University of Helsinki
Dissertationes Universitatis Helsingiensis 311/2025

Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

Use of pregabalin for alleviation of feline acute anxiety and fear associated with transportation and veterinary visits

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Athena, 107, Siltavuorenpenger 3A, Helsinki on the 3rd of October 2025, at 12 o'clock noon.

Helsinki 2025

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Publisher: University of Helsinki
Series: Dissertationes Universitatis Helsingiensis 311/2025

ISBN 978-952-84-1221-2 (print)
ISBN 978-952-84-1220-5 (online)
ISSN 2954-2898 (print)
ISSN 2954-2952 (online)

PunaMusta, Joensuu 2025

Ajattelen, siis olen.

Rakastan, siis elän.

Epäilen, siis tiedän.

–Petter Portin –

Abstract

Cats are popular pets and increasingly considered family members by their owners. However, cats often lack the preventive and acute veterinary care they would need due to difficulties of transporting the cat in a car to a veterinary clinic. Additionally, many owners find veterinary visits very stressful for their cats and themselves because of their cats' anxiety and fear.

The objective of the present thesis was, firstly, to find suitable variables for the evaluation of acute anxiety in cats when associated with transportation and veterinary visit. Secondly, the aim was to select and confirm a clinical dose of a novel cat-specific pregabalin oral solution. Furthermore, the goal was to confirm the clinical efficacy and safety of pregabalin at the selected dose during transportation and veterinary visits. In addition, the pharmacokinetic parameters of the novel pregabalin oral solution formulation were evaluated in healthy laboratory cats.

The two clinical studies were conducted in client-owned cats having a history of anxiety and fear associated with veterinary visits and/or travelling. In the pilot study (n = 11) the owners assessed that cats given 5 or 10 mg/kg pregabalin showed less vocalization, restlessness and panting during transportation compared to placebo. There was a good correlation between the owners' and an external observer's assessments of the overall treatment effect (0.63, $p < 0.01$), which reinforces the reliability of owners' ability to observe their cat's behaviour. The human registered pregabalin oral solution formulation used in this study was found difficult or very difficult to administer by 73% of the owners.

The next clinical study (n = 238) evaluated the effect of pregabalin 2.5 and 5 mg/kg compared to placebo. The study showed that pregabalin 5 mg/kg statistically significantly decreased both travel ($p < 0.01$) and veterinary visit ($p < 0.01$) related anxiety compared to the placebo. Additionally, the decrease of behavioural signs of anxiety assessed by the owner and the external observer showed significant clinical benefit with pregabalin. Furthermore, only a few cats showed transient mild tiredness and incoordination after treatment administration, and the cat-specific formulation was assessed as easy or very easy to administer by 79% of the owners.

The pharmacokinetic profile and bioavailability of the novel pregabalin oral solution formulation was studied in 6 healthy laboratory cats. The cats received pregabalin as single oral doses of 2.5, 5, and 7.5 mg/kg, a dose of 5 mg/kg on two consecutive days, and a single intravenous dose of 2.5 mg/kg. The mean half-life after oral administration of the 5 mg/kg dose was 14.7 h and the mean systemic bioavailability 94%. Pregabalin showed linear pharmacokinetics from 2.5 to 7.5 mg/kg. Exposures after a single dose and re-dosing with 5 mg/kg at 24 h were comparable.

In conclusion, a single oral dose 5 mg/kg of the novel pregabalin oral solution alleviates anxiety and fear related to transportation and veterinary visits in cats and is well tolerated. Furthermore, the developed scales to assess the treatment effect during car travel and veterinary visits were able to find significant difference between pregabalin and placebo in the owners', the external expert's and investigators' assessments. The novel pregabalin oral solution is absorbed rapidly, has high bioavailability and demonstrates a linear pharmacokinetic profile in cats.

List of abbreviations

5-HT	5-hydroksitryptamin (also called serotonin)
AAFP	American of Association of Feline Practitioners
AE	Adverse event
AS	Aggression score
ACTH	Adrenocorticotrophic hormone
$AUC_{0-24\,h}$	Area under the plasma drug concentration-time curve within 24 h after dosing
CI	Confidence interval
C_{max}	Peak drug concentration in plasma
CRO	Contract research organisation
CS	Compliance score
CSS	Cat stress score
EMA	European Medicine Agency
F	Bioavailability of drug
FAS	Fear, anxiety and stress
GABA	Gamma-aminobutyric acid
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
HRV	Heart rate variability
ISFM	International Society of Feline Medicine
ITT	Intention-to-treat
IV	Intravenous
κ	Cohen's weighted kappa coefficient
NNT	Number needed to treat
OR	Odds ratio
RM-ANCOVA	Repeated measures analysis of covariance
r_s	Spearman's rank order correlation coefficient
SD	Standard deviation
$t_{1/2}$	Half-life

T_{max}	Time to maximum drug concentration
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

Index

List of original publications

This thesis is based on the following publications:

I. Lamminen T, Korpivaara M, Suokko M, Aspegrén J, Palestrini C and Overall K (2021) Efficacy of a Single Dose of Pregabalin on Signs of Anxiety in Cats During Transportation—A Pilot Study. Front. Vet. Sci. 8:711816. https://doi: 10.3389/fvets.2021.711816

II. Lamminen T, Korpivaara M, Aspegrén J, Palestrini C and Overall K (2023) Pregabalin alleviates anxiety and fear in cats during transportation and veterinary visits – A clinical field study. Animals 2023, 13, 371. https://www.mdpi.com/2076-2615/13/3/371

III. Lamminen T, Doedée A, Hyttilä-Hopponen M and Kaskinoro J (2022) Pharmacokinetics of single and repeated oral doses of pregabalin oral solution formulation in cats. Journal of Veterinary Pharmacology and Therapeutics, 45, 385–391. https://onlinelibrary.wiley.com/doi/10.1111/jvp.13061

The publications are referred to in the text by their Roman numerals (I-III).

1. Introduction

Cats are popular pets, found in about 25% of households across the United States (Burns, 2019) and Europe (Fediaf, 2018). Increasing number of owners nowadays consider their cat a family member (review by Rodan, 2010). Even though cats are common pets in households there is concern that cats often lack both adequate preventative and acute veterinary care (Volk et al., 2011; 2014). One of the main reasons for this welfare concern is that cats are often too challenging to transport (Volk et al., 2011; 2014).

Many cat owners have difficulties in placing their cat into a carrier and to transporting the cat in a car to a veterinary clinic. Therefore, many cats are not brought to veterinary clinics for regular healthcare visits, such as vaccinations, or even when they have mild signs of a disease (Habacher et al., 2010). According to a veterinary care usage study by Volk et al. (2011), 40% of cats had not been seen by a veterinarian within the past year, compared to only 15% of dogs. Similar results are shown in another survey, which reported that about 52% of cat owners did not take their cats to routine veterinary health-checks, despite the recommendation of an annual preventive care visit (Burns, 2019). Anxiety and fear related to travel and veterinary visits are common in cats (Mariti et al., 2017; Grigg et al., 2019; Karn-Buehler and Kuhne, 2021) and human registered anxiolytic medicines have been used for the alleviation of the syndrome (Orlando et al., 2015; Stevens et al., 2016; Pankratz et al., 2017; Van Haaften et al., 2017; Hudec and Griffin, 2020; Kruszka et al., 2021; Crowe et al., 2022; Gurney and Gower, 2022; Ruviaro Tuleski et al., 2022; Spano et al., 2023). However, the use of medicinal treatment to alleviate undesirable behaviours of cats may still be uncommon among veterinarians (Raekallio et al., 2024).

Increasing the owners' and veterinarians' awareness of the high prevalence of anxiety in cats, and the signs they show when fearful, stressed or anxious, is important for improving the welfare of cats (Mariti et al., 2016; review by Ellis, 2018;). Also, knowledge regarding cat friendly handling techniques and various training methods to diminish anxiety in new situations is crucial (Pratsch et al.,

2018; review by Riemer et al., 2021; Caney et al., 2022). Furthermore, a lot can be done at the veterinary clinics to make the environment less frightening and to increase cat-friendliness (review by Riemer et al., 2021). In addition to improvements in clinic environments and cat handling, the development of new pharmaceutical solutions to alleviate anxiety in cats brings the possibility of increasing the comfort and well-being of feline patients which can hopefully enable easier access to regular veterinary care for cats. All this not only improves the welfare of cats but may also decreases caregiver burden and stress, and thus benefits even owners (Karn-Buehler and Kuhne, 2021; Caney et al., 2022). Additionally, the work of clinic personnel is easier and safer when the handling of cats is less challenging.

This thesis consists of three studies; a pilot clinical study in client-owned cats to evaluate the dose levels and efficacy of pregabalin in travel anxiety (I) followed by a larger scale clinical study to confirm the clinical dose as well as clinical efficacy and safety in pet cats with travel and veterinary visit related anxiety (II). Additionally, the variables to be used for evaluation of travel and veterinary visit related anxiety in cats were developed and tested in these clinical studies (I, II). The third study was a pharmacokinetic trial in a laboratory setting to investigate the pharmacokinetic parameters of pregabalin in cats, e.g., bioavailability, absorption and elimination (III).

The hypotheses of the studies were that pregabalin doses between 2 and 15 mg/kg show positive effects in travel anxiety (I); that either a dose 2.5 or 5 mg/kg shows statistically significant efficacy over the other dose and placebo in travel and veterinary visit anxiety (II); that the primary efficacy parameters are able to reliably detect the difference between active treatment and placebo (I, II); and that the laboratory study in cats produces solid pharmacokinetic data regarding the novel cat-specific pregabalin oral solution formulation (III).

2. Review of the literature

2.1 Mechanisms of anxiety and fear

Anxiety has been defined as a state of distress triggered by anticipated events or changes in an animal's environment, or internal conflicts, characterized by increased arousal and expectancy. Fear, on the other hand, is an emotional state triggered by a present or imminent danger where the animal's intent is to avoid the triggering cause (review by Steimer et al., 2002; book by Landsberg et al., 2024). Both anxiety and fear are characterized by the individual's experiences and personality (book by Overall, 2013). Fear and anxiety belong to normal behaviour of domestic cats and have in general an important role in survival (review by Steimer, 2002; book by Overall, 2013). However, an animal that is constantly experiencing anxiety and fear will be stressed and unable to cope or adapt to new situations, further reducing the cat's welfare. Anxious animals may also demonstrate a decreased ability to learn new behaviours (review by Steimer, 2002; book by Overall, 2013).

Several physiological responses are involved in anxiety and fear, including activation of the autonomic and neuroendocrine systems (book by Bowen and Heath, 2005). The autonomic response is associated with the arousal of the sympathetic nervous system resulting in a fight or flight response through the release of adrenaline and noradrenaline from the medulla of the adrenal gland. This involves reactions that prepare the body for action. Simultaneously, there is a decrease in parasympathetic activity in the body. The neuroendocrine system including the hypothalamic-pituitary-adrenocortical axis is activated in situations of anxiety, fear and stress and this causes an increase of plasma cortisol concentrations (Carlstead et al., 1993; review by Steimer, 2002; review by Charney, 2003; review by Herman et al., 2016; book by Landsberg et al., 2024). The brain's neural mechanisms react via noradrenergic neurons as a response to homeostatic challenge in stressful situations causing the release of corticotrophin releasing hormone from the hypothalamic paraventricular nucleus. Additionally, glutamate likely acts as an excitatory drive for corticotrophin releasing hormone. In the anterior pituitary, corticotrophin releasing hormone causes the release of the

adrenocorticotrophic hormone (ACTH) into systemic circulation. In the adrenal cortex, ACTH activates the synthesis of glucocorticoids, e.g., cortisol, which is released into the circulation and have an effect in various areas in the body (review by Charney, 2003; review by Herman et al., 2016). This pathway also has several inhibitory mechanisms, acting, e.g., via the negative feedback system initiated by increased cortisol levels in the blood stream, through GABAergic inputs, as well as other inhibitory actions by the brain (book by Bowen and Heath, 2005; review by Herman et al., 2016).

At the brain level, the control of anxiety and fear responses involve limbic structures, which include the amygdala, hippocampus, hypothalamus, *locus coeruleus*, and periaqueductal gray, as well as the parabrachial and paraventricular nucleus (Figure 1). All these structures are under the inhibitory control of the prefrontal cortex (book by Bowen and Heath, 2005; review by Płaźnik, 2011). In these brain structures, several neurotransmitters are involved in fear and anxiety responses with noradrenaline, serotonin (5-HT), gamma-aminobutyric acid (GABA), glutamate and dopamine being among the major ones involved.

In fearful and anxious situations, a physiological stress reaction is triggered and the noradrenergic system originating in the *locus coeruleus* is activated increasing the release of noradrenaline (review by Steimer, 2002). Elevation in noradrenergic transmission is associated with typical signs of anxiety including raised arousal, awareness and hypervigilance (book by Bowen and Heath, 2005). Thus, medicines causing a decrease in noradrenalin release are known to induce anxiolytic effect.

Similarly, 5-HT reuptake inhibitors have been shown to cause antianxiety effects in certain anxiety disorders and animal models. However, the role of 5-HT in fear and anxiety is not clear, as it may both enhance and inhibit fear, as well as have an impact on anxiety and the physiological stress response (review by Steimer, 2002). Variations in anxiety-related serotonergic activity among individuals may stem from differences in their past experiences, which influence the responsiveness of the serotonergic system and thereby affect the intensity of anxiety (book by Bowen and Heath, 2005). The serotonergic system has been localised to act in several areas

of brain, such as the amygdala, the hippocampus and the frontal cortex (review by Steimer, 2002).

Another neurotransmitter, GABA, is the most common inhibitory neurochemical involved in anxiolysis with receptors appearing, e.g., in the areas of the hippocampus, amygdala and striatum of the brain (review by Steimer, 2002). The GABA receptor is an important target for several anxiolytic medicines, such as the benzodiazepines including diazepam, alprazolam, oxazepam and lorazepam (review by Steimer, 2002).

Excitatory amino acid neurotransmitters, such as glutamate, are on the other hand involved in the induction of anxiety and fear as well as the flight reaction (review by Steimer, 2002). Thus, the inhibition of glutamate has an anxiolytic effect. Glutamate receptors are found throughout the central nervous system, and they are known to be important in cognition, learning and mood, which are areas where neuroplasticity is needed to adapt to environmental stressors (review by Pal, 2021).

The role of dopamine in anxiety is contradictory and it may act both as an inhibitory and excitatory neurotransmitter (review by Dong et al., 2024). It has an important role in neuromodulation, such as motor control, motivation, reward and cognitive function (review by Klein et al., 2019). Dopamine neurons may also be selectively activated during the stress response (book by Bowen and Heath, 2005). Dopamine release occurs primarily in the prefrontal cortex, hippocampus, hypothalamus and amygdala, and it is known to be an important neurotransmitter in many neurological and psychiatric disorders in humans, such as Parkinson's disease, schizophrenia and attention deficit/hyperactivity disorder (Klein et al., 2019).

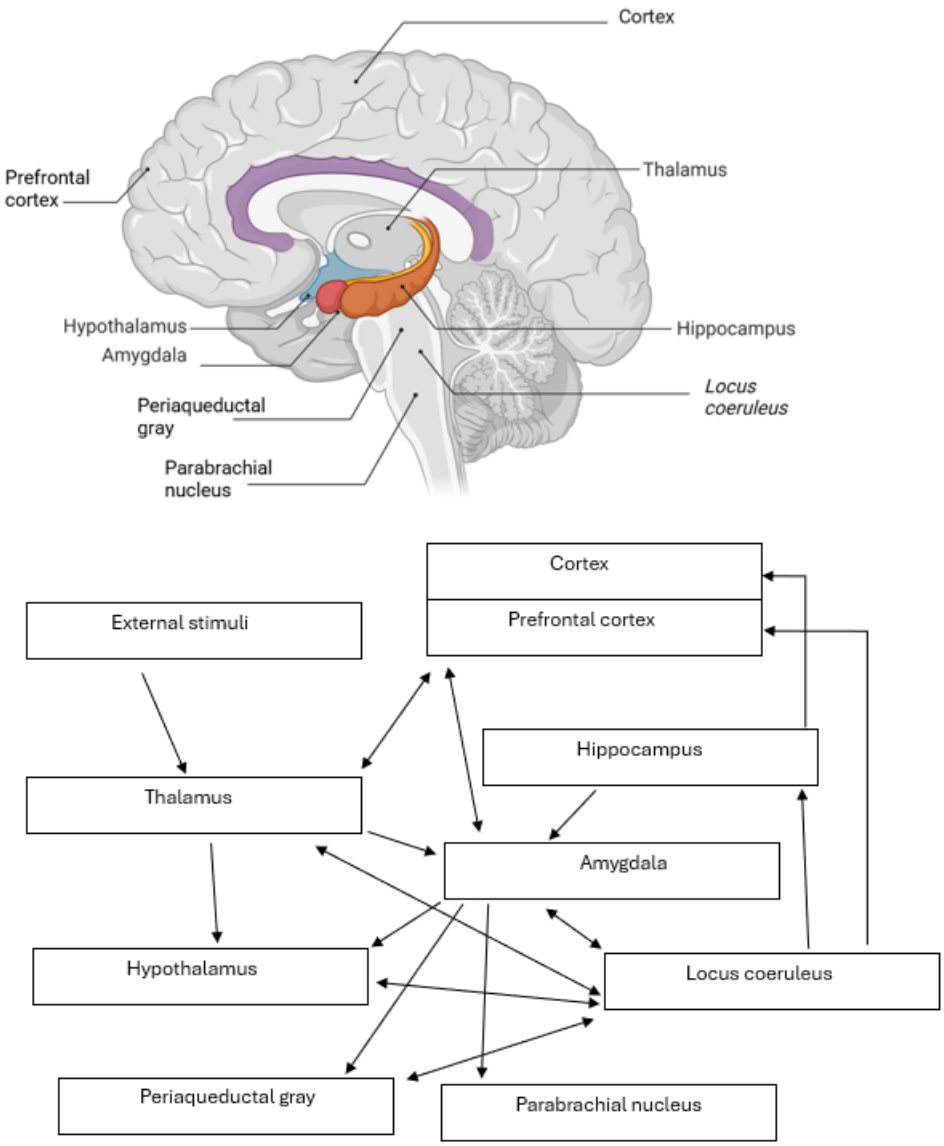

Figure 1 A simplified anatomical (human brains, Created in BioRender. Lamminen, T. 2024, BioRender.com/g87t755) and schematic view of major brain areas involved in fear and anxiety (modified from review by Steimer 2002). External stimuli are transmitted by the thalamus to the amygdala and cortex. The amygdala also receives information from the hippocampus. The amygdala activates the *locus coeruleus*, the hypothalamus and several regions or nuclei in the mid brain and medulla (like periaqueductal gray and parabrachial nucleus). The prefrontal cortex processes information and modulates the physiological, neuroendocrine, and behavioural responses via the amygdala.

2.2 Anxiety and fear in cats

Cats prefer familiar elements and environments and are easily disturbed when facing novel situations. Typically, fast movements, loud noises, odd smells and unpleasant handling cause anxiety and fear in cats (review by Rodan, 2010). Contexts where cats may show signs of fear and anxiety are veterinary clinic visits, cat shows, being placed in a carrier box, travelling, separation from the owner, thunderstorms or other loud noises, contact with strange cats, other animals or humans, or when restricted from doing what it wants (Schwartz, 2002; AAHA guideline by Hammerle et al., 2015; review by Amat et al., 2016; Mariti et al., 2017; Grigg et al., 2019; De Souza Machado et al., 2020; abstract by Lamminen and Aspegrén, 2021; review by Riemer et al., 2021; Caney et al., 2022; Furgala et al., 2022; Cannas et al., 2023; Girão et al., 2024).

When fearful or anxious, cats may show a variety of behaviours, which indicate its psychological state, such as extensive vocalisation, restlessness, resistance to handling, destructive behaviour, depression, hiding, urinary spraying or toileting outside of the litter box (AAHA guideline by Hammerle et al., 2015; Niblett et al., 2015; review by Amat et al., 2016; Bennett et al., 2017; Mariti et al., 2017; Moody et al., 2018; De Souza Machado et al., 2020; Tateo et al., 2021). In fearful situations cats may respond with aggressive behaviour and start to show aggression in similar occasions in the future (review by Rodan, 2010; Mariti et al., 2016). However, cats also often communicate and show their feelings in indirect ways, such as body postures, tail positions, facial expressions and ear positions, that are often subtle and may not be noticed by humans unless learned to understand. Flattening the ears to the side indicate that the cat is fearful and even defensive, and a tail that is tightly tucked near the body expresses fear and anxiety. If the tail, particularly the tip, is lashing vigorously from side to side, it typically indicates agitation, arousal or even an aggressive emotional state (review by Rodan, 2010; review by Ellis, 2018).

Owners do not always recognize signs of fear, anxiety or stress in their cats, unless the signs are prominent, common or potentially disturbing for the owner (Mariti et al., 2017; Karn-Buehler and Kuhne, 2021). In some cases, a cat's normal behaviour is inhibited due to anxiety, and this may be difficult for the owners to notice (Amat et al., 2016). General veterinary practitioners may also not be well aware of cats'

normal behaviour or their behavioural needs (Da Graça Pereira et al., 2014). By better understanding cats and learning to interpret their behavioural signs correctly, both owners and veterinary professionals can reduce their fear and anxiety (AAHA guideline by Hammerle et al., 2015; Mariti et al., 2016; review by Ellis, 2018; AAFP and ISFM guideline by Rodan et al., 2022; AAFP and ISFM guideline by Taylor et al., 2022).

Physiological changes associated with fear, anxiety and stress are related to the activation of the sympathetic nervous system and include increased heart and respiratory rates, increased blood pressure, dilated pupils, hyperthermia, urination and even evacuation of bowel and anal sacs (Belew et al., 1999; Abbott, 2005; review by Rodan, 2010; Quimby et al., 2011). Elevated blood cortisol, as a consequence of activation of the hypothalamic-pituitary-adrenocortical axis, is also a recognised indicator of stress in cats (Carlstead et al., 1993; Nibblett et al., 2015; review by Herman et al., 2016; Contreras et al., 2021). The stress leucogram, characterised by lymphopenia and neutrophilia, as well as hyperglycaemia are both caused by increased cortisol levels, and they may also be associated with stressful situations (Rand et al., 2002; review by Rodan, 2010; Stella et al., 2013; Nibblett et al., 2015). All these changes are connected to physiological reactions related to increased sympathetic tone in situations causing anxiety, fear or stress. The overall cascade of neuro-hormonal changes and feline responses to stimuli causing anxiety and fear is presented in Figure 2.

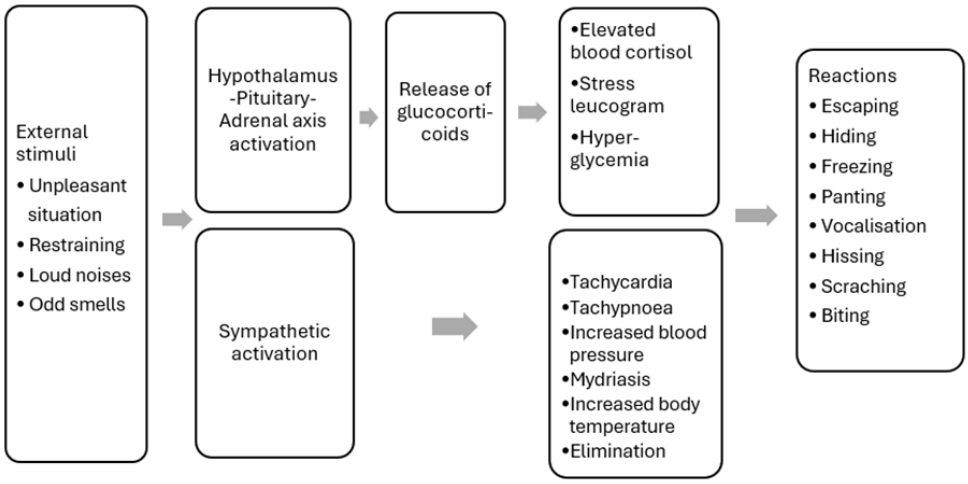

Figure 2 Schematic overview of neuro-hormonal changes and physiological responses in anxiety and fear in cats (modified from review by Steimer et al., 2002 and AAHA guideline by Hammerle et al., 2015).

2.2.1 Travel and veterinary visit related anxiety and fear in cats

Anxiety and fear associated with transportation and veterinary visits is a well-known challenge among cat owners (Quimby et al., 2011; Volk et al., 2011 Niblett et al., 2015; Mariti et al., 2017; Grigg et al., 2019; Karn-Buehler and Kuhne, 2021; Caney et al., 2022). As many as 59% of cats have been reported to show signs of distress during car transportation and 66% during visits at a veterinary clinic (Mariti et al. 2017). Another survey reported that 89% of the cats were perceived to be stressed during veterinary consultation as assessed by their owners based on the behavioural reactions and signs of the cat (Karn-Buehler and Kuhne, 2021).

Based on a cat owner survey conducted in the United Kingdom and the United States among 364 owners who completed the survey for 451 cats with travel anxiety, the most common signs during car transportation were vocalisation, abnormal activity and panting (abstract by Lamminen and Aspegrén, 2021). Most of the cats started to show signs of travel anxiety already when they were young, and the signs stayed similar during their life. Cats' travel anxiety may affect negatively to cat-owner relationship and caregiver burden among owners (abstract by Lamminen et al., 2023).

The results of a cat owner survey conducted in Italy among 1111 cat owners by Mariti et al. (2016) showed that most cats had decreased welfare during a clinic visit; the cats showed signs of anxiety and fear before entering the waiting room, when moved to the examination room, when they were on the examination table, as well as after returning home. All stressful experiences worsen the welfare of cats during clinic visits, and this can cause negative effects on traveling and handling also in other situations (Mariti et al., 2016).

The cat-owner relationship and the owner's own attitudes and behaviour can affect the evaluation of the stress of a cat. Based on a study by Karn-Buehler and Kuhne (2021) the owners that experienced veterinary clinic visit stressful also more often perceived their cat as stressed during the visit compared to those owners that did not feel stress. This study revealed that feline-friendly handling can influence, not only the cat's experience, but also the owner's feeling of stress during the visit. Empathy showed by the veterinarian decreases the stress experienced by the owner. In addition, it has been shown that cats' stress associated with clinic visits can be reduced by carrying out physical examinations and procedures with the owner present whenever possible (Griffin et al., 2020).

2.2.2 How anxiety and fear in cats can be studied

Behaviour of feline species has been studied for decades. Stanton et al. (2015) made a review of standardised ethograms in 95 published articles for the *felidae* species, including domesticated cats. It showed that even though there is variety of definitions created for each behaviour, many behaviours were very similar across different *felidae* species.

The first acknowledged cat specific behavioural score for pet cats was published by McCune (1994). This behaviour score examined the differences between shelter cats in their response when they were caged and was based on an earlier study where interaction and friendliness of kittens between 2 and 12 weeks of age was observed. The kittens were tested further at the age of one year for their response to a familiar person, a stranger and a novel object. Cats that were socialised as kittens and originated from a father known to be friendly were less distressed and friendlier

when approached and handled by unfamiliar people. The same rational was proven useful for explaining difference between cats in their ability to adapt to being caged.

Shortly after that, Kessler and Turner (1997) developed a behavioural scoring system for the assessment of stress in cats. The assessment tool was named the cat-stress-score (CSS), and it has been used since in several behavioural studies in cats. The CSS is a further modification of the score by McCune (1994) and it was developed based on a study on 140 pet cats during their two-week stay at the boarding cattery and their stress scores were compared with 45 control cats that had been at the shelter for several weeks. The CSS by Kessler and Turner (1997) describes the signs or positions of five cat body parts forming seven possible stress levels (Table 1). The stress level scores range from "fully relaxed" (score 1) to "terrorized" (score 7). The study population consisted of non-fractious cats and no clinical examination was conducted but the behaviour and posture of the cats was observed without touch or interfering. The study indicated that the overall CSS of the cats decreased during their two-week stay at the boarding cattery. When evaluating the later use of the CSS score in assessment of fear, anxiety and stress of cats during the clinic visit, the obvious limitations of the study include the lack of clinical examination procedures and absence of selection of the study population based on history of anxiety observed during the vet visits. Despite of this, the CSS has been used for the assessment of cats' signs of stress during transportation and veterinary examination in several studies (Orlando et al., 2015; Stevens et al., 2016; Van Haaften et al., 2017; Spano et al., 2023; DuPont et al., 2024).

Table 1 Cat-stress-score developed by Kessler and Turner (1997).

