

Analytical Method Development and Validation for the Determination of Isoniazid in, Isoniazid and Ethambutol Hydrochloride Tablets

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Abstract: A simple method using HPLC system for the quantification of Isoniazid, in a fixed-dose combination (FDC) of anti-tuberculosis drug product was developed and validated. The chromatographic separation of Isoniazid (INH) was carried out using Waters Symmetry C8 column (150× 4.6 mm: Particle size: 5 μm), UV detection at 262 nm, and anisocratic system with composed of mobile phase of Phosphate buffer solution (pH 6.8) and Acetonitrile in the ratio of 980:20, at a flow rate of 1.0 mL/min. The retention time of Isoniazid was about 3.8 minutes.

Keywords: Antituberculosis, Isoniazid, Ethambutol Hydrochloride, HPLC method

1. Introduction

According to the World Health Organization, tuberculosis has been widely spread in the world for thousands of years and is a major problem in healthcare systems. In 2013, about 9.0 million new cases (13% co-infected with HIV) and 1.5 million deaths were estimated (1).

Tuberculosis is transmitted by a single agent, *Mycobacterium tuberculosis* (MTB), isolated by Robert Koch in 1882 (2). Sixtythree years after the isolation of MTB, in 1945, the development of streptomycin made tuberculosis treatment possible. Before that, the only option was surgery. Until 1970, a combination of streptomycin, isoniazid (INH), and *p*-amino salicylic acid was used in tuberculosis treatment. Later, the inclusion of rifampicin (RIF) and pyrazinamide (PYZ) in the treatment substantially reduced the recurrence rate and treatment time (3). Combined treatment using various drugs is necessary for patient cure, without recrudescence, and for the prevention of drug-resistant mutants that may occur during treatment (4). Since 2010, the treatment for new cases of tuberculosis consists of a 6 month regimen of four first line drugs, Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PYR), and Ethambutol hydrochloride (EMB) (Figure 1; 5). The World Health Organization and International Union against Tuberculosis and Lung Disease recommend the use of a fixed-dose combination to ensure proper treatment. The fixed dose combination simplifies the prescription drug and the supply management and may even reduce the risk of mycobacterial resistance due to inappropriate treatment (4, 6).

A method for simultaneous determination of RIF, INH, and PYZ in tablets is described in the U.S. Pharmacopeia; however, EMB analysis is performed separately (7), making the method laborious and time-consuming. In a similar way, the International Pharmacopoeia describes a method for simultaneous determination of INH, PYZ, and EMB in tablets, but the RIF analysis is performed separately. In this

method, copper (II) acetate is added to the mobile phase to form a chromophoric adduct with EMB (8). Shewiyo et al. (9) developed a TLC method for the quantification of RIF, INH, PYZ, and EMB by derivatizing the Ethambutol molecule. Faria et al. (10) developed a capillary zone electrophoresis method for simultaneous analysis of RIF, INH, PYZ, and EMB in sachets with copper (II) sulfate addition. Wang et al. (11) described a pre-column derivatization method with phenethyl-isocyanate to quantify RIF, INH, PYZ, and EMB by HPLC. To evaluate the quality of antituberculosis drugs in fixed-dose combination tablets, a simple HPLC method for the determination of Isoniazid was developed and validated. The validated method was applied to the analysis of tablets containing INH (300mg) and EMB (800mg) in a fixed-dose combination.

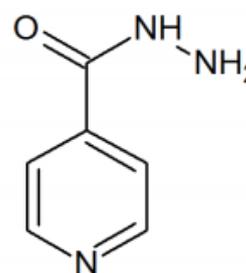


Figure 1: Structure of isoniazid

The molecular structure of isoniazid having the hydrazide group (-C(=O)NHNH₂). The IUPAC name is pyridine-4-carbohydrazide. This drug has a molecular formula C₆H₇N₃O and formula weight 137.14 grams/mole. The SMILES (simplified molecular-input line-entry system) notation is O=C(NN)c1ccncc1. Isoniazid is a first-line tuberculostatic agent that is part of the core of TB treatment regimens. It has been approved as prophylactic therapy for both latent and active tuberculosis infections. It is activated in the body by a bacterial catalase-peroxidase enzyme in

