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Formation of N-Oxide metabolites from isoniazid hydrazones of substituted benzaldehydes by hepatic washed pig microsomal preparations

Göknil Pelin COŞKUN [1] *, Elif Göksu ALGIN[1] Jülide Cansu KARAKOÇ[1] Başak TÜRK ERBUL[1] Mert ÜLGEN[1]

[1] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Acibadem Mehmet Ali Aydınlar University, 34684, İçerenköy, Istanbul, Türkiye.

* Corresponding Author. E-mail: goknilpelincoskun@gmail.com (G.P.C.); Tel. +90-537-064 45 42.

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ABSTRACT: Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*. It is highly transmissible through air droplets suspended in the air. Isoniazid has long been the first-choice drug for pulmonary and extra-pulmonary tuberculosis treatment and prophylaxis. It was first synthesized by Malley and Meyer in 1912. It is known that isoniazid is metabolized through hydrolysis, but there are less information on its metabolism into N-oxide metabolites in the presence of hydrazone structure. In this study, three different hydrazone structures ie 2-hydroxyphenyl, 4-methylphenyl and 4-bromophenyl derivatives were synthesized from the isoniazid with substituted aldehydes. Their purity were proven by chromatographic analysis. The potential N-oxide metabolites and the corresponding aldehyde hydrazones were synthesized and their structures were elucidated by spectroscopic methods. All the hydrazone derivatives were incubated with pig liver microsomal preparations fortified with NADPH. The formation of the metabolites were observed using LC-MS. The results indicated that 2-hydroxyphenyl and 4-methylphenyl derivatives produced N-oxide metabolite in the presence of enzyme and co-factors (NADPH) and 4-bromophenyl derivative produced N-oxide metabolite in the presence of enzyme but in the absence of co-factors. Hydrolysis was detected for only 4-methylphenyl and 4-bromophenyl derivatives (independently with enzyme and co-factor) but not with 2-hydroxyphenyl derivatives. These results indicated that the hydrazone derivatives might undergo metabolic oxidation reaction rather than hydrolytic reaction. The present study is the first example of N-oxide formation from hydrazide hydrazones bearing a pyridine ring.

KEYWORDS: Isoniazid; tuberculosis; N-oxide; hydrazine; metabolism

1. INTRODUCTION

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*. The disease commonly affects the lungs but may affect almost any organ system, including the lymph nodes, central nervous system, liver, bones, genitourinary tract, and gastrointestinal tract. The World Global Tuberculosis Report 2021 pronounced that tuberculosis became the second leading cause of death after COVID-19 pandemic. Tuberculosis is highly transmissible through respiratory droplets [1]. In the treatment of pulmonary and extra-pulmonary tuberculosis, isoniazid is the first line of drug therapy. It has the property of being one of the few drugs that is powerful against all tuberculosis bacteria. When administered, isoniazid rapidly and completely absorbs into the body. Metabolism occurs in the liver. The rate of this metabolism is determined by factors like age, sex, environment etc. Since hepatotoxicity and peripheral neuropathy are commonly seen adverse effects, it is usually administered together with pyridoxine [2]. The World Health Organization (WHO) estimates that since 2015, tuberculosis has outweighed human immunodeficiency virus infection and acquired immunodeficiency syndrome (HIV/AIDS) as the leading cause of death caused by an infectious disease. About 95% of tuberculosis cases are seen in the developing world. Asia, Africa and the eastern Mediterranean region are the hosts to the maximal number of cases in the world.

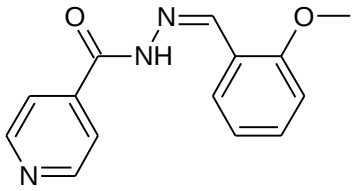
Hydrazones are synthesized from hydrazides and aldehydes in the presence of different solvents. Hydrazones may need to be catalyzed by a concentrated acid, seeing that they possess a sort of "imine"

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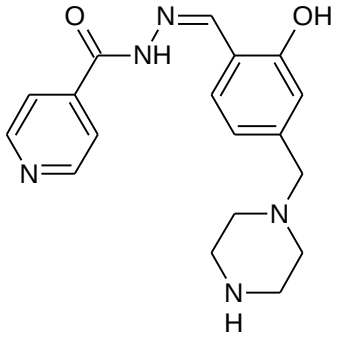
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function in their structure. The resulting products, just like imine structures, are stable enough to not be affected by water. Rollas and colleagues have stated that hydrazone and derivatives are multifaceted, that these compounds have the structure of R_1R_2C=NNH- and that they have an azomethine proton –NHN=CH- [3]. The nitrogen atoms of hydrazone exhibit nucleophilic, and the carbon atom exhibits both electrophilic and nucleophilic characteristics [4]. The literature studies presented many hydrazone derivatives of isoniazid. The studies indicated an augmented biological profile of these derivatives against *Mycobacterium tuberculosis* strains. Mainly, halogenated derivatives of isoniazid hydrazones showed remarkable biological activity. However, none of these studies showed metabolic stability or metabolic profile for these derivatives (**Figure 1**).

