Isoniazid, Thiacetazone and Pyridoxine HClcombined dosage forms.

INTRODUCTION:

Analytical method development and validation play an important role in the discovery, development and manufacture of pharmaceuticals and natural medicinal compounds. The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products.

There is a great need for development of new analytical methods for quality evaluation of new emerging drugs. Isoniazid (isonicotinyl hydrazine) (INH)[1,2] ,pyridine-4-carbohydrazide (Fig.1a), is the first-line anti-tubercular drug most widely used for treatment of tuberculosis. It is mainly metabolized in the liver, where acetyl isoniazid (AcINH) is formed by the action of N-acetyltransferase.INH inhibits the synthesis of mycolic acid in the mycobacterial cell wall and is used for the treatment of tuberculosis. Thiacetazone [2], N{4-[(ethanethioamidoimino) methyl]phenyl} acetamide (Fig.2a), a synthetic thiosemicarbazone, inactivates ribonucleotide reductase, used extensively in the treatment of tuberculosisin developing

countriesand has activity against M.Tuberculosis and leprosy.A recent study showed that thiacetazone is active against primary cultures of human prostate cancer cells.Chemical name of pyridoxine hydrochloride (PDX)[4]is 5-hydroxy-6-methyl-3,4-pyridinedimethanol hydrochloride. It is a water soluble vitamin administrated at a dose of 1050 mg/day to the patients accepting isoniazid in order to prevent peripheral neuropathy and CNS effects that are associated with the therapy with isoniazid.

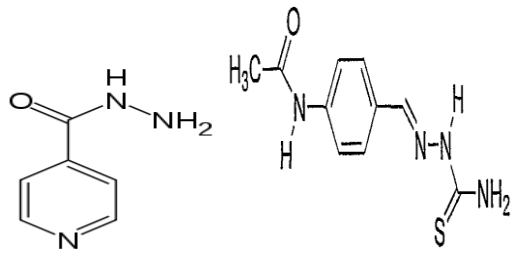


Fig: 1 a) Structure of Isoniazid

Fig: 1 b) Structure of Thiacetazone

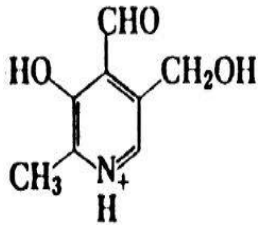


Fig: 1 c) Structure of pyridoxine

A recentliterature survey on the analytical methods of Isoniazid, Thiacetazone and Pyridoxine revealed that a few HPLC [3] methods are available for theirestimation in dosage forms. Some of these methods havecertain drawbacks like complexity in the composition of mobile phase and higher amount of buffer. To the best of our knowledge, none of the analytical method is available for simultaneous determination of combination of these drugs. Hence, we attempted to develop simple,fast, accurate and precise HPLC method for determination of these drugs. Solvents used are easily affordable by the laboratories. The proposed method can be used as alternative methods to thosereported by the earlier workers and provide good choice for the routine determination ofthe choosen drugs in their formulations.

EXPERIMENTAL WORK

Chemicals: The reference samples Isoniazid, Thiacetazone,PyridoxineHCl and HPLC grade water, HPLC grade Acetonitrile, HPLC grade Triethylamine, HPLC grade Ammonium Acetate were obtained from Chandra labs ,Kukatpally, Hyderabad.

Instrumentation

Chromatographic separation was performed by SHIMADZU, LC-20 AT VP, Manual injector with Hamilton Syringe, variable wavelength programmable UV detector and SPDM20A the output signal was monitored and integrated by Software version SPINCHROM CFR. CITIZEN Ultrasonicator was used forsonicating the mobile phase and samples. Standard and sample drugs were weighed by using SHIMADZU AYZ20 analytical balance and pH of the mobile phase was adjusted by using Global digital pH meter.

