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Cross-species differential plasma protein binding of Propoxazepam, a novel analgesic agent

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Aim. The purpose of this study was to characterize the Propoxazepam properties of binding to the human, dog and rat plasma proteins and to evaluate potential interspecies differences in binding affinity. **Methods.** The studies on Plasma protein binding were conducted using the Rapid Equilibrium Dialysis system with subsequent LC MS/MS analysis. The albumin binding of the substances was characterized by the percentage of bound drug. **Results.** Propoxazepam was highly bound to the plasma proteins in rats, dogs and humans. The extent of Propoxazepam binding in human plasma was similar to that observed in rat plasma whereas it was slightly lower in dog plasma, following dialysis for 8 hours. **Conclusions.** No concentration dependent effects on plasma protein binding were observed in the species investigated for Propoxazepam. Overall mean free fractions of Propoxazepam were 1.60 %, 2.90 % and 1.96 % in rats, dogs and humans, respectively. The results obtained in this work gave insights on the role of HSA in distributing Propoxazepam to target tissues and in determining their pharmacological properties. The results are crucial for the development of Benzodizepines-based therapeutics drugs.

Keywords: Propoxazepam, plasma protein, interspecies binding

Introduction

Propoxazepam, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepine-2-one, showed significant analgesic activity in the models of nociceptive and neuropathic pain [1], and is currently being tested in clinical trials. Similar to gabapentin and pregabalin, well-known anti-epileptic drugs used in general medical practice in the treatment of neuropathic pain, propoxazepam also has an anticonvulsant effect [2, 3], which ex-

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plains the analgesic component of the pharmacological spectrum. Both oral and intraperitoneally preparations are similar in activity. The *in vivo* studies proved that Propoxazepam is the potent drug in its class against acute and chronic pain.

Our data suggest that the mechanism of Propoxazepam anticonvulsant properties includes GABAergic and Glycinergic system [2, 3]. Antibradykinin and antileukotriene action, dopaminergic system, NMDA, and alpha-1 adrenergic receptors with the absence of antiprostaglandin component are involved in the mechanisms of Propoxazepam analgesic effect [4].

In recent years, the binding between drug and biomacromolecules has attracted more and more attention, as the binding interactions are considered to be the beginning of the biological effects. Drug-plasma protein binding (PPB) is one of the important parameters of drug efficacy and safety, and the determination of bound fraction is a necessary step in the drug discovery and clinical trials [5]. It determines the pharmacokinetic and pharmacodynamic characteristics of drugs and influences drug absorption, distribution, metabolism, excretion and toxicity [6]. Small molecular substances can be protected from some elimination pathways, such as enzymatic reactions in the liver or blood and glomerular filtration of the kidneys, by forming non-covalent complexes with plasma proteins [7]. As a drug reservoir, the bound drug fraction can maintain an effective concentration and prolong the duration of the drug action. By itself, PPB has limited value, but this combination, together with the free drug hypothesis and an appropriate *in vitro* model of the human disease, enables the identification of potentially efficacious compounds. The free drug assumption states that only the free drug concentration at a receptor is responsible for efficacy and that at steady-state and in the absence of active transport, a permeable compound will have the same unbound concentration on both sides of a biological membrane. Therefore, the free compound concentration at the receptor in the target tissue is expected to be equivalent to the unbound concentration in blood. A greater dose of the drugs with high affinity for plasma proteins is generally needed to reach therapeutic level; such drugs have a long half-life and probably increased toxicity. Conversely, the drugs with low plasma protein binding affinities are less limited in their ability to perfuse tissues and reach the site of action [8].

The drug plasma protein binding is a critical parameter measured during drug discovery because it is generally accepted that only free drug in plasma is available to elicit a pharmacological effect, and from a pharmacokinetic point of view, only free drug is available to be cleared [9]. Therefore to assess the free drug concentration in the species used for pharmacokinetic (e.g. rat and dog) and pharmacodynamic (e.g. mouse) studies, it is necessary to measure the protein binding in plasma from each of the relevant species. Additionally, the value of human plasma protein binding should be measured to facilitate the prediction of human pharmacokinetics and pharmacodynamics [10].