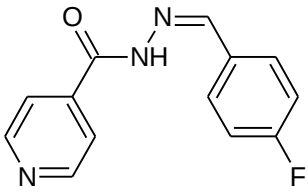


(Kumar, 2014, 0.28 µM IC_{50} value on tuberculosis strains)



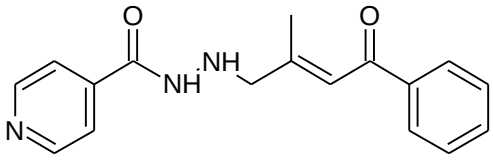
(Sriram, 0.56 mg/ml MIC value on tuberculosis strains)

[6]



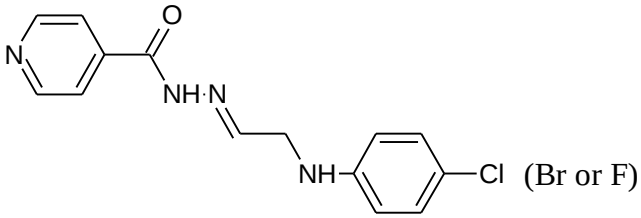
(Kumar, 2014, 0.65 µM IC_{50} value on tuberculosis strains)

[5]



(Pavan, 3.13 mg/ml MIC value on tuberculosis strains)

[7]



(Active on tuberculosis strains)

[8]

Figure 1. Antituberculostatic isoniazid hydrazone compounds

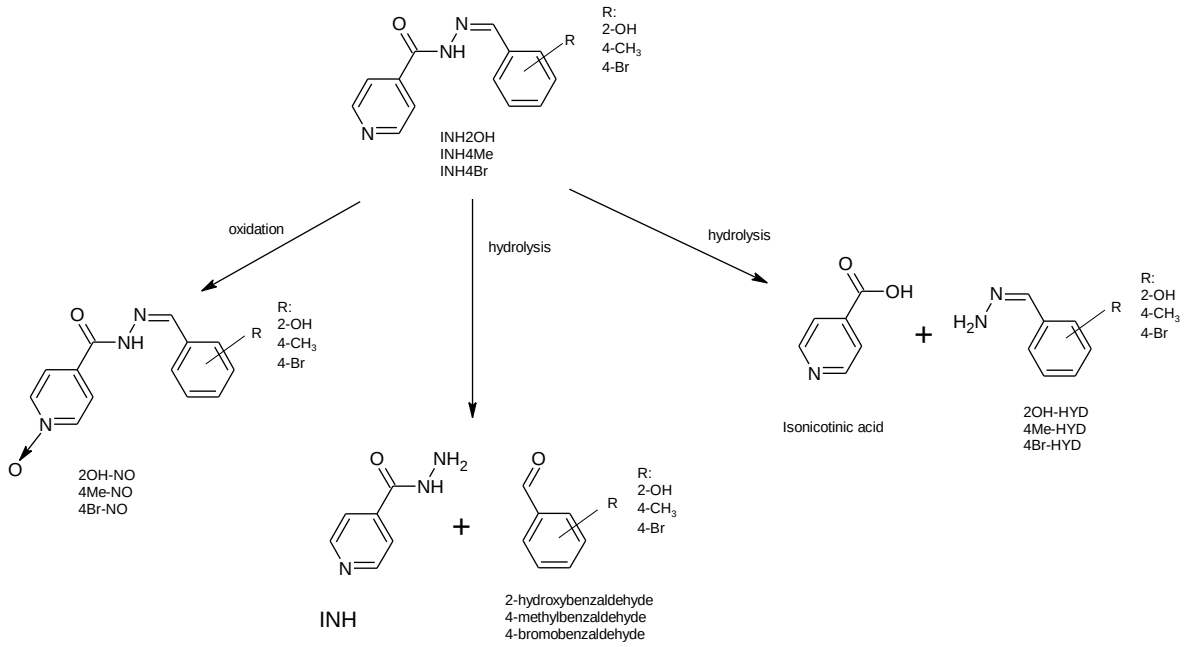
Literature search releaved that there is limited data available for the metabolic study of hydrazones in the literature. Kömürcü and co-workers showed the enzymatic hydrolysis of 4-amino-N'-[(Z)-(4-fluorophenyl)methylidene]benzohydrazide. The study indicated that compound was hydrolysed in the presence of microsomes and co-factors. The hydrolytic products were recorded as corresponding hydrazide and 4-fluorobenzaldehyde [9]. A prototype and model hydrazone compound was investigated by Ulgen et

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al. Compound N'-[(Z)-phenylmethylidene]benzohydrazide undergone enzymatic oxidation and hydrolytic reaction. N'-[(Z)-(4-hydroxyphenyl)methylidene]benzohydrazide metabolite was formed as oxidative product. In the case of hydrolysis, benzoic acid hydrazide and benzaldehyde was detected after the microsomal metabolism study of N'-[(Z)-phenylmethylidene]benzohydrazide [10]. In the present study, we have synthesized isoniazid hydrazones and their authentic oxidative and hydrolytic metabolites. We have studied in vitro microsomal metabolism of these compounds with pig liver microsomes. The results indicated a remarkable perspective on the metabolic profile of hydrazone structures.