Preparation of standard stock solution

Accurately weighed75mg of Isoniazid, 37.5mg of Thiacetazoneand 0.75mg of Pyridoxinein 100 ml of volumetric flask and dissolved in 10ml of mobile phase and volume wasmade up with mobile phase. From above stock solution 200µg/ml of Thiacetazoneand 400µg/ml ofIsoniazid and 4µg/ml of Pyridoxine is prepared by diluting 5.3ml to 10ml with mobile phase.

Preparation of sample solution

20tablets (each tablet contains 37.5mg of Thiacetazoneand 75mg of Isoniazid and 0.75mg of Pyridoxine) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of Thiacetazone(200µg/ml) and Isoniazid (400µg/ml) and Pyridoxine (4µg/ml) were prepared by dissolving weight equivalent to 37.5mg ofThiacetazoneand 75mg of Isoniazid and 0.75mg of Pyridoxineand dissolved in sufficient mobile phase. Then solution was filtered using 0.45µfilter and sonicated for 5 min and diluted to 100ml with mobile phase. Further dilutions were prepared in 5 replicates of 200µg/ml ofThiacetazoneand 400µg/ml of Isoniazid and 4µg/ml of Pyridoxinewas made by adding 5.3ml of stock solution to 10 ml of mobile phase.

Preparation of mobile phase

The mobile phase was prepared by mixing Ammonium Acetate: Acetonitrile (30:70). This solution was filtered through 0.45µ filter paper.

Preparation of samples for forced degradation studies

For acidic degradation study

1mL of 0.1N HCl was added to the flask containing tablet stock solutionand this solution was placed in water bath at 60°C for 1 hr. Then the solution was allowed to cool at room temperature and filtered using 0.45µ filter and Sonicated for 5 min and diluted to 100ml with mobile phase. Further dilution was preparedby adding 5.3ml of stock solution to 10 ml of mobile phaseThe acid stress study sample after 1 hour was prepared like zero hour solution. After 1 hr the samples were injected into the HPLC system.

For basic degradation study: 1mL of 0.1N NaOH was added to the flask containing tablet stock solution and this solution was placed in water bath at 60°C for 1 hr. Then the solution was allowed to cool at room temperature and filteredthrough0.45-micron syringe filter and Sonicated for 5 min and diluted to 100ml with mobile phase. Further dilution was preparedby adding 5.3ml of stock solution to 10 ml of mobile phaseand injected in to column.The base stress study sample after 1 hour was prepared like zero hour solution. After 1 hr the samples were injected into the HPLC system.

For oxidation studies: 1mL of 1.0% H_2O_2 was added to the flask containing tablet stock solutionand this solution was placed in water bath at 60°C for 1 hr. Then the solution was allowed to cool at room temperature and filteredthrough0.45-micron syringe filter and Sonicated for 5 min and diluted to 100ml with mobile phase. Further dilutions were preparedby adding 5.3ml of stock solution to 10 ml of mobile phaseAfter 1 hr the samples were injected into the HPLC system.

For Thermal degradation:

The drug substance was taken in petri dish and exposed to a temperature of 105ºC for 1 hr. Then the sample was taken and diluted with the diluent for further analysis. 10µL of the solution was injected and chromatograms were recorded.

For UV degradation study:

The drug substance was taken in petri dish and exposed to UV rays for 1 hrs. Then the sample was taken and diluted with the diluent for further analysis. 10µL of the solution was injected and chromatograms were recorded for the same.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

A gradient, rapid and simple RP-HPLC method was developed and validated for the simultaneous estimation ofsimultaneous estimation of Isoniazid, Thiacetazone and Pyridoxine HCl in tablet dosage form. Mobile phase consisting of Ammonium Acetate: Acetonitrile (30:70) set with gradient programming for 10min. Chromatographic conditions were optimized for mobile phase using Zodiac, C_{18} (250 × 4.6 mm, 5 µm) column at a flow rate of 1 ml/ min. Effluents were detected at 254nm in UV detector. Column compartment temperature was maintained at 25°C.The optimized chromatogram was show in **fig: 1.**

Method validation

The experimental method was validated according to the recommendations of ICH-guidelines for the parameters like specificity, system suitability, accuracy, linearity, precision, robustness, LOD & LOQ and also forced degradation studies for stability testing.

