

## AN ABSTRACT OF THE THESIS OF

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Title: Acute Exposure to Ethionamide in an *Fmo1/2/4* Null Murine Model

Abstract approved:

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The use of ethionamide has been increasing in drug regimens due to greater incidence of multidrug resistance tuberculosis around the world. Ethionamide is metabolized into antimicrobial relevant compounds by different flavin-containing monooxygenase (FMO) enzymes including FMO1, FMO2, and FMO3. FMOs are found in various locations in the body including the intestine, kidney, liver, and lung. In humans, active functional FMO2\*1 is found in approximately 50% of Sub-Saharan Africans and a truncated, inactive FMO2\*2, is found in all Caucasians and Asians. Polymorphisms in human FMO2 were investigated by comparing differences in metabolism of ethionamide in wildtype mice relative to *Fmo 1/2/4* null mice. All mice were capable of metabolizing ethionamide into ethionamide S-oxide and 2-ethyl-4-amidopyridine. Wildtype mice had higher plasma levels of metabolites than parent compound. In contrast, *Fmo 1/2/4* null mice had higher plasma levels of parent compound than metabolites. In both mouse populations, maximum ethionamide concentration peaked at 2 hours post-exposure. Increased metabolism of ethionamide in wildtype mice may deplete glutathione pools and induce oxidative stress leading to greater toxicity and adverse drug effects. This murine model is used to demonstrate the polymorphic

differences of FMO2 occurring in humans. Taking these differences into account, polymorphisms of drug metabolizing enzymes provide a basis for increasing specific and individualized drug treatment regimens in susceptible populations.

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Acute Exposure to Ethionamide in an *Fmo 1/2/4* Null Murine Model

by  
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A THESIS

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degree of

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Master of Science thesis of Amy L. Palmer  
presented on March 22, 2012.

APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Amy L. Palmer, Author

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## 1 Introduction

Tuberculosis (TB) is a pulmonary infection that has been recorded throughout history and continues to afflict the world populace. Infection is caused by *Mycobacterium tuberculosis*. Approximately one-third of the world is afflicted. In 2010, nearly nine million new cases of infection and 1.4 million deaths related to TB occurred globally [1]. In the United States alone, 11,182 cases were reported in 2010; a 3.1% decrease of incidence from the previous year. After the resurgence of TB infection (peaking in the 1990s) overall trends in the U.S. have decreased by 58% [1]. Because of global incidence of immune suppression as with HIV-AIDS, TB rates have increased [2].

Standard treatment against fully susceptible (non-resistant) TB is a combination of isoniazid (INH), rifampicin, and pyrazinamide given over two months, followed by INH and rifampicin for four more months [3]. This cocktail of medications is well tolerated in most individuals. Complicating TB control throughout the world is an increased incidence in multidrug resistant tuberculosis (MDRTB). Resistance to first-line antibiotics is produced by spontaneous unlinked chromosomal mutation [3].

MDRTB is believed to be exacerbated through misuse of proper antibiotic treatment by patients with inadequate physician observation.

Due to MDRTB, second-line drugs are being utilized more frequently. Ethionamide (ETA) is one of the more frequently prescribed second-line pharmaceuticals in this class and shares similarities in structure and antimicrobial function with INH. The

average daily dose for ETA is 250 mg/day orally but can be increased to 1 g if well tolerated [4]. Second-line anti-TB drugs, such as ETA, can exhibit adverse drug reactions including nausea, vomiting, gastrointestinal disorder, and hepatotoxicity [3, 5]. For ETA to be effective against infection, a minimum inhibitory concentration (MIC) of at least 0.3 µg/ml must be achieved in plasma [3, 4, 6].

ETA, like INH, requires metabolic activation before it becomes effective against TB, i.e., they are prodrugs. Both activated metabolites target the enoyl-acyl carrier protein reductase, *inhA* [7]. A single mutation in the *inhA* gene confers resistance to both INH and ETA affecting mycolic acid synthesis [8, 9]. INH is activated through *katG*, a catalase-peroxidase [10]. When *katG* is mutated, *Mycobacteria* become resistant to INH but not ETA suggesting a different activation enzyme for ETA [10].

Overexpression of *M. tuberculosis* gene Rv3855 (*EtaR*) generates ETA resistance [10, 11]. Rv3854c (*EtaA*) encodes a protein with homology to monooxygenases known to activate ETA [12]. When the *EtaA* gene is mutated, ETA resistance occurs [7].

The monooxygenase in *M. tuberculosis* is homologous to flavin-containing monooxygenases (FMOs, EC 1.14.13.8) found in humans and other mammals. There are five FMOs active in humans (FMO1-5). The role of these enzymes is to detoxify various compounds and make them more excretable. In some cases the metabolites generated are more harmful than the parent compound. The monooxygenase gene family catalyzes oxygenation of 'soft' nucleophiles such as N-, S-, and P- containing

drugs, pesticides, and dietary components [13-15]. FMOs in different families share 50-51% amino acid identity, suggesting semi-conserved ancestral lineage [13, 14]. FMO catalysis requires flavin adenine dinucleotide (FAD) as a prosthetic group, NADPH as a cofactor and molecular oxygen [13]. FMO is present in high concentrations in membranes of the endoplasmic reticulum. Various reactive oxygen species can be generated through the decomposition of FADOOH, such as superoxide anion radicals or hydrogen peroxides. These reactions may be the contributors to toxicity wherever FMOs are located.

FMO isoforms are expressed in a tissue-specific manner and at certain developmental stages. FMOs 1, 2, and 3 are the most important in the metabolism of xenobiotics. FMO1 has the broadest substrate specificity and, in humans, is located primarily in fetal liver as well as adult kidney, and intestine. FMO3 specificity is moderate and is the primary constituent of the liver. FMO2 is primarily located in the lungs and has the strictest binding specificity (mostly by size exclusion) [16]. This pulmonary enzyme is highly expressed in most mammals. In humans a divergence due to a single nucleotide polymorphism generates truncated inactive protein. In all Caucasians and Asians genotyped to date, full length active enzyme is not produced. In 2-7% of Hispanics and up to 50% of individuals of African descent, active FMO2 is produced in the lung [17, 18]. Variations amongst other drug metabolizing enzymes are known to be influenced by environmental and genetic factors. Unlike cytochrome P450s and other

phase II conjugating enzymes, environmental factors do not induce FMO expression [15, 16, 19]. Genetic variation is the primary factor to affect FMO expression.

Polymorphisms exist in the pulmonary FMO2 enzyme that differs between ethnic groups. A premature stop codon (1414T) in *FMO2* (*FMO2\*2*) removes the last 64 amino acids on the C-terminus, truncating the protein and rendering it inactive in people of Asian and Caucasian descent. In African Americans, 26% of the population and 5% of Hispanics, have at least one copy of the active allele (*FMO2\*1*) encoding for the full length protein [14, 17, 19, 20]. High percentage of Sub-Saharan Africans (50%) with *FMO2\*1* corresponds to the same region of high TB incidence [21]. These polymorphisms have the potential to induce varying therapeutic and adverse health effects or benefits from the same drug.

Oxidative activation of the thioamide ETA by FMO1, FMO2.1, and FMO3 occurs in humans and other mammals [5, 18, 22-24]. FMOs, including mycobacterial EtaA, can catalyze thioamide oxygenation in two steps, first to the sulfenic acid and second to the sulfinic acid. The third product detectable, carbodiimide, is thought to be a spontaneous breakdown product of the sulfinic acid (fig. 1). These derivatives deplete glutathione (GSH) in humans and mycothiol (MSH) in *M. tuberculosis* [12, 25]. A redox cycle depletes the GSH pools leading to oxidative stress and cell injury [12]. Toxicity of ETA stems from the biotransformation to reactive metabolites, such as ethionamide S-oxide (ETASO) and 2-ethyl-4-amidopyridine (ETAA), rather than the

parent compound [12]. Differences between individuals with an active FMO2 protein or a truncated version are expected to affect drug tolerance from one person to the next and perhaps therapeutic efficacy.

Activities of human FMOs are comparable to those found in mice [5]. The advantage of using a murine model is that it has utility in creating strategies for treatment of human *M. tuberculosis* through genetic tools. It is difficult to determine the role of FMOs in drug efficacy due to the lack of specific inhibitors for these enzymes and overlapping isoform specificity. In order to investigate the effects of FMO2 on the metabolism of ETA, a murine knockout model was utilized. Chromosomal engineering produced an *Fmo1* (-/-), *Fmo2* (-/-), *Fmo4* (-/-) mouse lineage [26], using a Cre/loxP system to effectively remove the genes from mouse embryonic stem cells. Hernandez *et al.*, has successfully demonstrated the use of these mice for characterizing FMO1 metabolism [27], but have not looked into any other FMO. These knockout mice can be utilized to further characterize metabolism of drugs specific to FMO2.

