JOURNAL OF
Veterinary Pharmacology and Therapeutics

J. vet. Pharmacol. Therap. **37**, 421–434. doi: 10.1111/jvp.12117.

Imepitoin as novel treatment option for canine idiopathic epilepsy: pharmacokinetics, distribution, and metabolism in dogs

C. RUNDFELDT*
A. GASPARIC† &
P. WLAŹ‡

Drug-Consult.Net, Magdeburg, Germany; †Vitilis, Weinböhla, Germany; ‡Department of Animal Physiology, Faculty of Biology and Biotechnology, Maria Curie-Sklodowska University, Lublin, Poland

Rundfeldt, C., Gasparic, A., Wlaź, P. Imepitoin as novel treatment option for canine idiopathic epilepsy: pharmacokinetics, distribution, and metabolism in dogs. *J. vet. Pharmacol. Therap.* **37**, 421–434.

Imepitoin is a novel anti-epileptic licensed in the European Union for the treatment of canine idiopathic epilepsy. The aim of this study was to characterize the pharmacokinetics of imepitoin in dogs and to evaluate the interaction with drug metabolizing enzymes. Upon administration of imepitoin tablets at a dose of 30 mg/kg to beagle dogs, high plasma levels were observed within 30 min following oral dosing, with maximal plasma concentrations of 14.9–17.2 μg/mL reached after 2–3 h. In a crossover study, co-administration of imepitoin tablets with food reduced the total AUC by 30%, but it did not result in significant changes in T_{max} and C_{max}, indicating lack of clinical relevance. No clinically relevant effects of sex and no accumulation or metabolic tolerance were observed upon twice daily dosing. Following single dose administration of 10–100 mg/kg, dose linearity was found. Administering [^{14}C] imepitoin, high enteral absorption of 92% and primary fecal excretion were identified. Plasma protein binding was only 55%. At therapeutic plasma concentrations, imepitoin did not inhibit microsomal cytochrome P450 family liver enzymes *in vitro*. In rats, no relevant induction of liver enzymes was found. Therefore, protein binding or metabolism-derived drug–drug interactions are unlikely. Based on these data, imepitoin can be dosed twice daily, but the timing of tablet administration in relation to feeding should be kept consistent.

(Paper received 10 November 2013; accepted for publication 21 January 2014)

PD Dr habil. Chris Rundfeldt, Drug-Consult.Net, Toepfferspark 2a, D-39108 Magdeburg, Germany. E-mail: chris.rundfeldt@t-online.de

INTRODUCTION

Recurrent seizures caused by idiopathic epilepsy are a common neurological problem in veterinary medicine. The prevalence of canine epilepsy is estimated to be 0.5–5% of all dogs in the general population (Podell *et al.*, 1995). In a recent study, the prevalence was found to be 0.62% in a large UK population with an increased prevalence in border terriers and German shepherds and reduced prevalence in West Highland white terriers (Kearsley-Fleet *et al.*, 2013). In humans suffering from idiopathic epilepsy, about 70–80% are able to reach a seizure free state with the use of anti-epileptic drugs (Kwan *et al.*, 2011). This sometimes requires combination therapy with two or more drugs. About 25% of human patients suffering from epilepsy continue to experience seizures and are considered to be drug resistant (Kwan *et al.*, 2011). In contrast, in dogs only 15% of treated cases achieve this level of control and up to 30% of canine patients do not experience significant seizure frequency reduction with the most commonly used anti-epileptics, phenobarbital, and potassium bromide (Wright *et al.*, 2012; Potschka *et al.*, 2013). Many of these patients are euthanized because of the severity of seizures or because of severe side effects from anti-epileptic drugs (Trepanier *et al.*, 1998; Berendt *et al.*, 2007). One possible difference in accessibility to seizure control between man and dogs may be the limited availability of treatment options for canine epilepsy (Potschka *et al.*, 2013). In fact, most anti-epileptic drugs which have been developed for humans are not suitable for treatment in dogs due to inadequate pharmacokinetics or adverse effects which are not acceptable (Speciale *et al.*, 1991; Schwartz *et al.*, 2011; Ziółkowski *et al.*, 2012). Even when administration of novel drugs developed for the control of human seizure disorders is attempted to improve seizure control in dogs, nearly half of the dogs refractory to phenobarbital and potassium bromide have remained poorly controlled (Potschka *et al.*, 2013). Therefore, the search continues for alternative anti-epileptic drugs that may be used in canine patients.

Imepitoin (AWD 131–138 or ELB 138; 1-(4-chlorophenyl)-4-morpholino-imidazolin-2-one, Fig. 1) is a new anti-epileptic drug recently approved in the European Union for the treatment of canine idiopathic epilepsy. It was developed from a series of imidazolinones due to its pronounced antiseizure activity in a large variety of rodent models of epileptic seizures, combined with a high tolerability in these models. In addition, potent anxiolytic activity could be demonstrated for imepitoin in rats (Rostock *et al.*, 1998; Rundfeldt & Löscher, 2014).

The first hint that imepitoin may be useful for the treatment of canine epilepsy was derived from a canine seizure model in which imepitoin was capable of elevating the threshold for pentylenetetrazole-induced convulsions in dogs (Löscher *et al.*, 2004). In this model, a dose as low as 5 mg/kg administered orally twice daily was capable of elevating the seizure threshold and increasing the dose to 40 mg/kg twice daily greatly enhanced the drug-induced effect while maintaining the tolerability. Upon repeated administration for 28 days, no development of tolerance was observed (Löscher *et al.*, 2004). While this animal model is not predictive for the treatment of canine idiopathic epilepsy as seizures are chemically induced only at the time of maximal plasma concentration, enabling the detection of anticonvulsant activity even for drugs with very short plasma kinetics which are not suitable for once or twice daily dosing, a first clinical study in epileptic dogs was initiated, to evaluate whether imepitoin may be useful for the treatment of canine idiopathic epilepsy. This study was conducted using a dose escalation approach. Comparing the anti-epileptic activity of dogs treated with imepitoin with historical data of dogs treated with phenobarbital, comparable anti-epileptic activity of imepitoin could be demonstrated, while the tolerability of imepitoin was clearly superior to phenobarbital. In this study, imepitoin was also dosed as add-on to phenobarbital. Similar clinical activity and superior tolerability were observed when compared to add-on treatment with potassium bromide (Löscher *et al.*, 2004; Rieck *et al.*, 2006). Following this pilot study, the clinical activity and safety of imepitoin was evaluated in prospective and controlled multicenter clinical studies in epileptic dogs founding the data basis for marketing approval of imepitoin in the European Union for the treatment of idiopathic canine epilepsy (Bialer *et al.*, 2013; Rundfeldt & Löscher, 2014).

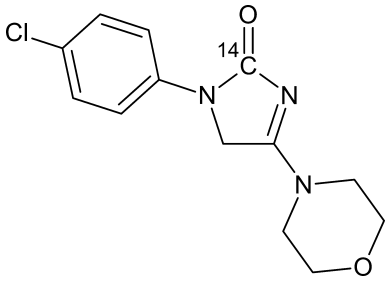

Fig. 1. Imepitoin (1-(4-chlorophenyl)-4-morpholino-imidazolin-2-one, AWD 131-138, ELB 138). For [^{14}C] labeling, the carbonyl group was used (indicated by '14'). The specific activity of [^{14}C] imepitoin was 195.5 μCi/mg. The radiochemical purity was 99.6–99.8%, as determined by HPLC.

The aim of this work was to evaluate the pharmacokinetic properties and the drug–drug interaction potential of imepitoin in dogs. For this purpose, a series of three experimental studies were conducted in beagle dogs to evaluate the pharmacokinetic parameters after single and repeated oral dosing of the marketed formulation of imepitoin under fasted and fed conditions, to evaluate the dose linearity of the plasma concentration–time profile of imepitoin after oral administration in enteric-coated gelatin capsules, and to characterize the route of excretion and the excretion balance of [^{14}C]-labeled imepitoin. In addition, to evaluate the drug–drug interaction potential, the *in vitro* and *in vivo* plasma protein binding of imepitoin and the in vitro inhibition potential as well as induction potential of hepatic drug metabolizing enzymes were examined.

MATERIALS AND METHODS

Animals and husbandry

For the food effect study, six male and six female healthy beagle dogs, aged 2–3 years, were obtained from the animal colony of Southwest Bio Labs (USA), with a body weight of 8.3–9.5 kg. Dogs were fed a commercial diet (Iams Minichunks, Iams, Dayton, OH, USA) and allowed free access to tap water. They were fed twice daily at 12-h intervals and were individually housed at controlled room temperature and humidity.

For all other experiments with dogs discussed in this work, healthy female and male beagle dogs (Harlan-Winkelmann, Borchen, Germany) at an age of approximately 3 years weighing 11–15 kg were used. Animals were housed during the study individually in standard dog kennels at ambient temperature. They were fed a standard diet (Brocken, Meradog, Kevalar, Netherlands), and had free access to tap water.

For enzyme induction experiments, male and female Wistar rats (Charles River Wiga, Sulzfeld, Germany) were held under controlled housing conditions (room temperature 25 °C, 12 h light/dark cycle with light on at 7 a.m., standard rodent polycarbonate cages with four or two rats per cage, standard bedding and rodent chow (ssniff, Lage, Germany)). Animals had free access to acidified drinking water.

All animal studies were conducted in agreement with the local law for animal protection and were approved by local authorities.

