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# Method Development and Validation for Simultaneous Estimation of Isoniazid and Ethambutol by using RP-HPLC in Bulk and Pharmaceutical Dosage form

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**A B S T R A C T**

A reverse phase high performance liquid chromatographic method was developed for the determination of Isoniazid and Ethambutol in bulk and pharmaceutical dosage form. The separation was carried out on a [Column: Inertsil C18 (4.6 x 250mm, 5µm, Make: Waters)] using a mobile phase mixture of buffer, acetonitrile in a isocratic elution at a flow rate of 1ml/min. The detection was made at 255 nm. The retention time of Isoniazid and Ethambutol was found to be 2.325 and 4.322 min respectively, Calibration curve was linear over the concentration range of 1µg - 5µg and 100µg-500µg of Isoniazid and Ethambutol. The propose method was validated as per the ICH guidelines. The method was accurate, precise, specific and rapid found to be suitable for the quantitative estimation of related substances in drug and pharmaceutical dosage form.

**Keywords:** Isoniazid and Ethambutol, HPLC, Validation studies

**A R T I C L E I N F O**

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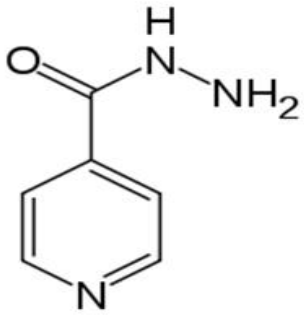
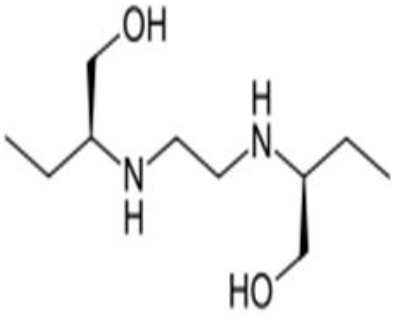
## 1. Introduction

Isoniazid and Ethambutol is an Antitubercular Agents is bacteriocidal against actively growing intracellular and extracellular *Mycobacterium tuberculosis* organisms. Specifically isoniazid inhibits InhA, the enoyl reductase

from *Mycobacterium tuberculosis*, by forming a covalent adduct with the NAD cofactor. Ethambutol inhibits arabinosyl transferases which are involved in cell wall biosynthesis. As a more potent successor to gabapentin. Pregabalin binds to the $\alpha_2\delta$ (alpha-2-delta) subunit of the voltage-dependent calcium channel in the central nervous system. Pregabalin decreases the release of neurotransmitters including glutamate, nor- epinephrine, substance P and calcitonin gene related peptide. However, unlike anxiolytic compounds, which exert their therapeutic effects through binding to $GABA_A$, Isoniazid and Ethambutol neither binds directly to these receptors nor augments $GABA_A$ currents or affects GABA metabolism



**Isonizide**



**Ethambutol**

## 2. Materials and Methods

**Buffer preparation:**

**Preparation of Phosphate buffer :( PH: 4.6):**

Weighed 6.8 grams of KH2PO4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 4.6 with ortho phosphoric acid.

**Standard Preparation:**

Weigh about 15mg of Isoniazid and Ethambutol standard and transfers in to 50ml volumetric flask add about 30ml of diluent sonicate to dissolve resulting solution was diluted with the mobile phase.

**Method development & Optimization:**

Using Mobile phase consisting of different buffers and methanol at different concentrations and different mobile phase's pH values are attempted .The peak was observed that the shape and retention time of voriconazole was found to be broad compared to the mixed phosphate buffer and acetonitrile composition of mobile phase. After selecting the best conditions based on peak performance , mixed phosphate buffer solution and acetonitrile in the ratio 35:65 and hplc using column is Symmetry C18 (150*4.6 *5µ), the run times of the proposed method was 10 mins with isocratic solution. Column temperature is 25°C,flow rate is 1ml/min, PDA Detector is mainly used this purpose ,after inject the standard solution volume was found to be 10µL. Retention times found were about 2.5 minutes for Pregabalin.

**Method validation:**

Isoniazid and Ethambutol standards taken to the 25mg was accurately weighed and transferred into a 25ml of volumetric flask containing HPLC grade Methanol s diluents. It was sonicated, dissolves completely and made volume up to the mark with the same solvent. The method was validated in accordance with ICH guidelines. The parameters assessed were precision, accuracy, linearity, specificity, robustness.

