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Metabolic stability of a hydrazone derivative: *N*′-[(4-chlorophenyl)methylidene)] -4-[(4-methylphenyl)sulfonyloxy]benzohydrazide

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Abstract

Metabolic stability, a key parameter in drug development, refers to a drug substance's resistance to metabolism. The failure rate can be significantly reduced by conducting metabolism studies for the drug candidate compound from the early stages. These studies are primarily carried out on *in vitro* microsomal enzymes, which play a crucial role in the process. Various analytical methods, predominantly liquid chromatography, can be used for analysis. In this context, we conducted metabolic stability studies of a hydrazone-sulfonate derivative compound previously synthesized and investigated in terms of biological activity. Metabolic stability was determined by LC-MS/MS on rat microsomes *in vitro*. Analyses were performed at 0, 5, 10, 15, 30, and 60. minutes during incubation. The analysis revealed that the stability of the compound was highly cofactor-dependent, maintaining its stability without cofactor and in a buffer medium.

Keywords: Metabolic stability, LC-MS/MS, hydrazone, sulfonate

1. Introduction

Determination of metabolic profiles of drug candidates is one of the most critical steps in drug discovery [1].

Previously, metabolism studies were rarely conducted until they reached early clinical stages. Inadequate metabolism and pharmacokinetic parameters were the primary reasons for failure in the process [2,3]. Nowadays, metabolic studies are conducted at all stages of drug development [4].

Drug candidates' favorable metabolic stability profiles are characterized by enhanced bioavailability and prolonged half-life [4].

The liver widely excretes most oral medications, mainly through hepatic cytochrome P (CYP)-mediated metabolism. Therefore, precise estimation of hepatic metabolism and first-pass clearance is essential for early drug development and discovery [5].

Metabolic stability is generally first determined *in vitro* in liver microsomes because these microsomes are rich in CYP450 enzymes [6]. Hepatocytes provide a clearer picture but have some disadvantages [7].

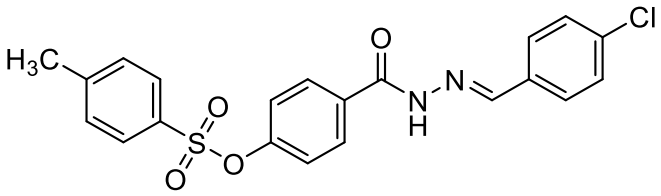

Figure 1. Structure of the tested compound

The National Center for Advancing Translational Sciences uses rat liver microsomes for *in vitro* experiments. This is because rats are involved after *in vitro* pharmacokinetics, efficacy, and toxicology studies *in vivo*. Furthermore, the data obtained can also correlate with human pharmacokinetic results [8].

Developing analytical methods can help achieve higher sensitivity, a higher detection limit, better resolution, precise and accurate quantification in metabolic stability studies [1,9].

This research investigates the metabolic stability of a tosyl-hydrazone derivate coded **3o**. The structure of **3o**

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(Fig. 1) molecule is *N*′-[(4-chlorophenyl)methylidene)]-4-[(4-methylphenyl)sulfonyloxy]benzohydrazide which our research group previously developed. It was synthesized from the starting material, ethylparaben. It was reacted with hydrazine hydrate to obtain the hydrazide compound. Then, the reaction with 4-chlorobenzaldehyde and tosyl chloride, respectively, resulted in the final compound. The inhibitory activity against some metabolic enzymes was also investigated [10].

2. Material and methods

2.1. Chemicals and materials

All the chemicals were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO). For the metabolic stability assay, 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. An Agilent 1260 Infinity II LC-MS/MS spectra equipped with G7115A 1260 DAD WR detector, G7104C 1260 Flexible Pump system, G7116A 1260 MCT Column Oven, G7129C 1260 Vialsampler autosample injection unit and G6465B LC/TQ MS/MS detector were used for both HPLC and mass analysis.

Retention times were recorded with an ACE C18 column (particle size: 3 µm, pore size: 100 Å). The column temperature was adjusted to 25ºC in the column compartment. The mobile phase consisted of an acetonitrile-water (90:10, v/v) mixture delivered at a 0.4 mL/min flow rate. The injection volume was 20 µL. The UV detector was operated at 254 nm.

2.2. Preparation of rat liver microsomes and incubation and extraction procedures

Wistar albino rat liver was used in this study. The animals were deprived of food overnight before sacrifice but were allowed water *ad libitum.* They were previously fed on a balanced diet. Hepatic-washed pig microsomes were prepared as described by Coskun et al. [11]. Incubations were carried out in a shaking water bath at 37ºC using a standard cofactor 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. Cofactors 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, six test tubes for the substrate (**3o**) were prepared (2 for the test, 4 for the controls), and cofactors (2 mL in each tube), the microsomal fraction (1 mL for each tube), and substrate (50 µL for each tube) was added respectively (Table 1 and Table 2). The incubation was continued for 60 min, and samples were collected from each tube in 0, 5, 10, 15, 30, and 60. min. At the end of 60. min, the reaction terminated, and media was extracted with dichloromethane (3×5 mL). The organic extracts were evaporated to dryness under a stream of nitrogen. The residues were reconstituted in methanol (200 µL) for LC-MS/MS. The reconstituted extracts were analyzed using the reverse-phase LC-MS/MS system described in the text.