The purpose of this study was to characterize the Propoxazepam properties of binding to human serum albumin using the equilibrium dialysis (Thermo-Scientific Rapid Equilibrium Dialysis) method, and to evaluate potential

interspecies differences in binding affinity that might help to explain the apparent pharmacokinetic discrepancy between humans and laboratory animals.

Materials and Methods

Chemical and tissue source

Propoxazepam (7-bromo-5-(o-chlorophenyl)-3-propiloxy-1,2-dihydro-3H-1,4-benzodiazepine-2-one) was synthesized according to the method described in [11]. 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) and were obtained principally from VWR International Ltd, Rathburn Chemicals Ltd, Sigma Aldrich Chemical Company Ltd and Fisher Scientific UK Limited.

Pooled plasma was from 13 male rats Han Wistar strain (BioIVT UK) and 4 male beagle dogs (Envigo UK Ltd). Fresh human blood was obtained from three male Labcorp volunteers who had taken no medication during the previous seven days. All procedures carried out on animals for the purposes of supplying blood and plasma samples were subject to the provisions of United Kingdom National Law, in particular the Animals (Scientific Procedures) Act 1986. Human blood was obtained at Labcorp via an informed consent process (which is subject to Local Research Ethics Committee approval).

Unbound Propoxazepam separation

PPB studies were conducted with plasma using the Rapid Equilibrium Dialysis (RED) system with subsequent LC MS/MS analysis. The duration of dialysis required to achieve equilibration was determined in an initial investigation, in human plasma, at the mid concentration of Propoxazepam (100 ng/mL). Spiked plasma and a volume of PBS were pre-incubated at 37 °C for 15 minutes. Human plasma spiked with Propoxazepam was then placed in the donor chamber and PBS in the acceptor chamber. Triplicate samples were then dialysed at 37 °C/5 % CO_2 for 0, 1, 2, 4, 6, 8, 16 and 24 hours. Aliquots of the buffer and protein solutions from each chamber with aliquots of stock spiked plasma were analyzed by LC-MS/MS and the concentration of Propoxazepam in each chamber were calculated. Additionally, the total recovery of Propoxazepam from the inserts was calculated.

PPB characteristics of Propoxazepam from In Vitro

Rat, dog and human plasma samples were spiked with Propoxazepam at 10, 100 and 1000 ng/mL. Spiked plasmas and a volume of PBS were pre incubated at ca. 37 °C for 15 minutes. Triplicate samples were then dialysed at 37 °C/5 % CO_2 for 8 hours (as determined in the previous experiment). Aliquots of the stock spiked plasma and the buffer and protein solutions from each chamber were analysed by LC-MS/MS and concentrations of Propoxazepam in each chamber were calculated, to enable calculation of the free fraction.

Analytical Methods for the buffer and plasma concentration of Propoxazepam

Matrix matched protein and buffer samples were analysed by LC-MS/MS following protein precipitation. Calibration standard working solutions were used to freshly prepare the calibration standards. An aliquot 50 µL (low range) and 10 µL (high range) of the calibration standards, quality control (QC) samples and blanks were added to a 2 mL 96-well microplate. An equivalent volume of water was added to the reagent blank sample. Internal standard solution (Propoxazepam; 10 ng/mL (low range) or 500 ng/mL (high range) in acetonitrile; 25 µL) was then added to the samples, calibration standards and QC samples. Acetonitrile (25 µL) was added to the blank samples. Acetonitrile (150 µL (low range) or 400 µL (high range)) was added to all samples. Aliquots of the supernatant solutions (80 µL (low range) or 20 µL (high range)) were then transferred to a clean 96-well microplate using an automated liquid handling device (Hamilton Microlab STAR). Acetonitrile (60 µL) was added to high range samples only. To all samples, 10 mM ammonium formate (aq.): formic acid (100:0.2 v/v) was added (120 µL). After centrifugation at 2000 × g for approximately 5 minutes at 5 °C, the samples were stored at 2–8 °C (nominally 4 °C) prior to analysis by LC-MS/MS.

