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Species differences metabolism *in vitro* model of a novel analgesic propoxazepam with polymodal mechanism of action

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Abstract

The aim of this study was to identify the Propoxazepam metabolites, formed by suspension of rat, dog, monkey and minipig cryopreserved hepatocytes. 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. The reported proportions of parent compound and its metabolites assume equivalent mass spectrometer detector response. The proportions of unchanged parent drug after 4 hour incubation with hepatocytes varied between 37.1 to 96.0 % of the total peak response. The most abundant metabolite observed across all species was oxidised Propoxazepam (3-hydroxy derivative) which accounted for between 2.5 to 46.5 % of the total peak response in the 4 hour samples. Four other minor metabolites were observed each representing <10 % of the total peak response. The primary route of *in vitro* metabolism was by oxidation, dealkylation and conjugation with glucuronic acid. Some species differences were noted. The presented data indicate the absence of reactive chemicals among the metabolites of Propoxazepam. Propoxazepam underwent moderate metabolism in monkey cryopreserved hepatocytes after 4 hour incubation. Less extensive metabolism was observed in rat and minipig hepatocytes after the same incubation period and limited metabolism was observed in dog hepatocytes.

Keywords: Propoxazepam; Rat; Dog; Monkey; Minipig; Metabolism

1. 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].

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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 ivo* 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 [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*.

For this reason, the current study aimed to comparative in vitro Propoxazepam metabolism using rat, dog, monkey and minipig hepatocytes by LC-MS/MS instrument. Our results will provide a reference for the clinical safety and rational use of this drug.

2. Material and methods

2.1. Chemicals and reagents

Propoxazepam and 3-hydroxy derivative (7-bromo-5-(2chlorophenyl)-3-hydroxy-1,3 dihydro-2H-1,4-benzodiazepin-2-one, major metabolite) were 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 analytical 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.

2.2. Cryopreserved hepatocytes

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. All animal hepatocytes were supplied as a pool of the male: rat Sprague Dawley (30), dog Beagle (3), monkey Cynomolgus (3), minipig Gottingen (3). Each vial used contained at least 5×10^6 cells. 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 s until all the contents had melted. The hepatocytes were re-suspended and cells were then centrifuged at 50 × g for 5 minutes at 20°C. The supernatant was then poured off and the cell pellet was then loosened and then resuspended in a total of ~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% for all species.

2.3. Dose Formulation and Incubation with pooled rat, dog, monkey and minipig cryopreserved hepatocytes

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

2.4. Metabolite identification

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].

2.5. Data Evaluation

Data were interrogated for the presence of metabolites by comparison of retention times with the reference standards and based on the accurate masses of potential metabolites using screening software (UNIFI version 1.9.4), and user defined search parameters. To confirm a suspected metabolite, the measured accurate mass of the peak detected in the sample used for structural elucidation had to be within 5 ppm of the theoretical mass in order to confirm the molecular formula. The peak response of the parent compound and potential metabolites were determined from the respective accurate mass extracted ion chromatograms (EIC). The peak response of each component was calculated as a percent of total evaluated response. Components of the profiles with a response greater than 1 % of the total evaluated response in at least one sample were reported. All calculations were carried out using Microsoft Excel. Data presented in the tables and appendices are computer generated and rounded appropriately for inclusion in the report. Consequently, manual calculation of group means from the data presented in the report tables will, in some instances, yield a minor variation from the reported value.

No statistical analyses were performed on this study

3. Results

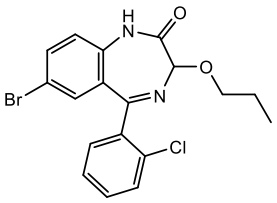
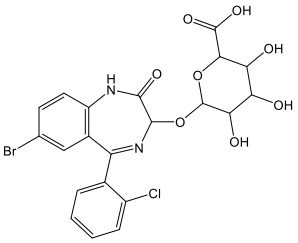
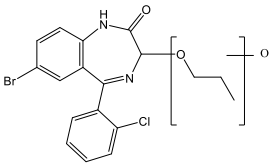
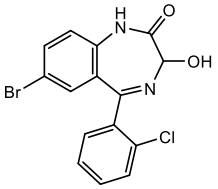
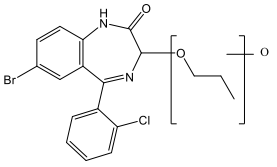
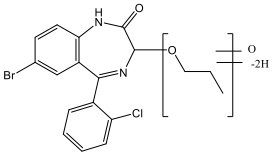
For all hepatocytes, cell viability was assessed using the trypan blue exclusion test [15]. The cell viability for all hepatocytes was in the range 74.7% to 90.1%. The metabolic viabilities of the hepatocytes used in the study were assessed by incubating 7 ethoxy[3-^{14}C]Coumarin (50 µM) in parallel with Propoxazepam as a positive control [16]. In all species, the Phase I metabolite 7-hydroxycoumarin (7-HC) was formed and subsequently conjugated to varying extents as indicated by the presence of the 7-hydroxy-coumarin glucuronide (7-HCG) and 7 hydroxycoumarin sulphate

(7-HCS). The changes in levels of parent 7-ethoxycoumarin 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 will be considered to be valid.

