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# Feline hepatic biotransformation of diazepam: Differences between cats and dogs

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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

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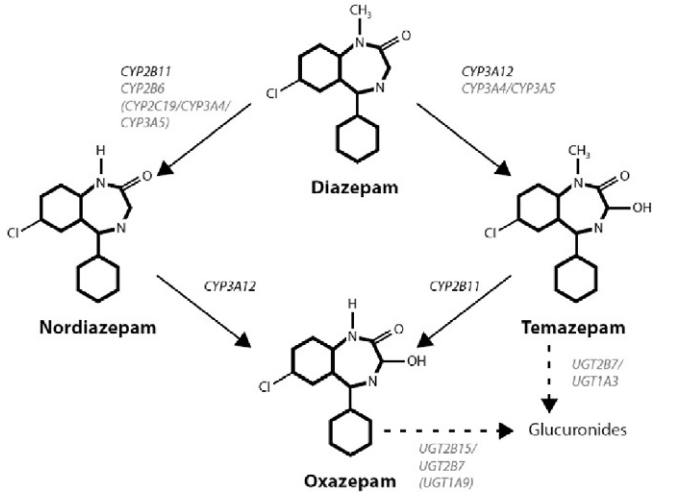


**Fig. 1.** Metabolism pathway of diazepam (Yang et al., 1999; Court et al., 2002; Shou et al., 2003; Acikgöz et al., 2009). The involved isozymes are given in black for dogs, and in gray for humans.

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)<br>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.