DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR ESTIMATION OF ISONIAZID IN BULK AND TABLET DOSAGE FORM

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ABSTRACT
The aim of present study is to develop simple, sensitive, accurate, rapid and precise reverse phase High Performance Liquid Chromatography (RP-HPLC) method for estimation of isoniazid in bulk drug and pharmaceutical dosage forms. The chromatographic separation was achieved on Neosphere C_{18} (150×4.6mm, i.d.3µ) column. The Analytical method was developed by using mobile phase consisting of 0.010 M potassium dihydrogen orthophosphate: methanol (85:15 v/v). The flow rate and detection wavelength was 1ml/min and 253 nm respectively. The retention time was 2.975±0.054min. The response of the drug was found to be linear in the range of 15-90 µg/ml (r^2>0.999). Isoniazid was subjected to acid, alkali, neutral, dry heat, photostability and oxidative degradation. The LOD and LOQ were 0.671 and 2.033 µg/ml respectively. There was no interference of any degradants and excipients in determination of drugs in marketed formulation and it could effectively separate the drug from its degradation products and it can be successfully employed for routine analysis.

INTRODUCTION [1, 2, 3]

Isoniazid (INH) chemically is an isonicotinic acid hydrazide. It is an antibacterial agent used primarily as a tuberculostic. It blocks the synthesis of mycolic acid, major components of the bacterial cell wall. The structure of INH is given in figure 1 & molecular formula is $C_6H_7N_3O$ and its molecular weight is 137.142 g/mol. The INH is a white crystalline powder it have solubility in water. It is practically insoluble in benzene and ether, solubility in chloroform is 1 %, in boiling alcohol 10 %.

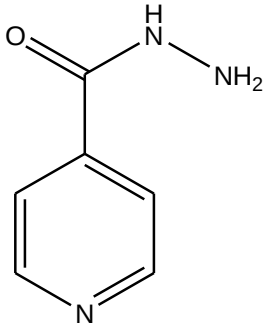

Figure 1: Structure of Isoniazid

INH is a prodrug and must be activated by bacterial catalase–peroxidase enzyme in mycobacterium tuberculosis called Kat G. Kat G catalyses the formation of isonicotinic acyl radical, which spontaneously couples with NADH to form the nicotinoyl-NAD adduct. This complex tightly binds to the enyol-acyl carrier protein reductase known as Inh A, thereby blocking the natural enyol – AcpM substrate and the action of fatty acid synthase. This process inhibits the synthesis of mycolic acids, which are required components of mycobacterial cell wall. A range of radicals are produced by Kat G activation of INH, including nitric oxide, which has also been shown to be important in the action of another antimycobacterial prodrug pretomanid. INH is bactericidal to rapidly dividing mycobacterial but it bacteriostatic if the bacteria are slowly growing. It inhibits the cytochrome p-450 and hence as a source of free radical. INH is a mild monoamine oxidase inhibitor.

According to literature survey several methods are reported on INH like RP-HPLC [4-10] UV spectroscopy [11-14], HPTLC [15-16], Mass spectroscopy [17], Tritrimetry [18-19], Polarography [20], Coulometry [21] and fluorimetry [22]. This paper describes the validation of RP-HPLC method for the assay of INH as bulk and pharmaceutical dosage form. The method was validated as per ICH guidelines.

MATERIALS AND METHODS:

Instrument used

The HPLC system used for study was JASCO HPLC System equipped with pump (Model PU 2080 Plus), Rheodyne sample injector of 50µl capacity, PDA detector (MD 2080) operated with Browin-PDA software (version 1.5)

Chemicals

Active pharmaceutical ingredient of INH was obtained from Lupin ltd. Aurangabad as a gift sample. Solonex tablet manufactured by (Macleoids Pharmaceuticals) labled to contain 300 mg INH was obtained from local pharmacy. HCL, NaOH, H_2O_2, Water, Orthophosphate, Methanol (HPLC grade) was purchased from local pharmacy.