Score	Body	Belly	Legs	Tail	Head
1 Fully relaxed	i: laid out on side or back a: not applicable	exposed, slow ventilation	i: fully extended a: not applicable	i: extended or loosely wrapped a: not applicable	laid on the surface with chin upwards or on the surface
2 Weakly relaxed	i: laid ventrally or half on side or sitting a: standing or moving, back horizontal	exposed or not exposed, slow or normal ventilation	i: bent, hind legs may be laid out a: when standing extended	i: extended or loosely wrapped a: tail up or loosely downwards	laid on the surface or over the body, some movement
3 Weakly tense	i: laid ventrally or sitting a: standing or moving, back horizontal	not exposed, normal ventilation	i: bent a: when standing extended	i: on the body or curved backwards may be twitching a: up or tense downwards, may be twitching	over the body, some movement
4 Very tense	i: laid ventral, rolled or sitting a: standing or moving, body behind lower than in front	not exposed, normal ventilation	i: bent a: when standing hind legs bent, front extended	i: close to the body a: tense downwards or curled forward, may be twitching	over the body or pressed to body, less or no movement
5 Fearful, stiff	i: laid ventral, or sitting a: standing or moving, body behind lower than in front	not exposed, normal or fast ventilation	i: bent a: bent near to surface	i: close to the body a: curled forward close to the body	on the plane of the body, less or no movement
6 Very fearful	i: laid ventral, or crouched directly on top of all paws, may be shaking a: whole body near to ground, crawling, may be shaking	not exposed, fast ventilation	i: bent a: bent near to surface	i: close to the body a: curled forward close to the body	near to surface, motionless
7 Terrorised	i: crouched directly on top of all paws, shaking a: not applicable	not exposed, fast ventilation	i: bent a: not applicable	i: close to the body a: not applicable	lower than the body, motionless

i = inactive, a = active

In addition to stress score, cats' attitude, compliance and aggression have been used to evaluate cat's behaviour during the veterinary clinic visit. Quimby et al. (2011) scored 30 non-fractious client owned cats' attitude at home and at a veterinary clinic during manipulation, which included measurements of blood pressure, heart rate, respiratory rate and rectal temperature. Cats' attitude (calmness, compliance, vocalisation) was evaluated by the investigator on a 2-point scale. The attitude scores decreased in 50% and increased in 20% of the cats in hospital environment compared to the home environment. 30% of the cats showed no change in their attitude scores. This may suggest that even though the typical physiological parameters indicated higher stress at the hospital environment, the cat's attitude score was not able to detect it. Many cats were reported to have objected the manipulation at home more than at the hospital. This may reveal that those cats had been ostensibly calmer and more compliant at the hospital due to freezing of the cat in an environment causing fear and anxiety, and the score had falsely taken it as improvement of the attitude. Additionally, the non-fractiousness of the population may have caused bias by selecting less-stress prone cats to the study.

Van Haaften et al. (2017) developed a compliance score (CS) and an aggression score (AS), which they used in addition to the CSS to assess 20 pet cats' behaviour during clinical examination. The CS was scored by using a 4-point scale and the AS using a 3-point scale. Clinical examination did not include any invasive procedures, such as blood sampling. The CSS was assessed by the owner and from video by an external observer, the CS by the investigator and the video observer, and the AS by the video observer. The study compared the effect of an anxiolytic treatment to a placebo group and all three scores were able to detect a statistically significant difference favouring the anxiolytic treatment group. However, it was noted that the video observer was not able to detect the differences between the treatment groups as often as the owner and the investigator. This may be due to the familiarity of the owners regarding their cats' typical behaviours and the hands-on feeling of the investigator during the examination procedure. On the other hand, the video observer is considered to be more objective compared to the owners and the investigator, that are typically more sensitive to bias. The cats participating to the study had history of showing signs of stress or fractious behaviour during

transportation or veterinary visit and represented thus suitable population to study these scoring systems (Van Haaften et al., 2017).

Griffin et al. (2020) used five variables (body position, tail position, ear position, eyes and vocalisation) as behavioural indicators of fear, anxiety and stress (FAS) to score 21 non-fractious cats' behaviour during clinical examinations with scoring them either present or absent. The examination did not include any invasive procedures. The study compared the FAS score when the owner was present and absent during clinical examination and found that FAS score as well as heart rate were elevated, and more FAS-related behaviours were seen when cats were separated from their owners. However, the investigators were not blinded regarding the owner absence or presence and thus may have been biased due to predisposing expectations (Griffin et al., 2020).

Different behaviour scoring systems used to evaluate cat's behaviour during veterinary visit are summarised in Table 2.

Table 2 Summary of behavioural scoring systems developed for veterinary visits in domestic cats.

Name	**Abbreviation**	**Description**	**Reference**
Cat-stress-score	CSS	Assessment of signs or position of five body parts of a cat forming seven possible stress levels: 1 Fully relaxed, 2 Weakly relaxed, 3 Weakly tense, 4 Very tense, 5 Fearful, stiff, 6 Very fearful and 7 Terrorised.	Kessler and Turner, 1997
Compliance score	CS	Assessment of a cat's behaviour during clinical examination: 0 No resistance to handling, 1 Minimally resistant to handling, 2 Struggling and difficult to handle, and 3 Extreme struggling with or without urination or defecation.	Van Haaften et al., 2017
Aggression score	AS	Assessment of a cat's behaviour during clinical examination: 0 No aggressive behaviours, 1 Hiss, growl or spit, and 2 Attempt to bite or swat.	Van Haaften et al., 2017
Fear, anxiety and stress score	FAS	Assessment of a cat's behaviour during clinical examination: 1 Presence or 0 Absence of crouched body position, descended tail below	Griffin et al., 2020

Name	Abbreviation	Description	Reference
		body and between back legs, ear deviated to side or back, moderate to wide pupil dilatation and vocalisation (growling, hissing, meowing).	
Cat's attitude score	-	Assessment of a cat's attitude during clinical examination: 0 Calm or 1 Agitated; 0 Compliant or 1 Struggling; 0 Quiet or 1 Vocalising/hissing.	Quimby et al., 2011

Behavioural scoring during the actual presence of a cat may be challenging for the investigator. It has been noted that veterinary professionals or owners do not always notice especially subtle signs of anxiety and fear (Da Graça Pereira et al., 2014; Amat et al., 2016; Mariti et al., 2017; Karn-Buehler and Kuhne, 2021). Therefore, video recordings have also been used in several studies as they can be assessed later to help the evaluation of cats' behaviour (Kronen et al., 2006; Nibblett et al., 2015; Orlando et al., 2015; Van Haaften et al., 2017; Pratsch et al., 2018; McGlone et al., 2019). Recordings have been made at home and at veterinary clinics (Kronen et al., 2006; Nibblett et al., 2015; Van Haaften et al., 2017) or during car transportation (Pratsch et al., 2018). Video recordings have been usually assessed by an independent trained observer.

In addition to observing cats' behaviour, anxiety and fear has been measured with help of physiological parameters, which may support veterinary professionals to recognize cats' anxiety, fear and stress in clinical situations or experimental settings. These measurable variables, such as heart rate, respiratory rate, body temperature and blood pressure have been shown to increase in situations that are stressful for cats (Belew et al., 1999; Abbott, 2005; review by Rodan, 2010; Quimby et al., 2011; Pratsch et al., 2018; Paz et al., 2022). In addition to the above-mentioned parameters, cortisol concentration in serum or plasma (Smith et al., 1999; Stella et al., 2013; Nibblett et al., 2015), saliva (Paz et al., 2022), urine (Carlstead et al., 1993), or hair and nails (Contreras et al., 2021) has been measured as an indicator of stress. Based on these studies, cortisol measurement has not shown consistent results as a stress biomarker. However, surgical stress and pain after ovariohysterectomy in cats have been observed to increase the blood cortisol

concentration (Smith et al., 1999). Additionally, nail cortisol concentrations showed potential for evaluation of chronic stress in cats (Contreras et al., 2021). Nibblet et al. (2015) noted that the familiarity of a person conducting clinical examination and the procedure itself may decrease serum cortisol concentrations in cats. On the other hand, collecting saliva samples for cortisol analysis has been reported to be challenging and not a practical method for stress measurement in cats (Pratsch et al., 2018; Paz et al., 2022).

The physiological measurements are not always easy to interpret as in addition to anxiety, fear and stress, many external and internal factors, such as the temperature of the surrounding space, diseases of the cardiac, respiratory and renal systems, as well as many endocrinological disturbances, influence them. Additionally, cortisol concentrations are impacted by diurnal rhythms, which may create additional demands for study conduct (Nibblet et al., 2015).

2.3 Treatment of anxiety in cats

2.3.1 Management and handling

To help a cat, owners and veterinarians are advised to employ actions such as training, using treats or toys, applying gentle handling, and providing a cat-friendly environment (review by Herron and Shreyer, 2014; AAHA guideline by Hammerle et al., 2015; Pratsch et al., 2018; review by Heath, 2020; Moody et al., 2020; review by Riemer et al., 2021; Caney et al., 2022; AAFP and ISFM guideline by Rodan et al., 2022; AAFP and ISFM guideline by Taylor et al., 2022). Even though a new environment can include stressful stimuli for the cat, earlier positive experiences and habituation may increase the coping abilities of the cat in a situation causing acute stress (review by Riemer et al., 2021; Cannas et al., 2023).

Training methods, such as classical conditioning and classic counterconditioning, can be used to reduce the frequency of unwanted behaviours (book by Overall, 2013; AAHA guideline by Hammerle et al., 2015). In training, palatable food treats are often the easiest and most powerful option in creating positive emotional response in unpleasant situations, like when injections are given, or rectal temperature is measured. However, treats do not work if the cat is already too scared in the situation, therefore training and palatable food should be used preferably when the

cat is experiencing the unpleasant situation for the first time, and not to wait until fear and anxiety are already learned (review by Herron and Shreyer, 2014).

Training needs to be done not only with veterinary clinic procedures, but also with transportation and carriers. Ideal carriers have a removable top half, and they have doors both on the top and in front of the box. Large enough size and soft blanket or bedding with familiar smell make the carrier more comfortable. Carrier training using positive reinforcement with highly palatable food treats during a gradual procedure has shown reduced behavioural signs of stress during car rides (Pratsch et al., 2018). It is advisable to keep the carrier at home open and in an easily accessible location so that the cat can use it as safe and cosy place to be. Habituation of the cat with the carrier is key for non-anxious travelling (AAFP and ISFM guideline by Rodan et al., 2022).

During clinic visits it is important to use gentle handling techniques, which give the cat time to acclimatize to the situation and reduce the fear and anxiety of the cat. A clinical examination method proceeding preferrable from head to tail and from less disturbing to more invasive procedures should be used. As many procedures as possible are advisable to be done in the cat's chosen location, whether it is in the bottom half of the carrier so that the cat can stay it it's safe place, on the owner's lap, on the floor or on the examination table (AAFP and ISFM guideline by Rodan et al., 2022). If the cat needs to be restrained, the use of towels is the safest and most comfortable way to do it (Rudolph, 2015; review by Riemer et al., 2021; AAFP and ISFM guideline by Rodan et al., 2022).

A cat-friendly veterinary practice environment should start in the reception area and waiting room (review by Riemer et al., 2021). If possible, cats should have a separate waiting room or area of the waiting room with visual barriers and enough space to hinder too close contacts to other animals. The waiting room should include tables or other horizontal surfaces where carriers can be placed, as cats prefer spots higher than the floor level. Owners could have towel or scarf with them to cover the carrier. In the waiting room, like throughout the clinic, wall and floor materials should not only be easy to keep clean but also such that they absorb sounds, and doors between rooms should be solid to diminish potentially aversive noise. It has been shown that even the background noise at the clinic elicits fear and

anxiety in cats (Furgala et al., 2022; Girão et al., 2024). Low voices and classical music or commercial recordings containing sounds preferred by cats should be used at the clinic if possible (review by Herron and Shreyer, 2014; Hampton et al., 2020; Paz et al., 2022). Additionally, olfactory stimulation is important to take into account; all surfaces in contact with an earlier patient should be cleaned with non-smelling chemicals to minimize exposure to possible pheromones that are associated with fear and alarm, as well as to the strong odour of detergents and disinfectants (review by Herron and Shreyer, 2014; review by Heath, 2020; AAFP and ISFM guideline by Taylor et al., 2022). If possible, a separate examination room allocated only to cats with a non-slippery examination table is preferred at a cat-friendly clinic (review by Herron and Shreyer, 2014; review by Riemer et al., 2021; AAFP and ISFM guideline by Taylor et al., 2022).

2.3.2 Non-pharmaceutical treatment options

Non-pharmaceutical products, such as pheromones, nutraceuticals and herbs, are used to decrease cats' anxiety and fear. The effectiveness of pheromones in cats on fear behaviour could not be demonstrated in a review by Frank et al. (2010). After publishing the review (Frank et al., 2010), positive findings in cats' behaviour have been observed in a few blinded and placebo-controlled studies during transportation (Shu and Gu, 2021) or veterinary visits (Pereira et al., 2016), whereas one study showed lack of effect (Conti et al., 2017). Currently pheromones are recommended to be used both during the car ride and at the clinic (review by Herron and Shreyer, 2014; review by Vitale, 2018; AAFP and ISFM guideline by Rodan et al., 2022; AAFP and ISFM guideline by Taylor et al., 2022). The commonly used commercial pheromones contain synthetic analogues of the F3 and F4 fractions of feline facial pheromones. Both these pheromones are secreted from glands on the head and cheeks; F3 is excreted mainly when the cat is rubbing objects and F4 in social situations including allorubbing between cats (review by Vitale, 2018).

Nutraceuticals are used as functional ingredients to support the behavioural needs of cats. Pet foods should contain all ingredients needed physiologically. However, a response to nutrient supplementation may be seen especially if there is a deficiency of certain nutraceuticals (review by Orlando, 2018). The most commonly used

nutraceuticals in behavioural supportation with positive effects reported are alpha-casozepine and alpha-lactalbumin, which are bovine milk protein derivates, theanine that has similar structure as glutamate neurotransmitter, and tryptophan, which is a dietary amino acid and a precursor to serotonin (Landsberg et al., 2017; review by Orlando, 2018). Additionally, herbal plants such as valerian (*Valeriana officinalis*) and catnip (*Nepeta cataria*) are often used, as they are believed to have anxiolytic or calming effects. Some cats are attracted to these plants and show behavioural signs such as head rubbing and rolling over, which are typically described as the "catnip response" (Bol et al., 2022).

In a published abstract reporting previous use of non-medicinal products in Finnish cat populations participating in two clinical anxiety studies (abstract by Lamminen et al., 2022), the most commonly used non-medicinal products were pheromones, valerian, tryptophan and alpha-casozepine. Based on a cat owner survey, published as a congress abstract by Lamminen and Aspegrén (2021), pheromones and other non-medicinal products were frequently used to alleviate signs of anxiety during transportation but were often assessed by the owners to have poor or only some effect.

2.3.3 Pharmaceutical treatment options in cats

In addition to training, environmental modification and non-pharmaceutical products, anxiolytic medication can be used to reduce anxiety during transportation and to enable patient-friendly, low-stress visits at the veterinary clinic (AAHA guideline by Hammerle et al., 2015; review by Denenberg and Dubé, 2018; review by Riemer et al., 2021).

A survey by Grigg et al. (2019) studied cat owners' attitudes in the USA regarding the use of psychoactive medications and non-pharmaceutical products for the treatment of behavioural problems in cats. The results showed lack of awareness of availability and benefits of medicinal treatments of feline behavioural problems among owners, even though the prevalence of behaviour problems of cats was high based on the owners' answers. Anxiety and fear of travelling, placing the cat into a carrier, and strangers were the most prevalent issues reported by 67% of the owners (Grigg et al., 2019).

Several anxiolytic medicines registered for human use have been studied in cats off-label. Before pregabalin, no registered medicinal treatment for the alleviation of anxiety and fear associated with travel and veterinary visit in cats was available.

2.3.3.1 Gabapentin

Gabapentin is a structural analogue of the inhibitory neurotransmitter GABA affecting via the alpha-2-delta auxiliary subunit of voltage gated calcium channels in neurons. Through that mechanism it reduces glutamate and other excitatory neurotransmitters in the central nervous system (book by De Risio, 2014; review by Offord and Isom, 2016). Gabapentinoids, gabapentin and pregabalin, are structurally related compounds with similar mechanism of action, which is however incompletely understood (review by Offord and Isom, 2016). Gabapentin has been registered in humans in 1990's for the treatment of epileptic seizures and later also for neuropathic pain in Europe and the United States, but it has not been registered as a veterinary medicine in any country.

In cats, gabapentin has been studied as a single dose pre-appointment medication for attenuating fear and stress responses before transport and veterinary clinic visits (Pankratz et al., 2017; Van Haaften et al., 2017; Hudec and Griffin, 2020; Kruszka et al., 2021; Crowe et al., 2022; Gurney and Gower, 2022; Ruviaro Tuleski et al., 2022; Spano et al., 2023; DuPont et al., 2024). Mean serum cortisol levels were lower when cats received gabapentin before a veterinary appointment compared to placebo (Versteg et al., 2024). However, in another study by Hudec and Griffin (2020) significant decreases in serum cortisol concentrations were not detected.

Gabapentin doses from 50 mg to 200 mg per cat have provided anxiolytic effects and increased compliance in handling the cat. These doses correspond to amount between 9.2 and 47.6 mg/kg. Currently doses around 20 mg/kg are most commonly used (Crowe et al., 2022; Gurney and Gower, 2022; Ruviaro Tuleski et al. 2022). These dose levels have not been observed to affect physiologic (heart rate, respiratory rate, blood pressure) or echocardiographic variables (Allen et al., 2021; Ruviaro Tuleski et al., 2022; Veronezi et al., 2022; De Lombaert et al., 2023), or ocular parameters (Crowe et al., 2022) in healthy cats. However, in a recent study

in cats with and without chronic kidney disease a significant decrease in blood pressure was observed after administration of gabapentin with dose 10 mg/kg compared to placebo (Quimby et al., 2024). The median change in blood pressure was -12 mmHg in both healthy cats and those with chronic kidney disease. Gabapentin also significantly alters gait analyses and postural reactions and could lead to false-positive results and incorrect identification of neurological lesions (De Azevedo et al., 2023; DuPont et al., 2024). Especially in geriatric cats dose reduction before neurologic examination should be considered (DuPont et al, 2024).

The stress reducing effect of consecutive administration of gabapentin during postoperative short-term hospitalisation has been studied in comparison to alprazolam and placebo (Papageorgiou et al., 2024). The results show that the administration of gabapentin with a dose of 100 mg per cat twice daily for two days, when the first administration was done pre-operatively, reduced anxiety after ovariohysterectomy. Decreases in serum cortisol levels were noted when compared to a placebo group (Papageorgiou et al., 2024). In another study with longer term administration, gabapentin with a dose of 10 mg/kg twice daily during a behaviour modification program showed positive results in shelter cats (Eagan et al., 2023). The median time to pass the program was 11 days and gabapentin predicted quicker progress, as well as a lower stress score and reappearance of anxiety symptoms compared to placebo (Eagan et al., 2023).

The effect of gabapentin has been additionally studied for controlling chronic pain related to osteoarthritis or other diseases (Guedes et al., 2018; Slovak and Costa, 2021) and management of post-operative acute pain in cats undergoing ovariohysterectomy (Steagall et al., 2018) in small-scale studies. Treatment with a gabapentin dose of 10 mg/kg given four times a day was associated with a decrease in pain scores in cats with osteoarthritis or other chronic pain (Slovak and Costa, 2021) and when given twice daily, reduction in owner-identified impaired activities was reported in osteoarthritic geriatric cats (Guedes et al., 2018). However, gabapentin (50 mg/cat twice before surgery) did not show clear benefits after ovariohysterectomy operation (Steagall et al., 2018).

Recently, one study reported significant isoflurane sparing effect in cats after the administration of gabapentin (100 mg/cat) two hours before anaesthesia (Chen et

al., 2023). In another study gabapentin 100 mg/kg given 100 minutes before premedication with acepromazine and methadone did not result in a significant decrease on propofol doses required for anaesthesia induction (Ferronatto et al., 2024).

Common adverse events (AE) reported in cats after gabapentin administration include incoordination, drowsiness, sedation, weakness, myorelaxation, muscle tremor, vomiting and hypersalivation (Van Haaften et al., 2017; Kruszka et al., 2021; Crowe et al., 2022; Ruviaro Tuleski et al., 2022; Spano et al., 2023; Papageorgiou et al., 2024). The symptoms typically resolved within eight hours after administration (Van Haaften et al., 2017).

The pharmacokinetics of gabapentin have been evaluated after oral administration in healthy cats (Siao et al., 2010; Adrian et al., 2018; abstract by Quimby et al., 2019) as well as in cats with chronic kidney disease (Quimby et al., 2022), and additionally after administration by the transdermal route (Adrian et al., 2018; Slovak and Costa, 2021). Gabapentin has a small volume of distribution and a low clearance in cats (Siao et al., 2010). After a single oral dose of 10 mg/kg given as an oral capsule, median (range) terminal half-life ($t_{½}$) was 3.56 (2.96–4.78) hours, maximum concentration (C_{max}) 12.42 (8.31–18.35) µg/ml, time to maximum concentration (T_{max}) 1.05 (0.74–2.11) hours, and bioavailability approximately 95% (Adrian et al., 2018). After single oral dose of 20 mg/kg, mean ± standard deviation (SD) $t_{½}$ was 4.1 ± 0.5 hours, median (range) C_{max} 242.0 (183.6–271.0) ng/ml/mg, and T_{max} 1.5 (1–2) hours in healthy cats, thus showing similar pharmacokinetics as after a dose of 10 mg/kg (Quimby et al., 2022). Cats with chronic kidney disease had significantly higher serum gabapentin concentrations after a 10 mg/kg dose than healthy cats that received 20 mg/kg (Quimby et al., 2022). Gabapentin at doses 5 and 10 mg/kg was absorbed via the transdermal route with a compounded lipoderm-based formulation (Slovak and Costa, 2021), but not with another compounded gel formulation administered with a dose of 10 mg/kg (Adrian et al., 2018).

2.3.3.2 Trazodone

Trazodone is an antidepressant registered for human use. It antagonises postsynaptic serotonin receptors and inhibits serotonin reuptake in the central nervous system, and additionally acts as a blocker of alpha-1 adrenergic receptors (review by James and Mendelson, 2004). A few clinical studies have been conducted in cats that have shown reduction of signs of transport- and veterinary visit-related anxiety with single oral pre-appointment doses of 50-100 mg per cat (Orlando et al., 2015; Stevens et al., 2016). Improvement in cats' signs of anxiety during transportation and ease of handling during clinical examination has been reported (Stevens et al., 2016). Typically, sleepiness and sedation are seen after the administration of trazodone at dose levels from 5 to 33.3 mg/kg (Orlando et al., 2015; Stevens et al., 2016; Fries et al., 2019; Tucker et al., 2023). Additionally, facial and head twitching has been reported after the administration of trazodone with a dose of 8 mg/kg (Brosnan et al., 2024). Pre-appointment transdermal administration of compounded trazodone decreased stress scores during travelling but not during clinical examination (Shih and Wang, 2024). However, behavioural response scores and owner-assessed overall experience scores showed significant reduction also during the examination.

The effects of trazodone at 50 mg/cat on echocardiographic variables, heart rate and blood pressure in healthy cats has been evaluated (Fries et al., 2019). There were no clinically relevant effects in heart rate or echocardiographic parameters, but systolic blood pressure was significantly decreased when measured approximately two hours after administration. In another study by Wu et al. (2025), similar effects on systolic blood pressure were seen with 100 mg/cat dose and signs of sedation and decreased respiratory rate with doses 50, 75 and 100 mg/cat, while no significant changes were reported in echocardiographic variables. Trazodone at 50 mg/cat administered with dexmedetomidine decreased the minimum alveolar concentration of isoflurane in cats (Brosnan et al., 2024). In a study by Tucker et al. (2023) the administration of trazodone at 5 mg/kg alone or in combination with gabapentin 10 mg/kg in blood donor cats resulted in significant sedation compared to placebo or gabapentin alone.

The pharmacokinetics of orally administered trazodone in cats have been studied alone and when combined with gabapentin (Tucker et al., 2023). Trazodone administered as compounded oral liquid formulation at a dose of 5 mg/kg demonstrated bioavailability of approximately 55% alone and 17% when administered together with a compounded oral liquid formulation of gabapentin with a dose of 10 mg/kg. Trazodone median (range) T_{max} was 0.17 (0.17–0.5) hours, mean ± SD C_{max} 1.67 ± 0.91 µg/ml and $t_{½}$ 5.12 ± 2.56 hours when administered alone. Combination with gabapentin did not significantly affect the pharmacokinetics or sedative effect of trazodone in cats but there was an overall tendency for lower plasma concentrations of trazodone when administered with gabapentin (Tucker et al., 2023).

2.3.3.3 Other anxiolytic medicines

Anecdotal data can be found regarding other antianxiety medicines used in cats for alleviation of fear and anxiety related to travelling, veterinary visits, as well as other behavioural problems related to anxiety and stress, such as urine spraying, psychogenic alopecia and inter-cat aggression.

Clomipramine is a tricyclic antidepressant and selective serotonin reuptake inhibitor used in humans to treat depression, anxiety, panic attacks, phobias, obsessive-compulsive disorders, and chronic pain. Clomipramine is registered for the treatment of separation anxiety in dogs (King et al., 2004). In cats, clomipramine has been studied for the treatment of urine spraying (King et al., 2004; Hart et al., 2005; Landsberg and Wilson, 2005), psychogenic alopecia (Sawyer et al., 1999; Mertens et al., 2006) and in other anxiety related disorders with multifactorial background (Seksel and Lindeman, 1998) with positive results. The recommended clinical dose given orally is 0.25-0.5 mg/kg every 24 hours (Seksel and Lindeman, 1998; King et al., 2004; Hart et al., 2005; Landsberg and Wilson, 2005; Mertens et al., 2006). In two pharmacokinetic studies by Lainesse et al. (2006; 2007) a canine registered clomipramine tablet with target dose 0.5 mg/kg had a bioavailability of approximately 90% in cats. Mean ± SD T_{max} in the first study in 5 laboratory cats was 6.2 ± 3.5 hours, C_{max} 87.5 ± 46.4 ng/ml and $t_{½}$ 12.3 ± 4.7 hours (Lainesse et al., 2006). In the second study in 76 client-owned cats,

the reported mean ± SD T_{max} was 3.0 ± 1.9 hours and C_{max} 84.0 ± 71.2 ng/ml (Lainesse et al., 2007). The pharmacokinetics of clomipramine shows marked interindividual variability in cats with significant sex-related differences resulting in higher metabolic ratios (Lainesse et al., 2006; 2007). This suggests higher demethylation or hydroxylation potential and clearance in females. Mild sedation and dilatated pupils were reported as AEs in the first pharmacokinetic study (Lainesse et al., 2006).

Another antidepressant, fluoxetine, is a selective serotonin reuptake inhibitor, which is used in humans for treatment of depression, obsessive-compulsive disorder, panic disorder and bulimia. It is registered in dogs for separation anxiety, but used also in many other behavioural indications, including other anxiety-related disorders, aggression, compulsive disorders and phobias (Kaur et al., 2016). In cats, fluoxetine has been studied for the treatment of urine marking (Pryor et al., 2001; Hart et al., 2005) with positive results, but used sometimes also in other anxiety related behavioural problems (Kaur et al., 2016). A pharmacokinetic study of compounded oral and transdermal fluoxetine formulations in cats by Ciribassi et al. (2003) showed good bioavailability when given orally at the dose 1 mg/kg. The mean ± SD T_{max} was 6.5 ± 2.9 hours, C_{max} 94.8 ± 34.3 ng/ml and $t_{½}$ 46.8 ± 6.5 hours. However, even though fluoxetine is absorbed through the skin of cats, bioavailability with the transdermal route is poor, approximately 10% (Ciribassi et al., 2003).

Dexmedetomidine, an alpha-2 adrenoreceptor agonist, is available in an injectable formulation and is registered as a sedative in humans, dogs and cats. Additionally, a dexmedetomidine oromucosal gel formulation has been approved in dogs for alleviation of acute anxiety and fear associated with noise. In cats, data of the anxiolytic effect of dexmedetomidine is scarce, as only few studies have been published in connection with veterinary visit related anxiety (abstract by Carson et al., 2020) and travel anxiety (abstract by Landsberg et al., 2018). In addition, a few pharmacological studies of cardiorespiratory effects, hemodynamic changes and the pharmacokinetics of dexmedetomidine administered transmucosally to cats are available (Smith et al., 2020; Santos et al., 2010). However, it is well known that cats are very sensitive for the emetic effect induced by dexmedetomidine, as well as

other alpha-2 adrenoreceptor agonists (Thawley and Drobatz, 2015), which is a disadvantage in use of medicines with this mechanism of action for anxiolysis in cats.

Venlafaxine is a dual serotonin and noradrenaline reuptake inhibitor registered for depression, generalized anxiety, social anxiety and panic disorders in humans. One clinical placebo-controlled pilot study has been published regarding the use of venlafaxine with 1 mg/kg/day for 60 days in the treatment of fear reactions and urine spraying (Metz et al., 2022). Cats with aggressive behaviours were also included. Compared to placebo, venlafaxine was efficacious in reducing a wide range of problem behaviours based on assessment by owners and veterinarians. Very few AEs were reported but some drowsiness was observed at the beginning of the treatment period (Metz et al., 2022).

Benzodiazepines, such as alprazolam and diazepam, have been used in cats for treatment of behaviour problems, such as anxiety, fear and urine spraying (review by Denenberg and Dubé, 2018; review by Erickson et al., 2021). Diazepam has been registered in the Netherlands for cats with indications of anxiety, excitement, unruliness, and convulsive states (Summary of product characteristics Diazepam 2, 5 and 10 mg tablets). However, it has been reported to cause hepatic failure with clinical signs as early as five days after administration in cats with doses ranging from 1 mg/cat every 24 hours to 2.5 mg/cat every 12 hours (Levy et al., 1994; Center et al., 1996). These findings have probably reduced its use in cats.

The stress reducing effect of alprazolam has been studied during postoperative short-term hospitalization as a comparator to gabapentin (Papageorgiou et al., 2024). The results showed decreased anxiety after ovariohysterectomy when alprazolam was administered with a dose of 0.125 mg per cat twice daily for two days (the first administration pre-operatively). Decrease of serum cortisol and glucose levels were noted when compared to a placebo group. Sedation and ataxia were observed in the majority of cats after alprazolam administration (Papageorgiou et al., 2024).

Additionally, amitriptyline, buspirone, and sertraline have been used anecdotally in cats for the treatment of behaviour problems, including fear, psychogenic alopecia

and urine spraying (Sawyer et al., 1999; Mealey et al., 2004; review by Denenberg and Dubé, 2018). None of these substances have been approved as veterinary medicines in Europe and the anxiolytic efficacy and more detailed safety evaluation in cats remain unclear until further studies are published.