Specificity

The specificity of the method was evaluated to ensure that there was no interference of excipients in the chromatogram ofIsoniazid, Thiacetazone and Pyridoxine HCl. The specificity was studied by injecting the placebo, diluting(blank) solution and standard solution of Isoniazid, Thiacetazone and Pyridoxine HCl. Spectral purities of Isoniazid, Thiacetazone and Pyridoxine HClchromatographic peaks were evaluated for the interference of excipients and the results were shown in the **Fig: 5, 6& 7.**

Linearity

The linearity of the chromatographic method was established by plotting a graph to concentration vs. peak area ofIsoniazid, Thiacetazone and Pyridoxine HCl and determining the correlation coefficients (R^2) of the three compounds. Linearity of Isoniazid, Thiacetazone and Pyridoxine HClstandard solution at concentration levels of 10%, 25%, 50%, 100%, 150%, and 200% were injected into the HPLC system.The calibration curves were linear ($r^2 \geq 0.99$) over a concentration range from 200µg/ml to 600µg/ml for Isoniazid, 100µg/ml to 300µg/ml for Thiacetazone, 2µg/ml to 6µg/ml for Pyridoxine were shown in **Table.No:1.**

Accuracy

The accuracy of the method was established by recovery studies. The known amount of standard was added at three different levels to pre-analyzed sample. Each determination was performed in triplicate at three different concentration levels 50%, 100% and 150%, taking in to percentage purity of added drug sample. The amount ofIsoniazid, Thiacetazone and Pyridoxine HCl was estimated by applying obtained values to the respective regression lines equation. Each concentration was analyzed 3 times and avg. recovery was measured. The results were shown in **Table.No:2.**

Precision: Precision of proposed method was evaluated by performing repeatability on same day and intermediate precision of two different days.Prepared sample preparations of Thiacetazone, Isoniazide and Pyridoxine as per test method and injected 5 times in to the column for repeatability. Prepared sample preparations of Thiacetazone, Isoniazide and Pyridoxine as per test method and injected 5 times in to the column on different days with in same laboratory conditions for Intermediate Precision.The results were expressed as % RSD and are less than 2, shown in **Table.No:3.**

Limit of detection (LOD) and Limit of quantification (LOQ)

Limit of detection and Limit of quantification were calculated from linearity plot. The LOD and LOQ of the proposed methods were calculated from the standard deviation (σ) of the response and the slope of the calibration curve (S) in accordance to the equations. The results were shown in**Table.No:4.**

LOD = 3.3 x σ/S and LOQ = 10 x σ/S.

Robustness

The robustness is the ability of method to remain unaffected by small changes in parameters. The robustness of the method was by purposely altering experimental conditions and % assay ofThiacetazone, Isoniazide and Pyridoxine HCl, peak tailing, theoretical plates, % RSD were calculated. To study the effect of flow rate, it was changed to 0.2 units from 1.0 ml/min i.e, 0.8 ml/min and 1.2ml/min and change in wavelength. The results showed that the developed method was robust and results were shown in**Table.No:5.**

Ruggedness

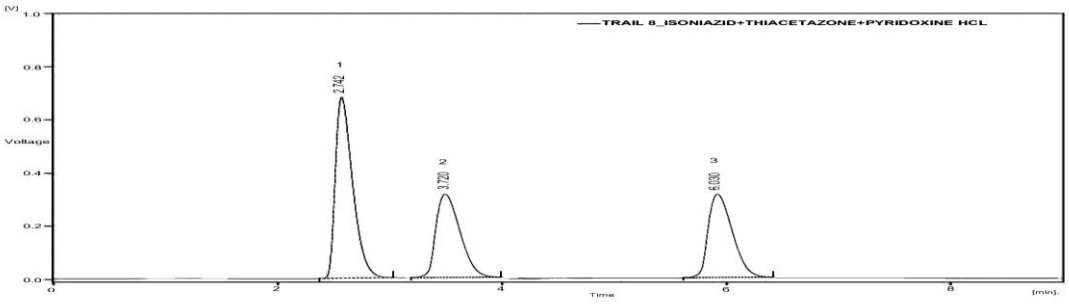
The ruggedness of the method was studied by determining the analyst to analyst variation by performing the Assay by two different analysts. The results were shown in **Table.No:6**