2. RESULTS AND DISCUSSION

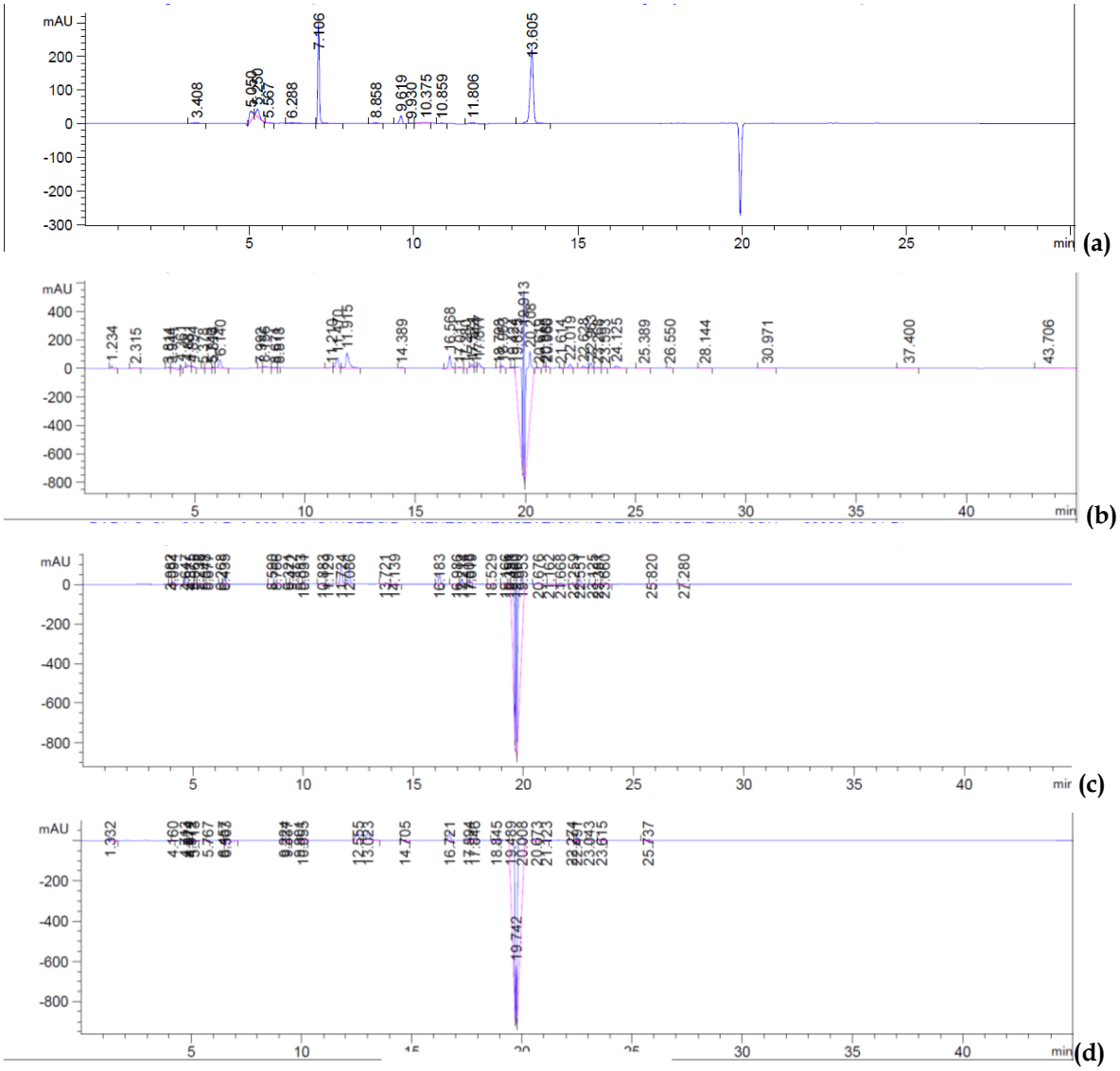
All the substrates [**INH2OH** N'-[(E)-(2-hydroxyphenyl)methyliden]pyridin-4-carbohydrazide, **INH4Me** N'-[(E)-(S4-methylphenyl)methyliden]pyridin-4-carbohydrazide and **INH4Br** N'-[(E)-(4-bromophenyl)methyliden]pyridin-4-carbohydrazide] were synthesized according to the previously reported methods [11, 12, 13]. N-oxides were synthesized with *m*-CPBA oxidation of corresponding hydrazones the presence of acetone. The reaction was started in cold conditions in order to keep the oxidizing agent stable. Then, the reaction continued by the addition of *m*-CPBA dropwise in acetone. Since N-oxides are known to be light sensitive, the reaction was performed in the dark. The reaction mixture was monitored by TLC. After a period of 30 days, the reaction was terminated and the spot corresponded to the N-oxide was analysed by TLC and LC-MS. The results from LC-MS analysis proved the formation of N-oxides although these were not stable in methanolic solution. The aldehyde hydrazones (the other hydrolytic metabolite) were synthesized according to reported method [9]. The in vitro microsomal metabolism studies were performed for all hydrazones. The expected metabolic profile of the substrates [INH2OH, INH4Me and INH4Br] were shown in **Scheme 1**.



