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ORIGINAL ARTICLE

Safety of torasemide in healthy adult dogs administered daily for 26 weeks

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Abstract

Thirty-two (16 males and 16 females) healthy young beagles were randomly divided into four groups of eight. The control group remained untreated. Torasemide (ISEMID®, Ceva Santé Animale) was orally administered, once daily, at 0.5 mg/kg from Days 1–5 then 0.25 mg/kg to Day 182, and at three times and five times this dosing regimen in two additional groups. Treated animals (predominantly at the higher dose levels) showed dryness of the oral mucosa, evidence of diuresis, decreased diet consumption, decreased bodyweight gain over the first 3 weeks, increased water consumption, increases in erythrocytes count, haemoglobin, calcium and magnesium, decrease in chloride, phosphorus, potassium and sodium, increases in urine pH, decreases in urine specific gravity and increases in serum aldosterone concentrations. Plasma concentrations of torasemide increased in a dose-dependent manner and showed no evidence of accumulation. There were also changes to electrocardiogram patterns and the macroscopic and microscopic appearance of the kidney and adrenal glands, but these changes were almost exclusively confined to the over-dosed groups. In conclusion, torasemide was found to be safe when administered to dogs at 0.25 mg/kg once daily for 26 weeks, and any changes were consistent with its known diuretic effects.

KEYWORDS
canine, cardiovascular disease, loop diuretic, torasemide

1 | INTRODUCTION

Congestive heart failure (CHF) is a clinical syndrome in dogs caused by heart disease characterized by sodium and water retention resulting in oedema (Hori et al., 2007).

Loop diuretics are commonly administered in the treatment and management of CHF in both humans and animals due to their ability to reduce intravascular hydrostatic pressure and reduce the clinical signs associated with oedema (Brater, 1996; Cosín & Díez, 2002; Hori et al., 2007; Peddle et al., 2012; Roush et al., 2014; Wargo & Banta, 2009). Torasemide is one such loop diuretic agent and has a pyridyl sulfonylurea structure (Uechi et al., 2003). Its primary site of action is the thick ascending limb of the loop of Henle where it promotes excretion of sodium, water and chloride via interaction with the sodium–potassium–chloride (Na^+, $2Cl^-$, K^+) cotransporter (Oyama et al., 2011). In addition, torasemide has been shown to have competitive antagonistic properties at the aldosterone receptor (Uechi et al., 2003; Wargo & Banta, 2009) without altering mineralocorticoid receptor (MR) expression and activity (Gravez et al, 2013; and Adam et al, 2015). The concept of torasemide having an

antialdosterone effect is controversial and remains to be proven. It has been suggested that torasemide could block aldosterone binding to the MR (Uchida et al., 1991). However, Gravez et al. (2013) demonstrated that torasemide does not bind the MR in rat cardiomyocytes and is not a MR antagonist. Adam et al. (2015) showed that torasemide but not furosemide inhibited human aldosterone synthase activity in transfected lung fibroblasts but this effect remains to be verified in other tissues and species by other groups. Considering that torasemide does not block the MR, its association with spironolactone in heart failure may be beneficial, through actions on complementary aldosterone pathways. It has been suggested that this additional antialdosterone property of torasemide may lead to blunting of diuretic resistance (Peddle et al., 2012) although this suggestion remains speculative.

Torasemide has been shown to have a greater bioavailability, longer half-life and longer duration of action than furosemide in human patients where it has been successfully used as an alternative to other diuretics such as furosemide (Brater, 1996; Buggey et al., 2015; Cosín & Díez, 2002; Vadivelan & Dabhi, 2013). Current evidence in human cardiac patients suggests clinical benefits of torasemide over furosemide, in particular on heart failure and cardiovascular readmissions, with no significant increase in adverse events (Cosín and Díez, 2002; Murray et al., 2001; Shah et al., 2018). It has also been successfully used as an alternative to furosemide in animals with CHF (Besche et al., 2020; Chetboul et al., 2017; Oyama et al., 2011; Peddle et al., 2012). In dogs, torasemide is reported to significantly reduce the risk of cardiac death or worsening of CHF versus furosemide (Besche et al., 2020; Chetboul et al., 2017). It has been shown to have at least 10 times the potency and twice the duration of action of furosemide (Ghys et al., 1985; Pelligand et al., 2020; Uechi et al., 2003). It is also excreted much more slowly than furosemide (Sogame et al., 1996) and is effective at low doses (Ghys et al., 1985; Paulin et al., 2016).

Although previous studies have been conducted on the effects of torasemide in dogs, they have either used high dose rates, short periods of administration or the results have been found to be equivocal (Hori et al., 2007; Okada et al., 1994; Wada et al., 1994; Uechi et al., 2003). The objectives of the present study were therefore to assess the safety of torasemide (ISEMID®, Ceva Santé Animale) in dogs after a 5-day loading period at 0.5 mg/kg followed by a 26-week administration period at 0.25 mg/kg and at multiples of three and five times this dose; to assess the reversibility of any changes after a 28-day recovery period; and to assess the toxicokinetics of torasemide.

2 | MATERIALS AND METHODS

2.1 | Animals

Beagles were used in this study as dogs are the target species for the test item. The animals were aged 9–10 months and weighed 8.1–11.8 kg (unneutered females) and 9.1–12.2 kg (unneutered males) prior to their first treatment. They had been vaccinated and received routine anthelmintic medication at least 14 days previously, and had not been used in any previous studies. The study was conducted in AAALAC certified facilities. The study protocol was reviewed and approved by the company's Institutional Animal Care and Use Committee before the inclusion of animals in the study.

