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Feline hepatic biotransformation of diazepam: Differences between cats and dogs

Cyrina D. van Beusekom [a,*], Jeroen J.M.W. van den Heuvel [b], Jan B. Koenderink [b], Frans G.M. Russel [b], Johannes A. Schrickx [a]

[a] Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM, Utrecht, The Netherlands

[b] Department of Pharmacology and Toxicology, Radboud University Medical Center, Radboud Institute for Molecular Life Sciences, Geert Grooteplein 21, 6500 HB, Nijmegen The Netherlands

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ABSTRACT

In contrast to humans and dogs, diazepam has been reported to induce severe hepatic side effects in cats, particularly after repeated dosing. With the aim to elucidate the mechanisms underlying this apparent sensitivity of cats to drug-induced liver injury, in a series of *in vitro* experiments, the feline-specific biotransformation of diazepam was studied with liver microsomes obtained from cats and dogs and the possible inhibition of the bile salt export pump (Bsep) was measured in isolated membrane vesicles overexpressing feline and canine Bsep. In line with previous *in vivo* studies, the phase I metabolites nordiazepam, temazepam and oxazepam were measurable in microsomal incubations, although enzyme velocity of demethylases and hydroxylases differed significantly between cats and dogs. In cats, the main metabolite was temazepam, which also could be glucuronidated. In contrast to dogs, no other glucuronidated metabolites could be observed. In addition, in the membrane vesicles an inhibition of the transport of the Bsep substrate taurocholic acid could be observed in the presence of diazepam and its metabolites. It was concluded that both mechanisms, the slow biotransformation of diazepam as well the inhibition of the bile acid efflux that results in an accumulation of bile acids in the hepatocytes, seem to contribute to the liver injury observed in cats following repetitive treatment with diazepam.

1. Introduction

Diazepam, a benzodiazepine, has been suggested for use in cats for the treatment of a variety of conditions, including muscle relaxation and anticonvulsive therapy (Podell, 1998; Podell, 2013), treatment of behavioral disorders and anxiety (Hart, 1996), and appetite stimulation (Agnew and Korman, 2014). Despite the therapeutic benefits, long-term application of diazepam is discouraged, as more than 50% of the treated cats have been shown to exhibit side effects such as depression and anorexia in the first days of treatment. Moreover, when treated for a longer period, all cats showed jaundice, associated with acute hepatic failure (Dez Hughes et al., 1996; Center et al., 1996; Park, 2012).

The underlying mechanism of acute liver injury caused by diazepam in cats is currently unknown, but it has been hypothesized that the feline-specific biotransformation of diazepam may be the cause (Park, 2012). In most animal species, diazepam is converted into three metabolites including nordiazepam (N-desmethyldiazepam), temazepam, and ultimately oxazepam, via phase I N-demethylation or hydroxylation reactions (see Fig. 1). All three metabolites are pharmacologically active. Nordiazepam appears to be the principal metabolite of diazepam in man and dogs, although species-specific differences in the relative quantities of these metabolites have been observed previously (Vree et al., 1979; Chenery et al., 1987; Seddon et al., 1989). The lipophilic metabolites temazepam and oxazepam are excreted as phase II glucuronides in the urine (Schwartz et al., 1965; Vree et al., 1979). Considering that the metabolites of diazepam are conjugated with glucuronic acid in humans and dogs, the known low feline glucuronidation capacity, associated with the expression of a pseudogene for UGT1A6 (Court and Greenblatt, 2000), may result in intrahepatic accumulation of diazepam and its metabolites and subsequently in liver injury after repeated dosing.

In humans, drug-induced liver injury (DILI) is also observed following an inhibition of the bile salt export pump (Bsep). Bsep, a member of the ATP-binding cassette transporters, is responsible for the active efflux of bile acids from the hepatocytes into the bile canaliculi. Inhibition of this transporter by drugs can cause cholestasis and subsequent cytotoxic effects (Kullak-Ublick and Meier, 2000).

The aim of the current study was to investigate both mechanisms that may contribute to the particular sensitivity of cats towards

* Corresponding author.
E-mail addresses: C.D.vanBeusekom@uu.nl (C.D. van Beusekom), Jeroen.vandenHeuvel@radboudumc.nl (J.J.M.W. van den Heuvel), Jan.Koenderink@radboudumc.nl (J.B. Koenderink), Frans.Russel@radboudumc.nl (F.G.M. Russel), J.A.Schrickx@uu.nl (J.A. Schrickx).