This study investigates ETA metabolism by these novel *Fmo* knockouts and compares their catalytic properties with wildtype C57BL/6J mice. This provides a model for the human, ethnic dependent genetic polymorphism and its role in the therapeutic efficacy and toxicity of ETA.

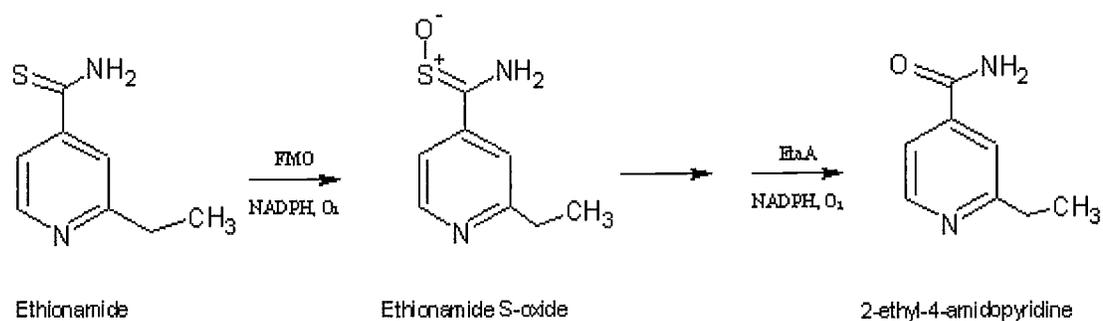


Fig. 1. Proposed metabolism of ETA. Ethionamide is actively metabolized by FMO2 to its more reactive species; ethionamide S-oxide and 2-ethyl-4-amidopyridine.

## 2 Materials and Methods

### 2.1 Chemicals

High performance liquid chromatography (HPLC) reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were HPLC grade. The vehicle for gavage suspension (Vet syrup®) was from FlavoRx (Washington, D.C.). ETASO was custom synthesized by Tjaden BioSciences (Burlington, IA). ETAA was a gift from Paul R. Ortiz deMontellano (University of California, San Francisco CA) [28].

### 2.2 Animals

Male and female C57BL/6J mice were obtained from Jackson Laboratory (Sacramento, CA). These mice were utilized as study controls and delivered at 11 weeks of age to allow for one week acclimation at the Laboratory Animal Resources Center (LARC) (Corvallis, OR) before study initiation. *Fmo1* (-/-), *Fmo2* (-/-), *Fmo4* (-/-) eight generation back-cross knockout mice, on a C57BL/6J background, originated from Elizabeth Shephard and Ian Phillips (University College, London, UK) [26] and were rederived by Harlan Laboratories (Hillcrest, UK) prior to shipment to Oregon State University. A breeding colony was established and maintained at LARC. Mice were provided pellet diet (Purina 5053) and water *ad libitum*. Mice were kept on a 12 hr light cycle under a controlled temperature between 70-72°F. The study was approved by the local Institutional Animal Care and Use Committee.

### 2.3 *In vivo* Single Dose Ethionamide Study

ETA was ground into fine powder and suspended in Vet Syrup (at 12.5  $\mu\text{g}/\mu\text{l}$ ) with 10 minutes of sonication and 30 seconds of vortexing. Mice were grouped by strain (C57BL/6J or *Fmo 1/2/4* null) and gender. Four replicates of each sex per time point were collected. A single dose of 125 mg ETA/kg body weight was administered orally by gavage. The suspension was vortexed before each gavage to ensure consistent concentration was achieved. At designated intervals (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 hr post-gavage) mice were euthanized by CO<sub>2</sub> asphyxiation and exsanguinated by direct cardiac puncture. Blood was mixed with 33 IU of sodium heparin to prevent clotting and was stored at room temperature until all samples were collected from mice that day. Plasma was prepared by centrifugation of whole blood at 2,000  $\times$  g for 10 minutes and was stored at -80°C.

### 2.4 Sample Extraction

Plasma preparations with volumes ranging between 90-200 $\mu\text{l}$  were spiked with 9.6  $\mu\text{g}/\text{ml}$  thiobenzamide (TBZA) as internal standard at room temperature in 1.7 ml microcentrifuge tubes. Samples were extracted with three aliquots (200  $\mu\text{l}$ ) of methanol (MeOH) with vortexing between each addition. The mixture sat at room temperature for 10 minutes, then centrifuged for 10 minutes at 4°C at 17,000  $\times$  g. Supernatant was transferred into new 1.7 ml microcentrifuge tubes and evaporated to dryness under nitrogen. Residues were resuspended with 100  $\mu\text{l}$  (H<sub>2</sub>O: acetonitrile (MeCN) 75:25). These samples were centrifuged for three minutes, room temperature

at 16,300  $\times$  g, to pellet any debris. Clear supernatant was transferred into 2.5 ml Waters (Milford, PA) HPLC vial with 150  $\mu$ l glass inserts for analysis.

Stability of ETA, ETASO, ETAA, and TBZA in bovine plasma (Sigma-Aldrich) was determined. Two hundred  $\mu$ l aliquots of spiked plasma (0.15 mM for each compound) was prepared and stored at  $-80^{\circ}\text{C}$  for four months and extracted as described above. Also the stability of ETA in vet syrup suspension was investigated. Aliquots of 200  $\mu$ l, containing 2500  $\mu$ g of ETA, were extracted by first diluting the suspension with 200  $\mu$ l of water to reduce the solution viscosity. The mixture was extracted with 1.2 ml of MeOH, creating a ratio of 3:1 solvent to aqueous, the same as the plasma precipitation. The resulting concentration of the solution was 15.1 mM (2510  $\mu$ g/ml) ETA. The solution was diluted 151-fold to equal 16.6  $\mu$ g/ml and placed into an HPLC vial for subsequent analysis.

## 2.5 Estimation of Ethionamide

HPLC analysis was performed on a Waters 2695 separations module equipped with a 2996 photo diode array detector and a Waters  $\text{dC}_{18}$  Atlantis $^{\circ}$  5  $\mu$ m, 3.9  $\times$  150 mm column. The flow rate was 0.8 ml/min. Solvents for the mobile phase consisted of 80%  $\text{H}_2\text{O}$  and 20% MeOH at a temperature of  $35^{\circ}\text{C}$  running isocratically for 20 minutes for each injection. Quantitation of ETA, ETASO, and ETAA was performed with standards generated from diluted stocks measured at 267 nm. Concentrations of metabolites were determined by ratios of analyte to internal standard. TBZA was

spiked into each plasma sample at 9.6 µg/ml before extraction. Results were analyzed with Empower software (Waters). A Broad spectra range was also collected from 210 nm and 400 nm to verify the identity of each analyte by absorption profiles.

## 2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Pharmacokinetic parameters for the four ethionamide treatment groups were compared using a one way analysis of variance (ANOVA). The comparisons of interest were wildtype male, wildtype female, knockout male, and knockout female. Comparison of genotypes and genders was performed by an unpaired t-test with Welch's correction. If the overall test differences were below  $p \leq 0.05$  then they were considered significant.

### 3 Results

#### 3.1 Linearity, Assay Precision, Accuracy, and Recovery Assessments

To establish the calibration of the assay, standard concentrations were formulated by purposely introducing ETA, ETASO, ETAA, and TBZA to either mobile phase or bovine plasma and extracting it using protein precipitation. The sensitivity of this assay for ETA was 0.25 µg/ml. ETASO was 0.32 µg/ml. ETAA was 0.20 µg/ml. The retention times were  $17.5 \pm 0.5$  min (ETA),  $13.0 \pm 0.5$  min (ETASO),  $11.5 \pm 0.5$  min (TBZA), and  $6.3 \pm 0.5$  min (ETAA) (fig.2a, b; 3a, b; 4a, b). The mean  $\pm$  standard deviation (SD) of the  $r^2$  for ETA from 15 standard curves (8 in plasma, 7 in mobile phase) was  $0.98 \pm 0.027$ ; ETASO was  $0.98 \pm 0.033$ ; ETAA was  $0.98 \pm 0.029$ . Results for the assay precision and recovery from spiked plasma and mobile phase are summarized in Table 1 and 2.

#### 3.2 Stability

All plasma samples collected from mice were promptly stored at  $-80^\circ\text{C}$  after collection. The stability of ETA, ETASO, ETAA, and TBZA compounds in stored samples was unknown, so 200 µl aliquots of bovine plasma were spiked with 24.9, 27.3, 22.53, and 20.58 µg/ml of ETA, ETASO, ETAA, and TBZA, respectively. After four months there was no degradation of the compounds (fig. 5a, b). The stability of ETA suspended in the vet syrup was also investigated. The age of the suspensions ranged from freshly made to over one year. The suspension solutions showed no degradation (fig. 5a, b).