System suitability

System suitability was performed by injecting six replicates of standards solution of BRZ (0.1mg/ml) and TM (0.05mg/ml) prepared by using stock solution. This method was evaluated by analyzing the repeatability of retention time, tailing factor, theoretical plates of the column. The results were shown in **Table.No:7**

Forced degradation of Isoniazid, Thiacetazone and Pyridoxine

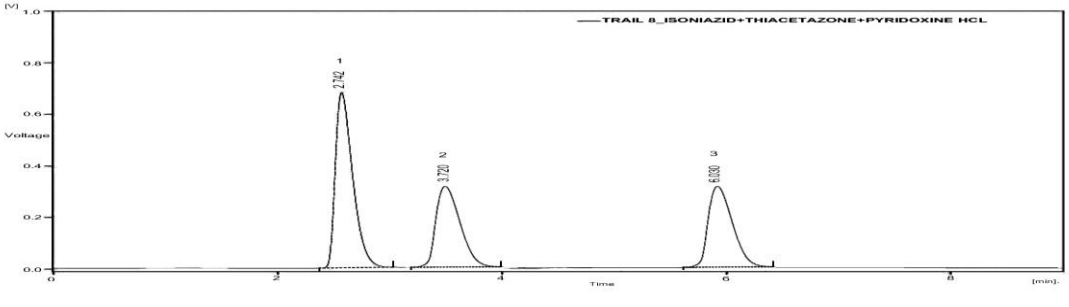
To determine the proposed method as a stability-indicating method, Isoniazid, Thiacetazone and Pyridoxinewere stressed under different conditions in forced degradation studies. Stock solutions of Isoniazid, Thiacetazone and Pyridoxineused to forced degradation studies were prepared by dissolving in methanol. The results were shown in**Table.No:8.**



Result Table (uncal – TRAIL 8_ISONIAZID+THIACETAZONE+PYRIDOXINE HCL)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.742	10468.629	867.406	43.61	55.1	0.17
2	3.720	4592.306	680.606	38.07	22.2	0.21
3	6.030	4762.244	689.943	18.32	22.7	0.31
	Total	18128.179	2177.955	100.00	100.00	

Fig.No: 1 Optimized Chromatogram of Isoniazid, Thiacetazone and Pyridoxine HCl



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	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
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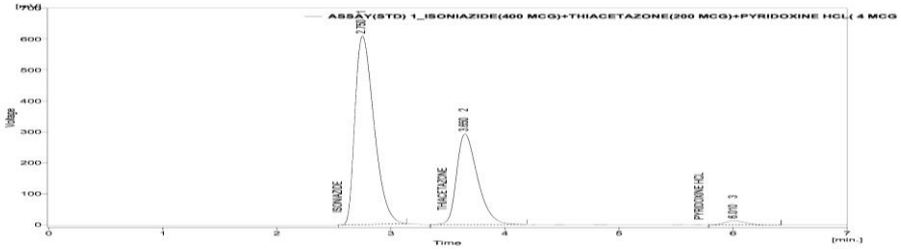
3	6.030	4762.244	689.943	18.32	22.7	0.31
	Total	18128.179	2177.955	100.00	100.00	