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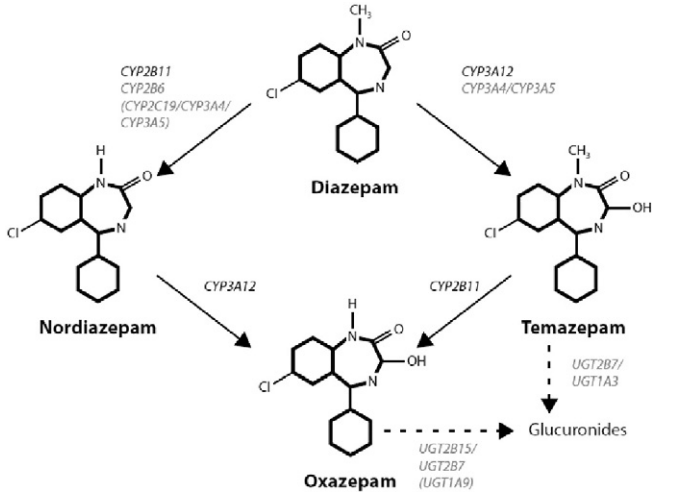

Fig. 1. Metabolism pathway of diazepam (Yang et al., 1999; Court et al., 2002; Shou et al., 2003; Acikgöz et al., 2009). The involved isozymes are given in black for dogs, and in gray for humans.

diazepam. To this end, two *in vitro* models were used: liver microsomal fractions were used to analyze the species-specific biotransformation comparing cats and dogs, and isolated membrane vesicles overexpressing feline or canine Bsep were used for functional transport assays as indicators of a potential Bsep inhibition.

2. Materials & methods

2.1. Chemicals and reagents

Adenosine triphosphate (ATP), adenosine monophosphate (AMP), alamethicin solution, cholic acid, cyclosporine A, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glycocholic acid, ketoconazole, magnesium chloride hexahydrate, β-nicotinamide adenine dinucleotide phosphate hydrate (NADP), taurocholic acid, troglitazone and uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diazepam, (*R,S*)-temazepam and (*R,S*)-oxazepam were purchased from BUFA B.V. (IJsselstein, The Netherlands). N-desmethyldiazepam (nordiazepam) and temazepam glucuronide were purchased from Lipomed (Arlesheim, Switzerland). Oxazepam glucuronide was purchased from Cerilliant Corporation (Round Rock, Texas, USA). Tauro[carbonyl-^{3}H]cholic acid (TCA) (5 Ci/mmol) was obtained from Perkin Elmer (Boston, MA, USA).

2.2. Tissue samples

Liver tissue was obtained from adult healthy European Shorthair cats (n = 8, five males and three females, aged from 11 to 13 months) directly after euthanasia and samples were immediately frozen in liquid nitrogen and stored at −70 °C. The cats had served as controls in a study for the development of FIV vaccines. Liver samples of Beagle dogs (n = 7, two males and five females, aged from 3.5 to 4.5 years) were taken from animals that had also served as controls in clinical trials and were euthanized at the end of this trial. Euthanasia was performed after anesthesia with medetomidine and ketamine, where after cats and dogs were euthanized with a high dose of pentobarbital. Animals were sacrificed with permission of the Animal Ethical Committee of Utrecht University according to the Dutch law on Animal Experiments.

2.3. Preparation of submitochondrial fractions

Submitochondrial fractions (commonly referred to as microsomes) containing predominantly microsomal proteins were isolated according to the procedure by Rutten et al. (1987) with minor modifications described in our previous study (van Beusekom et al., 2014). In brief, cat and dog liver samples of approximately 10 g were homogenized with 1.15% KCl, containing 0.1 mM EDTA at 4 °C. The homogenates were centrifuged at 9000 *g* for 25 min at 4 °C, and the supernatant obtained (S9-fraction) was centrifuged at 100 000 *g* for 1 h and 15 min at 4 °C. The microsomal pellet was re-suspended in 1.15% KCl 0.05 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20% glycerol. The microsomes were then quickly frozen in liquid nitrogen and stored in Eppendorf-cups at −70 °C until use (Rutten et al., 1987).

The protein concentrations of the microsomal fractions were determined by the method of Bradford (1976) and data were expressed as nmol/mg protein/min.

2.4. Phase I (N-demethylation and hydroxylation) assays of diazepam

For measuring phase I reactions pooled liver microsomes were used. Incubation mixtures contained 100 mM phosphate buffer (KH_2PO_4, pH 7.4), 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, and a concentration range of diazepam, in a total volume of 500 μl and a final concentration of 1% DMSO. Assays were tested for linearity in incubation time and protein concentration. For all substances, an incubation time of 10 min and a microsomal protein concentration of 0.5 mg/ml was within the linear range. Pre-incubations were performed for 5 min at 37 °C, and thereafter the enzymatic reactions were initiated by adding NADP to a final concentration of 1 mM. Samples remained at 37 °C in a heat block for the indicated

Table 1
Incubation conditions for the different substrates and HPLC retention times for the substrates and formed products.

