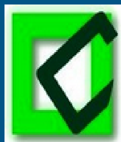
Asian Journal of Biomedical and Pharmaceutical Sciences
ISSN : 2249 - 622X

RESEARCH ARTICLE

Received on: 24-07-2014
Accepted on: 30-11-2014
Published on: 11-12-2014

Hemanth kumar A.K.
National Institute for Research in Tuberculosis (ICMR), Chennai, India
Email:
hemanthkumarak@nirt.res.in
Phone No: 91-44-28369650

QR Code for Mobile users

Conflict of Interest: None Declared !

DOI: 10.15272/ajbps.v4i38.555

Simple and Rapid High Pressure Liquid Chromatography Method for Estimation of Ethionamide in Plasma

Hemanth kumar A.K. *, Sudha. V., Geetha Ramachandran
National Institute for Research in Tuberculosis (ICMR), Chennai, India

Abstract

A high performance liquid chromatographic method for determination of Ethionamide (ETH) in human plasma was developed. The method involved deproteinisation of the sample with perchloric acid and analysis of the supernatant using a reversed-phase CN column (150mm) and a wavelength of 267 nm. The mobile phase consisted of Milli-Q water and Methanol (85:15, v/v) containing 0.05% Perchloric acid and 0.1% tetrabutyl N-ammonium hydroxide. The assay was specific for ETH and linear from 0.25 to 10.0µg/ml. The relative standard deviation of intra- and inter-day assays was lower than 10%. The average recovery of ETH from plasma was 91%. Due to its simplicity, the assay can be used for pharmacokinetic studies of ETH and to check patient adherence to tuberculosis treatment.

Keywords: Ethionamide - HPLC method - plasma – Anti-tuberculosis drug.

Cite this article as:
Hemanth kumar A.K. , Sudha. V., Geetha Ramachandran. Simple and Rapid High Pressure Liquid Chromatography Method for Estimation of Ethionamide in Plasma. Asian Journal of Biomedical and Pharmaceutical Sciences; 04 (38); 2014; 1-5.

INTRODUCTION

Ethionamide (ETH) is a second-line, orally administered drug that is used for the treatment of multi-drug resistant tuberculosis (MDR-TB) along with other anti-tuberculosis agents [1]. ETH is efficacious, relatively non-toxic, cheap, and easily available, which has been in use since the 1960s [2]. ETH is a structural analog of isoniazid [3, 4] and both compounds are known to inhibit mycolic acid biosynthesis [5]. The oral bioavailability of ETH is good; effective plasma levels are achieved in 1 to 2 hours [6].

Resistance to second-line drugs is associated with worse treatment outcomes since an inadequate or poorly administered treatment regimen allows a drug –resistant strain to become dominant in a patient infected with TB. Therefore monitoring ETH levels in plasma could be useful in the clinical management of patients with MDR-TB.

The published methods reported for the determination of ETH included titrimetry, spectrophotometry, electro-analysis, fluorimetry [7] and mass spectrometry [8]. A number of high performance liquid chromatography methods for the estimation of ETH in plasma have been reported [9-12]. These methods were either insufficiently sensitive or are time consuming and require highly sophisticated instrumentation, utilized complex mobile phase or gradient mobile phases and solid phase extraction cartridges. Therefore, there is still a need for a relatively sensitive, rapid, specific and simple method for the estimation of ETH in plasma.

The aim of the present study was to develop and validate a simple and rapid method for measuring ETH levels in plasma by HPLC, which is free of interference from certain anti-TB and antiretroviral drugs. After validation, the method was evaluated in samples collected from TB patients who were receiving ETH along with other anti-TB drugs as part of their regimen.

MATERIALS AND METHODS

Chemicals

Pure ETH powder was obtained from sigma Chemical Company, St.Louis, MO, USA. Methanol (HPLC grade), perchloric acid, tetrabutyl n-ammonium hydroxide, Acetonitrile (HPLC grade) were purchased from Qualigens (India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human plasma was obtained from a Blood Bank, Chennai, India.