Scheme 1: The potential metabolic pathways of substrates INH2OH, INH4Me and INH4Br

In vitro metabolism of INH2OH resulted in the formation of N-oxide metabolite. But, no hydrolytic product was detected for this substrate. The control experiments proved that the oxidation process in this case needs both microsomes and co-factors. Since the hydroxyl group on the phenyl ring can provide a polarity, this substrate may not need to any hydrolytic reaction and the N-oxidation was the only metabolic product observed (**Scheme 2**).

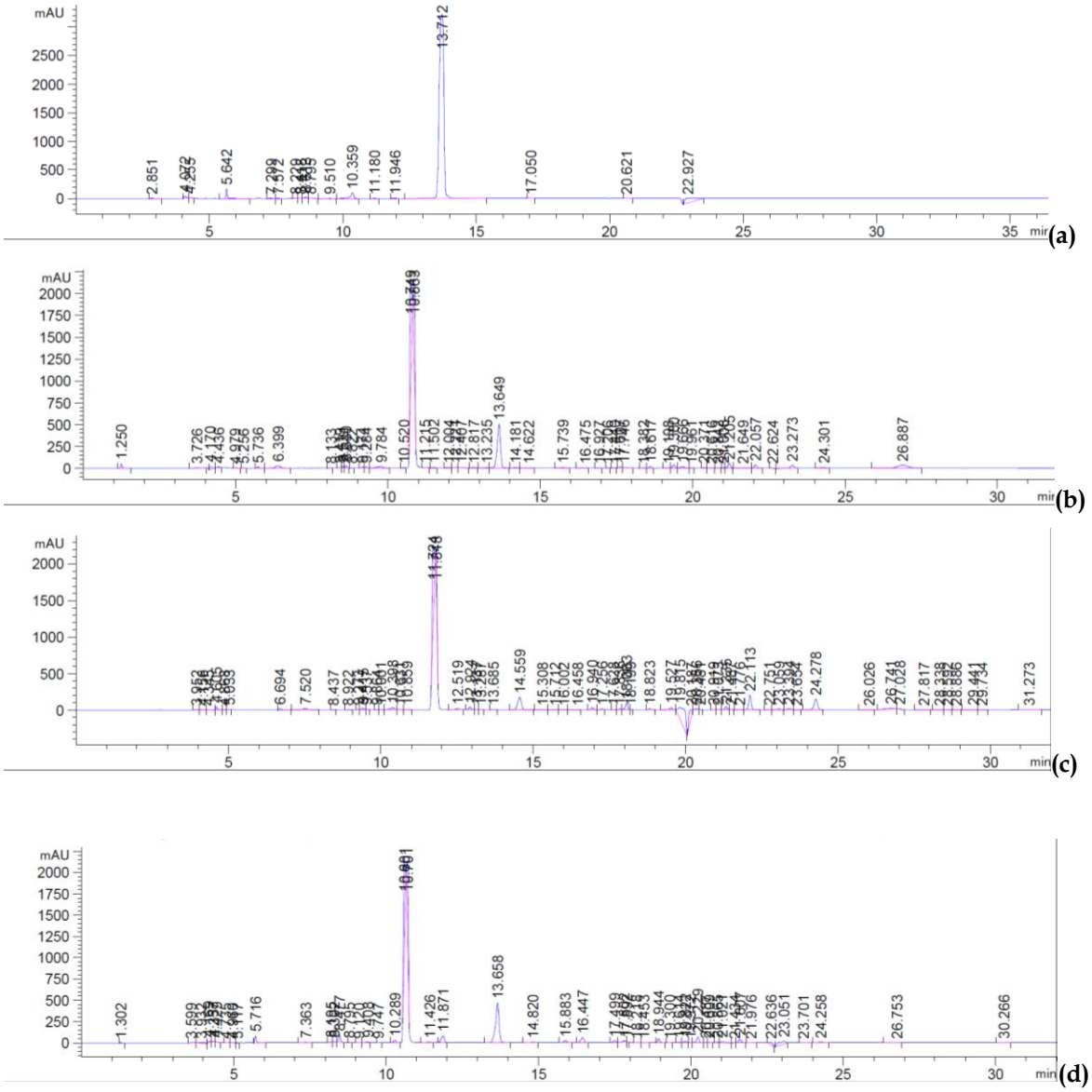
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Scheme 2. *In vitro* microsomal metabolic results of substrate INH2OH **(a)** HPLC chromatogram of INH2OH (substrate) and its authentic metabolites (Rt (min) INH: 5.05; 2OH-HYD: 7.10; 2OH-NO: 9.93; 2-hydroxybenzaldehyde: 13.60; INH2OH: 19.90) ; **(b)** INH2OH metabolism (in the presence of microsomes and co-factors) (Rt (min) metabolically formed 2OH-NO: 11.47, INH2OH: 19.91 and all other peaks resulted from microsomal environment); **(c)** INH2OH control mixture (denaturated microsome) (Rt (min) INH2OH: 19.90, all other peaks resulted from microsomal environment); **(d)** HPLC chromatogram of INH2OH control mixture (no co-factor) (Rt (min) INH2OH: 19.74, all other peaks resulted from microsomal environment).