## 3. Results and Discussion

A new method was established for simultaneous estimation of Isoniazid and Ethambutol by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Isoniazid and Ethambutol by using X terra C18 5µm (4.6*250mm) column, flow rate was 1ml/min, mobile phase ratio was Phosphate buffer (0.05M) pH 4.6: ACN (55:45%v/v) (pH was adjusted with ortho phosphoric acid), detection wave length was 255nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector 996, Empower-software version-2. The retention times were found to be 2.399mins and 3.907mins. The % purity of Isoniazid and Ethambutol was found to be 100.7% and 101.4% respectively. The system suitability parameters for Isoniazid and Ethambutol such as theoretical plates and tailing factor were found to be 1.3, 5117.5and 1.4, 3877.3 the resolution was found to be 8.0. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Isoniazid and Ethambutol was found in concentration range of 1µg-5µg and 100µg-500µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % mean recovery was found to be 100% and 100.5%, %RSD for repeatability was 0.2 and 0.4, % RSD for intermediate precision was 0.5 and 0.1 respectively. The precision study was precise, robust and repeatable.

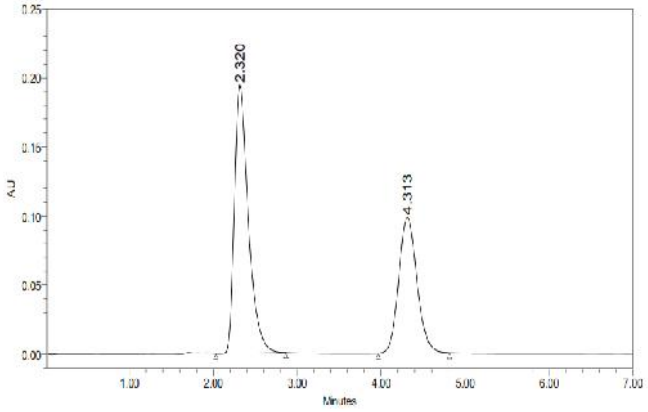



**Figure 1**

| | Name | Retention Time (min) | Area (µV*sec) | Height (µV) | USP Plate Count | USP Tailing | USP Resclution |
|---|---|---|---|---|---|---|---|
| 1 | Isoniazid | 2.320 | 2230876 | 194455 | 978.79 | 1.51 | |
| 2 | Ethambutol | 4.313 | 1486304 | 98590 | 1899.07 | 1.13 | 5.61 |

**Method Validation:**

**Precision:**

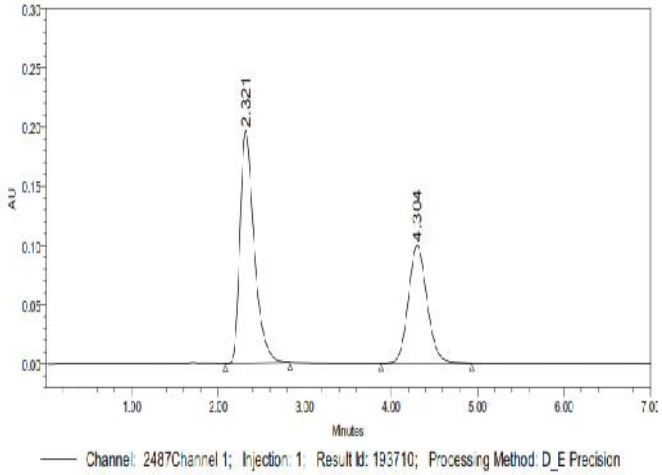



**Figure 2**

**Name: Isoniazid**

| | Name | RT | Area | Height (µV) |
|---|---|---|---|---|
| 1 | Isoniazid | 2.321 | 2235319 | 196999 |
| 2 | Isoniazid | 2.317 | 2240678 | 198254 |
| 3 | Isoniazid | 2.323 | 2249490 | 195128 |
| 4 | Isoniazid | 2.322 | 2245822 | 196164 |
| 5 | Isoniazid | 2.324 | 2251694 | 195887 |
| Mean | | | 2244601 | |
| Std. Dev. | | | 6656.8 | |

| | Name | RT | Area | Height (µV) |
|---|---|---|---|---|
| % RSD | | | 0.30 | |

**Name: Ethambutol**

| | Name | RT | Area | Height (µV) |
|---|---|---|---|---|
| 1 | Ethambutol | 4.304 | 1501417 | 100275 |
| 2 | Ethambutol | 4.300 | 1486940 | 100079 |
| 3 | Ethambutol | 4.308 | 1490656 | 98257 |
| 4 | Ethambutol | 4.310 | 1487329 | 98165 |
| 5 | Ethambutol | 4.314 | 1490384 | 98153 |
| Mean | | | 1491345 | |
| Std. Dev. | | | 5881.4 | |
| % RSD | | | 0.39 | |

**Accuracy:**

The spiked level was found to be at 50,100,150 and the % recovery was found to be 100.42, 99.34, and 98.95% respectively.