2.2 | Housing

The dogs were housed in an environmental controlled room (16–25°C) with a day length of 12 h light and 12 h darkness. The animals were pair-housed for at least 2 h per day with a floor area of 6.1 m^2 for up to four dogs. Animals were individually housed in an area of 1.2 m^2 for approximately 1 h in the morning, and 1 h in the evening, in order to allow for feeding and monitoring of feeding and diet consumption. A raised area of 0.3 m^2 was available in each individual pen. Pens had underfloor heating, and the floors were washed daily.

2.3 | Allocation to treatment groups

Thirty-two suitable beagles were ranked by bodyweight within sex, then randomly allocated to four treatment groups (four males and four females per group), using SPSS/PC software, so that the treatment groups were as homogenous as possible with respect

TABLE 1 Experimental design

				Number of animals			
				Main		Recovery	
		Dose level (mg/kg)		Day 1 to Day 182		Day 183 to Day 211	
Group No.	Group designation	Initial (Day 1 to Day 5)	Maintenance (Day 6 to Day 182)	Male	Female	Male	Female
1	Control	–	–	3	3	1	1
2	1X	0.5	0.25	3	3	1	1
3	3X	1.5	0.75	3	3	1	1
4	5X	2.5	1.25	3	3	1	1

to bodyweight. Group 1 animals (Control) remained untreated throughout the study. They were exposed to the same dosing procedure than test item treated dogs daily. In order to minimize bias, all personnel involved in data collection were masked to treatment assignment and were not involved in administration of the test item. Animals assigned to groups 2, 3 and 4 were treated daily over 26 weeks with the test item (ISEMID®, Ceva Santé Animale) at 1X, 3X or 5X the maximum therapeutic dose, respectively. At the end of the treatment period (Day 183), six dogs from each treatment group were humanely euthanized by an intravenous overdose of sodium pentobarbital following a sedation with a combination of xylazine with ketamine and subjected to a full necropsy. The remaining two dogs from each treatment group were allowed a recovery period of 28 days before also being euthanized and subjected to a full necropsy (Table 1, Experimental Design).

2.4 | Test item administration

Animals assigned to group 1 were untreated. Animals assigned to groups 2, 3 and 4 were administered an initial dose of test item (once daily *per os*) between Day 1 and Day 5. This was followed by a maintenance dose of test item (once daily *per os*) between Day 6 and Day 182 (refer to Table 1). Doses were calculated based on the most recent bodyweight and made up using a combination of whole 2 mg and 4 mg tablets, and whole or halved 1 mg tablets, rounded up to the nearest half mg. The tablet formulation used was the final commercial formulation as ISEMID® (Ceva Santé Animale), produced under Good Manufacturing Practice (GMP). All dosing was successful, and there was no evidence of regurgitation or vomiting after dosing.

2.5 | Diet

Animals were offered 300 g of Ssniff® dry diet (Dog maintenance, 10 mm, Ssniff Spezialdiäten GmbH, D-59494 Soest Germany) per day, just after test item administration, throughout the study. Any remaining diet was offered again for approximately 1 h towards the end of the working day. However, as there was evidence of inappetence in some treated animals, they were offered additional wet diet. In order to get comparable diet consumption results, all animals were offered an additional 100 g of Pedigree® wet diet per day from Day 37 for males/Day 36 for females for the remainder of the study. In order to increase the animals' interest in the diet, wet diet was mixed with the dry diet.

A qualitative assessment of diet consumption was made daily, from Day 1 to Day 210, according to the following scoring system:

Score 1: Animal takes intense interest in feed and consumes the whole amount within a short period;
Score 2: Animal takes interest in feed and consumes the majority of it within a short period;
Score 3: Animal takes modest interest in feed, but consumes the majority of it within a working day;
Score 4: Animal takes slight interest in feed, and majority of the feed remains until the end of the working day;
Score 5: Animal takes no interest in feed and does not consume it.

2.6 | Water

Animals were provided with ad libitum municipal water delivered using an automatic water dispenser, except during periods of water consumption monitoring. Water consumption was measured over a 24 h period, once during baseline monitoring, on Days 5, 10, and approximately every 2 weeks thereafter.

2.7 | Clinical observations and body weight

Animals were checked twice daily for signs of morbidity/mortality, and general clinical observations were performed towards the end of each working day by trained personnel. Detailed clinical observations were performed once during acclimatization, once during baseline monitoring, on Days 5, 10, and approximately every 2 weeks thereafter. This included measurement of rectal temperature and a detailed physical examination of the animals by a veterinarian. Animals were weighed on Days −7, −1, 5, 10, and approximately every 2 weeks thereafter.

2.8 | Cardiovascular measurements

Indirect blood pressure and electrocardiogram (ECG) measurements were recorded using MAC 1200 ST type ECG device with the Lead II position on all animals once during acclimatization, once during baseline monitoring, on Days 5, 10, and approximately every 2 weeks thereafter. ECG were recorded over a period of at least 30 s.

2.9 | Clinical pathology

Blood samples were collected by cephalic venipuncture (after an overnight fast) on Days −2, 5, 10, 23, 37, 65, 93, 135, 182 and 210 (recovery animals only). Haematology parameters included red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red cell volume, platelet count, mean platelet volume, reticulocyte count, white blood cell count, neutrophil, lymphocyte, monocyte, basophil, eosinophil and large unstained cells. Coagulation parameters included activated partial thromboplastin time and prothrombin time. Clinical chemistry values included glucose, total bilirubin, urea, cholesterol, creatinine,

phosphorus, sodium, potassium, calcium, magnesium, chloride, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, gamma glutamyltransferase, amylase, creatine kinase, bile acids, lactate-dehydrogenase, triglycerides and aldosterone.