2.4 Pregabalin

2.4.1 Pharmacodynamics

Pregabalin, similarly as gabapentin, is a structural analogue of the GABA neurotransmitter and binds to the alpha-2-delta subunit of the voltage-dependent calcium channels in the central nervous system (Li et al., 2011). Calcium channels control the release of neurotransmitters from the terminal ending of a presynaptic neuron. By binding to the calcium channel, pregabalin inhibits the cellular calcium influx that would be needed for the evacuation of neurotransmitters from vesicles to synaptic cleft (Figure 3). With this mechanism pregabalin decreases the release of glutamate and monoamine neurotransmitters, such as dopamine, serotonin and norepinephrine, which have been implicated to play a role in the pathophysiology of anxiety (review by Micó and Prieto, 2012; review by Frampton, 2014).

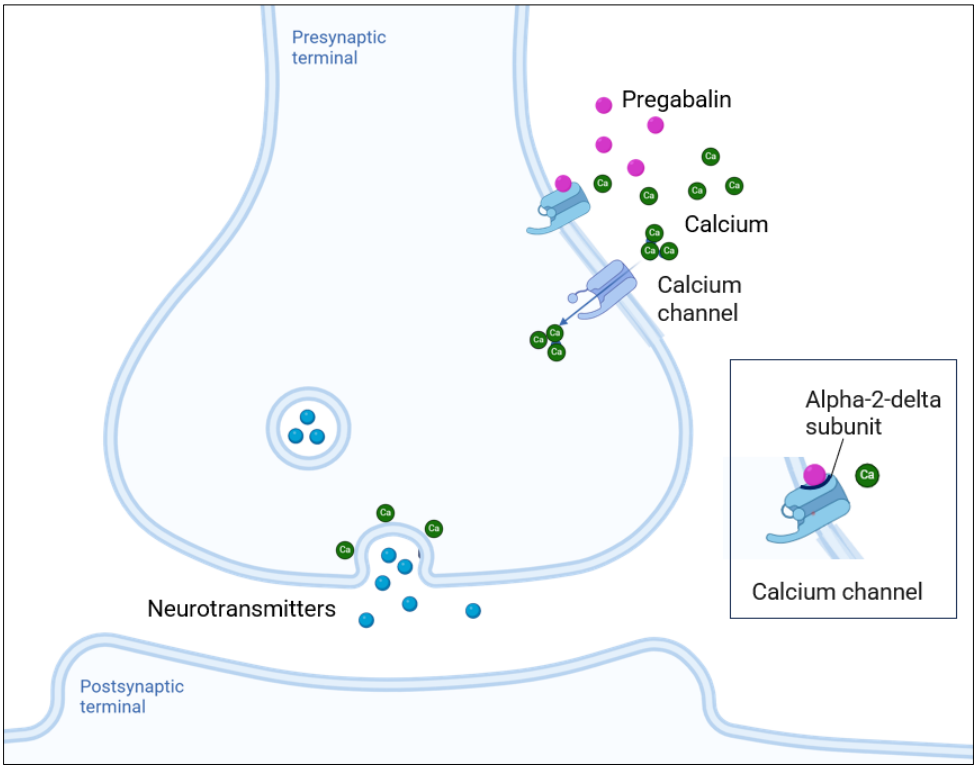

Figure 3 Calcium-channel function in a synapse (Created in BioRender. Lamminen, T. 2024, BioRender.com/g87t755).

Of the four separate variants of the alpha-2-delta protein, pregabalin has high affinity for type 1 and type 2 proteins in rats. Of the auxiliary subunits, alpha-2-delta-1 and alpha-2-delta-2 are shown to be the targets for pregabalin binding (Cole et al., 2005; review by Calandre et al., 2016). The alpha-2-delta-1 and -2 subunits are widely expressed in central nervous system (Cole et al., 2005, review by Dolphin, 2016). Extensive pregabalin binding was demonstrated in rats throughout the central nervous system, with high-level binding in the cortex, hippocampus, cerebellum, amygdala and dorsal horn of the spinal cord (Li et al., 2011). In addition to the central nervous system, auxiliary subunit types are found in the heart, lungs, skeletal muscle, testis, pancreas and prostate (review by Arikkath and Campbell, 2003; review by Dolphin, 2016). Besides the anxiolytic and analgetic effects, pregabalin may cause increase in food intake due to the inhibition of dopaminergic function in the hypothalamus (Ikeda et al., 2018).

At the brain level, the attenuation of fear-related activation of the amygdala and anterior insular cortex contributes to the anxiolytic effect of pregabalin as shown in rats (Wang et al., 2012) and healthy human volunteers (Aupperle et al., 2011). In rodent models, pregabalin has shown dose-dependent anxiolytic-like effects (Field et al., 2001; Lotarski et al., 2011; Wang et al., 2012).

Field et al. (2001) studied pregabalin in rats using two traditional anxiety models, X-maze and rat conflict test. In X-maze experiment animals are placed in the centre of X-maze and time spent in the open arm and total arm entries are measured during a 5-minute test period. In this model pregabalin has shown anxiolytic-like action as indicated by increased time spent on the open arms. The X-maze model is based on rodents' natural aversion to open spaces counterbalanced with their innate curiosity to explore new areas, and in theory less anxious rodents will spend more time in open arms compared to anxious ones (review by Kumar et al., 2013). In a rat conflict test animals are trained to press levers for food reward in a testing chamber, and periods when rats were unpunished, or punished with foot shock concomitantly with food delivery are measured. This model has demonstrated the positive dose-dependent anxiolytic effect of pregabalin by increasing lever pressing in the punished period (Field et al., 2001). The conflict tests create controversy between positive resources, such as food or drinking, and punishment, and anxiolysis can be seen in higher tolerance of punishment to get the positive resources (review by Kumar et al., 2013).

In a study by Lotarski et al. (2011), point mutated alpha-2-delta-1 mice and non-mutated mice were included in a Vogel conflict model, which was used to test whether a mutation that reduces binding of pregabalin causes different results in anxiolytic-like activity compared non-mutated animals, and whether pregabalin affects the activity of animals. In the model, a number of electric shocks during licking of milk-water mixture after water deprivation was measured. In this test the mean number of electric shocks was significantly higher in the non-mutated mice treated with pregabalin compared to vehicle but did not differ in mutated mice after pregabalin versus vehicle administration. These findings confirmed that binding to the alpha-2-delta-1 subunit is responsible for the anxiolytic-like actions of pregabalin (Lotarski et al., 2011).

Wang et al. (2012) used cerebral blood flow mapping to examine the effect of pregabalin in a rat model of electric foot shock-induced fear. In addition to evaluating the effect of the test compound on cerebral perfusion with the help of a radiolabelled substance and imaging, this test compares the effect of a compound and vehicle on the duration of ultrasonic vocalisation and freezing (lack of movement) of the test animals as response to electric shock. The results showed less ultrasonic vocalisation after pregabalin treatment compared to vehicle, but no differences in freezing between the treatment groups. In imaging, the effect of pregabalin on fear-related functional brain activation was supported by a significant drug × shock interaction in the amygdala, insula, cortex and thalamus. This study with functional brain mapping provided new endpoints for preclinical evaluation of anxiolytic drug candidates with potentially improved translational power compared to behavioural measurements alone (Wang et al., 2012).

All rodent models of anxiety have been created to help the development of new drugs for humans, and they typically include comparative data produced earlier with known anxiolytic compounds, such as benzodiazepines. Of the above-described models, X-maze and the rat conflict test (Field et al., 2001) as well as the Vogel conflict test (Lotarski et al., 2011) are preclinical models commonly used to evaluate the anxiolytic effect of new potential active substances. Rodent models are not models of specific indications, in which anxiolytic actives could be used, but merely mimic symptoms or behaviours naturally occurring in the species used in a certain test system (book chapter by Kraeuter et al., 2019). Even though the rodent models have not been validated for signs of feline anxiety, nor do they directly resemble situations where a cat feels anxious or fearful in a car or veterinary clinic, the basic anxiolytic effect of the active substance can be shown with these models.

2.4.2 Pharmacokinetics

Pharmacokinetics of pregabalin have been earlier described in several species including humans (Bockbrader et al., 2010), dogs (Salazar et al., 2009) and cats (Esteban et al., 2018).

In humans, pregabalin is rapidly absorbed following oral administration, with a T_{max} of 0.7-1.3 hours and approximately 90% bioavailability. Food slows down

pregabalin absorption, resulting in lower and delayed C_{max}. However, the total exposure remains the same with or without meal. Pregabalin's $t_{½}$ in humans range from 4.6 to 6.8 hours. Clearance of orally administered pregabalin is equivalent to renal clearance indicating negligible nonrenal elimination and kidney function affects its pharmacokinetics (Bockbrader et al., 2010).

In dogs, Salazar et al. (2009) reported a median (range) C_{max} of 7.15 (4.6–7.9) µg/ml, T_{max} of 1.5 (1–4) hours and $t_{½}$ of 6.90 (6.21–7.40) hours after single oral dose (4 mg/kg) of pregabalin human tablet formulation. The plasma concentrations in dogs were approximately 11 hours above the presumed minimal effective concentration preventing epileptic seizures in humans. According to the so-called free drug hypothesis, the protein binding of a drug likely influences on its pharmacokinetics and pharmacodynamics, and there are marked differences between species (review by Smith and Waters, 2019). In humans pregabalin has negligible binding to plasma proteins (Bockbrader et al., 2010), but protein blinding has not been studied in dogs or cats. However, the authors concluded that a pregabalin dose of 4 mg/kg twice daily could be adequate for chronic use in dogs even though protein binding was not known (Salazar et al., 2009).

In their study in cats, Esteban et al. (2018) administered a single oral pregabalin dose of 4 mg/kg in a gelatine capsule. The mean ± SD C_{max} was 8.3 ± 1.6 µg/ml, T_{max} 2.9 ± 1.2 hours and $t_{½}$ 10.4 ± 2.6 hours. Plasma concentrations in cats stayed above the minimum therapeutic concentration for seizure control in humans for approximately 17.6 hours. Due to moderate sedation and the high plasma levels in cats, the authors estimated that a lower dose, such as 1–2 mg/kg twice daily, might be a more acceptable starting dose for seizure control in cats with epilepsy (Esteban et al, 2018).

2.4.3 Clinical use of pregabalin

2.4.3.1 Pregabalin in cats

A few anecdotal case reports have been published of pregabalin in client-owned cats when used for treatment of chronic pain with doses of 1-5 mg/kg two to three times a day (Clark et al., 2017; Amengual Batle et al., 2019; Goich et al., 2019; Korff and

Williamson, 2020; Ertel and Dörner, 2024; Rusbridge, 2024) and refractory epilepsy with a dose of 1 mg/kg two times a day (Djani and Draper, 2019).

Some AEs related to sedation have been reported in published literature, typically similar to gabapentin. In their case report, Clark et al. (2017) described development of superficial dermatitis lesions on the face of the cat after a week's treatment period. The lesions healed when pregabalin treatment was stopped. The aetiology of the lesions remained unknown. Skin lesions have been reported in laboratory rats and monkeys during human pregabalin product development (FDA, 2004), but not earlier in clinical use in animals.

Recently a study reported significant isoflurane sparing effect in laboratory cats after administration of pregabalin with doses 5 and 10 mg/kg two hours before anaesthesia (Luo et al., 2024). Additionally, Madan et al. (2023) reported that oral pregabalin with a dose of 4 mg/kg and gabapentin with a dose of 10 mg/kg given before anaesthesia produced similar effects when used as adjunctive preanesthetic sedation agents in laboratory cats. There were no significant differences between pregabalin and gabapentin in change of physiologic parameters or sedation scores before and after anaesthesia. Need for rescue sedation for placing an IV catheter and the prevalence of delirium were low and at the similar level for both treatments (Madan et al., 2023).

Li et al. (2024) studied the effect of pregabalin on physiological and echocardiographic variables in laboratory cats at three dose levels: 2.5, 5 and 10 mg/kg. The results showed dose-dependent decrease in systolic blood pressure and mild to moderate sedation, but only minimal impact on pulse rate and respiratory rate. Pregabalin did not significantly affect echocardiographic variables (Li et al., 2024).

2.4.3.2 Pregabalin in other animals

In dogs, pregabalin has been studied for the treatment of neuropathic pain in chiari-like malformation and syringomyelia (Sanchis-Mora et al., 2019; Thoefner et al., 2020) and as post-operative analgesia in intervertebral disc disease (Schmierer et al., 2020). When compared to placebo, pregabalin was observed to reduce clinical signs and improve quality of life in syringomyelia dogs (Thoefner et al., 2020), as

well as to reduce mechanical hyperalgesia, cold hyperalgesia and allodynia (Sanchis-Mora et al., 2019). When given with opioids after surgical treatment of intervertebral disc disease, pregabalin reduced pain levels and increased mechanical nociceptive thresholds compared to opioids alone (Schmierer et al., 2020).

Additionally, use of pregabalin in idiopathic epilepsy in dogs as an adjunct to phenobarbital and potassium bromide has been studied with promising results in a small number of patients (Dewey et al, 2009).

2.4.3.3 Pregabalin in humans

Pregabalin is approved in humans for the treatment of neuropathic pain, generalized anxiety disorder and as an adjunctive therapy in epilepsy in the European Union (EMA, 2023), and for neuropathic pain associated with diabetic peripheral neuropathy, postherpetic neuralgia and spinal cord injury as well as for fibromyalgia, and as adjunctive therapy in epilepsy in the United States (FDA, 2023). In addition to the label indications, pregabalin has been widely used also in other pain syndromes, such as low back pain, chronic pelvic pain, spinal cord injury, traumatic nerve injury and phantom limb pain (Goodman and Brett, 2019). Pregabalin has also been studied in social anxiety disorder (Kawalec et al., 2015), and as pre-operative treatment for anxiety control in patients undergoing surgery (Torres-Gonzáles et al., 2020) with positive results.

3. Aims of the study

The overall aim of the present study was to demonstrate that the cat-specific pregabalin oral solution formulation effectively and safely alleviates acute anxiety and fear associated with transportation and veterinary visits in cats.

The more specific aims were as follows:

1. To find suitable variables for evaluation of acute anxiety associated with transportation and veterinary visits in cats (I, II).

2. To select and confirm the clinical dose of pregabalin in anxious cats during transportation and veterinary visits (I, II).

3. To confirm the clinical efficacy and safety of pregabalin with the selected dose in anxious cats during transportation and veterinary visits (II).

4. To evaluate the pharmacokinetic parameters of the cat-specific pregabalin oral solution formulation in healthy laboratory cats (III).

4. Materials and methods

4.1 Ethical approvals (I-III)

The clinical studies (I, II) were conducted in compliance with Good Clinical Practice (GCP) standards, as defined by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) Guideline number 9.

The clinical trial application was approved by the competent regulatory authority of each country, where studies I and II were conducted. Prior to enrolment, cat owners gave their written consent after they had been given information about the study. The owners were able to withdraw their cat from the study for any reason and at any time. Cats' health, welfare and treatment were followed and ensured by investigator veterinarians.

The pharmacokinetic study (III) was conducted according to Good Laboratory Practice (GLP) and following the Dutch Act on Animal Experimentation (December 2014). The study was approved by the Central Authority for Scientific Procedures on Animals and the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. under the authority of the Project License AVD2360020172866.

4.2 Cats (I-III)

Privately owned cats had to have a history of either being challenging to place and keep in a carrier or fearful and/or anxious when transported by car to be eligible for study I. These behaviours were verified at the baseline. Cats aged at least 1 year and weighing a maximum of 8 kg could be enrolled because the human formulation of oral pregabalin used in this study had a concentration of 20 mg/ml, and cats heavier than this would have had to be given large volumes, which could have led to poor compliance. All cats enrolled to studies I and II were identified as healthy or with mild systemic disease (American Society of Anaesthesiologists class I or II) by a veterinarian.

In study II, privately owned cats of any age were eligible to participate in the study if they had a history of being stressed, anxious, and/or fearful when transported by

car and during veterinary visits. Additionally, cats enrolled were required to score 3–5 at the screening in the owner's assessment of transportation (Table 4) and 3–5 in the investigator's assessment of the ability to perform clinical examinations (Table 5). Cats were excluded from participating if they were being treated with other psychoactive medications, homeopathic remedies, pheromonal products, supplements, or a special diet to control anxiety. Other reasons for exclusion were pregnancy, lactation, concurrent participation in other clinical study, and any other condition or situation which could disturb the conduct of the study, for example, owner's inability to administer the study treatment, make video recording, or transport the cat in a car.

Study III included six laboratory cats, three neutered males and three intact females. The domestic shorthair cats were 1–4.5 years old at the initiation of the study. The males weighed between 4.8 and 5.4 kg and the females between 4.4 and 4.6 kg. The animals were deemed healthy based on a physical examination and clinical chemistry and were trained for restraining prior to the start of the study. The cats were socially housed (same sex) except at times when they were separated for study procedures on the days of dosing the study treatment. At those occasions, animals were housed individually until approximately 4 hours after dosing.

4.3 Study design and treatments (I-III)

The clinical studies (I, II) were randomized, blinded and placebo-controlled single dose field trials.

4.3.1 Study I

Study I with a crossover design was conducted in 13 cats between June and October 2016 in Finland. The study included screening of suitable participants, health check, baseline assessments, four separate treatment days with wash-out periods of 8 ± 2 days between each treatment day and an end-of-study contact 1-3 days after the last treatment. The health check before the study started was done by a practicing veterinarian chosen by the owner.

At baseline, cats were given tap water orally using a syringe to mimic the study procedure. On the four treatment days, the cats were administered human labelled

formulation of pregabalin (Lyrica 20 mg/ml oral solution, Pfizer limited) on three treatment days and placebo, matched to the active treatment regarding odour and appearance, on one treatment day. The used pregabalin 20 mg/ml oral solution with strawberry taste was registered for human use. On the three first treatment days the cats received pregabalin at a dose 5 and 10 mg/kg and placebo in randomised manner. On the fourth treatment day the cats were given pregabalin with individually tailored dose level between 2 and 15 mg/kg based on the cat's response to the study treatment on the earlier treatment days. The investigators contacted the cat owners on the following day of each treatment to collect the AEs.

Study procedures and assessments during the treatment days were performed by the cat owners that were blinded to the study treatment. Additionally, external, independent, trained observers blinded to the owner assessment and the study treatment assessed the cats' behaviour during each treatment day compared to baseline based on video recordings, which were taken during the car ride and at home before and after transportation.

4.3.2 Study II

Study II with parallel groups was conducted in cats suffering from travel and veterinary clinic anxiety (n = 238). In total 22 veterinary clinics in five European countries (Finland, Germany, Hungary, Ireland, and Portugal) participated in the study that was conducted between September 2018 and May 2019.

The study included pre-screening of suitable participants, screening visit with baseline assessments, one treatment visit 5-10 days after the screening and an end-of-study contact 3-10 days after the treatment visit. The investigators were licensed veterinarians working at clinics that were willing to participate and able to recruit suitable patients from their patient populations. One external expert observer evaluated the cats' behaviour during transportation based on video recordings of screening and treatment visits. The investigators, owners, external expert, and the sponsor representatives were all blinded to the study treatment.

The study had two phases; phase 1 included three parallel groups with two dose levels of active treatment and a placebo. An interim analysis was made after enrolment of the first 90 cats (i.e., at the end of the first phase). In the interim

analysis the two pregabalin doses were evaluated against placebo and each other to find the appropriate dose for the proposed indications in cats. In phase 2 the selected clinical dose level was compared to placebo and the rest of the cats were randomized to either of these two treatments.

At baseline, cats were given tap water orally using a syringe to mimic the study procedure. The eligible cats in phase 1 were randomly assigned in a 1:1:1 ratio to receive either a single 2.5 or 5 mg/kg dose of the novel pregabalin oral solution (Bonqat 50 mg/ml, Orion Corporation) or placebo. The cats enrolled in phase 2 of the study were randomised to receive pregabalin with the selected clinical dose level 5 mg/kg or placebo in a ratio of 1:1. To ensure blinding during the study, the active and placebo treatments were identical in colour, odour and dosing volume. The used pregabalin oral solution was the final formulation corresponding to the one that is currently marketed. The product was flavoured with ethyl maltol tasting like burned sugar or cotton candy.

In both clinical studies the study treatments were administered without food directly into to the cat's mouth with a syringe at home by the owner who was trained to administer the study product. Study treatments were administered 90 ± 15 min before the cats were placed into a carrier for car transportation that lasted at least 20 min and ended at a veterinary clinic where a clinical examination was conducted.

4.3.3 Study III

Study III was conducted between February and June 2019 in healthy laboratory cats (n = 6) at a contract research organisation (CRO) specialised in nonclinical pharmacological and safety studies in laboratory animals. As the research aimed at registration of a new veterinary medicinal product, the EMA guideline for the conduct of pharmacokinetic studies in target animal species (EMEA, 2000) was followed.

The study consisted of five dosing periods separated by a 4-week washout period between each dosing due to the long elimination half-life of pregabalin in cats. The dosing periods included three dose levels 2.5, 5 and 7.5 mg/kg of the novel pregabalin oral solution as a single dose and the dose 5 mg/kg administered on two consecutive days to evaluate pharmacokinetics of the second dose if given 24 hours

after the first one. In addition to the doses given orally, an intravenous (IV) bolus injection of pregabalin 2.5 mg/kg was given via the cephalic vein to the same cats to estimate the bioavailability of oral solution. The injectable solution was prepared by dissolving pregabalin into sterile 0.9% sodium chloride on the day of dosing at the study laboratory. All administrations were done in a fasted state and in non-randomised order from lower to higher exposure level and starting from oral administrations to minimise the effect of the previous dose on plasma concentrations.

Blood samples (0.75 ml) from the jugular or cephalic vein were collected before pregabalin dosing and at defined time points up to 168 hours after dosing. In the period of dosing on two consecutive days, the samples post administration were collected after the second dose (Table 3). The total blood volume removed by several sampling within one week (168 hours) was well under limit of maximum blood volumes that can be taken from laboratory animals taking into consideration the 3-week recovery period between each test period. (Diehl et al., 2001).

Table 3 Pharmacokinetic study design.

Period	Dose level (mg/kg)	Dose route	Timepoints of plasma samples (hour)
1	2.5	PO	predose, 0.5, 1, 2, 3, 4, 8, 12, 24, 36, 96, 168
2	5	PO	predose, 0.5, 1, 2, 3, 4, 8, 12, 24, 36, 96, 168
3	7.5	PO	predose, 0.5, 1, 2, 3, 4, 8, 12, 24, 36, 96, 168
4	5*	PO	predose, 0.5, 1, 2, 3, 4, 8, 12, 24, 36, 96, 168
5	2.5	IV	predose, 0.083, 0.25, 0.5, 1, 2, 3, 4, 8, 12, 24, 36, 96, 168

*Dose given on two consecutive days with a 24-hour interval

4.4 Study variables (I-III)

4.4.1 Variables used for assessment of anxiolytic efficacy of pregabalin (I-II)

In the clinical studies (I-II) owners recorded their assessments of the variables to paper diaries and the data from the diaries were transferred later to an electronic database by investigators or sponsor personnel. Investigators reported their findings directly to an electronic database designed for clinical studies (Rave EDC, Medidata).

In study I the owner assessed efficacy during the treatment days compared to baseline on the following variables:

- The overall effect of the study treatment during each treatment day using the scale “excellent,” “good,” “some effect,” “no effect,” or “worse”.
- The ability to place the cat into a carrier at home with the scale “excellent,” “good,” “fair,” “poor,” “very poor,” or “not possible”.
- The ability to transportation of the cat in a car for at least 20 minutes with the scale “excellent,” “good,” “fair,” “poor,” and “very poor”.
- The signs of stress, anxiety, and/or fear when placing the cat into a carrier and during transport including vocalization, abnormal activity/restlessness/pacing, resistance/destructive behaviour, escaping/evading/hiding, inappropriate urination, inappropriate defecation, panting/intense breathing, vomiting, licking/self-grooming, freezing/decreased motor activity, salivation, and sweaty paws. The severity of these signs was rated each as “absent” (0), “mild” (1-2), “moderate” (3-4), or “severe” (5-6). The sum of the signs was calculated over all signs using their numerical severity rating when placing the cat into a carrier and during transportation.

Additionally, in study I the external observer using video recordings made the following assessments focusing on timepoints when the cat was placed into a carrier and transported in a car:

- The overall efficacy during each treatment day compared to baseline using the same 5-point ordinal scale as the owner. Baseline and treatment day videos of each cat were assessed separately and the change of ratings on treatment days compared to baseline was assessed in statistical analysis.

- The signs of anxiety and fear either by frequency (elimination, lip licking, shake off, swallowing, vomiting, yawning) or duration (active interaction, avoiding pet carrier, crouched position, ears flattened, eating, exploration, eyes closed, grooming, hiding, locomotion, not visible, oriented to the environment, passive behaviour, passive interaction, play, pupils dilatated, purring, panting, scratching, salivating, tail close to body, vocalisation, withdrawal) depending on their nature according to an ethogram created suitable for video assessment of cats.

The reliability of the external observer evaluating all the cats was assessed by a second observer, a board-certified behaviourist, scoring independently a random sample of 20% of the recordings.

In study II the following primary efficacy variables were assessed:

- The treatment effect during transportation lasting at least 20 minutes by the owner's assessment using a 5-point ordinal scale presented in table 4.
- The treatment effect during clinical examination by the investigator's assessment using a 5-point ordinal scale presented in table 5.

Table 4 Numerical scale for the owner's assessment of the treatment effect during transportation in a car lasting at least 20 minutes.

Score	Description
1	Excellent: Cat was calm and quiet during the whole transportation time, did not express signs of stress, anxiety and/or fear
2	Good: Cat was calm and quiet during most of the transportation time. Transient mild signs of stress, anxiety and/or fear (e.g. occasional vocalisation, salivation or locomotion) up to 25% of the transportation time
3	Fair: Cat showed moderate signs of stress, anxiety and/or fear (e.g. vocalisation, salivation, locomotion or other activity in bouts) up to 50% of the transportation time
4	Poor: Cat showed strong signs of stress, anxiety and/or fear (e.g. vocalisation, salivation, locomotion or other activity almost without interruption or in longer, more forceful bouts) up to 75% of the transportation time
5	Very poor: Cat showed extreme signs of stress, anxiety and/or fear (e.g. vocalisation, salivation, locomotion or other activity forcefully and without interruption) for 75-100% of the transportation time

Table 5 Numerical scale for the investigator's assessment of the treatment effect during the clinical examination at the clinic.

Score	Description
1	Excellent: Clinical examination could be easily performed without resistance or with insignificant resistance (no restraint needed). Cat was compliant and not frozen and did not express signs of stress, anxiety and/or fear.
2	Good: Minor resistance; clinical examination could be performed with the technician minimally restraining the cat by placing a hand on the head or back. Cat was compliant and not frozen and expressed mild signs of stress, anxiety and/or fear.
3	Fair: Moderate resistance or freezing. Cat expressed moderate signs of stress, anxiety and/or fear. Clinical examination could be performed with the veterinary technician using physical restraint involving stabilizing the cat and holding in place. Freezing is defined as a moderately tense body.
4	Poor: Strong resistance or freezing. Cat expressed strong signs of stress, anxiety and/or fear. Clinical examination could be performed without sedation with the veterinary technician more tightly restraining the cat (physically wrapping or scruffing cat). Freezing is defined as a very tense body, e.g. absence of movement except respiration.

Score	Description
5	Very poor: Extremely strong resistance. Cat expressed extreme signs of stress, anxiety and/or fear and responded to the clinical examination with avoidant and/or defensive behaviour to an extent that completing the examination required sedation.

As secondary efficacy variables in study II the owners assessed:

- The ability to place the cat into the carrier using a similar 5-point scale as used for transportation.
- The signs of stress, anxiety, and/or fear including vocalization, abnormal activity, resistance, destructive behaviour, escaping/hiding, withdrawn/crouching, freezing/decreased motor activity, urination, defecation, vomiting, panting, continuous licking, scaling, salivation, sweaty paws in a table format with the scale "none" (0), "only a few times" (1), "half of the time" (2), "most of the time" (3) or "continuously" (4) for assessment of extent of each sign. Rating occurred at several time points: during transportation, clinical examination, at home just after opening the carrier, and 1 and 3 hours after coming home. The sum of the signs of stress, anxiety and/or fear was calculated for each individual and timepoint, and the means of the treatment groups were compared to each other.
- The onset and end of any change or signs of effect in the cat's behaviour.

Additionally, in study II the external observer assessed:

- The treatment effect during transportation based on video recordings using the same 5-point ordinal scale as the owner.
- The signs of stress, anxiety, and/or fear during transportation according to the frequency (elimination, eyes closed, lip licking, purring events, shake off, swallowing, vomiting, yawning) and/or duration (crouched position, ears flattened, exploration, grooming, hiding, locomotion, pupils dilatated, purring, panting, scratching, salivating, sleeping, tail close to body, vocalisation, withdrawal, other), depending on the type of behaviour.

4.4.2 Other clinical variables (I-II)

In study I the owners assessed the cat's activity with the scale "very calm/sleeping," "calm," "neutral," "active," or "very active".

Heart rate and respiratory rate measured by auscultation and rectal temperature, as well as haematology and clinical chemistry values were assessed as part of clinical examination in study II at screening and treatment visits.

The usability of the product was assessed in both clinical studies (I-II) by the owner using the scale "very easy," "easy," "somewhat difficult," and "very difficult".

4.4.3 Variables used for assessment of clinical safety of pregabalin (I-II)

In both clinical studies (I-II) AEs were recorded by the investigators based on owner interview, diary markings, and video observations.

