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Synthesis, structure elucidation and *in vitro* microsomal metabolism of adamantane hydrazide-hydrazone derivatives

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ABSTRACT

The discovery of adamantane has led to the researchers to find different drug molecules which exhibit diverse biological activities. On the other hand, hydrazide-hydrazones possess strong anticancer, antibacterial, antienflamatory and antiseptic activites. However, we have limited knowledge on the metabolic profiles of adamantane hydrazide-hydrazones. The metabolic pathway of adamantane hydrazide-hydrazones emerged to be an important step for pre-clinical drug discovery studies. In this study, the metabolic profile of adamantane hydrazones was the main motivation for our research group. The synthesis of hydrazide-hydrazone derivatives as substrates of this study was performed via condensation of corresponding hydrazide with aldehyde and ketone and their authentic metabolites were also synthesized. Following their structures were elucidated with spectroscopic methods, their *in-vitro* microsomal metabolic study was performed. The results indicated that the aldehyde derivative is susceptible to metabolic hydrolysis, whereas ketone derivative is stable for metabolic changes. LC-MS results proved the metabolic hydrolysis.

Keywords: Adamantane, *in vitro* metabolism, hydrazide-hydrazone, hydrolysis.

INTRODUCTION

Adamantane was first discovered in Hodonin, Czechoslovakia in 1933 from crude oil. The discovery of this interesting ring opened new fields in chemistry including drug development. Adamantane ring were then derivatized with dif-

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ferent class of substituents leading a diverse biological activities [1]. Drugs having adamantane ring exhibit surprising clinical efficacy. The most common and well known drugs that have adamantane ring are amantadine, rimantadine, memantine, tromantadine and saxagliptin. These drugs are known to have different pharmacological activities ranging from antiviral to antidiabetic [2].

Hydrazide-hydrazone derivatives have an azomethine structure linked to an amide (-CH=N-NH-CO-) which plays a significant role in terms of pharmacological activites. Recent studies have showed their promising antimicrobial, anticancer, antituberculosis, antiviral and anticonvulsant activities [3-18].

The literature search indicated that there was limited number of studies related with adamantane hydrazide-hydrazones. Wassel and co-workers recently reported the synthesis and the carbonic anhydrase activity of adamantane hydrazide-hydrazones [19]. A hydrazone-adamantane structure with pentyl moiety showed excellent inhibitory activity and presented good pharmacokinetic properties. Both carbonic anhydrase and antimicrobial activity studies on adamantane-hydrazone derivatives were conducted by Pham and co-workers [3]. Although these compounds exhibited good biological activites, no metabolic study was performed showing their metabolic profile. There are very few studies on the metabolism of hydrazone compounds in the literature and these studies have been done on certain prototypes. Kömürcü et al. synthesized a number of hydrazide-hydrazones to investigate their antibacterial activity. They also investigated *in vitro* microsomal metabolites of 4-fluorobenzene derivative as a prototype following *in vitro* microsomal metabolism [20-21]. In another study by Ulgen et al, *in vitro* metabolism of benzoic acid benzylidenehydrazide was studied and it was observed that the compound produced the corresponding hydrolytic metabolites, namely benzoic acid hydrazide and benzaldehyde together with a p-hydroxylation metabolite which occured by oxidation from the para position of the benzylidene due to the strong electron withdrawing effect of the benzoyl ring [22]. Motiur Rahman et al. studied *in vitro* micromosal metabolism of 2-fluorescein hydrazones which are topoisomerase inhibitors. The study was performed on rat liver microsomes and metabolites were analyzed in the LC-MS system. They found out that the compounds undergone biotransformation to a hydroxyl derivative and tetra hydroxyl derivative. The metabolic products was recorded as hydroxylation and reduction. It was concluded that the hydrazone compounds needed co-factors for *in vitro* metabolism [23]. The literature data revealed limited knowledge on the microsomal metabolism of hydrazone derivatives. The hydrazone compounds were promising derivatives in medicinal chemistry field, therefore, it is vital to investigate their biological

profile before clinical studies. On the light of foregoing, we have designed and synthesized adamantane hydrazide-hydrazone derivatives from aldehyde and ketone. The ketone derivative compound is original molecule. The *in vitro* metabolism studies for both compounds were performed to establish the potantial stability of ketone substrate over the aldehyde hydrazone.

