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Formation of an *N-oxide* metabolite following metabolism of 1-(3-chlorobenzyl)[1,2,4] triazolo[4,3-a]quinoxaline by *in vitro* rat liver microsomal preparations

Göknil Pelin Coşkun[1*], Hasan Erdinç Sellitepe[2], Bahittin Kahveci[3], Mert Ülgen[1], İnci Selin Doğan[2]

1Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Acibadem Mehmet Ali Aydınlar University, 34684, İçerenköy, Istanbul, Turkey.
2Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Karadeniz Technical University, 61100, Trabzon, Turkey
3Department of Nutrition and Dietetics, Karadeniz Technical University, Faculty of Health Sciences 61080, Trabzon, Turkey

ABSTRACT

Metabolic formation of *N*-oxides has always been important because of their biological activity profiles. Many *N*-oxide derivatives today are registered on the market for their diverse clinical use. Tertiary amines and ring nitrogens are main structures in drugs and xenobiotics for metabolic production of *N*-oxides in biological systems. Recently a new class of quinoxaline derivatives were synthesized and their anti-inflammatory activity was studied. In the present study, we studied *in vitro* microsomal metabolism of 1-(3-chlorobenzyl)[1,2,4]triazolo[4,3-*a*]quinoxaline (substrate) selected as the most active compound out of these quinoxaline derivatives using rat liver microsomes. The preliminary results from LC-MS experiments revealed that this substrate underwent *N*-oxidation in the presence of microsomes and co-factors.

Keywords: *in vitro* metabolism, *N*-oxide, 1,2,4-triazolo[4,3-*a*]quinoxaline

*Corresponding author: Göknil Pelin Coşkun
E-mail: goknilpelincoskun@gmail.com
ORCIDs:
Göknil Pelin Coşkun: 0000-0001-5168-3866
Hasan Erdinç Sellitepe: 0000-0001-5339-6940
Bahittin Kahveci: 0000-0001-7394-0552
Mert Ülgen: 0000-0003-4913-4950
İnci Selin Doğan: 0000-0003-4949-1747
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INTRODUCTION

Medicinal chemistry held diverse structures as drug molecules throughout history. Heterocyclic ring systems are one of the leading structures for drug development research. Since their biological activities have tremendous importance on pharmacological treatments, their metabolic routes could always have a probability of producing active/toxic chemicals during biotransformation. Heterocyclic compounds with nitrogen are formerly thought to be substrates of *N*-oxide metabolites and are believed to have no further use in medicine [1]. After the development of minoxidil and chlordiazepoxide, these compounds gained importance in drug research [2]. This discovery had led to the development of many active *N*-oxide compounds from antiviral to antifungal activities [3-8]. *N*-oxides today keep an important place as metabolites of tertiary nitrogens and ring nitrogens in heterocyclic structures. They can sometimes be biologically active. *N*-oxides are known to be biologically unstable molecules and most of the time they revert back to its original compound. The instability of *N*-oxides sometimes create challenge in research and development studies[9]. Recent studies presented the formation of *N*-oxide metabolites from tertiary amines. Metabolic *N*-oxide formation depends on NADPH and species differences (rats, pigs, humans, etc.) [10]. An *N*-Oxide metabolite of lignocaine was observed following *in vitro* metabolism by rat liver microsomes [11]. Phenanthridine, a fused azaheterocyclic ring was also reported to produce the corresponding *N*-oxide by rat hepatic microsomes [12]. The *in vitro N*-oxidation of isomeric aromatic diazines to the corresponding *N*-oxides were also reported [13].

Recently, Doğan and co-workers carried out an important study on anti-inflammatory activity of novel quinoxaline derivatives. Quinoxaline hydrazine and iminoester derivatives were used in a cyclo-condensation reactions. Compounds were screened for their anti-inflammatory and cytotoxic activities using MTT assay. Among the synthesized compounds, 3-chlorobenzyl derivative was found to show the best inhibition on nitrite-reducing effect with 65 % compared to the standard. Therefore, we aimed to study the compound's metabolic profile on rat liver microsomes *in vitro* [14].