Urine samples for urinalysis were collected by urinary bladder catheterization at the same timepoints. Urine parameters included leukocyte, nitrite, pH, protein, glucose, urobilinogen, bilirubin, ketones, blood erythrocytes, specific gravity, sediment, colour and clarity.

2.10 | Toxicokinetics

Blood samples for toxicokinetic analysis of torasemide were collected prior to dosing on Day 1, then at 0 h (prior to dosing), 1, 2, 8, 12 and 24 h after dose administration on Days 5, 85 and 177. Plasma samples were stored frozen at approximately −20°C before determination of plasma torasemide concentrations using a LC-MS/MS method developed and validated previously in dog plasma (Pelligand et al., 2020). The limit of quantification in dog blood was 5 μg/L. Torasemide was stable in dog plasma for at least 184 days at −20°C. The following toxicokinetic parameters were calculated for each animal: C_{max}, t_{max}, $AUC_{0\text{-}t}$, C_{max}/Dose and $AUC_{0\text{-}t}$/Dose using WinNonlin software (version 5.2).

2.11 | Necropsy

A detailed macroscopic examination was performed on each animal. The following tissues were collected, trimmed and weighed: adrenal glands, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, spleen, testes, thymus, thyroids with parathyroids and uterus. The relative organ weight to the body and brain weight were calculated as recommended by the Society of Toxicologic Pathology to normalize animal-to-animal variability and to provide additional confidence in absolute weight data interpretation (Sellers et al., 2007).

In addition, samples of the following tissues were retained for histological examination: adrenal gland, aorta, brain (cerebral cortex, midbrain, cerebellum and medulla), epididymis, oesophagus, eye (with optic nerve), femur (with marrow), gallbladder, heart (ventricles, atria, ventricular septum and papillary muscle), kidney, lacrimal gland, large intestine (caecum, colon and rectum), liver, lungs (with bronchi), lymph node (mandibular and mesenteric), ovary, pancreas, pituitary, prostate, salivary gland, sciatic nerve, skeletal muscle (thigh), skin (including subcutis and mammary gland area), small intestine (including duodenum, ileum and jejunum with Peyer's patches), spinal cord (cervical, thoracic and lumbar), spleen, sternum (with marrow), stomach, testis, thymus, thyroid with parathyroid gland, tongue, trachea, urinary bladder, uterus (horns, body and cervix) and vagina.

Eyes, testes and epididymides were retained in modified Davidson's fixative. Lungs were infused with formalin. All other tissues were fixed in 10% formalin solution.

2.12 | Histopathology

A histopathological examination was performed on the retained tissues of all animals. The tissues and organs were cut at either 4 μm (soft tissues) or 6 μm (brain) by microtome and transferred to slides. Tissue sections were stained with haematoxylin-eosin/phloxine and examined by light microscopy. Data were collected using PROVANTIS (Version 7, Instem).

2.13 | Statistics

Data were collected and analysed using PROVANTIS (Version 7, Instem). Data were summarized and displayed as mean (SD). Comparisons between groups were made using ANOVA with Duncan's multiple range test when the ANOVA was significant ($p < .05$) or Mann–Whitney test as appropriate.

In addition to the statistical analysis, the clinical chemistry parameters were evaluated comparatively to published values (Kaneko et al., 1997).

2.14 | Standards

This study was a target animal safety study conducted at CiToxLAB, Hungary, according to VICH Guideline 43 (Guideline on Target Animal Safety for Veterinary Pharmaceutical Products), and the principles of Good Laboratory Practice ENV/MC/CHEM (98) 17.

3 | RESULTS

3.1 | Clinical observations

There was no mortality during the study, and no significant differences between the treatment groups and controls for rectal temperature, for either males or females. Evidence of diuresis and dryness of the oral mucosa was observed in animals assigned to all three treated groups from Day 4 until the end of the study. These same findings were not observed in the controls.

3.2 | Bodyweight

Statistical analysis of the bodyweight data revealed no significant differences ($p > .05$) between treatment groups and controls for either males or females over the course of the study.

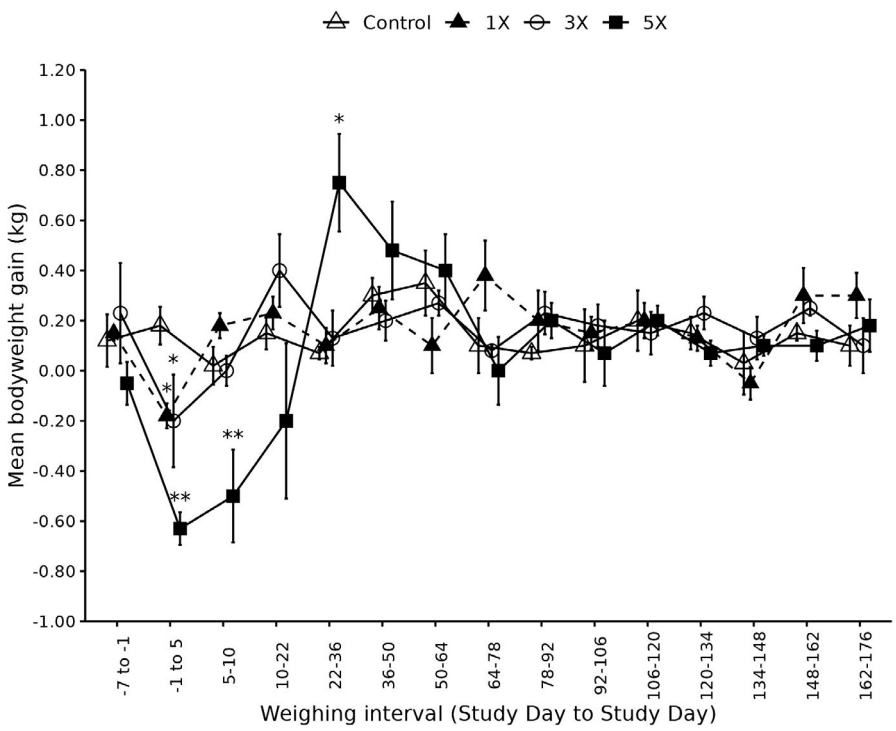

FIGURE 1 Mean and SEM bodyweight gain for male animals ($n = 4$ per group) during the study. Torasemide was administered once daily on Day 1 to Day 5 (at double the maintenance dose) and Days 6 to 182 (at a maintenance dose of 0.25 mg/kg (1X), 0.75mg/kg (3X) and 1.25 mg/kg [5X]). * $p < .05$, ** $p < .01$