A system suitability check was routinely performed at the start of each sample batch. The purpose was to check the chromatography of the system by evaluating the retention time window of the test substance, the internal standard and also to check the system response, ensuring that required sensitivity could be achieved. The system suitability as used throughout the sample analysis comprised of a Lower limit of quantification (LLOQ) extracted sample followed by an Upper limit of quantification (ULOQ) extracted sample, followed by an injection of a matrix blank.

Low range calibration curves in the range 0.1 to 500 ng/mL and high range curves in the range 10 to 50000 ng/mL were constructed by adding known amounts of C007/II and extraction of these standards alongside each batch of study samples.

The calibration curves were calculated by quadratic weighted (1/x2) least squares regression analysis by plotting peak area ratio versus analyte concentration.

In each analytical run, the following Quality controls (QC) samples were analysed in duplicate as a minimum: Low range: 0.3 ng/mL (LoQC), 6 ng/mL (Lo-MeQC), 40 ng/mL (MeQC), 200 ng/mL (Hi MeQC) and 400 ng/mL (Hi QC). High range: 30 ng/mL (LoQC), 600 ng/mL (Lo-MeQC), 4000 ng/mL (MeQC), 20000 ng/mL (Me-HiQC) and 40000 ng/mL (HiQC).

The calibration standards were accepted if the back-calculated concentration did not deviate by more than 30 % from the nominal concentration. No more than 25 % of the calibration standard levels were excluded from the calibration curve and at least six calibration standard levels remained. All sample results of an analytical run were accepted if QC accuracy did not exceed±25 % at each concentration.

Calculation of PPB of Propoxazepam

The extent of protein binding, determined using the RED equilibrium dialysis method, was calculated from the following equations:

$$\% \text{ Bound} = \frac{Cp - Cb}{Cp} \times 100$$

$$\% \text{ Free fraction} = \frac{Cb}{Cp} \times 100$$

$$\% \text{ Recovery} = \frac{(Cp \times Vp + Cb \times Vp)}{Cpi \times Vp} \times 100$$

where: Cp = concentration in protein compartment
Cb = concentration in buffer compartment
Cpi = initial concentration in spiking solution
Vp = volume in protein compartment
Vb = volume in buffer compartment

Statistics

Results were presented as mean±standard deviation (SD).

Results

The stability of Propoxazepam in plasma and 0.01 M phosphate buffered saline, pH 7.4 (PBS) was assessed prior to sample dialysis. Propoxazepam (100 ng/mL) was incubated for 0, 1, 2, 4, 6, 8, 16 and 24 hours at 37 °C in rat, dog and human plasma and PBS. Aliquots of plasma or buffer samples were then matrix matched (i.e. plasma diluted with an equal volume of PBS buffer and vice versa) prior to analysis by LC MS/MS. The concentration of Propoxazepam in plasma and buffer was determined at each time point. Samples were generated in triplicate. The results of the experiment are presented in Table 1. Absolute

Table 1. **Stability of Propoxazepam (100 ng/mL) following incubation for up to 24 hours at 37 °C in plasma and buffer.**

Species	Duration of incubation (h)	Mean concentration (ng/mL)±SD	% Time zero
Rat	0	89.6±2.2	100
	8	93.0±3.2	104
	16	87.8±1.2	97.9
	24	90.0±2.8	100
Dog	0	91.2±1.6	100
	8	89.8±0.6	98.5
	16	94.3±1.3	103
	24	93.5±0.6	103
Human	0	84.8±5.0	100
	8	92.3±3.0	109
	16	94.8±3.8	112
	24	91.8±1.7	108
Buffer	0	63.6±1.5	100
	8	73.7±3.1	116
	16	63.7±2.6	100
	24	80.6±0.8	127

