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## Development and Validation of RP-HPLC Method for Simultaneous Estimation of Isoniazid & Pyridoxine in Bulk and It Pharmaceutical Formulations

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### ABSTRACT

A new, simple, rapid, selective, precise and accurate isocratic reverse phase high performance liquid Chromatography assay method has been developed for simultaneous estimation of Isoniazid and Pyridoxine tablet formulations. The separation was achieved by using column Hypersil BDS, (250 x 4.6 mm, 5  $\mu$ ) (Make: Thermo), in mobile phase consisted of pH4.0 phosphate buffer and Acetonitrile in the ratio of 75:25 v/v. The flow rate was 1.0 mL/min, column oven temperature 30° C, the injection volume was 10  $\mu$ L, and detection was performed at 267 nm using a photodiode array detector (PDA), Run time 6 minutes. The retention time of Isoniazid and Pyridoxine, was noted to be 3.5 minutes and 4.3 minutes respectively, indicative of rather shorter analysis time. The method was validated as per ICH guidelines. The proposed method was found to be accurate, reproducible, and consistent.

**Keywords:** Liquid Chromatography; Isoniazid, Pyridoxine, combined dosage forms; Simultaneous estimation, Validation

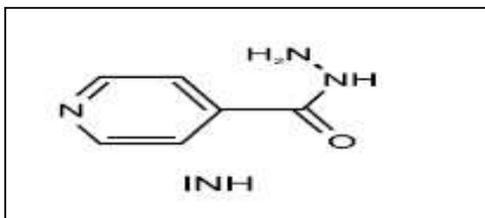
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## INTRODUCTION

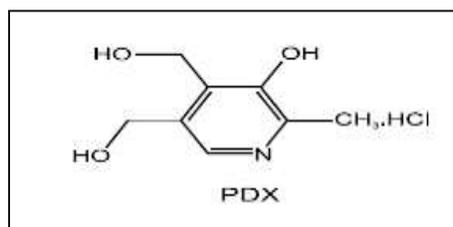
Isoniazid, also known as isonicotinyldiazide (INH), is an antibiotic used for the treatment of tuberculosis.[1] For active tuberculosis it is often used together with rifampicin, pyrazinamide, and either streptomycin or ethambutol.[2] For latent tuberculosis it is often used by itself.



**Figure 1: Structure of Isoniazid**

Pyridoxine, also known as vitamin B<sub>6</sub> or pyridoxol [4], is a form of vitamin B<sub>6</sub> found commonly in food and used as dietary supplement [3]. As a supplement it is used to treat and prevent pyridoxine deficiency, sideroblastic anaemia, pyridoxine-dependent epilepsy, certain metabolic disorders, problems from isoniazid, and certain types of mushroom poisoning [5]. It is used by mouth or by injection.

It is usually well tolerated occasionally side effects include headache, numbness, and sleepiness. Normal doses are safe during pregnancy and breastfeeding. Pyridoxine is in the vitamin B family of vitamins. It is required by the body to make amino acids, carbohydrates, and lipids. Sources in the diet include fruit, vegetables, and grain [6].



**Figure 2: Structure of Pyridoxine**

Literature survey reveals that few analytical methods have been reported for the estimation of Isoniazid and pyridoxine in pharmaceutical dosage form including UV-Vis spectroscopy [7-9], HPTLC [10-12] and high performance liquid chromatography HPLC [13-17]. Although reports are available on stability indicating HPLC methods, the information provided is incomplete as well as results are contrast. Hence we tried to develop stability indicating HPLC method for Isoniazid and pyridoxine. The present work describes a simple, stability indicating HPLC method for the determination of Isoniazid and pyridoxine in bulk and tablet dosage form according to ICH guidelines [18-19].

## MATERIALS AND METHOD

### Chemicals and Reagents

Milli-Q Water, Acetonitrile (HPLC Grade) and Potassium dihydrogen phosphate monohydrate (AR Grade) Triethyl amine, Hydrochloric acid and, sodium hydroxide (AR Grade), Hydrogen peroxide (AR Grade), orthophosphoric acid (GR Grade), were obtained from Qualigens Ltd., Mumbai. All other chemical of analytical grade were procured from local sources unless specified. All dilutions were performed in standard class-A, volumetric glassware.

### Instrumentation and Chromatographic Conditions

#### Instrumentation

Waters 2489 U.V-Visible detector/2695 Separation Module, equipped with Empower 2 software, Bandelin ultrasonic bath, pH Meter (Thermo Orion Model), Analytical Balance (Mettler Toledo Model) were use in the present assay.