In study II investigators also assessed the alertness (physical and mental activity) and potential sedation of the cat at the beginning of clinical examination. Additionally, owners scored the cat's activity at home ("active", "neutral", "calm" or "very calm/sleeping") and ability to stand up and walk ("normal", "calm", "mild incoordination", "moderate incoordination" or "severe incoordination") when opening the carrier, and 1 and 3 hours after coming home. Blood samples were collected at screening and treatment visits for haematological and clinical chemistry analysis.

4.4.4 Variables used for pharmacokinetic evaluation of pregabalin (III)

In study III, the CRO personnel used a separate electronic database designed for nonclinical studies for recording of the data on clinical signs (ToxData 8.0, PDS Pathology Data Systems Ltd.).

Plasma samples were analysed in a commercial GLP laboratory (Ardena Bioanalysis BV Assen) for the concentrations of pregabalin using a validated liquid

chromatography–tandem mass spectrometry method. Bioanalytical data was collected in a separate database (Analyst, MDS Sciex).

The parameters were estimated using pharmacokinetic software (Phoenix WinNonlin 6.4, Certara). A non-compartmental approach consistent with the oral or IV route of administration was used for parameter estimation. All values below the lower limit of quantification were assigned a value zero for pharmacokinetic purposes. Nominal sampling times were used in pharmacokinetic calculations, except where the deviation was > 5%; in this case, actual times were used.

The pharmacokinetic parameters that were analysed in the study were area under plasma concentration time curve within 24 hours after dosing ($AUC_{0-24\,h}$), C_{max}, T_{max}, $t_{1/2}$ and oral bioavailability (F), which complied with the EMA guideline on pharmacokinetic studies in the target animal species (EMEA, 2000).

The cats were observed twice daily for general health and additionally possible level of sedation, which was assessed using scores “no sedation”, “slight sedation”, “moderate sedation” or “deep sedation”.

4.5 Statistical analysis (I-III)

4.5.1 Study I

Due to the exploratory nature of study I, neither a formal statistical hypothesis was defined, nor a formal sample size calculation was performed. All cats that were enrolled into the study and received at least one dose of the study treatment were included in the analyses.

All efficacy variables were analysed in terms of absolute and/or change from baseline, and/or sum scores. Pregabalin was compared to placebo using a generalized linear model for ordinal data, with a cumulative logit as a link function, in which treatment and day were used as fixed effects. The correlation within cats was modelled as a repeated effect. This analysis provided a comparison of pregabalin and placebo over several days, taking into account the baseline assessments at screening. The statistical method considered that the same cats were measured multiple times.

Analyses were adjusted for a carry-over effect when a significant carry-over effect was found. The results were reported as odds ratio (OR) with 95% confidence interval (CI). The OR indicates how likely different outcomes are associated with the treatment. Differences were considered to be statistically significant with $p < 0.05$.

The correlation between owners' and external observer's assessments of the overall effect of the study treatment was calculated using Spearman's rank order correlation coefficient (r_s) that describes reliability of ordinal scales (De Raadt et al., 2021). The change from baseline in individual signs and the sum of signs of distress, anxiety, and/or fear assessed by the owner was reported with mean (95% CI) and analysed with a linear mixed model. The reliability of the external observer was assessed by a board-certified behaviourist using a randomly selected 20% of the recordings using r_s. The external observer's assessment of the duration or frequency of each sign was separately analysed with a linear mixed model. Baseline data were used as a covariate, when available. Differences were considered to be statistically significant with $p < 0.05$. Safety variables were reported descriptively.

4.5.2 Study II

The sample size of study II was estimated based on study I. The estimated sample size in each group was 81, with a 5% level Chi-square test having a 90% power to distinguish between an active treatment group and placebo. It was assumed that variation would be larger in this study than in study I due to several centres, countries, and possible dropouts. Thus, at least 90 cats were to be recruited for both the pregabalin group with clinical dose level and the placebo group by the end of the study.

Both primary variables were analysed with a generalized linear mixed model appropriate for a multinomial response variable, with a cumulative logit link function. Treatment was modelled as a fixed effect, and centre and centre-by-treatment interaction as random effects. The baseline score was included as a covariate. Equality of distribution between treatments was tested and OR reported as the result. Differences were considered to be statistically significant with $p < 0.05$. The used statistical method compared the effect of pregabalin, and placebo

treatments assessed with a scale including several categories. The method took into account variations between study centres and baseline measurements at screening visit to adjust for any initial differences. It was also checked whether the outcomes were distributed similarly across the treatment groups.

As a supportive analysis, both primary variables were also dichotomized into success/failure variables following the predefined plan, where "success" was defined as "excellent" or "good" in the 5-point scale. All other scores ("fair", "poor", "very poor") were regarded as "failure". Dichotomized variables were analysed with a logistic regression model, which can be used with data analysis, when the outcome is one of two possible categories.

The multinomial secondary variables were analysed with a similar model as the primary variables. Change from baseline in the owner's assessment of sum of signs of stress, anxiety, and/or fear was analysed with a linear mixed repeated measures analysis of covariance (RM-ANCOVA) model. Treatment, time and treatment-by-time interaction were fixed effects and subject, centre and centre-by-treatment interaction were random effects. Estimates for individual time points were done using contrasts. This statistical method was used to analyse how the total score of anxiety signs changed over time with pregabalin and placebo treatments taking into account the baseline assessment at the screening visit. The analysis considered both the specific treatments and times of measurement, as well as variations between individual pets and study centres. The differences in assessment scores at specific time points were compared to see how the treatment effect changed over time.

External observer assessment of signs of stress, anxiety, and/or fear were analysed descriptively. The inter-rater reliability between owner and external observer assessments of treatment effect during transportation was assessed with Cohen's weighted kappa coefficient (κ), which is commonly used for quantifying agreement between ratings with an ordinal scale (De Raadt et al., 2021) and therefore selected to this pivotal phase study. Differences were considered to be statistically significant with $p < 0.05$.

All safety variables were reported descriptively by treatment group. All randomized cats (intention-to-treat [ITT] population) were included in the safety analysis. As

the aim was to study the anxiolytic effect of pregabalin, a conservative approach was chosen where the cats were excluded from the ITT population in the efficacy analyses if signs of sedation were seen. The predefined criteria in the study protocol stated that cats showing sedation at the clinic when evaluated at the beginning of clinical examination, or cats that were very calm/sleeping and showed moderate or severe incoordination at two timepoints after coming home were excluded from the efficacy analysis.

An independent data monitoring committee was established to evaluate efficacy and safety data based on the interim analysis and to make the dose selection for phase 2 using the same rules of statistical analysis, as applicable. Adjustments were applied to all analyses that integrated the stage-wise results. The adjustments ensured to account possible biases that might have occurred at either phase of the study.

4.5.3 Study III

The sample size of study III was selected based on the regulatory guidance of pharmacokinetic studies conducted for marketing authorisation of a veterinary medicinal product (EMEA, 2000).

Descriptive statistics of the pharmacokinetic parameters (number, mean, SD and variation) for males, females, and males and females combined were generated using Phoenix WinNonlin 6.4. All cats that showed either spillage or salivation after dosing were excluded from the descriptive statistics of the pharmacokinetic parameters, because already minimal spillage of the formulation during dosing and salivation after dosing could affect dose accuracy. Clinical observations and level of sedation were analysed descriptively, as well.

5. Results

5.1 Demography of the cats (I-II)

In study I, 13 cats were enrolled of which seven were females and six males. All cats, except one female, were neutered. Their median (range) age was 3.3 years (1.0–8.9 years), and weight was 4.2 kg (4–7 kg). Two cats discontinued the study after the first treatment day; one of them received pregabalin with a dose of 5 mg/kg and the other with 10 mg/kg. Thus, total of 11 cats received all the study medications. In the statistical analysis 11 administrations of both 5 and 10 mg/kg, in total 22 administrations, were included for assessment of overall treatment effect. However, as the owner of one discontinued cat was able to put the cat into a carrier, but the other owner was not, the ability to place the cat into a carrier was conducted for 23 pregabalin administrations.

The median (range) actual dose was 5.1 mg/kg (4.8–5.3 mg/kg) and 10.1 mg/kg (8.1–10.7 mg/kg) for the 5 and 10 mg/kg doses, respectively. Lower (2.5 mg/kg), medium (7.5 mg/kg) and higher (12.5 mg/kg) dose levels were administered only for three, two and one cat, respectively, and therefore they were not used as separate dose groups nor included in efficacy and safety analysis.

A total of 243 client-owned cats entered study II. Five of them discontinued the study before treatment administration, thus 238 cats were randomly allocated to receive pregabalin 2.5 mg/kg (n = 29), 5 mg/kg (n = 108) or placebo (n = 101). 57.1% of the cats were females and 42.9% males. The median (range) age was 4.9 years (0.4–15.6 years) and weight 4.3 kg (1.8–10.3 kg). The cats represented several breeds, most commonly domestic cats (58.0%) and European shorthairs (24.8%). Most of the cats (84.5%) were neutered. The demographic and baseline characteristics were comparable in the treatment groups. The median (range) duration of car transportation was 22 (20–45) minutes and 22 (20–50) minutes for 5 mg/kg and placebo, respectively.

5.2 Determination of pregabalin dose in anxious cats (I-II)

In study I, no significant differences were found between the 5 and 10 mg/kg doses of pregabalin in cats with travel anxiety based on the owner's assessment of overall treatment effect, ability to put the cat into a carrier, ability to transport the cat or in the sum of signs when placing the cat into a carrier or during transportation. However, at the 5 mg/kg dose level less AEs were observed than with 10 mg/kg. In dose selection both efficacy and safety aspects are taken into consideration. The goal of dose determination is to select the lowest effective dose with as few adverse effects as possible and therefore the 5 mg/kg pregabalin dose was selected as the highest dose to be used in next clinical study.

In study II, a lower dose than 5 mg/kg was needed in order to evaluate if the anxiolytic effect could be seen with even a smaller dose. Thus, pregabalin doses 2.5 mg/kg, 5 mg/kg and placebo were included in phase 1. In the interim analysis, the 5 mg/kg dose showed significantly better efficacy compared to both 2.5 mg/kg and placebo. The safety profiles of both pregabalin dose levels were acceptable and did not differ from each other. Therefore, 5 mg/kg was assessed as the lowest effective dose level of the studied doses and was selected as the clinical dose.

5.3 Anxiolytic efficacy of pregabalin in cats (I-II)

In study I, no significant differences were found between pregabalin 5 and 10 mg/kg groups in efficacy analysis. Therefore, the efficacy results were reported as pregabalin groups combined.

Overall, 14/22 (administrations) of the pregabalin treated cats and 1/11 of the placebo treated cats scored "excellent" or "good" for the overall effect of the study treatment. Due to significant carry-over effect the statistical model was adjusted and the difference was not significant (OR = 2.4; 95% CI 0.6, 8.9; p = 0.21).

Of the cats treated with pregabalin, 19/23 scored "excellent" or "good" in the ability to place the cat into a carrier compared to 8/11 of the placebo treated cats. There was no statistically significant treatment effect (p = 0.48). The same concerns the ability to transport the cat in a car, in which 13/22 of the pregabalin treated cats and 3/11 of the placebo treated cats scored "excellent" or "good" (p = 0.06).

In the evaluation of individual signs of anxiety, fear, and stress during transportation, there was significantly less vocalization (mean −1.3; 95% CI −1.9, −0.7; $p < 0.01$), abnormal activity/restlessness/pacing (mean −0.7; 95% CI −1.2, −0.2; $p = 0.01$), and panting/intense breathing (mean −0.9; 95% CI −1.7, −0.1; $p = 0.03$) after pregabalin treatment compared to placebo. When placing the cat into the carrier vocalization was significantly less after pregabalin treatment (mean −0.6; 95% CI −1.0, −0.2); $p < 0.01$).

In study I, the owners' and the external observer's assessment on the overall effect of the study treatment correlated significantly ($r_s = 0.63$, $p < 0.01$). Based on the video assessment made by the external observer, cats showed significantly less vocalization ($p < 0.01$) and swallowing ($p < 0.01$) and more hiding ($p < 0.01$) and passive interaction ($p < 0.01$) when given pregabalin compared to placebo. The inter-observer reliability between the external observer and the board-certified behaviourist was significant for 17 of the 29 signs in which r_s ranged from 0.65 to 0.94 ($p < 0.05$). Four signs did not correlate significantly between the external observer and the board-certified behaviourist, and for the rest the scarce data did not allow statistical testing.

In study II, a significant difference favouring pregabalin 5 mg/kg over placebo was seen in both primary efficacy variables, the owner's assessment of the treatment effect during transportation (OR = 3.81; 95% CI 1.78, 8.14; $p < 0.01$) and the investigator's assessment of the treatment effect during clinical examination (OR = 3.42; 95% CI 1.83, 6.38; $p < 0.01$).

The treatment effect in the owners' assessment of the ability to place the cat into the carrier was significantly better for pregabalin than placebo ($p < 0.01$). The difference between pregabalin and placebo in the mean sum of signs at the treatment visit was significant during transportation ($p < 0.01$), clinical examination ($p < 0.01$) and when opening the carrier at home ($p = 0.02$) favouring pregabalin. Based on the owners' observations, vocalization, panting/intense breathing, resistance, and abnormal activity were the characteristic signs of anxiety with the greatest numerical decrease with pregabalin treatment versus placebo. Owners were able to detect the onset and end of any change or signs of effect in 45%

(49/108) of cats receiving pregabalin with the median (range) duration of changes being 7 hours (1.3–28.5).

In study II, the external observer's assessment of the treatment effect during transportation was significantly better for pregabalin compared with placebo ($p < 0.01$). The evaluations of the owners and the external observer were in agreement ($\kappa = 0.47$, $p < 0.01$). The external observer found the greatest numerical decreases in pregabalin treatment versus placebo in vocalisation, dilatation of pupils, flattening of the ears, lip licking and swallowing.

5.4 Evaluation of the suitability of variables for evaluation of acute anxiety associated with transportation and veterinary visit in cats (II)

Study II was designed and conducted to confirm the clinical efficacy of pregabalin. Based on the earlier findings and clinical relevance of both the transportation and clinical examination phases, the two primary parameters were selected to be included in the study. In the statistical analysis of transportation and clinical examination results a significant difference was detected between pregabalin and placebo in favour of pregabalin.

The clinical relevance of the treatment was evaluated by analysing the owner assessed change in the mean sum of signs at the treatment visit during both timepoints of the primary variables. The change in mean sum of signs was found to be significantly better with pregabalin treatment compared to placebo during transportation, clinical examination and when opening the carrier at home after the clinic visit. Additionally, the owners were able to notice decrease in vocalization, panting, resistance and abnormal activity after pregabalin treatment versus placebo.

The external observer's assessment of the treatment effect during transportation in study II was used as confirmation of the owners' assessment. Both the owner and the external observer used the same ordinal scale, which makes the comparison between them possible and shows that their evaluations were in an agreement ($\kappa = 0.47$, $p < 0.01$).

5.5 Other clinical variables (I-II)

In study I, cats were significantly less active after receiving pregabalin compared to placebo when placed into the carrier ($p < 0.05$); however, this effect was not observed during transportation ($p = 0.24$). In study II, seven cats of 108 receiving pregabalin with a dose of 5 mg/kg and 14 cats receiving placebo out of 101 cats required sedation at the treatment visit approximately 2 hours after the administration of the study treatment to complete the standardised clinical examination including blood sampling, however the difference was not statistically significant. The sedatives used (e.g., alpha-2 agonists and opioids), and their doses were similar to those used at the screening visit for the concerned cats, and no safety concerns were reported.

In study II the mean ± SD values of heart rate were numerically slightly lower at treatment visit (165.6 ± 29.7 beats/min) compared to screening (173.6 ± 36.4 beats/min) in pregabalin group. In respiratory rate the mean ± SD values in pregabalin group were 44.2 ± 15.3 breaths/min and 51.2 ± 24.2 breaths/min at treatment and screening visits, correspondingly. However, no statistically significant differences were observed in the changes of either variable. Additionally, rectal temperature, as well as haematology and clinical chemistry values, did not exhibit significant alterations between the screening and treatment visits.

Administration of the study product by the owners in study I, where human labelled pregabalin formulation was used, was assessed to be “somewhat difficult” (15/22) or “very difficult” (1/22) in 73% (16/22) of administrations, while some of the owners assessed it to be “easy” (3/22) or “very easy” (3/22). In study II approximately 79% (85/108) of cat owners assessed that it was ”very easy” (52/108) or ”easy” (33/108) to administer the novel pregabalin oral solution, while part of the owners found it “somewhat difficult” (18/108) and “very difficult” (5/108). The difference between usability assessments of the two formulations was significant ($p < 0.01$).

5.6 Clinical safety of pregabalin in cats (I-II)

No serious AEs were reported in either of the clinical studies (I-II).

In study I, the most common AE was transient incoordination, which was assessed as mild in seven and five cats and as moderate in one and seven cats with the doses of 5 and 10 mg/kg, respectively. Muscle tremor and anxiety were reported once each with 10 mg/kg. All AEs had resolved by the next day when the investigators contacted the owners. No AEs were reported with placebo.

In study II, the most common AE was mild transient incoordination (five events in four cats) and tiredness (three events in three cats). Additionally, proprioception abnormality and emesis were reported in some cats and muscle tremor, mydriasis, anorexia, weight loss and leucopoenia in single animals. These AEs had resolved by the next day. In study II, the investigators considered the majority of cats in both groups to have normal alertness. Only one cat in the pregabalin group was considered to show signs of mild sedation by the veterinarian and was thus excluded from the efficacy analysis. Based on the owners' assessment, two additional cats were excluded from the efficacy analysis, according to the predefined criteria in the study protocol, as they both scored to be very calm or sleeping and having moderate incoordination at least in two time points after coming home. In the sensitivity analysis, when the three cats with mild signs of sedation were included, the efficacy results were similar to the ones in the main analysis. The owners assessed that 3/101 (3%) cats were very calm or sleeping in the pregabalin group and 1/87 (1%) in the placebo group after coming home from the veterinary clinic. The ability to stand up and walk was assessed normal in 57/101 (56%), 59/101 (58%), and 74/101 (73%) of cats in the pregabalin group and 78/87 (90%), 79/87 (91%), and 83/87 (95%) of cats in the placebo group at home when opening the carrier and 1 and 3 hours after coming home from treatment visit, respectively. These parameters were not assessed in the cats that had required sedation to complete the standardised clinical examination at the treatment visit.

There were no notable changes in the laboratory values between the screening and treatment visits in either the pregabalin or placebo groups with the exception of one cat in each group. The clinically relevant findings, leukopenia in one cat in the pregabalin group and thrombocytopenia in one cat in the placebo group, were reported as AEs.

5.7 Pharmacokinetics of pregabalin oral solution in cats (III)

The pharmacokinetic parameters of pregabalin in cats after administration of single oral doses of 2.5, 5 and 7.5 mg/kg, and the parameters of the dose 5 mg/kg administered on two consecutive days with a 24 h interval, are listed in Table 6.

Table 6 Pharmacokinetic parameters of pregabalin after single doses of 2.5, 5 and 7.5 mg/kg and 5 mg/kg on two consecutive days of pregabalin 50 mg/ml oral solution formulation in fasted cats.

Parameter[a]	**Pregabalin 5 mg/kg (n=4)**[b]	**Pregabalin 5 mg/kg twice (n=5)**[b]	**Pregabalin 2.5 mg/kg (n=6)**	**Pregabalin 7.5 mg/kg (n=5)**[b]
C_{max} (µg/ml)	10.1 ± 0.8	12.9 ± 2.6	5.7 ± 0.7	19.1 ± 3.1
T_{max} (hours)	0.5-1	0.5-4	0.5-3	0.5-1
AUC_{0-24h} (h* µg/ml)	129 ± 3.0	157 ± 21.7	65 ± 7.9	200 ± 17.0
$t_{½}$ (hours)	14.7 ± 2.7	15.6 ± 3.6	12.0 ± 3.2	12.1 ± 2.6
F (%)	94.3 (87.3-102)	NA	95.6 (75.4-130)	89.4 (81.0-95.3)

[a] Mean ± SD values, except range for T_{max} and mean (range) for F.
[b] Animals with incomplete dosing, due to spillage or salivation after dosing, excluded.

No clinical signs or sedation were noted after single oral dosing at 2.5 and 5 mg/kg. Following single dosing at 7.5 mg/kg, mild signs of sedation and mildly uncoordinated movements were reported in two cats, and salivation directly after dosing in one cat. Following the first dosing with a repeated oral dose 5 mg/kg, mydriasis of both eyes in all six cats was observed, and salivation was noted in two cats.

6. Discussion

6.1 Anxiety variables in cats

The 5-point scales for the primary efficacy parameters, treatment effect during transportation assessed by the owners and treatment effect during clinical examination assessed by the investigators, were able to distinguish satisfactory and unsatisfactory anxiolytic effect from each other in cats (II). Thus, the used numerical ordinal scales were deemed reliable to assess anxiolytic effect in cats during transportation and veterinary examination.

The anxiety variables used in studies I and II differ from the CSS published by McCune (1994) and later modified by Kessler and Turner (1997). The CSS is the anxiety score that has been used in several pilot scale behavioural studies in cats after it was published (Pankratz et al., 2017; Van Haaften et al., 2017; Crowe et al., 2022; Gurney and Gower, 2022). However, the CSS was evaluated to be a quite complicated score to be used in a pivotal scale clinical study including over twenty investigators, hundreds of client-owned cats, and a similarly large group of individual cat owners, who needed to make their part of the assessments. Therefore, it was decided to develop and use a less complex, but still accurate enough scoring system for treatment effect during transportation and clinical examination.

The development of the transportation scale was started in the pilot study I, as no dedicated score was available. The development was continued for the pivotal study II based on the pilot study experience, and a simple 5-point scale with short written descriptions of each score was formed as an easy and guided way for owners to assess the level of travel anxiety in a large-scale clinical field study.

In study II, also the treatment effect during clinical examination was evaluated by the investigator. The published scores describing cats' signs of anxiety or behaviour during clinical examination (Van Haaften et al., 2017; Griffin et al., 2020) or cats' attitude during manipulation (Quimby et al., 2011) used in earlier studies in client-owned cats were not found suitable for this project. These scores were limited to one aspect each and were not satisfactory to be used alone as such. One aspect to consider in a clinical study is to limit the number of variables to the minimum

needed and to avoid any overlapping variables to make the study procedures as smooth as possible for the participating owners and investigators. Therefore, a new scoring system for clinical examination including detailed description of each score was created based on scales used earlier for similar purpose (Mills et al., 2006; Van Haaften et al., 2017). The new scale was adapted to be suitable for general practitioners unaccustomed to evaluating anxiety in cats.

Neither of the primary parameter scores in study II has been validated. For validation of published survey instruments measuring behaviour in domestic cats, internal consistency, test-retest reliability, inter and intra-rater reliability and factor-analysis have been used (Duffy et al., 2017; Klinck et al., 2018; Mikkola et al., 2021). An unvalidated behavioural scale based on observations of the cat's behaviour is indirect and subjective. Thus, the reliability can be affected by placebo effect and the assessor's subjectivity. On the other hand, randomisation and blinding in study II were the tools used to minimise bias of the assessment scores as they are essential for maintaining the reliability of the evaluations in clinical studies.

To some extent, the variability in behaviour can be mitigated by having multiple individuals assess the behaviour using the same scale, as long as each person observes the cat in different contexts (book chapter by Richard and Haynes, 2002). The inter-rater reliability of the used transportation scale in studies I and II was evaluated by comparing the assessments by an external expert based on video recordings and the owner assessments during the car ride. The focus was on the evaluation of the owners' ability to see and rate the anxiety level of their own cat (I, II). As both owners and the external expert used the same scale of treatment effect during transportation, the correlation between them could be calculated. In study I, strong positive correlation was detected between the owners' and external expert's assessments. In study II, a moderate agreement between their assessment was found. In both studies the correlations were statistically significant, which suggests a satisfactory level of confidence for the owners' assessment of treatment effect during the car ride. The reliability of the scoring system would have been improved by the intra-rater reliability evaluation, which would have involved the external expert reviewing and assessing a set of video recordings for a second time.

However, that was not conducted due to the limited timeframe of the development project.

In addition to an external expert assessment, physiological stress variables such as blood or saliva cortisol concentrations, heart rate, respiratory rate, body temperature and blood pressure could be used as validation tools for an anxiety scoring system. In study II heart and respiratory rates were measured during clinical examinations at screening and treatment visits. The mean values of both variables were numerically slightly lower at treatment visit in the pregabalin group, however these differences were not statistically significant. Evaluating heart rate variability (HRV) would have provided more precise indicator of stress compared to a single measurement of heart rate. HRV describes the variation between successive heartbeat intervals, reflecting changes in the activity of the autonomic nervous system (Grigg et al., 2021). Nevertheless, the measurement of HRV would have necessitated the use of a heart rate monitor over an extended period or electrocardiography, which were not included in this larger-scale clinical study conducted by general practitioners.

There were no notable changes in the rectal temperature, blood glucose concentrations or leucogram between screening and treatment visits in study II. Other physiological values, such as blood pressure and cortisol levels were not measured in the study. Blood and saliva cortisol have been used as indicators of acute stress in cats (Smith et al., 1999; Stella et al., 2013; Nibblett et al., 2015; Paz et al., 2022). However, cortisol measurement has not always provided valuable data as a stress indicator (Nibblett et al., 2015; Paz et al., 2022). This may be due to other issues such as endocrinological disturbances, further diseases, infections and diurnal changes that can affect to the cortisol concentrations besides the stress (review by Jones and Gwenin, 2020).

The investigator's assessment of treatment effect during clinical examination in study II was not compared to the external behaviour expert evaluation. The clinic visit was not video recorded as veterinary professionals were evaluated to be more experienced in the assessment of behaviour of cats. Additionally, the written description of each score, the limited number of investigators, and their thorough training can be assumed to increase the reliability of the investigators' assessment.

In studies I and II also other anxiety variables were assessed; the owners evaluated the ability to place the cat into the carrier with a 6-point scale in study I and a 5-point scale in study II. The last score of the 6-point scale was “not possible”, which was left out from the 5-point scale as it was noted that the main problem of the study population was transportation, not placement into the carrier. In study II, the statistically significant difference between pregabalin and placebo suggested that also this variable can be used to distinguish an anxiolytic effect.

Additionally, signs of stress, anxiety, and/or fear were assessed by the owners and the external observer in both studies I and II with similar descriptive scales including a list of signs of anxiety. A comparable scale has been earlier used in an anxiety study in dogs (Korpivaara et al., 2017). For studies I and II, the variable was adjusted to be more suitable for cats by including earlier reported signs of feline fear and anxiety in the used scale (Hammerle et al., 2015; Niblett et al., 2015; Bennett et al., 2017; Mariti et al., 2017; Moody et al., 2018). Owners may not recognise all signs of stress in their cats to the same extent as a trained behavioural expert (Mariti et al., 2017). However, when owners are instructed about the behaviours to observe, their awareness increases and reported findings can be regarded as reliable (Beata et al., 2007; Gruen and Sherman, 2008; Kendall and Ley, 2008; Ogata and Dodman, 2011). Thus, the reliability of the owner reported signs of anxiety was probably increased by using a scale with a list of signs where they could select the appropriate signs and assess their frequency (I, II).

In addition to the owners, an external expert also assessed signs of stress, anxiety, and/or fear during transportation based on video recordings from studies I and II. The expert used an ethogram developed for study I and refined it based on the experience gained for study II. This ethogram is comparable to those published before (Stanton et al., 2015) and after studies I and II (Nicholson and O’Carrol, 2021; Kappel et al., 2024). Recently, artificial intelligence-based systems have been developed to analyse animal behaviour, reducing the need for laborious human observation, which can introduce biases due to subjectivity and inconsistency (review by Farhat et al., 2024). These advancements likely offer new possibilities for analysing feline anxiety based on video recordings in the future.

6.2 Determination of pregabalin dose in cats

Study I provided evidence that pregabalin decreases signs of anxiety associated with car transportation in cats when used with the doses 5 and 10 mg/kg. The dose 5 mg/kg demonstrated a better safety profile than 10 mg/kg. In study II the interim analysis results indicated superior efficacy at the 5 mg/kg dose compared to 2.5 mg/kg and placebo. The anxiolytic properties of the pregabalin oral solution with a dose of 5 mg/kg were measurable, statistically significant, and clinically relevant in cats with acute anxiety and fear associated with transportation and veterinary visits. The dose used did not have safety concerns or a clinically relevant sedative effect.

The selected dose for travel and veterinary visit anxiety is at the higher end of the dose range 1-5 mg/kg that has been reported earlier to be used in client-owned cats. However, only anecdotal data can be found of these doses and the therapy areas have been related to chronic pain (Clark et al., 2017; Amengual Batle et al., 2019; Goich et al., 2019; Korff and Williamson, 2020; Ertel and Dörner, 2024; Rusbridge, 2024) and refractory epilepsy (Djani and Draper, 2019), not to anxiety. In these indications pregabalin needs to be administered chronically and twice or even three times a day, and thus lower dose levels compared to single use anxiolytic doses are reasonable. Additionally, pregabalin is used in both chronic pain and epilepsy usually as an additional medicine to other conventional treatments, which also explains the low dose levels. There are no earlier publications regarding anxiolytic doses of pregabalin in cats.

6.3 Clinical efficacy of pregabalin in cats

The results of study II confirm that the novel pregabalin oral solution given at 5 mg/kg is effective in alleviating acute anxiety and fear associated with transportation and veterinary visits in cats, as both primary efficacy endpoints, the owner-assessed treatment effect during transportation and the investigator-assessed treatment effect during clinical examination, were met. The veterinarians were able to more easily perform the clinical examination after pre-visit medication with pregabalin compared to the placebo. Anxious cats were also more likely to remain calm and quiet during transportation when treated with pregabalin before

the start of the car ride compared to the placebo. Additionally, the statistically significant difference between pregabalin and placebo when placing the cat into a carrier in study II indicated that the cats may benefit from alleviation of anxiety also in other stressful situations related to travelling.

