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# Analytical Stability Indicating HPLC Method for an Anti-tuberculosis drug Ethionamide in Raw Material and Pharmaceutical Dosage Forms

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***SUMMARY*. A simple, sensitive and stability indicative method for the simultaneous quantification of ethionamide (ETA) in raw material and pharmaceutical dosage forms by high performance liquid chromatography (HPLC) was developed. Chromatographic separation was archieved on a ODS-3 column (250 × 4.0 mm, 5 $\mu$m) with acetonitrile-0.05% trifluoroacetic acid (30:70) as mobile phase with isocratic elution, at wavelength 270 nm and at room temperature (22 ºC). The developed method was validated according to the International Conference on Harmonization (ICH) guidelines regarding and it was found to be suitable for the quality control and stability studies of ETA. The proposed method showed adequate linearity. The recovery at 80, 100, and 120% of working concentration level was within 98.0 to 102.0%. The validated method is highly selective, simple, accurate, cost effective, and it is applicable for stability studies and routine quality control analysis in the pharmaceutical industries.**

*RESUMEN*. Se desarrolló un método simple, sensible e indicativo de la estabilidad para la cuantificación simultánea de etionamida (ETA) en la materia prima y las formas de dosificación farmacéuticas por cromatografía líquida de alta resolución (HPLC). La separación cromatográfica fue obtenida con una columna de ODS-3 (250 × 4,0 mm, 5 $\mu$m) con una solución al 0,05% de ácido trifluoroacético y acetonitrilo (30:70) como fase móvil con elución isocrática, a una longitud de onda de 270 nm y a temperatura ambiente (22 ºC). El método desarrollado se validó de acuerdo con la Conferencia Internacional de Armonización (ICH) y se encontró que era adecuado para los estudios de control de calidad y la estabilidad de ETA. El método propuesto demostró una linealidad adecuada. La recuperación a 80, 100, y 120% del nivel de concentración de trabajo estuvo dentro del 98,0 al 102,0%. El método validado es altamente selectivo, simple, preciso, de bajo costo y aplicable para estudios de estabilidad y análisis de control de calidad de rutina en la industria farmacéutica.

## INTRODUCTION

Ethionamide (ETA, 2-ethylpyridine-4-carbothioamide) is a nicotinamide derivate and structural analogue of isoniazid (Fig. 1). It is an orally administered second line anti-tuberculosis drug in chemotherapy of drug-susceptible tuberculosis patients. ETA is included in the 17th edition of the WHO Model List of Essential Medicines and the 3rd edition of the WHO Model List of Essential Medicines for Children [1,2].

Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* that affects populations worldwide. The emergence of drug-resistant tuberculosis has become a serious problem for health organizations and government agencies. Anti-tuberculosis (TB) drug resistance is a major public health problem that threatens progress made in TB care and control worldwide. Patients are treated with second-line drugs as ETA in treatment with other five anti-tuberculosis drugs during at least 24 months. So the adequate analysis of the potency and stability of these drugs plays a fundamental role in the correct treatment [2].

ETA is a yellow crystalline, nonhygroscopic compound with a faint to moderate sulfide odor and a melting point in the range of 158-164 °C. It is practically insoluble in water and ether, but soluble in methanol and ethanol [3].

Infections caused by *Mycobacterium avium* intracellular complex and drug resistant mycobacterium are increasingly common in differ-

*KEY WORDS:* anti-tuberculosis drug, ethionamide, HPLC, pharmaceutical dosage forms, raw material.

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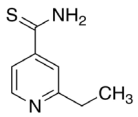


**Figure 1**. The structure of ETA ($C_8H_{10}N_2S$) M.W.: 166.24.

ent part of the world and a fueled with spread of Acquired Immunodeficiency Syndrome (AIDS), as a result of the second line antimicrobial agents such as ETA are being used much more frequently. *In vitro* ETA is active against most of the Mycobacteria including *Mycobacterium tuberculosis* [4].

Several methods have been reported in literature to determine ETA in biological matrixes such as human plasma but very few in pharmaceutical preparations. The official pharmacopoeias present spectrophotometric method for the quantitative determination of ETA from the bulk and dosage forms but this method isn't enough for detecting degradation products or particular impurities. Therefore, in this study, a simple, accurate, cost effective and applicable reversed phase HPLC method was developed and validated in order to be used as quality control and stability control tool routine quantitative analysis of ETA in raw material and pharmaceutical dosage forms [4-10]. The applicability of this developed method was validated according to the International Conference on Harmonization (ICH) guidelines Q2 (R1) [11].