#### Mobile phase preparation

Mixed 750ml of pH 4.0 phosphate buffer and 250 ml of Acetonitrile solvent was degassed in ultrasonic water bath for 5 minutes. Filter through 0.45  $\mu$  filter under vacuum filtration.

#### Standard preparation:

Accurately 60mg of Isoniazid is weighed and transferred into 100ml volumetric flask, about 7ml of diluent was added and sonicated for 5 minutes to dissolve it. Accurately 20 mg of Pyridoxine is weighed and transferred to a volumetric flask of 10 ml capacity. Drug is dissolved in little amount of water and volume is made up to the mark with water. 1ml of Pyridoxine solution is pipetted out and transferred to Isoniazid solution. Now, the volume is made up to the mark with water. The solution was filtered through 0.45 $\mu$ m membrane filter.

#### Sample preparation:

Weigh accurately 428 mg of sample and dissolve in a little amount of water and sonicated for 5 minutes. After the sample complete dissolution made up the final volume with water up to 50 ml. The concentration of solution is 6000  $\mu$ g/ml of Isoniazid and 200  $\mu$ g/ml of Pyridoxine. Pipette out 5 ml of this solution transfer in to 50 ml volumetric flask and make up the volume to 50 ml with water. The solution was filtered through 0.45 $\mu$ m membrane filter. The concentration of solution is 600  $\mu$ g/ml of Isoniazid and 20  $\mu$ g/ml of Pyridoxine.

#### Chromatographic conditions

Hypersil BDS C18 (250 x 4.6 mm, 5 $\mu$ ) Column was used for analysis at 30°C column temperature. The mobile phase was pumped through the column at a flow rate of 1.0mL/min. The sample injection

volume was 10  $\mu$ L. The photodiode array detector was set to a wavelength of 267nm for the detection and Chromatographic runtime was 6 minutes.

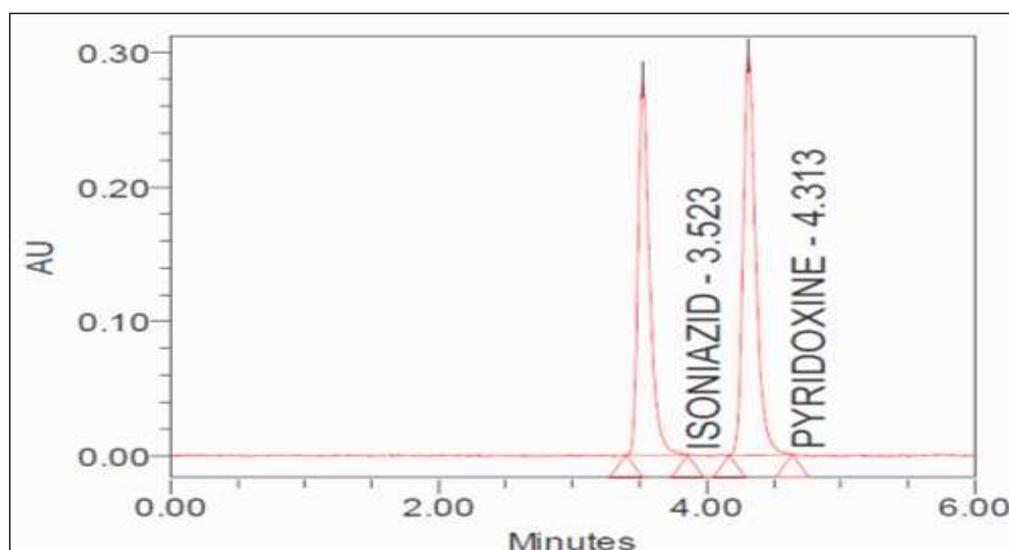
## RESULTS AND DISCUSSION

### Method development

To develop a suitable and robust LC method for the determination of Isoniazid and pyridoxine, different mobile phases were employed to achieve the best separation and resolution. The method development was started with Hypersil BDS C-8, (150  $\times$  4.6 mm, 5  $\mu$ ) with the following mobile phase. Phosphate buffer (adjusted to p<sup>H</sup>: 4.0 with OPA): Acetonitrile (50: 50). Detector wavelength 267 nm, column temperature 25° C, Injection volume 10  $\mu$ L and Flow rate 1.5 ml/min used. Peak shape was not good, Due to asymmetry in peak and lesser retention time and no elution of second peak. So, another trial was made with change in flow rate.

