EXPERIMENTAL ARTICLES

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METABOLIC PROFILE AND MECHANISMS REACTION OF RECEPTOR GABA-TARGETED PROPOXAZEPAM IN HUMAN HEPATOCYTES

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The aim of this study was to identify the Propoxazepam metabolites, formed by suspension of human cryopreserved hepatocytes using the method accurate mass LC-MS/MS analysis. A suitable chromatographic method was developed for the profiling of Propoxazepam and its metabolites. Samples were analyzed using a Waters Vion high resolution LC-MS/MS instrument and data examined using Waters Unifi software to determine the identity of the most abundant metabolites. Following a 4 hour incubation with human hepatocytes, Propoxazepam accounted for 96.0% of the profile. The most abundant metabolite formed was oxidized Propoxazepam (3-hydroxyderivative), which accounted for approximately 2.5% of the total peak response in the 4 hour sample. Two minor components were also observed, each accounting for <10% of the total peak response. Glucuronic conjugates have not been found under these conditions. All metabolites formed represented less than 10% of the total chromatographic peak response. The data obtained indicate the absence of reactive electrophilic derivatives among the metabolites of Propoxazepam.

Key words: Propoxazepam; human hepatocytes; metabolism; LC-MS/MS analysis.

Introduction

A number of 3-substituted 1,4-benzodiazepines have been synthesized at the A.V. Bogatskiy Physico-Chemical Institute of the National Academy of Sciences of Ukraine, and their structure–activity relationships studied. Their pharmacological effect was unusual, because unlike most drugs in this class, in the models of nociceptive and neuropathic pain these substances showed significant analgesic activity [1]. One of them, Propoxazepam, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one, is considered a promising drug and is undergoing preclinical studies [2]. Similar to gabapentin and pregabalin, which are well-known drugs used in general medical practice in the treatment of neuropathic pain [3], propoxazepam also has an anticonvulsant effect [4, 5], which explains the analgesic component in the pharmacological spectrum of compound. Our data suggest that the mechanism of propoxazepam analgesic and anticonvulsant properties includes GABAergic and glycinergic systems [6, 7].

Metabolism (biochemical transformation) is an essential biological process that converts xenobiotics including medicines to water-soluble substances, and eliminates toxic compounds from the body. As a result of metabolism process, the parent compound can be converted to more (activation) or less (deactivation) active. To investigate how metabolites act in body, screening systems require *in vitro* and *in vivo* metabolism methods. Human *in vivo* toxicity data for new chemicals are usually not full and require preliminary data, obtained in a variety of *in vitro* models. One key initiative is the National

Toxicity Program (NTP), a U.S. federal government organization, started in 1978 to coordinate toxicological testing programs for the strengthening of toxicological sciences and development and validation of methods for testing of metabolites with potential toxicity. Various *in vitro* approaches have been described in the literature to screen for toxicity [7, 8].

Hepatocytes suspension can be used for short-term assays in a collagen-coated 24-well plates [9] or culture tubes [10]. In addition, hepatocytes demonstrated strong correlation between *in vitro* and *in vivo* hepatotoxicity studies [11]. Activity of different cell enzymes (cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT), and sulfotransferase (ST) have been characterized for their ability to be cultured *in vitro*.

The study was aimed to determine Propoxazepam metabolism in human hepatocytes. The metabolic profile of Propoxazepam and its metabolites analyzed by LC-MS/MS instrument. Our results will provide a reference for the clinical safety and rational use of this drug.

Materials and Methods

Propoxazepam and 3-hydroxy derivative (7-bromo-5-(2chlorophenyl)-3-hydroxy-1,3dihydro-2H-1,4-benzodiazepin-2-one, major metabolite) was synthesized according to the method described in [12]. Using the IR, mass spectroscopy and X-ray diffraction analysis the structure of the substance was determined and approved. Chemical purity was confirmed by elemental analysis (99%). General purpose reagents and solvents were of AnalaR® grade (or a suitable alternative). LC/MS grade reagents were used for preparation of mobile phases with developed chromatographic conditions for parent compound and reference standard. Samples were analyzed using reverse phase LC-MS analysis to generate high and low energy mass spectra (MSE). Each sample was analysed using accurate mass LC-MS to determine relative levels of parent compound at each time-point to determine the profile of metabolites formed. Appropriate no cells control samples were also analyzed.