The statistical methods that were used in study II to analyse efficacy took into account the baseline values taken before any treatment was applied. These baseline values were applied to adjust the analysis, ensuring that any observed effects were due to the treatment rather than pre-existing differences. The methods also considered variation between different study centres in a multicentre, multi-country trial.

In study I, vocalisation decreased substantially during transportation after pregabalin treatment compared to placebo and was likely the easiest sign for the owners to assess. Restlessness, panting and swallowing were also significantly reduced. In study II, the signs with the numerically greatest change after pregabalin treatment were in agreement with the signs of fear and anxiety reported in cats also in other studies (Niblett et al., 2015; Bennett et al., 2017; Mariti et al., 2017; Moody et al., 2018). In study II, a significant decrease in the sum score of signs of stress, anxiety, and/or fear compared to the baseline was noted, especially during transportation and clinical examination and to a lesser extent also when opening the carrier after coming home. Based on earlier studies, it is known that these situations are very stressful for cats (Quimby et al., 2011; Volk et al., 2011; Niblett et al., 2015; Mariti et al., 2016; 2017). These findings suggest the clinical relevance of the treatment effect in anxious cats.

In study II, owners were asked to record the onset and end of any change or sign of effect. However, it was noticed that it did not give reliable information on the duration of the actual anxiolytic effect. In general, owners were able to detect the onset and end of changes only for less than half of the cats receiving pregabalin. Based on the limited data from the study, it seems that the duration of effect of the cat-specific pregabalin formulation could be approximately 7 hours. This estimate is supported by the long half-life of pregabalin reported in study III and in an earlier publication in cats (Esteban et al. 2018). Currently, evaluation made based on cat owners' estimate of onset and end of any change or sign of effect in study II, and the

pharmacokinetic values from study III are the best approximation available about the duration of the effect.

Effect size and clinical significance in research can be evaluated e.g. with OR, which describes how likely different outcomes are associated with the treatment, or by describing a magnitude of change in clinically relevant signs of the disease. Additionally, in human medicine assessment of number of patients needed to be treated (NNT) to prevent one additional bad outcome (review by Davis et al. 2021) can be used to describe the effect size. NNT characterises epidemiologically the effectiveness of an intervention. However, it is used more in human than veterinary medicine, perhaps due to access to larger data pools of human research.

In study II, the effect size was depicted with OR, which shows that the anxious cats were more likely to remain calm and quiet during transportation and more likely to be easier to handle during the clinical examination after medication with pregabalin compared to placebo. At the same timepoints the change in the sum of anxiety signs was also significantly greater with pregabalin treatment. In study I, the clinical relevance was shown with significant decreases in the key signs of anxiety during the car ride after pregabalin administration compared to placebo.

In treatment of neuropathic pain in humans, the combined NNT for 50% pain relief of pregabalin based on 25 randomised controlled trials was 7.7 (CI 6.5–9.4) with doses 150-600 mg/day (meta-analysis by Finnerup et al., 2015). NNT of pregabalin in alleviation of anxiety has not been published in humans, nor was it calculated based on the data of study II.

6.4 Clinical safety of pregabalin in cats

No major safety concerns were observed in cats related to a single dose administration of pregabalin oral solution (I, II). The reported AEs consisted primarily of central nervous system signs, such as mild transient incoordination, tiredness proprioception abnormality and emesis. Additionally, muscle tremor, mydriasis, anorexia, weight loss and leucopoenia were reported in single animals. The typical AEs were similar in both studies and were considered to be related to the pharmacological action of pregabalin. As the reported AEs were short in

duration and investigators assessed the severity to be mild, these pharmacological risks related to a single dose of pregabalin were regarded acceptable.

In addition to the central nervous system, alpha-2-delta auxiliary subunits of voltage-gated calcium channels are found in the skeletal and heart muscles, lungs, pancreas and male reproductive glands, where they regulate muscle contraction and hormone secretion (review by Arikkath and Campbell, 2003; review by Dolphin et al. 2016). Thus, peripheral action of pregabalin might potentially affect cardiac and skeletal muscle contractility as well as hormone secretion. However, no significant effects on muscles, cardiovascular parameters, pulmonary function, or glucose and testosterone secretion have been observed in clinical studies in cats with single dose (study I and II; Li et al. 2024) or safety pharmacological studies in dogs, rats or monkeys with high doses and long-term administration (FDA, 2004; Taha et al., 2020). Thus, the observed transient and mild decrease in heart rate as well as incoordination and muscle tremors in cats after single dose of pregabalin are assumed to be most likely due to central nervous effects. Besides to the AE reporting in the clinical studies, the activity or alertness level of the treated cats was followed more closely (II). The owner assessments of the cats' activity and ability to stand up and walk after coming home were generally in line with the reported AEs. Although the owners noted that some cats in the pregabalin group were very calm or sleeping after coming home, a similar trend could also be seen in cats treated with placebo. This may be related to the finding that tiredness is a normal reaction in cats after stressful events and disruption of their normal daily routines (Stella et al., 2013). Signs of decreased activity, tiredness and incoordination after pregabalin administration have been reported earlier in cats (Esteban et al., 2018), dogs (Sanchis-Mora et al., 2019; Thoefner et al., 2020) and humans (Zaccara et al., 2011). Comparable safety findings have been reported also in cats after gabapentin administration (Pankratz et al., 2017; Van Haaften et al., 2017; Hudec and Griffin, 2020; Kruszka et al. 2021; Ruviaro Tuleski et al. 2022).

Safety of pregabalin in cats seem to be generally comparable to observations in humans, in which pregabalin has been extensively studied and used in chronic indications for two decades. Similar AE profile as with cats has been observed in humans with dizziness, somnolence and ataxia being the most commonly noticed

signs. Additionally, blurred vision, dry mouth, peripheral oedema without association to cardiovascular or renal function as well as increased appetite and weight gain with no deterioration of glycemia in diabetic patients have been reported (Toth, 2014; EMA, 2023; FDA, 2023). Furthermore, in connection to long term use in humans, withdrawal syndrome has been described in case of sudden discontinuation of pregabalin treatment. Typically, these signs may last for one to two days and include insomnia, dysphoria, anxiety, hallucinations, suicidal thoughts, tremors, sweating and tachycardia (review by Toth, 2014; review by Ishikawa et al., 2021). Pregabalin has also been noted to cause drug dependence and abuse in humans (FDA, 2023). In cats or other animals withdrawal syndrome or dependence have not been studied or reported even though practitioners commonly recommend that long-term off-label use of pregabalin should not be discontinued abruptly in dogs or cats. However, the novel pregabalin oral solution formulation has been registered in cats only for single dose administration.

More safety data would be needed to evaluate use of pregabalin in long-term use, as well as in specific cat populations, like in cats with kidney dysfunction or hyperthyroidism. This kind of evaluation would need a discrete approach to ensure the safety of the patients as so far pregabalin has only been studied and used as a single dose in cats that are healthy or have a mild systemic disease.

6.5 Usability of the product

The human pregabalin oral solution formulation including strawberry flavour was used in study I with poor usability results. This result indicated that a better and more acceptable flavouring agent, as well as lower dosing volume was needed for the new formulation intended for cats. Cats are known to prefer animal proteins and fats, meat, liver, fish, milk, amino acids and sour/acidic flavours (Thombre et al, 2004). As organic flavouring agents cause challenges in restricted hygiene areas dedicated for the production of medicines, artificial flavouring agents are preferred in commercial manufacturing of medicinal products. Therefore, several artificial flavoured formulations were tested before clinical studies I and II during the research phase in laboratory cats to select a suitable flavouring agent. Based on

these tests, ethyl maltol was chosen to be used in the final pregabalin formulation for cats.

The usability of the cat-specific oral solution formulation was good (II). Most likely the oral solution formulation, very small dosing volume of 0.1 ml/kg, and the use of the flavouring agent are the reasons for easy administration. Cats are not reported to favour sweet aromas (Thombre et al., 2004), however, ethyl maltol which has a taste and smell of burned sugar may act more like a masker of the bitterness of pregabalin than a sweetener of the formulation.

6.6 Pharmacokinetics of pregabalin oral solution in cats

Pregabalin was quickly absorbed after a single dose of 5 mg/kg of the oral solution in a fasted state (III). The absolute oral bioavailability was excellent at the clinical dose 5 mg/kg. The systemic exposure to pregabalin, in terms of AUC and C_{max} showed linear pharmacokinetics at the studied dose range of 2.5–7.5 mg/kg and the interindividual variability was low (III). This allows a reliable dose-dependent effect in clinical use.

After re-dosing with 5 mg/kg at 24 hours, the exposure, with regards to AUC, C_{max} and $t_{1/2}$, was comparable with the exposure following single dosing suggesting no clear signs for accumulation. However, studying just two consecutive doses of pregabalin provides only limited data regarding potential for accumulation. Using several consecutive administrations of pregabalin is not indicated in travel and veterinary visit anxiety. Therefore, the dosing on two consecutive days was sufficient for to get this indication registered.

Pregabalin has a relatively large volume of distribution in cats. This means that pregabalin is highly distributed into tissues, as is also described for humans (Bockbrader et al., 2010). Protein binding of pregabalin in cats has not been studied, however it is not known to bind to plasma proteins in mice, rats, monkeys or humans (FDA, 2004).

The total exposure in terms of AUC and C_{max} (III) were in line with earlier published data in cats (Esteban et al., 2018). On the contrary, T_{max} was shorter with the cat-specific oral solution used in our study than with the capsule formulation

administered by Esteban et al. (2018). This could result in a quicker clinical effect after dosing of the cat-specific formulation. The longer T_{max} with capsule formulation might have been caused by slower release of the active agent compared to the oral solution and thus somewhat slower absorption. However, both pharmacokinetic studies were done in a small number of cats and showed some inter-individual variation, which may be considered to be the major limitations of these studies.

Pregabalin is quite slowly eliminated from the body of cats with total plasma clearance of 0.03 l/h/min (EMA, 2021). Pregabalin $t_{1/2}$ in cats (III) was approximately twice as long as the one reported in humans (Bockbrader et al., 2010) and dogs (Salazar et al., 2009). This finding was supported by Esteban et al. (2018). In practice, this likely influences on the duration of effect after administration in cats compared to human use. In humans, pregabalin is primarily eliminated by kidneys and thus the dose in patients with reduced renal function needs to be adjusted depending on the creatinine clearance (EMA, 2023; FDA, 2023). Even though exact data of elimination route in cats is missing, most likely kidneys are the potential organ to excrete pregabalin in this species as well. No studies have been published about the elimination of pregabalin in cats with renal dysfunction, but with gabapentin significantly higher serum concentrations were measured in cats with chronic kidney disease after a 10 mg/kg dose than in healthy cats that received 20 mg/kg (Quimby et al., 2022). This suggests that a dose should be reduced in a cat population with renal dysfunction and based on the similar mechanism of action and excretion pattern, the same also likely applies to pregabalin.

Interactions with other medicines have not been studied in cats, but the effects of pregabalin are expected to be potentiated in case other medicines causing central nervous system depression, such as sedatives, are used. Thus, dose adjustment should be considered based on the clinical judgement (EMA, 2021). In study II, sedatives were used in a limited number of cats with no clinically abnormal findings. However, larger data would be needed to make any conclusions regarding the clinical interactions. In humans, no clinically relevant pharmacokinetic interactions or effect on clearance have been observed between pregabalin and

other anticonvulsant, pain and anxiolytic medicines, alcohol, insulin or diuretics (EMA, 2023). Based on the above, administration of a single dose of pregabalin in cats is unlikely to cause any major interactions with other medicines or affect on clearance. However, this remains to be studied further.

As the research targeted on registration of a new veterinary medicinal product, the study was conducted according to the EMA guideline (EMEA, 2000) setting frames to the obligatory final formulation pharmacokinetic studies. Formal sample size calculation was not performed but the number of purposed bred laboratory cats was estimated to be the minimum required to properly characterise the pharmacokinetic features of the novel formulation in cats and on the other hand prevent unnecessary numbers of laboratory animals to be used.

The EMA guideline also defines that clinically healthy animals under well-defined and controlled conditions are recommended to be included in the study and the Good Laboratory Practice guidance (OECD, 2021) should be preferably followed. The pharmacokinetic data of at least three doses need be provided based on adequate number and timing of sampling to allow determination of absorption, distribution and elimination of the medicine. Additionally, requirements for analytical procedures and pharmacokinetic calculations are described in the guidance. The frames of these regulatory requirements, especially related to the necessity to study at least three dose levels and to take several samples per an animal, create practical obstacles to studying pharmacokinetics at an adequate level in clinical studies. However, as an additional data the pharmacokinetic evaluation of pregabalin plasma concentrations in clinical patient cats would have provided valuable information in more variable population and probably also a hint of an anxiolytic plasma concentration. Thus, adding pharmacokinetic samples to study II would have increased the generalisability of the pharmacokinetic data within wider cat population.

6.7 Limitations of the studies

The major limitations of study I were the small sample size and the observed carry-over effect. Learning and habituation by the cat and the owner due to several treatment days with one-week intervals probably led to the observed carry-over

effect (I), which is often seen as an inherent problem in crossover studies (Ravina et al., 2005). The treatment order in study I was randomized to reduce bias caused by a possible carry-over effect, but it did not prevent it. When studying clinical behaviour, a parallel study design and larger study population might be more suitable. In a crossover study the carry-over could have been diminished with longer wash-out periods and the random inclusion of more than one placebo treatment day in the study. Study I was a pilot study with a limited number of patients, yet the dose selected to be further evaluated based on the data retrieved from it was confirmed with a larger number of animals and a parallel study design in the next phase of the development program (study II).

A joint limitation in both clinical studies (I, II) was the use of unvalidated scoring systems in assessment of the primary efficacy variables. Additionally, neither study included a more objective physiological stress variable, such as measurement of blood or saliva cortisol, to complement the subjective behavioural scoring system. Combining these methods would have strengthened the reliability of the results. Use of unvalidated subjective scoring systems brings a risk of systematic bias to the studies. Especially the owner subjectivity is difficult to prevent due to expected variation among the large number of owners. Veterinarians and external behavioural experts are smaller group of professionals, and it is easier to train them for correct and more precise use of the scoring system. Thus, it could be expected them to make the assessment more objectively than the owners. On the other hand, in both clinical studies all individuals responsible for assessing efficacy were blinded, which can be expected to equalise bias in both the treatment and control groups.

Placebo effect is a typically seen bias in all clinical trials. The mechanisms of the effect in animal studies have been explained through classical conditioning, expectancy, endogenous opiates and effect of human contact (review by McMillian, 1999). Behavioural studies with observational variables are prone to placebo effect even though standard preventive measures, such as use of control group and blinding all participants of the study, are used. The placebo effect was also seen in study II, where 27% and 30% of cats in the placebo group were assessed to have successful treatment effect by owners and investigators, correspondingly. Similar

effect was also seen in the external expert assessment. This level of placebo effect is in agreement with publications of other clinical studies (Munana et al., 2010; Conzemius and Evans, 2012; Korpivaara et al., 2017). In study II, the assessor's expectancy is likely the most important mechanism behind the placebo effect. Additionally, familiarity of the investigator and clinical examination procedure at the clinic could have somewhat reduced the anxiety through conditioning. However, as nontreated group was not included in the study, the true bias caused by placebo effect cannot be reliably estimated.

Study II was a multicentre, multi-country trial representing Northern, Southern, Eastern and Western European countries. That allowed a diverse collection of data including differences in local practices, environmental factors and population characteristics, and thus attempted to decrease the selection bias and bring some confidence on the study results. Additionally, both sexes were evenly presented, and the study population exhibited a relatively broad range of body weights and ages. However, majority of the cats were in the age group around five years, and the variation of different cat breeds was limited, as most of the cats were categorised to be domestic, i.e. not any known breed or mixed breed, or European shorthair. Some cat breeds that have undergone intensive selective breeding have low genetic diversity (Leroy et al., 2013). In humans it is known that genetic differences may lead to divergences in pharmacodynamic or pharmacokinetic processes between individuals (review by Rojgar, 2020), which may be seen as variable effect of medication or differences in adverse effects. Some deficiencies in drug metabolism have been identified also in cats, such as insufficient glucuronidation (review by Court, 2013) and individual genetic variation in metabolising enzymes (Lee et al., 2018). However, as in humans only minor part of pregabalin is metabolised through methylation pathway and majority is excreted unchanged, metabolic differences do not affect to the drug concentrations. In cats the metabolism of pregabalin is unknown, but minor effects could be expected, unless cats are metabolising pregabalin to a substantial extent trough the glucuronidation pathway.

Study II was performed in relatively healthy cats. Therefore, it lacked information regarding use of pregabalin in specific populations, such as cats with renal dysfunction, as well as interactions with other medicines. Further limitations are

that study II did not provide robust data regarding the duration of effect of pregabalin in anxious cats nor pharmacokinetic data in pet cats for evaluation of anxiolytic plasma concentrations. In this study, owners were asked to record the onset and end of any change or sign of effect, which does not give reliable information on the duration of the actual anxiolytic effect. To receive more information of the duration of effect, the cats should be followed more closely, and they should likely be predisposed to anxiety causing triggers periodically to see behavioural changes that could suggest decrease in anxiolytic potency of the treatment. However, study II was the first large scale study conducted on client owned cats with the objective of confirming the anxiolytic efficacy and clinical safety of the treatment. Therefore, the study procedures were limited to those that were relevant, and all potential disturbing factors, such as extra triggers of anxiety, were minimised. Additionally, the study focused on gathering key clinical efficacy and safety data for the registration of a new veterinary medicinal product, which restricted the inclusion of additional research questions and variables.

A thorough pharmacokinetic evaluation of the anxiolytic plasma concentration of pregabalin in cats in study II would have required multiple samples collected consecutively, which is usually impossible in clinical studies. Furthermore, taking into consideration that the cats were anxious and fearful, several samplings during the clinic visit would be even more unfeasible. In a clinical setting one blood sample taken at the estimated T_{max}, or at the same timepoint when the primary variable is assessed, could be considered practical to evaluate plasma concentration in client-owned cats. This kind of approach could have been taken also in study II. If the sampling had been done at the clinic immediately after investigator's assessment of the anxiolytic effect, it could have been used in a pharmacokinetic-pharmacodynamic analysis with e.g. Bayesian method (Lunn et al., 2002). Thus, it could potentially have provided information regarding the anxiolytic plasma level in cats in addition to knowledge of individual exposures. However, pharmacokinetic samples were not collected in study II mainly due to practical reasons. These included the need for sample storage under -70°C temperature at the study sites and frequent temperature-controlled shipments of frozen samples from each of the 22 sites across Europe during the nine-month recruitment period, and sample bioanalysis, within a limited stability time of pregabalin in the samples. Thus,

further clinical studies would be required to assess the anxiolytic plasma concentration of pregabalin in cats. However, it should be noted that this has not been determined in humans or other animal species either.

Due to the practical obstacles related to clinical studies, the pharmacokinetics of the novel pregabalin oral solution was studied in small number of laboratory cats. This caused the major limitations of study III, which are the small sample size and lack of pharmacokinetic data in diverse feline population. Study III was a typical pharmacokinetic study conducted according to the EMA guideline on pharmacokinetic studies in the target animal species (EMEA, 2000) for registration of a veterinary medicinal product. Six animals including both sexes are typically considered to be the minimum required to adequately characterise pharmacokinetic parameters, taking into consideration also the need to reduce the use of laboratory animals as much as possible due to ethical reasons. However, the relevance of the study was somewhat diminished due to a reduced number of cats in the final analysis, caused by incomplete exposure resulting from salivation. Yet, the variation of parameters within reasonable limits allowed interpretation of the results.

The healthy laboratory cats do not represent the variety of clinical population that could have systemic diseases, like diabetes, hyperthyroidism, or kidney dysfunction. Thus, further studies would be needed also in these patient populations to get more information regarding plasma concentrations in cats with systemic diseases.

6.8 Clinical implications and future studies

The clinical studies (I-II) have been the first ones published with pregabalin in anxious cats. Their results indicate that pregabalin with the clinical dose of 5 mg/kg is anxiolytic in cats showing signs of travel and veterinary visit related fear and anxiety. Based on these results a new pregabalin product has been registered in the European Union as the first anxiolytic product in cats. This new product has an in detail determined dose and well evaluated safety profile compared to the human anxiolytic medicines used off-label in cats. Furthermore, the adverse effects reported for a registered veterinary medicine are followed by the marketing

authorisation holder and changes in the safety profile are updated in the product literature and informed to the users in clinical practice.

The studies I, II and III were mainly conducted according to well established and traditional study designs and many of the study hypotheses could be based on earlier publications on gabapentin as an anxiolytic medicine used off-label in cats. However, in addition to the use of pregabalin as a new therapeutic substance, there are some aspects that bring new information to the field of veterinary medicine and contribute to advancing the veterinary behavioural medicine. Firstly, the novel efficacy variables for travel and clinical examination were developed and tested for the first time (I, II). Even though they are not formally validated, they seem to be able to separate the cats that benefitted of pregabalin treatment from those that did not or had received placebo. Secondly, the adaptive seamless design has not been typically used in veterinary clinical studies. In study II it was used, and it enabled selection of a clinical dose from two active dose levels as well as confirmation of the dose and its safety and efficacy is the same clinical trial. The conservative way of research usually forces to conduct these phases in separate clinical studies, which lengthens the research time and requires more investments.

The presented studies focused on one indication area, alleviation of acute anxiety. Due to the limited variation of the study population, future studies in various subpopulations with a wider range of ages, breeds and co-morbidities are warranted. Additionally, evaluating the use of pregabalin in cats for other indications besides alleviating anxiety would be an interesting topic for further research. Based on its use in humans (Goodman and Brett, 2019), treating chronic pain in conditions that include a neuropathic component would be a compelling area for future studies. In cats such diseases could include spinal cord injury, osteoarthritis, feline idiopathic cystitis, chronic gingivostomatitis, feline hyperesthesia syndrome and feline orofacial pain syndrome (review by Epstein, 2020). Additionally, the use of pregabalin in cats as an adjunctive therapy in epilepsy, if needed, could be a possible new area for use based on data in human use. These new indication areas would need further assessment of effective dose levels, dosing interval in chronic use, as well as clinical and safety data in larger scale studies to confirm that the benefit-risk ratio is positive. Furthermore,

interactions with other medicines used in these chronic conditions as well as safety and pharmacokinetics in cats with possible concomitant diseases or dysfunctions would need additional evaluation.

After a new medicine is registered and available for clinical use, academic studies using the product typically follow. These studies are highly appreciated as they can provide data from diverse patient populations, test alternative efficacy variables, and evaluate different indication areas. Since medicines are often initially studied in limited populations during development, mandatory follow-up of adverse event reporting from clinical patients provides necessary safety data from larger and more variable populations. All this gathered information enhances the knowledge for users, the veterinarians working with clinical patients.

7. Conclusions

The overall conclusion of this study was that the cat-specific pregabalin oral solution formulation effectively and safely alleviates acute anxiety and fear associated with transportation and veterinary visits in cats.

Based on publications I-III, it can be concluded that:

1. The developed scoring system with the numerical 5-point ordinal scale to assess the treatment effect during car transportation was able to find significant differences between pregabalin and placebo in both the owners' and the external expert's assessments. Additionally, the owners' and external behaviourist's assessments agreed significantly (I, II). The veterinarian's assessment of the treatment effect was able to show a statistically significant difference between the pregabalin and placebo groups during clinical examination at the clinic (II). Therefore, both variables could be considered reliable for the assessment of anxiety in pet cats (I, II).

2. The efficacy of the 5 mg/kg pregabalin dose was significantly superior compared to the lower 2.5 mg/kg dose in acute anxiety and fear associated with transportation and veterinary visits (II). The anxiolytic efficacy of the 5 and 10 mg/kg doses during transportation did not differ significantly, but less AEs were observed after administration of the 5 mg/kg dose compared to 10 mg/kg (I). Thus, pregabalin 5 mg/kg dose was selected as a clinically anxiolytic but non-sedative dose in anxious cats for transportation and veterinary visits.

3. The anxiolytic properties of pregabalin with the 5 mg/kg dose were measurable and statistically significant compared to placebo, as well as clinically relevant in cats with acute anxiety and fear associated with transportation and veterinary visit (II). There were no clinical safety concerns with the 5 mg/kg dose (II). Pregabalin decreased the major signs of anxiety and fear associated with car transportation and veterinary visits in cats (I, II). Thus, the clinical efficacy and safety of the selected pregabalin dose for the proposed indications was confirmed.

4. The pharmacokinetic profile of the novel pregabalin 50 mg/ml oral solution formulation for cats was described in healthy laboratory animals (III). The results show the fast absorption, linear pharmacokinetic profile and high oral bioavailability of the formulation.

8. Acknowledgements

This work was carried out at Orion Corporation. Orion has been my employer for over 20 years, and I am grateful for this opportunity to make my thesis based on the studies conducted for the marketing authorisation of Bonqat 50 mg/ml oral solution for cats. That project was my main work in 2018-2021.

I am deeply grateful to my supervisors Docent, PhD Marja Raekallio from the University of Helsinki and Director of Animal Health Clinical Development, PhD Mirja Huhtinen from Orion, as well as Professor emerita, PhD Outi Vainio who acted as my supervisor until her retirement from Helsinki University. Your knowledge, support and encouragement have led me forward during these years.

My gratitude extends to other members of my thesis committee: Docent Timo Lotta and Docent Ulrike Lyhs. Your guidance and expertise have greatly helped my journey in academic world.

I want to thank my pre-examiners, Professor Anouck Haverbeke from Ghent University and Professor Jonathan Elliott from the Royal Veterinary College for review of this thesis and their valuable comments. Furthermore, I would like to thank Professor Simona Cannas for accepting the invitation to be my opponent.

My warm thanks to all my co-authors of the three articles included in this thesis: Mira Korpivaara, Minna Suokko, John Aspegrén, Minja Hyttilä-Hopponen, Janne Kaskinoro, Anne Doedée, Clara Palestrini and Karen Overall. Your input has been great and invaluable. I want to also thank Timothy Wilson for revising and correcting the language of this thesis.

This work would not have been possible without my fellow-workers at Orion. Development of new medicines is always teamwork. My deepest gratitude goes particularly to the colleagues and friends in the project and clinical study teams that year after year worked devotedly towards a shared goal. My warmest thanks to Ken, Jaana, Minja, Heidi, Noora, Tarja, Johanna, Taru, Suvi, Mira, Minna, John, Helena, Riikka, Iris, Seppo, Marjukka, Sanna, Anssi, Mikko, Catarina, Sari and last but not the least Mari, my “best forever” Clinical Study Manager, who always had the situation under control. I am also grateful to my former and current superiors at

Orion; Mirja and Lasse, you have understood and encouraged me to pursue my dream of making a doctoral dissertation and helped to pave the way for it to come true.

My heartfelt thanks go to all the veterinarians who acted as investigators and all cats and their owners who participated to the studies. Without your interest for clinical trials and valuable work during them, the development of new veterinary medicines would not be possible.

I also want to mention Markus “Mako” Sandholm, the late professor of pharmacology in the old College of Veterinary Medicine at Hämeentie. His inspiring lectures in a semicircular lecture hall in the early 1990’s got me interested in pharmacology, and that interest began to bloom when I joined Orion and started my career in the development of veterinary medicines.

Olen kiitollinen äidilleni sekä isälleni, joka menehtyi pandemian aikana. Olette antaneet minulle halun ja ilon oppia uutta. Se tuo elämään paljon sisältöä ja ennenkokemattomia mahdollisuuksia. Lisäksi olen saanut teiltä hyvät geenit, jotka ovat auttaneet minua sekä pitkäjänteisessä puurtamisessa että vastapainoa antaneessa kestävyysurheilussa. Kiitos kaikesta!

Thank you, my dear sisters Maija, Essi, Leena and my brother Heikki. Your example and company are valuable and always carry me forward. I can trust that you are always there. Thanks also to my colleagues and friends, Mia and Mari. We have known each other from the very first day at Hämeentie and the joint years and experiences have brought us close together.

Finally, I thank with all my heart my family: Arto, Ida and Anna. I am sorry that many times I have been physically present, but my mind has wandered around my work. Still, you have been able to keep me grounded in everyday life and your company. You girls are wonderful. Believe in yourself and do what you most want, and you will achieve your goals. Arto, my loved husband, friend and life-mate – thank you for listening, for the discussions, understanding and help with computers. Without you this thesis project would not have happened.

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ORIGINAL RESEARCH
published: 01 September 2021
doi: 10.3389/fvets.2021.711816

Efficacy of a Single Dose of Pregabalin on Signs of Anxiety in Cats During Transportation—A Pilot Study

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OPEN ACCESS

Edited by:
Janice Siegford, Michigan State University, United States

Reviewed by:
Judith L. Stella, Animal and Plant Health Inspection Service (USDA), United States
Louise Anne Buckley, University of Edinburgh, United Kingdom

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Specialty section:
This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science

Received: *19 May 2021*
Accepted: *28 July 2021*
Published: *01 September 2021*

Citation:
Lamminen T, Korpivaara M, Suokko M, Aspegrén J, Palestrini C and Overall K (2021) Efficacy of a Single Dose of Pregabalin on Signs of Anxiety in Cats During Transportation—A Pilot Study. Front. Vet. Sci. 8:711816. doi: 10.3389/fvets.2021.711816

Objectives: The aim of this clinical pilot study was to evaluate the dosage, efficacy, and clinical safety of a single oral dose of pregabalin in cats that experience fear and anxiety when placed into a carrier and transported by car.