Preparation of standard solution

Standard stock solution of INH was prepared by dissolving 30 mg of drug in 100 ml of methanol to get working standard solution having concentration of 300 µg/ml.

Determination of absorption maxima

Three ml of the standard stock solution was further diluted using methanol to get concentration of 30 µg/ml. This solution was scanned in UV –Visible range 200-400 nm against distilled water as blank. And the drug shows considerable absorbance at 253 nm which was chosen as detection wavelength.

Chromatographic conditions

The chromatographic column used was neosphere C18 (150×4.6 mm, i.d.3µ). Mobile phase used was 0.010 M Potassium dihydrogen orthophosphate: Methanol (85:15 v/v) the detection wavelength was 253 nm, the flow rate was 1ml/min and the injection volume 50 µl.

System sutability test

The system sutability was assessed by injecting standard solution in to chromatographic system containing 60 µg/ml concentrations. The parameters and their results are shown in table 1.

Table 1: Results of system suitability test

Parameters	**Isoniazid**
Detection wavelength (nm)	253
Linearity range (µg/ml)	15-90
Correlation coefficient (r^2)	0.999
Linear regression Equation[a] (y = mx + c)	
Intercept (c)	-11230
Slope (m)	31752
Retention time	2.965±0.051
Peak area	1899762
Throtical plates	2371.7
Asymmetry	2.25

Preparation of calibration curve

The standard stock solution was used for preparation of linear concentration of INH. Aliquots of 0.5-3 ml from stock solution of INH were transferred in a series of 10 ml volumetric flask and volume was made up to the mark with methanol. Six replicates per concentration were injected and chromatograms were recorded. The peak area of drug was recorded and calibration curve was plotted of peak area against concentration of drugs. Linear response was observed in the concentration range of 15-90 µg/ml. The correlation coefficient was found to be 0.999 for INH

Validation of method [23]

Method was validated as per ICH guideline

Linearity: Aliquots of 0.5- 3.0 ml from stock solution of INH (300 µg/ml) were transferred in a series of 10 ml volumetric flasks and the volume was made up to the mark with the methanol. Six replicates per concentration were injected and chromatograms were recorded. The peak area of drug was recorded and calibration curve was plotted of peak area against concentration of drug. Linear response was observed in the concentration range of 15-90 µg/ml. The results obtained are shown in table 2. An excellent correlation exists between peak area and concentration of drug within the concentration range indicated above. Calibration curve for INH was shown in figure 2.

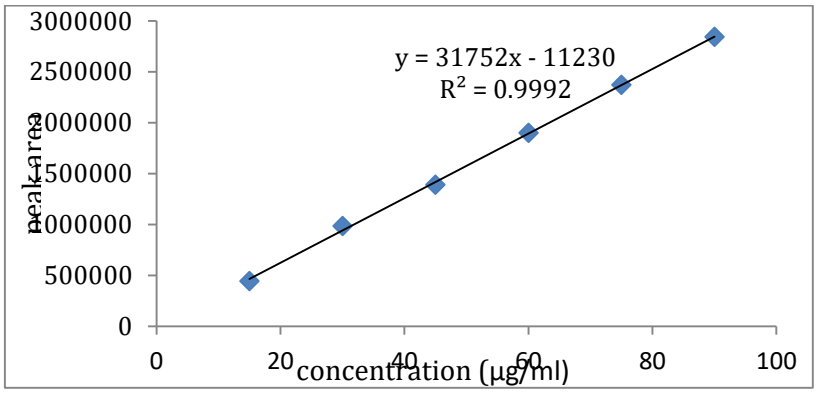

Figure 2: Calibration curve of Isoniazid

Table 2: Observations for Calibration Curve

Sr. No.	Concentration (µg/ml)	Mean Peak Area
1	**15**	**442976**
2	**30**	**985504**
3	**45**	**1390140**
4	**60**	**1900756**
5	**75**	**2372629**
6	**90**	**2842557**

Accuracy: Accuracy expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value found in acceptable ranges. In mean recovery, study were performed at three different concentration ranges as 80%, 100% and 120% is maintained by standard known sample. The percent recovery for INH was calculated. The results are shown in table 3.