SD: Standard deviation

Table 2. **In vitro determination of time to equilibration of Propoxazepam (100 ng/mL) following dialysis of spiked human plasma against buffer for 0, 6, 8, 16 or 24 hours (concentration, mean±SD).**

Duration of dialysis (hours)	Plasma cmpt (ng/mL)	Buffer cmpt (ng/mL)	Protein bound (%)	Free fraction (%)	NSB (%)**	Recovery (%)
0*	85.4±3.7	0.00±0.00	100±0	0.00±0.00	5.18±4.10	94.8±4.1
6	87.6±7.0	1.99±0.09	97.7±0.1	2.28±0.09	2.25±3.28	101±8
8	87.8±6.6	1.85±0.10	97.9±0.1	2.11±0.11	2.55±4.41	101±7
16	81.3±5.9	2.20±0.11	97.3±0.3	2.72±0.32	4.73±4.09	96.7±6.6
24	87.2±4.2	2.14±0.12	97.5±0.0	2.46±0.02	1.28±1.64	101±5

NSB: Non-specific binding cmpt:
Compartment SD: Standard deviation

* 0 hour buffer samples below LLOQ
** Negative NSB values reported as zero

Propoxazepam concentrations are presented, together with concentrations expressed as a percentage of the initial value at 0 hour.

In plasma from all species and PBS, no notable degradation of Propoxazepam was observed after incubation for up to 24 hours at 37 °C. These results demonstrated that reported data from the protein binding for these species were not biased.

The equilibration time of unbound Propoxazepam (100 ng/mL) and degree of non-specific binding were initially investigated in human plasma over a 24 hour incubation period. The results of this experiment are shown in Table 2 and indicated that a dialysis time of 8 hours was sufficient for the equilibration of unbound Propoxazepam between the two compartments.

Table 3. **Determination of protein binding of Propoxazepam at (10, 100 and 1000 ng/mL) following dialysis of spiked rat, dog and human plasma against phosphate buffered saline (PBS) for 8 hours.**

Species	Nominal Propoxazepam at concentration (ng/mL)	Mean protein bound±SD (%)	Mean free fraction±SD (%)
Rat	10	98.4±0.2	1.56±0.17
	100	98.5±0.1	1.50±0.13
	1000	98.3±0.2	1.73±0.15
	Overall mean±SD	**98.4±0.1**	**1.60±0.12**
Dog	10	96.9±0.3	3.06±0.34
	100	97.1±0.2	2.89±0.22
	1000	97.2±0.3	2.76±0.31
	Overall mean±SD	**97.1±0.2**	**2.90±0.15**
Human	10	98.0±0.1	1.95±0.08
	100	98.0±0.1	1.96±0.09
	1000	98.0±0.1	1.98±0.10
	Overall mean±SD	**98.0±0.0**	**1.96±0.01**

SD: Standard deviation

The equilibration time of unbound Propoxazepam (100 ng/mL) and degree of non-specific binding were investigated in human plasma over an 8 hour incubation period. The results of this experiment are shown in Table 3 and indicated that a dialysis time of 8 hours was sufficient for the equilibration of unbound Propoxazepam between the two compartments. An incubation time of 8 hours was therefore used for all subsequent experiments. Post-dialysis recoveries in these experiments (range: 94.8-103 %) indicated that non-specific binding was generally negligible. Protein binding data for Propoxazepam at concentrations of 10, 100 and 1000 ng/mL in rat, dog and human plasma are presented in Table 3.