In vitro metabolism of INH4Me also resulted with the formation N-oxide metabolite which was required both microsomes and co-factors. Hydrolytic products were observed in all test and control experiments. In the case of this substrate, as the methyl group on the phenyl ring can decrease the polarity, the hydrolytic pathway will be dominant. A very small amount of hydrolytic product was also observed in the absence of enzymes and co-factors (**Scheme 3**).

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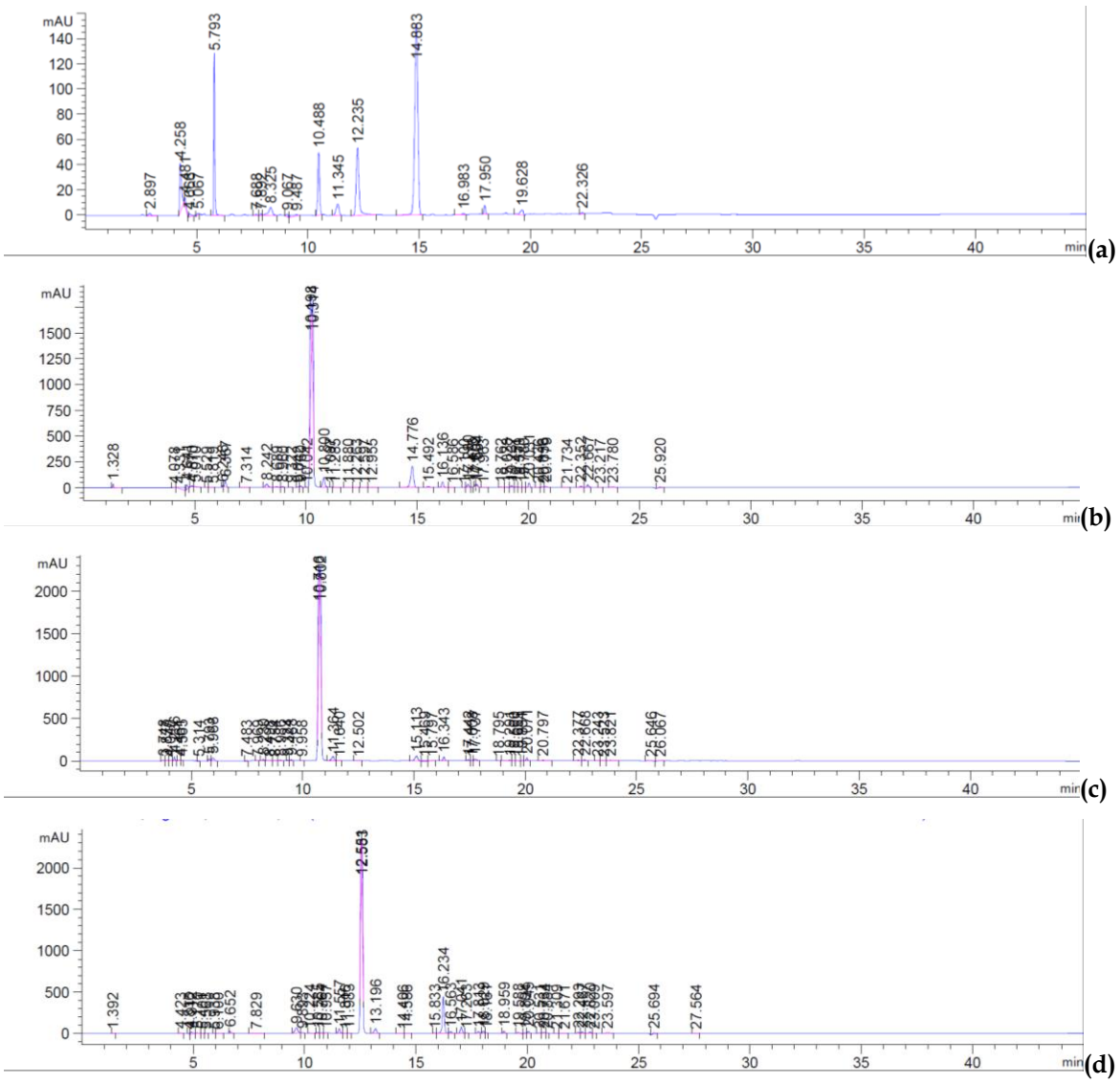