METHODOLOGY

General

All the chemicals were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO). 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 column (particle size: 3 μm, pore size: 100Å). The column temperature was adjusted to 25°C in the column compartment. The mobile phase consisted of acetonitrile-water (60:40, v/v) mixture and was delivered at a flow rate of 0.6 ml/min. The injection volume was 20 μL. The UV detector was operated at 254 nm.

Chemistry

The substrate was synthesized previously by Doğan et al. [14] Figure 1.

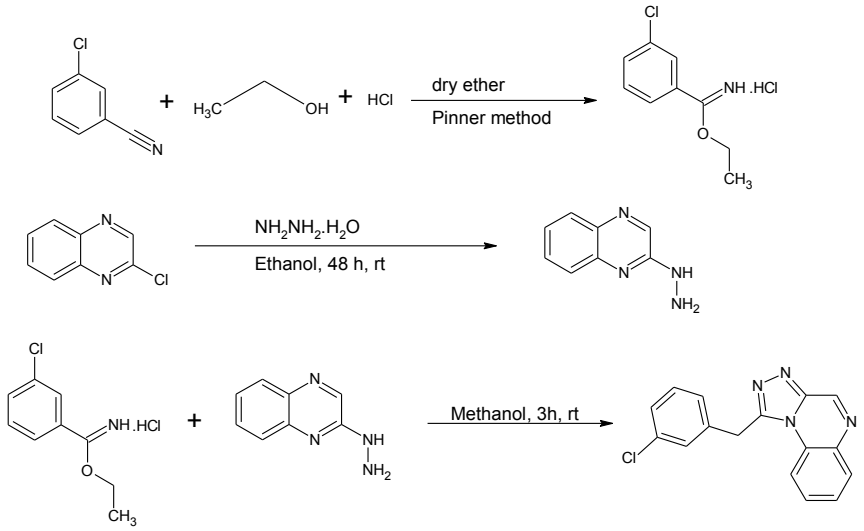


Figure 1. Synthesis method for the substrate by Doğan et al.

Preparation of rat liver microsomes and incubation/extraction procedures

Wistar albino rat liver microsomes were 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 rat microsomes were prepared as described by Schenkman and Cinti [15] and Ulgen [16]. 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 (Table 1). 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 [S] 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 stream of nitrogen. The residues were reconstituted in methanol (200 μl) for LC-MS. The reconstituted extracts were analyzed 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 μmole
G6P disodium	3.04 mg	10 μmole
G6P dehydrogenase	1.40 μl	1 unit
$MgCl_2$ (50% w/w)	8.00 μl	20 μmole

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, was 2 ml. Control tubes were also prepared (Table 2).

Table 2. Incubation protocol

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

Denaturation of microsomes

For control experiments, microsomes were denaturized 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 denaturized microsomes were used for control experiments.

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.

LC-MS analysis

An acetonitrile/water (60:40, v/v) mobile phase mixture was used. The substrate and metabolically formed *N*-oxide 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 standards were recorded. A DAD detector was also used to compare UV spectra of standard and metabolic products.

Autoxidation studies

The substrate (2 μM) was dissolved in methanol (50 μl). Then, phosphate buffer (0.2 M, pH 7.4) (3ml) was added in the same incubation conditions with test experiments. The incubation was continued for 30 min, terminated and extracted with dichloromethane (3x5 ml). The organic extracts were evaporated to dryness under a stream of nitrogen. The residues were reconstituted in methanol (200 μl) for LC-MS. The reconstituted extracts were analyzed using the reverse-phase LC-MS system described in the text.

RESULTS and DISCUSSION

The aim of this present study is first to observe and prove any qualitative *in vitro* microsomal metabolite formation of the anti-inflammatory drug candidate, 1-(3-chlorobenzyl)[1,2,4]triazolo[4,3-*a*]quinoxaline. The only possible metabolite formation from the substrate was hydroxylation of aromatic ring, or oxidation of ring nitrogen.