All cryopreserved hepatocytes were obtained from BioIVT (formerly Bioreclamation IVT) and delivered stored frozen in liquid nitrogen. On receipt, the hepatocytes were transferred to permanent storage in liquid nitrogen. Human hepatocytes were supplied as a mixed-gender pool. The required vials of cryopreserved hepatocytes were removed from storage in liquid nitrogen. They were then immersed in a water bath set at 37 °C ± 1 °C for approximately 75-90 sec. The contents of each vial were then decanted directly into a tube which contained 15 mL of pre-warmed (37 °C ± 1 °C) *in vitro* GRO™ hepatocyte thawing medium per vial of hepatocytes with further re-suspension by gentle tube inverting followed by centrifugation at 50 g for 5 min at 20 °C. The supernatant was then poured off and the cell pellet was then loosened and then re-suspended in a total of ca 1.5 mL of supplemented Williams' Medium E per vial of thawed hepatocytes. Cells were kept on ice while not being actively prepared. Supplemented Williams' Medium E comprised Williams' Medium E (500 mL) supplemented with 10 mM dexamethasone (5 µL) and cell maintenance 'cocktail B' (solution of penicillin-streptomycin, ITS (insulin, transferrin, selenium complex) + BSA and linoleic acid), GlutaMAX™, and HEPES, 20 mL) which had been pre-gassed with (95% O_2/CO_2) for 30 minutes at room temperature. The cell suspensions were then made up to give required cell density at 1×10^6 cells/0.990 mL with supplemented Williams' Medium E. Trypan blue exclusion methods were used to determine the cell viability and density. Cell viability in the hepatocyte preparations was ≥70%.

The incubation components were mixed together in untreated 12-well plates as follows: Supplemented Williams' Medium E containing 1.52×10^6 viable cells in 1485 µL; Well plates were maintained at 37 °C ± 1 °C using a heated well plate incubator; Propoxazepam (15 µL of 10 mM solution in DMSO: Super pure water (1:1, v/v)) for hepatocyte-containing samples. Following the final addition of Propoxazepam, the well plates were then placed on a tilting mini rocker-shaker in an incubator (maintained at 37 °C ± 1 °C, 5% CO_2/95% O_2) to commence the incubation. At the requisite incubation period, for Propoxazepam samples, an aliquot (500 µL) was removed from each incubate and transferred to an aliquot of chilled acetonitrile to stop the reaction (incubation : acetonitrile ratio = 1:1 (v/v)). Each sample was vortexed and then sonificated in an ultrasonic bath for approximately 10 minutes to fully disrupt the cells. The samples were then stored at −70 °C ± 10 °C prior to transfer to the test site for analysis.

For the main incubation, a stock solution of Propoxazepam was prepared nominally at 2 mM in DMSO, followed by 1:1 dilution

with ultrapure water to give 1 mM stock solution. The 1 mM stock solution gave a final concentration of 10 μM in the final incubation and 0.5% (v/v) organic content. A portion of the stock solution was added to separate portions of the incubation mixture. For the remainder of the Propoxazepam samples after 4 hours incubation, the residual incubates, a further 1 mL aliquot of chilled acetonitrile added, mixed with a pipette and 2×1 mL aliquots were transferred into microcentrifuge tubes and treated as described above. These additional samples were prepared for the development of a suitable analytical method check/method development for the analysis of parent compound and metabolites. True' time zero incubations were prepared in parallel. Hepatocyte suspensions of each species (495 μL) were placed in a clean microcentrifuge tube followed by the immediate addition of 500 μL of chilled acetonitrile and vortex-mixing. Propoxazepam (5 μL) was then added to the hepatocyte suspension/acetonitrile mixture and then sonicated in an ultrasonic bath for approximately 10 minutes to fully disrupt the cells. The following control incubations were conducted in 12-well plates, in duplicate, in parallel to the main incubation: Incubation of Propoxazepam for 4 hours in the absence of hepatocytes.