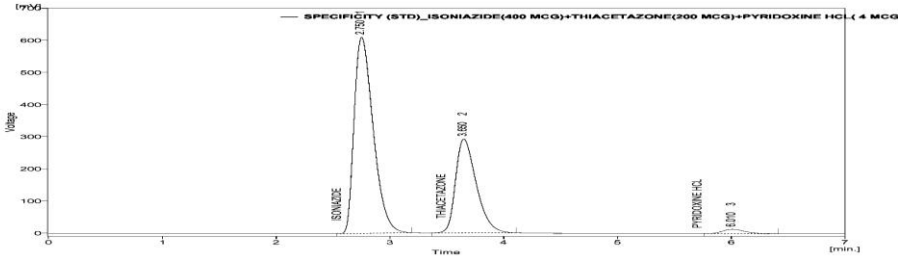
Fig.No: 2. Sample chromatogramof Isoniazid, Thiacetazone and Pyridoxine HCl



Result Table (Uncal - ASSAY(STD) 1_ISONIAZIDE(400 MCG)+THIACETAZONE(200 MCG)+PYRIDOXINE HCL(4 MCG))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.750	7094.113	610.186	64.59	66.6	0.18
2	3.650	3715.515	292.983	33.83	32.0	0.20
3	6.010	173.685	13.262	1.58	1.4	0.21
	Total	10983.414	916.430	100.00	100.0	

Fig.No: 3 Standard chromatogramof Isoniazid, Thiacetazone and Pyridoxine HCl



Result Table (Uncal - SPECIFICITY (STD)_ISONIAZIDE(400 MCG)+THIACETAZONE(200 MCG)+PYRIDOXINE HCL(4 MCG))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.750	7131.877	610.875	64.82	66.6	0.18
2	3.650	3696.958	292.682	33.60	31.9	0.20
3	6.010	174.492	13.289	1.59	1.4	0.21
	Total	11003.327	916.845	100.00	100.0	

FigNo: 4. Specificity standard sample

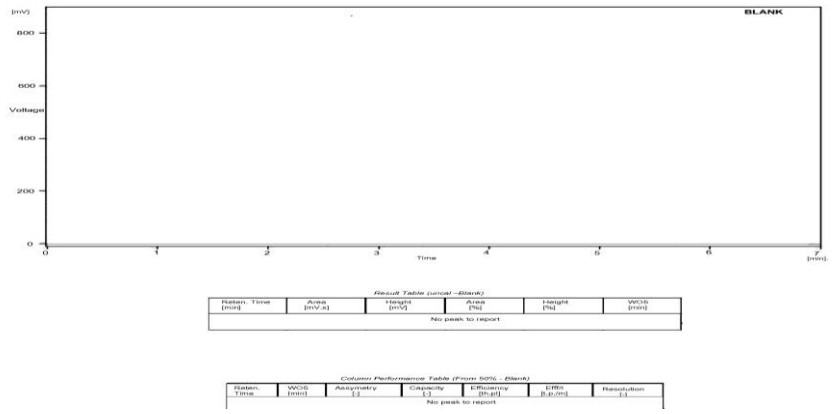


Fig.No:5 Specificity Blank

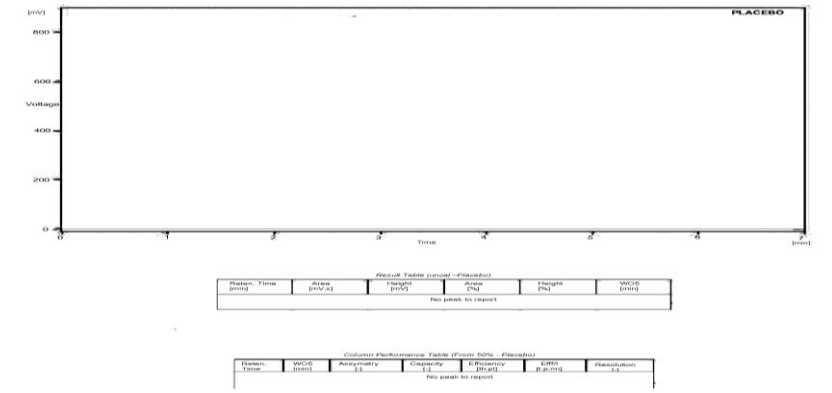
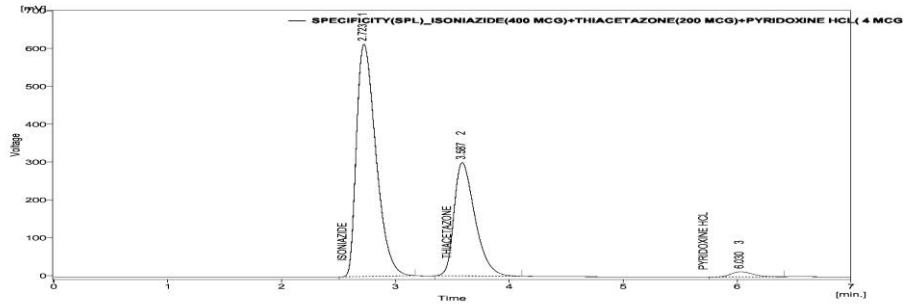