Table 3: Results of Accuracy

Level of % recovery	Amount taken (µg/ml)	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	% R.S.D.*
50	30	15	45.07	100.16	0.25
100	30	30	59.85	99.75	0.35
150	30	45	74.39	99.18	0.99

Precision: Precision express that the standard deviation or relative standard deviation (coefficient of variation). The precision were determined at three different parameter such as repeatability, intraday precision and interday precision was performed in specified time. The results are shown in table 4.

Table 4: Analysis of Precision

Intra-day precision			
Concentration (µg/ml)	**Average Area**	**S.D.**	**% R.S.D.***
45	1407056	0.78	0.79

60	1893218	0.30	0.30
75	2359959	0.57	0.57
Inter-day precision			
45	1406691	1.21	1.22
60	1888267	0.43	0.43
75	2354050	0.54	0.54

*n = 3

Limit of detection (LOD) and Limit of quantitation (LOQ): LOD express as lowest amount of analyte in sample the can be estimated but not quantified, LOQ express as the lowest concentration of analyte in sample that can be quantitatively determined by precision and accuracy. This can be calculated by following formula.

$LOD = 3.3\sigma/S$ $LOQ = 10\sigma/S$

Where,

σ = the standard deviation of the response

S = slope of calibration curve

The results are shown in table 5.

Table 5: Results of LOD and LOQ

Drug	LOD (µg/ml)	LOQ (µg/ml)
Isoniazid	0.671	2.033

Robustness: Robustness of the developed method was determined by small but deliberate changes in chromatographic conditions such as flow rate (± 1 ml/min) and mobile phase composition (± 2% methanol). No marked change in chromatogram as well as peak areas of drug which demonstrated robust nature of the developed method. Results of robustness study are presented in table 6.

Table 6: Results of Robustness studies

Drug Used	% R.S.D.* Found For Robustness Study			
	Flow Rate (1 ml/min)		Wavelength (253nm)	
	+1	-1	+1	-1
Isoniazid	0.53	0.27	0.60	0.79

*Average of three determinations

Analysis of tablet formulation: Twenty tablets were weighed accurately; the average weight was determined and then ground to a fine powder. Powder equivalent to 30 mg of INH was weighed; transferred to a 100 ml volumetric flask containing about 60 ml of methanol and sonicated for 15 min. Then volume was made up to the mark with the water; filtered through whatman filter paper no. 41. From this solution 1 ml was taken and transferred to 10 ml volumetric flask, and volume was made up to the mark using methanol to get solution having concentration 30 µg/ml and was injected and chromatogram was obtained. The injections were repeated six times and the peak areas were recorded. The amount of drug present in sample was estimated form the calibration curve. The % drug content was found to be 99.10±1.58. The results obtained are shown in table 7.

Table 7: Analysis of tablet formulation

Drug	Label Claim (mg/tablet)	Concentration injected (µg/ml)	Conc. recovered (µg/ml)	% Drug content	% R.S.D.*
Isoniazid	300	30	29.73	99.10	1.59

Force degradation studies [24, 25]

Acid hydrolysis: Hydrochloric acid (0.1 N, 1 ml) was added to 2 ml stock solution (300 µg/ml) of INH in 10 ml volumetric flask. Then the mixture was refluxed at 80^0 C for 1 hr and neutralized with sodium hydroxide solution and diluted with solvent to obtain final concentration 60 µg/ml. Then injected in stabilized chromatographic conditions. Representative chromatogram for acid treated INH was depicted in figure 3.