Mycobacterium tuberculosis called KatG. This prodrug inhibits the synthesis of mycolic acids, which are essential for the mycobacterial cell wall [7]. It interferes with the metabolism of vitamin B-6 in some patients [8]. For active tuberculosis infections, isoniazid is often used in combination with other tuberculosis medications, in order to prevent the development of drug resistance. Isoniazid was selected for analysis because it is first-line tuberculostatic drug that is a small molecule with properties suitable for penetration into the central nervous system and appropriate for treatment of tuberculosis infection in the central nervous system.

2. Experimental

Chemicals, Standards, and Samples

Isoniazid reference standard was purchased from the Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q-Plus system (EMD Millipore Corp., Billerica, MA). Acetonitrile HPLC grade (make: Qualigens), Methanol HPLC grade (make: Qualigens), Disodium hydrogen orthophosphate (make: Rankem), and Ortho phosphoric acid were purchased from Merck. One batch of COMBUNEX 800 MG TAB (J901441) of fixed-dose combination tablets manufactured by Lupin Limited was tested (marketed product).

Instrumentation and Analytical Conditions

The HPLC analyses were carried out on a Waters 2690, composed of a quaternary pump, autosampler, and PDA. Empower software was used for data acquisition and analysis. Separations were performed on a Waters symmetry C8 column (150 × 4.6 mm id, 5 µm particle size) at 30°C with UV detection at 262 nm. Mobile phase was a mixture of 20mM phosphate buffer (pH adjusted to 6.8 with phosphoric acid) and acetonitrile (980:20). Isocratic elution was carried out with flow rate of 1.0 mL/minute and the injection volume was 50 µL.

Preparation of Standard Solutions

Reference standard solution was prepared by accurately weighing and transferring 2 mg of Isoniazid reference standard into a 20 mL volumetric flask. Added 2 mL of methanol and sonicated for 10 minutes. Allowed the flask to attain room temperature and diluted up to the mark with phosphate buffer solution.

Linearity standard stock solution was prepared by accurately weighing and transferring 10 mg Isoniazid reference standard into a 20 mL volumetric flask. Added 2 mL of methanol and sonicated for 10 minutes. Allowed the flask to attain room temperature and diluted up to the mark with phosphate buffer solution.

Accuracy standard stock solution was prepared by accurately weighing and transferring 100 mg Isoniazid reference standard into a 100 mL volumetric flask. Added 10 mL of methanol and sonicated for 10 minutes. Allowed the flask to attain room temperature and diluted up to the mark with phosphate buffer solution.

Method Validation

The method was validated according to Brazilian Guideline RE No. 899/2003 (12) and International Conference on Harmonization Guidance for Industry Q2 (R1) Validation of Analytical Procedures: Text and Methodology (13).

(a) **Linearity-** Linearity was assessed by five-point calibration curve (covering 50 to 150% level of sample concentration, i.e., 100 µg/mL). Linearity level solutions were prepared by diluting linearity standard stock solution to obtain solutions having theoretical concentration of 50.0, 75.0, 100.0, 125.0, 150.0, µg/mL. Calibration curve of concentration versus area was plotted, and the obtained data was subjected to regression analysis using the least-squares method.

(b) **Precision-** To evaluate the intraday precision of the method, six samples at 100% of the test concentration were analyzed. Intraday ($n = 6$) precision was calculated and expressed as RSD.

(c) **Accuracy-** Recovery was investigated by means of standard addition in a placebo matrix. The placebo matrix was prepared from a homogenous mixture of the formulation components in the absence of the active principle (Isoniazid). The amount of excipients in the simulated formulation was defined based on the percentage specifications outlined by Kibbe (14). Aliquots of 5, 10, and 15 mL of the accuracy standard stock solution was pipetted out into 100 mL volumetric flasks containing about 34 mg of placebo matrix obtaining three concentration levels (50, 100, and 150%) relative to the theoretical concentrations of Isoniazid (100 µg/mL). At each level, the solutions were prepared in triplicate, and the % recovery was calculated.