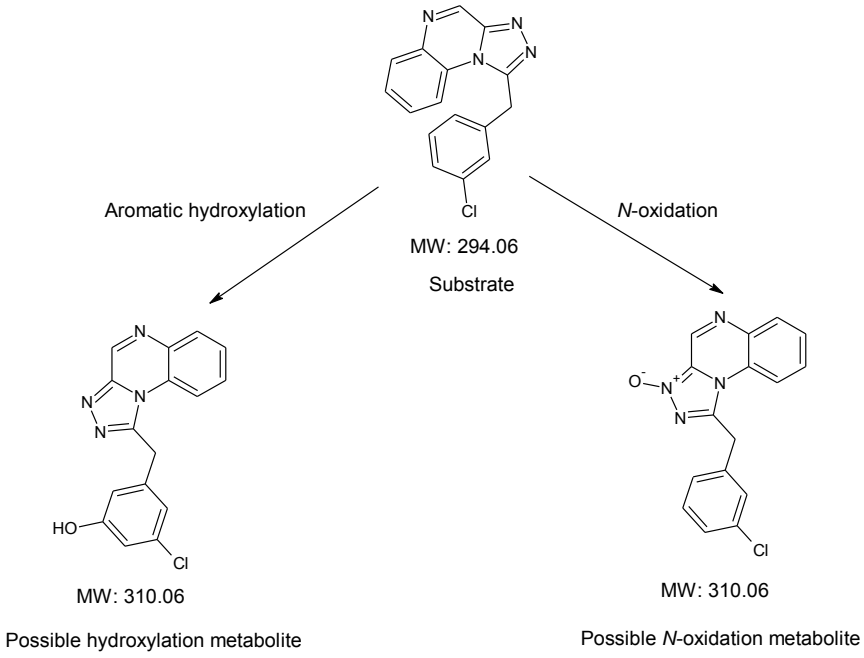
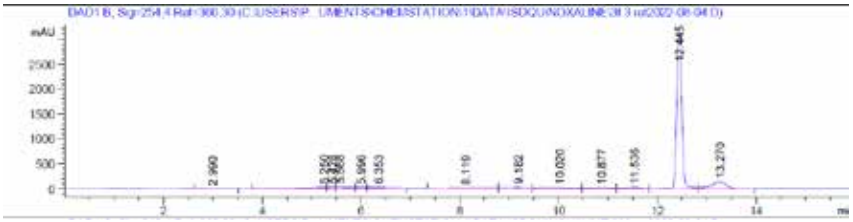


Figure 2. Potential metabolic pathways of the substrate

LC-MS

Several attempts were made in order to analyze substrate with LC-MS. Acetonitrile/water (60:40, v/v) was found to be the best mobile phase for the LC-MS analysis of substrate and therefore thought to be give the best retention time for the analysis of any possible oxidative metabolites (Figure 3).

Following the metabolic study of the substrate, the *N*-oxide formation was only observed in test experiments and not in control experiments. The metabolite was more polar than the substrate. It was clearly understood from this study that the compound undergoes *N*-oxidation in the presence of NADPH.



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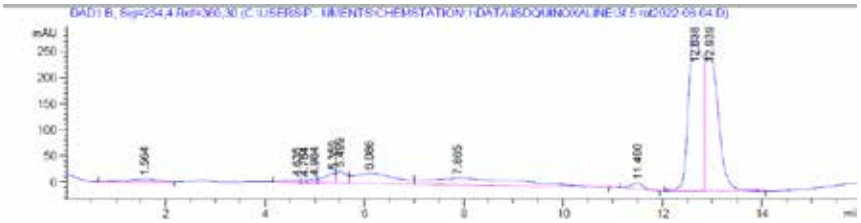