## MATERIALS AND METHODS

### Chemicals and reactives

Acetonitrile (HPLC grade) and trifluoroacetic acid were supplied by J.T.Baker. Water HPLC grade was supplied by Sintorgan S.A. (Argentina). Ethionamide and Ethionamide tablets (ETIOCAT) were gifted by local pharmaceutical industry. All solutions were filtered through a 0.45 µm nylon membrane (Microclar, Argentina) and degassed before use through Vacuum Degassing Millipore Apparatus.

### Equipment

HPLC system used was a UFLC Prominence Liquid Chromatography (Shimadzu Corporation, Japan) equipped with a LC-20AT low pressure gradient HPLC quaternary Pump, a DGU-20A5 Prominence Degasser, a CTO-10AS Column oven and a SPD-20A Prominence UV/VIS Detector. Chromatograms were processed using LCsolution Software (Shimadzu Corporation).

### Chromatographic conditions

Separations were carried out using an ODS-3 column $C_{18}$ (250 × 4.6 mm i.d.), 5 µm particle size (GL Sciences). The column temperature was set at room temperature. The mobile phase consisted of acetonitrile-0.05% trifluoroacetic acid (A) solution (30:70) as mobile phase. A solution was prepared mixing 0.5 mL of trifluoroacetic acid and 1 L of water, which was filtered and degassed through a 0.45 µm nylon membrane with a Vacuum Degassing Millipore Apparatus. The flow rate was set at 0.8 mL/min. The UV detection was carried out at 270 nm and the injection volume was 20 µL.

### Analytical method development

For developing this new chromatography method, ETA solubility was checked in water, acetonitrile and mobile phase (acetonitrile and A solution 30:70). As ETA was freely soluble in mobile phase, a solution of ETA standard was scanned on UV spectrofotometric to identify the maximum absorbance of wavelength. Maxima UV absorbance was obtained at 270 nm and was chosen as detection point. An isocratic method was carried out. Efficiency (N), retention factor (k') and tailing factor (T) are calculated according to USP 35 pharmacopoeia specifications [11]. Injected blank (mobile phase) and placebo served to verify that no inherencies because of it. ETA Standard solution was performed and injected in order to check the system suitability, the relative standard deviation for ETA in not more than 2.0 %.

### Preparation of standard solutions

#### *Stock Standard solution of ETA*

The stock solution of ETA Reference standard was prepared dissolving 25.0 mg of ETA Reference standard in 50.0 mL of mobile phase containing 0.5 mg/mL. The solution was sonicated during 10 min.

#### *Standard solution of ETA*

Ten mL of stock standard solution of ETA was diluted to 100.0 mL with mobile phase containing 0.05 mg/mL of the analyte. The prepared solution was then filtered through a 0.45 µm nylon membrane before analysis.

### Sample preparation

Twenty five mg of ETA, raw material, was transferred into a clean and dried 50.0 mL volumetric flask. It was dissolved in 30 mL of mobile phase, sonicated during 10 min and finally diluent was added up to volume; 5.0 mL of this so-

lution was diluted to 50.0 mL with mobile phase and mixed.

### Pharmaceutical dosage form

Ten tablets of ETA were crushed into powder.

### Stock solution of ETA pharmaceutical dosage form

Fifty mg of the powder (equivalent to approximately 25.0 mg of ETA) was transferred to a 50.0 mL volumetric flask, dissolved in 30 mL of mobile phase and sonicated during 10 minutes, then was diluted to volume.

### Sample solution

Five mL of this solution was transferred to a 50.0 mL volumetric flask, diluted with mobile phase and mixed. Finally all solutions were filtered through a 0.45 µm nylon membrane before analysis.

## RESULTS AND DISCUSSION

The development and validation of analytical methods for the determination of active ingredients in raw material and in pharmaceutical dosages forms is essential for quality control and patient safety.

To our information, there is no report available in literature about the use of a HPLC-UV method for the analysis of ETA assay as active pharmaceutical ingredient (API) and in pharmaceutical dosage forms. This method presents high efficiency and high reproducibility.

### HPLC-UV method optimization

A conventional column ODS-3 C18, 5 µm, 250 × 4,6 mm i.d., is employed in this HPLC method for quantification of ETA. In this study, high resolution and sensitivity in the analysis of ETA were achieved in a short time and less consumption of solvents using a simple UV-detector.

The optimization of the chromatographic method was found to be suitable and provided adequate results in terms of chromatographic parameters. The retention time of ETA was found to be between 3.3-3.5 min. The theoretical plates were not less than 2000 and the tailing factor was no more than 2.0. The system suitability parameters were appropriate for use (Fig. 2).

The specificity of the method for the determination of ETA in raw material and pharmaceutical dosage form was determined by accelerated stress conditions (alkaline, acid, oxidative, photolytic, thermolytic and hydrolytic degradation).