Substrate	Product formed	Incubation time	Protein concentration	Retention time (phase I protocol)	Retention time (phase II protocol)
(*R,S*)-oxazepam		60 min	0.5 mg/ml		21.4 min
	R-oxazepam glucuronide				7.4 min
	S-oxazepam glucuronide				8.3 min
(*R,S*)-temazepam		30 min	0.5 mg/ml		22.6 min
	R-temazepam glucuronide				12.9 min
	S-temazepam glucuronide				13.3 min
Diazepam		10 min (phase I) 30 min (phase II)	0.5 mg/ml	24.6 min	24.1 min
	Oxazepam			14.2 min	21.4 min
	Temazepam			17.7 min	22.6 min
	Nordiazepam			18.7 min	22.7 min

Mobile phase: (A) 20 mM phosphate buffer (KH_2PO_4, pH 4.5); (B) acetonitrile.
Protocol phase I: start with 25% B for 4 min, balance up to 40% B in 5 min, stay at 40% B for 6 min. Gradually decrease back to 25% B in 1 min and stay at 25% B for 4 min. Balance up to 60% B again in 1 min, stay at 60% B for 9 min, and decrease back to 25% in 2 min.
Protocol phase II: start with 25% B for 15 min, balance up to 60% B in 5 min, stay at 60% B for 7 min, then gradually decrease back to 25% B in 1 min.

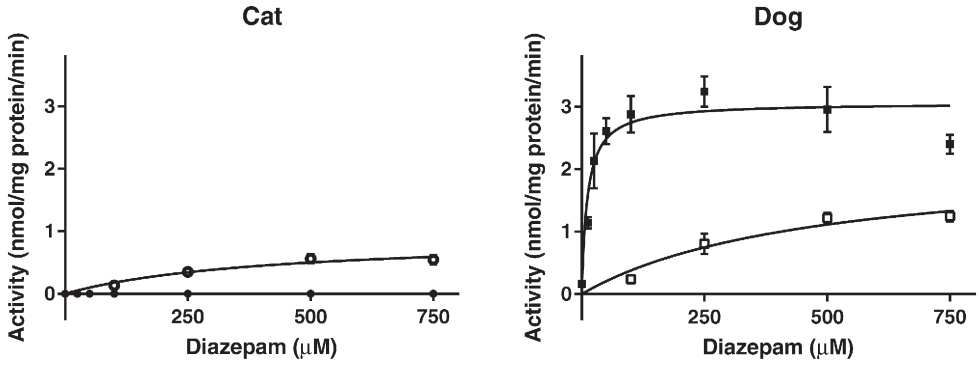

Fig. 2. Enzyme kinetics for demethylation of diazepam to nordiazepam (●,■), and hydroxylation of diazepam to (*R*,*S*)-temazepam (○,□) in pooled cat (circles) and dog (squares) liver microsomes. Nordiazepam production in cats was below the LOQ.

time and were shaken regularly in order to ensure an equal temperature within the incubating mixtures. Reactions were terminated by addition of ice-cold acetonitrile, followed by putting the samples on ice. Samples were centrifuged at 13,000 *g* for 5 min and the supernatants were directly injected onto the HPLC column. All measurements to determine K_m and V_{max} values were performed in duplicate and in at least three independent experiments. Blanks were obtained from incubations without NADP.

2.5. Phase II (glucuronidation) assays of diazepam and its metabolites

Glucuronidation assays were performed with pooled liver microsomes. Incubation mixtures contained 100 mM phosphate buffer (KH_2PO_4, pH 7.4), 5 mM $MgCl_2$, alamethicin (50 μg/mg protein) and a concentration range of diazepam, nordiazepam, (*R*,*S*)-temazepam or (*R*,*S*)-oxazepam, in a total volume of 500 μl and with a maximum of 5% DMSO or 1% methanol as final concentration. Assays were tested for linearity in incubation time and protein concentration. The chosen incubation times were 30 min for diazepam and (*R*,*S*)-temazepam, and 60 min for nordiazepam and (*R*,*S*)-oxazepam, all with a microsomal protein concentration of 0.5 mg/ml. Pre-incubations were performed for 5 min at 37 °C, where after the glucuronidation reactions were initiated by adding UDPGA with a final concentration of 5 mM. Samples remained at 37 °C in a heat block for the indicated time and were shaken regularly in order to ensure an equal temperature within the incubating mixtures. Reactions were terminated by addition of ice-cold acetonitrile, followed by a rapid cooling step. Samples were centrifuged at 13,000 *g* for 5 min and the supernatants were directly injected onto the HPLC column. All measurements to determine K_m and V_{max} values were performed in duplicates and in at least three independent experiments. Blanks were obtained from incubations without UDPGA.

2.6. HPLC conditions

The HPLC system was a Prominence HPLC and consisted of an auto injector, two pumps, a column oven, an UV-detector (set at a wavelength of 230 nm), all from Shimadzu ('s-Hertogenbosch, The Netherlands), and a C18 column (250 × 4.6 mm, 5 μm, RP, Gemini, Phenomenex). The mobile phase consisted of two solutions: (A) 20 mM phosphate buffer (KH_2PO_4, pH 4.5); (B) acetonitrile. The gradient for the phase I incubations started with 75% A and 25% B for 4 min, whereafter it was balanced up to 40% B in 5 min. The concentration of 40% B remained for 6 min and gradually decreased back to 25% B in 1 min. The purpose of using 40% B was the separation of temazepam and nordiazepam, because with higher acetonitrile concentrations these peaks merged. The concentration of 25% B remained for 4 min and was increased to 60% B in 1 min and remained for 9 min, to accelerate the retention time of diazepam. Thereafter the concentration of B was decreased to 25% in 2 min. The retention times of all substrates and metabolites are shown in Table 1. The gradient for the phase II incubations started with 75% A and 25% B for 15 min, whereafter it was balanced up to 60% B in 5 min. The concentration of 60% B remained for 7 min and gradually decreased back to 25% B in 1 min.