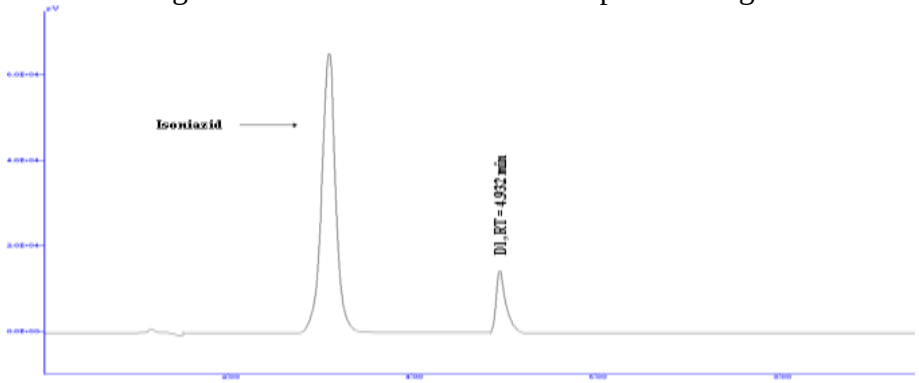

Figure 3: Acid hydrolysis for INH (D1, RT =4.932 min)

Alkaline hydrolysis: Sodium hydroxide (0.1 N, 1 ml) was added to 2 ml stock solution (300 µg /ml) of INH in 10 ml volumetric flask. Then the mixture was refluxed at 80˚ C for 1 hr and neutralized with hydrochloric acid and diluted with solvent to obtain final concentration 60 µg/ml. Then injected in stabilized chromatographic conditions. Chromatogram for alkali treated INH was depicted in figure 4.

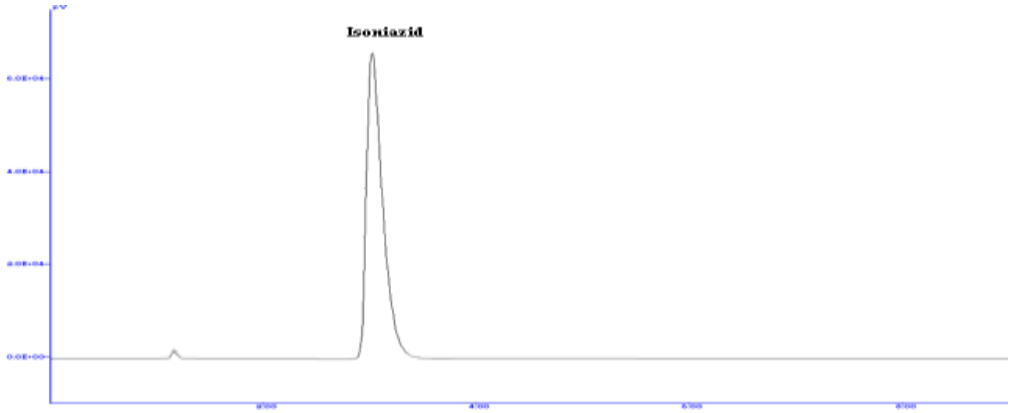

Figure 4: Base hydrolysis for INH

Neutral hydrolysis: Distilled water (1 ml) was added to 2 ml stock solution (300 µg/ml) of INH in 10 ml volumetric flask. The mixture was refluxed at 80˚ C for 1 hr and diluted with solvent to obtain final concentration 60 µg/ml. Then injected into HPLC system and analyzed under stabilized chromatographic conditions and the chromatogram for INH are shown in figure 5.