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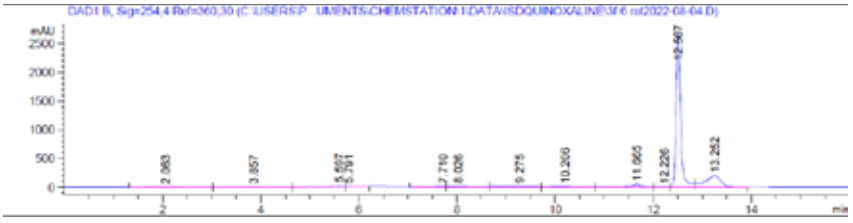
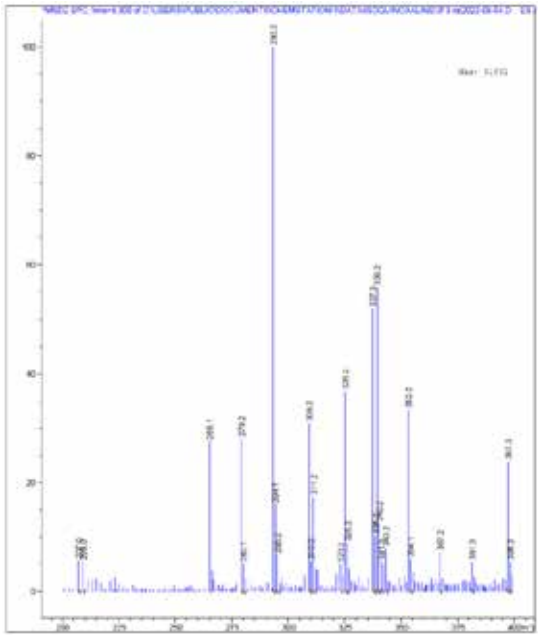


(c)

Figure 3. HPLC chromatogram obtained from (a) metabolism of substrate (in the presence of microsomes and co-factors) (Rt (min) metabolically formed N-oxide: 8.11, substrate:12.44 and all other peaks resulted from microsomal environment); (b) control with boiled (denatured) microsomes (Rt (min) substrate: 12.63-12.93 and all other peaks resulted from microsomal environment) and (c) control with no co-factor (Rt (min) substrate: 12.50 and all other peaks resulted from microsomal environment) with rat microsomal preparations

The MS spectrum for all samples revealed the formation of *N*-oxide metabolite. The m/z peaks in the spectrum were detected in negative ions in 8.3 min. The same spectrum was not detected in all the control experiments. The results revealed that the *N*-oxide formation can only be observed with both microsomes and co-factors in biological conditions (Figure 4-5).



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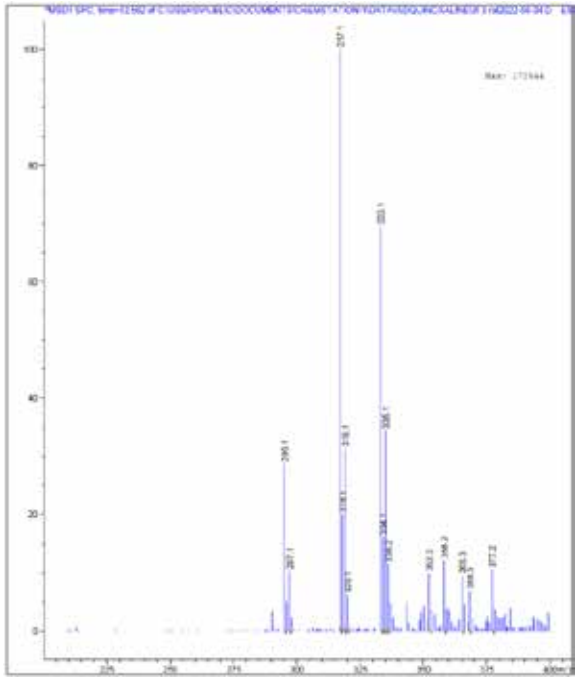

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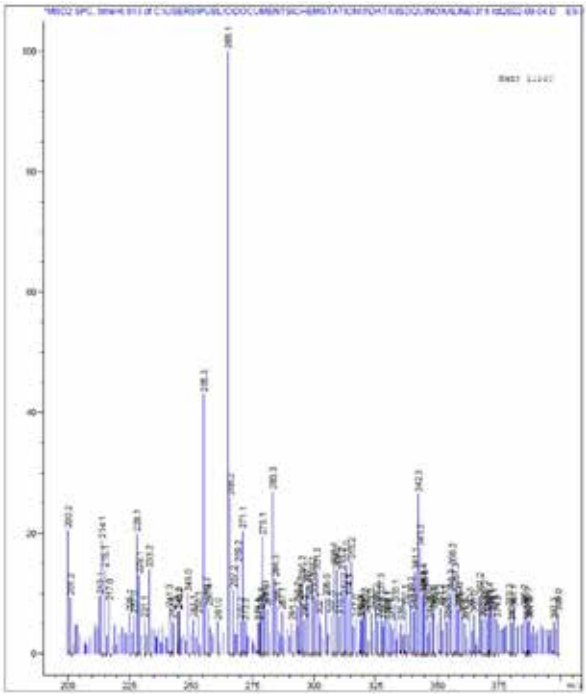