Positive control samples incubating 7-ethoxy[3^{14}C]coumarin (7-EC) at a concentration of 50 μM for 4 hours in duplicate with hepatocytes. All samples were stored at −70 °C ± 10 °C. The samples incubated with test item were then transferred (frozen on dry ice, at −80 °C) to test site for characterization of parent and metabolites in the incubation medium by LC-MS/MS analysis [13]. The percentage of sample radioactivity associated with 7-EC and known metabolites, 7-hydroxycoumarin glucuronide (7-HCG), 7 hydroxycoumarin sulphate (7-HCS) and 7 hydroxycoumarin (7-HC) was determined for all samples

The nature and identity of Propoxazepam and its metabolites, present in representative aliquots of the incubation medium from human samples at 'true' zero and 4 hours as well as parent were investigated using accurate mass spectrometry LC-MS/(MS) analysis. A suitable chromatographic method was developed for the profiling of Propoxazepam and its metabolites. Samples were analysed using a Waters Vion high resolutions LC-MS/MS and data examined using Waters Unifi software to determine the identity of the most abundant metabolites. Samples were analyzed using reverse phase LC-MS analysis to generate high and low energy mass spectra (MSE). Each sample was analyzed using accurate mass LC-MS to determine relative levels of parent compound at each time-point, and determine the profile of metabolites formed. Appropriate no cells control samples were also analyzed. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for detection and characterization of reactive metabolites due to the high sensitivity and selectivity of this combined technique and its ability to separate, detect, an identify metabolites in the presence of endogenous materials [14].

No statistical analyses were performed on this study.

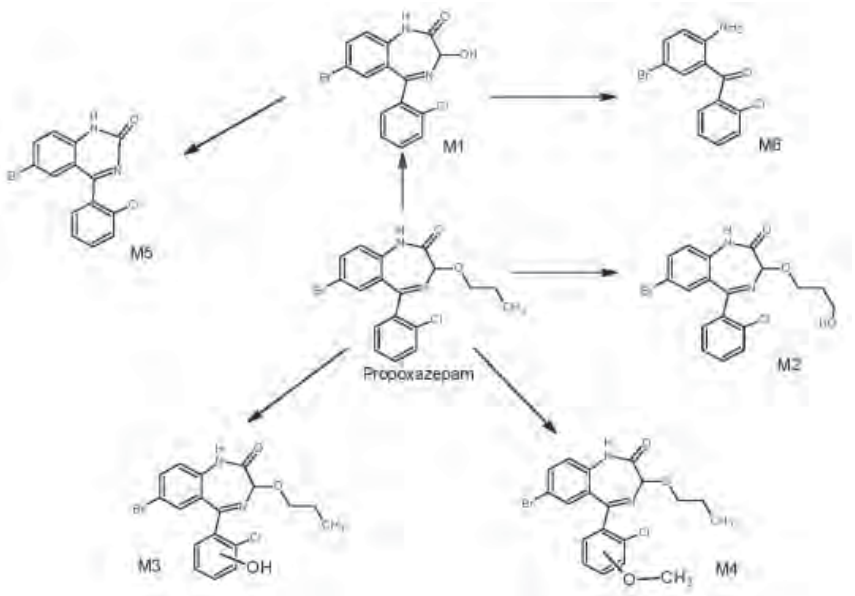


Fig. 1. **Theoretical scheme of Propoxazeam metabolism routes**

Results and Discussion

Based on the concept of regioselectivity and stereoselectivity of xenobiotic biochemical transformation [15], we proposed theoretically possible metabolism route of propoxazepam.

According to the results of previous studies [16], Propoxazepam metabolism leads to formation, at least, the following metabolites: 3-hydroxyderivative (M1) and aromaticaly oxidized (hydroxylated) parent compound (M3) as well as its methoxyderivative (M4) and oxidized in the alcoxyradical (M2). The presence of corresponding quinazolinone (M5) and benzophenone (M6) is also possible.

In this metabolite profiling data are comparative only, as these values were not generated using quantitative analytical methods. The proportions of parent compound and its metabolites assume equivalent mass spectrometer detector response. As the ionization characteristics of the parent compound and its metabolites are likely to be different; the actual proportions of these components cannot be confirmed, but the data can be considered to be comparable across metabolites. In addition to the accurate masses associated with the parent compound, the LC-MS data from the study samples were also examined for the possible metabolite transformations.