### 3.3 Peak Levels of Ethionamide and its Metabolites in Plasma

A total of 144 mice were utilized to assess the time course for appearance of ETA and its metabolites in wildtype and knockout mice. Nine time points were collected ranging from 0.25-3.5 hrs at 30 minute intervals, excluding the first 15 minute interval. Each mouse was gavaged with a dose of 125 mg/kg and then euthanized at the correct time post-ingestion. A 2 hr time point was included as a control for the vet syrup vehicle. The time course was repeated four times for each gender and genotype (C57BL/6J or *Fmo 1/2/4* null). The variability of maximum plasma concentrations of ETA and its metabolites differed greatly between individuals (fig. 7a, b; 8a, b).

#### 3.3.1 Kinetics of ETA Metabolism in Wildtype C57BL/6J Mice

Pharmacokinetic measurements from wildtype mice are summarized in Table 3. The mean concentration maximum ( $C_{max}$ ) found in wildtype males for ETA, ETASO, and ETAA was 22.26, 28.98, and 2.36  $\mu\text{g/ml}$  respectively. The time required to reach peak concentration of ETA, ETASO, and ETAA, was 1.5 hrs post-gavage. The area under the curve from time 0.25-3.5 hr ( $AUC_{0.25-3.5}$ ) for ETA, ETASO, ETAA was 36.05, 52.33, and 3.99  $\mu\text{g}\cdot\text{hr/ml}$ . The mean  $C_{max}$  with female mice for ETA, ETASO, and ETAA was 15.33, 21.74, 2.95  $\mu\text{g/ml}$  and the  $T_{max}$  ranged from 0.5 – 1.5 hrs depending on the compound.  $AUC_{0.25-3.5}$  for ETA was 18.12  $\mu\text{g}\cdot\text{hr/ml}$ ; ETASO was 36.40  $\mu\text{g}\cdot\text{hr/ml}$ ; ETAA was 2.95  $\mu\text{g}\cdot\text{hr/ml}$ . In male wildtype mice a single peak in ETA concentrations was observed while in females there were two peaks in maximum

concentrations. Collectively, this data demonstrates differences between genders in C57BL/6J mice.

### 3.3.2 Kinetics of ETA metabolism in Knockout *Fmo 1/2/4* Mice

Pharmacokinetic measurements from knockout mice are also summarized in Table 3. The mean  $C_{\max}$  measured in males for ETA, ETASO, and ETAA was 40.56, 16.92, 1.41  $\mu\text{g}/\text{ml}$ . The  $T_{\max}$  ranged from 1-2 hrs depending on the compound. The  $\text{AUC}_{0.25-3.5}$  was 83.48, 42.39, 3.39  $\mu\text{g}\cdot\text{hr}/\text{ml}$  for ETA, ETASO, and ETAA. The mean  $C_{\max}$  with females was 50.97 (ETA), 19.37 (ETASO), and 1.27  $\mu\text{g}/\text{ml}$  (ETAA). In females, the  $T_{\max}$  was one hour post-gavage for each compound of interest. The  $\text{AUC}_{0.25-3.5}$  was 83.05 (ETA), 38.21 (ETASO), and 2.71  $\mu\text{g}\cdot\text{hr}/\text{ml}$  (ETAA). Taken together, this data demonstrates differences between genders in *Fmo 1/2/4* null mice.

### 3.4 Strain Differences in Metabolic Profiles

Differences between wildtype and knockout mice were observed in metabolic profiles, representative HPLC tracings are shown in fig. 9a, b and fig. 10a, b. The differences between the  $C_{\max}$  for ETA with wildtype mice (average 17.07  $\mu\text{g}/\text{ml}$ ) and knockout mice (average 39.75  $\mu\text{g}/\text{ml}$ ) was significant ( $p = 0.003$ ) (fig. 11a). There are no significant differences between genotypes when observing the metabolites of ETA (ETASO and ETAA).

Mean ETASO plasma concentrations from the time course study are shown by gender in fig. 11b. The differences between  $C_{\max}$  of males (average 21.15  $\mu\text{g/ml}$ ) and females (average 14.17  $\mu\text{g/ml}$ ) was significant ( $p = 0.018$ ). Also the ETASO plasma concentration with female mice peaked at 30 minutes while the male ETASO plasma concentration peaked at 1.5 hours.

To investigate significant differences between genotypes and gender in these mice, an ANOVA was calculated for each compound of interest. The mean ETA time course ANOVA comparing wildtype male, wildtype female, knockout male, and knockout female, showed a significant difference in at least one of the treatment groups ( $p = 0.0005$ ). There was no difference between treatment variances (Bartlett's test  $p=0.0861$ ). The mean ETASO time course ANOVA, comparing the four treatment groups, also showed a significant difference in at least one of the groups ( $p = 0.006$ ). There was no difference in variances (Bartlett's test  $p= 0.2507$ ). The mean ETAA metabolite showed no significant differences between the four groups ( $p = 0.3516$ ) There was also no difference in variances (Bartlett's test  $p= 0.2479$ ). Comparing the genotype and gender of the treatment groups established differences for levels of ETA and ETASO in blood.

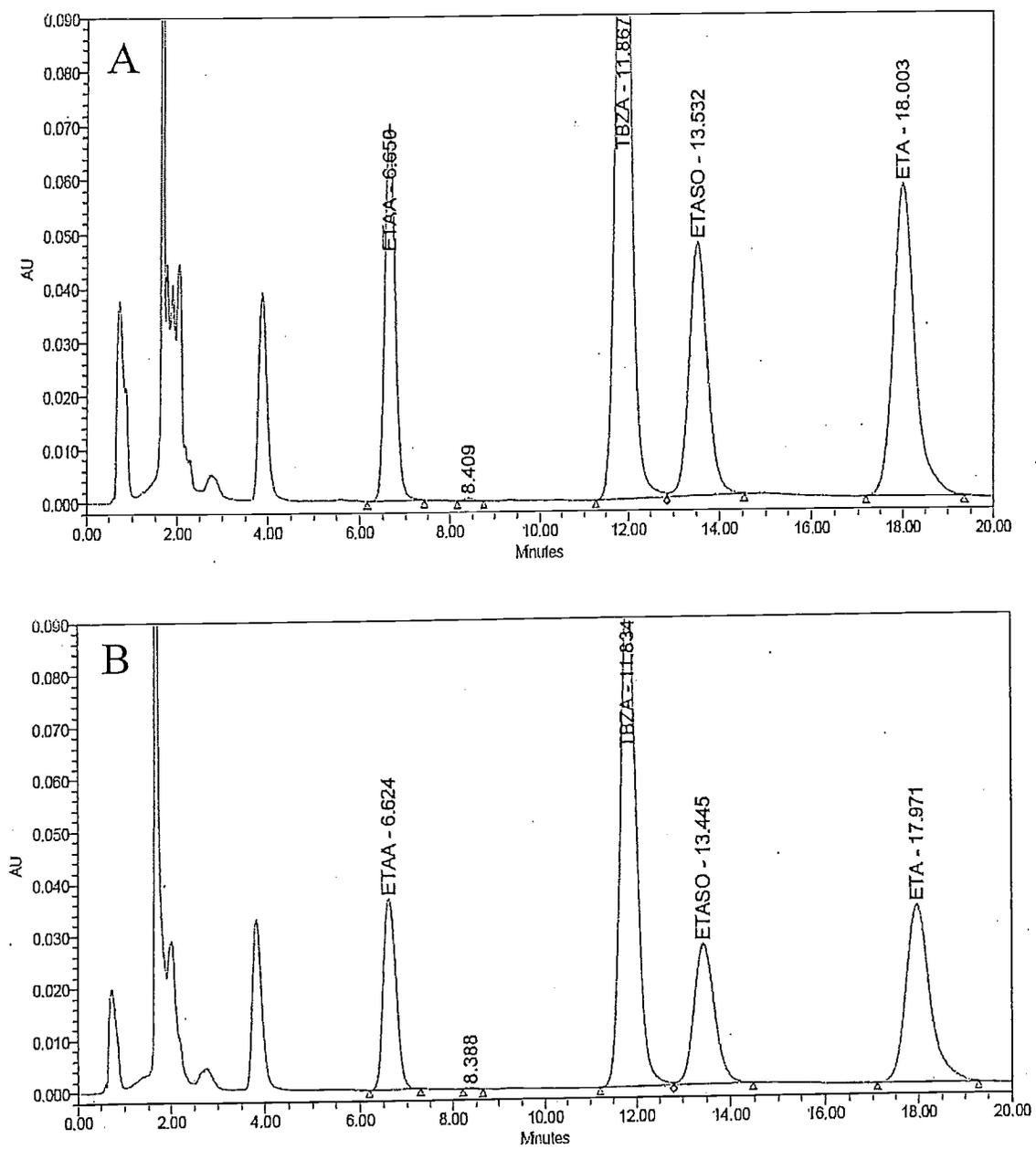


Fig. 2. HPLC chromatogram tracings monitored at UV 267 nm extracted from bovine plasma. a) 0.3 mM and b) 0.2 mM of ETA, metabolites, and TBZA.