Single dose and repeated (BID) dosing of imepitoin tablets to male and female beagle dogs under fed and fasted conditions

To evaluate the pharmacokinetic parameters after oral administration of 30 mg/kg imepitoin as finished product (100 or 400 mg imepitoin per tablet, tablets with break score, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany), a 2 × 2 crossover design was selected comparing plasma concentrations and PK parameters in six female and six male fasted and fed young beagle dogs. In each period, three male and three female dogs were administered in fasted stage, and

three males and three females were administered in fed stage, with a 15-day washout phase between periods. Fed animals were dosed approximately 0.5 h after food had been offered, while fasted dogs were fasted approximately 7.5 h before dosing. In fasted animals, food was offered 4 h after dosing. The drug was dosed individually according to the body weight of each dog, using tablets and half tablets of both available strengths to obtain the dose closest to the intended dose of 30 mg/kg. The actual calculated dose administered ranged from 26.5 to 31.3 mg/kg. On day 0 and day 5, dogs received a single dose in the morning followed by serial blood sampling, while on days 1–4, dogs received two daily doses with a 12-h dosing interval in between. On day 0 and 5 of each period, blood for plasma samples were collected from the jugular vein of each dog prior to dosing (0) and at 0.5, 1, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 8, 12, and 24 h postdosing using heparinized syringes. Plasma was prepared by centrifugation of the samples for 10 min at 2000 ***g*** at 4 °C, and the samples were stored at −20 °C until analysis.

Dose linearity of imepitoin in dogs after oral dosing

To evaluate the pharmacokinetic parameters and dose linearity of imepitoin after oral dosing, an ascending dosage regimen administering single doses with at least 2 weeks washout between doses to three male and three female beagle dogs was selected. Dogs were administered 10, 20, 50, or 100 mg/kg imepitoin as micronized powder filled in enteric-coated (gastric juice-resistant coating) gelatin capsules. Blood samples were collected using heparinized syringes predose, 0.5, 1, 2, 4, 6, 8, 10, 24, 30, 48, 72, and 96 h after administration. The administration form of enteric-coated capsules was selected since at this early stage of development, imepitoin tablets had not been developed. Plasma was prepared immediately after withdrawal by centrifugation of the samples for 10 min at 2000 ***g*** at 4 °C. Samples were stored at −20 °C until analysis. To evaluate dose linearity, the maximal plasma concentration (C_{max}, mean ± SEM) and the drug exposure calculated as area under the plasma concentration–time curve (AUC) were calculated. Due to availability of plasma samples with levels below limit of detection in each animal, AUC could be determined as AUC_{zero}, that is, the area from zero before dosing until the first time point with concentration below limit of detection, set at zero. Mean values of both, AUC_{zero} and C_{max} were plotted against the dose, and a linear regression was performed to determine dose linearity.

Quantification of plasma imepitoin concentrations using a validated HPLC-MS method

For quantification of imepitoin in dog plasma from the dose linearity study, samples were spiked with 1-(4-chlorophenyl)-4-hexamethylenimine-1-yl-imidazoline-2-one as internal standard followed by plasma protein precipitation using acetonitrile. Chromatography was performed using a Luna C8 column sized 50 × 4.6 mm, grain size 5 μm at a flow rate of 0.5 mL/min with a mobile phase of 80% acetonitrile and 20% 2.5 mM ammonium acetate (pH 6.5). The injection volume was 5 μL, and analysis was performed using a tandem mass spectrometry detector with polarization set to positive mode. The selected precursor and product ions for imepitoin were at m/z 280 and 237, respectively; the respective precursor and product ions for the internal standard were at m/z 292 and 249, respectively. The lower limit of quantification (LOQ) was 5 ng/mL, and the lower limit of detection was 2 ng/mL. The standard curve was linear in the range of 5.0–5000 ng/mL. The above-described method had been fully validated and had been used previously to determine plasma concentrations of imepitoin from clinical studies in dogs (Rieck *et al.*, 2006).

For analysis of plasma samples generated during the single and multiple dose PK study to investigate the influence of prandial state and to investigate possible gender differences, the method was modified and optimized for sensitivity by the use of a solid-phase extraction replacing the protein precipitation and by optimization of the mobile phase. For solid-phase extraction, 100 μL of dog plasma was mixed with 0.5% formic acid solution containing the internal standard and was applied to solid-phase extraction using a Strata-X 10 mg extraction column (Phenomenex, Aschaffenburg, Germany). Elution was performed with 10 mM ammonium acetate/acetonitrile/methanol (30:60:10) mixture as eluent. The elution fluid was directly injected into the HPLC system (5 μL injection volume) using a phenyl/hexyl column sized 50 × 3 mm with 3 μm grain size (Phenomenex, Aschaffenburg, Germany) and a mobile phase of 50% 10 mM ammonium acetate and 50% acetonitrile. For detection, a Sciex API 5000 (AB Sciex Germany GmbH, Darmstadt, Germany) with electrospray in positive ionization mode was used. Using this approach, a LOQ of 2 ng/mL was reached.

Pharmacokinetics and excretion balance of [^{14}C] imepitoin in dogs after oral and intravenous dosing

To determine the pharmacokinetic parameters, route of excretion and excretion balance of radiolabeled imepitoin, three male and three female dogs were orally dosed with micronized imepitoin filled in enteric-coated gelatin capsules and spiked with [^{14}C]-labeled compound dissolved in DMSO to a nominal dose of radioactivity of 0.792 MBq/kg body weight and a dose of 20 mg/kg body weight imepitoin. After a washout period of at least 4 weeks, three female dogs were also dosed intravenously with a dose of 1 mg/kg imepitoin administered as solution in 70% DMSO and 30% physiological saline, at a volume of 0.25 mL/kg body weight, containing 0.792 MBq/kg body weight. Due to the low solubility, the intravenous dose had to be limited to 1 mg/kg, because a higher dose could not be reliably kept in a tolerable solution for intravenous dosing. To enable comparison of both dosing routes, the amount of radioactivity dosed was identical, that is, 0.792 MBq/kg.

To determine the route of excretion and the excretion balance, animals were kept in metabolic cages after dosing throughout the study period, and feces and urine (as well as

cage wash) were collected continuously for up to 264 h. The following sampling intervals were evaluated: 0–8, 0–24, 0–48, and 0–264 h. Cage-washing fluid, containing predominantly urine washed from the metabolic cage wall, was added to the urine fraction to determine urinary excretion. By comparing the excreted fraction in urine after oral dosing with respective data after intravenous dosing, an extrapolation of the absorbed fraction after oral dosing could be carried out.

To evaluate the pharmacokinetics of [^{14}C] imepitoin, 10 mL of blood samples was collected using heparinized syringes predose, 0.5, 1, 2, 4, 6, 8, 10, 24, 30, 48, 72, 96, 120, 168, and 192 h after oral dosing. After intravenous dosing, in addition, blood samples were taken and analyzed 5 and 15 min after dosing.

All plasma, feces, urine, and cage-washing samples were subjected to liquid scintillation counting (LSC) for determination of total radioactivity. For this purpose, aliquots of urine, cage wash, and plasma (500 μL each, duplicate samples) were measured without further processing. Homogenization of the feces was carried out after adding 1.5-fold of the feces weight of water followed by thorough mixing by Ultra-Turrax homogenizer (IKA-Werke GmbH & Co., Staufen, Germany). Triplicate aliquots of 150 mg of feces homogenates were transferred for combustion in an oxidizer (OX 500, Zinsser, Germany). Released carbon dioxide containing [^{14}C] carbon dioxide was trapped in 20 mL of a CO_2-binding scintillation cocktail (Oxisolve 500) and directly used for LSC. All samples were measured in a liquid scintillation counter (Wallac 1409; Wallac Distribution GmbH, Freiburg, Germany). Plasma, urine, and cage-washing samples were counted in mini vials containing 4 mL of Hionic Fluor® (Packard, Frankfurt, Germany). Feces samples were counted after combustion. Means of duplicate or triplicate samples were recorded to determine content of radioactivity. As the total content of plasma cannot be determined, it was estimated to be 5.2% of the individual body weight, as proposed by Altman *et al.* (1959).

Pharmacokinetic analysis, biostatistics

All pharmacokinetic parameters, that is, time to maximal concentration T_{max}, maximal plasma concentration (C_{max}), area under the concentration–time curve (*AUC*), terminal half-life, volume of distribution (*Vz*) and clearance (Cl), were calculated by noncompartment analysis using the software WinNonLin® (Pharsight Corporation, Mountain View, CA, USA). The terminal elimination rate constant (λ_z) was assessed by linear regression. Terminal half-life ($t_{½}$) was calculated as $\ln 2/\lambda_z$. Areas under the concentration–time curve from the time of dosing extrapolated to infinity using the calculated terminal half-life (AUC_{inf}) or the areas under the concentration–time curve from the time of dosing to the first data point with plasma concentration below level of detection (AUC_{zero}) were calculated by the linear trapezoidal rule.

Dose linearity is investigated by linear regression of AUC_{zero} using arithmetic mean values. Descriptive statistics was used including mean, standard deviation, and standard error. To evaluate differences between treatment regimes in the single and multiple dose pharmacokinetic study in fed and fasted dogs, paired comparisons of geometric mean pharmacokinetic parameter were tested for significant difference using *t*-test.

Plasma protein binding of imepitoin

To evaluate the fraction of imepitoin which is bound to protein, two approaches were selected. The protein binding was evaluated after *in vitro* incubation in dog plasma and also after oral dosing to fasted beagle dogs. To determine the fraction of imepitoin bound to plasma protein, an ultracentrifugation approach was selected as described by Pacifici and Viani (1992). This was necessary due to the high lipophilicity of imepitoin resulting in high nonspecific binding on the filters and membranes which are required for ultrafiltration or equilibrium dialysis, as has been shown in experiments to establish a validated method (data not shown). After ultracentrifugation, the upper area of the supernatant was used for analysis of the unbound fraction of imepitoin. This portion of the supernatant is essentially free of protein in the plasma of fasted individuals and contains only a low amount of chylomicrons. To facilitate analysis, [^{14}C]-labeled imepitoin (Amersham International plc, Little Calfont, UK) was used in these experiments (Fig. 1).

For *in vitro* protein binding, 4.5 mL samples of heparinized plasma collected from beagle dogs that were fasted for 12 h (pool of three animals), fasted rats (pool of five animals), and male human volunteers (fasted for 10 h) were used. Samples were spiked with [^{14}C]-labeled imepitoin stock solution dissolved in 70% DMSO and 30% phosphate-buffered saline (concentration 1 mg/mL) to achieve a final drug concentration of 0.1, 1, or 10 μg/mL imepitoin in plasma. To allow protein binding, samples were incubated for 15 min at 37 °C prior to ultracentrifugation.