Methods: Thirteen client-owned cats were enrolled in a blinded, randomized, crossover study with three treatment days approximately 1 week apart. The cats were assigned to receive pregabalin oral solution at dosages of 5 and 10 mg/kg and placebo in a randomized order, one treatment per week. Treatment was administered ∼90 min before placing the cat into a carrier and starting transportation. Efficacy was assessed by the owners using a categorical scale and, based on video recordings, by an external observer, both blinded to the treatment.

Results: Owners assessed that cats given pregabalin displayed less vocalization, restlessness, and panting during transportation than did cats given placebo. Correlation between owners' and external observer's assessment of the overall treatment effect was good (0.63, $p < 0.01$), which confirms the owners' ability to observe reliably their own cat's behavior. Transient mild ataxia was the most common adverse event reported. The human commercial formulation used in this study was found difficult or very difficult to administer by 79% of the owners.

Conclusions and Relevance: Based on results of this pilot study, a single oral dose of pregabalin was well tolerated and decreased signs of anxiety and fear associated with car transportation in cats, as evaluated by blinded owners and external observer. The use of pregabalin prior to traveling may improve cat welfare and compliance for transportation. Further studies are needed to investigate the use of oral pregabalin in cats to alleviate signs of anxiety and fear associated with transportation and sequelae, like veterinary visits, and to develop a more user-friendly formulation.

Keywords: feline, fear, travel, carrier, clinical, pregabalin, anxiety, transport

INTRODUCTION

Cats are common pets, occupying approximately 25% of households in the United States (1) and Europe (2). However, there is concern that cats often lack both adequate preventative and acute veterinary care (3, 4). One of the main reasons for this welfare concern is that cats are often too challenging to transport (3, 4). Based on a survey by Grigg et al. (5), anxiety and fear related to travel or to the carrier used for travel has been reported as the most prevalent behavior problem by 67% of the cat owners. In another owner survey, Mariti et al. (6) showed that 59% of cats were reported to exhibit signs of distress during car travel and 66% during veterinary visits. Pre-visit anxiolytic medications can be prescribed to make transport and veterinary visits less stressful for feline patients. The goal of pharmacologic intervention is to reduce anxiety during transportation and to enable patient-friendly, low-stress, physical examination (7). Currently, no licensed anxiolytic drugs are available for cats in the EU or the USA. Recently, studies concerning extra-label use of trazodone (8) and gabapentin (9) to reduce transport- and veterinary examination-related anxiety in cats have been published.

Pregabalin is a structural analog of neurotransmitter gamma-aminobutyric acid (GABA). Its mechanism of action is similar to gabapentin, but it is more potent and has favorable pharmacokinetic properties (10). The mode of action of pregabalin differs from benzodiazepines and other anxiolytic agents. It binds to the alpha-2-delta subunit of the voltage-dependent calcium channel in the central nervous system and decreases the release of several neurotransmitters, including glutamate and monoaminergic neurotransmitters, which are implicated in the pathophysiology of anxiety (11). A dose-dependent anxiolytic effect of pregabalin has been demonstrated in rodent models (12–14), and in humans (15) but not yet described in cats.

The objective of this pilot study was to evaluate the dosage, efficacy, and clinical safety of pregabalin in cats that are challenging to place into a carrier and/or distressed when transported by car.

MATERIALS AND METHODS

This study was a randomized, blinded, placebo-controlled, single dose, crossover study. The study procedures were performed at home by the cat owners. The study protocol was approved by the competent regulatory authority in Finland (Finnish Medicines Agency Fimea). The study was conducted according to the study protocol and the principles of Good Clinical Practice as defined by the Veterinary International Conference on Harmonization (VICH) Guideline (GL) number 9, and informed consent was obtained in writing from the owners prior to enrolment. The welfare, treatment, and care of the study animals were ensured by veterinary supervision, and the owners were able to contact the investigating veterinarians at any time during the study.

Animals

Client-owned cats were screened during a phone interview by the investigating veterinarians at Orion Pharma, using an owner-directed questionnaire (**Supplementary Table 1**). All cats admitted to the study were identified as healthy (American Society of Anesthesiologists status I or II) by a practicing veterinarian chosen by the owner. Cats aged at least 1 year and weighing a maximum of 8 kg were eligible for the study. Because the human formulation of oral pregabalin used here had a concentration of 20 mg/ml, cats heavier than this would have had to be given large volumes, which could have led to poor compliance. Cats had to have a history of either being challenging to place and keep in a carrier or fearful and/or anxious when transported by car. These behaviors were verified at the baseline. Cats were excluded from participating if they were being treated with other psychoactive medications, homeopathic remedies, pheromonal products, supplements, or a special diet to control anxiety. Other reasons for exclusion were pregnancy, lactation, concurrent participation to any other clinical study, and any other condition or situation which could disturb the conduct of the study, for example, owner's inability to administer the study products or conduct the car transportations.

Treatments

At baseline, behavioral assessments and a health check were performed. Cats were also given tap water orally using a syringe to mimic the study procedure. If eligible, a cat was enrolled and treated on three separate days, with wash-out periods of 8 ± 2 days between each treatment day (**Figure 1**). The cats received pregabalin oral solution (Lyrica, Pfizer), at a dosage of 5 and 10 mg/kg, and placebo, matched to the active treatment regarding taste, odor, and appearance. The treatments were administered in a randomized order using a three-period, three-treatment Williams crossover design. Randomization was conducted by an independent randomization specialist before the study start using computer software. The dosages were selected based on a previous non-clinical study in laboratory cats conducted by Orion Pharma. The study treatments were administered directly into to the cat's mouth with a syringe at home by the owner, who was blinded to the study treatments. The treatments were administered without food, but a small treat could be given to the cat after dosing. The owner was trained by the investigators to administer the study product and received written dosing instructions and a diary to report the study treatment administrations, assessments, and observations. An end-of-study contact followed 1 to 3 days after the last treatment day.

Assessments

At baseline and during the three treatment days, 90 ± 15 min after study treatment administration, the cats were placed into a carrier and transported in a car for 20 min. Treatment administration, placing the cat into the carrier, transportation, opening the carrier after return, and a short time at 60 min after return were video recorded by the owner. Individuals making efficacy assessments, that is, owners and an external observer, were blinded to the study treatment.

The owner assessed the efficacy during the treatment days compared to baseline on the following variables: the overall effect of the study treatment during each treatment day, the ability

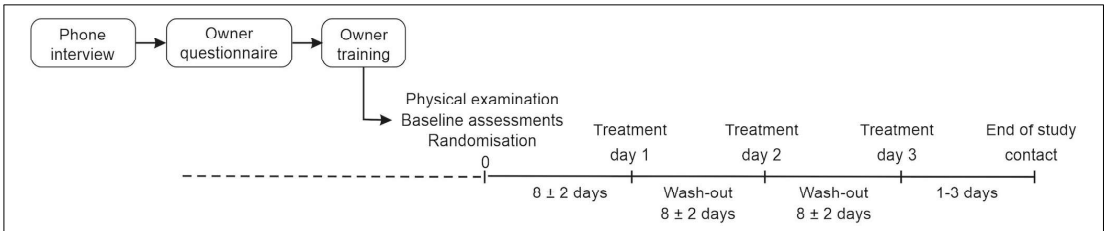

FIGURE 1 | Study design of the crossover pilot clinical study. Pregabalin at a dose of 5 and 10 mg/kg and placebo were given in a randomized order to cats on three treatment days.

TABLE 1 | Rating of the signs of distress, anxiety, and/or fear by the cat owner.

Numerical rating	Severity
0	Absent
1	Mild
2	Mild
3	Moderate
4	Moderate
5	Severe
6	Severe

to perform the procedures (placing the cat into the carrier at home and transportation of the cat in a car), and signs of stress, anxiety, and/or fear when placing the cat into a carrier and during transport. Additionally, the owners assessed the cat's activity and the usability of the product. Adverse events were recorded by the investigators based on owner interview and video observations.

Categorical scales were used in the assessments. Treatment effect was scored as "excellent," "good," "some effect," "no effect," or "worse." The ability to place the cat into the carrier was scored as "excellent," "good," "fair," "poor," "very poor," or "not possible." For transportation in a car, a scale of "excellent," "good," "fair," "poor," and "very poor" was used.

The signs of distress, anxiety, and/or fear included vocalization, abnormal activity/restlessness/pacing, resistance/destructive behavior, escaping/evading/hiding, inappropriate urination, inappropriate defecation, panting/intense breathing, vomiting, licking/self-grooming, freezing/decreased motor activity, salivation, and sweaty paws. The severity of these signs was rated each as "absent," "mild," "moderate," or "severe" (**Table 1**). The sum of the signs was calculated over all signs using their numerical severity rating.

To assess the potential sedative effect of the treatment, the cat's activity was scored as "very calm/sleeping," "calm," "neutral," "active," or "very active." The usability of the product was assessed using a scale of "very easy," "easy," "somewhat difficult," and "very difficult."

An external, independent, trained observer blinded to the owner assessment and the study treatment assessed the overall efficacy during each treatment day compared to baseline based on video recordings. The external observer scored the effect of the study treatment using the same rating scale as the owner. In addition, signs of anxiety and fear were assessed by the external observer either by frequency or duration depending on their nature according to an ethogram she created suitable for video assessment of the cats (**Table 2**).

Statistics

Due to the exploratory nature of this pilot study, neither a formal statistical hypothesis was defined nor a formal sample size calculation was performed. All cats entered into the study that received at least one dose of the study treatment were included in the analyses. Commercially available software SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA) was used.

All efficacy variables were analyzed in terms of absolute and/or change from baseline, and/or sum scores. Pregabalin was compared to placebo using a generalized linear model for ordinal data, with a cumulative logit as a link function, in which treatment and day were used as fixed effects. The correlation within cats was modeled as a repeated effect. Analyses were adjusted for a carry-over effect when a significant carry-over effect was found. The results were reported as odds ratio (OR) with 95% confidence interval (CI). The correlation between owners' and external observer's assessments of the overall effect of the study treatment was calculated using Spearman's rank-order correlation coefficient (r_s). The change from baseline in individual signs and the sum of signs of distress, anxiety, and/or fear assessed by the owner was reported with mean (95% CI) and analyzed with a linear mixed model. The reliability of the external observer was assessed by a board-certified behaviorist using randomly selected 20% of the recordings using r_s. The external observer's assessment of the duration or frequency of each sign was separately analyzed with a linear mixed model. Baseline data were used as a covariate, when available. Differences were considered to be statistically significant with $p < 0.05$.

RESULTS

No significant differences were found between pregabalin 5 and 10 mg/kg groups in efficacy analysis (**Supplementary Table 2**). Therefore, the efficacy results are reported as pregabalin groups combined.

TABLE 2 | The ethogram used by the external observer for video assessment of cats.

Signs assessed by duration (seconds)	Description
Active interaction	Any behavior performed when interacting with the owner including active physical contact, sniffing, rubbing, close visual inspection, and gentle oral examination such as licking
Avoiding pet carrier	Get stuck, try to escape/break free while the owner tries to put the cat into the pet carrier (active resistance, but not aggressive—not biting, no scratching, no hissing)
Crouched position	Crouching. A pronounced lowering of the posture
Ears flattened	Ears flattened and back
Eating	Eating food
Exploration	Motor activity directed toward physical aspects of the environment, including sniffing, and gentle oral examination such as licking
Eyes closed	Sitting, standing, or lying down (the head does not rest on the ground) with eyes closed
Grooming	Action of cleaning the body surface by licking, nibbling, picking, rubbing, scratching, etc., directed toward the animal's body (self-grooming)
Hiding	Hiding
Locomotion	Walking around without exploring the environment
Not visible	Not visible (during these periods, activities like vocalizing, scratching, and chewing were identified and recorded by the sound of the activity)
Oriented to the environment	Sitting, standing, or lying down (the head does not rest on the ground) with obvious orientation toward the physical or social environment, including sniffing, close visual inspection, distant visual inspection (vigilance or scanning)
Passive behavior	Lying down with the head on ground without any obvious orientation toward the physical or social environment
Passive interaction	Sitting, standing or lying down during owner interaction or manipulation
Play	Any vigorous or galloping gaited behavior directed toward a toy; including chewing, biting, shaking from side to side, scratching or batting with the paw, chasing rolling balls, and tossing using the mouth. Although the cat may take the objects into its mouth, destruction is not included in this category
Pupils dilated	Mydriasis
Purring	Purring
Panting	Increased frequency of inhalation and exhalation often in combination with opening of mouth
Scratching	All active behaviors resulting in physical contact with the cage/door, including scratching the cage/door with the paws, jumping on the cage/door, handling with the forelimbs
Salivating	Salivation
Tail close to body	Lowered position of tail close to the body
Vocalization	Any form of vocalization, including: meowing, moaning, mewing, etc.
Withdraw	Avoiding interaction with the owner by running, moving away, very clearly turning away, or looking away
(Continued)	

TABLE 2 | Continued

Signs assessed by frequency (count)	Description
Elimination	Defecation or urination in sitting or standing position
Lip licking	Part of tongue is shown and moved along the upper lip
Shake off	Shaking the body to release stress
Swallowing	Swallowing
Vomiting	Vomiting
Yawning	Yawning

Animals

Fifteen cats were screened for the study. Two cats were withdrawn before administration of the first study treatment, leaving 13 cats that were enrolled. Seven cats were female and six were male. Their median (range) age was 3.3 years (1.0 to 8.9 years), and weight was 4.2 kg (4 to 7 kg). All cats, except one female, were neutered. Two cats discontinued the study after the first treatment day: one because the cat could not be placed into the carrier, and the other which had access to outdoors. Thus, 11 cats received all the study medications and the 2 cats received only one dose of pregabalin. The mean (min, max) actual dose was 5.1 mg/kg (4.8 mg/kg, 5.3 mg/kg) and 10.0 mg/kg (8.1 mg/kg, 10.7 mg/kg) for 5 and 10 mg/kg doses, respectively. In 29% (10/35) of the administrations the cat salivated or spilled out part of the dose. However, the overall treatment compliance was good as 71% (25/35) of the treatment administrations were successful.

Owner Assessments

Overall, 64% (14/22 dosings) of the pregabalin treated cats (5 and 10 mg/kg groups combined) and 9% (1/11) of the placebo treated cats scored "excellent" or "good" for the overall effect of the study treatment (**Figure 2**).

The owners' assessment of the treatment effect (pregabalin groups combined vs. placebo) was significant [OR 4.2 (95% CI 1.1–16.6); $p = 0.04$]. However, a statistically significant carry-over effect was observed, as the cats randomized to receive pregabalin in the first or second period scored better results in the following period(s). When the model was adjusted for carry-over, the treatment effect was no longer significant [OR 2.4 (95% CI 0.6–8.9); $p = 0.21$].

In cats treated with pregabalin (groups combined), 83% (19/23) scored "excellent" or "good" in the ability to place the cat into the carrier compared to 73% (8/11) of the placebo treated cats. There was no statistically significant treatment effect [OR 1.3 (95% CI 0.6–3.0); $p = 0.48$].

In the ability to transport the cat in a car, 59% (13/22 dosings) of the pregabalin treated cats (groups combined) and 27% (3/11) of the placebo treated cats scored "excellent" or "good." The treatment effect was not statistically significant [OR 2.8 (95% CI 1.0–8.3); $p = 0.06$].

Figure 3 presents model-based estimates of mean (95% CI) change from baseline of sum of owner's assessment of signs of

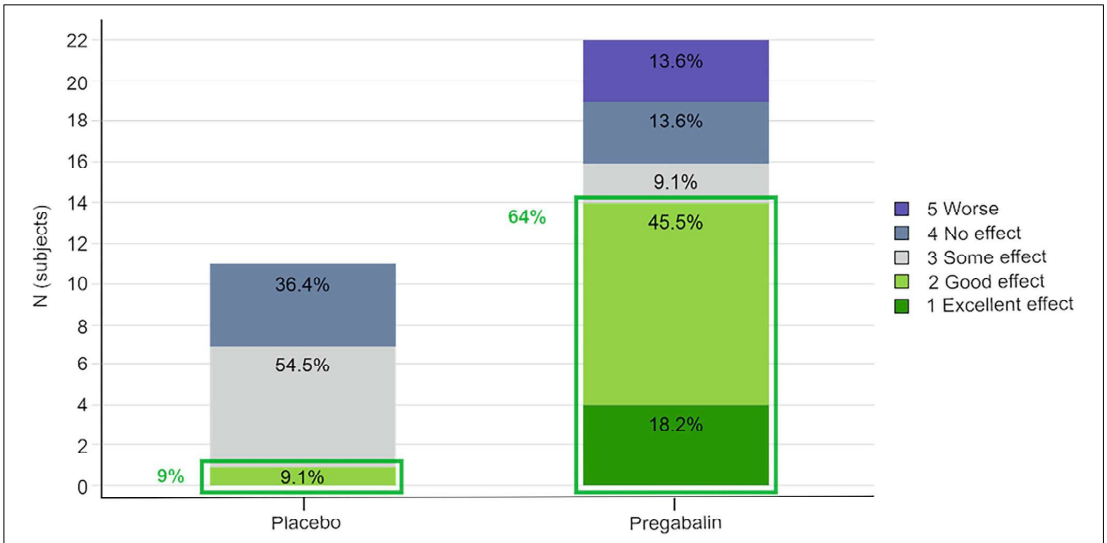

FIGURE 2 | Owner's assessment of the effect of the study treatment. Pregabalin 5 and 10 mg/kg groups combined.

distress, anxiety, and/or fear. A significant ($p < 0.01$) decrease in sum of owner-rated signs of distress from baseline was seen when placing the cat into the carrier for both pregabalin and placebo, but this effect was seen only for pregabalin during transportation. A significantly larger decrease from the baseline in the sum score was seen for pregabalin compared to placebo for placing the cat into the carrier with mean decrease of −2.5 [(95% CI −4.3 to −0.7); $p = 0.01$]. This effect was not seen during transportation [−3.8 (95% CI −8.7 to 1.1); $p = 0.12$].

In the evaluation of individual signs of anxiety, fear, and stress during transportation, there was significantly less vocalization [mean −1.3 (95% CI −1.9 to −0.7); $p < 0.01$], abnormal activity/restlessness/pacing [mean −0.7 (95% CI −1.2 to −0.2); $p = 0.01$], and panting/intense breathing [mean −0.9 (95% CI −1.7 to −0.1); $p = 0.03$] after pregabalin treatment compared to placebo. There was significantly less vocalization after pregabalin treatment when placing the cat into the carrier [mean −0.6 (−1.0 to −0.2); $p < 0.01$].

After receiving pregabalin, cats were statistically significantly less active compared to placebo when placed into the carrier [OR 4.1 (95% CI 1.0–16.2); $p < 0.05$]; however, this effect was not found during transportation [OR 2.8 (95% CI 0.5–16.3); $p = 0.24$].

The owners assessed the administration of the product to be "difficult" or "very difficult" in 74% (25/34) of all administrations, while 26% (9/34) reported it to be "easy" or "very easy." The volume of the administered product varied from 0.5 to 3.5 ml based on the dose and cat's weight. The difficulties in administration were not related to the dosing volume.

External Observer Assessments

There was a significant correlation between the owners' and the external observer's assessment on the effect of the study treatment ($r_s = 0.63$, $p < 0.01$). The owners tended to assess the study treatment to be effective more often than did the external observer. Based on the video assessment, cats showed significantly less vocalization [mean −111.0 (95% CI −169.8 to −52.1); $p < 0.01$] and swallowing [mean −5.8 (95% CI −8.7 to −3.0); $p < 0.01$] and significantly more hiding [mean 0.2 (95% CI 0.1–0.3); $p < 0.01$] and passive interaction [mean 24.7 (95% CI 9.3–40.1); $p < 0.01$] when given pregabalin compared to placebo.

The inter-observer reliability was statistically significant for 17 of the signs in which r_s ranged from 0.65 to 0.94 ($p < 0.05$) and insignificant for 4 of the signs with r_s 0.10–0.59. For the remaining signs, sparse data precluded meaningful analysis.

Safety

No serious adverse events were reported. The most common adverse event was transient ataxia, which was assessed as mild in 7 (58%) and 5 (42%) cats and as moderate in 1 (8%) and 7 (58%) cats with the doses 5 and 10 mg/kg, respectively. Muscle tremor and anxiety were reported once each (8%) with 10 mg/kg. There was no connection with the actual dose (mg/kg) and severity of ataxia. All adverse events had resolved by the next day when the investigators contacted the owners. No adverse events were reported with placebo.

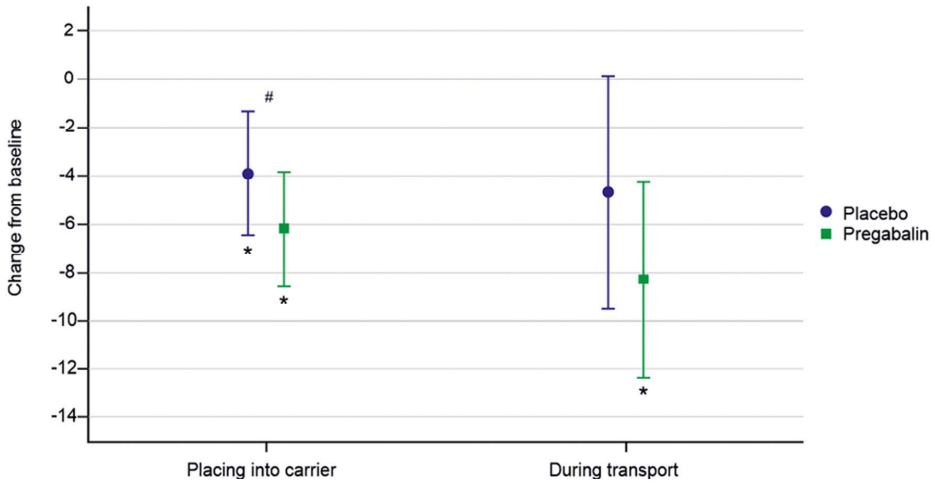

FIGURE 3 | Model-based estimates of mean (95% CI) change from baseline in owner's assessment of signs of distress, anxiety, and/or fear. Pregabalin 5 and 10 mg/kg groups combined. *Indicates a significantly different value compared to baseline. #Indicates a significant difference between treatments.

DISCUSSION

Despite the recommendation by the veterinary associations that every pet should have at least one visit annually for preventive care (4, 16), ∼45% of cat owners do not take their cat to a veterinarian (17), most likely due to difficulty in transporting the cat (3, 4). As a significant decrease in some signs of anxiety and fear was observed, this pilot study suggests that a single dose of pregabalin given by the owner at home could make the transportation easier for the cat and the owner and thus enable regular veterinary visits. However, as this was a small study, the results warrant a clinical study in a larger population of anxious cats.

Vocalization decreased substantially after pregabalin treatment compared to placebo during transportation and was likely the easiest for the owners to assess. Restlessness and panting were also significantly reduced. According to the external observer's assessment, vocalization and swallowing decreased the most when receiving pregabalin. These findings are in agreement with the signs of fear and anxiety reported for cats also in other studies (18–20).

As the external observer assessed the cat's behavior using a predefined ethogram, somewhat different signs were assessed compared to the owner. However, both the owners and external observer noted the same improvement in vocalization, which was the most prevalent sign observed. Hiding was only observed in a few cats, so the small increase in hiding (less than a second) in cats receiving pregabalin, although statistically significant, is likely not clinically or biologically relevant.

Owners assessed the treatment effect for the ability to place the cat into the carrier separate from that of the car transportation. When comparing pregabalin to placebo, a hint of positive treatment effect could be seen for transportation, which was supported by significant decrease in some signs of anxiety and fear after pregabalin treatment. In this population, based on the baseline data, it is clear that the main problem was transportation and not placement into the carrier. The demanding study design included several car rides for the cat with 1-week intervals, so owners who struggled with placing cats into carriers appear to have elected not to participate. A parallel design study with only one treatment period per cat would likely relieve this impediment. In general, a high number of trips has been reported to decrease owner willingness to participate in clinical studies (21).

Generally, owner observations of their pet's behavior, when they are instructed about the behaviors to observe, can be regarded as reliable (22–25). This pattern was shown in this pilot study. There was good correlation between owners' and the external observer's assessments of the overall treatment effect. Given the objectivity of the blinded external observer's assessment when evaluating the videos and the positive inter-observer reliability, owner assessment was deemed reliable and valid.

The two doses of pregabalin did not differ from each other significantly regarding efficacy; however, in safety assessments some differences were observed. Overall, pregabalin was well-tolerated, and the 5 mg/kg dose demonstrated a superior safety profile to the higher dose in the number and severity of adverse events.

The most frequently reported adverse event was transient ataxia, which is also reported with gabapentin (9). Esteban et al. (26) have studied pharmacokinetics of a single dose of pregabalin

in oral capsules in healthy cats. In that study a dose of 4 mg/kg produced plasma concentrations in the cats that were reported to be similar to those considered efficacious for control of seizures in human patients with epilepsy. However, 4 of the 6 cats in that study showed signs of moderate sedation (26). Sedation is a known side effect in human patients treated with pregabalin (27). Signs related to the potential sedative effect, like ataxia and decreased activity, were observed also in our study. However, the signs associated with any sedative effect in our clinical study in client-owned cats were mostly mild, which may be due to the presence of travel-related anxiety. In the study by Esteban et al. (26) the cats were non-anxious, healthy, laboratory animals accustomed to handling and their familiar surroundings, which may be the reason for more severe signs of sedation at the lower dose reported in the study.

The usability of the formulation licensed for humans used in this study was poor, as most of the owners assessed it to be difficult or very difficult to administer. The administered volume did not seem to constitute the main difficulty in administration, but the taste and/or odor of the commercial strawberry flavor used in the human formulation may not be pleasant for cats, who generally prefer fish, liver, meat, yeast, and sour/acidic flavors (28).

The major limitations of the study are the small sample size and the observed carry-over effect. Learning/habituation by the cat and the owner due to several treatment days with 1-week intervals probably led to the observed carry-over effect, which is seen as an inherent problem in crossover studies (29). When studying clinical behavior, a parallel study design and larger study population might be more suitable. In a crossover study the carry-over could have been diminished with longer wash-out periods and including more than one placebo treatment day randomly in the study.

Conclusions

This clinical pilot study showed that pregabalin decreases some signs of anxiety and fear associated with car transportation in cats. Such effects may improve the welfare of cats and aid owners in bringing their cat to the veterinarian. The clinical safety of pregabalin in client-owned cats was good, but the user-friendliness of the commercial human formulation used in this study was poor. A feline-specific formulation and further clinical data are warranted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was conducted in Finland and the clinical trial application was approved by the Finnish Medicine Agency (Fimea). The Directive 2010/63/EU on the protection of animals used for scientific purpose does not apply to veterinary clinical trials required for the marketing authorization of a veterinary medicinal product. Separate Ethical Committee approval was therefore not needed. The study was conducted according to the principles of Good Clinical Practice as defined by the VICH GL 9. Informed written owner consent for the use of each animal was obtained prior to any study-specific procedures. The welfare, treatment and care of study animals were ensured by veterinary supervision.

AUTHOR CONTRIBUTIONS

TL, MK, and MS contributed to the conception, design, and conduct of the study. JA contributed to the design of the study and performed the statistical analysis. CP contributed to the design and conduct of the study. KO contributed to the conception and design of the study. TL wrote the first draft. MK, MS, JA, CP, and KO contributed to writing and editing. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This study was sponsored by Orion Pharma.

ACKNOWLEDGMENTS

The authors thank the study team and the cat owners. The authors also thank Stig Sundberg for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.711816/full#supplementary-material

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Conflict of Interest: This study received funding from Orion Pharma, which is the employer of TL, MK, MS, and JA. The funder had the following involvement with the study: TL, MK, and MS: conception, design and conduct of the study, and writing. JA: design, statistical analysis and writing. CP (design, conduct of the study and writing) and KO (conception, design of the study and writing) are paid consultants of Orion Pharma.

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Article

Pregabalin Alleviates Anxiety and Fear in Cats during Transportation and Veterinary Visits—A Clinical Field Study

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Simple Summary: Cats are often anxious during travel and veterinary visits which can lead to a lack of veterinary care. In this study, a novel pregabalin 50 mg/mL oral solution was tested in 209 cats suffering from anxiety. The cats were given either flavored pregabalin solution or an identical placebo solution without pregabalin 90 min before transporting them in a car for at least 20 min to a veterinary clinic. The effect of the treatment during transportation was evaluated by the cat owner and during clinical examination by the veterinarian. Neither the cat owner nor the veterinarian knew which treatment the cat had received. Both travel- and veterinary-visit-related anxiety were significantly decreased in cats that had received pregabalin. Treatment was well tolerated. Only few cats showed slight incoordination and tiredness for a short time. The owners found a small volume o flavored oral solution user-friendly. It was also well accepted by the cats. This study showed that a single oral dosage of the novel pregabalin solution alleviates anxiety and fear related to transportation and veterinary visits in cats, thus aiding both owners and veterinarians by enabling cat-friendly handling and improving the welfare of cats in stressful situations.