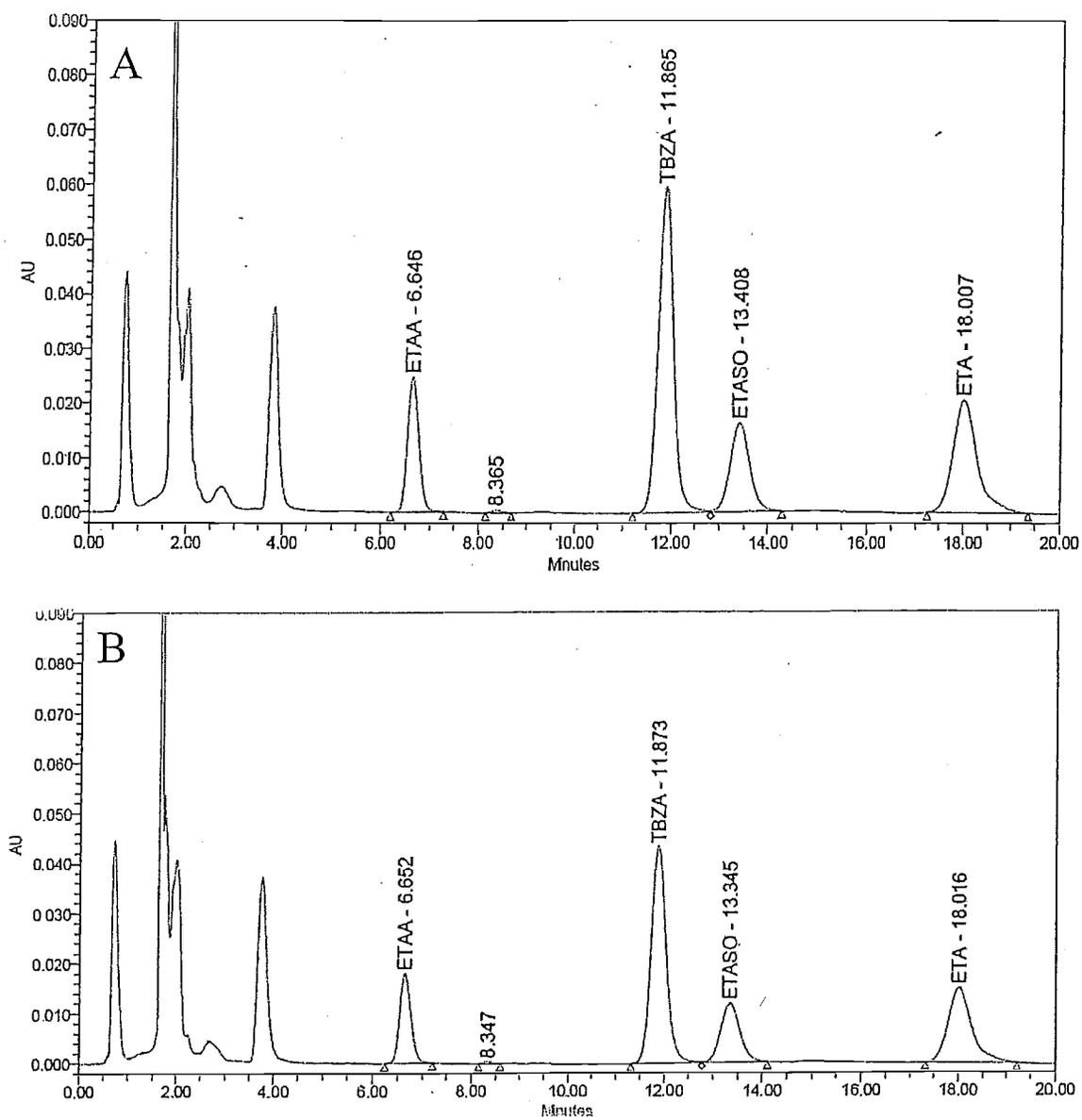


Fig. 3. HPLC chromatogram tracings monitored at UV 267nm extracted from bovine plasma. a) 0.1 mM and b) 0.07 mM of ETA, metabolites, and TBZA.

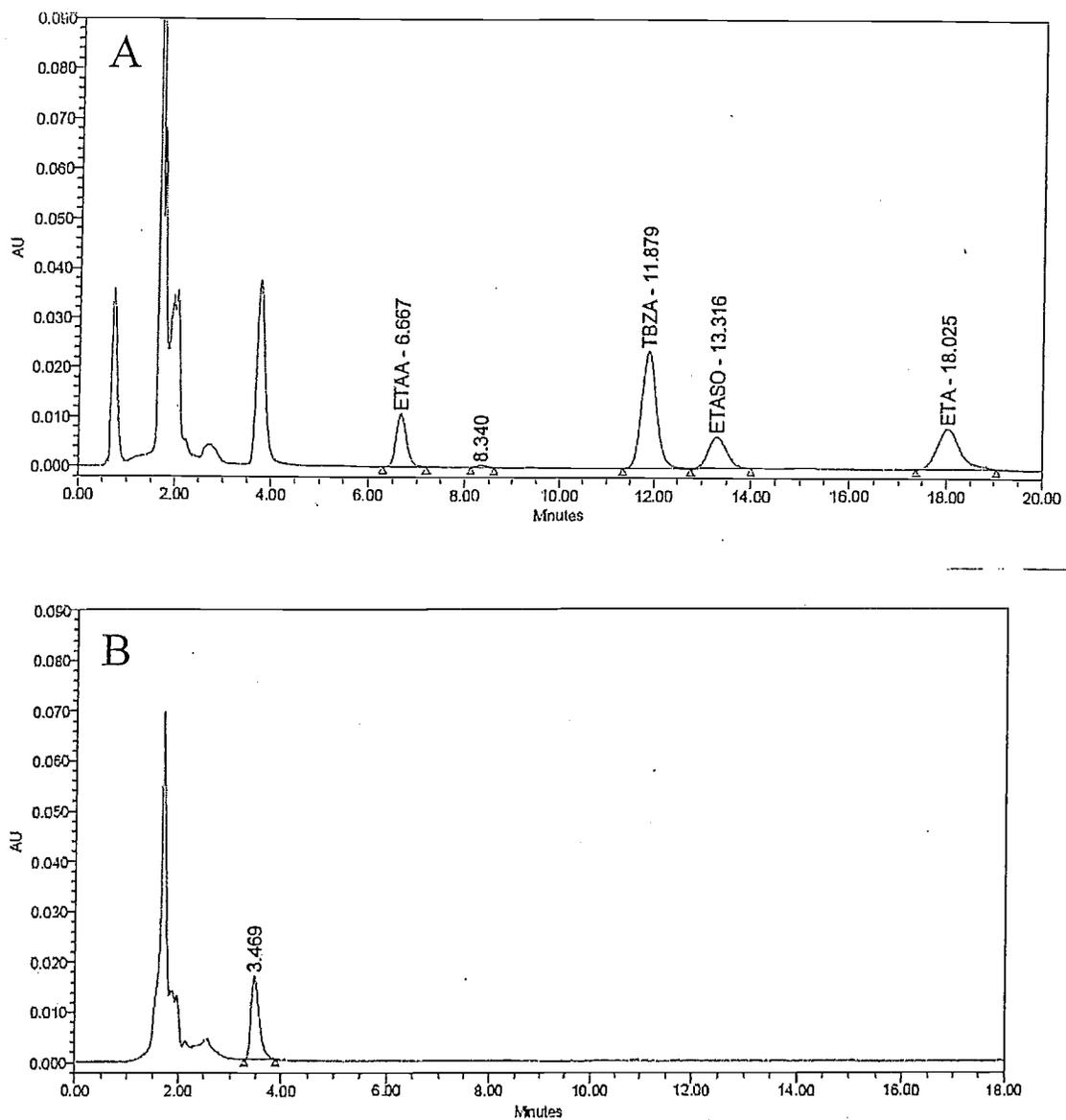


Fig. 4. HPLC chromatogram tracings monitored at UV 267 nm extracted from bovine plasma. a) 0.04 mM of ETA, metabolites, TBZA, and b) a blank plasma sample with no added compounds.