Scheme 3. *In vitro* microsomal metabolic results of substrate INH4Me **(a):** HPLC chromatogram of INH4Me (substrate) and its authentic metabolites (Rt (min) INH: 5.64; 4Me-NO: 9.51; INH4Me: 10.35; 4-methylbenzaldehyde: 13.71, 4Me-HYD: 22.92) **(b):** INH4Me metabolism (in the presence of microsomes and co-factors) (Rt (min) metabolically formed INH: 3.72; metabolically formed 4Me-NO: 9.78; INH4Me: 10.74; metabolically formed 4-methylbenzaldehyde: 13.64 and all other peaks resulted from microsomal environment). **(c):** INH4Me control mixture (denaturated microsome) (Rt (min) metabolically formed INH: 3.95; INH4Me: 11.72; metabolically formed 4-methylbenzaldehyde: 14.55, all other pears resulted from microsomal enviroment). **(d):** INH4Me control mixture (no co-factor) (Rt (min) 3.59: metabolically formed INH: 3.59; INH4Me: 10.60; metabolically formed 4-methylbenzaldehyde: 13.65, all other peaks resulted from microsomal environment).

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In vitro metabolism of INH4Br also revealed the formation of corresponding N-oxide. Surprisingly, this metabolite was observed in the control experiment in the absence of co-factors. It was understood that the oxidation product of INH4Br does not require co-factor. Hydrolytic products were observed in all test and control experiments (**Scheme 4**).



Scheme 4. *In vitro* microsomal metabolic results of substrate INH4Br **(a):** HPLC chromatogram of substrate INH4Br (substrate) and its authentic metabolites (Rt (min) INH: 4.25; 4Br-NO: 9.48; INH4Br: 10.48; 4-Br-HYD: 12.23; 4-bromobenzaldehyde: 14.88) **(b):** INH4Br metabolism (in the presence of microsomes and co-factors) (Rt (min) metabolically formed INH: 4.07; metabolically formed 4Br-NO: 8.95, INH4Br: 10.39; metabolically formed 4-bromobenzaldehyde: 14.77 and all other peaks resulted from microsomal environment). **(c):** INH4Br control mixture (denaturated microsome) (Rt (min) metabolically formed INH: 2.7; INH4Br: 10.7, metabolically formed 4-bromobenzaldehyde: 15.11, all other pears resulted from microsomal enviroment). **(d):** INH4Br control mixture (no co-factor) (Rt (min) metabolically formed INH: 4.42; metabolically formed 4Br-NO: 10.15; INH4Br: 12.58; metabolically formed 4-bromobenzaldehyde: 15.92, all other peaks resulted from microsomal environment).

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3. CONCLUSION

Tuberculosis is a morbid disease and caused tremendous negative effects on human health. The research on developing new drugs on tuberculosis is a therefore major field of medicinal chemistry. The literature data revealed the antimicrobial activity of hydrazone derivatives of isoniazid. In the present study, we have synthesized some previously reported hydrazone derivatives of isoniazid and studied their in vitro microsomal metabolism. Although these were promising drug candidates against tuberculosis, there is no detailed metabolic study reported for these compounds. Here we investigated the substituent effects of these substrates on the N-oxidation and hydrolysis reactions. The formation of oxidation and hydrolysis were discussed. Our study showed that the substrate with hydroxyl derivative showed only N-oxidative metabolite while other substrates gave both N-oxidative and hydrolytic metabolites. Surprisingly, the substrate with bromine substituent produced N-oxidation metabolite in the absence of co-factors. This study is a light on the future drug development process of investigating anti tuberculostatic agents.

4. MATERIALS AND METHODS

4.1.General

All the chemicals (including *m*-Chloroperoxybenzoic acid (*m*-CPBA)) were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO). Reactions were monitored by TLC on silica gel plates purchased from Merck (Merck Co., Darmstadt, Germany). Melting points of the synthesized compounds were determined in a Stuart SMP50 Automatic Melting Point apparatus and these are uncorrected. The purity of the compounds was confirmed by TLC, LC-MS An Agilent 1260 Infinity II LC-MS spectra equipped with G7114A 1260DAD detector, G7311B 1260 Quad Pump system, G1328C 1260 manual injection unit and G6125B LC/MSD detector was used for both HPLC and mass analysis. Retention times were recorded with ACE C18 coloumn (particle size: 3 µm, pore size: 100A). The column temperature was adjusted to 25ºC in the column compartment. The mobile phase consisted of acetonitrile-water (gradient elution) mixture and delivered at a flow rate of 0.8 ml/min. The injection volume was 20 µL. The UV detector was operated at 254 nm. Rfx100 values were recorded on benzene:MeOH (9:1,v/v).