Post dialysis recoveries in this experiment were >94 %. The mean free fractions (± SD) of Propoxazepam at 10, 100 and 1000 ng/mL were 1.56±0.17 %, 1.50±0.13 % and 1.73±0.15 % in rat plasma, respectively, with an overall mean of 1.60±0.12 %. In dog plasma, the mean free fractions of Propoxazepam at 10, 100 and 1000 ng/mL were 3.06±0.34 %, 2.89±0.22 % and 2.76±0.31 %, respectively, which gave an overall mean of 2.90±0.15 %. Dialysis of human plasma at Propoxazepam concentrations of 10, 100 and 1000 ng/mL gave mean free fractions of 1.95±0.08 %, 1.96±0.09 % and 1.98±0.10 %, respectively, with an overall mean value of 1.96±0.01 %. These data indicated that Propoxazepam was very highly bound to plasma proteins in rat, dog and human. The extent of Propoxazepam binding in human plasma was similar to that

observed in rat plasma and the extent of binding of Propoxazepam was slightly lower in dog plasma, following dialysis for 8 hours. No notable concentration dependent effects on the extent of plasma protein binding were observed in the concentration range tested for any of the species investigated.

Discussion

Propoxazepam is a novel analgesic and the process of drug development involves non-clinical and clinical studies. Non-clinical studies are conducted using different protocols including animal studies [12]. Drug plasma protein binding is a critical parameter measured during the drug discovery because it is generally believed that only free drug in plasma is available to elicit a pharmacological effect, and from a pharmacokinetic point of view, only free drug is available to be cleared [9].

Interspecies differences in PPB can lead to a reduced or increased drug-safety margin [13]. The regulatory authorities, including The State Expert Center of the Ministry of Health of Ukraine, recommend the determination of PPB in animal and human plasma before commencing Phase I trials and to use it as the supporting data set when evaluating drug–drug interactions [14]. Therefore, PPB is considered to be an important parameter throughout the ongoing drug development program. Comparison of the human PPB data for a variety of drug discovery compounds indicates that the compounds tend to be slightly more bound to the human plasma proteins, than to the plasma proteins from animals. There are, however, the examples of compounds that do show significant variation in the amount of unbound drug in plasma from pre-clinical species compared to that in human plasma and it can have major implications [15]. For the purposes of general screening, the human or rat measurements can be used as a single parameter. However, it is recommended to measure PPB always in the species of greater interest, whether it is for understanding pharmacokinetics in pre-clinical species or pharmacodynamics in disease models and generation of dose to human predictions. It is generally accepted that the distribution, metabolism, excretion and toxicity of ligands are correlated with their affinities toward the protein, especially serum albumin. Accurate determination of free levels of highly plasma protein bound drugs is technically challenging, which makes the comparison between the species for these drugs extremely difficult. In the results reported herein, the methods used allowed comparison between dog, rat, and human plasma protein binding. This permitted the appropriate interpretation of the pharmacology and potential for human risk of Propoxazepam. Human serum albumin (HSA) hydrolyzes different compounds [16] and considering the fact that Propoxazepam is ether, it was necessary to check whether its hydrolysis occurs in a medium containing plasma proteins of humans, dogs and rats.

Following incubation in plasma and buffer at 37 °C for up to 24 hours, LC MS/MS analyses indicated that Propoxazepam was stable in these matrices under the experimental conditions employed (Table 1). Therefore, Propoxazepam is not a serum albumin substrate.

Propoxazepam was highly bound to plasma proteins in rats, dogs and humans (Table 3).

The extent of Propoxazepam binding in human plasma was similar to that observed in rat plasma whereas the extent of Propoxazepam binding was slightly lower in dog plasma, following dialysis for 8 hours. No concentration dependent effects on plasma protein binding were observed in the species investigated for Propoxazepam. Overall mean free fractions of Propoxazepam were 1.60 %, 2.90 % and 1.96 % in rats, dogs and humans, respectively (Table 2). HSA has two major drug binding sites located in domain-I (warfarin site) and domain-III (diazepam site) and one minor site along with several tetradecanoic acid binding sites [17]. Propoxazepam is a benzodiazepine derivative, therefore, domain III (diazepam site) is a binding site on the HSA molecule.