Table 1. Assay precision, recovery, and accuracy for ETA, ETASO, and ETAA determination in spiked plasma

	Spiked Conc. ( $\mu\text{g/ml}$ )			Measured Conc. (mean $\pm$ SD) ( $\mu\text{g/ml}$ )			Recovery (%) <sup>b</sup>			Accuracy (%) <sup>c</sup>		
	ETA	ETASO	ETAA	ETA	ETASO	ETAA	ETA	ETASO	ETAA	ETA	ETASO	ETAA
49.8	54.7	45.1	51.3 $\pm$ 0.33	56.0 $\pm$ 0.32	45.8 $\pm$ 0.10	103.0	102.4	101.6	2.9	2.4	1.6	
33.2	36.4	30.0	31.6 $\pm$ 0.25	34.0 $\pm$ 0.25	28.9 $\pm$ 0.19	95.2	93.4	96.3	-4.8	-6.6	-3.7	
16.6	18.2	15.0	15.7 $\pm$ 0.15	16.0 $\pm$ 0.15	14.8 $\pm$ 0.03	94.6	87.9	98.7	-5.4	-12.1	-1.3	
11.6	12.8	10.5	11.1 $\pm$ 0.10	12.3 $\pm$ 0.06	10.4 $\pm$ 0.02	106.0	96.1	99.0	-4.3	-3.9	-1.0	
6.6	7.3	6.0	5.0 $\pm$ 0.09	6.5 $\pm$ 0.03	5.8 $\pm$ 0.02	75.8	89.0	96.7	-24.2	-11.0	-3.3	

<sup>b</sup> Measured/spiked x 100%

<sup>c</sup> (Measured - spiked)/spiked x 100%

Table 2. Assay precision, recovery, and accuracy for ETA, ETASO, and ETAA determination in solvent

Spiked Conc. ( $\mu\text{g}/\text{mL}$ )			Measured Conc. (mean $\pm$ SD) ( $\mu\text{g}/\text{mL}$ )			Recovery (%) <sup>b</sup>			Accuracy (%) <sup>c</sup>		
ETA	ETASO	ETAA	ETA	ETASO	ETAA	ETA	ETASO	ETAA	ETA	ETASO	ETAA
49.8	54.7	45.1	49.8 $\pm$ 0.34	54.7 $\pm$ 0.28	45.0 $\pm$ 0.12	100.0	100.0	99.8	0.0	0.0	-0.2
33.2	36.4	30.0	33.8 $\pm$ 0.27	36.6 $\pm$ 0.22	30.1 $\pm$ 0.09	101.8	100.5	100.3	1.8	0.6	0.3
16.6	18.2	15.0	16.5 $\pm$ 0.14	18.5 $\pm$ 0.10	15.1 $\pm$ 0.04	99.4	101.6	100.7	-0.6	1.7	0.7
11.6	12.8	10.5	10.2 $\pm$ 0.05	11.3 $\pm$ 0.04	10.1 $\pm$ 0.02	87.9	88.3	96.2	-12.1	-11.7	-3.8
6.6	7.3	6.0	6.2 $\pm$ 0.05	6.8 $\pm$ 0.04	5.9 $\pm$ 0.01	93.9	93.2	98.3	-6.1	-6.9	-1.7

<sup>b</sup> Measured/spiked x 100%

<sup>c</sup> (Measured - spiked)/spiked x 100%

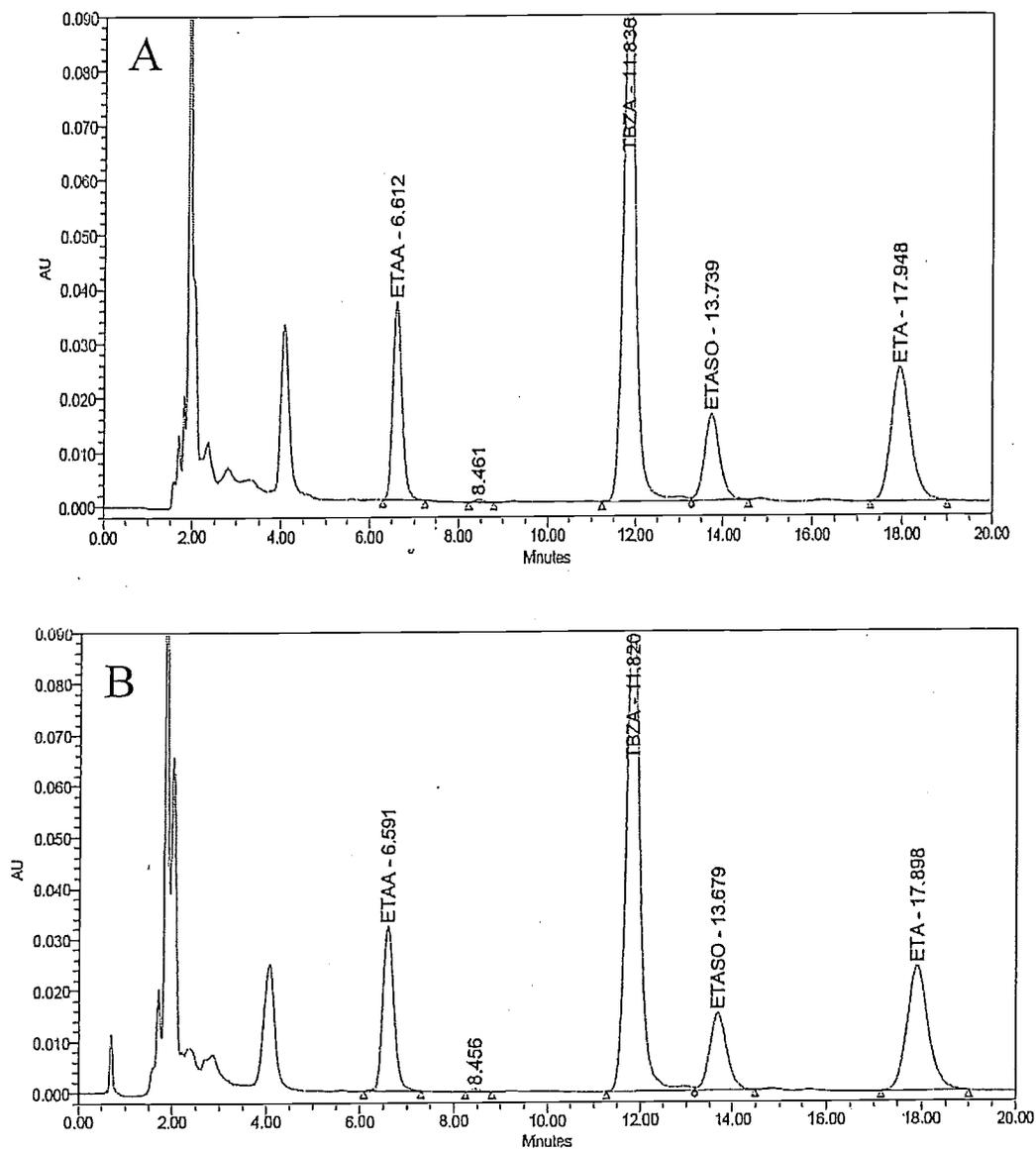


Fig. 5. HPLC chromatogram tracings monitored at UV 267 nm extracted from bovine plasma. a) spiked bovine plasma containing ETA and its metabolites with the internal standard thiobenzamide. b) A second aliquot of spiked bovine plasma containing the same compounds. The compound concentrations were 0.15 mM. These samples were stored over four months at  $-80^{\circ}\text{C}$ .