(d) **Specificity-** The specificity of the method was evaluated by assaying peak purities of Isoniazid using the UV spectra recorded by a PDA. In addition, the specificity of the assay method was assessed in relation to the placebo formulation and to the diluted solution both were injected into the chromatograph to observe possible interfering peaks.

Analysis of Fixed Dose Combination Tablets

Twenty tablets containing 600 mg of Isoniazid were crushed to powder, and an accurately weighed portion of the powder, equivalent to about 10 mg of Isoniazid was transferred into a 100 mL volumetric flask. Added 10 mL of methanol to the volumetric flask and sonicated for 30 minutes. Allowed the solution to attain room temperature, diluted up to the mark with phosphate buffer and again sonicated for 10 minutes. Filtered a portion of the sample solution through 0.45 µm nylon filter discarding the first 5 mL of filtrate.

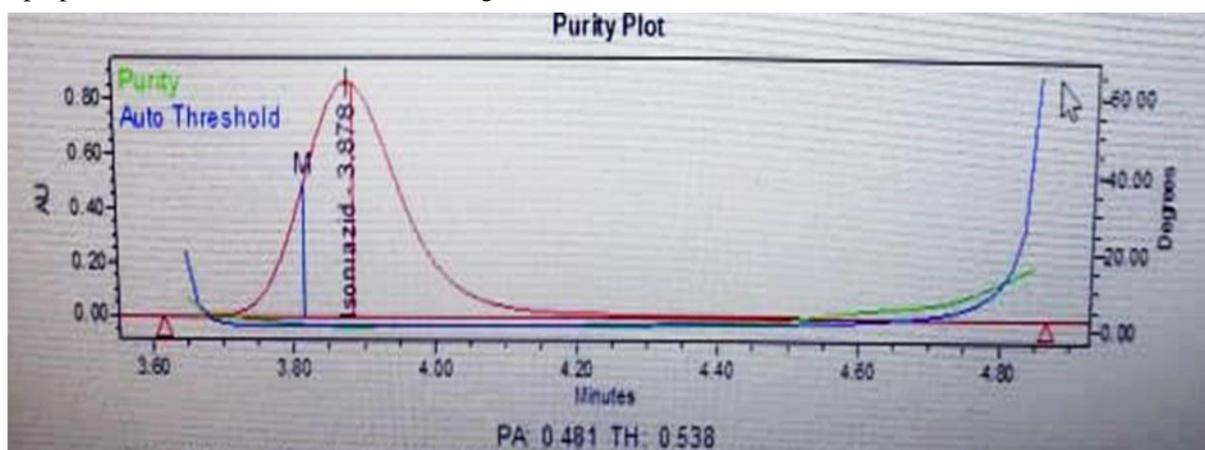
3. Results and Discussion

Optimization of the Chromatographic Conditions

PDA scan was performed from 200 to 400 nm and INH shows good UV absorption at 262 nm wavelength. The retention time and tailing factor were studied to choose the ideal buffer. The tailing factor was calculated at 5% peak height according to the U.S. Pharmacopeia (7). The buffer preparation was also evaluated. Buffer with different pH values of 6.5, 6.7, 6.8 and 7.0 was evaluated, aiming to avoid the interference at the retention time of Isoniazid. Initially, 10 and 20 mM dibasic sodium phosphate buffers were tested, with or without triethylamine addition. A 0.2% triethylamine buffer and an ammonium acetate buffer with copper (II) acetate were also tested. The most appropriate results for the described parameters were obtained with buffer pH 6.8 without using triethylamine. The buffer pH was tested every day during one week, and it was constant; even then it was prepared every couple of days. Considering the polarity differences among the two drugs, exploratory gradient runs (0–100% acetonitrile in 60 min) were performed for each drug to estimate the retention time and to determine the acetonitrile and aqueous solvent proportions. From the obtained chromatograms, it