An extracted ion chromatogram (EIC) for Propoxazepam and 3-hydroxyderivative (M1) in a standard solution are presented in Fig. 2. EICs for selected 4 hour incubations are presented in Fig. 3. The peak response for each component was determined from the relevant EICs and was expressed as a percentage of the total evaluated peak response. These results are presented in Table 2.

In addition to the parent compound 3 metabolites were detected across the samples analyzed, these were assigned as M1, M2 and M2N based on retention time. Only Propoxazepam was observed following incubation of the test substance for 4 hours in the absence of hepatocytes, indicating that it was stable under the test conditions and absence spontaneous nonenzymatic (chemical) transformation.

Proposed structures, parent masses and characteristic fragment ions of metabolites observed in matrix samples are presented in Table 1.

A summary of representative accurate mass data is presented in Table 2.

Consequently Propoxazepam underwent moderate biotransformation in human cryopreserved hepatocytes after 4 hour incubation. Overall three metabolites were identified (M1, M2, M2N). All these metabolites formed represented less than 10% of the total chromatographic peak response.

The changes in levels of parent 7-ethoxycoumarin (positive control) and formation of associated Phase I and Phase II metabolites indicated that all hepatocytes were metabolically viable and were capable of integrated Phase I/II metabolism under the incubation conditions used on this study. Therefore, the results generated for the incubation of these hepatocytes with Propoxazepam considered valid.

Earlier we have analyzed the Propoxazepam metabolites formed in the organism of animals using the synthesized [2^{14}C]Propoxazepam [13,

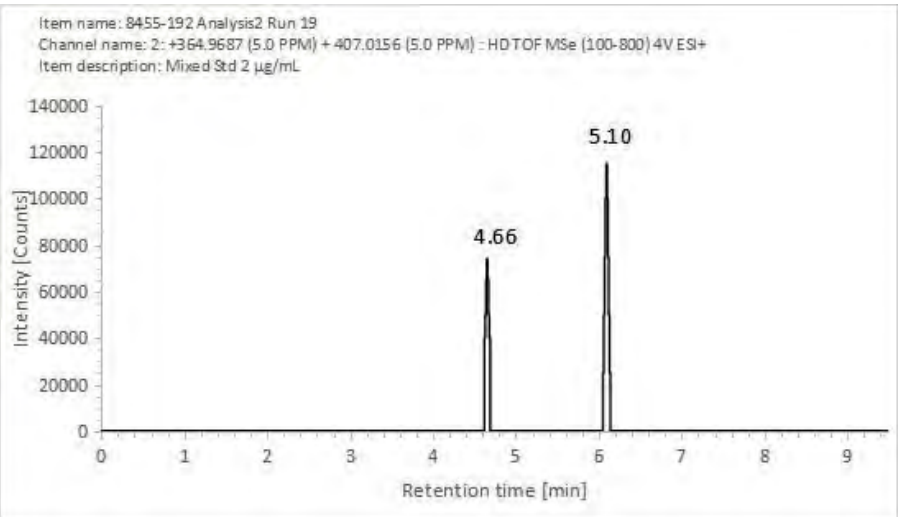


Fig. 2. **Extracted ion chromatogram from the analysis of a 2 µg/mL standard solution of Propoxazepam (retention time 6.10 min) and 3-hydroxyderivative (retention time 4.66 min)**