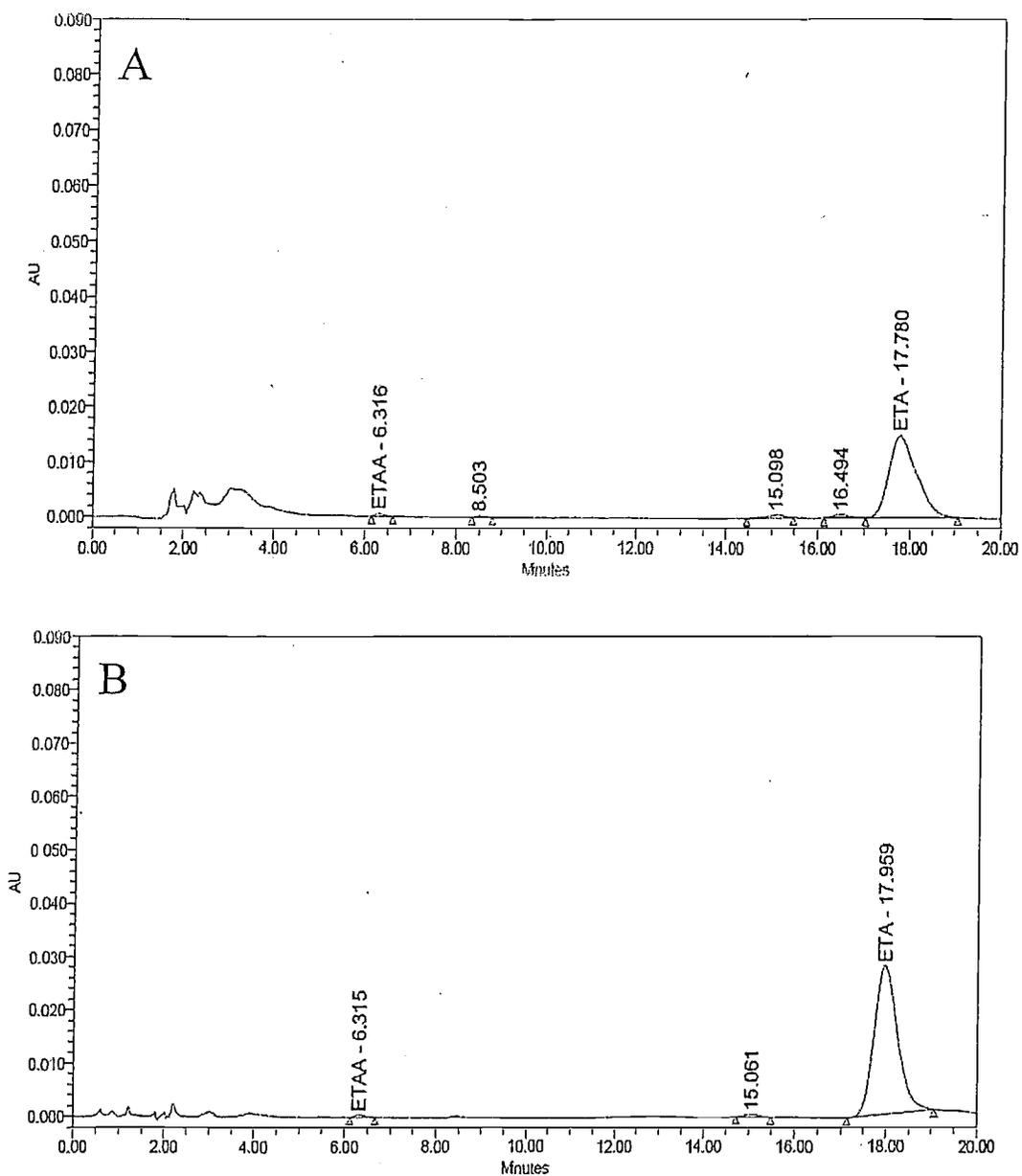


Fig. 6. HPLC chromatogram tracings monitored at UV 267 nm extracted from vet syrup. a) fresh ETA at 0.1 mM in vet syrup suspension, extracted the same day it was mixed, and b) 0.1 mM of ETA in vet syrup suspension stored at  $-80^{\circ}\text{C}$  for over one year.

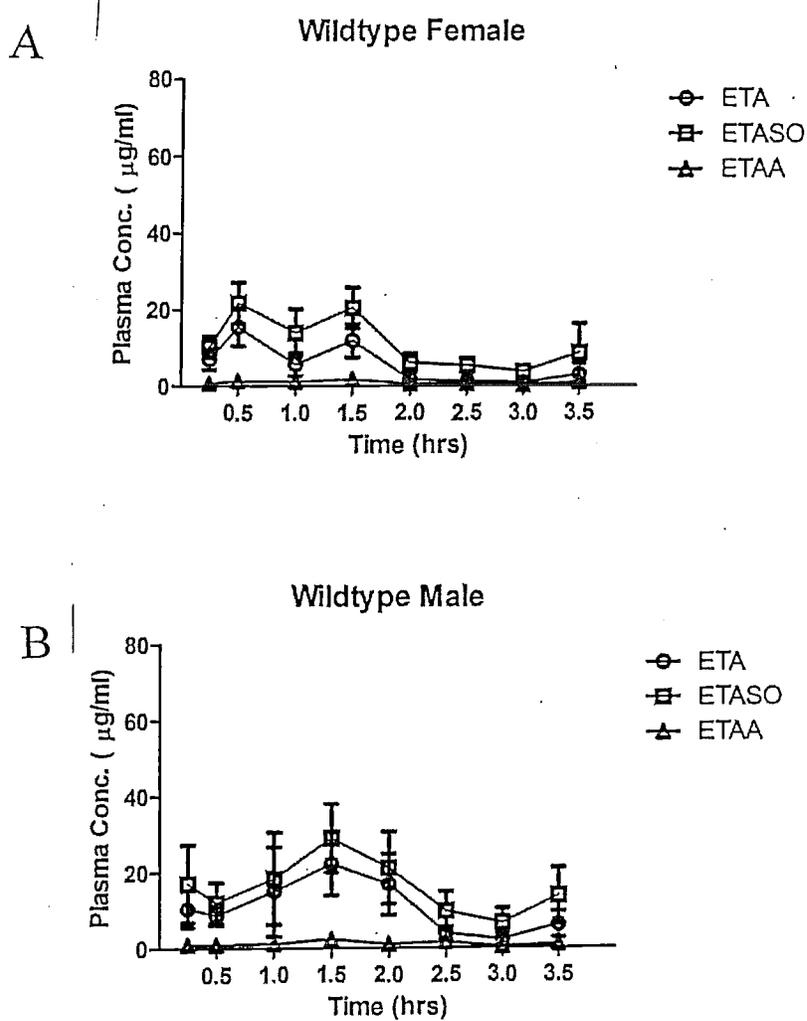


Fig. 7. Time course for metabolism within Wildtype mice. Metabolic graphs depicting a) the means of wildtype females for ETA, ETASO, and ETAA and b) the means of wildtype males for ETA, ETASO, and ETAA. The charts are based on time versus plasma concentration ( $\mu\text{g/ml}$ ), error bars are standard deviation.

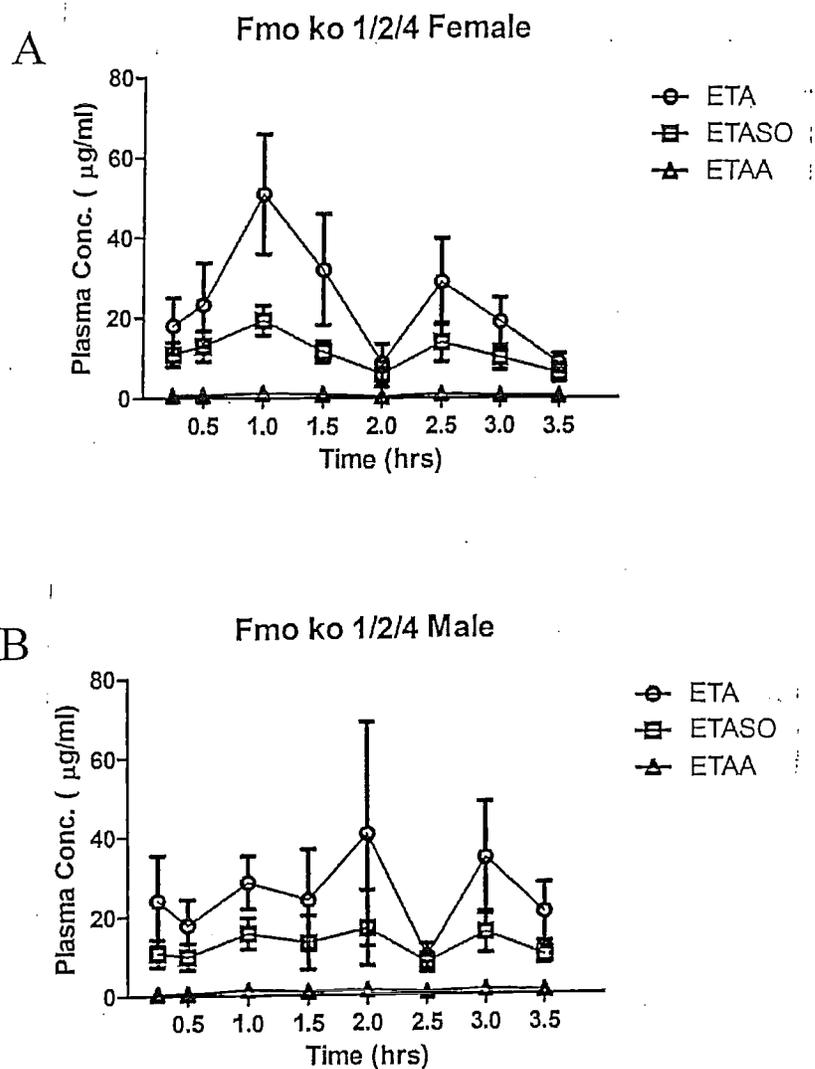


Fig. 8. Time course for metabolism within knockout mice. Metabolic graphs depicting a) the means of knockout females for ETA, ETASO, and ETAA and b) the means of knockout males for ETA, ETASO, and ETAA. The charts are based on time versus plasma concentration ( $\mu\text{g/ml}$ ), error bars are standard deviation.

Table 3. Pharmacokinetic parameters of ethionamide and its metabolites in mice.