**Acceptance Criteria:**

The % Recovery for each level should be between 98.0 to 102.0%.

**Linearity**

Linearity Results (for Ethambutol): (for Isoniazid):

**Table 4:** Linearity results of Isoniazid and Ethambutol

| | SampleName | Name | RT | Area | Height (µV) |
|---|---|---|---|---|---|
| 1 | Linearty 1 | Isoniazid | 2.309 | 1810101 | 145957 |
| 2 | Linearty 1 | Ethambutol | 4.307 | 1164173 | 75128 |
| 3 | Linearty 2 | Isoniazid | 2.322 | 2044287 | 176935 |
| 4 | Linearty 2 | Ethambutol | 4.317 | 1342535 | 87703 |

| | SampleName | Name | RT | Area | Height (µV) |
|---|---|---|---|---|---|
| 5 | Linearty 3 | Isoniazid | 2.324 | 2367133 | 206622 |
| 6 | Linearty 3 | Ethambutol | 4.323 | 1555931 | 101999 |
| 7 | Linearty 4 | Isoniazid | 2.336 | 2602279 | 228576 |
| 8 | Linearty 4 | Ethambutol | 4.340 | 1777973 | 117084 |
| 9 | Linearty 5 | Isoniazid | 2.345 | 2869778 | 259346 |
| 10 | Linearty 5 | eEthambutol | 4.340 | 1942319 | 129409 |

**Acceptance Criteria:**

Correlation coefficient should be not less than 0.999

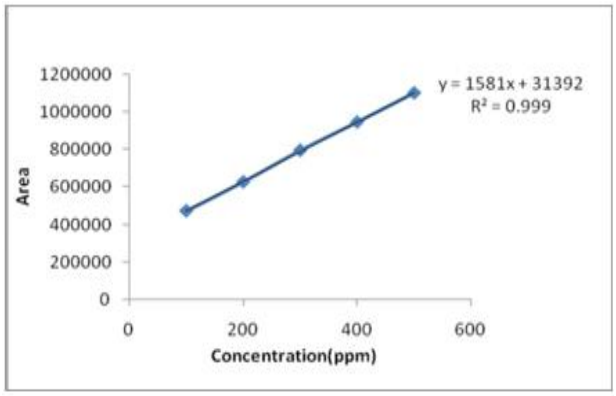



**Figure 3:** Calibration curve of Ethambutol

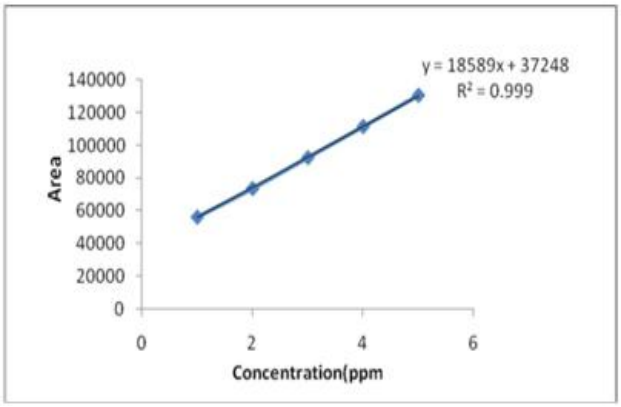
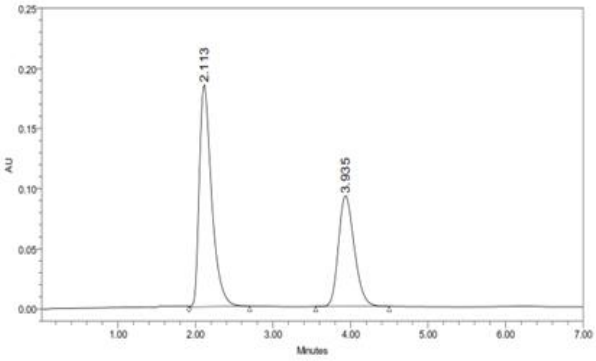