Chromatographic System

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), Photo diode array detector (SPD-M10Avp) and auto sampler (SIL-HTA) with built in system controller. Class VP-LC workstation was used for data collection and acquisition. The analytical column was a CN, 150 mm × 4.6 mm ID, 5 um particle size (Lichrospher 100 CN, Merck, Germany) protected by a compatible guard column.

The mobile phase consisted of Milli-Q water and Methanol (85:15, v/v) containing 0.05% Perchloric acid and 0.1% tetrabutyl N-ammonium hydroxide. Prior to preparation of the mobile phase, the solvents were degassed separately using a Millipore vacuum pump. The PDA detector was set at a wavelength of 267 nm. The chromatogram was run for 6 minutes at a flow rate of 1.5 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis vs. concentration curve. The linearity was verified using estimates of correlation coefficient (r).

Preparation of standard solution

A stock standard (1 mg/ml) was prepared by dissolving ETH in 0.1N hydrochloric acid. The working standards of ETH in concentrations ranging from 0.25 to 10.0µg/ml were prepared in pooled plasma.

Sample preparation

To 200 µl each of calibration standards and test samples, 400µl of acetonitrile was added and the contents were vortexed vigorously, centrifuged at 10,000 rpm for 10 min; 300µl of the organic phase was evaporated to dryness. The dried residue was reconstituted in 100µl of mobile phase and 50µl was injected into the HPLC column.

Method Validation

Accuracy and Linearity

The accuracy and linearity of ETH standards were evaluated by analysing a set of standards ranging from 0.25 to 10.0µg/ml. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

Precision

In order to evaluate the precision of the method, plasma samples containing varying concentrations of ETH were analyzed in duplicate on three consecutive days.

Recovery

Known concentrations of ETH (0.25, 0.5, & 5.0µg/ml) were prepared in pooled human plasma and were spiked with lower and higher concentrations of standards. The percentage of drug recovery from plasma samples was calculated by dividing sample differences with the added concentrations. Recovery experiments were carried out on three different occasions.

Specificity

Interference from endogenous compounds was investigated by analysing blank plasma samples. Interference from certain anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, cycloserine and certain antiretroviral

drugs, namely, nevirapine, efavirenz, zidovudine, didanosine, stavudine, lamivudine, saquinavir, lopinavir, ritonavir and indinavir at a concentration of 10µg/ml was also evaluated.

Limits of Detection (LOD) and Quantitation (LOQ)

Limits of Detection (LOD) and Quantitation (LOQ) were calculated mathematically according to ICH guidelines. LOD was calculated using the formula 3.3 * σ/S, where σ is the standard deviation of Y-axis intercepts and S is the slope of the calibration curve. LOQ was calculated using the formula 10.0 * σ/S, where σ is the standard deviation of Y-axis intercepts and S is the slope of the calibration curve.

Samples

The method was applied to estimate ETH in four patient samples. These patients were attending the NIRT clinic and were receiving ETH (250 mg) along with other second line anti-TB drugs. Two milliliters of blood was collected at two hours after drug administration in a heparinised vacutainer tube. Plasma was separated and stored at -20°C until assay.

RESULTS AND DISCUSSION

Several HPLC methods have been described to measure ETH levels in plasma. Conte and others have measured ETH in plasma epithelial lining fluid (ELF) and alveolar cells (AC) by new column chromatographic mass spectrometric technique. A study that examined the pharmacokinetics of ETH administered under fasting condition or with orange juice, food or antacids employed a HPLC method for estimation of ETH (8). A Fluorimetric method for the determination of ETH in pharmaceutical preparations & biological fluids such as urine and plasma was developed using the native fluorescence of eosin in acidic medium due to complex formation (7).

The method described here has several advantages over the published methods, such as (i) the volume of sample required is very less compared to the existing methods (200 µl), (ii) the volume of the organic solvent used in this method, namely acetonitrile is also low (400 µl), (iii) simple extraction procedure and (iv) the retention time is 4.3 minutes and this reduces the run time thereby more number of samples can be processed. Hence the present method is rapid, and sample pre-treatment is simple without any loss of analyte. The method described here could be advantageous for pharmacokinetic studies and therapeutic drug monitoring in MDR-TB patients who are receiving ETH along with other second line anti – TB drugs.