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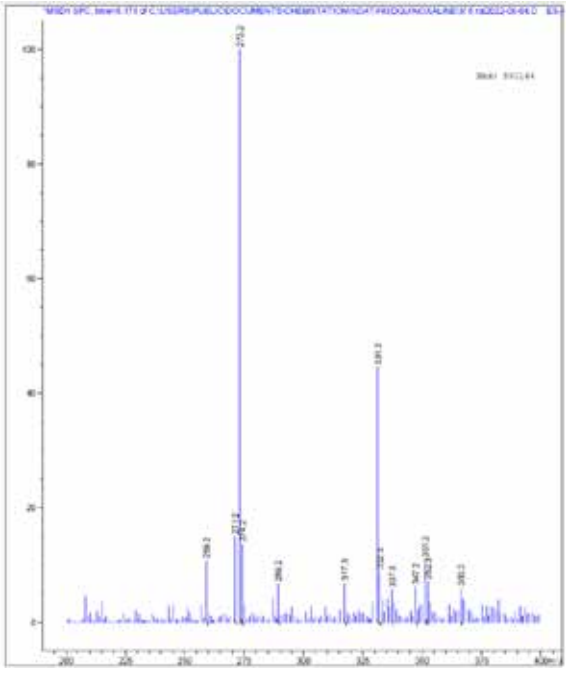


(d)

Figure 4. Mass Spectrum results; (a) Mass Spectrum of substrate test (min 8.3, negative ion 309 was observed for *N*-oxide metabolite); (b) Mass spectrum of substrate test (min 12.56, positive ion 295 was observed for substrate); (c) Mass spectrum of substrate control with denaturized microsomes (no *N*-oxide ion peak was observed); (d) Mass spectrum of substrate control with no co-factor (no *N*-oxide ion peak was observed)