### Stress conditions

In order to develop a stability indicating HPLC method for the quantification of ETA, the active pharmaceutical ingredient (API) and the pharmaceutical dosage form were stressed under various conditions to conduct forced degradation studies.

Stressed decomposition was performed on a stock solution of ETA and then on a stock solution of ETA pharmaceutical dosage form in comparison with standard. Acid-, alkaline-induced degradation were performed in 0.1 N and 1.0 N NaOH, and 0.1 N and 1.0 N HCl, respectively, with refluxed during 120 min and were neutralized with 1.0 mL of 0.1 N HCl and 1.0 mL of 0.1 N NaOH, respectively. Oxidative degradation was performed in a 10% solution of hydrogen peroxide and refluxed during 120 min. As the degradation was higher than expected, the test was repeated with a 3% solution of hydrogen peroxide. Photolytic degradation was carried out by exposing to natural light for 24 h. For thermolytic degradation the stock solution was placed in a glass ball at a water bath at 80 °C and refluxed during 120 min and hydrolytic degradation with distilled water and refluxed during 120 min. All solutions were transferred

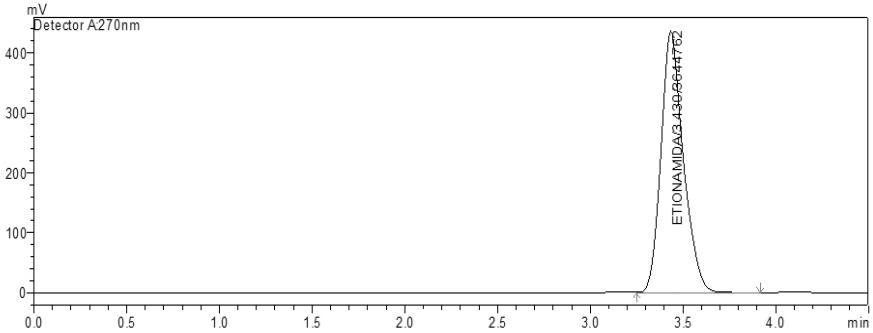


**Figure 2**. Representative chromatogram of ETA reference standard. Experimental conditions are given in the text.

| Stress conditions | Assay % | Resolution between peaks (%) |
|---|---|---|
| Sample in normal conditions | 101.0 | - |
| Stressed with 0.1 N NaOH and neutralized with 0.1 N HCl | 98.1 | - |
| Stressed with 0.1 N HCl and neutralized with 0.1 N NaOH | 97.6 | - |
| Stressed with 1.0 N NaOH and neutralized with 1.0 N HCl | 88.9 | - |
| Stressed with 1.0 N HCl and neutralized with 1.0 N NaOH | 87.3 | - |
| Stressed with 10% hydrogen peroxide solution | 84.4 | 1.4 |
| Stressed with 3% hydrogen peroxide solution | 90.1 | 1.5 |
| Exposed to sunlight for 24 h | 98.7 | - |
| Exposed at 80.0 ºC during 120 min | 98.3 | |
| Stressed with distilled water | 99.4 | |

**Table 1**. Specificity results.

quantitatively to a 50.0 mL volumetric flask and diluted to volume with mobile phase [10-13].

**Non-interference of placebo**

To check the non-interference action of placebo, a solution was prepared in the same way as the stock solution of ETA pharmaceutical dosage form with the presence of all inactive ingredients of the ETA formulation but without the active ingredient. All determinations were carried out during 15 min. Degradations were observed in acid, alkali and oxidative conditions with a slight decrease in ETA title and they are showed in Table 2.

**System suitability**

Chromatographic parameters were evaluated on ten replicates and the results (%RSD) are shown in Table 2.

**Robustness**

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

To determine the robustness of the method the experimental conditions were deliberately altered by change in column temperature ± 2 °C, change in flow rate ± 0.2 mL/min, change in mobile phase pH ± 0.2 units and change in wavelength ± 2 nm. The system suitability parameters are passed for all the conditions and the results for assay were evaluated. The RSD of mean values under these conditions was calculated in all cases. The results indicate that the proposed method is robust under the experimental condition tested.

**Method validation**

The validation of the developed HPLC method was accomplished following the International Conference on Harmonization (ICH) guidelines. The evaluated parameters were specificity, linearity, precision, accuracy, range and robustness [12].