For next trial flow rate was changed to 1.0 ml/min from 1.5 ml/min remaining chromatographic conditions are same. Peak shape was not good, Due to tailing in peaks and asymmetry, as drugs are highly polar column was changed to C18 and another trial was made with change in mobile phase ratio.

For next trial column was changed to Hypersil BDS C18 (250X4.6mm, 5 $\mu$ m) from Hypersil BDS C-8, (150  $\times$  4.6 mm, 5  $\mu$ ) remaining chromatographic conditions are same. Peak shape was satisfactory in both standard and sample preparations. Retention time of Isoniazid and pyridoxine, were found to be 3.5 and 4.3 min acceptable. The chromatogram of Isoniazid and pyridoxine standard using the proposed method is shown in **(Fig-3.)** System suitability results of the method are presented in **Table-1.**



**Figure 3: A typical HPLC Chromatogram showing the peak of Isoniazid and pyridoxine**

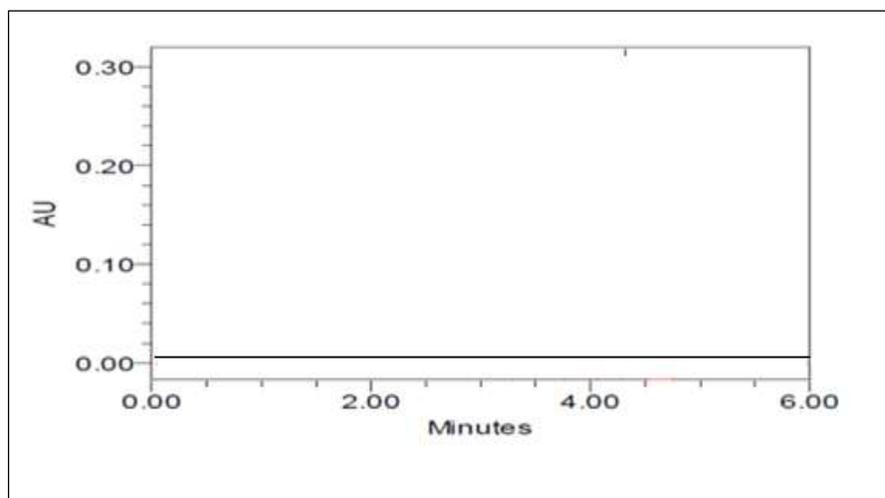
## Method validation

The developed RP-LC method extensively validated for assay of Isoniazid and pyridoxine using the following Parameters.

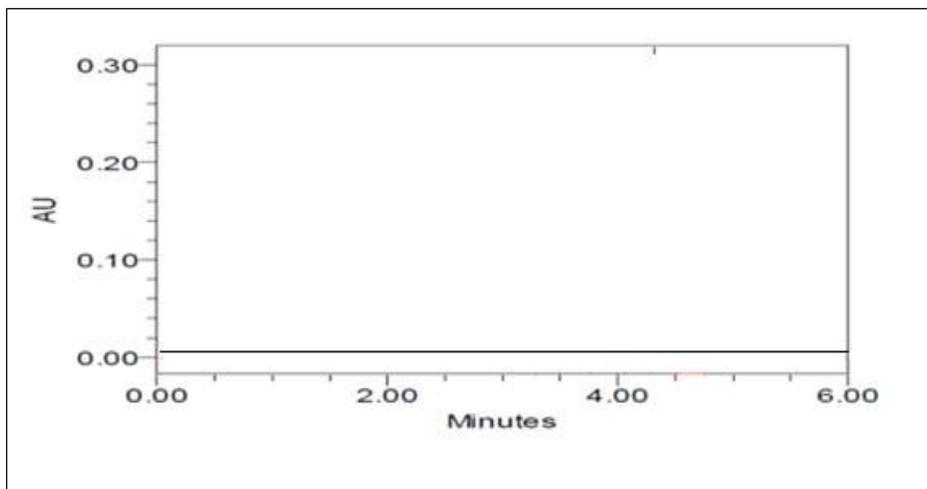
### Specificity

#### Blank and Placebo interference

A study to establish the interference of blank and placebo were conducted. Diluent and placebo was injected into the chromatograph in the defined above chromatographic conditions and the blank and placebo chromatograms were recorded. Chromatogram of Blank solution (**Fig. no.-3**) showed no peaks at the retention time of Isoniazid and pyridoxine peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of Isoniazid and pyridoxine in tablets. Similarly Chromatogram of Placebo solution (**Fig. no.-4**) showed no peaks at the retention time of Isoniazid and pyridoxine peak. This indicates that the Placebo used in sample preparation do not interfere in estimation of Isoniazid and pyridoxine in Isoniazid and pyridoxine tablets. The chromatogram of Isoniazid and pyridoxine Blank using the proposed method is shown in **Fig- 4**. The chromatogram of Isoniazid and pyridoxine Placebo using the proposed method is shown in **Fig-5**.