4.1.1.Synthesis of substrates and potential metabolites

4.1.1.1.Synthesis of N'-[(E)-(Substitutedphenyl)methyliden]pyridin-4-carbohydrazide [INH2OH, INH4Me and INH4Br]:

Isoniazid (0.01 mol) was dissolved in ethanol (20 ml) and equimolar amount of substituted benzaldehyde and refluxed for 24 hours. The reactions were monitored by TLC. After the completion of the reaction, the precipitate was filtered off and crystallized from ethanol. N′-[(E)-(2-hydroxyphenyl)methylidene]pyridine-4-carbohydrazide [INH2OH]: white colored compound. Yield: 75%. Melting Point: 216.5 ºC. Soluble in chloroform, DMSO. Rf x 100 value: 16 (Benzene:MeOH 9:1) (T: 21ºC). Rt value (min): 19.833min. Mass Spectrum: [M+1] calculated 242, found [M+1] 242.1 m/z. ; N′-[(E)-(4-methylphenyl)methylidene]pyridine-4-carbohydrazide [INH4Me]: white colored compound. Yield: 79%. Melting point: 189 ºC. Soluble in chloroform, DMSO. Rf x 100 value: 27.08 (Benzene:MeOH 9:1) (T: 21ºC). Rt value (min): 6.268 min. Mass Spectrum: [M+1] calculated 240, found [M+1] 240.1 m/z. N′-[(E)-(4-bromophenyl)methylydene]pyridine-4-carbohydrazide [INH4Br]: white colored compound. Yield: 82% .Melting point: 214.6 ºC. Soluble in chloroform, DMSO. Rf x 100 value: 7.69 (Benzene:MeOH 9:1) (T: 21□C) Rt value (min): 12.488 min. Mass Spectrum: [M-1] calculated 304, found [M-1] 304.0 m/z.

4.1.1.2.Synthesis of N′-[(E)-(substitutedphenyl)methylydene]pyridine-4-carbohydrazide-N-oxide [2OH-NO, 4Me-NO and 4Br-NO]:

Compounds INH2OH, INH4Me and INH4Br (0.001 mol) were dissolved in acetone and *m*-chloroperbenzoic acid (*m*-CPBA) (0.0012 mol) was added drop by drop in cold (0-5 ºC). The reaction mixture was covered with aluminum foil and placed in room temperature and allowed to stir. The reaction was completed in 30 days and kept in dark at all times. After the completion of the reaction, the formed N-oxide was found in liquid media and evaporated under vacuum. The compound was further purified by column chromatography. N′-[(E)-(2-hydroxyphenyl)methylydene]pyridine-4-carbohydrazide-N-oxide [2OHNO]: off white to yellow colored compound. Yield: 29%. Melting point: 170 ˚C. Soluble in chloroform, DMSO. Rf x 100 value: 66.66 (Hexane:Methylacetate 8:2). Rt value (min): 9.797 min. Mass Spectrum: [M+1] calculated 257,

found [M+1] 257.2 m/z for. N'-[(E)-(4-methylphenyl)methylydene]pyridine-4-carbohydrazide-N-oxide [4MeNO]: off white to yellow colored compound. Yield: 25%. Melting point: 126.5 °C. Soluble in chloroform, DMSO. Rf x 100 value: 13.28 (Benzene:MeOH 9:1). Rt value (min): 10.521 min. Mass Spectrum: [M+1] calculated 257, found [M+1] 257.2 m/z for. N'-[(E)-(4-bromophenyl)methylydene]pyridine-4-carbohydrazide-N-oxide [4BrNO]: off white to yellow colored compound. Yield: 28%. Melting point: 126 °C. Soluble in chloroform, DMSO. Rf x 100 value: 11.11 (Benzene:MeOH 9:1). Rt value (min): 9.49 min. Mass Spectrum: [M+1] calculated 322, found [M+1] 322 m/z.

4.1.1.3.Synthesis of (1E)-(substitutedbenzylidene)hydrazine [2OH-HYD, 4Me-HYD and 4Br-HYD]:

Substituted benzaldehyde (0.01 mol) was dissolved in ethanol and hydrazine solution (5 ml) was added at room temperature. The reaction was monitored by TLC. After 24 hours, the precipitate was filtered off and crystallized from ethanol.