***Linearity***

The linearity of the analytical method is the ability to obtain test results that are directly proportional to the concentration of analyte within a given range. Linearity was calculated by the least-square regression analysis. A standard

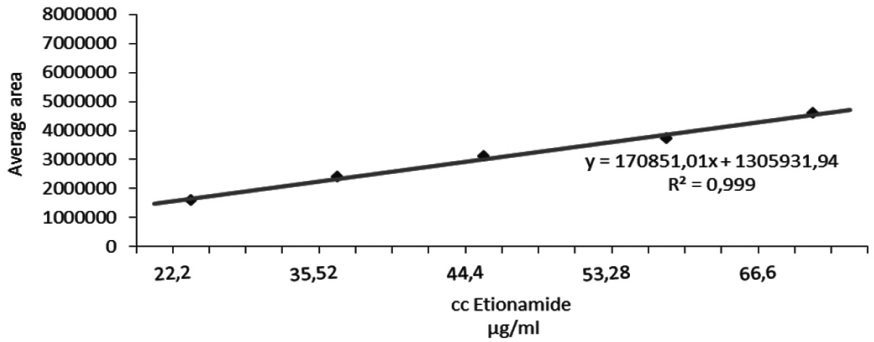


**Figure 3**. Calibration curve of ETA.

curve ranging from 25 to 75 µg/mL (50.0-150.0%) was constructed by diluting appropriate amounts of ETA solution (500 µg/mL) with mobile phase (Fig. 3). Peak Areas were plotted against concentrations and linear regression analysis was performed.

The regression equation for ETA was found to be *y =170851.01x + 1305931.94* and the correlation coefficient and regression coefficient were not less than 0.999. In Addition, the analysis of residuals show that the values are randomly scattered around zero, which shows good fit to the linear model.

***Accuracy***

Accuracy of the analytical method is the closeness of test results obtained by the method to the true value (standard value). Accuracy was carried out for drug-matrix solutions and calculated from recovery studies in samples spiked with ETA at levels of 80, 100, and 120 % of the nominal analytical concentration. Preparations of each level were assayed by triplicate. The overall results of percent recoveries of ETA are displayed in Table 3.

| Level | Recovery (%) | %RSD |
|---|---|---|
| 80% | 100.9 | 0.14 |
| 100% | 101.1 | 0.35 |
| 120% | 100.5 | 0.24 |
| Correlation coefficient | 0.9998 | |

**Table 3**. Summary of accuracy.

***Precision***

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurements. In this study, the precision was evaluated by system precision, intra-day (intermediate precision) and inter-day (Repetition precision).

***System precision***

The system precision is checked by using standard solution to ensure that the analytical system is working properly. The retention time and area response of six determinations should be measured and calculate relative standard deviation. Recorded chromatograms, calculated the relative standard deviation of the retention time of ethionamide peak at 3.5 min is less than 0.5% and relative standard deviation of the area response of ethionamide peak obtained from six injections of standard solution is 0.2%. Hence it was concluded that the system precision parameter meets the requirement of validation.

***Intra-day and inter day precision***

The precision of the assay method was evaluated by carrying out six times of same sample as per analytical procedure against reference standard solution. The percentage of relative standard deviation of six assay values was calculated. Different analyst from the same laboratory evaluated the intermediate precision of the method (Table 4).

| Sample | intra-day precision | | inter-day precision | |
|---|---|---|---|---|
| | % assay | %RSD | % assay | %RSD |
| 1 | 100.1 | 0,18 | 100.2 | 0.17 |
| 2 | 99.6 | 0.13 | 99.7 | 0.07 |
| 3 | 100.2 | 0.27 | 99.9 | 0.18 |
| 4 | 100.2 | 0.25 | 100.1 | 0.28 |
| 5 | 100.4 | 0.24 | 100.2 | 0.39 |
| 6 | 99.9 | 0.21 | 99.8 | 0.06 |
| Average | 100.06 | | 99.98 | |
| %RSD | 0.28 | | 0.21 | |

**Table 4**. Results table of precision.

In all cases, RSD values were acceptable within 2% limit specification, indicating that the proposed method is repeatable.

***Range***

The range of analytical method is the interval between the upper and lower levels of analyte that has been demonstrated to be determinated with a suitable accuracy and linearity. Derived the specified ranges from accuracy and linearity studies and observed results, the range of this method is adequate.

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

The detection limit is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated under the stated experimental conditions. The quantitation limit is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. They were determined using the standard deviation of the response and the slope of the calibration curve.

The LOD and LOQ of ETA by the proposed method were found 0.37359 and 1.13209 µg/mL, respectively.

## CONCLUSION

A simple, rapid, highly sensitive and efficient HPLC-UV method has been developed for the quantification of ethionamide in a tablets pharmaceutical formulation as well as in active ingredient. The advantages of the method are simplicity of the methodology, with shorter time of analyses and improved accurate and specificity. In conclusion, this method is suitable to be used in a pharmaceutical laboratory for quality control or stabilities indicating studies of ethionamide in raw material or formulation products.

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