Abstract: Cats frequently suffer from anxiety related to travel and veterinary visits. One sequela is avoidance of veterinary visits and lack of adequate veterinary care. The objective of this study was to test clinical efficacy and safety of a novel formulation of a pregabalin 50 mg/mL oral solution for alleviation of anxiety and fear in cats during transport and veterinary visits. A total of 209 client-owned cats were given either a flavored pregabalin oral solution at the dosage of 5 mg/kg (n = 108) or an identical placebo (n = 101) approximately 90 min before placing them into the carrier and transporting them in a car for at least 20 min to a veterinary clinic. The treatment effect using a 5-point numerical rating scale was evaluated during transportation by the owner and during clinical examination by the veterinarian, both blinded to the treatment. In addition, to verify the owner assessment, an external expert blinded to the treatment and owner assessment evaluated the transportation video recordings using the same rating scale as the owner. Pregabalin 5 mg/kg statistically significantly decreased both travel- ($p < 0.01$) and veterinary-visit- ($p < 0.01$) related anxiety compared to the placebo. The external expert's evaluation was in agreement with the owners' assessment confirming the treatment effect during transportation ($p < 0.01$). Treatment was well tolerated with only a few cats showing transient slight incoordination and tiredness. The flavored oral solution formulation with a small dosing volume of 0.1 mL/kg was found by the owners to be user-friendly and was well-accepted by the cats. This study demonstrated that a single oral dosage of the novel pregabalin oral solution alleviates anxiety and fear related to transportation and veterinary visits in cats, thus providing practical aid for both owners and veterinarians to enable cat-friendly handling and improving the welfare of cats in situations they often perceive as very stressful.

Keywords: feline; pregabalin; anxiety; fear; veterinary visit; clinical examination; transportation

Citation: Lamminen, T.; Korpivaara, M.; Aspegrén, J.; Palestrini, C.; Overall, K.L. Pregabalin Alleviates Anxiety and Fear in Cats during Transportation and Veterinary Visits—A Clinical Field Study. *Animals* **2023**, *13*, 371. https://doi.org/10.3390/ani13030371

Academic Editor: Mandy Paterson

Received: 31 October 2022
Revised: 22 December 2022
Accepted: 18 January 2023
Published: 21 January 2023

1. Introduction

Anxiety and fear associated with transportation and veterinary visits is a well-known challenge among cat owners [1–4]. Based on the results of a cat owner survey by Mariti et al. (2016) [5], most cats show impaired welfare during all stages of a clinic visit: before entering the waiting room, moving to the examination room, on the examination table, and after returning home. Distress worsens with every further experience and has a compounded negative effect on traveling and handling in other situations [5]. As many cats aggressively resist being placed into a carrier and show signs of distress when transported and during veterinary visits, many cat owners defer taking their cat to the veterinarian.

According to a veterinary care usage study by Volk et al. (2011) [2], 40% of cats had not been seen by a veterinarian within the past year, compared to only 15% of dogs. Similar results are shown in another survey, in which 44.9% of cat owners did not take their cats to a veterinarian, despite the recommendation of an annual preventive care visit [6]. Therefore, cats are likely to be more seriously ill before veterinary care is sought.

Pregabalin is a structural analogue of the neurotransmitter gamma-aminobutyric acid (GABA) and binds to the alpha-2-delta subunit of the voltage-dependent calcium channel in the central nervous system [7]. It decreases the release of glutamate and monoamine neurotransmitters involved in the pathophysiology of anxiety [8,9]. At the brain level, attenuation of fear-related activation of the amygdala and anterior insular cortex contributes to the anxiolytic effect of pregabalin [10,11]. In rodent models, pregabalin has shown dose-dependent anxiolytic-like effects [11–13]. In a pilot study in client-owned cats, good clinical safety and a significant decrease in signs of anxiety and fear associated with car transportation was reported [14].

Two pharmacokinetic studies of pregabalin in laboratory cats have been published [15,16] showing good absorption and bioavailability, as well as a linear pharmacokinetic profile. Lamminen et al. (2022) [16] reported the bioavailability of 94%, mean maximum plasma concentration of 10.1 μg/mL reached between 0.5 and 1 h, area under the curve of 129 h*μg/mL, and a mean half-life of 14.7 h after administration of the oral solution formulation used in this clinical study with a dose 5 of mg/kg. No safety concerns were reported in healthy laboratory cats.

The objective of this study was to confirm clinical efficacy and safety with the newly developed flavored oral solution formulation in cats showing signs of distress, anxiety, and/or fear during transportation and veterinary visits.

2. Materials and Methods

The study was a randomized, double-blind, placebo-controlled, parallel-group, multicenter clinical field study conducted at 22 veterinary clinics in five European countries (Finland, Germany, Hungary, Ireland, and Portugal) between September 2018 and May 2019. The randomization of the study treatment was made before the study started by an independent randomization expert using computer software. The investigators, owners, external expert, and the sponsor representatives were all blinded to the study treatment. The investigators were licensed veterinarians who were willing to participate and able to recruit suitable patients from their patient populations to the study. These veterinarians were trained for the study procedures and their working time used for the study tasks was financially compensated.

The study was conducted in compliance with Good Clinical Practice (GCP) as defined by the Veterinary International Conference on Harmonization (VICH) Guideline (GL) number 9. The GCP is an acknowledged international ethical and scientific quality standard, and gives assurance about the integrity of the data and animal welfare. The General Data Protection Regulation was fully followed during the study.

The clinical trial application was approved by the competent regulatory authority of each country, and the study protocol was written in accordance with animal welfare standards and requirements. Owner informed consent was obtained in writing from cat owners prior to enrollment. Owners were informed that cats would randomly be assigned

to placebo and treatment groups, that all cats would receive physical and laboratory examinations as part of their participation in the study, and possible risks associated with the use of sedatives, if required, were explained. The owners were permitted to withdraw their cat from the study for any reason, at any time. Owners acted as rapporteurs and assessors of the cats' behaviors for parts of the study as described below, but no data were collected on the owners separate from that for the guardianship of the cats. The health, welfare, treatment, and care of the study animals were ensured by veterinary supervision at each participating clinic and also monitored by sponsor personnel not affiliated with the study site, but trained as clinical study monitors, to ensure humane care of the study animals according to GCP standards.

2.1. Animals

Client-owned cats were recruited by the investigators from clientele of their participating veterinary clinics and through advertisements in social media. Cats of any age were eligible to participate to the study if they had a history of being stressed, anxious, and fearful when transported by car and during veterinary visits. They could enter the study after being assessed by a veterinarian as healthy or with mild systemic disease (American Society of Anesthesiologists class I or II). Additionally, cats enrolled were required to score 3–5 at the screening in the owner's assessment of transportation (Table 1) and 3–5 in the investigator's assessment of the ability to perform clinical examinations (Table 2). Cats were excluded from participating if they were being treated with other psychoactive medications, homeopathic remedies, pheromonal products, supplements, or a special diet to control anxiety. Other reasons for exclusion were pregnancy, lactation, concurrent participation to any other clinical study, and any other condition or situation which could disturb the conduct of the study, for example, owner's inability to administer the study treatment, make video recording, or transport the cat in a car.

Table 1. Numerical rating scale for the owner's assessment of the treatment effect based on the cat's stress, anxiety, and/or fear during the transportation in a car. Modified from Lamminen et al. (2021) [14].

Score	Description
1	Excellent: Cat was calm and quiet during the whole transportation time, did not express signs of stress, anxiety, and/or fear.
2	Good: Cat was calm and quiet during most of the transportation time. Transient mild signs of stress, anxiety, and/or fear (e.g., occasional vocalization, salivation, or locomotion) up to 25% of the transportation time.
3	Fair: Cat showed moderate signs of stress, anxiety, and/or fear (e.g., vocalization, salivation, locomotion, or other activity in bouts) up to 50% of the transportation time.
4	Poor: Cat showed strong signs of stress, anxiety, and/or fear (e.g., vocalization, salivation, locomotion, or other activity almost without interruption or in longer, more forceful bouts) up to 75% of the transportation time.
5	Very poor: Cat showed extreme signs of stress, anxiety, and/or fear (e.g., vocalization, salivation, locomotion, or other activity forcefully and without interruption) for 75–100% of the transportation time.

Since these visits either were annual health visits or mimicked them, all cats received a physical and laboratory examination as part of their participation in the study. All cats were monitored for clinical safety of the study treatment (active or placebo) that they received, as pregabalin was not yet approved for this target species.

Table 2. Numerical rating scale for the investigator's assessment of the treatment effect based on the cat's stress, anxiety, and/or fear during the clinical examination at the clinic. Modified from Mills et al. (2006) [17] and van Haaften et al. (2017) [18].

Score	Description
1	Excellent: Clinical examination could be easily performed without resistance or with insignificant resistance (no restraint needed). Cat was compliant and not frozen and did not express signs of stress, anxiety, and/or fear.
2	Good: Minor resistance; clinical examination could be performed with the technician minimally restraining the cat by placing a hand on the head or back. Cat was compliant and not frozen and expressed mild signs of stress, anxiety, and/or fear.
3	Fair: Moderate resistance or freezing. Cat expressed moderate signs of stress, anxiety, and/or fear. Clinical examination could be performed with the veterinary technician using physical restraint involving stabilizing the cat and holding in place. Freezing is defined as a moderately tense body.
4	Poor: Strong resistance or freezing. Cat expressed strong signs of stress, anxiety, and/or fear. Clinical examination could be performed without sedation with the veterinary technician more tightly restraining the cat (physically wrapping or scruffing cat). Freezing is defined as a very tense body, e.g., absence of movement except respiration.
5	Very poor: Extremely strong resistance. Cat expressed extreme signs of stress, anxiety, and/or fear and responded to the clinical examination with avoidant and/or defensive behaviour to an extent that completing the examination required sedation.

2.2. Treatments

At screening, cats were given tap water orally with a syringe to mimic study procedures, and baseline assessments were performed. Eligible cats were randomly assigned in a 1:1 ratio to receive either a single 5 mg/kg dose of flavored pregabalin oral solution (Bonqat®50 mg/ml, Orion Corporation, Espoo, Finland) or a placebo. To ensure blinding, the study treatments were identical in color and odor with the same small dosing volume of 0.1 mL/kg. The study treatments were given at home by the owner who was trained to administer the study product.

2.3. Assessments

The assessments were done both at screening and treatment visits that were conducted at the interval of 5–10 days (Figure 1). Study treatment (or water at the screening visit) was administered 90 ± 15 min before the cats were placed into a carrier and transported in a car for at least 20 min to the veterinary clinic. A video was recorded during the car transportation. At the clinic, a standardized clinical examination (Table 3) was performed by the investigator, who was the participating clinic veterinarian. The clinical examination was designed to correspond to and coincide with a routine annual health check. To ensure adequate patient population and reliable results, all procedures and assessments were performed in a similar manner at both the screening and treatment visits.

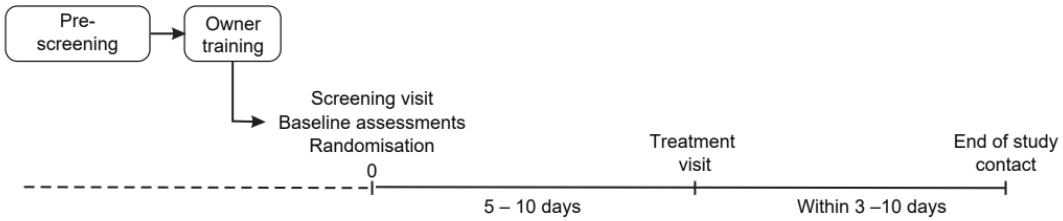

Figure 1. Study design of the clinical field study.

Table 3. Standardized clinical examination performed by the investigator at the veterinary clinic with specified order of performance in increasing invasiveness.

Order	Procedure
1	Cat carrier placed on examination table.
2	Carrier opened, and cat allowed to exit the carrier within approximately 1 min.
3	Cat removed from carrier, if it did not come out on its own, and placed on mat on the table. Assessment of alertness was done at the treatment visit.
4	Cat stroked dorsally on the table.
5	Heart and lungs auscultated. Heart rate and respiratory rate recorded.
6	Head held, eyes checked visually (primarily without any device).
7	Head held, ears checked visually (primarily without any device).
8	Head held, external neck and salivary glands and lymph nodes examined.
9	Head held, lip lifted – gums checked – capillary refill time (CRT).
10	Head held, mouth opened and checked.
11	Cat's neck held – prescapular lymph nodes checked, hand ran down cat to check skin, popliteal lymph nodes checked.
12	While cat is standing belly palpated.
13	Proprioception checked for front and hind limbs.
14	Tail lifted, anus visualised.
15	Rectal temperature measured.
16	Blood sampling.

2.4. Primary Efficacy Variables

The two primary efficacy variables were the owner's assessment of the treatment effect during transportation and the investigator's assessment of the treatment effect during clinical examination. A 5-point scale was used for both variables (Tables 1 and 2).

2.5. Secondary Efficacy Variables

As secondary efficacy variables, the owners assessed the ability to place the cat into the carrier using a similar 5-point scale. Additionally, the owners assessed signs of stress, anxiety, and/or fear (vocalization, abnormal activity, resistance, destructive behavior, escaping/hiding, withdrawn/crouching, freezing/decreased motor activity, urination, defecation, vomiting, panting, continuous licking, scaling, salivation, sweating paws). Each sign was rated for the extent to which it was expressed according to Table 4. Rating occurred at several time points: during transportation, clinical examination, at home just after opening the carrier, and 1 and 3 h after coming home. The sum of signs of stress, anxiety and/or fear was calculated for each individual and timepoint, and the means of the treatment groups were compared to each other. The owners also assessed the onset and end of any change or signs of effect in the cat's behavior. Additionally, they assessed the usability of the product utilizing a scale of "very easy", "easy", "somewhat difficult" and "very difficult".

Table 4. Numerical rating scale for the owner's assessment of the extent of signs of distress, anxiety, and/or fear. Modified from Lamminen et al. (2021) [14].

Score	Description
0	None
1	Only a few times
2	Half of the time
3	Most of the time
4	Continuously

2.6. External Expert Assessments

An external expert observer, blinded to the study treatment and owner assessment, evaluated the treatment effect during transportation based on video recordings using the same 5-point scale as the owner. Additionally, the external observer assessed the signs of stress, anxiety, and/or fear during transportation according to the frequency and/or duration, depending on the type of behavior (Table 5).

Table 5. The signs assessed by the external observer in video assessment of cats. Modified from Lamminen et al. (2021) [14].

Signs Assessed by Frequency	Signs Assessed by Duration
Elimination	Crouched position
Eyes closed	Ears flattened
Lip licking	Exploration
Purring events	Grooming
Shake off	Hiding
Swallowing	Locomotion
Vomiting	Pupils dilated
Yawning	Purring
	Panting
	Scratching
	Salivating
	Sleeping
	Tail close to body
	Vocalization
	Withdraw
	Other

2.7. Safety Assessments

As safety variables, the investigator assessed the alertness (physical and mental activity) and potential sedation of the cat at the beginning of clinical examination. Additionally, the owner scored at home the cat's activity and ability to stand up and walk when opening the carrier, and 1 and 3 h after coming home (Table 6). Blood samples were collected at screening and treatment visits for hematological (e.g., blood cell counts, anaemia parameters, and white blood cell differential) and clinical chemistry (e.g., kidney and liver parameters, and electrolyties) analysis, and adverse events were recorded throughout the study.

Table 6. Numerical rating scale for the owner's assessment of the cat's activity, and ability to stand up and walk. Modified from Korpivaara et al. (2017) [19] and Korpivaara et al. (2022) [20].

Score	Description of the Cat's Ability to Stand up and Walk	Description of the Cat's Activity
1	Normal: Cat is able to stand up and walk normally.	Active: e.g., mobile, pacing, trying to evade or hide when approached, hissing, crying, growling.
2	Calm: e.g., cat is able to stand up, can walk almost normally once it is moving although it may move more slowly. Walk may involve occasional mild staggering/incoordination.	Neutral: e.g., attentive, walking, standing, sitting normally, or normally lying down, behaves as usual.
3	Mild incoordination: e.g., cat can stand when encouraged or lifted up, mild staggering/incoordination when walking.	Calm: e.g., sitting or lying down, snoozing, may or may not look at observer, reacts to touch.
4	Moderate incoordination: e.g., cat can stand when encouraged or lifted up, hesitates to move, walking involves clear staggering/incoordination, may fall down when walking.	Very calm/sleeping: e.g., lying down or curled up, ignoring observer, eyes closed, does not react to touch or stimulation (e.g., when lifted up).
5	Severe incoordination: Cat is unable to stand up and walk.	

2.8. Statistics

Sample size was estimated based on a previously conducted pilot study [14]. The estimated sample size in each group was 81, with a 5% level Chi-square test having a 90% power to distinguish between an active treatment group and placebo. It was assumed that variation would be larger in this study than in the pilot study due to several centers, countries, and possible dropouts. Thus, at least 90 cats were to be recruited for both the pregabalin group and the placebo group by the end of the study.

Both primary variables were analyzed with a generalized linear mixed model appropriate for a multinomial response variable, with a cumulative logit link function. Treatment was modeled as a fixed effect, and center and center-by-treatment interaction as random effects. The baseline score was included as a covariate. As a supportive analysis, both primary variables were also dichotomized into success/failure variables following the predefined plan, where "success" was defined as "excellent" or "good" in the 5-point rating scale. All other scores ("fair", "poor", "very poor") were regarded as "failure". Dichotomized variables were analyzed with a logistic regression model. The inter-rater reliability between owners and external observer assessments of treatment effect during transportation was assessed with Cohen's weighted kappa coefficient (κ).

The multinomial secondary variables were analyzed with a similar model as the primary variables. Change from baseline in owner's assessment of sum of signs of stress, anxiety, and/or fear was analyzed with a linear mixed repeated measures analysis of covariance (RM-ANCOVA) model. Treatment, time and treatment-by-time interaction were fixed effects and subject, center and center-by-treatment interaction were random effects. Estimates for individual time points were done using contrasts. External observer assessment of signs of stress, anxiety, and/or fear were analysed descriptively.

All safety variables were reported descriptively by the treatment group. Differences were considered to be statistically significant with $p < 0.05$. All randomized cats (intention to treat [ITT] population) were included in the safety analysis. As the aim was to study the anxiolytic effect of pregabalin, a conservative approach was chosen where the cats were excluded from ITT population in the efficacy analyses if signs of sedation were seen. The predefined criteria in the study protocol stated that cats showing sedation at the clinic when evaluated at the beginning of clinical examination, or cats that were very calm/sleeping and showed moderate or severe incordination at two timepoints after coming home were excluded from the efficacy analysis.

3. Results

3.1. Animals

A total of 214 client-owned cats entered the study. Five of them discontinued the study before treatment administration, thus 209 cats (one from Germany, 18 from Ireland, 23 from Finland, 57 from Hungary, and 110 from Portugal) were randomly allocated to receive either pregabalin 5 mg/kg (n = 108) or placebo (n = 101). The mean (SD) actual dose of pregabalin was 5.6 (5.3) mg/kg, dose volume was 0.5 (0.3) mL per cat, and the median (range) duration of car transportation was 22 (20–45) min and 22 (20–50) min for 5 mg/kg and placebo, respectively. The demographic and baseline characteristics were comparable in the treatment groups (Table 7).

Table 7. Demographic and baseline characteristics.

Variable	Pregabalin(n = 108)	Placebo(n = 101)	Total(n = 209)
Sex, n (%)			
Male	51 (47)	36 (36)	87 (42)
Female	57 (53)	65 (64)	122 (58)
Age (years)			
Mean (sd)	5.3 (3.8)	5.7 (3.4)	5.5 (3.6)
Median (range)	4.7 (0.4–14.9)	5.5 (0.6–15.6)	4.9 (0.4–15.6)

Table 7. *Cont.*

Variable	Pregabalin(n = 108)	Placebo(n = 101)	Total(n = 209)
Weight (kg)			
Mean (sd)	4.1 (1.2)	4.3 (1.2)	4.4 (1.2)
Median (range)	4.1 (2.3–7.6)	4.3 (2.1–10.3)	4.2 (2.1–10.3)
Neutered, n (%)			
Yes	87 (81)	87 (86)	174 (83)
No	21 (19)	14 (14)	35 (17)
Signs of severe anxiety at baseline (at least 3 signs at least half of the time), n (%)			
During transportation	76 (72)	74 (73)	150 (73)
At the veterinary clinic	76 (72)	68 (67)	144 (70)

3.2. Primary Efficacy Variables

A statistically significant difference favoring pregabalin 5 mg/kg over placebo was seen in both primary efficacy variables, the owner's assessment of the treatment effect during transportation (OR 3.8 [95% CI 1.8–8.1], $p < 0.01$) and the investigator's assessment of the treatment effect during clinical examination (OR 3.4 [95% CI 1.8–6.4], $p < 0.01$) (Figure 2).

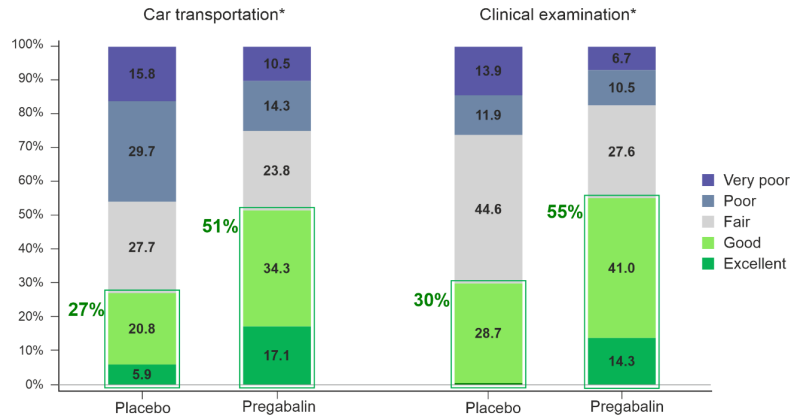

Figure 2. Distribution of responses (percentage of cats) for owner's assessment of the treatment effect during transportation and investigator's assessment of the treatment effect during clinical examination. * Indicates a statistically significant difference between pregabalin 5 mg/kg and placebo treatments.

Cat owners assessed pregabalin to more often have "excellent" or "good" effect during the transportation in 51% (54/105) of cases compared to in 27% (27/101) of placebo cases ($p < 0.01$). The investigators' assessment revealed more often "excellent" or "good" treatment effect during the clinical examination in cats treated with pregabalin, 55% (58/105), compared to cats receiving placebo, 30% (30/101), ($p < 0.01$).

3.3. Secondary Efficacy Variables

The treatment effect in the owners' assessment of the ability to place the cat into the carrier was statistically significant favoring pregabalin over placebo (OR 6.0 [95% CI 2.0–17.9], $p < 0.01$). The mean sum of signs of anxiety and/or fear decreased from the screening visit with both treatments. The difference between pregabalin and placebo in the mean sum of signs at treatment visit was statistically significant during transportation (−2.9 [95% CI −4.3 to −1.5], $p < 0.01$), clinical examination (−2.8 [95% CI −4.2 to −1.5], $p < 0.01$) and when opening the carrier at home (−1.8 [95% CI −3.3 to −0.4], $p = 0.02$) favoring

pregabalin (Figure 3). Based on the owners' observations, vocalization, panting/intense breathing, resistance, and abnormal activity were the signs with the greatest numerical decrease with pregabalin treatment versus placebo. Owners were able to detect the onset and end of any change or signs of effect in 45% (49/108) of cats receiving pregabalin with the median duration of changes being 7 h (range of 1.3–28.5). The reported range is wide, mostly because some owners with the clinic visits taking place during evening hours evaluated the end of possible changes the next morning.

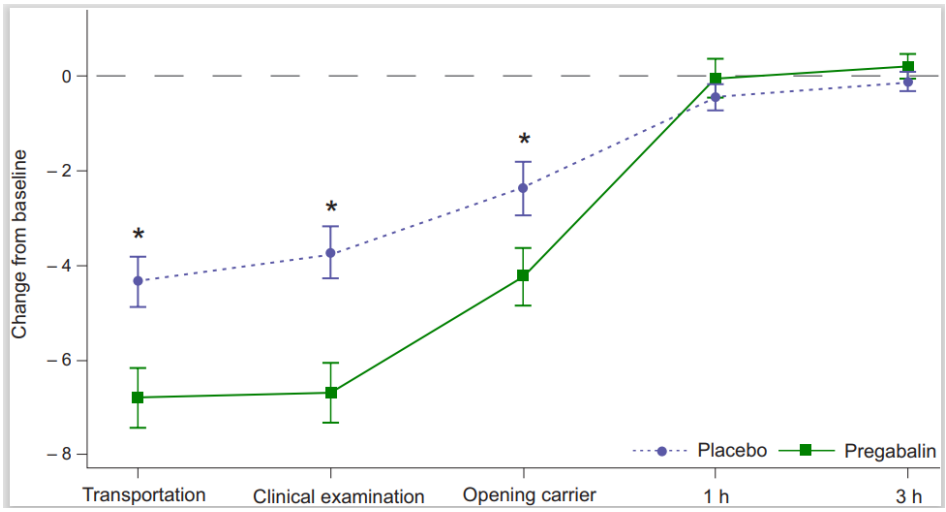

Figure 3. Mean (±SEM) change from baseline in the sum of signs of anxiety and/or fear during transportation, clinical examination, at home when opening the carrier, and 1 and 3 h after coming home. * Indicates a statistically significant difference between pregabalin 5 mg/kg and placebo treatments.

The treatment compliance was good as 95% (103/108) of the pregabalin administrations were successful. Approximately 79% (81/103) of cat owners assessed that it was "very easy" or "easy" to administer the flavored pregabalin oral solution.

3.4. External Expert Assessments

The external expert observer's assessment of the treatment effect during transportation confirmed the owners' assessment as pregabalin was statistically significantly better compared to placebo (OR 3.4 [95% CI 1.8–6.4], $p < 0.01$). The treatment effect was assessed "excellent" or "good" in 54% (54/101) of cats with pregabalin and in 43% (42/97) with placebo ($p < 0.01$). The owners' and external observer's agreement was moderate ($\kappa = 0.47$, $p < 0.01$). The external observer found the greatest numerical decrease in pregabalin treatment versus placebo in vocalization, dilation of pupils, flattening of ears, lip licking, and swallowing. In a total of eight cats, the lack of evaluable video material caused the absence of external observer assessment of these cats. Challenges with the video material in general included too dark of an environment during car rides taking place in the evening and technical problems with the video camera (e.g., running out of battery, missing audio, and incorrect focusing of the camera in the carrier). To mitigate these challenges observed early in the study, the owners were advised to use light colored bedding in the carrier, schedule the clinic visit to occur at daytime, if possible, and to increase attention on the function and the focusing of the video camera to improve video quality.

3.5. Safety

The investigators considered the majority of cats in both groups to have normal alertness. Only one cat in the pregabalin group was considered to show signs of mild sedation by the veterinarian and was thus excluded from the efficacy analysis. Based on the owners' assessment, two additional cats were excluded from the efficacy analysis, according to the predefined criteria in the study protocol, as they scored to be both very calm or sleeping and having moderate incoordination at least in two time points after coming home. In the sensitivity analysis, when the three cats with mild signs of sedation were included, the efficacy results were similar to the ones in the main analysis.

The owners assessed a few more cats being very calm or sleeping in the pregabalin group (3%, 3/101) compared to placebo (1%, 1/87) after coming home from the veterinary clinic at the treatment visit. The ability to stand up and walk was assessed normal in 56% (57/101), 58% (59/101), and 73% (74/101) of cats in pregabalin group and 90% (78/87), 91% (79/87), and 95% (83/87) of cats in placebo group at home when opening the carrier and 1 and 3 h after coming home on treatment visit, respectively.

There were few adverse events reported in the study, the most common being mild transient incoordination (five events in four cats) and tiredness (three events in three cats). These adverse events had resolved by the next day.

Seven cats in the pregabalin group and 14 cats in placebo group required sedation at the treatment visit approximately 2 h after administration of the study treatment to complete the standardized clinical examination including blood sampling. The sedatives used (e.g., alpha-2 agonists medetomidine, dexmedetomidine or xylazine, and opioids butorphanol or methadone), and their dosages were similar to those used at the screening visit for the concerned cats, and no safety concerns were reported. There were no notable changes in the laboratory values between the screening and treatment visits in either the pregabalin or placebo groups with the exception of one cat in each group. The clinically relevant findings, leucopenia in one cat in the pregabalin group and thrombocytopenia in one cat in the placebo group, were reported as adverse events.

4. Discussion

The study results confirm that the novel pregabalin oral solution given at 5 mg/kg is effective in alleviating acute anxiety and fear associated with transportation and veterinary visits in cats, as both primary efficacy endpoints, the owner-assessed treatment effect during transportation and the investigator-assessed treatment effect during clinical examination, were met. Anxious cats were 3.8 times more likely ($p < 0.01$) to remain calm and quiet during transportation after treating with pregabalin approximately 1.5 h before the start of the car ride compared to the placebo. The veterinarians were 3.4 times more likely ($p < 0.01$) to easily perform the clinical examination after pre-visit medication with pregabalin compared to the placebo.

According to the baseline data, most cats entering the study had been highly distressed during transportation and clinical examination, as more than 67% of cats showed at least three severe signs of anxiety (i.e., present at least half of the time) at those timepoints at screening. A clear and clinically relevant treatment effect was confirmed by decreased signs of anxiety and fear during transportation and veterinary visit after pregabalin treatment based on the owners' and external observer's assessments. The signs with a numerically greatest change after pregabalin treatment, vocalization, panting, resistance, abnormal activity, dilation of pupils, flattening of ears, lip licking, and swallowing, are described in the literature as typical signs of stress and anxiety in cats [3,4,21–23].

The decrease in the sum of signs of stress, anxiety, and/or fear compared to the baseline was noted, especially during transportation and clinical examination and to a lesser extent also when opening the carrier after coming home. Based on earlier studies, it is known that these timepoints are very stressful for cats [1–5]. The signs of stress, anxiety, and/or fear were low in both frequency and extent at 1 h and 3 h timepoints after coming home from screening and treatment visits, most likely because the cats felt safe at home and were able

to settle down after a stressful experience when in a comfortable and familiar place. This is contrary to the findings of an earlier study [5]. No statistically significant difference in the change from baseline of the mean sum of signs was seen between treatment and screening visits at 1 h and 3 h timepoints. A placebo effect was seen in the mean sum of signs during transportation, clinical examination, and when opening the carrier after coming home, and similarly also in the primary efficacy variables at the same timepoints. The placebo effect is a common phenomenon observed in double blinded placebo controlled studies in dogs and cats [19,24–26].