was observed that anisocratic elution would be sufficient. To check the retention time of drug, isocratic chromatographic runs with a high proportion of aqueous solvent were performed to analyze INH and an isocratic chromatographic run with a high proportion of organic solvent was also performed to analyze INH. Mobile phase composition of buffer and acetonitrile tested with 80:20, 90:10, 95:5, 98:2, and 99:1 (v/v). Mobile phase flow rates of 1.0, 1.2, and 1.5 mL/min were also considered. The best R (>4.0) and tailing factor of about 1.2 for Isoniazid was obtained with the buffer:acetonitrile, 98:2 (v/v) ratio which was in agreement with the U.S. Pharmacopeia monographs. The chromatograms with the spectra (200–400 nm) are presented in Figures 2, 3, 4 and 5. The developed method allowed the quantification of the drug, Isoniazid with only 10 minutes run time and using a simple and rapid procedure, which allows its application to routine Quality Control (QC) analysis. The retention time of Isoniazid peak was found to be about 3.8 minutes.

Note: No response was observed for Ethambutol using optimized chromatographic conditions for the quantification of Isoniazid.



Purity Angle: 0.481; Purity Threshold: 0.538; Peak Purity: Pass

Figure 2: PDA spectra of Isoniazid Reference Standard

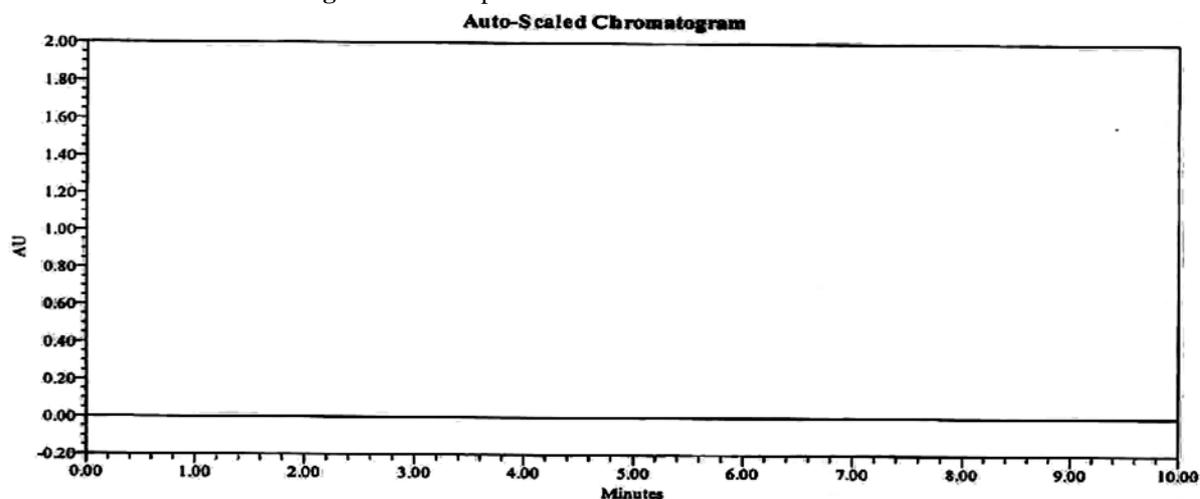


Figure 3: Chromatogram of Blank solution

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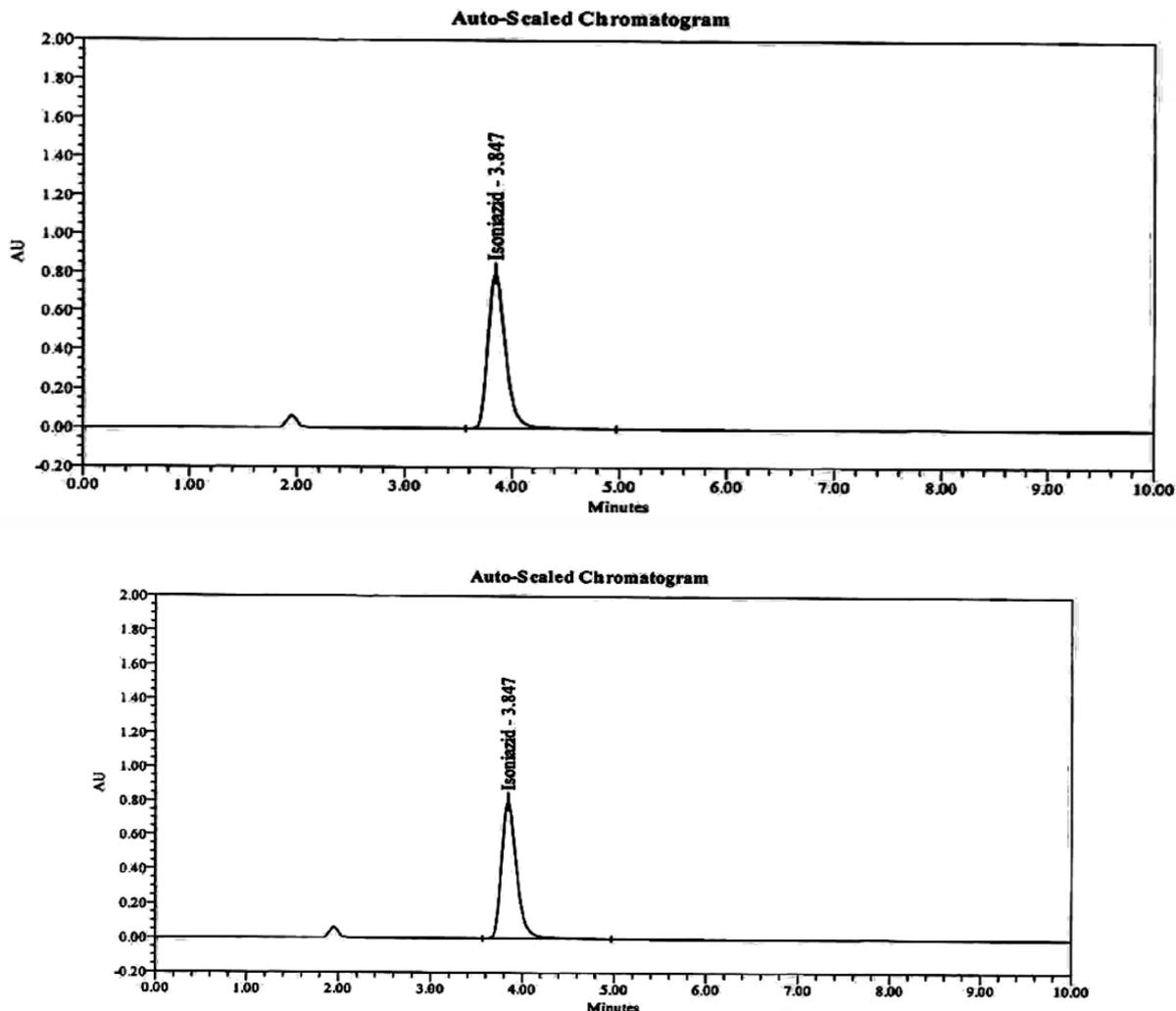


Figure 5: Chromatogram of Sample solution

Method Validation

Specificity: The method showed adequate specificity because the spectra of the Isoniazid obtained with sample solution was superimposable to the spectra of the peak obtained with reference standard. The ratio (sample/standard values) of peak purity Threshold is 0.538. No interfering peaks were observed at the same retention time of Isoniazid when the placebo and the diluted solutions were analyzed.

Linearity: A linear correlation was found between the peak areas and concentrations of Isoniazid in the assay range. In order to verify the method linearity (16), statistical tests such as residue normality (Ryan-Joiner test), homocedasticity (Levene’s test), and lack of fit (ANOVA) were performed at the 95% confidence level. The regression

analysis data and statistics test P-values are shown in Table 1. The regression coefficient (r^2) obtained was higher than 0.99 for Isoniazid, and no significant deviation of linearity was detected in the assay range, because P-values were higher than 0.05.