For *in vivo* protein binding, plasma samples from three male and three female dogs which had been dosed in the above-described pharmacokinetics and excretion balance study using [^{14}C] imepitoin were collected and analyzed. Blood samples were collected from each animal 2, 4, and 8 h postdosing using a heparinized syringe. Plasma was prepared immediately after withdrawal and was stored at 4 °C until ultracentrifugation on the following day.

To determine protein binding in *in vitro* samples and in samples from the *in vivo* dosing, plasma samples were stored at 37 °C for 15 min before ultracentrifugation, and total radioactivity was measured. Subsequently, the samples were submitted to ultracentrifugation at 250 000 *g* for 16 h at 37 °C (Beckmann Ultracentrifuge L5-65B, Palo Alto, CA, USA). Thereafter, consecutive 0.2 mL samples of the protein-free upper layers in the ultracentrifuge tube were carefully transferred into mini tubes avoiding perturbation for determination of the radioactivity. The first three samples of the upper layer were used to determine protein binding, while the remaining plasma containing the protein pellet was dried and analyzed to calculate the recovery. Radioactivity in all samples was determined by

liquid scintillation counting (LSC) using HionicFluor® (Packard, Frankfurt, Germany) as scintillator fluid and a liquid scintillator counter (Wallac 1409, Wallac GmbH).

In addition to the protein binding experiments, the reversibility of protein binding was investigated to evaluate potential irreversible (covalent) binding of imepitoin to plasma proteins, using human plasma as test matrix. Samples of 100 μL were spiked with [^{14}C] imepitoin and incubated for 0, 1, and 2 h at 37 °C. Incubation was stopped by administration of 200 μL of 0.9 M trichloroacetic acid (TCA) in 80% methanol. Precipitated protein was sedimented by centrifugation 250 *g*, 15 min and washed five times with 0.5 M TCA in methanol. Finally, the washed protein pellet was dissolved in 0.4 mL NaOH (2 M) overnight at 80 °C, neutralized, and subjected to quantification of radioactivity using LSC.

Interaction of imepitoin with drug-metabolizing cytochrome P450 liver enzymes

Anti-epileptics and especially phenobarbital are known to have a high drug–drug interaction potential. Drug-metabolizing liver enzymes are the main target for metabolic drug interactions and both inhibition, as well as induction can result in substantial drug–drug interactions (Johannessen & Landmark, 2010). To evaluate this interaction potential, liver enzyme induction and inhibition were evaluated.

In vitro enzyme inhibition. Assays were performed in 96-well microtiter plates based on the method described by Crespi and Stresser (2000). Briefly, the formation of fluorescent metabolites catalyzed by different recombinant human cytochrome P450 isoenzymes (CYPs) in the presence of multiple concentrations of the test item was investigated. For the verification of the functional state of materials and assay conditions, CYP-specific inhibitors were investigated along with the test item, and the IC_{50} values obtained for standard inhibitors were compared with historical data. Recombinant human CYPs (SUPERSOMES™), substrates, and standard inhibitors were purchased from NatuTec GmbH (Frankfurt, Germany), Mo Bi Tec GmbH (Göttingen, Germany), or Sigma (Deisenhofen, Germany). Incubations and data acquisition were accomplished using a fluorescence reader (FLUOstar, BMG Labtechnologies GmbH, Offenburg, Germany). Data evaluation and determination of IC_{50} values were performed with the software MPM/ADMET (BD Biosciences, Heidelberg, Germany). IC_{50} values were obtained by curve fitting from data of two parallel incubations on one plate using the nonparametric smoothing spline function (Nychka *et al.*, 1998).

A stock solution of imepitoin was prepared in acetonitrile and diluted in the assay mixtures to reach a final acetonitrile concentration of 2%. Concentrations of the test item reached from 0.046 to 100 μM (0.229 to 500 μM for testing of CYP1A1 interaction). As reference drugs with CYP isoform-specific inhibitory potential the following agents were selected: ketoconazole for CYP1A1 and for all three binding sites of CYP3A4, furafylline for CYP1A2, tranylcypromine for CYP2A6 and CYP2C19, quercetin for CYP2C8, sulphaphenazole for CYP2C9, and chinidine for CYP2D6. The incubation time was optimized for each enzyme and ranged from 10 to 45 min, as described previously (Crespi & Stresser, 2000).

In vivo enzyme induction potential. The potential of imepitoin to induce drug-metabolizing liver enzymes was evaluated in rats after repeated once daily oral dosing over a period of 7 days. Five groups of four male and four female rats were orally treated with 3, 10, 30, or 100 mg/kg imepitoin or corresponding vehicle solution. In addition, four groups of two male and two female rats were administered once daily orally phenobarbital 40 mg/kg suspended in corn oil administered i.p., dexamethasone 20 mg/kg suspended in corn oil and administered i.p., or corn oil administered as i.p. reference group. The volume of administration was 5 mL/kg for oral dosing or 2 mg/kg for i.p. dosing. The animals were euthanized in the morning of day 8. Livers were immediately removed and perfused with ice-cold saline to remove blood. Liver samples were then collected, processed, and centrifuged stepwise at 9000 ***g*** and 100 000 ***g*** to isolate both cytosol and microsomes. The microsome pellet was resuspended in phosphate buffer. To cytosolic samples foreseen for analysis of *N*-acetyltransferase (NAT) activity, dithioerythritol (1 mM final concentration) was added as reducing agent, and all samples were immediately frozen at −80 °C for further analysis (Orishiki *et al.*, 1994).

To evaluate potential enzyme induction, monooxygenase assays were performed incubating aliquots of liver microsomes with respective substrates. Activities of six monooxygenases were measured: ethylresorufin O-deethylase (EROD, for CYP1A), ethoxycoumarin O-deethylase (ECOD, for CYP2A and others), pentylresorufin O-dependentylase (PROD, for CYP2B), dextromethorphan O-demethylase (DXDM, for CYP2D1), nitrophenol hydroxylase (NPH, for CYP2E1), and erythromycin *N*-demethylase (ERDM, for CYP3A). Denaturation of the enzyme with trichloroacetic acid, methanol, and sodium hydroxide, respectively, stopped the reactions and the formed metabolites were measured with photometric (NPH, ERDM), fluorometric (EROD, ECOD, PROD), and gas chromatographic (DXDM) methods as described previously. Samples with inactivated microsomes (denaturation reaction) served as blanks (Walter *et al.*, 2003). In addition, the content of total CYP enzyme in microsomes was determined using a modified spectrophotometric method developed by Greim (1970) as described previously (Walter *et al.*, 2003).

The conjugative enzymes NAT1 and NAT2 were recorded for activity using aliquots of cytosol fractions with the substrate p-aminobenzoic acid (NAT1, 0.1 mM) or procainamide (NAT2, 2.5 mM) and an acetyl CoA-regenerating system (0.1 mM acetyl CoA, acetyl phosphate 5 mM, phosphotransacetylase 0.5 U/mL). The reaction was stopped and the metabolites p-acetamidobenzoic acid and *N*-acetylprocainamide were extracted and measured by HPLC or GC (Walter *et al.*, 1996).

Median enzyme activities were compared with vehicle-treated groups as well as reference drug-treated groups, calculating the ratio after repeated dose treatment compared with the vehicle-treated animals.

RESULTS

Single dose and repeated (BID) dosing of imepitoin tablets to male and female beagle dogs under fed and fasted conditions

Imepitoin administered as a single administration followed by twice daily dosing for 4 days and again a single dose administration on day 5 of imepitoin tablets at a nominal dose of 30 mg/kg under fed and fasted conditions resulted in comparable pharmacokinetic profiles in male and female beagle dogs. Imepitoin plasma concentration was detected already in the first sample after dosing indicating immediate start of absorption, and peaked at 2–3 h. The time to reach the peak plasma concentration was independent of the prandial state. After reaching the C_{max} concentration, plasma concentration rapidly declined throughout 12 h postdosing and approached the lower limit of quantification at 24 h postdosing. No accumulation was visible following twice daily dosing for 4 days (see Fig. 2a,b for plasma time curves in fasted and fed dogs on day 0 and day 5).

Comparing the prandial state, it could be seen that total systemic exposure, as indicated by AUC_{inf}, was 30% greater for the fasted dogs ($P < 0.03$). The calculated terminal half-life was significantly less in fasted dogs ($T_{½}$ 1.5 vs. 2.0 h, $P < 0.03$), as was the volume of distribution Vz (663 vs. 1129 mL/kg $P < 0.05$), and clearance Cl (320 vs. 417 mL/kg·h $P < 0.03$). The latter three differences are likely artifacts, as the prandial state cannot be expected to influence metabolism, distribution, and excretion of this compound.

Comparing single and multiple dosing, several PK parameters were reduced after 4 days of BID administration, including T_{max} (1.8 vs. 2.4 h) and AUC_{inf} (61.661 vs. 101.407 h·ng/mL) and this difference reached level of significance ($P < 0.03$) (Table 1, significantly different pairs are given in bold face). Comparing male and female dogs in this study, the only PK parameter that differed by sex was C_{max} which was 28% greater for females ($P < 0.03$). That effect was independent on the prandial state. In contrast, no gender effect was visible on AUC. An overview on the pharmacokinetic parameters of this study is given in Table 1 enabling pairwise comparison of prandial state, gender, and single vs. repeated dosing as well as combinations thereof (Table 1). If more than one parameter was combined, no significant difference was obtained, indicating that in fact the observed differences are small.

Dose linearity of imepitoin in dogs after oral dosing

The dose linearity of imepitoin, administered as micronized powder filled in enteric-coated capsules, was evaluated in six fasted beagle dogs, dosed in an ascending order with at least 14 days washout time in between. The data from male and female dogs were evaluated together as in this study no clinically relevant gender difference could be observed.