2.7. Inhibition of Bsep transport activity by diazepam and its metabolites

Membrane vesicles, consisting of everted cellular membranes, were produced by transfected HEK293 cells overexpressing feline Bsep as described in our previous study (van Beusekom et al., 2013). Bsep transport activity was determined by measuring ATP-dependent uptake of [^{3}H]labeled taurocholic acid (TCA) into the vesicles. To this end, membrane vesicles (7.5 μg protein) were incubated in a 30 μl transport medium with a final concentration of 10 mM Tris Base (pH 7.4), 250 mM sucrose, 10 mM $MgCl_2$, 4 mM adenosine triphosphate (ATP) or adenosine monophosphate (AMP), and 1 μM [^{3}H]taurocholic acid. To this medium a concentration range of diazepam or one of its metabolites was added and the mixture was incubated in 96-well plates at 37 °C for 5 min. The reaction was stopped by placing the 96-well plate on ice-water and by adding 150 μl ice-cold washing buffer (10 mM Tris Base

Table 2
Michaelis–Menten kinetics for nordiazepam and temazepam after incubation with the substrate diazepam in pooled cat (n = 8) and dog (n = 7) liver microsomes.

Substrate diazepam, formed metabolite	Cat		Dog	
	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)
Nordiazepam	<LOQ	<LOQ	11.6 ± 2.7*	3.1 ± 0.1*
Temazepam	410 ± 160	0.9 ± 0.2	470 ± 170	2.2 ± 0.4*

Data represent the mean ± SD of at least three independent experiments with samples performed in duplicate. LOQ = Limit of quantification.
* Denotes a significant difference ($P < 0.05$) between cat and dog values.

Table 3
Michaelis–Menten kinetics for different formed glucuronides in pooled cat (n = 8) and dog (n = 7) liver microsomes.

Substrate and formed glucuronide	Cat		Dog	
	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)
Temazepam				
R glucuronide	441 ± 84	0.51 ± 0.04	94 ± 29*	0.25 ± 0.02*
S glucuronide	920 ± 490	0.030 ± 0.009	79 ± 11*	3.04 ± 0.09*
Oxazepam				
R glucuronide	ND	ND	320 ± 50	4.1 ± 0.4*
S glucuronide	<LOQ	<LOQ	450 ± 95*	4.9 ± 0.6*

Data represent the mean ± SD of at least three independent experiments with samples performed in duplicate. ND = could not be determined. LOQ = Limit of quantification.
* Denotes a significant difference ($P < 0.05$) between cat and dog values.