(2-hydroxybenzylidene)hydrazine [2OH-HYD]: off white compound. Yield: 87%. Melting point: 242 °C. Soluble in chloroform, DMSO. Rf x 100 value: 21.30 (Benzene:MeOH 9:1). Rt value (min): 11.29 min. Mass spectrum: [M-1] calculated 136, found [M-1] 136 m/z. (4-methylbenzylidene)hydrazine [4Me-HYD]: off white compound. Yield: 88%. Melting point: 155 °C. Soluble in chloroform, DMSO. Rf x 100 value: 70.83 (Benzene:MeOH 9:1). Rt value (min): 22.65 min. Mass spectrum: [M+1] calculated 135, found [M+1] 135 m/z. (4-Bromobenzylidene)hydrazine [4Br-HYD]: off white compound. Yield: 89%. Melting point: 103 °C. Soluble in chloroform, DMSO. Rf x 100 value: 39.66 (Benzene:MeOH 9:1). Rt value (min): 12.37 min. Mass spectrum: [M+1] calculated 200, found [M+1] 200.4 m/z.

4.1.2.Preparation of pig liver microsomes and incubation and extraction procedures

Adult male Suffolk white pig was used in this study. ß-Nicotinamide dinucleotide phosphate (disodium salt, NADP) and glucose-6-phosphate (disodium salt, G-6-P) were purchased from Sigma. Glucose-6-phosphate dehydrogenase suspension (Reinheit grade II, 10 mg per 2 ml; G-6-PD) was obtained from Sigma Aldrich. Dichloromethane was obtained from Merck. The animals were deprived of food overnight prior to sacrifice, but were allowed water ad libitum. They were previously fed on a balanced diet. Hepatic washed pig microsomes were prepared as described by Schenkman and Cinti [14] and Ulgen [15]. Incubations were carried out in a shaking water-bath at 37 °C using a standard co-factor solution consisting of NADP (2 µmole), G-6-P (10 µmole), G-6-PD suspension (1 unit) and aqueous $MgCl_2$ (50% w/w) (20 µmole) in phosphate buffer (0.2M, pH 7.4, 2 ml) at pH 7.4. Co-factors were pre-incubated for 5 min to generate NADPH, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (5 µmole) in methanol (50 µl). Briefly, seven test tubes for each substrate [INH2OH, INH4Me, INH4Br] were prepared (3 for test, 4 for controls) and co-factors (2ml in each tube), microsomal fraction (1 ml for each tube) and substrate (50 µl for each tube) were added respectively (Table 1). The incubation was continued for 30 min, terminated and extracted with dichloromethane (3x5 ml). The organic extracts were evaporated to dryness under a steream of nitrogen. The residues were reconstituted in methanol (200 µl) for LC-MS. The reconstituted extracts were analysed using the reverse-phase LC-MS system described in the text.

Table 1: Contents of the co-factor solution for each tube

NADP disodium	**1.57 mg**	**2 µmol**
G6P disodium	3.04 mg	10 µmol
G6P dehydrogenase	1.40 µL	1 unit
$MgCl_2$ (50% w/w)	8.00 µL	20 µmol

The materials above were prepared right before incubation, by dissolving in 2 mL of phosphate buffer for one incubation tube. The G-6-P dehydrogenase enzyme was added to the co-factor solution right before pre-incubation. All co-factors were "pre-incubated" at a 37°C water bath for 5 minutes, to cause NADPH creation. The amount of microsomal preparation added into each incubation tube was 1 ml, and the co-factor solution, 2 ml. Control tubes were also prepared (**Table 2**).

Table 2: Incubation protocol

Test Tube	No	Substrate	Microsome	Co-factor
Test	1	Present	Present	Present
Test	2	Present	Present	Present
Test	3	Present	Present	Present
Control	4	Present	Denatured	Present
Control	5	Present	Denatured	Present
Control	6	Present	Present	Buffer
Control	7	Present	Present	Buffer
		50 µL in each tube	1 mL in each tube	2 mL in each tube

For control experiments, microsomes were denaturated using boiling water. The necessary amount of freshly de-frosted microsomes were taken in a test tube and it was placed in boiling water for 5 mins. After the heat-denaturation, the denatureated microsomes were used for control experiements.

4.1.3.Extraction of substrates and metabolites from the biological system

At the end of the incubation period, the tubes having the unchanged substrate and metabolites, were placed immediately on an ice bath. The enzymatic process was stopped with the addition of dichloromethane, extracted and evaporated under nitrogen. The extracts were analyzed by LC-MS.

4.2.LC-MS analysis

An acetonitrile/water (gradient elution) mobile phase mixture was used. The substrates and metabolic standards were separated according to their mass/charge ratio and their molecular ion peaks were determined in the mass spectroscopy section and the retention times (Rt) of the substrate and metabolic standarts were recorded. A DAD detector was also used to compare UV spectra of standard and metabolic products.

Ethics Approval and Consent to Participate

The pig livers were donated by Acibadem University, Animal Laboratory Centre from the Project by Dr. Mehmet Emin Aksoy; laparoscopic and robotic surgery, with the 2021-01 ethical approval number. The liver tissue was obtained from the euthanized pig at the end of course.

Human and Animal Rights

No humans were used in this study. All animal research procedures were followed in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) and/or the declaration of Helsinki promulgated in 1964 as amended in 1996.

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Conflict of interest statement: The authors declare no conflicts of interest, financial or otherwise.

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