Under these chromatographic conditions, ETH was well separated as seen in the representative chromatograms (Fig1-3). Sufficient resolution was obtained with shorter chromatographic run time of 6 minutes. In view of its potent antimycobacterial activity, ETH is used in the treatment of tuberculosis along with second – line anti-tuberculosis drugs. It therefore becomes necessary to rule out interference of anti-tuberculosis drugs in the quantitation of ETH and establish the specificity. No endogenous substances or anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, kanamycin, Para-amino salicylic acid, ofloxacin, levofloxacin, cycloserine or antiretroviral drugs such as nevirapine, efavirenz, zidovudine, didanosine, stavudine, lamivudine, saquinavir, lopinavir, ritonavir and indinavir interfered with the ETH chromatogram at a concentration of 10µg/ml.

In the present method, ETH concentrations ranging from 0.25-10.0µg/ml respectively was checked for linearity. These concentrations span the range of clinical interest. The calibration curve parameters of ETH from six individual experiments for standard concentrations ranging from 0.25-10.0µg/ml showed a linear relationship between peak height and concentrations (Fig 1). The mean correlation coefficient, intercept and slope values were 0.99731778, - 4263.2925 and 3922.5847 and 0.6890 + 0.2504, respectively. Blank plasma samples did not give any peak at the retention time of ETH (Fig 2). The highest and lowest concentration of ETH gave a discrete peak at 4.3 minutes (Fig 3 & 4).

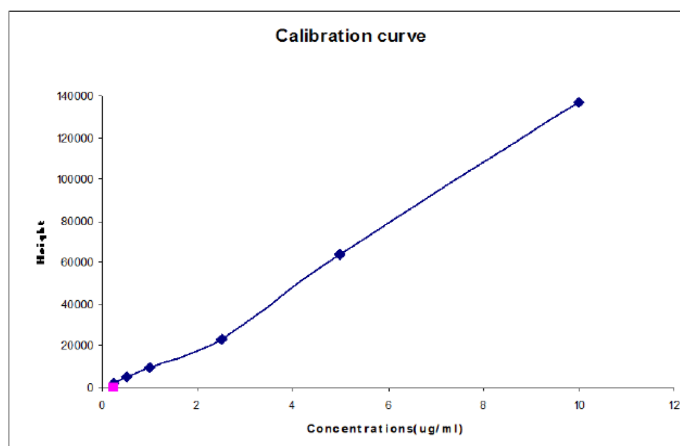

Figure 1: Calibration graph for plasma standard concentrations (0.25µg/ml to10µg/ml)