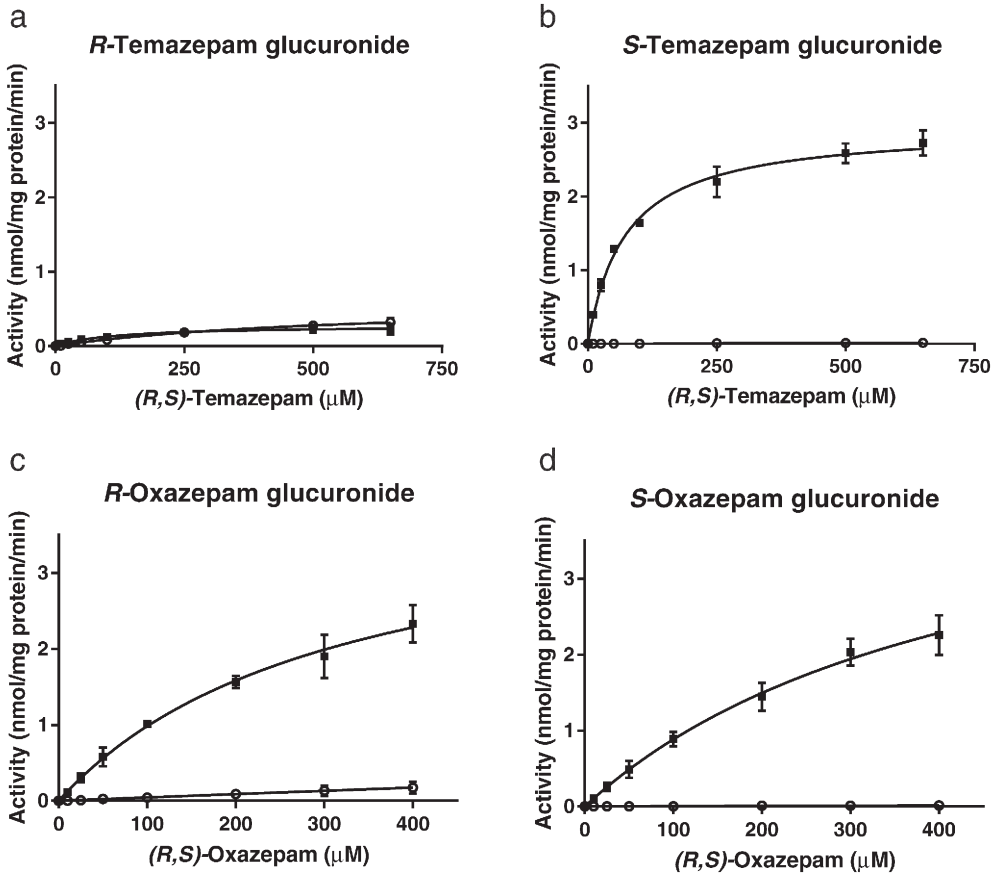

Fig. 3. Enzyme kinetics for the glucuronidation of (*R,S*)-temazepam and (*R,S*)-oxazepam in pooled cat (○) and dog (■) liver microsomes. (a) formation of *R*-temazepam glucuronide; (b) formation of *S*-temazepam glucuronide; (c) formation of *R*-oxazepam glucuronide; (d) formation of *S*-oxazepam glucuronide. Data represent the mean ± SD of at least three independent experiments with samples performed in duplicate.

pH 7.4, 250 mM sucrose). The reaction mixture was transferred to a 96-well Multi-Screen HTS filter plate (Millipore, Ireland) and filtered by means of a Multi-Screen HTS vacuum manifold filtration device (Millipore, Etten-Leur, The Netherlands). Filters were washed twice with 200 μl washing buffer, where after they were separated from the plate. Scintillation fluid was added and the radioactivity that was retained on the filter was measured by a liquid scintillation analyzer (Tri-carb 2900 TR, Packard). ATP-dependent transport was calculated by subtracting the uptake in presence of AMP from that in presence of ATP. The Bsep transport activity of TCA was set on 100%.

2.8. Statistical analysis

Data were expressed as means ± SD of at least three independent experiments with samples performed in duplicate. Data of the microsomal incubations were analyzed by nonlinear regression analysis according to the Michaelis–Menten equation using GraphPad Prism 6.04 software (San Diego, California, USA). Data of Bsep inhibition obtained in the experiments with isolated membrane vesicles were fitted according to a one-site binding competition model with the same software. Subsequently, K_m, V_{max} and IC_{50} values were calculated and statistically evaluated using an independent two-sample Student's *t*-test with $P < 0.05$ denoting a significant difference.

3. Results

3.1. Phase I (N-demethylation and hydroxylation) assays of diazepam

As a quality control for the enzymatic activity of the microsomal fractions, the cytochrome P450 (CYP) activity was tested by testosterone hydroxylation (6β-OH TST), as described in our previous study (van Beusekom et al., 2014). The 6β-OH TST activity in the microsomes from cats was approximately one-third of the activity in the microsomes from dogs (data not shown).

Incubations of the microsomes with a concentration range of diazepam resulted in the formation of the metabolites nordiazepam and temazepam. Cat liver microsomes produced mainly temazepam, while in dog microsomes relatively more nordiazepam was found. Data were fitted according to Michaelis–Menten enzyme kinetics, and the calculated K_m and V_{max} are presented in Fig. 2 and Table 2. The significant decrease in nordiazepam production at high concentrations of diazepam in the canine liver microsomes might be attributable to an auto-inhibition of the demethylation pathway by diazepam itself similar to what Kuroha et al. (2002) observed for the 1′-hydroxylation of midazolam. The velocity of temazepam production was significantly different between cat and dog microsomes, with V_{max} values of 0.9 ± 0.2 and 2.2 ± 0.4 nmol/mg protein/min ($P < 0.05$), respectively. The level of nordiazepam formed was below the limit of quantification (LOQ, which was 1 μM for nordiazepam) in cats, while in dogs a high velocity of 3.1 ± 0.1 nmol/mg protein/min was achieved with a low K_m value of 11.6 ± 2.7 μM. In dogs, after more than 10 min of incubation with concentrations of diazepam ranging from 25 to 500 μM, the formation of oxazepam was observed, which could not be detected in cat liver microsomes (data not shown).