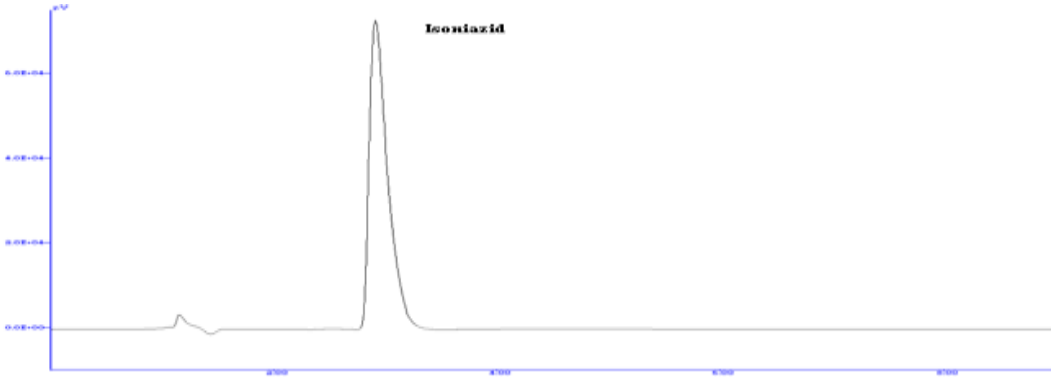

Figure 5: Neutral hydrolysis for INH

Oxidative degradation

Hydrogen peroxide (3 %, 1 ml) was added to 2 ml stock solution (300 µg/ ml) of INH in 10 ml volumetric flask. The mixture was refluxed at 80˚ C for 1 hr and diluted with solvent to obtain final concentration 60 µg/ml. Then injected into HPLC system and analyzed under stabilized chromatographic conditions and the chromatogram for INH was shown in figure 6.

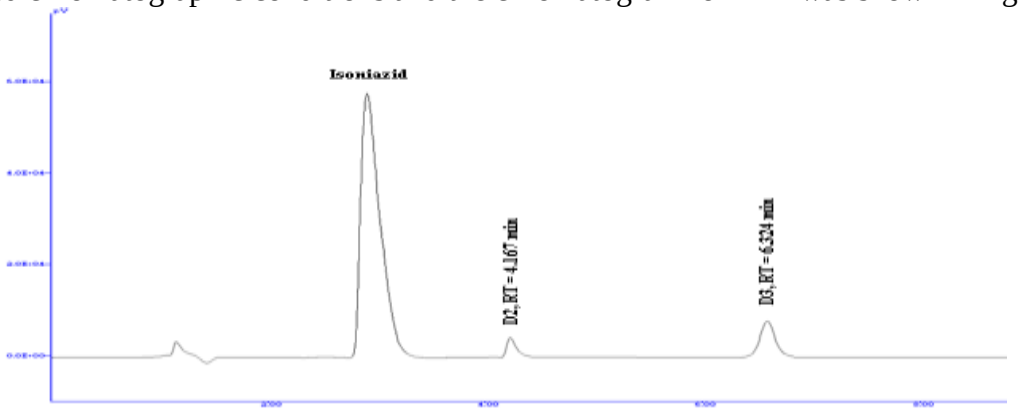

Figure 6: Oxidative degradation for INH

Dry heat degradation: Susceptibility to dry heat was studied by exposing the solid drug to 80º C for a period of 8 hrs. The sample was withdrawn at appropriate times, dissolved in solvent and diluted with solvent to get 60 µg/ml as final concentration. The resulting solution was then injected in stabilized chromatographic conditions. Decrease in the peak area of drug as compared to control sample indicated degradation of drug under thermal stress condition. About 8.84% degradation exhibited by INH without formation of degradation product and the chromatogram was depicted in figure 7.

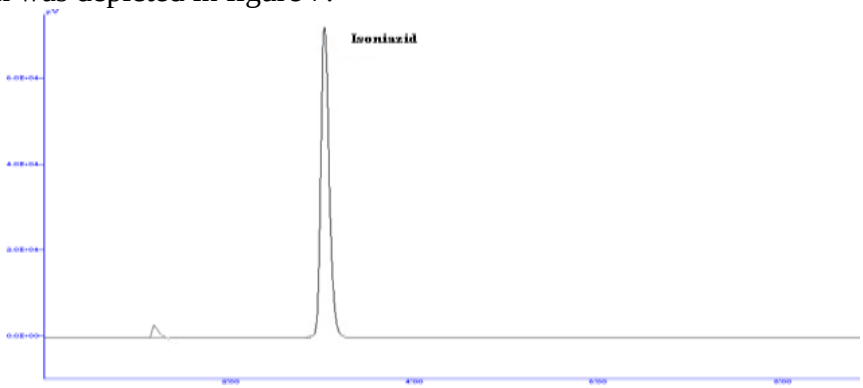

Figure 7: Photolytic degradation for INH

Photolytic degradation: The study was performed by spreading the drug substance in petridish as thin film and kept in photostability chamber equipped with ultraviolet light (200 watt h sequre /meter) for 24 hrs. Suitable control was kept in dark for comparison for the same period. Sample was weighed, diluted with solvent to obtain concentration 60 µg /ml and then was injected in stabilized chromatographic conditions. The drug was found to undergo photolysis with 11.67% degradation with decrease in the peak area. No additional degradation peak was observed. Chromatogram of INH was depicted in figure 8.