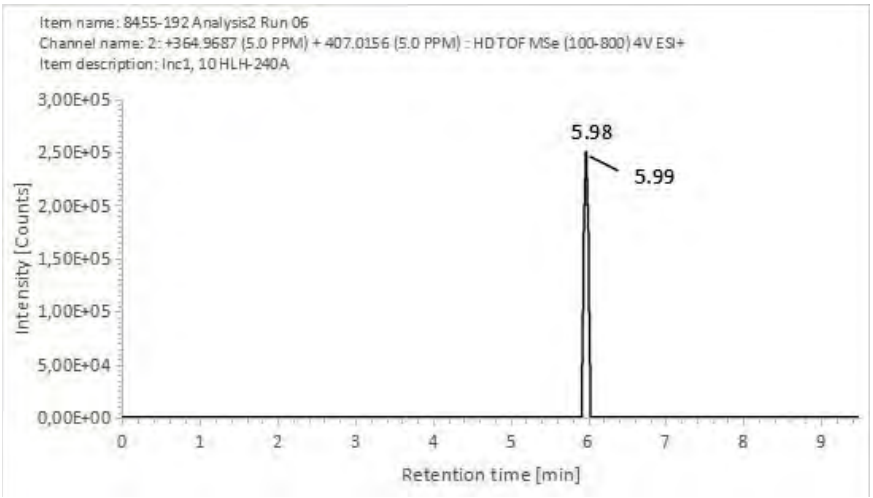
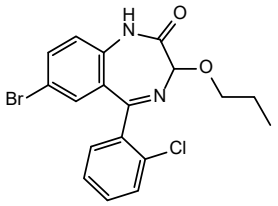
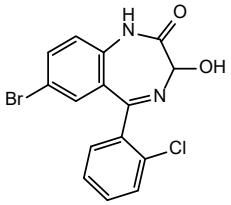
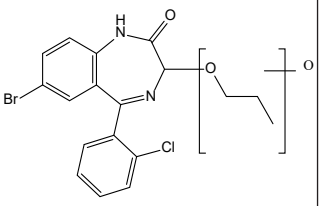
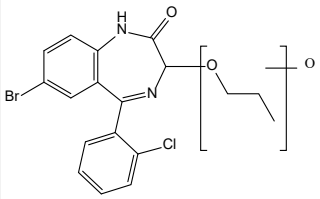


Fig. 3. **Extracted ion chromatogram of human hepatocyte incubation of Propoxazepam (4 hours). Retention time: 4.53 min Propoxazepam+O (M2); 4.55 min 3-hydroxyderivative (M3); 4.78 min Propoxazepam+O (M4); 5.98 min Propoxazepam**

Table 1. **Summary of protonated molecular ions and characteristic fragment ions for Propoxazepam and identified metabolites**

Peak	Retention Time, min	$[M+H]^+$	Structure	Characteristic Fragment Ions
Parent Propoxazepam	6.12	407		347*, 319, 273, 239, 205
M1 (3-hydroxyderiva-tive)	4.69	365		347*, 273, 194
M2 (Propoxazepam +O)	4.66	423		347*, 319, 239, 205
M2N (Propoxazepam +O)	4.91	423		347*, 319, 239, 205

Note: Retention times are representative and may differ from extracted chromatograms

Table 2. **Summary of representative accurate mass data for Propoxazepam and identified metabolites**

Peak	Measured *m/z*	Theoretical *m/z*	Proposed neutral formula	ΔmDa	Δppm
M2	423.0101	423.0106	$C_{18}H_{16}BrClN_2O_3$	−0.5	−1.2
M1	364.9676	364.9687	$C_{15}H_{10}BrClN_2O_2$	−1.1	−3.0
M2N	423.0107	423.0106	$C_{18}H_{16}BrClN_2O_3$	0.1	0.2
Propoxazepam	407.0155	407.0156	$C_{18}H_{16}BrClN_2O_2$	−0.1	−0.2

Note: values are representative unless otherwise stated ΔmDa = (Measured Mass — Theoretical Mass)*1000 Δppm = (ΔmDa/Theoretical Mass)*1000

17]. Using UPLC-MS/MS method [18] it was found that M1, M2, M3, M4 metabolites are formed in rat organism. Thus, only metabolites M1 and M2 are similar for human and rat.

The molecule of Propoxazepam has some reaction centers, which undergo transformation during metabolism. In theory any structural fragment or functional group can be a reaction center, but in particular enzymatic reaction it is formed by the limited quantity of atoms, what is due to the nature of reaction center and the nature of its nearest environment. The particularities of structural selectivity and stereochemical changes are valuable criteria for determination of reaction mechanism.

Each reaction of Propoxazepam metabolites formation has its own mechanism (Fig. 1). So the main metabolite M1 is formed during the oxidative dealcylation of the aliphatic moiety of molecule, which is facilitated by the transformation of stable ether group to ester one, which is more capable for hydrolysis, during which the formation of 3-hydroxyderivative takes place (Fig. 4).