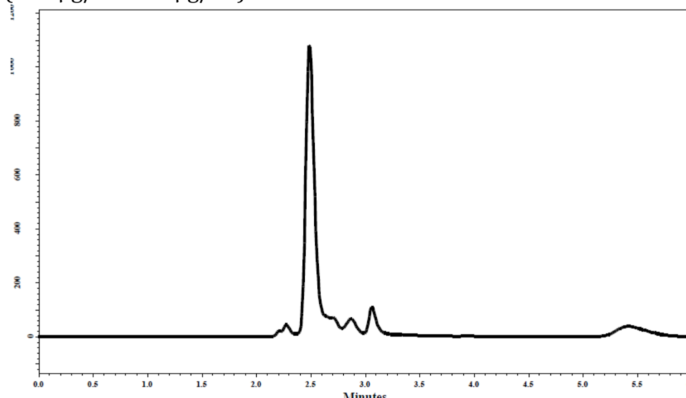

Figure 2: Chromatogram of extracted blank plasma

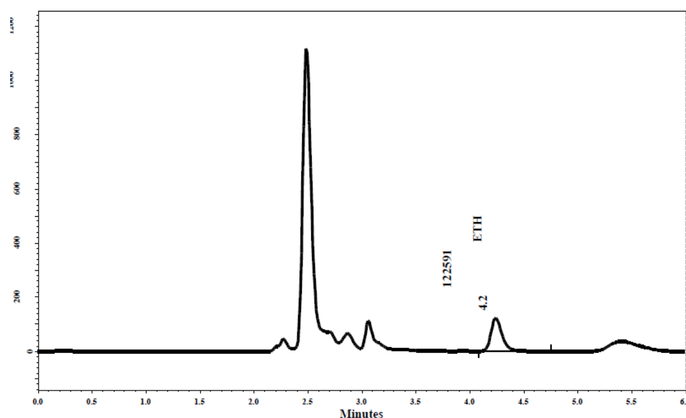

Figure 3: Chromatogram of extracted Ethionamide plasma standard 10.0µg/ml

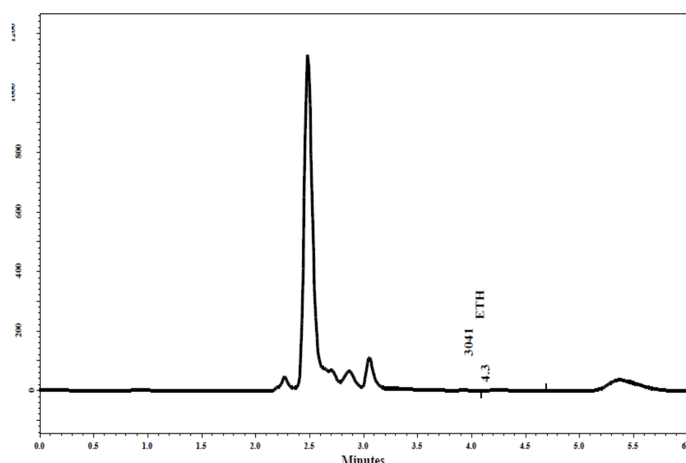

Figure 4: Chromatogram of extracted Ethionamide plasma standard 0.25µg/ml

The linearity and reproducibility of the various standards used for constructing calibration graphs for plasma ETH are given in Table 1. The intra-day and inter - day relative standard deviation (RSD) for standards containing 0.25-10.0µg/ml for ETH ranged from 3.3 to 9.2 % and 4.2 to 9.1 % respectively (Table 1).

	Mean peak height ±SD (RSD%)	
Concentrations (µg/ml)	**Intraday (n=6)**	**Inter day (n=6)**
10.0	137015± 4575 (3.3)	127068±5383 (4.2)
5.0	64354±5944 (9.2)	61340±5422 (8.8)
2.5	23419±1204 (5.1)	25141±1554 (6.2)
1.0	9769±418 (4.3)	10085±554 (5.5)
0.5	5358±459 (8.6)	5457±496 (9.1)
0.25	2515±101 (4.0)	2414±136 (5.6)

Table 1: Linearity and Reproducibility of plasma ETH standards

The reproducibility of the method was further evaluated by analyzing plasma samples containing different concentrations of ETH. The RSD for these samples ranged from 2.0 to 4.1 % (Table 2). The % variations from the actual concentrations ranged from 92 to 109 %. . The LOD and LOQ calculated mathematically according to ICH guidelines were 0.05 and 0.16ug/ml respectively (13). The method reliably eliminated interfering material from plasma, yielding a recovery for ETH that ranged from 86 to 96 % (Table 3).

	Sample 1	Sample 2	Sample 3
	0.32	1.26	0.74
	0.31	1.28	0.72
	0.34	1.22	0.76
MEAN	0.32	1.25	0.74
SD	0.013	0.025	0.016

Table 2: Precision of plasma ETH assay

BASE	ADDED (µg/ml)	ACTUAL (µg/ml)	OBTAINED (µg/ml)	RECOVERY (%)
2.5	0.25	2.75	2.34	86
1.25	0.25	1.5	1.41	94
0.25	0.25	0.5	0.48	96

Table 3: Recovery

The method described was applied for the determination of ETH concentration in plasma from 4 patients who received a single oral dose of 250 mg ETH (Fig 5). A mean plasma peak concentration of 1.3 µg/ml was obtained at two hours, the range being 0.68 to 2.1 µg/ml. The assay spans the concentration range of clinical interest.