Precision: The contents of Isoniazid in the intraday are presented in Table 2. RSD values lower than 2.0% for intraday analysis ensure the precision of the method.

Accuracy: Accuracy was investigated by means of a standard addition experiment. The % recovery values for Isoniazid were found to be between 100.06–102.87%. RSD values at each level were less than 2.0%, indicating that the method has adequate accuracy and precision for the determination of Isoniazid drug.

Table 1: Overview of the linearity data and statistics test results for Isoniazid (INH)

Conc. in $\mu\text{g/mL}$	Observed area	Regg.	y – Regg.	(y - Regg) ²
50.40000	4336928	4298227.2020	38700.7980	1497751765.8367600
75.60000	6443828	6449893.1030	-6065.1030	36785474.4006104

100.80000	8596317	8601559.0040	-5242.0040	27478605.93602290
126.00000	10627100	10753224.9050	-126123.9050	15907239412.4493000
151.20000	13003620	12904890.8060	98730.1940	9747651207.2776600

Slope	85383.5675
Intercept	-5104.6
% Intercept	-0.0594
R2	0.9994
R	0.99971
Residual sum of square	27216906466

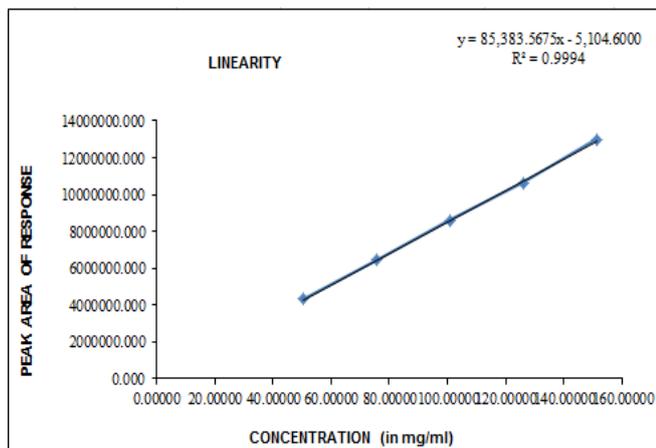


Table 2: Contents of Isoniazid (INH) in the intraday precision study

Sample Preparation	Sample weight (mg)	Peak Area	% Assay results
Sample-1	45.51	9166226	100.75
Sample-2	45.52	9185691	100.94
Sample-3	45.56	9173082	100.72
Sample-4	45.59	9167113	100.59
Sample-5	45.58	9149657	100.42
Sample-6	45.54	9139973	100.40
% RSD		0.18	0.21

Table 3: % Recovery of Isoniazid (INH) in the Accuracy study

Sample	Peak Area	Amount Added ($\mu\text{g/mL}$)	Recovered	% Recovery	% RSD
Sample-50% -1	4505378	52.58	52.61	100.06	0.18
Sample-50% -2	4519302	52.58	52.77	100.36	
Sample-50% -3	4519015	52.58	52.77	100.36	
Sample-100% -1	9210939	105.16	107.56	102.28	0.08
Sample-100% -2	9206254	105.16	107.5	102.23	
Sample-100% -3	9197359	105.16	107.4	102.13	
Sample-150% -1	13837089	157.74	161.58	102.43	0.21
Sample-150% -2	13895027	157.74	162.26	102.87	
Sample-150% -3	13867049	157.74	161.93	102.66	

4. Conclusions

The developed method proved to be selective, linear, accurate and precise for the quantification of Isoniazid with the run time of only 10 minutes in the fixed dose combination of Isoniazid and Ethambutol Hydrochloride Tablets. The method is simple, robust and reliable and may be used for QC analysis of pharmaceutical formulations containing fixed dose combination (Isoniazid and Ethambutol Hydrochloride).

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