The owners and external expert used the same 5-point scale for assessment of the treatment effect during transportation. The calculated agreement between their assessments was highly significant. Owners generally may not recognise all the signs of stress in their cats to the same extent as a trained behavioural expert [4]. In addition, owners and experts observed the cat from different viewpoints as the owners know their own pet and might look at the cat subjectively while the external observer evaluates the cats more objectively, purely rating the cats behaviour.

Pregabalin was well tolerated in cats, with mild and transient incoordination and tiredness as the most frequently reported adverse events. The owner assessments of the cats' activity and ability to stand up and walk after coming home are generally in line with the safety findings. Allthough the owners noted some cats in the pregabalin group were very calm or sleeping after coming home, a similar trend could also be seen in cats treated with the placebo. This may be related to the finding that tiredness is a normal reaction in cats after stressful events and disruption of their normal daily routines [27].

Sedatives were used at the clinic, a decision made by the cat's veterinarian to complete the annual exam, with informed consent and without safety concerns in seven cats after receiving pregabalin before the visit. This outcome suggests that healthy cats given a pre-appointment dose of pregabalin may be sedated during the following veterinary visit with commonly used sedatives, even though the number of cats sedated after a single dose of pregabalin is small. In this study, the doses of sedatives used at the treatment visit were similar to the doses used at the screening visit. However, as any central nervous system depressants may potentiate the effects of pregabalin, an appropriate dose adjustment should always be considered based on the clinical assessment. In humans, anxiolytic medicines are used as premedication in day surgery [28], and pregabalin was used safely to control both preoperative and intraoperative anxiety in patients undergoing anesthesia and surgery [29]. In cats, reduction of distress by applying a low-stress protocol during transportation to the veterinary clinic was shown to decrease the time to reach sedation and to reduce the required dose of an induction agent [30].

Pregabalin is currently approved in the European Union for use in humans for treatment of generalized anxiety, neuropathic pain, and epilepsy [31]. Recently pregabalin has been approved also in cats for alleviation of acute anxiety and fear related to transportation and veterinary visits [32] based on the results of this and other studies [14,16]. Currently, no other anxiolytic medicines are registered for travel- and veterinary-visit-related anxiety in cats. Gabapentin was studied and used in clinical practice to some extent [18,33–35] and there is one report of clinical use of trazodone [36]. Compared to gabapentin, pregabalin is a more potent molecule enabling similar efficacy with a much smaller dose. Pregabalin was reported to have more favorable pharmacokinetic properties with faster absorption and linear kinetic profile in humans [37]. Similar findings have also been reported in cats [15,16,38,39]. As the mode of action of both gabapentinoids is alike, their clinical efficacy and safety profile seem to be close to each other. However, both efficacy and safety depend on the used dose, which in cats is accurately studied and selected for pregabalin [40] but not so closely explored for gabapentin.

Limitations of the present study include the lack of data on pregabalin use in cats with moderate or severe systemic diseases due to the inclusion criteria. This deficit leads to scarce information related to interactions with other medicines. Additionally, there was an absence of invaluable video material in eight cats due to practical challenges related to

video recording during transportation, which may have complicated the external expert's evaluations. Further limitations are that the study does not provide robust data regarding the duration of effect of pregabalin in anxious cats nor the pharmacokinetic data in pet cats for evaluation of anxiolytic plasma concentrations. In this study, owners were asked to record the onset and end of any change or sign of effect, which does not give reliable information on the duration of the actual anxiolytic effect. In general, owners were able to detect the onset and end of changes only for less than half of cats receiving pregabalin. Based on the limited data from the present study, it seems that the duration of effect of the novel cat-specific pregabalin formulation could be approximately 7 h. This estimate is supported by the pharmacokinetic parameters of pregabalin reported in cats [15,41], suggesting that cats have a higher degree of absorption and slower elimination compared to humans [42] and dogs [43]. More detailed studies are required to verify this finding.

5. Conclusions

The anxiolytic properties of the novel pregabalin oral solution with a dose of 5 mg/kg were measurable, statistically significant, and clinically relevant in cats with acute anxiety and fear associated with transportation and veterinary visits. The dose used was safe without a significant sedative effect. The owners found the cat-specific formulation with a small dosing volume easy to administer. The addition of this new product, with proven safety and efficacy in cats, provides practical aid for both owners and veterinarians for fear-free handling, and thus it improves the welfare of cats.

Author Contributions: Conceptualization T.L., M.K., J.A., C.P., and K.L.O.; methodology, T.L., M.K., J.A., C.P., and K.L.O.; formal analysis, J.A.; investigation, investigator veterinarians and C.P. who performed the video assessments as an external expert; writing—original draft preparation, T.L.; writing—review and editing, T.L., M.K., J.A., C.P., and K.L.O.; All authors have read and agreed to the published version of the manuscript.

Funding: The study was sponsored by Orion Corporation Orion Pharma.

Institutional Review Board Statement: The study was conducted in five European countries (Finland, Germany, Hungary, Ireland, and Portugal), and the clinical trial application was approved by the competent regulatory authority of each country (Finnish Medicines Agency approval number Vetkl-nro 05/2018, Regierung von Oberbayern ROB-55.2-2532.Vet_03-18-77, Hungarian Directorate of Veterinary Medicinal Products 02.2/4170-3/2018, Irish Health Products Regulatory Authority CT22016/002 and Portuguese National Authority for Animal Health 58/ECVPT/2018). Regulatory authority review of the study protocol included detailed ethical evaluation. The study was found to be in accordance with applicable animal welfare standards and requirements. The Directive 2010/63/EU on the protection of animals used for scientific purpose does not apply to veterinary clinical trials required for the marketing authorization of a veterinary medicinal product. Separate local Ethical Committee approvals, in addition to the individual country regulatory approvals, were therefore not sought. The study was conducted according to the principles of GCP as defined by the VICH GL 9. The health, welfare, treatment, and care of study animals were ensured by veterinary supervision and also monitored by a person not affiliated with the study site, trained to ensure humane care of the study animals according to GCP standards.

Informed Consent Statement: Written informed consent from the owner of each animal was obtained prior to any study-specific procedures.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank the participating investigators and cat owners and the study team.

Conflicts of Interest: The study was sponsored by Orion Corporation Orion Pharma. TL, M.K. and J.A. are employees of Orion Corporation. C.P. and K.L.O. are paid consultants of Orion Corporation. K.L.O. is a member of the Editorial Board of Animals. She has no role in managing or reviewing this manuscript.

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Received: 28 December 2021 | Revised: 31 March 2022 | Accepted: 2 April 2022

DOI: 10.1111/jvp.13061

JOURNAL OF Veterinary Pharmacology and Therapeutics WILEY

ORIGINAL ARTICLE

Pharmacokinetics of single and repeated oral doses of pregabalin oral solution formulation in cats

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Funding information
The study was sponsored by Orion Corporation Orion Pharma.

Abstract

The study was designed to determine the pharmacokinetic profile and bioavailability of a novel pregabalin 50 mg/ml oral solution formulation (Bonqat®, Orion Corporation Orion Pharma) in 6 healthy laboratory cats. The cats received pregabalin as single oral doses of 2.5, 5, and 7.5 mg/kg, dose 5 mg/kg on two consecutive days, and a single intravenous dose of 2.5 mg/kg. The washout period between each administration was four weeks. The cats were monitored for clinical signs and level of sedation, and blood samples were taken before pregabalin dosing and at pre-defined time points up to 168 h after dosing. Plasma concentrations of pregabalin were determined using a validated liquid chromatography–tandem mass spectrometry method. The mean maximum plasma concentration of 10.1 μg/ml was reached between 0.5 and 1 h after oral administration of the clinical dose 5 mg/kg. The mean half-life after oral administration of dose 5 mg/kg was 14.7 h and the mean systemic bioavailability was 94%. Pregabalin showed linear pharmacokinetics from 2.5 to 7.5 mg/kg. Exposures after a single dose and re-dosing of 5 mg/kg at 24 h were comparable. Pregabalin was well tolerated with mild sedation and mildly uncoordinated movements observed in few cats at dose 7.5 mg/kg. As a conclusion, study results show rapid absorption, linear pharmacokinetics, and high oral bioavailability of pregabalin without safety concerns after administration of oral solution in cats.

KEYWORDS
cat, pharmacokinetics, pregabalin, repeated oral dose, single oral dose

1 | INTRODUCTION

Anxiety and fear in cats associated with transportation and veterinary visits is a well-known welfare challenge among cat owners (Mariti et al., 2017; Niblett et al., 2015; Quimby et al., 2011; Volk et al., 2011). To help the cat, owners and veterinarians are advised to employ actions such as training, using treats, applying gentle handling, and providing a cat-friendly environment (Hammerle et al., 2015; Moody et al., 2020; Pratsch et al., 2018; Riemer et al., 2021; Rodan et al., 2011). In addition to behavioural and environmental modification, anxiolytic medication can be used to reduce anxiety during transportation and to enable patient-friendly, low-stress and physical examination (Hammerle et al., 2015). The feline specific oral solution formulation of pregabalin with the strength of 50 mg/ml was developed to alleviate cats' anxiety and fear during transportation and veterinary visits. The anxiolytic efficacy of the novel pregabalin formulation with the dose 5 mg/kg has been demonstrated to significantly and clinically relevantly alleviate anxiety in cats (Lamminen, Korpivaara, Aspegrén, Palestrini, Overall, unpublished data).

Pregabalin is a structural analogue of the gamma-aminobutyric acid (GABA) neurotransmitter and binds to the alpha-2-delta subunit of the voltage-dependent calcium channel in the central nervous system (Li et al., 2011). It decreases the release of glutamate and monoamine neurotransmitters, which have been implicated to play a role in the pathophysiology of anxiety (Frampton, 2014; Mico & Prieto, 2012). In rodent models, pregabalin has shown dose-dependent anxiolytic-like effects (Field et al., 2001; Lotarski et al., 2011; Wang et al., 2012). Pregabalin is approved in the EU for treatment of generalized anxiety in humans (EMA, 2021a) and for alleviation of acute anxiety and fear related to transportation and veterinary visit in cats (EMA, 2021b).

Pharmacokinetics of pregabalin have been earlier described in several species including humans (Bockbrader, Radulovic et al., 2010), dogs (Salazar et al., 2009) and cats (Esteban et al., 2018). In their study in cats, Esteban et al. (2018) used a capsule formulation with a dose 4 mg/kg, which produced plasma concentrations reported to be similar to those considered efficacious for the control of seizures in human patients with epilepsy. Pharmacokinetics of the oral solution formulation developed for cats have not been reported earlier.

The objective of this study was to determine the pharmacokinetics and bioavailability of the novel formulation of pregabalin 50 mg/ml oral solution after single doses of 2.5, 5 and 7.5 mg/kg and dose 5 mg/kg on two consecutive days in 6 healthy adult cats.

2 | MATERIALS AND METHODS

All procedures were conducted in accordance with the Dutch Act on Animal Experimentation (December 2014), approved by the Central Authority for Scientific Procedures on Animals and the Animal Welfare Body of Charles River Laboratories Den Bosch B.V. and conducted under the authority of the Project License AVD2360020172866.

2.1 | Animals and housing

Six laboratory cats, three neutered males and three intact females, were included in the study. The domestic shorthair cats were 1–4.5-year-old at the initiation of the study. Males weighed between 4.8 and 5.4 kg and females between 4.4 and 4.6 kg. The animals were deemed healthy based on a physical examination and clinical chemistry and were trained for restraining and food regime prior to the start of the study. Veterinary care was available throughout the study.

Cats were socially housed (same sex) except at times when they were separated for study procedures on the days of dosing the study treatment. At those occasions, animals were housed individually until approximately 4 h after dosing.

The cats were housed in two connected stainless steel cages with litter box. For environmental enrichment, cats were provided with items such as balls in the cages. Environment temperatures of 21–22°C with a relative humidity of 40%–82% and a 12-h light/12-h dark cycle were maintained. The cats were provided commercially available pelleted complete cat feed (IAMS cat adult chicken, IAMS Company) in the morning ad libitum for approximately up to 4 h. Water was available ad libitum all the time. On the day of dosing, the cats were fasted until 4 h after dosing, after which the feed was availabe for a period of approximately up to 4 h.

2.2 | Study design

The study was conducted according to Good Laboratory Practice and included 5 dosing periods separated by a 4-week washout period between each dosing. The cats received single doses of 2.5, 5 and 7.5 mg/kg of pregabalin (Bonqat 50 mg/ml oral solution, Orion Corporation Orion Pharma) into the mouth in fasted state. The clinical dose 5 mg/kg was also studied after oral dosing on two consecutive days to evaluate pharmacokinetics of the second dose if given 24 h after the first one, as the product was planned to be used in practice as a single dose that is given at the maximum on two consecutive days, not for longer treatment periods. In addition, an intravenous (IV) bolus injection of dose 2.5 mg/kg of pregabalin was given via cephalic vein using a butterfly needle to the same cats as a reference to estimate the bioavailability of pregabalin oral solution. Injectable solution was prepared by dissolving pregabalin into sterile 0.9% sodium chloride (Dechra Veterinary Products, the Netherlands) on the day of dosing at the study laboratory. A validated analytical procedure was used to verify that the target concentration, 5 mg/ml, was achieved.

2.3 | Blood collection

Blood samples (0.75 ml) were collected from the jugular or cephalic vein by puncture into K_2-EDTA tubes (Greiner Bio-One GmbH). In oral administration periods, the samples were collected on the following time points: predose, 30 min, 1, 2, 3, 4, 8, 12, 24, 36, 96 and 168 h after dosing. In the period of dosing on two consecutive days, the samples post administration were collected after the second dosing. In the IV administration period, the samples were collected at predose, 5, 15, 30 min, 1, 2, 3, 4, 8, 12, 24, 36, 96 and 168 h after the IV injection. The cephalic vein that was used for IV dosing was not used for blood sampling.

Samples were centrifuged at approximately 2000 *g* for 10 min at 2–8°C. Plasma was transferred into polypropylene tubes (Micronic) on ice and frozen at ≤−75°C in an upright position, protected from light at all times.

2.4 | Bioanalytical method

Plasma samples were analysed in a laboratory (Ardena Bioanalysis BV Assen) for the concentrations of pregabalin using a validated

liquid chromatography–tandem mass spectrometry method. Quality control (QC) and calibration standard data were acceptable according to the requirements of the Food and Drug Administration Guidance for Industry, and the European Medicines Agency guidance on bioanalytical method validation.

Pregabalin (TRC) was used as the reference standard and $^{2}H_{6}$-labelled pregabalin (Cerilliant Corporation) as the internal standard (IS). The analyte and IS were extracted from cat K_{2}-EDTA plasma using solid phase extraction cartridges (Oasis HLB 30 mg 1 cc; Waters). The purified samples were analysed by a Prominence HPLC or Nexera UPLC system (Shimadzu) using an XBridge C18 column (4.6 × 100 mm, 3.5 μm; Waters) coupled with an API4000 mass spectrometer (AB Sciex).

The lower limit of quantification (LLOQ) was 5.00 ng/ml. The upper limit of quantification was 5000 ng/ml or 20,000 ng/ml, depending on the linearity of the range.

The calibration standard mean precision (CV) varied between 1.4% and 6.7% and the mean accuracy was between 93.2% and 104.0%. The mean precision (CV) and accuracy of the QC samples were 4.7% and 105.6% at 0.015 μg/ml, 3.2% and 104.8% at 0.40 μg/ml, 7.6% and 100.1% at 4.0 μg/ml and 3.1% and 106.9% at 16.0 μg/ml respectively.

2.5 | Pharmacokinetic analysis

Pharmacokinetic parameters were estimated using pharmacokinetic software (Phoenix WinNonlin 6.4, Certara). A non-compartmental approach consistent with the oral or IV route of administration was used for parameter estimation. All values below the LLOQ were assigned a value zero for pharmacokinetic purposes. Nominal sampling times were used in pharmacokinetic calculations, except where the deviation was >5%; in this case, actual times were used. Minimal spillage of the formulation during dosing and salivation after dosing could affect dosing accuracy. Therefore, all cats that showed either spillage or salivation after dosing were excluded from the descriptive statistics of the pharmacokinetic parameters. Descriptive statistics (*N*, mean, standard deviation and variation) for males, females and males and females combined were generated using Phoenix WinNonlin 6.4.

2.6 | Clinical observations

The cats were observed for general health twice daily throughout the study and during the dosing days for any clinical signs predose, 2, 4 and 12 h after dosing. Any observed signs were graded for severity as slight, moderate, severe or very severe. In addition, possible level of sedation was recorded at predose, 2, 4 and 8 h on the dosing days and graded according to Table 1. The scores were modified from the sedation scale published by Lamont et al. (2012). The differences between the sedation levels were discussed in a prestudy meeting with the involved technicians conducting the evaluation to allow for reproducible scoring.

3 | RESULTS

The pharmacokinetic parameters of pregabalin in cats after administration of a single oral dose of 5 mg/kg, and the parameters of the same dose on two consecutive days, are listed in Table 2. Figure 1 illustrates the mean plasma concentrations of doses 2.5, 5 and 7.5 mg/kg after a single oral administration of pregabalin in fasted cats, and Figure 2 correspondingly after a single and two consecutive doses of 5 mg/kg. The figures include data until 24 h to enlighten the plasma concentrations related to the effect of the treatment based on level of sedation. Table 3 presents the pharmacokinetic parameters in cats after IV administration of a single dose of 2.5 mg/kg. Variation of maximum plasma concentration (C_{max}) and area under plasma concentration curve (AUC) evaluated by coefficient of variation percentage ranged between 2% and 20%. As no sex differences were detected in pharmacokinetic parameters, the results are reported as combined. To accurately determine the pharmacokinetic properties of the formulation, only animals with the complete dosing, without any spillage or salivation after it, were included in the descriptive statistics of the pharmacokinetic parameters and the Figures 1 and 2.

No clinical signs or sedation were noted after single oral dosing at 2.5 and 5 mg/kg. Following single dosing at 7.5 mg/kg, mild signs of sedation and mildly uncoordinated movements were reported in two cats, and salivation directly after dosing in one cat. Following the first dosing of repeated oral dose 5 mg/kg, mydriasis of both eyes in all six cats was observed, and salivation was noted in two cats.

4 | DISCUSSION

In this study, we investigated the pharmacokinetics of a novel pregabalin oral solution formulation in cats. Pregabalin was quickly absorbed after a single dose of 5 mg/kg with time to maximum

TABLE 1 Scoring of the level of sedation

Category	Description
No sedation	No signs of depression, drowsiness or ataxia
Slight sedation	Mild signs of depression, drowsiness or ataxia. Decreased reaction to stimuli
Moderate sedation	Severe ataxia, reluctant to move, may attain sternal recumbency
Deep sedation	Depressed, drowsy and sleepy, no resistance to positioning on lateral recumbency

TABLE 2 Pharmacokinetic parameters of pregabalin after single doses of 2.5, 5 and 7.5 mg/kg and 5 mg/kg on two consecutive days of pregabalin 50 mg/ml oral solution formulation in fasted cats

Parameter[a]	Pregabalin 5 mg/kg ($N = 4$)[b]	Pregabalin 5 mg/kg twice ($N = 5$)[b]	Pregabalin 2.5 mg/kg ($N = 6$)	Pregabalin 7.5 mg/kg ($N = 5$)[b]
C_{max} (μg/ml)	10.1 ± 0.8	12.9 ± 2.6	5.7 ± 0.7	19.1 ± 3.1
T_{max} (h)	0.5–1	0.5–4	0.5–3	0.5–1
AUC_{0-24h} (h*μg/ml)	129 ± 3.0	157 ± 21.7	65 ± 7.9	200 ± 17.0
$t_{½}$ (h)	14.7 ± 2.7	15.6 ± 3.6	12.0 ± 3.2	12.1 ± 2.6
F (%)	94.3 (87.3–102)	NA	95.6 (75.4–130)	89.4 (81.0–95.3)

Abbreviations: AUC_{0-24h}, area under plasma concentration time curve within 24 h after dosing; C_{max}, peak plasma concentration; *F*, oral bioavailability; NA, not available; SD, standard deviation; $t_{1/2}$, elimination half-life; T_{max}, time to maximum concentration.

[a]Mean ± SD values, except range for T_{max} and mean (range) for *F*.

[b]Animals with incomplete dosing, due to spillage or salivation after dosing, excluded.

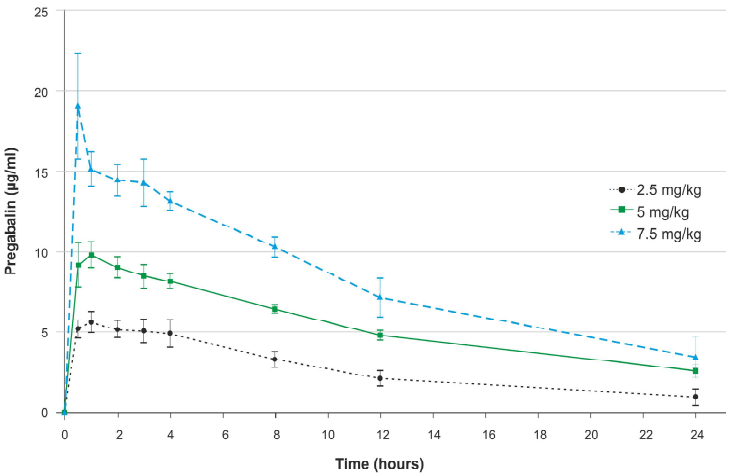

FIGURE 1 Pregabalin mean (±standard deviation) concentrations achieved in plasma after an accurate single dose of 2.5 ($N = 6$), 5 ($N = 4$) and 7.5 ($N = 5$) mg/kg of pregabalin 50 mg/ml oral solution formulation in fasted cats

concentration (T_{max}) ranging between 0.5 and 1 h after the administration of the oral solution in a fasted state. The absolute oral bioavailability was excellent, average 94% at the clinical dose 5 mg/kg. The systemic exposure to pregabalin, in terms of AUC and C_{max}, showed linear pharmacokinetics at the studied dose range of 2.5–7.5 mg/kg and the interindividual variability was low. This allows reliable dose-dependent effect in clinical use. After re-dosing of 5 mg/kg at 24 h, the exposure, in terms of AUC, C_{max} and elimination half-life ($t_{1/2}$), was comparable with the exposure following single dosing suggesting no clear signs for accumulation. Albeit dosing on two consecutive days is sufficient for pregabalin indication of reducing anxiety in cats for a specified event, it is only limited data to determine the pharmacokinetic properties after chronic dosing. Pregabalin has a relatively large volume of distribution in cat, as the value is higher than the extracellular fluid volume (Davies and Morris, 1993). This means that pregabalin is highly distributed into tissues, as is also described for humans (Bockbrader, Radulovic et al., 2010).

The total exposure in terms of AUC and C_{max} are in line between our study and earlier published data in cats (Esteban et al., 2018). On the contrary, T_{max} is shorter with the cat-specific novel oral solution used in our study than with the capsule formulation administered in the study by Esteban et al. (2018). This could result in a quicker clinical effect after dosing of the cat-specific formulation.

Pregabalin is quite slowly eliminated from the body of cats. Based on the results of our study, pregabalin $t_{1/2}$ in cats is approximately twice as long as the ones reported in humans (Bockbrader, Radulovic et al., 2010) and dogs (Salazar et al., 2009). This finding is supported by Esteban et al. (2018). The $t_{1/2}$ of pregabalin in cats seems to be clearly longer than the $t_{1/2}$ of gabapentin in the same species (Adrian et al., 2018; Siao et al., 2010). In practice, this can influence the dosing interval and probably also the duration of effect after administration.

Gabapentin is currently used off-label as an anxiolytic medication in cats (Van Haafen et al., 2017). However, pregabalin is a newer and more potent gabapentinoid compared with gabapentin and has been recently approved for alleviation of acute anxiety and fear associated with travel and veterinary visits in cats (EMA, 2021b). Even though not studied in cats, in mice, rats and monkeys pregabalin has been shown to cross the blood–brain barrier (EMA, 2021a), which

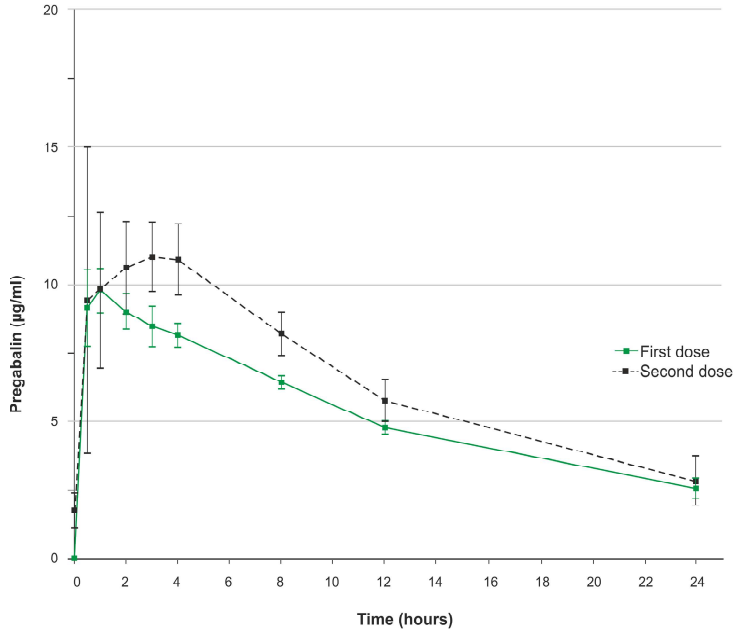

FIGURE 2 Pregabalin mean (±standard deviation) concentrations achieved in plasma after an accurate single dose of 5 mg/kg ($N = 4$) or after a dose of 5 mg/kg ($N = 5$) given on two consecutive days as pregabalin 50 mg/ml oral solution formulation in fasted cats

TABLE 3 Pharmacokinetic parameters of pregabalin after single intravenous (IV) dose of 2.5 mg/kg of pregabalin in fasted cats

Parameter[a]	Pregabalin 2.5 mg/kg IV ($N = 6$)
V_{ss} (L/kg)	0.4 ± 0.02
Cl (ml/h/kg)	30 ± 7.5
$t_{½}$ (h)	12.3 ± 3.1
AUC_{0-24h} (h*µg/ml)	69 ± 7.9

Abbreviations: AUC_{0-24h}, area under plasma concentration time curve within 24 h after dosing; Cl, plasma clearance; SD, standard deviation; $t_{1/2}$, elimination half-life; V_{ss}, volume of distribution at a steady state.
[a]Mean ± SD values.

allows the anxiolytic effect by decreasing of release of excitatory neurotransmitters. Pregabalin has been also reported to have more favourable pharmacokinetic properties than gabapentin in humans with nonsaturable absorption, linear pharmacokinetics and clear dose–response relationship (Bockbrader, Wesche et al., 2010). The same seems to apply also to cats based on the results of our studies (data on file, Orion Pharma) and earlier published data in this species (Adrian et al., 2018; Esteban et al., 2018; Siao et al., 2010).

The anxiolytic plasma concentrations of pregabalin have not been determined in humans or in cats. Based on the clinical study in anxious cats (unpublished data), pregabalin given with the clinical dose 5 mg/kg seems to have a duration of effect of approximately 7 h. In that study, plasma concentrations were not measured. In this pharmacokinetic study, conducted with the same oral solution formulation as the clinical trial, the mean plasma concentrations up to 24 h were comparable with previously published plasma concentrations up to at least 12 h in cats (Esteban et al., 2018). These plasma concentrations were considered to be efficacious for seizure control in humans (Arroyo et al., 2004; Berry and Millington, 2005) and dogs (Dewey et al., 2009; Salazar et al., 2009). However, these data do not give reliable information on the duration of the actual anxiolytic effect or anxiolytic plasma concentrations in cats.

There were no safety concerns detected in this study in laboratory cats with pregabalin. With the highest dose 7.5 mg/kg, some mild signs of sedation were seen in few cats. Similar signs of tiredness and incoordination have been reported as adverse events in clinical use in cats (Lamminen, Korpivaara, Aspegrén, Palestrini, Overall, unpublished data), dogs (Sanchis-Mora et al., 2019; Thoefner et al., 2020) and humans (Zaccara et al., 2011). A corresponding adverse event profile has been reported also with gabapentin in cats (Van Haafen et al., 2017).

5 | CONCLUSIONS

Our study describes the pharmacokinetic profile of the novel pregabalin 50 mg/ml oral solution formulation in cats. The results show fast absorption, linear pharmacokinetic profile and high oral bioavailability of the formulation. No safety concerns were observed with pregabalin in healthy laboratory cats.

ACKNOWLEDGMENT

The authors thank the laboratory staff and the study team.

CONFLICT OF INTEREST

TL, JK and MH-H are employees of Orion Corporation.

AUTHOR CONTRIBUTIONS

TL, JK and MH-H have contributed to conception of the study. JK and MH-H have designed the study and contributed to interpretation of the data. AD has contributed to the design of the study and to the acquizition, analysis and interpretation of the data. TL made the first draft and all authors have contributed to the writing of the manuscript. All authors have read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Lamminen, T., Doedée, A., Hyttilä-Hopponen, M. & Kaskinoro, J. (2022). Pharmacokinetics of single and repeated oral doses of pregabalin oral solution formulation in cats. *Journal of Veterinary Pharmacology and Therapeutics*, *00*, 1–7. https://doi.org/10.1111/jvp.13061