Propoxazepam and its metabolites have high affinities for certain binding sites on human plasma proteins similar to other benzodiazepines (Diazepam, Chlordiazepoxid, Tetrazepam and Oxazepam). It can result in displacing other drugs from the protein and is also one of the mechanisms, by which the multi-drug interactions occur.

The extraordinary ability of HSA to reversibly bind various drugs in plasma makes it possible to control the patterns of their distribution to different target tissues during plasma circulation [18]. However, strong binding has been correlated with an increase in the release time and subsequent diminishing of the therapeutic values of drugs whereas weak binding has been correlated with poor absorption of drugs, their low delivery rates and delay in reaching the action sites.

The values of drug and plasma protein binding are also important for establishing potential drug safety margins for human exposure and for selecting the final dose range for human trials. With regard to pharmacological response, the unbound benzodiazepine concentration is probably more important than the total drug level. Therefore, high protein binding of Propoxazepam suggests that this factor must be considered in the planning and interpretation of pharmacodynamic and pharmacokinetic clinical studies. The PPB information [19] must also be entered in the Medicine information leaflets.

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Міжвидові відмінності зв'язування із білками плазми Пропоксазепаму – нового анальгетика

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Ціль. Мета дослідження — охарактеризувати зв'язувальні властивості Пропоксазепаму з білками плазми людини, собаки і щура та оцінити потенційні міжвидові відмінності в афінності зв'язування. **Методи.** Дослідження проводили з використанням системи швидкого рівноважного діалізу з наступним LC MS/MS аналізом. Зв'язування речовин із альбуміном характеризували відсотковим вмістом зв'язаного лікарського засобу. **Результати.** Пропоксазепам сильно зв'язується з білками плазми крові щура, собаки та людини. Ступінь зв'язування Пропоксазепаму в плазмі людини був аналогічний тому, що спостерігався в плазмі щурів і був трохи нижчим у плазмі собак після діалізу протягом 8 годин. **Висновки.** У видів, досліджених щодо Пропоксазепаму, не спостерігалося залежних від концентрації ефектів зв'язування з білками плазми. Загальні середні вільні фракції Пропоксазепаму становили 1,60 %, 2,90 % та 1,96 % у щурів, собак та людей відповідно. **Результати,** отримані в цій роботі, дозволили зрозуміти роль білків плазми у розподілі Пропоксазепаму у тканинах-мішенях та у визначенні

їх фармакологічних властивостей. **Результати** мають вирішальне значення розробки терапевтичних препаратів похідних бензодізепіну.

Ключові слова: Пропоксазепам, білки плазми, міжвидове зв'язування.

Межвидовые различия связывания с белками плазмы пропоксазепама - нового анальгетика

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Цель. Цель исследования — охарактеризовать связывающие свойства Пропоксазепама с плазмой человека, собаки и крысы и оценить потенциальные межвидовые различия в аффинности связывания. **Методы.** Исследования проводили с использованием системы быстрого равновесного диализа с последующим LC MS/MS анализом. Связывание веществ с альбумином характеризовали процентным содержанием связанного лекарственного средства. **Результаты.** Пропоксазепам очень сильно связывается с белками плазмы крови крысы, собаки и человека. Степень связывания Пропоксазепама в плазме человека была аналогична той, что наблюдалась в плазме крыс, в то время как степень связывания пропоксазепама была немного ниже в плазме собак после диализа в течение 8 часов. **Выводы.** У видов, исследованных в отношении Пропоксазепама, не наблюдалось зависящих от концентрации эффектов на связывание с белками плазмы. Общие средние свободные фракции Пропоксазепама составляли 1,60 %, 2,90 % и 1,96 % у крыс, собак и людей, соответственно. **Результаты,** полученные в этой работе, позволили понять роль белков плазмы в распределении Пропоксазепама в тканях-мишенях и в определении их фармакологических свойств. **Результаты** имеют решающее значение для разработки терапевтических препаратов на основе бензодизепинов.

Ключевые слова: Пропоксазепам, белок плазмы, межвидовое связывание.

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