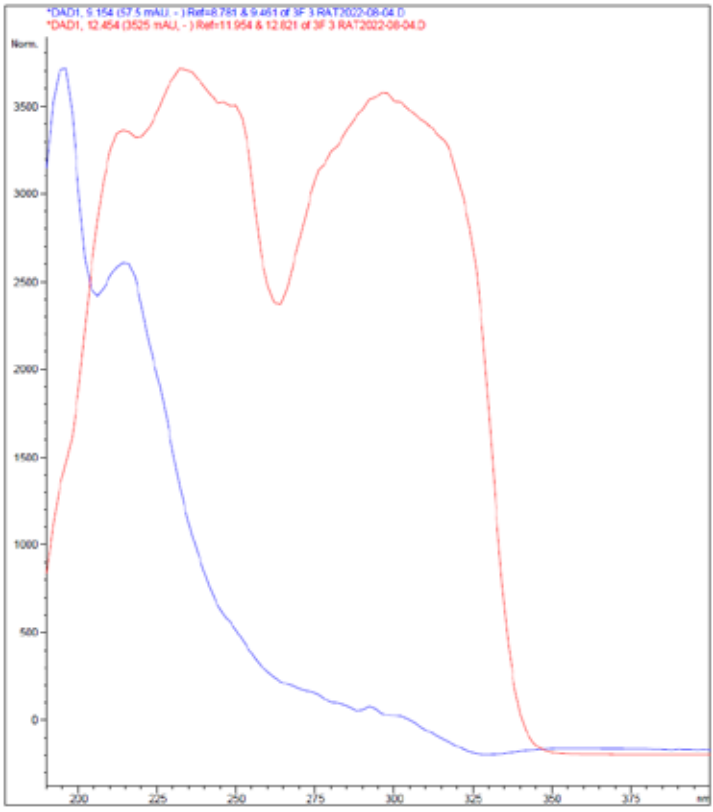
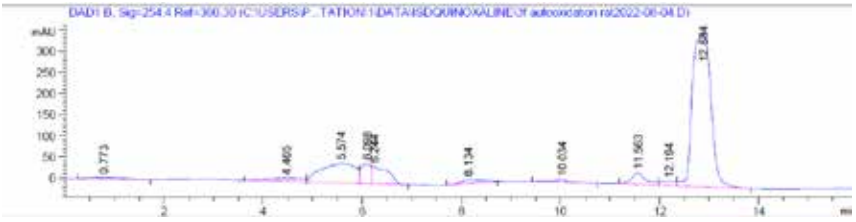
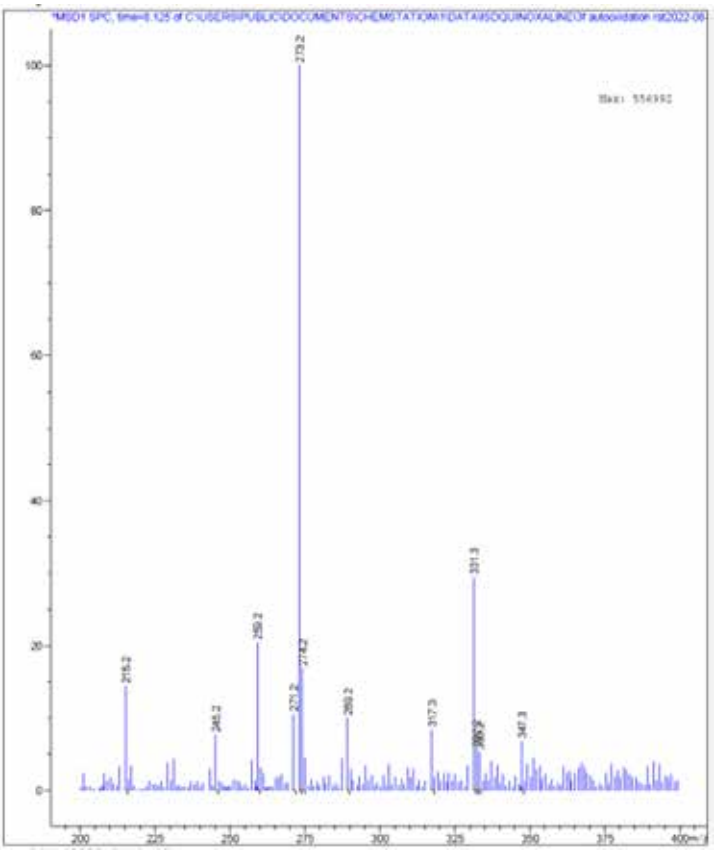


Figure 5. Comparison of UV spectra of metabolically formed *N*-oxide and substrate

In order to understand the metabolic profile of the substrate, an autoxidation experiment was also performed. The results indicated that the substrate was stable in buffer. No oxidation or any other metabolite was detected in LC-MS analysis.



(a)



(b)

Figure 6. Autoxidation of substrate with buffer (a) HPLC chromatogram of autoxidation of substrate with buffer; (b) Mass spectrum of autoxidation of substrate with buffer in min 8 (no *N*-oxide was observed)

The current preliminary study indicated that the drug candidate 1-(3-chlorobenzyl)[1,2,4]triazolo[4,3-*a*]quinoxaline (substrate) was converted into the corresponding *N*-oxide by rat liver microsomes *in vitro*. The LC-MS results indicated that the compound most probably undergoes metabolic *N*-oxidation rather than hydroxylation (Figure 3-4). The amount of the metabolically formed *N*-oxide was relatively low, therefore the *N*-oxide formation was more favorable than aromatic hydroxylation. As a result, we assumed that *N*-oxidative product was formed in the presence of NADPH. The UV spectrum of this *N*-oxide was also different from that of the substrate (Figure 5). The autoxidation experiment showed that the substrate was stable in buffer (Figure 6). No further metabolite was detected in LC-MS analysis. Experiments are under way to synthesize the corresponding *N*-oxide and further confirm its metabolic formation by comparison.

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 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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