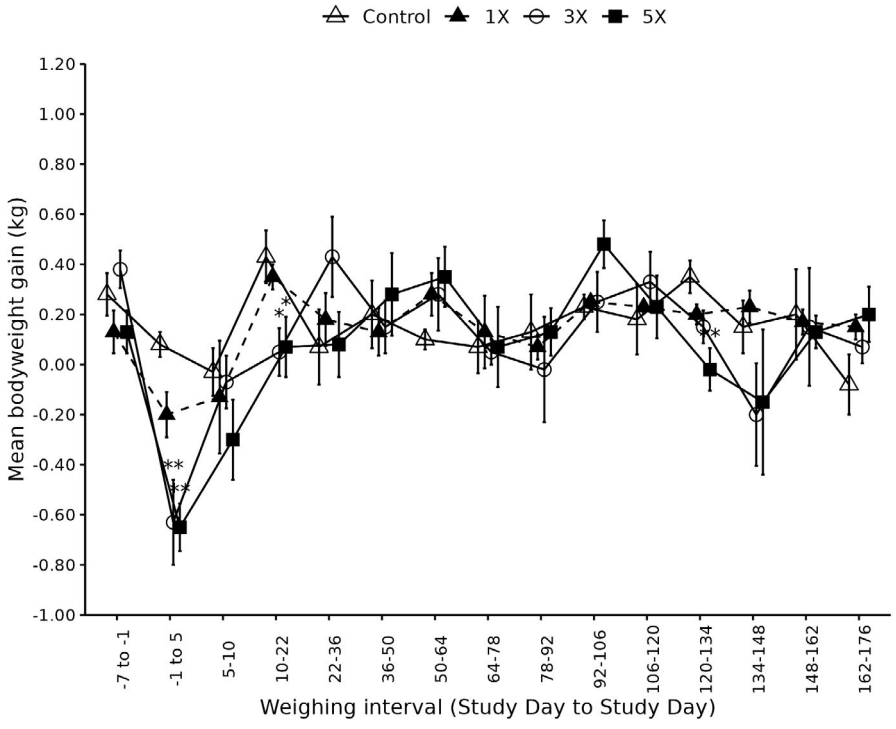

FIGURE 2 Mean and SEM bodyweight gain for female animals ($n = 4$ per group) during the study. Torasemide was administered once daily on Day 1 to Day 5 (at double the maintenance dose) and Days 6 to 182 (at a maintenance dose of 0.25 mg/kg (1X), 0.75mg/kg (3X) and 1.25 mg/kg [5X]). * $p < .05$, ** $p < .01$

However, when the rates of bodyweight gain during the weighing intervals were compared, male dogs in the all treated groups and female dogs in the 3X and 5X groups gained significantly less weight than control dogs between Day −1 and Day 5. Mean bodyweight gain data are graphically presented in Figures 1 and 2.

From Day 36 onwards, there were no significant differences ($p > .05$) in bodyweight gain between treatment groups and controls for either males or females, with the exception of 5X females on Days 120–134 ($p < .01$).

3.3 | Diet consumption

Untreated control animals were assigned scores of 1 at almost all assessments, meaning they showed an intense interest in their diet

TABLE 2 Qualitative assessment of food consumption during the treatment period (Day 1 to Day 182)

Treatment group (Dose Level)	No. of observations				
	Score 1	Score 2	Score 3	Score 4	Score 5
Group 1 (0X)	1,449	5	1	1	0
Group 2 (1X)	1,399	19	28	5	5
Group 3 (3X)	1,303	55	73	15	10
Group 4 (5X)	1,221	98	95	35	7

Note: Score 1: Animal takes intense interest in feed and consumes the whole amount within a short period;

Score 2: Animal takes interest in feed and consumes the majority of it within a short period;

Score 3: Animal takes modest interest in feed, but consumes the majority of it within a working day;

Score 4: Animal takes slight interest in feed, and majority of the feed remains until the end of the working day;

Score 5: Animal takes no interest in feed and does not consume it.

The number in each cell represents the number of times; this score was assigned to animals in this treatment group.