For the phase I incubations a final concentration of 1% DMSO was used to dissolve diazepam, similar to the method used for the phase II incubations with temazepam and oxazepam. As Nishiya et al. (2010) demonstrated that organic solvents influence the inhibition of recombinant CYP3A4 activity, which is necessary for the metabolism of diazepam, we evaluated this potential effect and found up to a

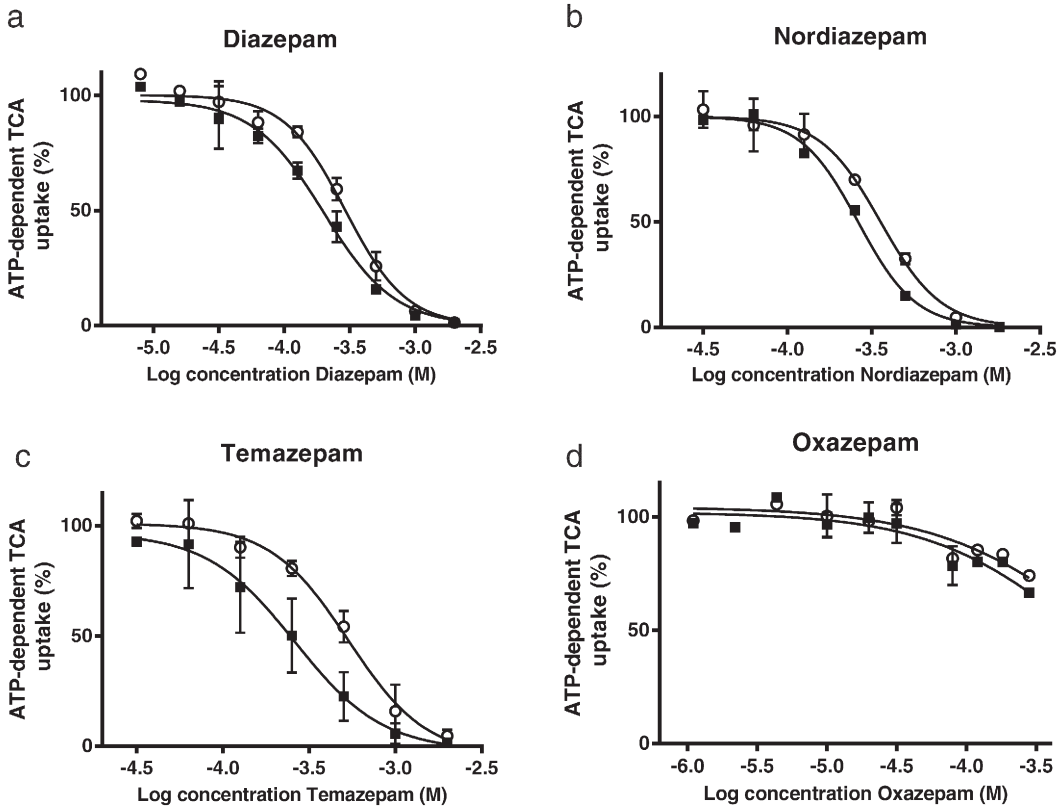

Fig. 4. Diazepam and its metabolites inhibit the uptake of 1 μM [^{3}H]TCA in membrane vesicles (7.5 μg protein) expressing cat (○) and dog (■) Bsep. Vesicles were incubated in 10 mM Tris Base buffer (pH 7.4) containing 250 mM sucrose, 10 mM $MgCl_2$ and 4 mM ATP or AMP, at 37 °C for 5 min. ATP-dependent transport was calculated by subtracting transport in presence of AMP from that in presence of ATP. Values are expressed as mean ± SD of percentage uptake of at least three independent experiments with samples performed in duplicate.

concentration of 1% DMSO had no significant effect on the rate of biotransformation.

3.2. Phase II (glucuronidation) assays of diazepam and its metabolites

In incubations with microsomes from cats, no glucuronide conjugates were formed after an incubation period of 30 min with concentrations of diazepam ranging from 250 μM up to 1 mM. Incubations with nordiazepam for 60 min resulted also in the lack of any measurable production of glucuronides. The same results were obtained in incubations with canine microsomes.

Incubations with a concentration range of (*R*,*S*)-temazepam resulted in the formation of *R*-temazepam and *S*-temazepam glucuronides, and incubations with a concentration range of (*R*,*S*)-oxazepam resulted in the formation of *R*-oxazepam and *S*-oxazepam glucuronides. All data were obtained within the linear phase of product formation for incubation time and protein content. The data could be fitted according to Michaelis–Menten enzyme kinetics, and the calculated K_m and V_{max} values are presented in Table 3.

Table 4
Inhibitory potency of diazepam and its metabolites.

Inhibitor	IC_{50} (μM)	
	Cat	Dog
Diazepam	293 ± 23	200 ± 19*
Nordiazepam	375 ± 22	268 ± 18*
Temazepam	533 ± 66	262 ± 60*
Oxazepam	–	–

Measured by the uptake of 1 μM [^{3}H]TCA into membrane vesicles (7.5 μg protein) expressing cat and dog Bsep. Data represent the mean ± SD of four independent experiments.
* Denotes a significant difference ($P < 0.05$) between cat and dog values.

Cat liver microsomes hardly glucuronidated racemic (*R*,*S*)-temazepam, and a very low V_{max} for *S*-temazepam glucuronide was calculated (Fig. 3). Surprisingly, a relatively high formation of *R*-temazepam glucuronide (V_{max} of 0.51 ± 0.04 nmol/mg protein/min) could be observed in cat microsomal incubations.