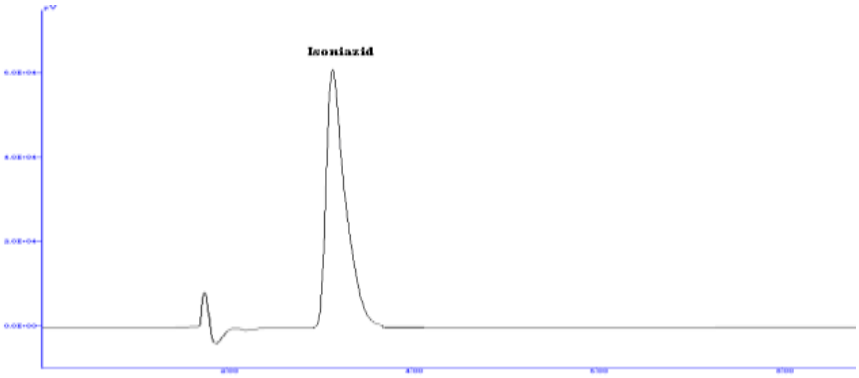

Figure 8: Thermal degradation for INH

RESULTS AND DISCUSSION:

The stability indicating RP-HPLC method for simultaneous estimation of INH in bulk and tablet dosage form was developed and validated. The performed method was simple, precise, accurate and economic, and validated as per ICH guideline. The method was developed by using mobile phase 0.01 M pottasium dihydrogen orthophosphate:methanol (85:15) v/v. The retention time was found to be 2.975±0.054 min for INH and flow rate 1 ml/min. The system suitability parameter was determined and values are in acceptable ranges. The linearity range was found to be 15 -90 µg/ml for INH and calibration curve was plotted as peak area verses concentration. The correlation coefficient (r^2) value for INH was 0.999. The accuracy study was performed in three different level 80%, 100% and 120%. The mean percent recovery of INH was found to be 100.16%, 99.75% and 99.18% respectively and they were found in limit the method was found to be accurate. The precision study mean % RSD was found to be less than 2, indicated that the method was precise. The LOD was found to be 0.671 µg/ml. The LOQ was found to be 2.033 µg/ml for INH. The percent assay was found to be 99.10 % for INH. The percent degradation was performed in acid, base , neutral, oxidative, photolytic and thermal conditions was found to be 16.24 13.04 %, 11.32 %, 20.49%, 11.67%, and 8.84% for INH. The results are shown in table 8.

Table 8: Results of Force Degradation Study

Sr. No.	Mode of degradation	% Recovery of active substance	% degradation
1.	Acid (1 N HCl)	83.76	16.24
2.	Alkali (0.1 N NaOH)	86.96	13.04
3.	Neutral (H_2O)	88.68	11.32
3.	Oxidation (3 % H_2O_2)	79.51	20.49
4.	Dry heat	91.16	8.84
5.	Photolysis	88.33	11.67

CONCLUSION

The developed and validated stability indicating RP-HPLC method for determination of INH in tablet dosage form was found to be simple, precise, selective and accurate. The stress degradation studies demonstrated susceptibility of the drug to acid, base, neutral hydrolysis, oxidative, thermal and photolytic stress conditions. The degradation products produced during the stability study were well separated from the pure drug signifying the specificity of

developed procedure. All the analyzed validation parameters showed acceptable data with satisfactory correlation co-efficient and lower % RSD. The developed method can be utilized by industry for quantitative estimation of drug as bulk and in tablet dosage form.

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