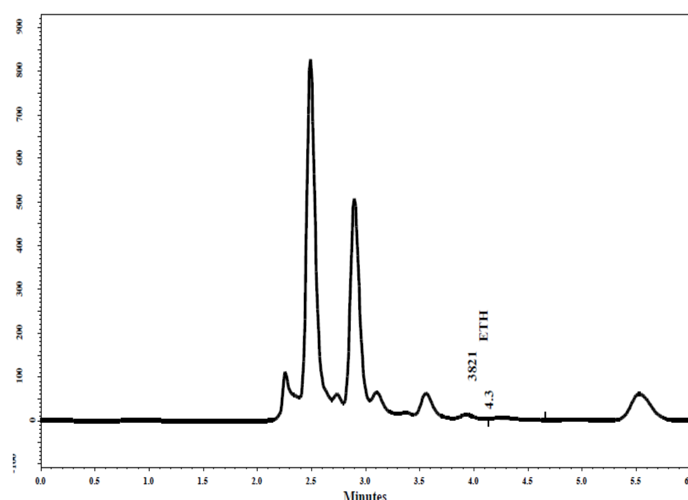

Figure 5: Chromatogram of extracted samples from TB patient

CONCLUSION

In conclusion, a sensitive, specific and validated method for quantitative determination of ETH plasma is described. The chromatogram yields a well resolved peak for ETH with good inter and inter day precision. The rapid, accurate and reproducible method utilizes a single step extraction, easy sample preparation and small sample size makes this assay highly suitable for pharmacokinetic studies of ETH in patients with MDR-TB.

ACKNOWLEDGEMENT

The authors acknowledge the technical and secretarial assistance rendered by Ms. Seema and Ms. Leelaavathi. A.

REFERENCES

1. John E. Conte, Jr., Jeffrey A. Golden, Mari Mcquitty, Juliana Kipps, Emil T. Lin, and Elisabeth Zurlinden. Effect of AIDS and Gender on Steady-State Plasma and Intrapulmonary Ethionamide Concentrations. Antimicrob Agents and Chemother, 2000; 44(5): 1337-1341
2. Ongaya V.A, Githui W.A, Meme H, Kiiyukia C, Juma E. High Ethionamide Resistance In Mycobacterium Tuberculosis Strains Isolated In Kenya. African Journal of Health Sciences, 2012; 20(1-2): 37-41
3. Crofton J., Chaulet P., maher D. et al. Guidelines for the management of multidrug-resistant tuberculosis. World Health Organisation, Geneva, Switzerland.1997; 1-49
4. Blanchard J.S. Molecular mechanisms of drug resistance in mycobacterium tuberculosis. Annu. Rev. Biochem. 1996 ; 65:215-239.
5. Takayama K., Wang L. and David H. L. Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of Mycobacterium tuberculosis. Antimicrob. Agents Chemother. 1972; 2: 29-35
6. Delgado, J.N.; Remers, W.A. In Textbook of Organic Medicinal and Pharmaceutical Chemistry; 10th ed.; Wilson & Gisvold: Philadelphia, PA, 1998: p 206
7. M. I. Walash, A. M. EI-Brashy, M. E. S. Metwally and A. A. Abdelal. Fluorimetric Determination of ethionamide in Pharmaceutical Preparations and Biological Fluids. Journal of the Chinese Chemical Society, 2004 ;(51): 1059-1064
8. Conte, J. E., Jr., E. Zurlinden, and E. Lin. Column liquid chromatographic-mass spectrometric method for the determination of Ethionamide in human plasma, bronchoalveolar lavage fluid, and alveolar cells. J. Chromatogr., in press.
9. Auclair B, Nix DE, Adam RD, James GT, Peloquin CA . Pharmacokinetic of Ethionamide Administered under fasting conditions, or with Orange juice, Food or Antacids. Antimicrob Agents and Chemother, 2001: 45 : 810-814.
10. Peloquin CA, James GT, McCarthy E. Improved high-performance liquid chromatographic assay for the determination of ethionamide in serum. J Chromatogr. 1991; 563(2): 472-475.
11. Jenner, P. J. and G. A. Ellard. High performance liquid chromatography determination of Ethionamide and prothionamide in body fluids. J. Chromatogr. 1981; 222: 245-251.
12. Thee S , Hesseling A.C and Rosenkranz B . Pharmacokinetics of Ethionamide in Children. Antimicrob.Agents Chemother. 2011; 55:4594-4600.
13. International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. ICH harmonised tripartite guideline. Validation of Analytical Procedures: Text and Methodology Q2 (R1), 2005.