Fig.No:6 Specificity Placebo sample



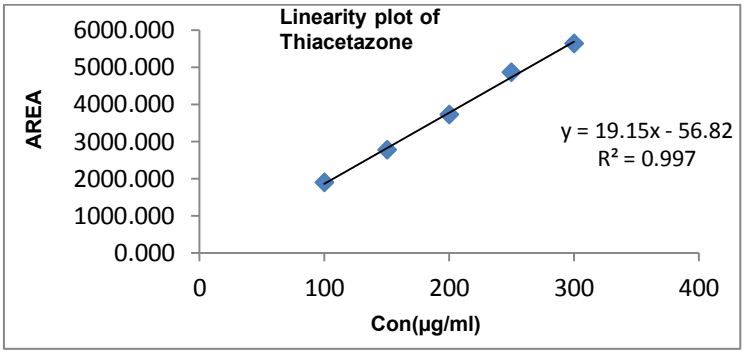
Result Table (Uncal - SPECIFICITY(SPL)_ISONIAZIDE(400 MCG)+THIACETAZONE(200 MCG)+PYRIDOXINE HCL(4 MCG))

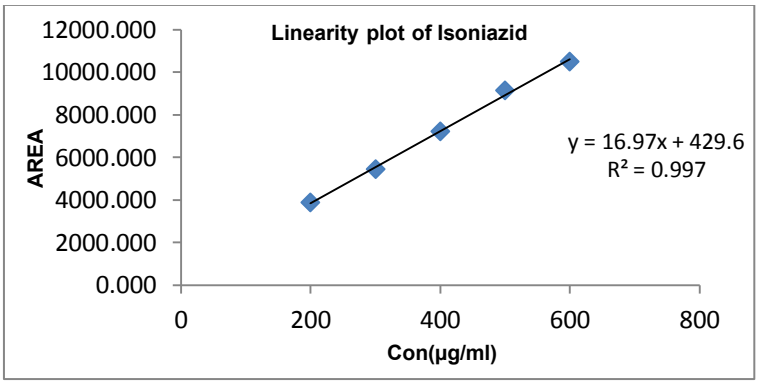
	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.723	7284.448	613.873	64.61	66.2	0.19
2	3.587	3810.855	299.327	33.80	32.3	0.20
3	6.030	178.698	13.449	1.59	1.5	0.21
	Total	11274.001	926.648	100.00	100.0	

Fig.No:7 Specificity Sample chromatogram

Table.No:1 Results of Linearity of Thiacetazone, Isoniazid and Pyridoxine

% of Injected solution	Thiacetazone		Isoniazid		Pyridoxine	
	Con. (µg/ml)	Average Peak area	Con. (µg/ml)	Average Peak area	Con. (µg/ml)	Average Peak area
50	100	1888.774	200	3861.797	2	95.156
75	150	2772.315	300	5418.568	3	134.434
100	200	3715.489	400	7193.541	4	186.885
125	250	4862.619	500	9141.993	5	236.854
150	300	5632.602	600	10489.31	6	281.75
Slope	19.15		16.97		47.56	
Coefficient of regression(r^2)	0.997		0.997		0.998	