**Figure 4:** Calibration curve of Isoniazid

**Robustness:**



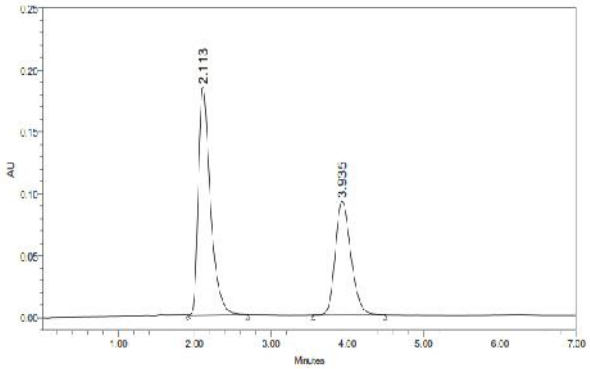
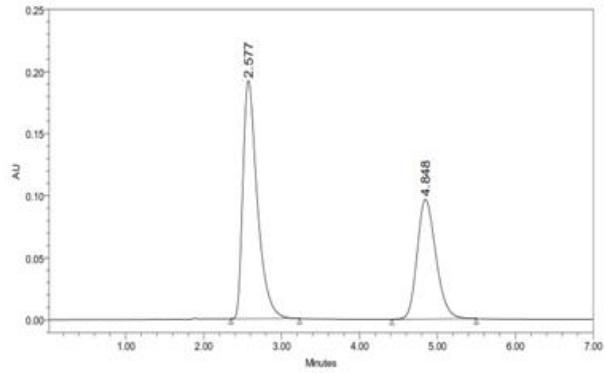




**Figure 4:** Chromatogram for Robustness more flow



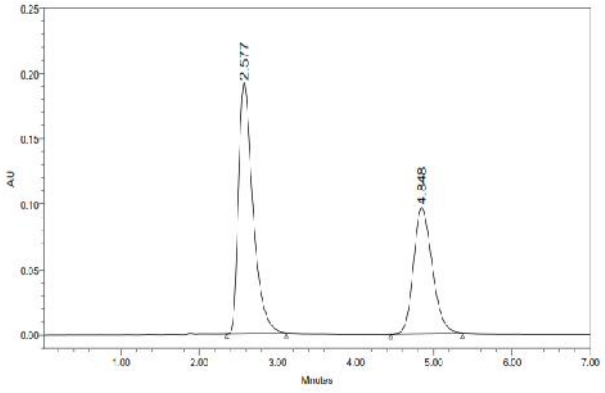




**Figure 5:** Details of Robutness less flow

**Table 1:** Details of Instrument

| S.No | Instrument | Model No. | Software | Manufacturer's name |
|---|---|---|---|---|
| 1 | HPLC Alliance | Waters 2695 | Empower | Waters |
| 2 | PDA Detector | Waters 996 | UV Win 5 | Lab India |
| 3 | UV double beam spectrophotometer | UV 3000 | - | Satorius |
| 4 | Digital weighing | BSA224SCW | - | Lab India |
| 5 | Balance | AD102U | - | - |

**Table 2:** Accuracy results of Isoniazid

| % Concentration (at specification level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 353867 | 5 | 5.0 | 101.3% | 100.0% |
| 100% | 4735088 | 10 | 9.94 | 99.4% | |
| 150% | 5911798 | 15 | 14.8 | 99.2% | |

**Table 3:** Accuracy results of Ethambutol

| %Concentration (at specification level) | Area | Amount Added (mg) | Amount Found (mg) | % Recovery | Mean Recovery |
|---|---|---|---|---|---|
| 50% | 2332744 | 5 | 5.10 | 101.8% | 100.0% |
| 100% | 3132697 | 10 | 9.99 | 99.9% | |
| 150% | 3918997 | 15 | 14.9 | 99.1% | |

## 4. Conclusion

Hence it can be concluded that the proposed HPLC method is sensitive and reproducible for the determination of related substances in Isoniazid and Ethambutol. The major advantage of this method was short retention time.

## 5. Acknowledgements

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**Division of KDPL)**, Kothapet, Hyderabad, India, using the instruments and kits.

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