Only Propoxazepam was observed following control incubation of the test substance for 4 hours in the absence of hepatocytes, indicating that it was stable under the test conditions.

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

Table 1 Protonated molecular ions and characteristic fragment ions for Propoxazepam and identified metabolites

Peak	**Retention Time (Minutes)**	**$[M+H]^+$**	**Structure**	**Characteristic Fragment Ions**	**Species**
Parent Propoxazepam	6.12[b]	407		347*, 319, 273, 239, 205	Rat, Dog, monkey, Minipig
M1 (Propoxazepam - C_3H_6(cleavage) +$C_6H_8O_6$)	3.80[b]	541		347, 319	Rat, Dog, Monkey, Minipig
M2 (Propoxazepam +O)	4.66[b]	423		347*, 319, 239, 205	Rat, Dog, Monkey, Minipig
M3 (3-Hydroxy propoxazepam)	4.69[b]	365		347*, 273, 194	Rat, Dog, Monkey, Minipig
M4 (Propoxazepam +O)	4.91[b]	423		347*, 319, 239, 205	Rat, Dog, Monkey, Minipig
M5 (Propoxazepam - H_2+O)	5.13[b]	443[#] 421		319	Rat, Monkey, Minipig

Note: Retention times are representative and may differ from extracted chromatograms; [b] Retention time from analysis of a 10µM cyno monkey 4h incubation sample; # Major ions observed as a sodium adduct * in source fragment

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

Table 2 Representative accurate mass data for Propoxazepam and identified metabolites

Peak	Measured *m/z*	Theoretical *m/z*	Proposed Neutral Formula	ΔmDa	Δppm
M1	540.9998	541.0008	$C_{21}H_{18}BrClN_2O_8$	-1.0	-1.8
M2	423.0101	423.0106	$C_{18}H_{16}BrClN_2O_3$	-0.5	-1.2
M3	364.9676	364.9687	$C_{15}H_{10}BrClN_2O_2$	-1.1	-3.0
M4	423.0107	423.0106	$C_{18}H_{16}BrClN_2O_3$	0.1	0.2
M5	442.9769	442.9769	$C_{18}H_{14}BrClN_2O_3$	0.0	0.0
C007/II	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

Following the incubation of Propoxazepam with cryopreserved hepatocytes in suspension for up to 4 hours, moderate or limited metabolism of the test compound was observed for all species. The parent compound, Propoxazepam, and 5 further components were detected across the samples analysed. Graphical of metabolites profile presented in Figure 1.