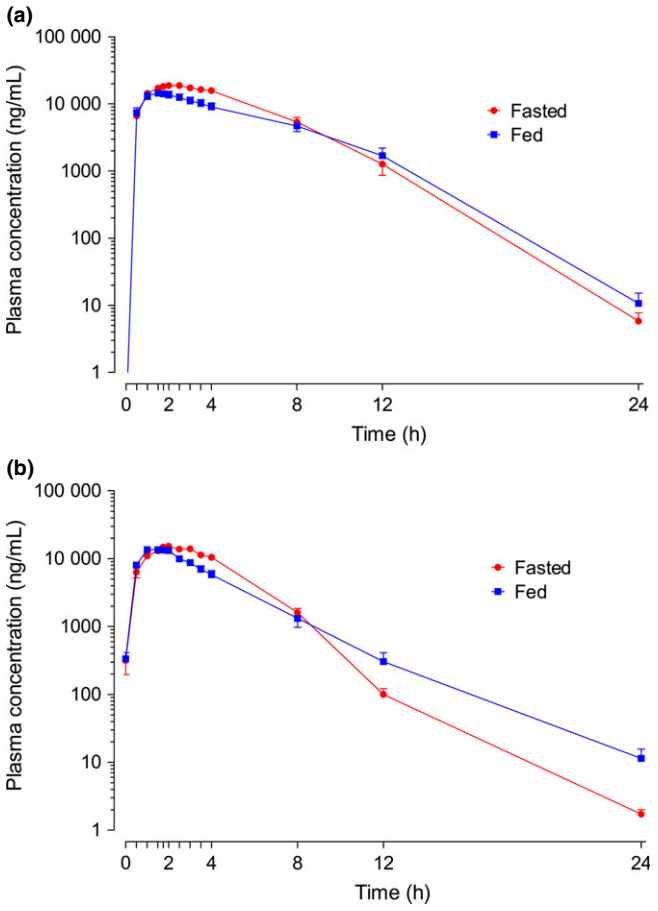

Fig. 2. Pharmacokinetic profile of imepitoin, dosed as immediate release tablets at a nominal dose of 30 mg/kg to six male and six female young beagle dogs under fasted (filled circles) and fed (filled squares) conditions. Panel A: pharmacokinetics after single dose administration (day 0). Panel B: pharmacokinetics after 4 days BID dosing (12-h dosing interval) on day 5. Displayed is mean ± SEM of 12 dogs per time point.

Due to the administration of the compound in enteric-coated gelatin capsules, absorption started after a lag time which varied between animals and from dosing to dosing resulting in a relatively large interindividual variability. Individual lag times observed ranged from 0.5 to 10 h. Due to the fact that for the 50 mg/kg dose, two capsules had to be administered, while for the 100 mg/kg dose, four capsules were given, in some animals two plasma level peaks were observed. A lag time of 0.5 h was associated with dogs who had managed to bite a single capsule in the 100 mg/kg dose level. Pharmacokinetic parameters of this study are listed in Table 2. The averaged geometric mean maximal plasma levels and AUC levels were smaller than expected from the fed/fasted study with dosing imepitoin tablets, but this was most likely due to the variability in lag time. Dose linearity for male and female dogs between 10 mg/kg and 100 mg/kg could be shown with reliable R^2 of 0.9980 for AUC_{zero} and 0.9939 for C_{max} (Fig. 3).

Table 1. Pharmacokinetic parameters of imepitoin

Effect	T_{max} [h]	Terminal $T_{½}$ [h]	C_{max} [ng/mL]	AUC_{inf} [h·ng/mL]	*Vz*/f [mL/kg]	Cl/f [mL·h/kg]
Fasted	2.17	**1.47**	17 168	**90 301**	**663**	**320**
Fed	2.02	**1.95***	14 892	**69 238***	**1129***	**417***
Females	1.89	1.72	**18 055**	82 868	833	350
Males	2.30	1.70	**14 160***	75 456	899	380
Day 0	**2.44**	1.75	17 190	**101 407**	**704**	**285**
Day 5	**1.75***	1.66	14 873	**61 661***	**1062***	**468***
Females, fasted	2.17	1.47	18 420	92 374	646	311
Females, fed	1.60	1.97	17 698	74 347	1074	395
Males, fasted	2.17	1.48	16 003	88 300	680	329
Males, fed	2.44	1.92	12 532	64 486	1187	440
Day 0, fasted	2.27	1.55	19 756	115 740	556	249
Day 0, fed	2.60	1.96	14 958	88 850	892	325
Day 5, fasted	2.06	1.39	14 921	70 467	789	410
Day 5, fed	1.44	1.94	14 825	53 960	1430	535

Statistical summary of pharmacokinetic parameters obtained from the pharmacokinetic study dosing imepitoin at a nominal dose of 30 mg/kg to six male and female dogs single dose and multiple doses for 4 days under fed and fasting conditions, using a crossover design. Paired comparisons of geometric mean pharmacokinetic parameter were tested for significant difference using *t*-test. Significantly, different pairs are displayed in bold face. In cases where one parameter was compared (fasted–fed, males–females, day 0–day 5), data are obtained from 12 dogs. In cases where two parameters were compared, data obtained from six dogs were obtained. For two parameter comparison, no statistically significant difference could be obtained.

*Pairs of mean in bold differ significantly ($P < 0.03$).

Table 2. Pharmacokinetic parameters of imepitoin dosed to six beagle dogs at ascending doses of 10–100 mg/kg

Dose [mg/kg]	C_{max} [ng/mL]	T_{max} [h]	AUC_{zero} [ng·h/mL]	$T_{lag\ obs}$ [h]
10	1815 ± 920	8.67 ± 3.1	14 035 ± 8566	3.67 ± 0.92
20	3502 ± 1073	10.7 ± 4.0	33 551 ± 15 410	5.67 ± 0.95
50	6407 ± 1752	18.0 ± 3.8	73 081 ± 21 441	4.17 ± 1.05
100	13 554 ± 3333	8.67 ± 3.1	154 269 ± 49 882	2.25 ± 0.94

Pharmacokinetic parameters of imepitoin dosed to six beagle dogs (three male and three female dogs) single dose as micronized powder filled in enteric-coated gelatin capsules in an ascending dose design with at least 14 days washout between doses. Displayed is mean ± SEM of six animals.

Pharmacokinetics and excretion balance of [^{14}C] imepitoin in dogs after oral and intravenous dosing

While oral administration of imepitoin revealed a slow absorption from the GI tract and a relatively rapid degradation as evidenced in the pharmacokinetic studies, the extent of absorption and the route of excretion can be only evaluated if the compound is dosed as radiolabeled compound to enable determination of radioactivity in plasma, urine, and feces. In such studies, total radioactivity derived from the parent compound is measured. Therefore both the parent compound and related metabolites are evaluated in total and expressed as ng equivalent. Both oral dosing of [^{14}C] imepitoin at a nominal dose of 20 mg/kg in three male and three female dogs and intravenous dosing of a dose of 1 mg/kg in three female dogs were well tolerated, and no clinical adverse events were visible.

Regardless of the route of administration, the excretion was predominantly fecal, resulting in fecal excretion of 63% of intravenously dosed radioactivity within 264 h, while 41% were excreted via urine. In male dogs, similar values were obtained with fecal excretion of 56% and 38% excretion via urine within 264 h following oral dosing. In females dosed orally, the rate of fecal excretion was a bit higher reaching 78% for the same interval. The excretion was rapid, with in total 76–97% of radioactivity being excreted within 48 h, but due to the fact that feces were not excreted from every dog within the first 48 h, only the 264 h values could be directly compared. Complete excretion of radioactivity was observed within 264 h (Table 3).

The fraction of [^{14}C] dose excreted in urine after oral administration compared with the urinary excretion rate of i.v. dosed compound was used to assess absorption rate. Urinary excretion after oral dosing amounted to > 26.7% in males and > 38.2% in females, indicating that at least this amount had been absorbed prior to excretion. If the intravenous route data were taken as reference, indicating 41.52% urinary excretion after i.v. dosing compared with 38.2% in female rats after oral dosing, a nearly complete absorption of 92% after oral dosing can be expected.

In these animals, the plasma concentration vs. time profile of total radioactivity was also evaluated. Values are expressed as ng equivalent (ng-eq) per mL plasma, based on the dosed specific radioactivity. Measured levels represent the content of

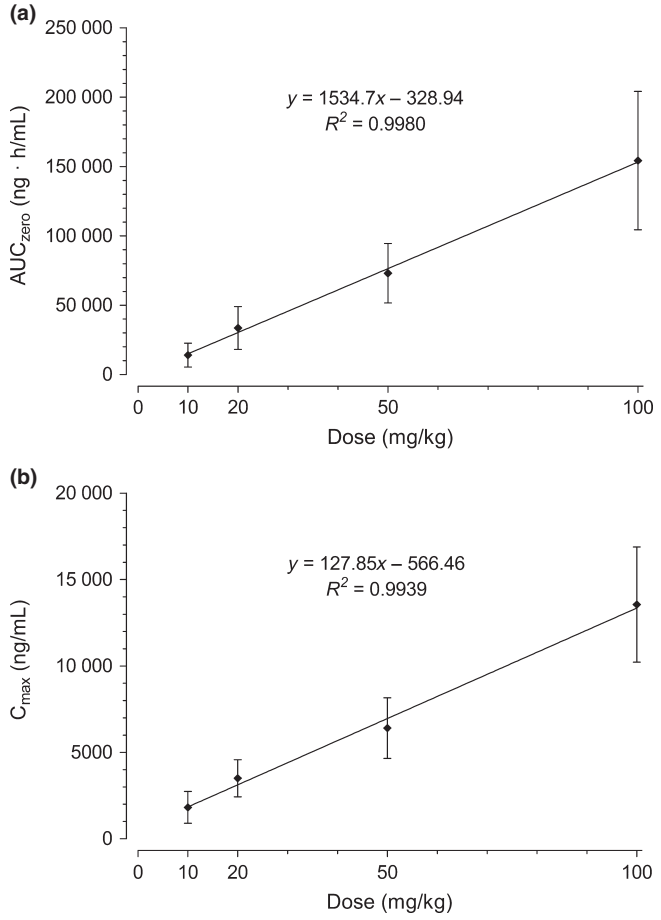

Fig. 3. Dose linearity of key pharmacokinetic parameters of imepitoin dosed to six beagle dogs (three male and three female dogs) single dose as micronized powder filled in enteric-coated gelatin capsules in an ascending dose design with at least 14 days washout between doses. Displayed is mean ± SEM of six animals. Panel A: AUC_{zero} for the dose range 10–100 mg/kg. The correlation coefficient obtained from linear regression of the mean values is given in the figure. Panel B: maximal plasma concentration. The correlation coefficient obtained from linear regression of the mean values is given in the figure.