METHODOLOGY

All the chemicals were purchased from Sigma Aldrich and Merck. All the substrates and potential metabolites were synthesized according to the reported procedures [3, 24].

Melting point was recorded on a Stuart SMP50 Automatic Melting Point apparatus and uncorrected. The structures of adamantane derivatives were confirmed by FT-IR, LC-MS spectra. FT-IR analysis were performed with Thermo Scientific Nicolet IS10 device. LC-MS separation of adamantane derivatives were performed by an Agilent 1260 Infinity II LC-MS chromatographic system comprised of a G7115A 1260DAD WR detector, a G7311B 1260 Quad Pump system, a G1328C 1260 Manual Injection unit and a G6125B LC/MSD detector. An ACE C18 column was used as a stationary phase. NMR spectra were recorded on Bruker 400 MHz (Billerica, MA) for ^{1}H-NMR. Data are reported as follows: chemical shift, multiplicity (b.s.: broad singlet, d: doublet; m: multiplet, s: singlet, and t: triplet), coupling constants (Hz), integration. Adult male Suffolk white pigs were used in this study. ß-Nicotinamidedinucleotidephosphate (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.

Experimental

Synthesis

Methyl adamantane-1-carboxylate: This compound was synthesized according to method presented in the literature [3]. Yield: 97% mp: 35-36°C. FT-IR υmax (cm^{-1}): 2927, 2850 (C-H), 1732 (C=O), 1450, 1425 (C-H), 1238 (C-O) [8].

Adamantane-1-carbohydrazide (M1): This compound was synthesized according to method presented in the literature [3]. Yield: 95% mp: 147-148°C. FT-IR υmax (cm^{-1}): 3332, 3275 (N-H), 2908, 2848 (C-H), 1616 (C=O), 1521 (N-H), 1456, 1367 (C-H) [8].

N'-[(1*E*)-1,1-Diphenylmethylidene]adamantane-1-carbohydrazide: (S1) Adamantane-1-carbohydrazide (M1) (0.01 mol) was dissolved in ethanol and equimolar amount of benzophenone was added. The reaction mixture was refluxed

for 2 hours and monitored by TLC. After the reaction completed, the mixture was evaporated under atmospheric pressure and solid product was crystallized with methanol. M.p 204-205°C. Yield: 88% FT-IR υmax (cm^{-1}): 3367 (N-H), 2903, 2874 (C-H), 1689 (C=O), 1521 (N-H), 1489 (C=N). ^{1}H-NMR (300 MHz, DMSO-d_6):1.4-2.1 (m, 17H, Adamantane and DMSO), 7.4-7.8 (m, 10H, Ar-H), 8.8 (s, 1H, CH=N). ^{13}C-NMR (75 MHz, DMSO-d6): 27.78, 36.26, 38.56, 39.52, 39.53, 39.74, 39.95, 40.15, 40.36, 40.57, 127.76, 128.67, 128.97, 130.21, 130.30, 130.47, 137.28 (CH=N), *the C=O peak was not detected due to noise*. MS-APCI (m/z): [M+1] 359. LC-MS: [M+1] 359.

N'-[(4-Chlorophenyl)methylidene]adamantane-1-carbohydrazide (S2): This compound was synthesized according to method presented in the literature [3]. M.p 205-207°C. Yield: 82% FT-IR υmax (cm^{-1}): 3289 (N-H), 2903, 2850 (C-H), 1647 (C=O), 1602 (C=N),), 1521 (N-H), 1489, 803 (C-Cl). MS-APCI (m/z): [M+1] 317; [M+2] 319. LC-MS: [M+1] 317.

[(4-Chlorophenyl)methylidene]hydrazine (M6): 4-chlorobenzaldehyde (M5) (0.001 mol) was refluxed with hydrazine hydrate (5ml) in the presence of ethanol. After the reaction completed, the mixture was evaporated under atmospheric pressure and solid product was crystallized with ethanol. M.p 59-61°C. Yield: FT-IR υmax (cm^{-1}): 3065 (C-H), 1605 (C=N). MS-APCI (m/z): [M+1] 155. LC-MS: [M+1] 155.