There is a possibility that one of intermediates (either M7 or M8) in this scheme is metabolite M2N (Table 2).

This process differs from the process of 3C-hydroxylation of 1,4-benzodiazepindes with methylene group in the position "3" of heteroring, which is catalyzed with the corresponding monooxygenazes CYP3A4 and CYP2C19 [19]. In this case takes place the stereoselective formation of 3-hydroxymetabolites due to the asymmetrical carbon in position "3".

M2 metabolite formation proceeds through the aliphatic oxydation of methylene group. In the simplest way nonactive carbon of the alcyl group undergoes hydroxylation, catalyzed by CYP. The penultimate position ω-1 is the preferred site, but hydroxylation can also take place at the ultimate position or in another positions in case of spatial difficulties of with specific cytochromes as in case of Propoxazepam. Based o the isotope exchange data [20] during the aliphatic hydroxylation the reaction mechanism involves the inclusion of singlet oxygen in the bond carbon-oxygen.

The enzymatic oxidation of Propoxazepam, which takes place only *in vivo*, passes through the epoxides stage. It can be the way of direct intrusion of oxygen in the bond carbon-hydrogen with the formation of corresponding phenol, or by joining of oxygen to the formally-aromatic double bond with subsequent epoxide isomerization to phenol [15]. Aromatic structures hydroxylation according to the electrophilic substitution does through the joining of CYP450-activated oxygen to π-electron system and the orientation of the formed compound is determined with the nature of already existing substituent [19] — either to ortho- or para- positions.

Methoxyderivative of Propoxazepam(M4) belongs to rare enough metabolites of xenobiotics. The mechanism of its formation is unknown but possibly the first step is aromatic hydroxylation followed by methylation performed by methyltransferase [15].

Both in present study and in previous one [13] metabolites M5 and M6 haven`t been found (Fig. 1). We have earlier identified in the rat organism the processes of narrowing of seven-membered heterocyclic ring of 1.4-benzodiazepines to six-membered quinazolinone (M5) and the mechanisms of this transformation were described [19, 21]. The hydrolytic cleavage to the corresponding 2-aminobensophenones had also been detected in the organism of rats [22].

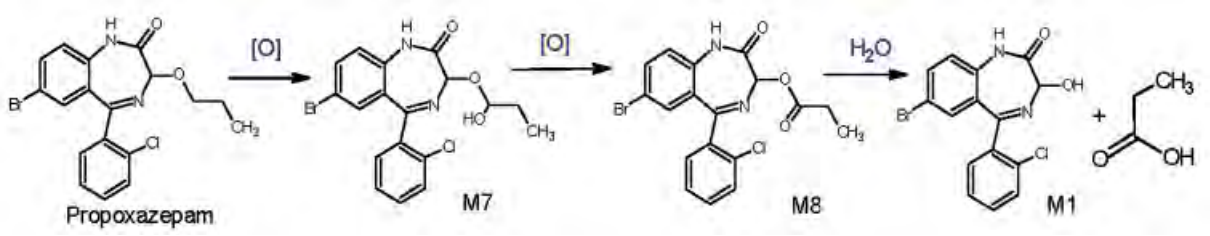


Fig. 4. **Scheme of Propoxazepam transformation to 3-hydroxyderivative (M1)**

Despite the presence of both UDP-glucuronosyltransferase (EC 2.4.1.17) and sulfotransferase (EC 2.8.2.29) in human hepatocytes, catalyze the conversation of 7-ethoxycoumarine (positive control) to glucuronide and sulfate, the corresponding conjugates of 3-hydroxymetabolite haven`t been found. The possible explanation of this is that this reaction goes through phenolic types of conjugates but in our studies such metabolites (M3) doesn`t form.

The common purpose of metabolism is adding of ionozable groups and formation of compounds with lower lipophilicity. This allows the faster elimination (detoxication) of xenobiotics from the body. Metabolites are commonly less toxic than the corresponding parent drug, though in some cases the more reactive electrophilic compounds, possessing higher toxicity are formed (bioactivation).