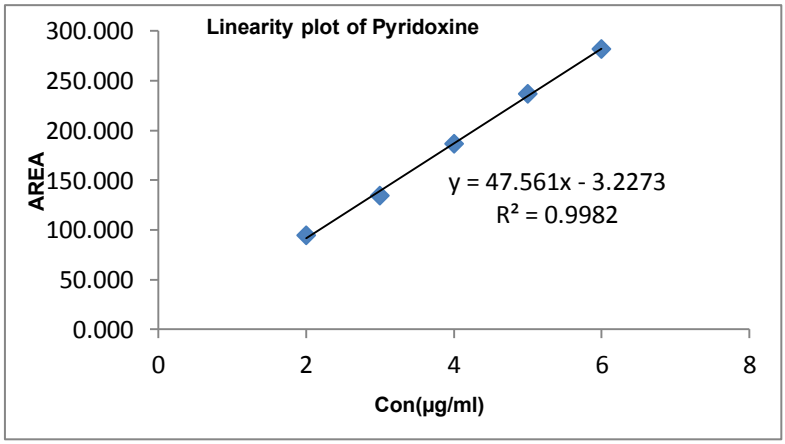


Table.No:2 Recovery results of Isoniazid, Thiacetazone and Pyridoxine HCl

Recovery level	Accuracy of Isoniazid					Average % Recovery
	Amount taken(µg/ml)	Area	Average area	Amount recovered(µg/ml)	%Recovery	
50%	200	39025.24	19040.006	197.27	98.64	98.71%
	200	38216.24				
	200	38733.28				
100%	400	71015.041	36333.575	395.37	98.84	
	400	69842.041				
	400	71053.760				
150%	600	104115.726	54720.328	592.03	98.67	
	600	103073.86				
	600	102986.629				

Recovery level	Accuracy of Thiacetazone					Average % Recovery
	Amount taken(µg/ml)	Area	Average area	Amount recovered(µg/ml)	%Recovery	
50%	100	1902.289	1904.006	98.62	98.62	98.66%
	100	1895.232				
	100	1914.498				
100%	200	3695.512	3633.575	197.18	98.59	
	200	3658.506				
	200	3542.706				
150%	300	5489.148	5471.328	296.35	98.78	
	300	5503.529				
	300	5423.306				

Recovery level	Accuracy of Pyridoxine					Average % Recovery
	Amount taken(µg/ml)	Area	Average area	Amount recovered (µg/ml)	%Recovery	
50%	2	95.958	97.206	1.97	98.60	98.63
	2	97.479				
	2	98.181				
100%	4	171.99	172.603	3.95	98.67	
	4	173.509				
	4	172.309				
150%	6	283.199	277.539	5.92	98.64	
	6	276.093				
	6	273.324				

Table.No:3 Results of Precision for Isoniazid, Thiacetazone and Pyridoxine

Drug	Repeatability		Intermediate Precision			
	Mean±SD	% RSD	Day-1		Day-2	
			Mean±SD	% RSD	Mean±SD	% RSD
Isoniazid	7195.410 ± 86.888	1.21	7222.353 ± 81.438	1.13	7318.444 ± 77.640	1.06
Thiacetazone	3791.750 ± 66.684	1.76	3831.676 ± 51.306	1.63	38416.176 ± 594.564	1.34
Pyridoxine	174.530 ± 2.544	1.46	174.682 ± 2.335	1.34	176.163 ± 2.138	1.21

Table.No:4**:** Results of LOD and LOQ of Thiacetazone, Isoniazide and Pyridoxine.