(Diphenylmethylidene)hydrazine (M4): Benzophenone (M2) (0.001 mol) was refluxed with hydrazine hydrate (5ml) in the presence of ethanol. After the reaction completed, the mixture was evaporated under atmospheric pressure and solid product was crystallized with ethanol. M.p 96-97°C. Yield: 74% FT-IR υmax (cm^{-1}): 3422, 3262 (N-H), 3187, 3054 (=C-H), 1580 (C=C), 1491 (C=N). MS-APCI (m/z): [M+1] 197. LC-MS: [M+1] 197.

LC-MS analysis

A methanol/water mixture was used with a gradient elution as a mobile phase. The substrates and their potential metabolites were separated according to their mass/charge ratio and their molecular ion peaks were determined. The retention times (Rt) of the substrate and possible metabolites were recorded. A DAD detector was also used to compare UV spectra of standard and metabolic products.

Biological studies

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 microsomes were prepared as described by Schenkman and Cinti [25] and Ulgen [26].

Incubation and extraction procedures

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 (5 μl). Incubation was continued for 30 min, terminated and extracted with dichloromethane (3x5 ml). Organic extracts were evaporated to dryness under a steream of nitrogen [10]. The residues were reconstituted in 200 μl of methanol for LC-MS. The reconstituted extracts were analysed using the reverse-phase LC-MS system described in the text.

RESULTS AND DISCUSSION

Chemistry

The ester and hydrazide derivatives of adamantane were synthesized according to the reported method [3]. Both IR and LC-MS results were in accordance with the expected data. Briefly, adamantane carboxylic acid was reacted with methanol in the presence of concentrated sulphuric acid to give methyl adamantane-1-carboxylate. IR and LC-MS studies proved to formation of ester derivative. The carbonyl peak was shifted from 1701 to 1732 cm^{-1} confirming the ester carbonyl in IR studies (Table 1).

Table 1. Chromatographic and spectroscopic properties of the substrates and their potential metabolites

Compound (abbreviation)	M.w. (g/mol)	Molecular ion peak [M+1] (m/z)	IR peak (C=O) (cm^{-1})	IR peak (C=N) (cm^{-1})	LC-MS retention time (min)
S1	358.49	359	1689	-	
S2	316.83	317	1647	-	4.56
M3	150.24	151	1732	-	NT
M1	194.27	195	1616	-	3.39
M4	196.25	197	-	1516	3.95
M6	154.60	155	-	1501	6.61

LC-MS results indicated the molecular weight of the compound. To synthesize hydrazide derivative, methyl adamantane-1-carboxylate was heated with hydrazine-hydrate in the presence of methanol. The formation of the hydrazide (adamantane-1-carbohydrazide) was also proved with IR and LC-MS studies. The carbonyl peak resulting from hydrazide formation shifted from 1732

cm^{-1} to 1616 cm^{-1} in IR studies. LC-MS and ^{1}H-NMR spectra also proved to formation of hydrazide (Figure S1 and Figure S2). The substrate synthesis was performed using simple addition reaction steps. Both N'-[1,1-diphenylmethylidene]adamantane-1-carbohydrazide and N'-[(4-chlorophenyl)methylidene] adamantane-1-carbohydrazide were synthesized in the presence of ethanol and addition of benzophenone and 4-chlorobenzaldehyde respectively. The formation of hydrazone structure were proved with IR and LC-MS studies; following the presence of hydrazide C=O and azomethine –CH=N- peaks in IR and moleculer weight in LC-MS. Finally, we performed the synthesis of possible metabolites; (diphenylmethylidene)hydrazine and [(4-chlorophenyl)methylidene] hydrazine. The synthetic procedures for compounds were already reported in literature; therefore we simply performed the synthesis and proved the formation of the compounds by comparison with previous chromatographic and spectroscopic data.

LC-MS

Adamantane 1-carboxylic acid and 1-adamantanecarbohydrazide have no detectable UV absorbtion. Therefore, we were only able to determine the compounds by their MS spectrum. In order to determine the suitable mobile phase for the seperation of both substrate and their potential metabolites, few experimental procedures were performed. Hydrazone functional groups generally do not need any buffer medium in liquid chromatography methods. However, a buffer was necessary for aldehyde/ketone hydrazones (M4 and M6) and other possible metabolites. First attempts were made by using buffer medium as mobile phase. Acetonitrile/phosphate buffer and methanol/phosphate buffer systems were used. The seperation of the substrate and possible metabolites were achieved. Unfortunately, we were unable to identify the molecular ions in MS detector due to the strong shielding effect of triethylamine in the buffer solutions. Therefore, we immediately developed a buffer free mobile phase system. After several attempts, a gradient elution with a methanol/water mixture was found to be the best system for the separation of the substrates and their potential metabolites.