Table 3. Excretion balance after oral and intravenous dosing

Time interval [h]	Urine + Cage wash	Feces	Total
Fraction excreted in males mean [% of oral dose] (CV [%])			
0–8*	0.58 ($n < 3$)	(no feces)	0.58 ($n < 3$)
0–24	16.70 (20.9)	34.41 (92.2)	51.11 (66.2)
0–48	21.86 (20.5)	75.65 (12.3)	97.51 (6.4)
0–264	26.74 (27.6)	78.01 (10.8)	104.74 (2.8)
Fraction excreted in females mean [% of oral dose] (CV [%])			
0–8*	7.95 ($n < 3$)	0.09 ($n < 3$)	8.04 ($n < 3$)
0–24*	22.96 ($n < 3$)	40.62 ($n < 3$)	63.58 ($n < 3$)
0–48	30.88 (23.3)	45.64 (22.0)	76.52 (20.8)
0–264	38.20 (25.4)	55.64 (8.4)	93.84 (8.4)
Fraction excreted in females mean [% of intravenous dose] (CV [%])			
0–8*	30.62 ($n < 3$)	(no feces)	30.62 ($n < 3$)
0–24*	37.58 (19.1)	54.74 ($n < 3$)	74.07 (33.4)
0–48*	39.87 (19.9)	68.33 ($n < 3$)	85.43 (37.2)
0–264	41.52 (21.8)	62.91 (21.3)	104.43 (4.4)
[^{14}C]Absorption$_{urine}$ [mean%] (CV [%]†: 91.92 (15.5)			

Excretion of [^{14}C] imepitoin in three male and three female dogs after oral dosing of 20 mg/kg filled in gelatin capsules, 0.792 MBq/kg, and i.v. dosing of 1 mg/kg dissolved in 70% DMSO in saline, 0.792 MBq/kg, to three female dogs. Displayed are cumulative excretion data in% excretion of total administered dose, given as mean and coefficient of variance (CV). In some intervals, samples were not obtained from all three dogs, and therefore, no CV could be calculated (indicated by *).
†The total enteral [^{14}C] absorption in% of administered dose was approximated from comparison of renally excreted fractions of the administered [^{14}C] dose following oral and intravenous administration in female dogs in crossover design.

total radioactivity, composed of active compound as well as the sum of all metabolites and degradation products. While the intravenous dose was 1 mg/kg, compared with the oral dose of 20 mg/kg, the total radioactivity administered was identical for both routes. Assuming nonsaturating metabolism, it is therefore feasible to scale the data obtained from i.v. dosing to the oral dose of 20 mg/kg. The plasma time profiles are displayed in Fig. 4.

While the data obtained from oral dosing indicate that the absorption phase was slow with peak levels only reached after 4–8 h, the decline of plasma radioactivity was biphasic with a more rapid decline within the first 48 h covering 80–90% of the area under the concentration–time curve, followed by a slow but steady decline for the next few days, with a calculated terminal half-life of 111.1 h. While the limit of quantification was not reached within 196 h, the remaining levels were very low. The pharmacokinetic data obtained in plasma correlate well with the excretion balance. In fact, within 48 h, 76.5–97.5% of administered radioactivity had been excreted and appeared in urine or feces.

After intravenous dosing, the pharmacokinetics of the radioactive label was dominated by an early rapid distribution and excretion phase lasting the first 10–12 h, followed by a slower decline of the remaining plasma levels (Fig. 4).

Plasma protein binding of imepitoin

Plasma samples obtained from the excretion balance study with oral and intravenous dosing of [^{14}C] imepitoin were also used to evaluate plasma protein binding *in vivo,* and the obtained binding data were compared with respective data obtained *in vitro* using frozen plasma samples from rats, dogs, and humans, enabling across species comparison. Due to the high lipophilicity of imepitoin resulting in unspecific binding to surfaces and filter membranes, plasma protein binding was determined using ultracentrifugation as adequate method. Comparing the *in vitro* protein binding of [^{14}C] imepitoin to dog plasma at a concentration range of 0.1–10 μg/mL with the binding to human and rat plasma, it becomes evident that imepitoin has low protein binding potential across species. In dogs, the fraction amounted to 56–57%, regardless of the concentration tested, while in rats, the binding was even less with 50–51%. Protein binding in human plasma was similar to

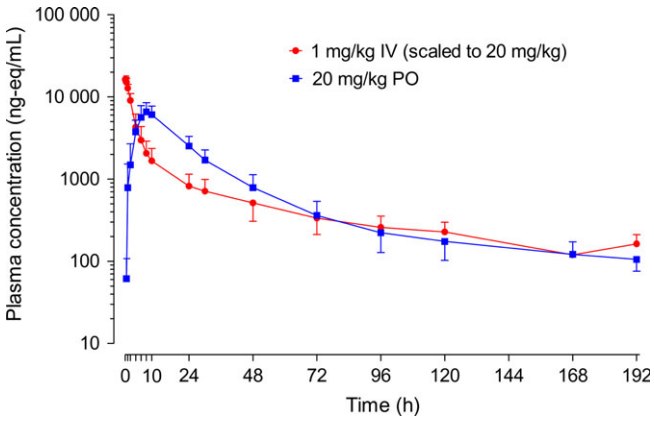

Fig. 4. Plasma pharmacokinetics of total radioactivity following oral dosing of 20 mg/kg filled in gelatin capsules, 0.792 MBq/kg, and i.v. dosing of 1 mg/kg dissolved in 70% DMSO in saline, 0.792 MBq/kg. Displayed is mean ± SEM of six dogs (three male and three female dogs) after oral dosing as well as mean ± SEM of three female dogs following intravenous dosing. For visualization purposes, the pharmacokinetic data obtained after intravenous dosing of 1 mg/kg were scaled to a dose of 20 mg/kg, to enable easy comparison. Note that the administered radioactivity was identical in both dosing arms and only radioactivity was quantified.

Table 4. *In vitro* protein binding, species comparison

	In vitro protein binding (%) mean, ± SEM		
Concentration [μg/mL]	Human [n = 4]	Dog [n = 3]	Rat [n = 3]
10	57 ± 3.0	56 ± 0.75	50 ± 1.1
1	59 ± 2.7	56 ± 0.75	50 ± 0.64
0.1	57 ± 2.5	57 ± 1.4	51 ± 0.35

In vitro protein binding using heparinized pooled plasma of 12-h-fasted male and female dogs (pool of three animals), rats (pool of five animals), and male human volunteers (individual plasma) (fasted for 10 h). Samples were spiked with [^{14}C]-labeled imepitoin stock solution (concentration 1 mg/mL) to achieve a final drug concentration of 0.1, 1, or 10 μg/mL imepitoin in plasma. To allow protein binding, samples were incubated for 15 min prior to ultracentrifugation.

dogs, amounting to 57–59% (Table 4). Protein binding was found to be completely reversible. After incubation for up to 2 h at 37 °C, plasma proteins were precipitated with trichloroacetic acid and washed resulting in complete reversal of protein binding with less than 0.5% of radioactivity found in the washed precipitate, indicating lack of formation of covalent binding to plasma proteins.

Using plasma samples of dogs dosed with 20 mg/kg [^{14}C] imepitoin orally or 1 mg/kg i.v., similar plasma protein binding values were obtained as compared to the *in vitro* data in samples taken 2 h after dosing, that is, at the time of expected peak plasma concentration or even before the peak concentration after oral dosing, representing a time window, where predominantly parent compound can be expected in plasma. At this time, the plasma protein binding amounted to 59–69% (Table 5). At later time points, that is, at 8 h which represents a time where in addition to parent compound metabolites can be expected in plasma, the plasma protein bound fraction was similar reaching 62–72%, indicating that main metabolites may also have a low plasma protein binding (Table 5).

Table 5. *In vivo* protein binding in dogs

	In vivo protein binding (%) mean, ± SEM		
Time after dosing [h]	Male, 20 mg/kg p.o.	Female, 20 mg/kg p.o.	Female, 1 mg/kg i.v.
2	60 (n < 3)	69 (n < 3)	59 ± 0.69
4	63 ± 1.62	66 ± 0.69	66 ± 2.66
8	67 ± 2.71	62 ± 7.68	72 ± 1.33

In vivo protein binding of [^{14}C] imepitoin in three male and three female dogs after oral dosing of 20 mg/kg filled in gelatin capsules, 0.792 MBq/kg, and i.v. dosing of 1 mg/kg dissolved in 70% DMSO in saline, 0.792 MBq/kg, to three female dogs. Displayed is mean ± SEM of three dogs at different time points after dosing. For the 2-h time point after oral dosing, insufficient plasma was obtained for two of three dogs. Protein binding was determined by ultracentrifugation.

Interaction of imepitoin with hepatic drug-metabolizing enzymes: enzyme inhibition and induction

To get further insight in the interaction potential of imepitoin, the potential to inhibit microsomal liver enzymes was evaluated using human recombinant cytochrome P450 isoforms in an *in vitro* setting. Imepitoin did not influence the activities of human recombinant cytochrome P450 isoforms CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 up to the highest concentration tested (100 μM). A slight potential for inhibition was found for CYP1A1 only (IC_{50} 55 μM). Based on these in vitro data obtained with human recombinant isoenzymes, compared to maximal plasma concentrations measured in dogs, an inhibition of CYP1A1-dependently eliminated co-medications cannot be completely ruled out. However, as the therapeutically relevant plasma concentrations are below 55 μM, and as a major CYP1A1-dependent elimination pathway is not known for anti-epileptics and other drugs used in dogs, a clinically relevant inhibition of CYP-dependently eliminated co-medications is not likely. Reference compounds exerted the expected strong enzyme inhibition in the same system (see Table 6 for details).