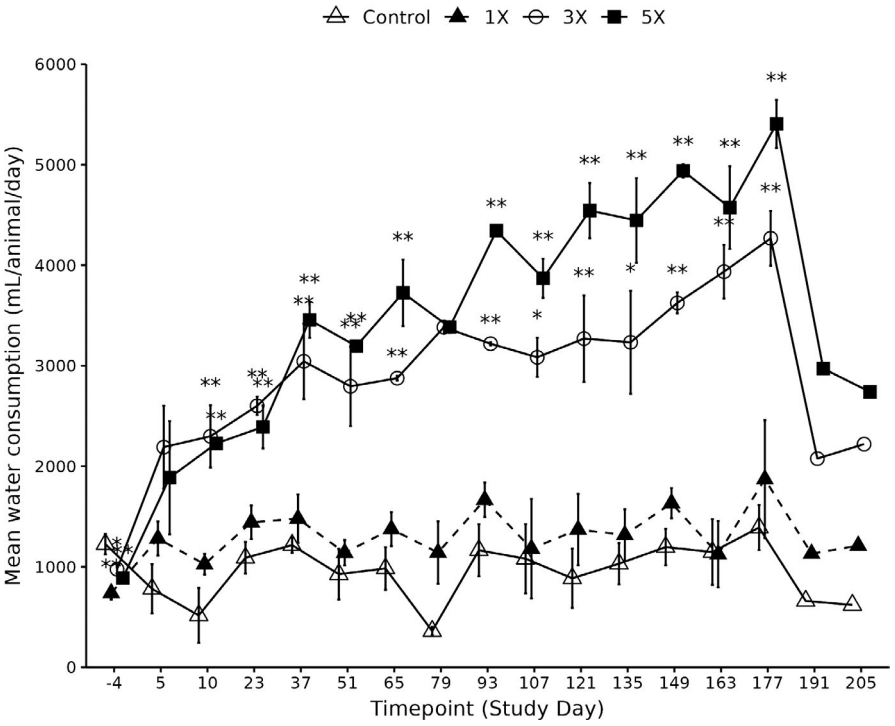

FIGURE 3 Mean and SEM water consumption for male animals ($n = 4$ per group) during the study. Torasemide was administered once daily on Day 1 to Day 5 (at double the maintenance dose) and Days 6 to 182 (at a maintenance dose of 0.25 mg/kg (1X), 0.75mg/kg (3X) and 1.25 mg/kg [5X]). Data collected on Days 191 and 205 were from a single recovery animal for each group and are therefore not mean values. * $p < .05$, ** $p < .01$

and consumed the whole amount within a short period, throughout the study. Scores of 2, 3, 4 and 5 were assigned almost exclusively to animals in the treated groups. A summary of the results of diet consumption is presented in Table 2. The overall incidence of reduced appetite (i.e. scores of 2–5) was calculated as 0.5% in the controls, 4% in the 1X, 11% in 3X and 16% in 5X animals, indicating a dose-dependent effect on appetite.

3.4 | Water consumption

Water consumption was significantly higher ($p < .05$) in the 3X and 5X groups than the control group for both sexes until Day 177 with some exceptions. During the recovery period, water consumption appeared to return towards control levels for the higher dose groups (see Figures 3 and 4).

3.5 | Cardiovascular measurements

Although there were some significant differences ($p < .05$) between treated and control animals for blood pressure measurements during the study, no patterns or effects related to torasemide treatment were observed (Figures S1–S4). Analysis of the electrocardiogram (ECG) data showed QT intervals were longer ($p < .05$), in treated animals compared to controls with a higher incidence in 5X males (Figures

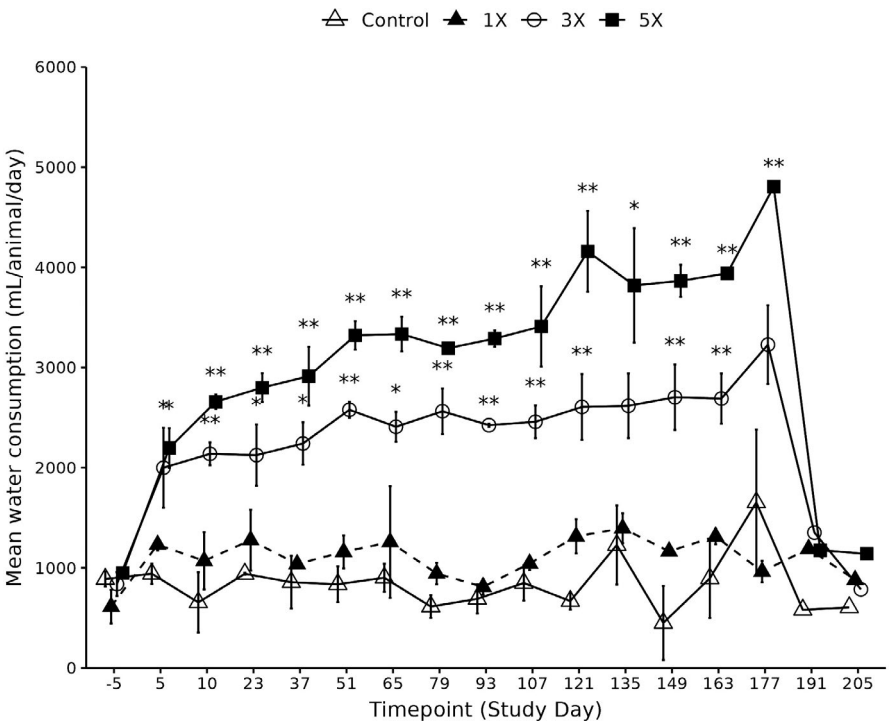

FIGURE 4 Mean and SEM water consumption for female animals ($n = 4$ per group) during the study. Torasemide was administered once daily on Day 1 to Day 5 (at double the maintenance dose) and Days 6 to 182 (at a maintenance dose of 0.25 mg/kg (1X), 0.75mg/kg (3X) and 1.25 mg/kg [5X]). Data collected on Days 191 and 205 were from a single recovery animal for each group and are therefore not mean values. * $p < .05$, ** $p < .01$