Table 1. Contents of the cofactor 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 cofactor solution right before pre-incubation. All cofactors were "pre-incubated" at a 37°C water bath for 5 minutes to create NADPH. The amount of microsomal preparation added to each incubation tube was 1 mL, and the cofactor solution was 2 mL. Control tubes were also prepared.

Table 2. Incubation protocol

Test Tube	No	Substrate[1]	Microsome[2]	Cofactor[3]
Test	1	Present	Present	Present
Test	2	Present	Present	Present
Control - Denatured microsomes	3	Present	Denatured	Present
Control - Denatured microsomes	4	Present	Denatured	Present
Control - No cofactor	5	Present	Present	Buffer
Control - No cofactor	6	Present	Present	Buffer

[1]50 µL in each tube
[2]1 mL in each tube
[3]2 mL in each tube

For control experiments, microsomes were denatured using boiling water. The necessary amount of freshly defrosted microsomes was taken in a test tube and placed in boiling water for 5 minutes. After the heat denaturation, the denatured microsomes were used for control experiments.

2.3. Autooxidation studies

The substrate (2 µM) was dissolved in methanol (50 µL). Then, phosphate buffer (0.2 M, pH 7.4) (3 mL) was added in the same incubation conditions as the test experiments. The test was performed according to the protocol as mentioned earlier. The reconstituted extracts were analyzed using the reverse-phase LC-MS/MS system described in the text.

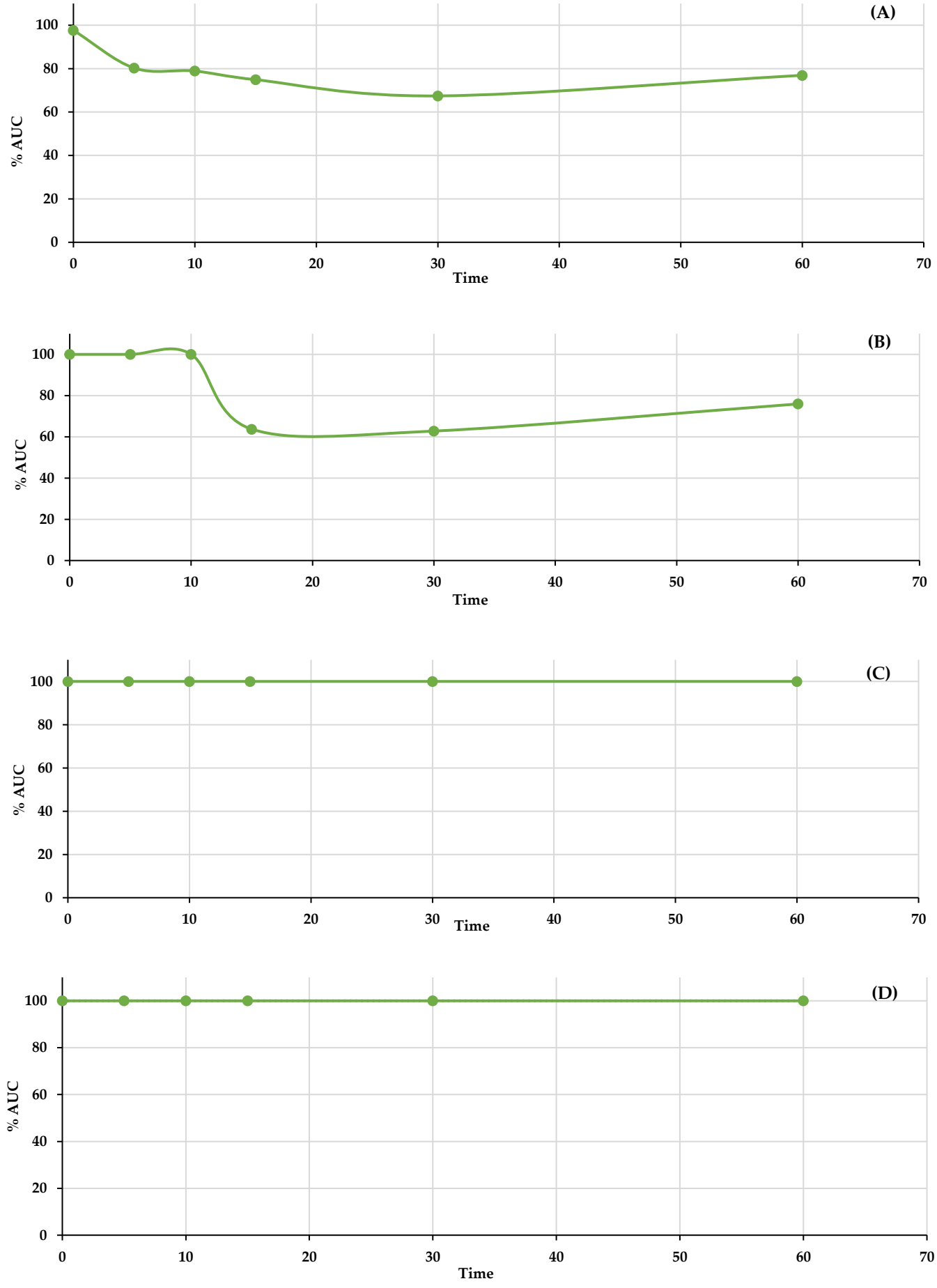

Figure 2. Metabolic stability results on **3o** ($t_{1/2}$ > 60 mins) (A) The test conditions. (B) The control conditions (denaturated microsome). (C) The control conditions (cofactor-free). (D) The control conditions (autooxidation).