**Figure 4: HPLC Chromatogram showing the no interference of Blank for Isoniazid and pyridoxine**



**Figure 5: HPLC Chromatogram showing the no interference of placebo for Isoniazid and pyridoxine**

**Table 1: System suitability parameters for Isoniazid and pyridoxine by proposed method**

Parameters	Isoniazid	pyridoxine
Resolution	4.76	
Retention time (min)	3.523	4.313
No. of Theoretical plates	8483	10752
Tailing factor	1.46	1.32

### Precision

The method precision study for six sample preparations in marketed samples showed a RSD of 0.19% for Isoniazid. Similarly the method precision study for six sample preparations in marketed samples showed a RSD of 0.13% for pyridoxine.

**Table 2: Method Precision studies for Isoniazid and pyridoxine by proposed method**

S.No	Sample weight (mg)	Isoniazid sample areas	PyridoxineSample areas	%Assay of Isoniazid	%Assay of Pyridoxine
1	428.00	1695114	1937856	100.06	100.03
2	428.00	1696810	1937691	100.16	100.03
3	428.00	1698009	1933196	100.23	99.79
4	428.00	1690639	1932382	99.79	99.83
5	428.00	1693871	1936346	99.98	99.96
6	428.00	1690160	1939318	99.73	100.11
Avg. assay				99.99	99.95
%RSD				0.19	0.13

### Accuracy

A series of solutions were prepared by spiking the placebo and API in the range of about 50% to 150% of test concentration in triplicate and injected into HPLC system and analyzed as per the test method. The percentage recoveries with found in the range of 98.64 to 99.04 for Isoniazid and The

percentage recoveries with found in the range of 99.46 to 99.72 for pyridoxine. From the data obtained which given in **Table-:3** and **Table-:4** the method was found to be accurate.

**Table 3: Recovery studies for Isoniazid by proposed method**

Spiked level	Amount added (ppm)	Amount found (ppm)	%Recovery	%Mean recovery
50%	297.000	296.33	98.77	98.64
50%	297.000	296.61	98.87	
50%	297.000	296.59	98.86	
50%	297.000	293.36	97.78	
50%	297.000	296.34	98.78	
50%	297.000	296.50	98.83	
100%	594.000	594.88	99.14	99.04
100%	594.000	594.93	99.15	
100%	594.000	593.00	98.83	
150%	891.000	888.74	98.74	98.77
150%	891.000	888.02	98.66	
150%	891.000	888.38	98.70	
150%	891.000	890.60	98.95	
150%	891.000	891.29	99.03	
150%	891.000	887.29	98.58	

**Table 4: Recovery studies for pyridoxine by proposed method**

Spiked level	Amount added (ppm)	Amount found (ppm)	%Recovery	%Mean recovery
50%	9.900	9.97	99.70	99.46
50%	9.900	9.98	99.08	
50%	9.900	9.89	98.90	
50%	9.900	9.89	98.90	
50%	9.900	9.97	99.70	
50%	9.900	9.98	99.80	
100%	19.800	19.92	99.60	99.71
100%	19.800	19.93	99.65	
100%	19.800	19.98	99.90	
150%	29.700	29.97	99.90	99.72
150%	29.700	29.94	99.80	
150%	29.700	29.89	99.63	
150%	29.700	29.94	99.80	
150%	29.700	29.90	99.60	
150%	29.700	29.90	99.60	