In vitro metabolic experiments were performed for two different substrates. Substrate 1 (S1) was synthesized from ketone, benzophenone derivative. Substrate 2 (S2) was synthesized from an aldehyde derivative. Both of the substrates have hydrazone functionality. Literature data mostly suggested the hydrolytic degredation of hydrazone compounds in *in vitro* metabolism studies [5]. Here in this study, we found a stable hydrazone compound, consisting of two large hydrophylic phenly ring. Following the metabolic experiments, no

hydrolytic change was observed for S1 (Figure 1). However, S2 resulted with the hydrolysis of hydrazone structure (Figure 2). The control experiments with denaturated microsomes or in the absence of co-factors were also carried out to establish whether hydrolysis was enzymatic and co-factor dependent. For S2, enzymatic hydrolysis was observed both in the presence of enzyme and co-factors and in the absence of co-factors, indicating that the reaction was enzymatic and co-factor independent (Figure 3). In the control experiment with denaturated enzyme but with co-factors, no hydrolytic metabolite was observed. The authentic and metabolically formed hydrolytic products were compared with their MS spectra (Figure 4).

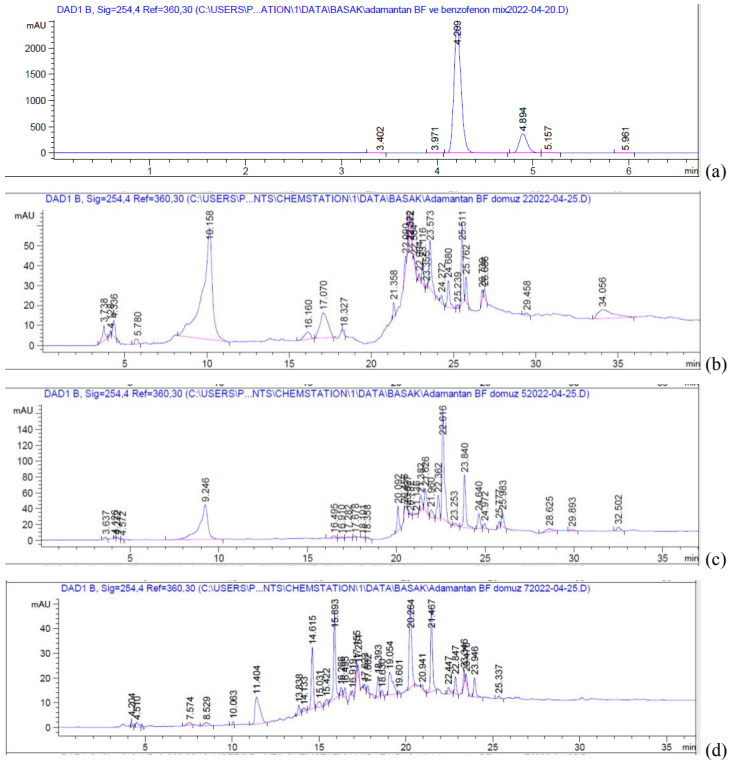

Figure 1. HPLC chromatogram of (a) S1 and its potential metabolites; S1 (4.209 min) and M2 (4.894 min); (b) HPLC chromatogram from test incubation mixture; HPLC chromatogram of S1 test: S1 (10.15 min), the other peaks resulted from microsomal mixture, no hydrolytic product was observed (c) HPLC chromatogram from control experiment with denaturated microsomes; HPLC chromatogram of S1 control (no microsome): S1 (9.24 min), the other peaks resulted from microsomal mixture, no hydrolytic product was observed (d) HPLC chromatogram from control experiment without co-factors; HPLC chromatogram of S1 control (no co-factor): S1 (11.404 min), the other peaks resulted from microsomal mixture, no hydrolytic product was observed.