In addition to enzyme inhibition, the potential for enzyme induction was also assessed using a rodent model for enzyme induction. For this model, four groups of rats were exposed once daily for 7 days to imepitoin at the dose levels 3, 10, 30, and 100 mg/kg, while control groups were exposed once daily for 7 days with well-known enzyme inducing compounds, namely phenobarbital (40 mg/kg p.o.), β-naphthoflavone (40 mg/kg i.p.), and dexamethasone (20 mg/kg i.p.). After preparation of liver microsomes for determination of cytochrome isoenzymes and cytosol for quantification of N-acetyltransferases, the activities of 6 cytochrome P450 isoforms of rodents, namely CYP1A, CYP2A, CYP2B, CYP2D1, CYP2E1, and CYP3A and two cytosolic N-acetyltransferases, NAT1 and NAT2, were evaluated. Phenobarbital as well as β-naphthoflavone treatment resulted in nearly a

Table 6. Inhibition of the specific cytochrome P450 isoforms by imepitoin

	Inhibition of the specific cytochrome P450 isoform IC_{50} [μM]								
Isoenzyme	1A1	1A2	2A6	2C9	2C19	2D6	3A4		
Binding site							BQ	BFC	DBF
Standard inhibitor	0.23[1]	0.77[2]	0.49[3]	0.36[4]	2.8[3]	0.02[5]	0.40[1]	0.11[1]	0.05[1]
Imepitoin	55	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.

Inhibiton of specific human cytochrome P450 isoforms *in vitro*. Displayed are mean IC_{50} values for each enzyme. For human CYP3A4, 3 different binding sites are reported which were individually addressed by the specific ligands BQ (benzyloxyquinoline), BFC (7-benzyloxy-4-(trifluoromethyl)-coumarin), and DBF (dibenzylfluorescein). For reference, the following standard inhibitors were tested in the same assay: [1]ketoconazole, [2]furafylline, [3]tranylcypromine, [4]sulphaphenazole, and [5]chinidine. n.e.: no inhibitory effect up to the highest concentration of imepitoin tested (100 μM). Note that data obtained using human enzymes may not be fully representative for dogs due to substantial species differences (Martignoni *et al.*, 2006).

doubling of total cytochrome P450 enzyme content in hepatic microsomes, indicating strong enzyme induction, while the effect of dexamethasone was less pronounced. Imepitoin had no appreciable effect on the total enzyme content (Table 7). Enzyme activity after repeated doses of imepitoin and reference treatments was evaluated. The reference drugs evaluated had a strong effect on selected enzymes. In phenobarbital-treated rats, the activity of the CYP2B enzyme was increased 17.7-fold indicating strong enzyme induction. In addition, other enzyme activities were also increased by phenobarbital, but to a lesser extent, that is, 5.5-fold for CYP1A, 3.5-fold for CYP3A, and 2.6-fold for CYP2A (Fig. 5). Effects on other enzymes including NAT were minimal. Treatment with *β*-naphthoflavone resulted in a strong induction of CYP1A (25.1-fold) and CYP2A (15.8-fold), with a minimal effect on CYP2B (3.8-fold). Dexamethasone had a moderate effect on the activity of CYP3A, resulting in a sixfold increase; effects on other enzymes were small resulting in a 3.8-fold increase in CYP1A activity. In addition, dexamethasone had the strongest effect of all drugs tested on NAT2, resulting in a 1.8-fold activity compared with untreated controls. In contrast, imepitoin did not induce CYP2A, CYP2D1, CYP2E1, CYP3A, as well as NAT1 and NAT2. A minor effect at the highest dose tested, that is, 100 mg/kg, was seen on CYP2B, resulting in a 2.5-fold increase, and also on CYP1A, resulting in a 2.5 and 3.7-fold increase at the highest two doses tested. Compared with the reference drug effects, the magnitude of effect, however, remained low and restricted to the highest doses tested (Fig. 5).

Table 7. Total cytochrome P450 content

	Total cytochrome P450 [nmol/mg protein]	
Treatment group	Female rats median (range)	Male rats median (range)
Oral vehicle	0.751 (0.602–1.156)	1.089 (0.994–1.191)
Imepitoin 3 mg/kg p.o.	0.838 (0.604–1.037)	1.053 (0.837–1.231)
Imepitoin 10 mg/kg p.o.	0.756 (0.659–0.941)	1.142 (1.016–1.282)
Imepitoin 30 mg/kg p.o.	0.852 (0.768–0.864)	1.165 (0.759–1.306)
Imepitoin 100 mg/kg p.o.	0.870 (0.687–1.453)	1.218 (1.065–1.262)
Phenobarbital 40 mg/kg p.o.	1.492 (1.182–1.802)	1.886 (1.451–2.321)
Corn oil (i.p. vehicle)	0.808 (0.732–0.902)	0.874 (0.787–1.087)
β-naphthoflavone 40 mg/kg i.p.	1.389 (1.388–1.389)	2.136 (1.989–2.283)
Dexamethasone 20 mg/kg i.p.	0.953 (0.903–1.002)	0.953 (0.899–1.007)

Microsomal content of total cytochrome P450 enzyme from male and female rats treated with imepitoin, phenobarbital, *β*-naphthoflavone, or dexamethasone for 7 days. Given are median and range for $n = 4$ rats per sex and treatment group. For the three positive control groups, the group size was only two males and two females per group.

DISCUSSION

Canine idiopathic epilepsy is currently treated using phenobarbital as drug of first choice. Other drugs used are potassium bromide (as add on to phenobarbital or in some cases also as monotherapy) and benzodiazepines, which are primarily used to control status epilepticus (Podell *et al.*, 1998; Boothe *et al.*, 2012). Other first-generation anti-epileptics used in human medicine, including carbamazepine, valproic acid, and phenytoin, are of very limited utility for veterinary medicine, as these drugs lack adequate pharmacokinetics in dogs and elimination is in part further accelerated as a result of enzyme induction (Frey & Löscher, 1985). As epileptic seizures can occur at any time during the day, the protective drug action is required to be persistent throughout the day to allow good seizure control. These drugs are eliminated so rapidly that a therapeutic value cannot be expected (Frey & Schwartz-Porsche, 1985).

Both phenobarbital and potassium bromide are now considered almost obsolete for treatment of epilepsy in human due to the limited safety profile, and a variety of novel anti-epileptics have reached the market. Several of these novel drugs have been also evaluated for the treatment of canine epilepsy. In a recent review, the pharmacokinetics of second-generation anti-epileptics including felbamate, gabapentin, levetiracetam, oxcarbazepine, and topiramate were evaluated and these drugs were all found to be eliminated too rapidly to support the use in dogs (Ziółkowski *et al.*, 2012).

Imepitoin has shown clinical efficacy in dogs with idiopathic epilepsy and hence is a unique novel therapeutic opportunity

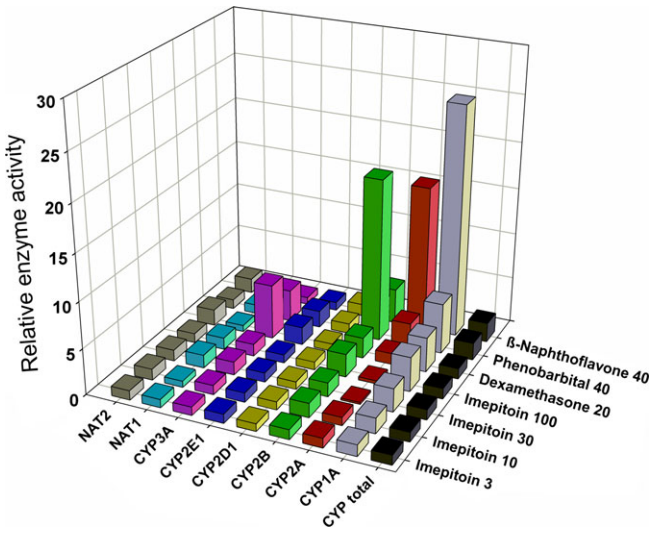

Fig. 5. Relative enzyme induction of different drug-metabolizing liver enzymes. Displayed are enzyme activities relative to the respective control groups (set at 1). While the basal level of CYP activity was lower in female rats compared with male rats, no difference was found for the relative drug-induced potentiation. Therefore, data from male and female rats were averaged after calculation of the individual activity.

(Löscher *et al.*, 2004; Rieck *et al.*, 2006; Bialer *et al.*, 2013). It has recently obtained marketing authorization for the treatment of idiopathic epilepsy in EU (EMA, 2012). The dosage regimen which has shown clinical efficacy was twice daily dosing, both for monotherapy and add on to phenobarbital in drug-resistant dogs (Löscher *et al.*, 2004; Rieck *et al.*, 2006). We have now evaluated the pharmacokinetic profile of this novel anti-epileptic drug in more detail, comparing single and multiple dosing, and dosing under fasted and fed conditions in male and female young beagle dogs using a crossover design. Upon administration as imepitoin tablets at a nominal single dose of 30 mg/kg, a rapid increase in plasma level was observed, indicating immediate release and rapid initiation of absorption after dosing (Fig. 2a,b). However, peak plasma levels were reached only after 2–3 h, indicating a prolonged absorption period. Indeed, imepitoin has a very low solubility in aqueous media (Heinecke & Thiel, 2001), and this results in a slow dissolution in the gastrointestinal environment. The compound is more readily soluble in acidic media such as gastric fluid, supporting the immediate release, and initiation of absorption of active substance after swallowing of the tablets (Heinecke & Thiel, 2001). Imepitoin immediate release tablets therefore behave like a modified release formulation with immediate release followed by a protracted absorption over 2–3 h, causing a flattening in the PK profile. The absorption time of 2–3 h nicely correlates with the average small intestine passage time of dogs of 183 min (Lidbury *et al.*, 2012). Administering radiolabeled compound demonstrated that the enteral absorption after oral dosing was nearly complete. Comparing the pharmacokinetics of fasted and fed dogs, a statistically significant increased exposure was observed if dosed under fasting conditions, but this was associated with a significantly shortened terminal half-life (Table 1). However, the clinical relevance of this difference may be limited. While under fasting conditions, a higher maximal plasma level is reached, the decline was found to be more rapid. The therapeutically relevant trough plasma level at 12 h was higher in fed condition dosed dogs than in fasted dogs, both after single dose administration and after repeated dosing (Fig. 2a,b). Nevertheless, it may be appropriate to keep the timing of tablet administration in relation to feeding consistent, once dosing was initiated. The terminal half-life of imepitoin was found to be in the range of 1.5–2 h. Twice daily dosing has been proven to be efficacious in dogs with idiopathic epilepsy. This demonstration of efficacy at twice daily dosing with a relatively short plasma half-life may be related to the physicochemical properties of imepitoin. The compound has a high lipophilicity and a high affinity to membranes and surfaces, as well as a very low solubility in aqueous media including plasma (Heinecke & Thiel, 2001). It is therefore possible, that the compound enriches in cell membranes and the time course of pharmacological activity is not directly coupled to the plasma concentration. In fact, no clear correlation between plasma levels and anti-epileptic activity could be found in a clinical study (Rieck *et al.*, 2006). While in some animals, seizures could be controlled with low doses (and low plasma levels), other dogs required higher plasma levels, and again other animals could not be satisfactory controlled. In addition, the pharmacokinetic data obtained in the studies reported here, involving young beagle dogs, may not be fully representative for the pharmacokinetics in typical epilepsy patient dogs. Indeed, Löscher *et al.* (2004) reported a terminal half-life of 6.2 h in a different strain of beagle dogs which were aged 6–9 years. In this study, repeated dosing resulted therefore in increased exposure compared with single dose.