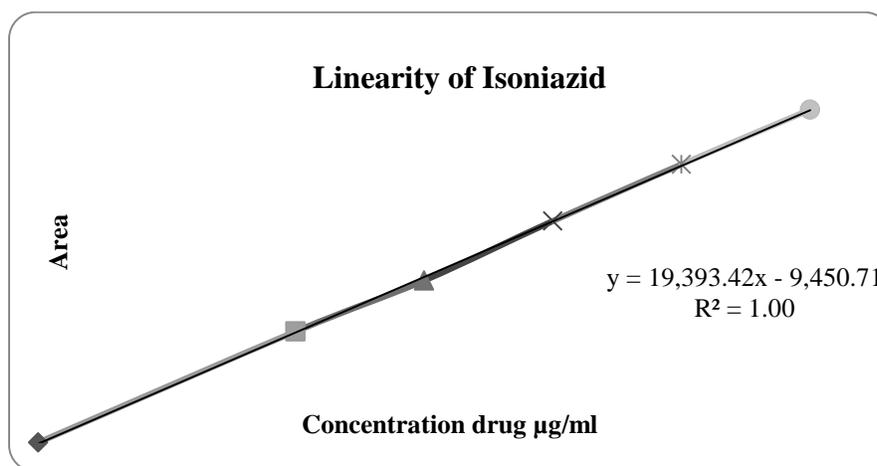
#### Linearity of detector response

The standard curve was obtained in the concentration range of 300-600 µg/ml for Isoniazid and 10-30 µg/ml for pyridoxine. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient [r<sup>2</sup>] of standard curve were calculated and given in

**Figure-5** For Isoniazid and **Figure-6** for pyridoxine to demonstrate the linearity of the proposed method. From the data obtained which given in **Table-5** For Isoniazid and **Table-6** for pyridoxine the method was found to be linear within the proposed range.

**Table 5: Linearity studies for Isoniazid by proposed method**

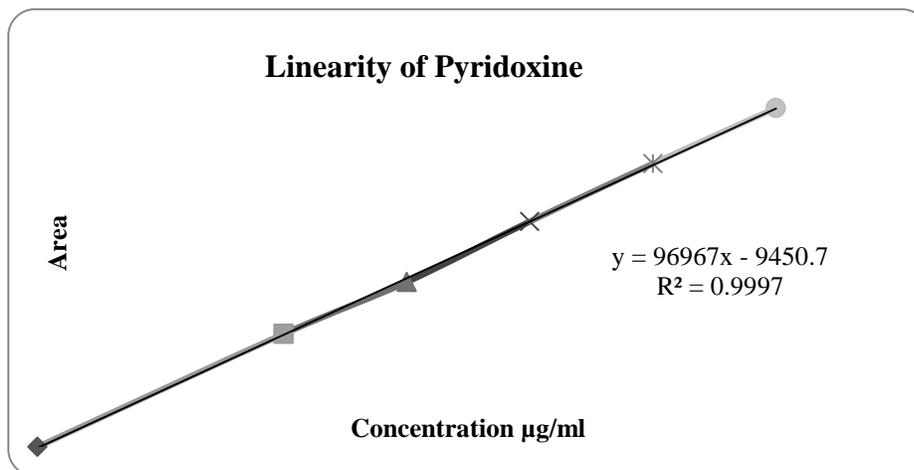
Linearity Level	Concentration(ppm)	Average area	Statistical Analysis	
50	300	845937	Slope	19393
75	450	1260271	Y-intercept	-9450
100	600	1688250	Correlation Coefficient R <sup>2</sup>	1.000
125	750	2108591		
150	900	2527361		



**Figure 5: Calibration curve for Isoniazid**

**Table 6: Linearity studies for pyridoxine by proposed method**

Linearity Level	Concentration (ppm)	Average area	Statistical Analysis	
50	10	967137	Slope	96967
75	15	1412064	Y-intercept	-9450
100	20	1931162	Correlation Coefficient R <sup>2</sup>	0.999
125	25	2426790		
150	30	2902853		



**Figure 6: Calibration curve for pyridoxine**

## CONCLUSION

An RP-HPLC method for simultaneous estimation of Isoniazid and pyridoxine was developed and validated as per ICH guidelines. The results obtained indicate that the proposed method is rapid, accurate, selective, and reproducible. Linearity was observed over a concentration range of 300-600 $\mu\text{g/ml}$  for Isoniazid and 10-30 $\mu\text{g/mL}$  for pyridoxine. The method has been successfully applied for the analysis of marketed tablets. It can be used for the routine analysis of formulations containing any one of the above drugs or their combinations without any alteration in the assay. The main advantage of the method is the common chromatographic conditions adopted for all formulations. Therefore, the proposed method reduces the time required for switch over of chromatographic conditions, equilibration of column and post column flushing that are typically associated when different formulations and their individual drug substances are analyzed. We have developed a fast, simple and reliable analytical method for determination of Isoniazid and pyridoxine in pharmaceutical preparation using RP-LC. As there is no interference of blank and placebo at the retention time of Isoniazid and pyridoxine. It is very fast, with good reproducibility and good response. Validation of this method was accomplished, getting results meeting all requirements. The method is simple, reproducible, with a good accuracy and precision. It allows reliably the analysis of Isoniazid and pyridoxine in bulk, its different pharmaceutical dosage forms.

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