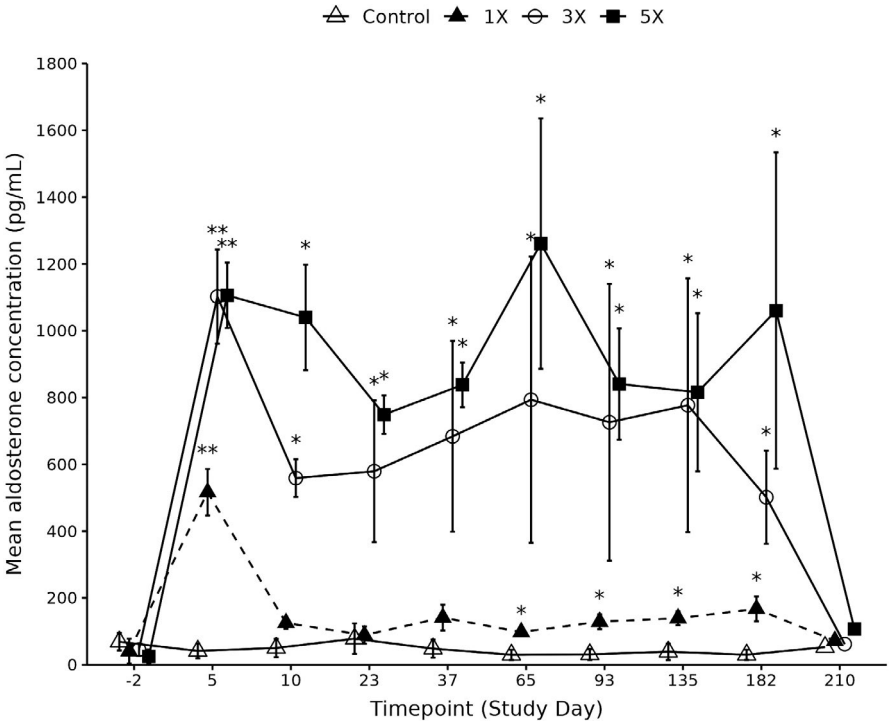

FIGURE 5 Mean and SEM serum aldosterone concentration measurements for male animals ($n = 4$ per group) during the study. Torasemide was administered once daily on Day 1 to Day 5 (at double the maintenance dose) and Days 6 to 182 (at a maintenance dose of 0.25 mg/kg (1X), 0.75mg/kg (3X) and 1.25 mg/kg [5X]). Data collected on Day 210 were from a single recovery animal for each group and are therefore not mean values. * $p < .05$, ** $p < .01$

S5–S6). In addition, P wave amplitude was higher ($p < .05$) in treated animals compared to controls (Figures S7–S8). During the recovery period, both parameters appeared to be close to control values.

3.6 | Clinical pathology

For haematology variables, a torasemide treatment-related increase was observed for red blood cell count (RBC), haemoglobin (HGB) concentration and haematocrit (Hct; Figures S9–S14). Overall, statistical significance ($p < .05$) was noted for RBC at the majority of time points in the 5X group, in some cases in the 3X group and sporadically in the 1X group when compared to controls. A similar trend was observed for HGB concentration with statistically significant ($p < .05$) differences at the majority of time points in the 5X group and sporadically in the 3X and 1X groups when compared to controls. Again, a similar pattern was noted for Hct. During the recovery period, RBC, HGB and Hct values appeared to return close to

values in the control group. For other parameters such as mean cell volume, reticulocyte per cent and lymphocyte percentage, statistically significant differences were observed in some sexes of some treated groups compared to the control group at varying time points. However, because there was no dose-related effect, they were considered to be of no clinical significance. In addition, no treatment-related changes were observed for coagulation parameters.

For clinical chemistry variables, torasemide treatment-related increase in concentration was observed for urea, creatinine, glucose, total protein, albumin and total bilirubin.

The effect on urea and creatinine appeared to be more pronounced for males than females. In male animals, statistical significance ($p < .05$) was observed at all time points for urea and most time points for creatinine when comparing each treated group with the control group. For the most part, a dose-dependent increase was observed for these parameters. All urea values were above the upper reference value (3.33 mmol/L) whereas all creatinine values remained below the upper reference value (132.6 mmol/L; Figures S15–S18).

For total protein, a statistically significant ($p < .05$) elevation was observed at a number of time points in both the 3X and 5X groups for both sexes. The peak effect was noted on Day 5 for both sexes of the 5X group. Individual values were within the reference range in both sexes, except on Day 5, when both males and females 5X and males 3X group mean were higher than the reference maximum value (71 g/L). Similar patterns were noted for glucose, albumin and total bilirubin (Figures S19–S26).

During the recovery period, values returned to within the reference ranges for most of clinical chemistry variables mentioned above. For the most part values in treated animals returned close to values in the control group.

Upon examination of the clinical chemistry electrolytes, in general a drug treatment-related increase was noted for serum calcium and magnesium while a torasemide-related decrease was noted for phosphorus, chloride, potassium and sodium (Figures S27–S38). Statistically significant ($p < .05$) differences were observed for the most part in the 3X and 5X groups, for both sexes. Most of the individual values were within or closed to the reference ranges, except in treated dogs dipping below the reference range for all dose groups (chloride) and the male 5X group (sodium). Following the recovery period, the majority of these electrolytes showed a good indication of recovery.

A torasemide treatment-related increase was observed for aldosterone for both sexes (Figures 5 and 6) with statistically significant ($p < .05$) differences to controls at all time points in the 3X and 5X groups, and on several occasions for males and on one occasion for females in the 1X group. During the recovery period, aldosterone values in treated animals returned closer to those observed in control animals.

Overall, the urine specific gravity results showed a dose-dependent decrease. Statistically significant ($p < .05$) differences were noted at some time points for males and females, predominantly in the 3X and 5X groups (Figures S39–S40). Although no clear dose response was observed for urinary pH, higher mean urinary pH values ($p < .05$) were measured for both sexes in the 3X and 5X groups, at most time points between Day 10 and Day 182, compared to the control group (Figures S41–S42). After the treatment period, the majority of animals showed some indication of recovery for these two urinary parameters.