In dog liver microsomes, a fast formation of *S*-temazepam glucuronide was observed after incubation with racemic (*R*,*S*)-temazepam, with a V_{max} of 3.0 nmol/mg protein/min (Fig. 3), which is approximately 100 times higher than the value observed in cats. In contrast, a limited formation of *R*-temazepam glucuronide was found (V_{max} = 0.25 ± 0.02 nmol/mg protein/min) and this value is significantly ($P < 0.05$) lower than the comparative value in cats. Correspondingly, the canine K_m value for *R*-temazepam glucuronide was lower than the feline K_m.

Cats showed also a very low formation of both *R*- and *S*-oxazepam glucuronides after incubation with racemic (*R*,*S*)-oxazepam, where *S*-oxazepam glucuronide was even below the LOQ (for *S*-oxazepam the LOQ was 0.1 μM). Dogs showed a much higher formation of both glucuronides, with a V_{max} of 4.1 ± 0.4 and 4.9 ± 0.6 nmol/mg protein/min for *R*- and *S*-oxazepam glucuronide, respectively.

3.3. Inhibition of Bsep transport activity by diazepam and its metabolites

The inhibitory effects of diazepam, nordiazepam, temazepam and oxazepam were determined on [^{3}H]TCA uptake in membrane vesicles overexpressing feline or canine Bsep. Diazepam and its metabolites all decreased the uptake of TCA and data were fitted according to a one-site competition model (Fig. 4). IC_{50} values were calculated and are given in Table 4. Calculation of an IC_{50} value for the highly lipophilic metabolite oxazepam was not possible, due to solubility problems at higher concentrations. Inhibitory potencies were significantly different between cats and dogs, where dogs had slightly lower IC_{50} values for all substrates than cats.

Table 5
Elimination half-lives of diazepam and its metabolites in cats and dogs.

	Cats		Dogs	
Diazepam	3.5 h	Driessen et al. (1987)	1.33 h	Vree et al. (1979)
	5.5 h	Cotler et al. (1984)	1.0 h	KuKanich and Nauss (2012)
			2.07 h	Platt et al. (2000)
			3.2 h	Loscher and Frey (1981)
			7.6 h	Klotz et al. (1976)
Nordiazepam	21 h	Cotler et al. (1984)	2.4 h	KuKanich and Nauss (2012)
			3.6 h	Loscher and Frey (1981)
			10 h	Vree et al. (1979)
Temazepam	4.5 h	Driessen et al. (1987)	3 h	Vree et al. (1979)
Oxazepam	–		3 h	Vree et al. (1979)
			5.7 h	Loscher and Frey (1981)
			6.2 h	KuKanich and Nauss (2012)

4. Discussion

Diazepam has been used effectively for the treatment of a variety of indications in cats, but after repeated dosing, severe side effects have been observed in feline patients, which have not been described for man or dogs. Livers of treated cats showed centrilobular hepatic necrosis, profound biliary duct proliferation and hyperplasia, and suppurative intraductal inflammation (Dez Hughes et al., 1996; Center et al., 1996). As the mechanisms involved in this sensitivity of cats is currently unknown, we investigated two different hypotheses: (i) incubations with liver microsomes should indicate whether or not diazepam is differently metabolized in cats than in dogs, and (ii) transport studies with isolated membrane vesicles should indicate if diazepam or its metabolites inhibit the bile efflux transporter Bsep, resulting in an accumulation of bile acids in hepatocytes, a phenomenon that is implicated in drug-induced liver injury in humans.

The cat is well-known for its limited hepatic capacity to form glucuronide conjugates in comparison to dogs and other species (for review see: van Beusekom et al., 2014; Court and Greenblatt, 2000). Our current data suggest that cats also have a low capacity to form glucuronide conjugates of diazepam and its phase I metabolites. Interestingly, apart from the differences found in the phase II biotransformation assays, striking differences were also observed in the phase I biotransformation reactions between cats and dogs.

Diazepam was converted into temazepam in the liver microsomes from both cats and dogs with a comparable affinity (K_m) and capacity (V_{max}). However, temazepam was the principle phase I metabolite formed in the feline liver microsomes, while formation of nordiazepam was observed in the canine liver microsomes with a higher efficiency (V_{max}/K_m) than for the temazepam formation. Moreover, dog liver microsomes produced oxazepam that could not be detected in the feline samples. Temazepam and nordiazepam formation from diazepam is catalyzed in dogs by mainly CYP3A12 and CYP2B11, respectively (Shou et al., 2003; Lu et al., 2005). Further biotransformation of temazepam into oxazepam in dogs is catalyzed by CYP2B11. The lack of quantifiable formation of nordiazepam and the lack of observable formation of oxazepam in the feline liver microsomes suggests that the feline CYP2B orthologue gene has different characteristics, expression levels and/or a different substrate spectrum.