Likewise, in a clinical study in epileptic dogs dosed 10–15 mg/kg imepitoin twice daily, the trough levels observed 12 h after dosing on day 21 of treatment were comparable to trough levels observed on day 5 of this pharmacokinetic study, although the dose administered in the pharmacokinetic study was more than double as high, that is, 30 mg/kg. The pharmacokinetic parameters obtained from studies conducted in young beagle dogs have been reported by others to not be fully representative for the general dog population as beagle dogs are known for a somewhat more rapid elimination compared with other breeds (Blaisdell *et al.*, 1998; Paulson *et al.*, 1999).

The pharmacokinetics in male and female dogs was also compared. While the maximal plasma concentration reached was statistically significant lower in male dogs, the difference was only 21.5% and did not translate in a comparable difference in exposure as assessed by AUC_{inf} (Table 1). Therefore, no clinically relevant sex difference in kinetics could be identified. The single dose kinetics was also compared with the kinetics after twice daily dosing for 4 days. There was no clinically relevant difference in C_{max} after multiple dosing. T_{max} and AUC differed significantly following 5 day dosing compared with single dose. A shorter T_{max} indicates early start of or more rapid absorption. This difference, that is, maximal plasma levels reached after 1.75 vs. 2.44 h may be of limited clinical

relevance for repeated dosing. The AUC was lower following repeated dosing. The reason for this reduced exposure remains open. The data on metabolic liver enzyme induction do not indicate that the metabolism of imepitoin is induced. The lack of metabolic adaptation can be also derived from other studies where imepitoin was dosed twice daily to dogs at a dose level of 5 or 40 mg/kg for 28 days, to evaluate the development of tolerance toward the anticonvulsant activity (Löscher *et al.*, 2004). No metabolic adaptation could be observed even at the high dose of 40 mg/kg twice daily. A lack of clinically relevant metabolic adaptation is also supported by peak and trough plasma levels taken during a clinical study on days 21, 90, and > 120 (Rieck *et al.*, 2006). At an individual dosing concentration of 10–15 mg/kg twice daily, no decline in peak or trough plasma levels was visible, but instead the median levels increased slightly over time, ruling out metabolic adaptation during longer treatment. The reduced AUC following repeated dosing may be therefore of limited clinical relevance.

Anti-epileptic drug treatment is an individual treatment, aiming at reducing the seizure frequency and ultimately obtaining a seizure free state. An epileptic seizure may occur, if endogenous or exogenous stimuli result in surpassing a synchronization threshold for a given neuronal network in an epileptic dog. While any dog can experience an epileptic seizure if the stimulus is strong enough, such as after a head trauma (Friedenberg *et al.*, 2012), the threshold for synchronization is lowered in dogs with idiopathic epilepsy. It is therefore the aim of any successful anti-epileptic treatment to elevate the threshold for synchronization until seizure control is obtained. A common approach is to start treatment with a low dose of an anti-epileptic and titrate the dose up until sufficient seizure control is obtained. Imepitoin was shown to elevate the synchronization threshold in a dose-dependent manner as evaluated in the pentylenetetrazole model of canine seizures with a dose of 5 mg/kg resulting already in a significant increase at the time of peak plasma concentration of about 1400–1700 ng/mL. A much stronger elevation of threshold was observed following a dose of 40 mg/kg given twice daily, associated with a peak plasma concentration of about 7500–17 000 ng/mL at the time of seizure induction. In the pharmacokinetic study following administration of 30 mg/kg as imepitoin tablets, a peak plasma concentration of 14 000–18 000 ng/mL was reached (Löscher *et al.*, 2004). This high peak plasma level may not be needed in all dogs to reach seizure control. Indeed, in a clinical study, titration was stopped in some dogs already at 10 mg/kg, while in others, it was continued up to 20 mg/kg or higher (Rieck *et al.*, 2006). A prerequisite for easy and successful titration is dose linearity of the pharmacokinetics. We have therefore evaluated the pharmacokinetics of imepitoin dosed 10 mg/kg to up to 100 mg/kg. Data indicate that both maximal plasma level and exposure correlated well with the dose (see Fig. 3). This dose linearity in combination with the rapid onset of release of imepitoin tablets facilitates dose adjustment and leads to predictive results.

Monotherapy is not always successful in canine epilepsy making add-on treatment a frequent condition (Kearsley-Fleet *et al.*, 2013). Therefore, the pharmacokinetic interaction potential of any novel anti-epileptic is of high interest. Imepitoin was found to have a very low protein binding both *in vitro* and *in vivo*, indicating that neither the parent compound nor any metabolite is highly protein bound. This makes an interaction on the level of plasma protein binding with highly protein bound drugs an unlikely event. Imepitoin also did not inhibit most human cytochrome P450 family liver enzymes with the exception of the enzyme CYP1A1, where a weak inhibition was found only at high concentrations with an IC_{50} of 55 μM. The primary metabolism of imepitoin was shown to be catalyzed by this enzyme at least in human-derived tissue, therefore a competitive inhibition would be expected if imepitoin itself is a substrate for this enzyme (Gasparic, 2005). Furthermore, known anti-epileptics used in dogs including phenobarbital are not primarily metabolized by CYP1A1, making drug interaction not a likely condition. Likewise, no strong induction potential for cytochrome P450 liver enzymes was found in a rat study testing a series of inducible cytochrome enzymes. While a modest induction was visible only for the CYP1A family, the extent of induction was much lower than that of the known inducer *β*-naphthoflavone (Fig. 5). The lack of pharmacokinetic interaction with phenobarbital is also supported by pharmacokinetic data obtained in a clinical study (Rieck *et al.*, 2006). While only limited pharmacokinetic data are available preventing an exact analysis, no clinically relevant interaction was visible in dogs which were treated with imepitoin add on to phenobarbital, which is known to be a strong enzyme inducer. Similar imepitoin peak and trough plasma levels were recorded in 19 dogs treated add on to phenobarbital as compared to dogs treated with monotherapy only (Rieck *et al.*, 2006). This lack of interaction with phenobarbital is favorable, as phenobarbital is notorious for drug interaction and even levetiracetam, known to have a very low interaction potential, was cleared more rapidly in dogs treated with phenobarbital as compared to naïve dogs (Moore *et al.*, 2011).

Treatment of canine epilepsy requires chronic drug administration. The typical patient with newly diagnosed epilepsy will be treated for many years. One potential complication for chronic drug treatment is chronic kidney disease resulting in reduced renal clearance, which is a frequent finding in aged dogs (Bartges, 2012). Imepitoin is extensively metabolized after oral intake. Therefore, the primary clearance route of imepitoin is metabolism, involving CYP1A1 as the primary enzyme (Gasparic *et al.*, 2001; Gasparic, 2005). This enzyme is known to be expressed in many organs including lungs, small intestine, and kidney, in addition to being expressed in the liver (Sakakibara *et al.*, 2013). Therefore, neither reduced kidney function nor impaired liver function is likely to greatly influence the pharmacokinetics of imepitoin. We now also demonstrate that the primary route of excretion of metabolites of imepitoin, as assessed in a mass balance study involving dosing of radiolabeled imepitoin, is the fecal route, that is, most likely as biliary excretion. In this study, between 56% and 78% of total radioactivity was excreted via the fecal route, while 27–38% were excreted urinary after oral dosing (Table 3). While

the terminal half-life of radioactivity was long, the majority of excretion was observed within the first 48 h, leaving only a small fraction for further excretion. Based on these data, it can be expected that renal insufficiency will also have no clinically relevant effect on the clearance of imepitoin metabolites; however, respective data in renally impaired dogs are not yet available.

In conclusion, imepitoin, administered as 100 or 400 mg immediate release tablets, was found to have predictable pharmacokinetics in dogs with rapid onset from immediate release followed by a prolonged absorption. No clinically relevant gender difference or food effect was found enabling twice daily dosing independent of the feeding schedule or gender. Due to the lack of accumulation upon multiple dosing, a treatment will result in dose proportional plasma levels on the first day of treatment, but a twice daily dosing schedule should be strictly obeyed to preserve pharmacological activity throughout the day. Due to the dose linearity and short half-life, any dose adjustment will result in rapid change in plasma levels enabling a rapid onset of action or rapid reduction in dose if needed, such as in the unexpected case of intolerance. The compound has a very low interaction potential. No interaction with highly protein bound or enzyme-inducing drugs is assumed. From the available data, no therapeutic plasma level can be proposed, and further clinical experience is required to better understand the therapeutic potential of this drug. Due to the safety profile, a co-medication with other drugs including phenobarbital in drug-resistant dogs is a valid option and should be explored further in the future.