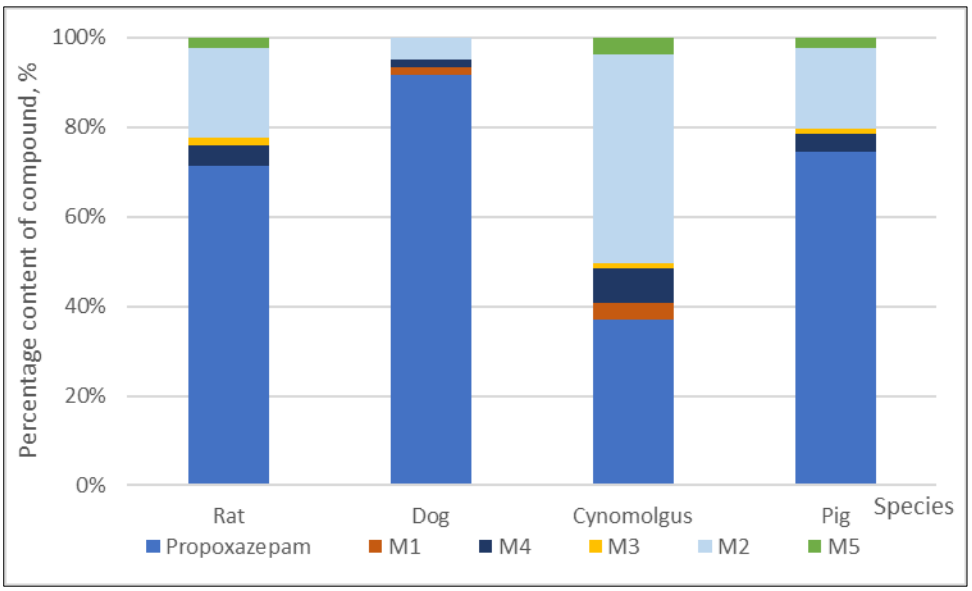


Figure 1 Comparison of metabolites across species. % of total responce

4. Discussion

Many new chemical entities are metabolically unstable due to metabolism mediated by various drug metabolizing enzymes, including CYP450 and several conjugative enzymes, especially UDP-glucuronosyltransferases [17]. Metabolic stability is considered an important property of drug and is usually quantified by monitoring the disappearance of the parent compound over time in an *in vitro* system to determine the metabolic half-life and intrinsic clearance) [18]. Results obtained from in vitro metabolic stability experiments are used to estimate and predict clearance; in *vivo* at the early stages of drug discovery. Metabolism of a drug candidate is often a primary mechanism of clearance. It may also lead to the production of metabolites with pharmacological or toxicological activity.

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 (Table. 1).

Propoxazepam underwent moderate metabolism in monkey cryopreserved hepatocytes after 4 hour incubation. Less extensive metabolism was observed in rat and minipig hepatocytes after the same incubation period and limited metabolism was observed in dog hepatocytes. Overall five metabolites were identified: four phase I metabolites (M2, M3, M4 and M5) and one phase II metabolite (M1).

The major metabolite formed in all species was Propoxazepam +O (M4) accounting for between 2.5% and 46.5% of the total chromatographic peak response. Additional phase I metabolites were formed via oxidation or dealkylation. Phase II metabolism was also observed via conjugation with glucuronic acid. All these additional metabolites formed represented less than 10% of the total chromatographic peak response. Some differences between responses and metabolites detected were observed between species.

In the rat hepatocyte incubation samples, Propoxazepam was the only component observed in the 0 hour samples, accounting for 100 % of the chromatographic peak area. Following a 4 hour incubation time with rat hepatocytes, Propoxazepam accounted for 71.3 % of the profile. The most abundant metabolite formed was oxidised Propoxazepam (M4). This accounted for ca. 20 % of the total peak response. Four minor components were also observed, each accounting for <10 % of the total peak response.

In the dog hepatocyte incubation samples, Propoxazepam was the only component observed at 0 hour, accounting for 100 % of the chromatographic peak area. Following a 4 hour incubation with dog hepatocytes, Propoxazepam accounted for 91.8 % of the profile. The most abundant metabolite formed was oxidised Propoxazepam (M4). This component accounted for ~4.8 % of the total peak response in the 4 hour sample. Three minor components were also observed, each accounting for <10 % of the total peak response. In the Cunomolgus monkey hepatocyte incubation samples, Propoxazepam was the only component observed at 0 hour, accounting for 100 % of the chromatographic peak area. Following a 4 hour incubation with monkey hepatocytes, Propoxazepam accounted for 37.1 % of the profile. The most abundant metabolite formed was oxidised Propoxazepam (M4). This component accounted for ~46.5 % of the total peak response in the 4 hour sample. Four minor components were also observed, each accounting for <10 % of the total peak response.

In the minipig hepatocyte incubation samples, Propoxazepam was the only component observed at 0 hour, accounting for 100 % of the chromatographic peak area. Following 4 hour incubation with minipig hepatocytes, Propoxazepam accounted for 74.4 % of the profile. The most abundant metabolite formed was oxidised Propoxazepam (M4). This component accounted for ~18.1 % of the total peak response in the 4 hour sample. Four minor components were also observed, each accounting for <10 % of the total peak response. 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). The presented data indicate the absence of reactive chemicals among the metabolites of Propoxazepam.

5. Conclusion

The metabolism of Propoxazepam across species using in vitro models (i.e. cryopreserved hepatocytes in suspension) was assessed. The reported metabolite profiling data are comparative only, as these values were not generated using quantitative analytical methods. The reported proportions of parent compound and its metabolites assume equivalent mass spectrometer detector response. The proportions of unchanged parent drug after 4 hour incubation with hepatocytes varied between 37.1 to 96.0 % of the total peak response. The most abundant metabolite observed across all species was oxidised Propoxazepam (M4) which accounted for between 2.5 to 46.5 % of the total peak response in the 4 hour samples. Four other minor metabolites were observed each representing <10 % of the total peak response. The primary route of in vitro metabolism was by oxidation (M2, M4 and M5), dealkylation (M3) and conjugation with glucuronic acid (M1). Some species differences were noted.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have no financial interest to declare in relation to the content of this article. The Article Processing Charge would be paid for by the authors.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

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