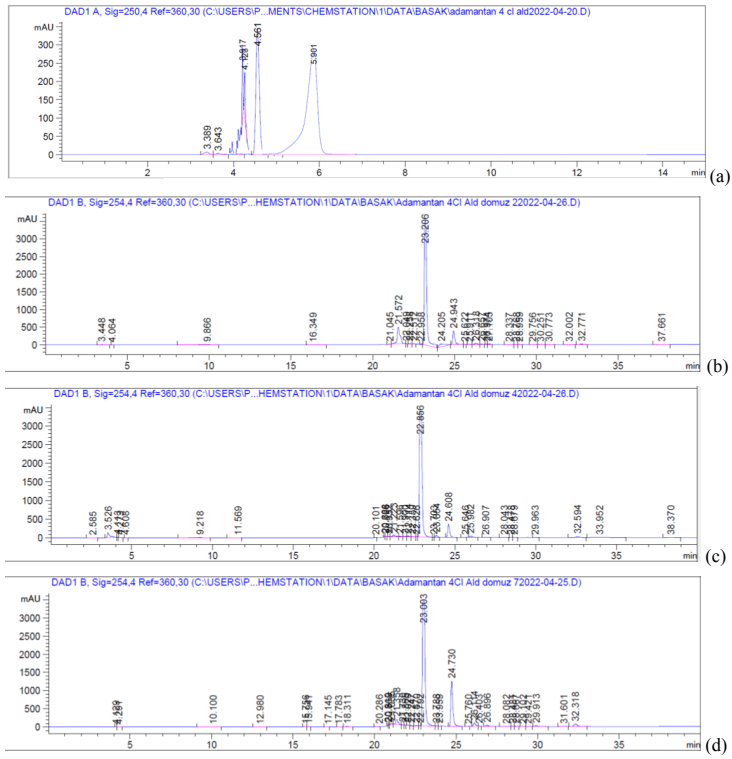

Figure 2. HPC chromatogram of (a) substrate 2 and its potential metabolites; HPLC chromatogram of S2 (4.561 min), M5 (5.901 min), M1 (3.917 min) (b) HPLC chromatogram from test incubation mixture; HPLC chromatogram of S2 test: S2 (23.206 min), M5 (24.943 min), M1 (NT), the other peaks resulted from microsomal mixture (c) HPLC chromatogram from control experiment with denaturated microsomes; HPLC chromatogram of S2 control (no microsome): S2 (22.856 min), M5 (NT), M1 (NT), the other peaks resulted from microsomal mixture (d) HPLC chromatogram from control experiment without co-factors; HPLC chromatogram of S2 control (no-cofactor): S2 (23.003 min), M5 (24.730), M1 (NT), the other peaks resulted from microsomal mixture.

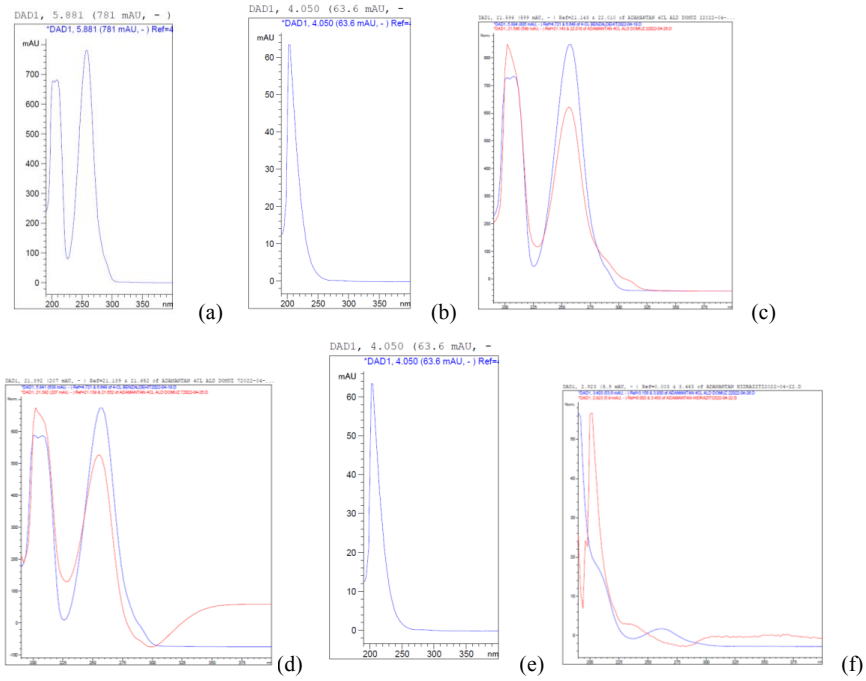

Figure 3. (a) UV spectra of M5 standard; (b) UV spectra of M1 standard; (c) Comparison of UV spectra from standard M5 and metabolically formed S1 test (22.010 min); (d) Comparison of UV spectra from standard M5 and metabolically formed M5 and S2 control with no co-factor (21.652 min); (e) M1 standard UV; (f) Comparison of UV spectra from standard M1 and metabolically formed S2 test (3.463 min); (d), (e), and (f) UV spectrum of S2 with pig microsomes fortified with NADPH