One of isoforms of cytochrome P450 (CYP3A4) is the predominant isoform in liver and metabolizes more than 50% of the clinical drugs commonly used. However, CYP3A4 is also responsible for the formation of reactive metabolites of nitrobenzodiazepines, such as flunitrazepam, nimetazepam, nitrazepam, and clonazepam [23]. Flunitrazepam was reported to induce hepatotoxicity and nitrazepam and clonazepam were reported to cause drug-induced liver injury [24]. In the reductive pathways from nitro to the reduced amine catalyzed by P450 and/or reductase, several reactive metabolites including nitroso- and N-hydroxylamine derivatives could be produced. Such reactive metabolites seem to bind covalently to nucleophile targets of proteins and nucleic acids, leading to the cytotoxic effects [25]. On the other hand, electrophilic N-hydroxylamine reacts with intracellular molecules, which induce various types of toxicity including hepatotoxicity.

Reactive metabolites are often short-lived and are not usually detectable in circulating blood/plasma. *In vivo* (acute and chronic toxicity, gene mutations) approaches are generally employed to examine the bioactivation potential of drug candidates, which may provide some indirect but valuable information that drug undergo bioactivation to form reactive electrophilic species. LD_{50} of Propoxazepam is greater than 5000 mg/kg and, therefore, it belongs to the category V of relatively non-toxic substances according to the GHS [26]. In our studies [27], the possible induction of gene mutations by the effects of Propoxazepam on the *S. typhimurium TA98* (frameshift mutation type) and *TA 100* (mutations of the base substitution) without and with metabolic activation (fraction S9) showed that Propoxazepam did not the ability to induce gene mutations in the test system used by us. The metabolic activation was also not effective, that is, Propoxazepam is neither a “direct” nor an “indirect” mutagen for Ames strains. Thus, the data obtained during the microplate version of the Ames test (Muta-ChromoPlate kit) on the strains *Salmonella typhimurium TA 98* and *TA 100* indicate that mutagenic activity of Propoxazepam in the concentrations studied is not revealed.

Conclusions

Under *in vitro* conditions Propoxazepam showed relatively high metabolic stability with ~90 % of unchanged compound after 4 hours incubation with human hepatocytes. The main detected metabolites detected were 3-hydroxyderivative and oxidized in alcoxy moiety. At the same time, no glucuronic or sulfate conjugates were found. The presented data indicate the absence of reactive chemicals among the metabolites of Propoxazepam.

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МЕТАБОЛІЧНИЙ ПРОФІЛЬ ТА МЕХАНІЗМИ РЕАКЦІЇ ГАМК-РЕЦЕПТОР ОРІЄНТОВАНОГО ПРОПОКСАЗЕПАМУ В ГЕПАТОЦИТАХ ЛЮДИНИ

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Мета. Виявлення метаболітів Пропоксазепаму, що утворюються суспензією кріоконсервованих гепатоцитів людини, за допомогою методу точного масового LC-MS/MS аналізу.

Методи. Для профілювання Пропоксазепаму та його метаболітів розроблено відповідний хроматографічний метод. Зразки аналізували за допомогою приладу WatersVion високої роздільної здатності LC-MS/MS, отримані дані обробляли за допомогою програмного забезпечення WatersUnifi для визначення та ідентифікації найбільш поширених метаболітів.

Результати. Після 4-годинної інкубації з гепатоцитами людини Пропоксазепам становив %96,0 складу від вихідної кількості препарату. Найпоширенішим метаболітом, який утворився, був окиснений Пропоксазепам (3-гідроксипохідне), що становило приблизно 2,5% загальної пікової реакції у 4-годинному зразку. Також спостерігали два мінорні компоненти, кожен з яких становив менше 10% загальної площі піків. Глюкуронові кон'югати за цих умов не було ідентифіковано. Усі утворені метаболіти становили менше 10% загальної хроматографічної пікової відповіді.

Висновки. Отримані дані свідчать про відсутність серед метаболітів Пропоксазепаму реакційно здатних електрофільних похідних.

Ключові слова: Пропоксазепам; гепатоцити людини; метаболізм; LC-MS/MS аналіз.