2.4. Extraction of substrates and metabolites from the biological system

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

2.5. LC-MS/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. The retention times of the substrates and metabolic standards were recorded. A DAD detector was also used to compare the UV spectra of standard and metabolic products.

2.6. *In silico* prediction of metabolic pathways

For *in silico* prediction, a virtual preliminary metabolic evaluation software, SMARTCyp 3.0, was used [12].

3. Results and discussion

Metabolic stability is one of the most important pharmacokinetic parameters affecting the *in vivo* efficacy of drug candidates. Metabolism studies are common to many stages of the development of active pharmaceutical ingredients.

In this study, we performed metabolic stability studies of a compound (**3o**) whose biological activity was previously screened.

Sulfonyl group-bearing compounds such as sulfone, sulfonate, sulfonamide, and sulfamate are essential in medicinal chemistry [13]. Such compounds are most commonly hydrolyzed from the sulfonyl group in phase 1, resulting in more polar metabolites [14,15].

Hydrazones are also of interest to medicinal chemists. If the metabolism based on this functional group is scrutinized, it is indicated that possible metabolic pathways exist, such as hydrolysis of the *N*-acylhydrazone group and aromatic hydroxylation [16–19]. Studies have shown that aroyl hydrazones are stable in phosphate buffer saline at 37 °C but undergo rapid degradation in plasma [20–22].

Compound **3o** (Fig. 1) was tested for its metabolic stability. The compound was found to be stable in the microsomal environment, and half-life is over 60 mins in a 60 mins assay experiment. Control experiments were performed to understand whether the stability depends on microsomes, cofactors, or buffers. Metabolic stability was affected in both microsomes and cofactors; however, a similar decrease was observed in denatured microsomes in LC-MS/MS experiments. It can be concluded that metabolic stability depends highly on cofactors. The compound was stable in cofactor-free and buffer environments (Fig. 2).

Metabolic stability is one of the most difficult parameters to estimate *in silico* due to the extreme complexity of the processes involved in xenobiotic transformations [23]. Nonetheless, researchers have made many online free predicting tools available to users [24,25]. The compound's metabolic stability was also calculated by SMARTCyp 3.0 software [12].

SMARTCyp predicted that the methyl group in the tosyl moiety would be most reactive for all three metabolic enzymes, Cyp 3A4, Cyp 2D6, and Cyp 2C9 (scores = 55.8, 63.9, 63.8, respectively). Table 3 shows the possible metabolic sites with energies less than 999, with similarities between 0.7 and 1.

Table 3. Metabolic site prediction scores

No	SMARTCyp Score*					
	3A4		2D6		2C9	
1	C.1	: 55.8	C.1	: 63.8	C.1	: 63.8
2	C.11	: 71.5	C.21	: 96.3	C.21	: 94.7
3	C.20	: 72.9	C.3	: 98.6	C.20	: 97.0
4	C.12	: 75.6	C.20	: 99.8	C.20	: 97.4
5	C.21	: 75.8	C.11	: 103.0	C.11	: 99.8
6	C.3	: 78.0	C.4	: 105.5	C.4	:103.1
7	C.4	: 78.7	C.12	: 106.7	C.12	: 103.5

*Lower scores indicate a higher probability of being metabolized result

4. Conclusion

Herein, the metabolic stability of a hydrazone-sulfonate derivative compound was evaluated using rat liver microsomes *in vitro*. The metabolic stability of the compound depends on its certain functional groups. Hydrazones are expected to be more stable than hydrazines as the terminal nitrogen, which is a metabolic soft spot, is protected by an imine group. This study gave a good perspective on the stability of hydrazone group. On the other hand, sulfonates are a type of ester known to be metabolically unstable. However, the overall results showed that two functional groups enhanced the stability of the entire compound. Control experiments to detect metabolic soft spots in the compounds were also performed in this study. Co-factor and microsome-free environments showed that the compound requires both microsomes and co-factors to be metabolized. On the other hand, autooxidation was also tested in a buffer environment, which proves that the compound does not provide any metabolites in an enzyme and co-factor-free environment. Overall results clearly show that the compound was stable in both the buffer and the cofactor-free environment, which shows that metabolic stability is highly dependent on cofactors. *In silico* calculations

indicated that the methyl group may be a possible site for metabolism and be considered a metabolic soft spot in the upcoming studies.

Ethics approval and consent to participate

The rat 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 rats at the end of the 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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