Pharmacokinetic parameter	Value for mice <sup>a</sup>															
	C57BL6/J Male				C57BL6/J Female				Fmo 1/2/4 null Male				Fmo 1/2/4 null Female			
	No. of mice/ timepoint	ETA	ETASO	ETAA	No. of mice/ timepoint	ETA	ETASO	ETAA	No. of mice/ timepoint	ETA	ETASO	ETAA	No. of mice/ timepoint	ETA	ETASO	ETAA
$C_{max}$ ( $\mu\text{g/ml}$ )	4	22.3	29.0	2.4	4	15.5	21.7	3.0	4	40.6	16.9	1.4	4	51.0	19.4	1.3
$t_{max}$ (hr)	4	1.5	1.5	1.5	4	0.5	0.5	1.5	4	2.0	2.0	1.0	4	1.0	1.0	1.0
$AUC_{0-25h}$ ( $\mu\text{g}\cdot\text{hr/ml}$ )	4	36.1	52.3	4.0	4	18.1	36.4	3.0	4	83.5	42.4	3.4	4	83.1	38.2	2.7

<sup>a</sup> Shown as mean values

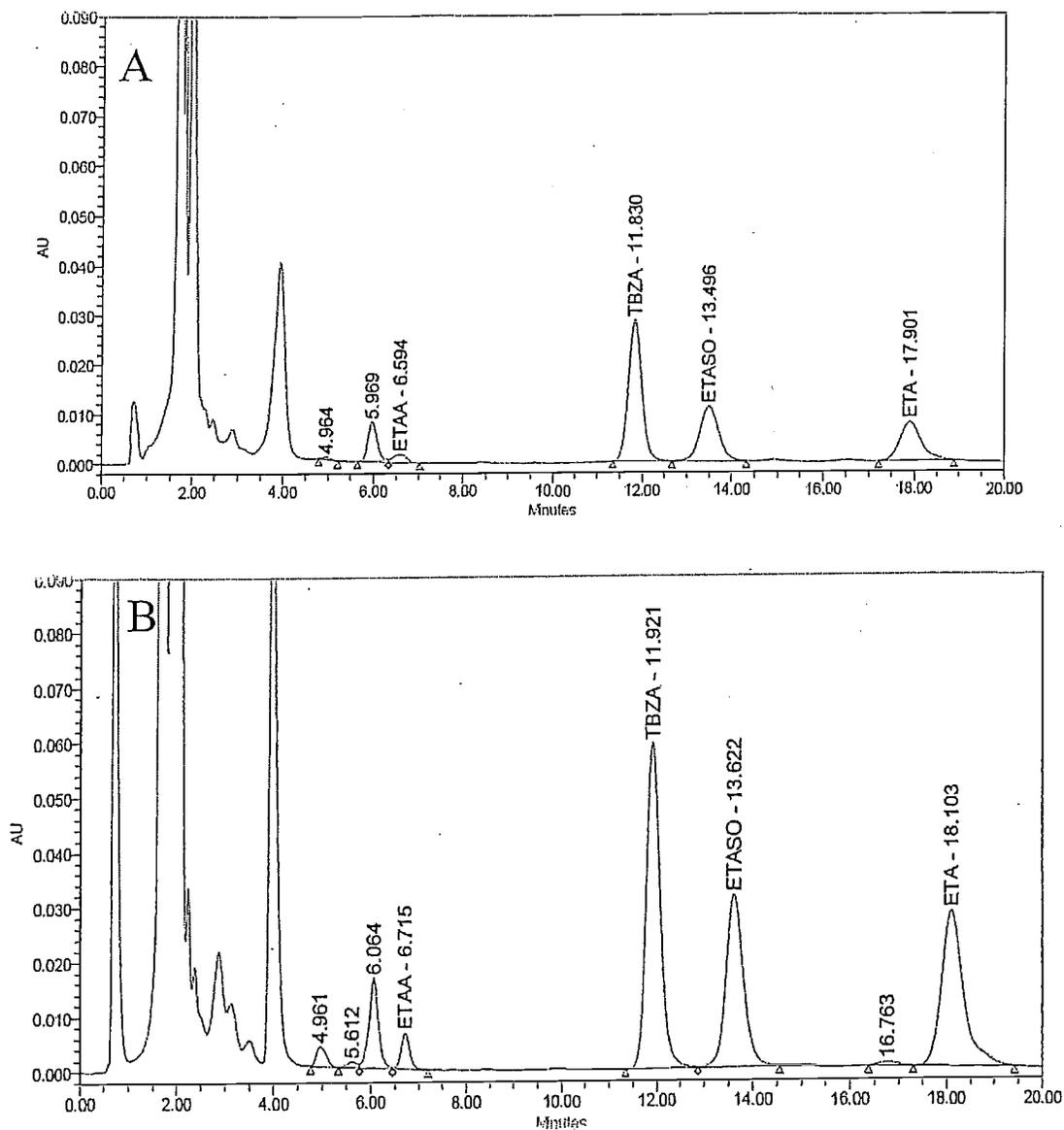


Fig. 9. Representative examples of HPLC analysis within wildtype mice. a) a single female wildtype mouse and b) a single male wildtype mouse. All time points are depicted at 1.5 hours.

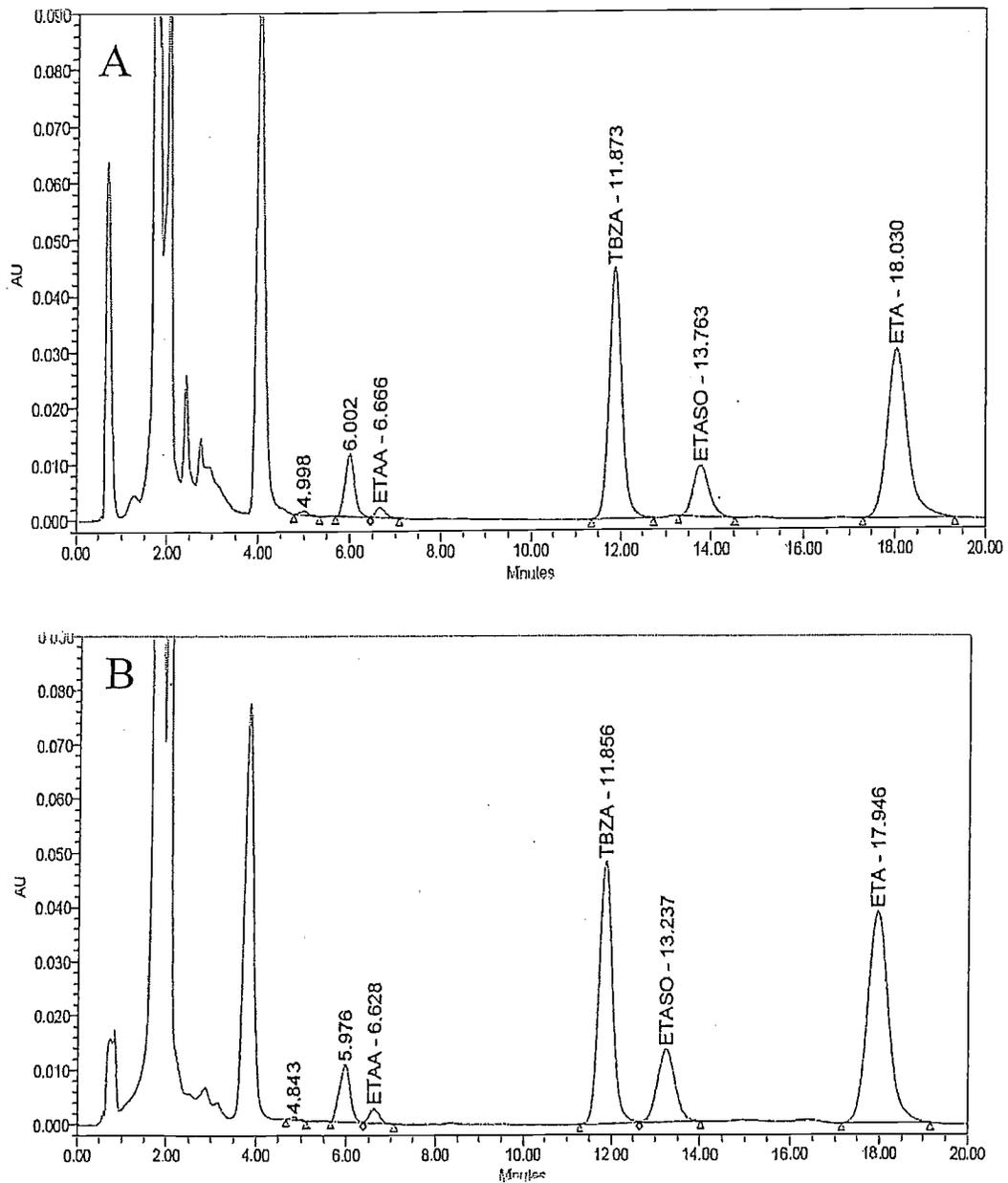


Fig. 10. Representative examples of HPLC analysis within knockout mice. a) a single female knockout mouse and b) a single male knockout mouse. All time points depicted are at 1.5 hours.