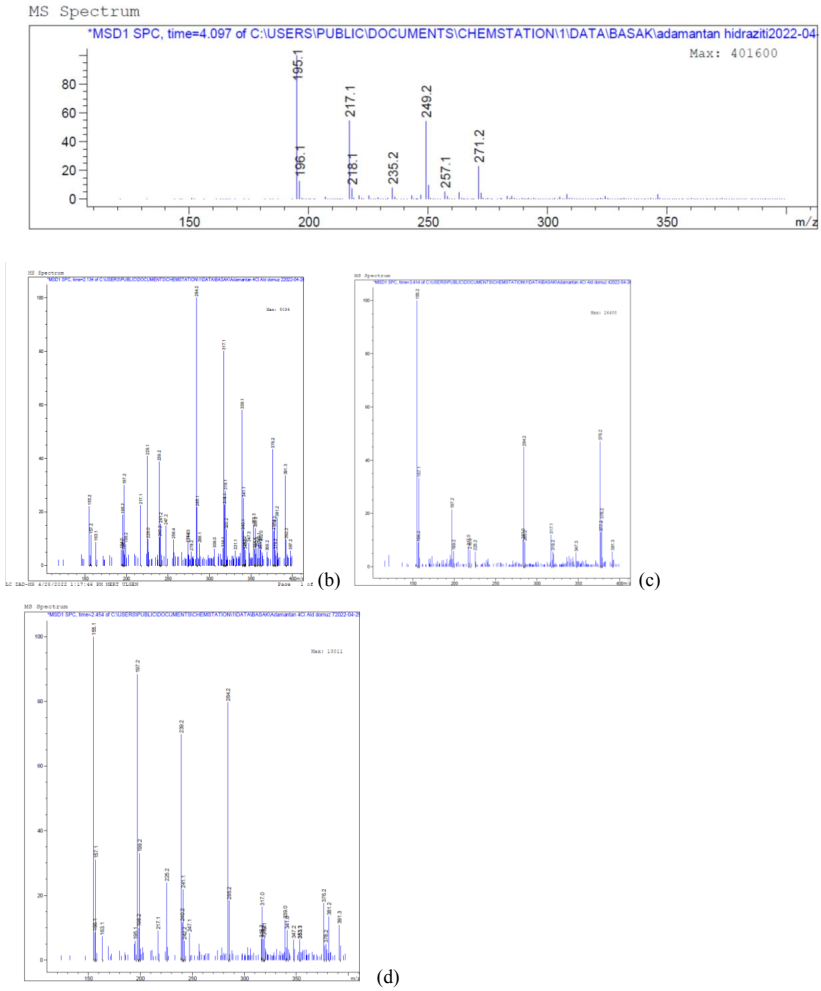

Figure 4. (a) MS spectrum of M1 (m/z=195); (b) MS spectrum of S2 test (2.13 min); (c) MS spectrum of S2 control (with denaturated microsome) (3.41 min); (d) MS spectrum of S2 control (with nocofactor) (2.45 min); (b), (c) and (d) Mass spectrum of S2 with pig microsomes fortified with NADPH

The UV visibility and a certain UV detection was not applicable for adamantane hydrazide as the compound have no UV absorbance. The formation of metabolites was determined with an MS detector. Another challenge within this study was the ionization of aldehyde standard was not observed. Even if we tried different mobile phases and also a gradient elution, aldehyde was not observed in MS detector. Finally a UV detector was used for aldehyde (M5) and an MS detector was used for hydrazide (M1) seperately. On both detectors, we succesfully showed the formation of hydrolysis only on S2 but not with S1 (Figure 5).