% of Injected solution	Thiacetazone		Isoniazid		Pyridoxine	
	Con. ((µg/ml))	Average Peak area	Con. ((µg/ml))	Average Peak area	Con. ((µg/ml))	Average Peak area
50	100	1888.774	200	3861.797	2	95.156
75	150	2772.315	300	5418.568	3	134.434
100	200	3715.489	400	7193.541	4	186.885
125	250	4862.619	500	9141.993	5	236.854
150	300	5632.602	600	10489.31	6	281.75
Standard deviation of intercept	22.72		144.10		8.09	
Slope	19.15		16.97		47.56	
Limit of detection	3.91		28.02		0.56	
Limit of quantification	11.86		84.91		1.07	

Table.No:5**:** Resultsof Robustness

Parameter	**Thiacetazone**		**Isoniazid**		**Pyridoxine**	
	Retention time(min)	**Tailing factor**	**Retention time(min)**	**Tailing factor**	**Retention time(min)**	**Tailing factor**
Flow rate						
0.8ml/min	3.872	1.775	2.983	1.886	6.095	1.426
1.0 ml/min	3.656	1.825	2.768	1.943	6.065	1.426
1.2ml/min	3.523	1.805	2.613	1.934	6.010	1.384
Wavelength						
252nm	3.673	1.793	2.765	1.898	6.030	1.532
254nm	3.654	1.780	2.763	1.780	6.020	1.447
256nm	3.687	1.805	2.754	1.971	6.040	1.580

Table.No:6 Results of Ruggedness

Thiacetazone	**%Assay**	**Isoniazid**	**%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.No:7 (a) Results of system suitability parameters of Isoniazid

S.No.	**Isoniazid**			
	Retention time	**Peak area**	**Tailing factor**	**Theoretical plates**
1	3.650	7144.041	1.857	2254
2	3.620	7101.615	1.859	2254
3	3.650	7094.113	1.914	2158
4	3.587	7269.612	1.875	2345
5	3.603	7240.482	1.889	2139
6	3.65	7262.734	1.919	2124
Avg	3.6267	7185.433		
Stdev	0.0276	81.449		
%RSD	**0.76**	**1.13**		

(b) Results of system suitability parameters Thiacetazone

S.No.	Thiacetazone			
	Retention time	Peak area	Tailing factor	Theoretical plates
1	3.650	3658.506	1.750	2974
2	3.620	3682.527	1.748	2974
3	3.650	3765.615	1.825	2877
4	3.587	3815.211	1.775	2561
5	3.603	3810.629	1.780	2798
6	3.65	3754.698	1.828	2685
Avg	3.6267	3747.864		
Stdev	0.0276	64.947		
%RSD	**0.76**	**1.73**		

(c) Results of system suitability parameters Pyridoxine

S.No.	Pyridoxine			
	Retention time	Peak area	Tailing factor	Theoretical plates
1	3.650	176.509	1.458	4685
2	3.620	172.775	1.423	4685
3	3.650	173.685	1.313	4613
4	3.587	174.747	1.426	4892
5	3.603	171.136	1.447	4608
6	3.65	176.431	1.319	4584
Avg	3.6267	174.214		
Stdev	0.0276	2.111		
%RSD	**0.76**	**1.21**		

Table.No:8Results of Force Degradation studies of Pyridoxine

Conditions	Sample weight(mg)	Peak Area	% Assay	% Degradation
Sample Control	142.897	1734.685	100.98	-
Alkali Degradation	140.23	1432.296	95.12	5.86
Acid Degradation	144.36	1591.423	92.52	8.46
Thermal Degradation	139.98	1516.135	94.26	6.72
Per Oxide Degradation	140.63	1723.775	100.87	0.11
UV Degradation	143.25	1541.546	92.23	8.75

CONCLUSION

The validation study showed that the developed method was accurate, rapid, precise, reproducible, economical and convenient with acceptable correlation co-efficient and standard deviations which make the proposed RP-HPLC method valuable for simultaneous Isoniazid, Thiacetazone and Pyridoxine HCl in tablet dosage form. So the developed method can be used conveniently for analysis of quality control, stability and further studies.

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