CONFLICT OF INTEREST

PD Dr. habil. Chris Rundfeldt is co-inventor in a medical use patent for use of imepitoin in canine epilepsy. He is also scientific advisor to Boehringer Ingelheim. The other authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

REFERENCES

Altman, P.L., Grebe, R.M. & Katz, D.D. (1959) *Handbook of Circulation*. Saunders, Philadelphia.

Bartges, J.W. (2012) Chronic kidney disease in dogs and cats. *Veterinary Clinics of North America: Small Animal Practice*, **42**, 669–692.

Berendt, M., Gredal, H., Ersboll, A.K. & Alving, J. (2007) Premature death, risk factors, and life patterns in dogs with epilepsy. *Journal of Veterinary Internal Medicine*, **21**, 754–759.

Bialer, M., Johannessen, S.I., Levy, R.H., Perucca, E., Tomson, T. & White, H.S. (2013) Progress report on new antiepileptic drugs: a summary of the Eleventh Eilat Conference (EILAT XI). *Epilepsy Research*, **103**, 2–30.

Blaisdell, J., Goldstein, J.A. & Bai, S.A. (1998) Isolation of a new canine cytochrome P450 CDNA from the cytochrome P450 2C subfamily (CYP2C41) and evidence for polymorphic differences in its expression. *Drug Metabolism and Disposition*, **26**, 278–283.

Boothe, D.M., Dewey, C. & Carpenter, D.M. (2012) Comparison of phenobarbital with bromide as a first-choice antiepileptic drug for treatment of epilepsy in dogs. *Journal of the American Veterinary Medical Association*, **240**, 1073–1083.

Crespi, C.L. & Stresser, D.M. (2000) Fluorometric screening for metabolism-based drug–drug interactions. *Journal of Pharmacological and Toxicological Methods*, **44**, 325–331.

EMA (2012) CVMP Assessment Report for Pexion® (imepitoin) (EMEA/V/C/002543/0000), http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/veterinary/002543/WC500140842.pdf (date last accessed: 5 April 2013).

Frey, H.H. & Löscher, W. (1985) Pharmacokinetics of anti-epileptic drugs in the dog: a review. *Journal of Veterinary Pharmacology and Therapeutics*, **8**, 219–233.

Frey, H.H. & Schwartz-Porsche, D. (1985) Pharmacologic principles in the treatment of epilepsy in the dog and cat. *Tierarztliche Praxis*, **13**, 541–549.

Friedenberg, S.G., Butler, A.L., Wei, L., Moore, S.A. & Cooper, E.S. (2012) Seizures following head trauma in dogs: 259 cases (1999–2009). *Journal of the American Veterinary Medical Association*, **241**, 1479–1483.

Gasparic, A. (2005) Investigations on biotransformation of AWD 131-138. Doctoral thesis, Faculty of Bioscience, Pharmacy and Psychology, University of Leipzig, Leipzig, Germany,

Gasparic, A., Schupke, H., Olbrich, M., Heinecke, K., Hempel, R., Hermann, R. & Kronbach, T. (2001) Morpholine ring oxidation of AWD 131-138, a novel anxiolytic and anticonvulsant, is catalysed by CYP1A1/2. *Drug Metabolism Reviews*, **33** (Suppl. 1), 89.

Greim, H. (1970) Increased synthesis and inhibited breakdown during the increase in microsomal cytochromes P-450 and b-5 by phenobarbital. *Naunyn-Schmiedebergs Archiv fur Pharmakologie*, **266**, 260–275.

Heinecke, K. & Thiel, W. (2001) Identity and physicochemical properties of 1-(4-chlorophenyl)-4-morpholino-imidazolin-2-one, AWD 131-138. *Pharmazie*, **56**, 458–461.

Johannessen, S.I. & Landmark, C.J. (2010) Antiepileptic drug interactions — principles and clinical implications. *Current Neuropharmacology*, **8**, 254–267.

Kearsley-Fleet, L., O'Neill, D.G., Volk, H.A., Church, D.B. & Brodbelt, D.C. (2013) Prevalence and risk factors for canine epilepsy of unknown origin in the UK. *Veterinary Record*, **172**, 338.

Kwan, P., Schachter, S.C. & Brodie, M.J. (2011) Drug-resistant epilepsy. *New England Journal of Medicine*, **365**, 919–926.

Lidbury, J.A., Suchodolski, J.S., Ivanek, R. & Steiner, J.M. (2012) Assessment of the variation associated with repeated measurement of gastrointestinal transit times and assessment of the effect of oral ranitidine on gastrointestinal transit times using a wireless motility capsule system in dogs. *Veterinary Medicine International*, **2012**, 938417

Löscher, W., Potschka, H., Rieck, S., Tipold, A. & Rundfeldt, C. (2004) Anticonvulsant efficacy of the low-affinity partial benzodiazepine receptor agonist ELB 138 in a dog seizure model and in epileptic dogs with spontaneously recurrent seizures. *Epilepsia*, **45**, 1228–1239.

Martignoni, M., Groothuis, G.M. & de Kanter, R. (2006) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opinion on Drug Metabolism and Toxicology*, **2**, 875–894.

Moore, S.A., Munana, K.R., Papich, M.G. & Nettifee-Osborne, J.A. (2011) The pharmacokinetics of levetiracetam in healthy dogs concurrently receiving phenobarbital. *Journal of Veterinary Pharmacology and Therapeutics*, **34**, 31–34.

Nychka, D., Haaland, P., O'Connell, M. & Ellner, S., (1998) FUNFITS, data analysis and statistical tools for estimating functions. In *Lecture Notes in Statistics*, Vol. 132. Case Studies in Environmental Statistics.

Eds Nychka, D., Piegorsch, W.W. & Cox, L.H., pp. 159–179. Springer-Verlag, New York.

Orishiki, M., Matsuo, Y., Nishioka, M. & Ichikawa, Y. (1994) *In vivo* administration of H2 blockers, cimetidine and ranitidine, reduced the contents of the cytochrome P450IID (CYP2D) subfamily and their activities in rat liver microsomes. *International Journal of Biochemistry*, **26**, 751–758.

Pacifici, G.M. & Viani, A. (1992) Methods of determining plasma and tissue binding of drugs. Pharmacokinetic consequences. *Clinical Pharmacokinetics*, **23**, 449–468.

Paulson, S.K., Engel, L., Reitz, B., Bolten, S., Burton, E.G., Maziasz, T.J., Yan, B. & Schoenhard, G.L. (1999) Evidence for polymorphism in the canine metabolism of the cyclooxygenase 2 inhibitor, celecoxib. *Drug Metabolism and Disposition*, **27**, 1133–1142.

Podell, M., Fenner, W.R. & Powers, J.D. (1995) Seizure classification in dogs from a nonreferral-based population. *Journal of the American Veterinary Medical Association*, **206**, 1721–1728.

Podell, M., Smeak, D. & Lord, L.K. (1998) Diazepam used to control cluster seizures in dogs. *Journal of Veterinary Internal Medicine*, **12**, 120–121.

Potschka, H., Fischer, A., von Rüden, E.L., Hülsmeyer, V. & Baumgärtner, W. (2013) Canine epilepsy as a translational model? *Epilepsia*, **54**, 571–579.

Rieck, S., Rundfeldt, C. & Tipold, A. (2006) Anticonvulsant activity and tolerance of ELB 138 in dogs with epilepsy: a clinical pilot study. *The Veterinary Journal*, **172**, 86–95.

Rostock, A., Tober, C., Dost, C., Rundfeldt, C., Egerland, U., Stark, B., Schupke, H., Kronbach, T., Lankau, H.-J., Unverferth, K. & Engel, J. (1998) AWD 131-138, ADD-233089. Anxiolytic, anticonvulsant. *Drugs of the Future*, **23**, 253–255.

Rundfeldt, C. & Löscher, W. (2014) The pharmacology of imepitoin: the first partial benzodiazepine receptor agonist developed for the treatment of epilepsy. *CNS Drugs*, **28**, 29–43.

Sakakibara, H., Ohura, T., Kido, T., Yamanaka, N., Tanimura, N., Shimoi, K. & Guruge, K.S. (2013) Organ-specific distribution of 7-chlorinated benz[*a*]anthracene and regulation of selected cytochrome P450 genes in rats. *Journal of Toxicological Sciences*, **38**, 137–143.

Schwartz, M., Munana, K.R. & Olby, N.J. (2011) Possible drug-induced hepatopathy in a dog receiving zonisamide monotherapy for treatment of cryptogenic epilepsy. *Journal of Veterinary Medical Science*, **73**, 1505–1508.

Speciale, J., Dayrell-Hart, B. & Steinberg, S.A. (1991) Clinical evaluation of gamma-vinyl-gamma-aminobutyric acid for control of epilepsy in dogs. *Journal of the American Veterinary Medical Association*, **198**, 995–1000.

Trepanier, L.A., Van Schoick, A., Schwark, W.S. & Carrillo, J. (1998) Therapeutic serum drug concentrations in epileptic dogs treated with potassium bromide alone or in combination with other anticonvulsants: 122 cases (1992–1996). *Journal of the American Veterinary Medical Association*, **213**, 1449–1453.

Walter, R., Siegmund, W. & Scheuch, E. (1996) Effects of interferon-γ and streptolysin O on hepatic procainamide *N*-acetyltransferase and various microsomal cytochrome P450-dependent monooxygenases in rats. *Immunopharmacology and Immunotoxicology*, **18**, 571–586.

Walter, R., Ullmann, C., Thummler, D. & Siegmund, W. (2003) Influence of propiverine on hepatic microsomal cytochrome p450 enzymes in male rats. *Drug Metabolism and Disposition*, **31**, 714–717.

Wright, H.M., Chen, A.V., Martinez, S.E. & Davies, N.M. (2012) Pharmacokinetics of oral rufinamide in dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **35**, 529–533.

Ziółkowski, H., Jaroszewski, J.J., Ziółkowska, N. & Jasiecka, A. (2012) Characteristics of selected second-generation antiepileptic drugs used in dogs. *Polish Journal of Veterinary Sciences*, **15**, 571–582.