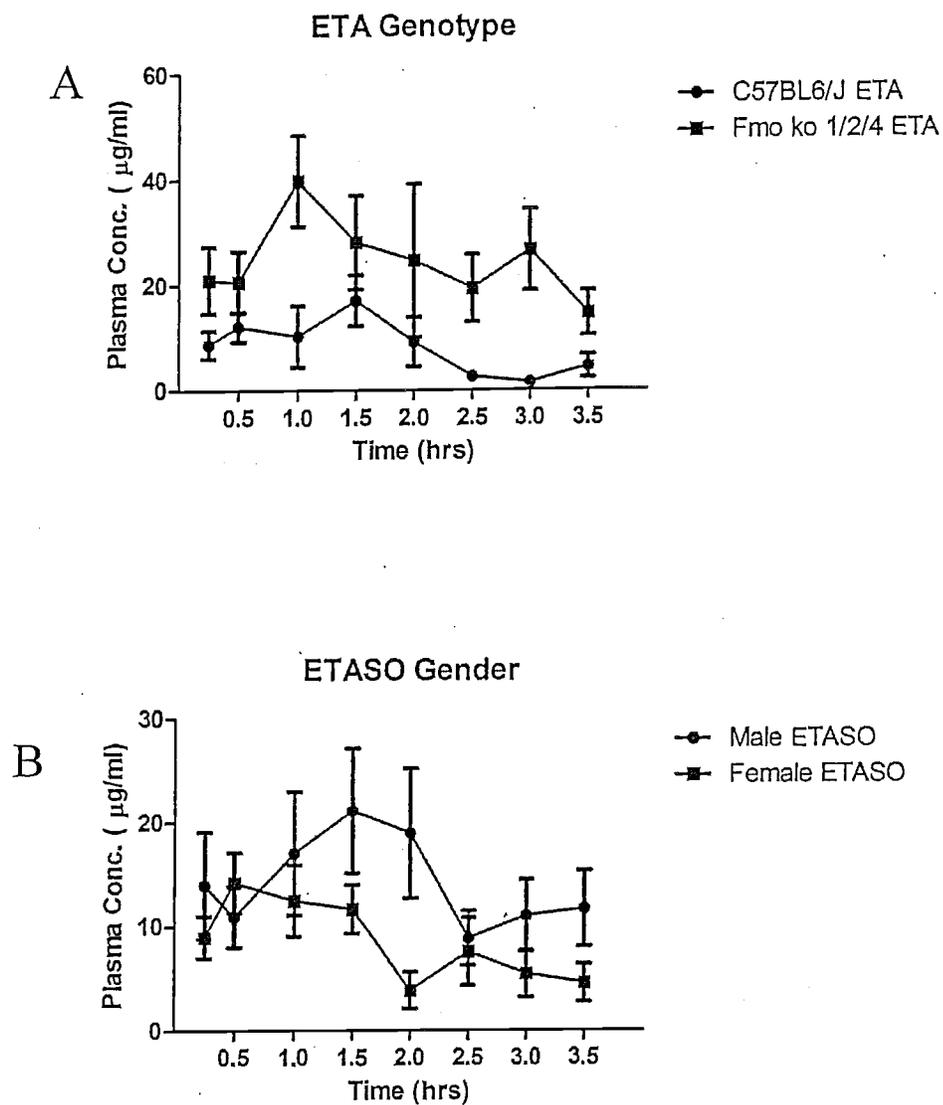


Fig. 11. Time course for metabolism in mice. Metabolic graphs depicting a) the means of wildtype mice for ETA against knockout mice and b) the means of male mice for ETASO against female mice. The charts are based on time versus plasma concentration ( $\mu\text{g/ml}$ ), error bars are standard Deviation

## 4 Conclusions

One-third of the world population is infected with *M. tuberculosis*, with millions more acquiring infection annually. The increased incidence of resistance to first-line antibiotics, including isoniazid and rifampicin, causes health organizations to recommend second-line antibiotics as first-line intervention [3, 4]. Second-line antibiotics possess less efficacy at clearing the infection resulting in protracted treatment regimens of six months to, in the case of MDRTB, over a year [3]. Drug tolerance of ethionamide, due to adverse physical side effects, results in treatment noncompliance in a significant percent of the population [3].

Toxicity from ETA is thought to occur through its S-oxygenation from FMO-mediated activation, much like *M. tuberculosis* bioactivation through EtaA [12]. In humans, multiple FMOs are able to carry out this reaction, including FMO1 (intestine/kidneys), FMO2.1 (lungs), and FMO3 (liver). Murine FMO activation of ETA is similar to FMO activation in humans [13]. Polymorphisms in these enzymes can lead to differing levels of toxicity between ethnic groups, mainly when comparing individuals of African/Hispanic descent and Caucasians. Being able to manipulate gene expression in mice by removing certain key gene clusters makes them a good model for understanding ETA metabolism [26, 27, 29].

To compare the metabolic differences of ETA between individuals with a functional FMO2 and individuals with a truncated, inactive FMO2, a novel *Fmo 1/2/4* null

mouse was utilized. This knockout mouse, created by Hernandez *et al.* [26], mimics the human lung environment of Caucasians and Asians where there are no active FMOs present. Mice differ from most mammals. FMO1 and 2 present are in the lung, the deletion of multiple FMO genes results in a better human model than an FMO2-only knockout. The wildtype C57BL/6J mice will have active FMO2 in the lung and best represent FMO expression among individuals of African and Hispanic descent.

Our HPLC analysis of FMO-dependent ETA oxygenation has demonstrates ETA was metabolized yielding the sulfenic acid (ETASO) and to a lesser degree, the carbodiimide (ETAA) (fig. 9a, b; 10a, b). The ETA plasma concentrations reached the MIC for *M. tuberculosis* and above for this drug in all mice ensuring that 125 mg/kg is an appropriate dose to treat infection [3, 4, 6]. Maximum concentrations of ETA in plasma measured in mice having functioning FMO2 averaged 22.26 and 15.33  $\mu\text{g/ml}$  (males and females respectively). In comparison, concentrations of ETA in plasma of mice having no functional FMOs were between two and three times higher (Table 3). The plasma levels of metabolites follow this trend as well. Approximately four times more parent compound is found in knockout females than wildtype, and approximately two times more in knockout males than wildtype (Table 3). This demonstrates that FMOs play an important role in ETA metabolism.

Peak concentrations of ETASO in males (mean 21.2  $\mu\text{g/ml}$ ) were two times greater than in females (mean 14.2  $\mu\text{g/ml}$ ). In female wildtype mice, ETASO peaked 30 minutes post-gavage; however with males ETASO peaked 1.5 hr post-gavage. The

difference between genders in mice can be attributed to the tissue distribution of FMOs. Female mice have substantial FMO3 localized in liver while males have markedly lower amounts. By removing specific FMOs in the knockout mouse, this difference was accentuated. This difference in FMO3 expression between male and female mice does not occur in humans. With the presence of ETA metabolism in knockout males an alternative activating enzyme capable of S oxygenation, such as cytochrome P450, could be at work.

The ratio of plasma levels between knockout females and males remained at approximately two times more parent compound than the first metabolite. Plasma levels of wildtype mice varied between 1.5 (males) and 2 times (females) greater amounts of ETASO than parent compound. The relative yield of ETASO in wildtype mice allows for greater depletion of the glutathione pool. Potential generation of more reactive oxygen species gives rise to increased oxidative stress in those individuals. Less conversion of ETA in individuals with no functional FMO2 can lower potential oxidative stress.

Taking into account the variations in enzymatic activity between ethnic groups can lead to the development of better drug regimes (“personalized medicine”). Decreased adverse side effects lead to more successful therapeutic compliance and bacterial clearance. The murine model applied in this study is adequate for modeling the genetic polymorphism for pulmonary FMO2 in humans.

Results from this study raise some issues that can be addressed in the future. With this model a closer investigation of oxidative stress in the lungs of these mice can determine, more accurately, ETA toxicity. Hepatotoxicity and gastrointestinal discomfort may result from ETA metabolism by FMO3 located in the liver; it is possible that side effects could be avoided by changing the route of administration. Since TB occurs primarily in the lungs, a delivery system of this drug could be devised through inhalation. A smaller dose of drug delivered by inhalation would avoid the GI tract by initially targeting the lung, decreasing overall toxicity. Future studies could be conducted utilizing this mouse model during active infection of TB to observe the efficacy and host/pathogen relations between the bioactivation of ETA by mouse FMOs compared to the extent of bioactivation by *M. tuberculosis* monooxygenase EtaA by ETA administration.

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