In humans, CYP2B6 has a major role in the formation of nordiazepam from diazepam (Yang et al., 1998; Yang et al., 1999; Acikgöz et al., 2009). A search through nucleotide databases using the BLASTN program (NCBI) did not retrieve results to obtain a full-length feline cDNA orthologue to human CYP2B6 cDNA (data not shown). Interestingly, another drug that is metabolized by CYP2B6 is ketamine, which is poorly metabolized in cats (Hanna et al., 1988). In conclusion, cats seem to have a CYP2B enzyme with characteristics different from dogs (CYP2B11) and humans (CYP2B6) and hence further studies should address the clinical relevance of this observation.

The results of the phase II (glucuronidation) assays showed that diazepam and nordiazepam were not glucuronidated, neither by feline nor by canine liver microsomes. In contrast, glucuronide conjugates of temazepam and oxazepam were found in microsomal incubations of both species. *S*-temazepam, *R*-oxazepam and *S*-oxazepam glucuronides were formed with a much lower affinity and capacity in feline than in the canine liver microsomes. Surprisingly, a relatively high formation of *R*-temazepam glucuronide could be observed in cat microsomal incubations.

As yet, the UGT isoforms that catalyze the conjugation of diazepam phase I metabolites in dogs or cats are not known. In humans, the UGT2B isoforms UGT2B15 and UGT2B7, and to a lesser extent, UGT1A9, catalyze the conjugation of *S*- and *R*-oxazepam, respectively (Court et al., 2002; He et al., 2009). We have previously suggested that cats have a very limited UGT2B7-like activity that is substantiated here and the current data indicate that UGT2B15 homologue activity is also low in the cat. Temazepam was suggested to be also a substrate for UGT1A3 (Oechsler and Skopp, 2010). The formation of *R*-temazepam glucuronide in feline liver microsomes may therefore depend on an active UGT1A3 orthologous gene in cats which might be the feline UGT1A02 described in earlier investigations (Court and Greenblatt, 2000). This is also indicated by the previous finding of the high extent of formation of glucuronide conjugates of the human UGT1A3 substrate telmisartan in feline liver microsomes (Yamada et al., 2011; Ebner et al., 2013).

A comparison of the data from our *in vitro* studies conducted with liver microsomes with previous *in vivo* studies show many similarities, but also various quantitative differences. The appearance of temazepam, oxazepam and nordiazepam in the blood plasma of cats and dogs after IV administration of diazepam is in line with our findings, although the formation of oxazepam and nordiazepam was much lower than expected from these *in vivo* findings (Cotler et al., 1984; Driessen et al., 1987; Vree et al., 1979).

In cats, as in dogs, the metabolite with the highest plasma concentration was nordiazepam, while in our microsomal incubations only minor amounts of nordiazepam were measurable. This suggests that extrahepatic biotransformation processes may account for the high plasma levels of nordiazepam and/or that its long elimination half-life of 21 h (Cotler et al., 1984) contribute to the high plasma levels after repeated application.

A comparison of the elimination half-lives of diazepam and its metabolites in blood plasma is presented in Table 5, indicating that in general the half-lives are longer in cats than in dogs. As all metabolites are biologically active, this would imply that in cats a longer dosage interval would be acceptable to achieve the desirable therapeutic effect while reducing the risk of undesirable side effects associated with a slow biotransformation in feline hepatocytes.

In addition to the differences in biotransformation, we had hypothesized that the typical liver injury observed in cats might be attributable to the inhibition of Bsep by diazepam or its metabolites, as this is a common mechanism observed in humans experiencing drug-induced liver injury by cholestasis (Kubitz et al., 2012; Bjornsson and Jonasson, 2013). Therefore, we studied the inhibitory potential of diazepam and

metabolites against Bsep-mediated taurocholate transport. The inhibitory potency of diazepam and its metabolites was rather comparable between the feline and canine Bsep, and less pronounced than that of common marker inhibitors for Bsep such as cyclosporine A or troglitazone (van Beusekom et al., 2013). However, it cannot be excluded, that the inhibition of the excretion of bile acids such as taurocholic acid, contribute to the liver injury observed in cats following the repeated application of diazepam.

In conclusion, in cats a different pattern of phase I metabolites of diazepam is observed in comparison to dogs. In addition, the glucuronidation capacity of diazepam hydroxyl metabolites is lower in cats, as expected, although the formation of the temazepam glucuronide remains a reparable finding. This supports previous observations that cats have a low glucuronidation capacity but selected drugs are substantially glucuronidated also in cats, most likely by an UGT1A3 isoform. Nevertheless, a low glucuronidation capacity increases the risk for an accumulation of drugs and their metabolites in liver cells, a process that may result in an inhibition of Bsep and hence an additional accumulation of bile acids within hepatocytes. This sequence of effects may explain the clinical observation of drug-induced liver injury in cats. Amendment of the dose, and particularly the dosing interval of diazepam and other drugs known to induce liver injury, might reduce the risk for such undesirable side effects. Therefore, we encourage investigations of specific drug biotransformation and transport mechanisms within the feline species for each individual drug, to prevent this species from toxicity.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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