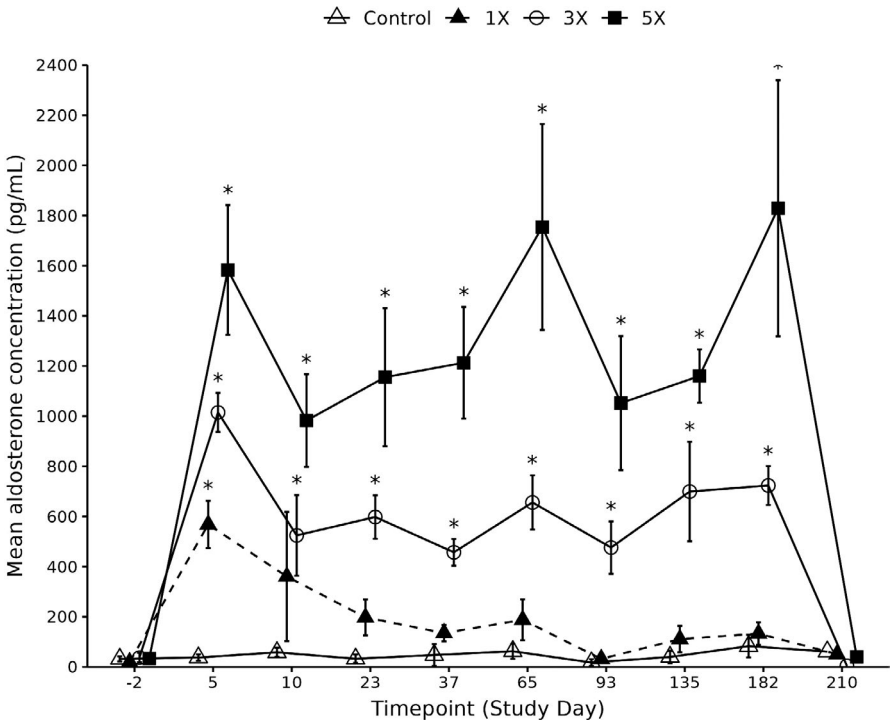

FIGURE 6 Mean and SEM serum aldosterone concentration measurements for female animals ($n = 4$ per group) during the study. Torasemide was administered once daily on Day 1 to Day 5 (at double the maintenance dose) and Days 6 to 182 (at a maintenance dose of 0.25 mg/kg (1X), 0.75mg/kg (3X) and 1.25 mg/kg [5X]). Data collected on Day 210 were from a single recovery animal for each group and are therefore not mean values.
* $p < .05$

TABLE 3 Mean (standard deviation) weight of adrenal glands and kidneys at necropsy on Day 183

Treatment Group (Dose Level)	Gender	Adrenals (g)	Adrenal/ Bodyweight	Adrenals/Brain	Kidneys (g)	Kidneys/ Bodyweight	Kidneys/Brain
Group 1 (0X)	Male	1.243 (0.083)	0.0102 (0.0015)	1.545 (0.139)	68.55 (16.62)	0.560 (0.118)	85.12 (20.62)
Group 2 (1X)		1.439 (0.010)	0.0112 (0.0013)	1.726 (0.261)	59.55 (2.10)	0.463 (0.032)	71.11 (6.02)
Group 3 (3X)		1.748 (0.393)	0.0130 (0.0032)	2.217 (0.460)	83.12 (7.89)	0.615 (0.067)	105.41 (6.96)
Group 4 (5X)		**1.917 (0.421)**	0.0156 (0.0035)	**2.470 (0.495)**	73.06 (6.77)	0.596 (0.061)	94.31 (5.79)
Group 1 (0X)	Female	1.617 (0.374)	0.0133 (0.0021)	2.130 (0.536)	46.54 (5.18)	0.384 (0.006)	61.17 (8.20)
Group 2 (1X)		1.592 (0.071)	0.0133 (0.0010)	2.277 (0.035)	49.16 (3.81)	**0.411 (0.010)**	70.29 (3.70)
Group 3 (3X)		1.948 (0.049)	0.0182 (0.0027)	2.800 (0.076)	59.71 (3.16)	**0.556 (0.065)**	85.77 (1.72)
Group 4 (5X)		2.044 (0.320)	**0.0190 (0.0040)**	2.630 (0.617)	59.59 (11.62)	**0.553 (0.121)**	76.73 (20.06)

Note: Bold indicates statistical significance at $p < .05$.

TABLE 4 Incidence of test item-related microscopic findings in the kidneys after the treatment and recovery period

	End of treatment period ($n = 6$ animals per group)				End of recovery period ($n = 2$ animals per group)			
Kidneys	Control	1X	3X	5X	Control	1X	3X	5X
Multifocal interstitial nephritis	0	0	6	6	0	0	2	2
Dilatation of tubules	0	0	6	6	0	0	1	2
Subscapular cysts in the renal cortex	0	0	3	5	0	0	1	2

TABLE 5 Incidence of test item-related microscopic findings in the adrenals after the treatment and recovery period

	End of treatment period ($n = 6$ animals per group)				End of recovery period ($n = 2$ animals per group)			
Adrenal glands	Control	1X	3X	5X	Control	1X	3X	5X
Hypertrophy/hyperplasia of zona glomerulosa	0	0	6	6	0	0	0	0

3.7 | Organ weights

Statistical analysis of the organ weight data showed statistically significant ($p < .05$) increases in both the absolute mean weight of the adrenal glands and the mean weight of the adrenal glands when adjusted for brain weight, in male animals in the 5X group compared to control male animals. When adjusted for bodyweight, the mean weight of the adrenal glands of female animals in the 5X group was significantly greater ($p < .05$) than control female animals (see Table 3).