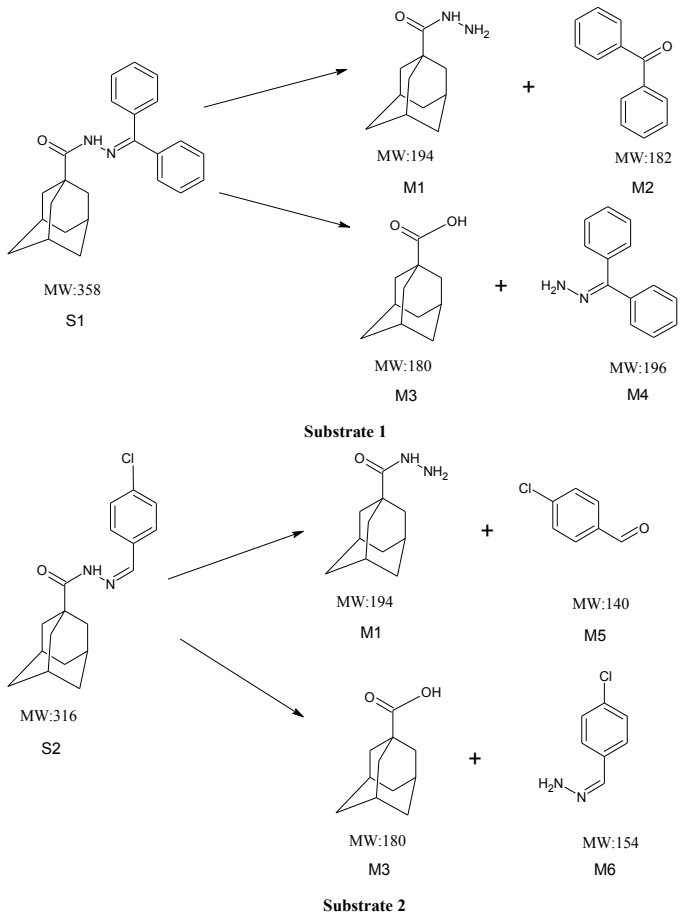

Figure 5. Potential metabolic profile of S1 and S2 and molecular weights of metabolites (see text for abbreviations)

The metabolic stability of ketone-derived hydrazone and aldehyde-derived hydrazone covers the novelty of this study. To the best of our knowledge, there are no comparation metabolic study was performed before. However, some studies revealed the metabolic stability of some different hydrazone compounds. Kovarikova [27] and co-workers investigated the the stability of aromatic hydrazones in plasma and in some different biological enviroment. Three different aroylhydrazones (pyridoxal isonicotinoyl hydrazone, salicylaldehyde isonicotinoyl hydrazone and pyridoxal 2-chlorobenzoyl hydrazone) were synthesized in this study and metabolic stability of those compounds were analysed. The results reveiled that the compounds are prone to degredation to their corresponding aldehyde and hydrazide intermediates in the presence of plasma and therefore, their half-life is short.

Another metabolic stability test for hydrazone molecules was presented by Kalia and co-workers; indicating that the hydrazone structures (the ones with aldehyde derivatives) are susceptible to hydrolysis in biological conditions. The synthesized compounds are derivatives of alkyl or acyl hydrazones. This study also its first representative of the comparition of oxime and hydrazone stability in biological conditions. The authors reveiled that the oximes are more stable than hydrazones; which also correletes with our current results [28].

Currently, no metabolic stability test were presented in the literature containing ketone-derived hydrazone compounds. It can be clearly understood that, even in the phyological contidions, aldehyde-derived hydrazones are hydrolized to their corresponding hydrazide and aldehyde. The existance of one proton in hydrazone, most propably favors the hydrolysis. Within this study, the metabolic enzymes clearly facilitates the hydrolysis on aldehyde-derived hydrazone compound. However, when hydrogen is exchanged with a larger group like phenly, the compound kept its stability both in physological conditions and in enzymatic enviroment.

In conclusion, the present study indicates that the hydrolytic profile of the hydrazone structures mostly depends on their steric hindrance for metabolic resistance. S1 was observed to be more stable to metabolic hydrolysis compared to S2, its aldehyde derivative hydrazone. It was also observed that the hydrazone formation depends on enzymes and do not require the co-factors for hydrolysis. These results indicated the metabolic stability of S1 for future studies.

ETHICAL STATEMENT

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. At the end of the training, liver tissue was obtained from the euthanized pig.

Human And Animal Rights

No humans were used in this study. All animal research procedures were followed in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

CONFLICT OF INTEREST

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

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