When adjusted for bodyweight, the mean kidney weight of female animals in each of the treated groups was significantly greater ($p < .05$) than female control animals (Table 3).

When adjusted for bodyweight, the mean heart weight of males assigned in the 5X group was statistically lower ($p < .05$) than the control animals (Table S1).

3.8 | Macroscopic examinations

There were no visible lesions on the kidneys of animals assigned to the control and 1X groups. However, few to many cortical subcapsular cysts (1–4 mm in diameter) were present in the kidneys of 2/6 animals in the 3X group and 5/6 animals in the 5X group at the end of the treatment period. Of the animals that were allowed a 28-day recovery period after treatment, cortical cysts were also present in 1/2 animals in the 3X group and in 2/2 animals in the 5X group (data not shown).

3.9 | Microscopic examinations

3.9.1 | Main study

There were no microscopic findings in the kidney tissues of animals in the control group and 1X group. However, a number of abnormalities were observed in the kidney tissues of animals of both sexes in the 3X and 5X groups (Table 4). These findings were characterized as multifocal interstitial inflammation, dilatation of cortical tubules and subcapsular cysts. Mononuclear inflammatory cells with fibroblasts were also noted. Dilatation of cortical tubules was accompanied by expansion of the lumina and lined with flattened tubular epithelium. Single or multiple subcapsular cysts were surrounded by connective tissue. The severity of the interstitial inflammation and tubular dilatation was classified as moderate in only one of the six animals in the 3X group, but was classified as moderate in four of the six animals in 5X group. Multiple cortical cysts were observed in only two of

the six animals in the 3X group but were observed in five of the six animals in the 5X group.

There were no microscopic findings in the adrenal tissues of control animals and 1X animals. However, a number of abnormalities were observed in the adrenal tissues of animals of both sexes assigned to 3X and 5X groups (Table 5). This included minimal to moderate reactive hypertrophy/hyperplasia of the zona glomerulosa. The changes were characterized by thickening of the zona glomerulosa, with enlargement (hypertrophy) and an increased number of diffuse cortical cells (hyperplasia), without atypia or increased mitosis.

Minimal to mild cortical atrophy was observed in the thymus glands of some animals in the 3X and 5X groups only. This correlated with gross observations and was thought to reflect individual stress conditions in the affected animals, rather than being a direct effect of the test item.

3.9.2 | Recovery animals

There were no microscopic findings in the kidney tissues of animals in the control group and 1X group that were allowed a recovery period prior to necropsy. However, test item-related histopathological findings were observed in the kidney tissues of animals in the 3X and 5X groups that were allowed a 28-day recovery period (Table 4). The severity of the multifocal interstitial inflammation was classified as minimal in both animals in the 3X group but was classified as mild to moderate in the two recovery animals in the 5X group.

Microscopic examination of the interstitial nephritis showed an increased population of fibroblasts compared with the main cohort of animals in which inflammatory mononuclear cells were predominantly seen. The tubular dilatation in 3X dose group animals was minimal, as opposed to the minimal to moderate classification seen in the main cohort of animals. Cysts were still present in animals in the 3X and 5X groups even after the recovery period.

No changes to the adrenal glands were observed at the light microscopic level in animals allowed a recovery period prior to necropsy.

3.10 | Torasemide plasma concentrations

On Day 5 (following administration of the initial doses of torasemide ranging from 0.5 to 2.5 mg/kg) and on Days 85 and 177 (following administration of maintenance doses of torasemide ranging from 0.25 to 1.25 mg/kg), there appeared to be a dose-dependent increase in the plasma concentration and exposure to torasemide (Figures S43–S46). Although not statistically tested, there was no evidence of accumulation of torasemide over the course of the study. A summary of toxicokinetic parameters is presented in Table 6.

4 | DISCUSSION

As a diuretic, torasemide has the effect of removing excessive fluid from the body. The dryness of the mucosa (oral cavity) and diuresis observed in treated animals in the current study might therefore be

TABLE 6 Summary of mean (standard deviation) toxicokinetic parameters of torasemide calculated during the study

Gender	Day	Group	C_{max} (μg/L)	t_{max} (h)	$AUC_{0\text{-}t}$ (h* μg/L)	C_{max}/Dose (μg/L)/(mg/kg)	$AUC_{0\text{-}t}$/Dose (h* μg/L)/(mg/kg)
Female	5	1X	4932 (802)	1 (0)	42292 (14234)	9864 (1605)	84584 (28469)
		3X	16856 (2311)	1 (0)	111131 (20894)	11237 (1540)	74087 (13929)
		5X	33604 (3652)	1 (1)	259442 (88220)	13441 (1461)	103777 (35288)
	85	1X	2913 (968)	2 (1)	24311 (3950)	11651 (3871)	97246 (15800)
		3X	9382 (2740)	2 (1)	86385 (27089)	12509 (3654)	115180 (36119)
		5X	16916 (2692)	2 (1)	171618 (26349)	13533 (2153)	137294 (21079)
	177	1X	2606 (452)	2 (1)	24829 (2256)	10423 (1806)	99314 (9026)
		3X	9556 (2683)	1 (0)	83290 (23251)	12741 (3578)	111053 (31001)
		5X	15809 (5722)	2 (1)	175643 (33380)	12648 (4578)	140514 (26704)
Male	5	1X	4781 (491)	1 (1)	53858 (18280)	9562 (982)	107716 (36559)
		3X	14324 (4273)	2 (1)	159215 (57847)	9550 (2848)	106143 (38565)
		5X	35093 (4202)	1 (0)	209376 (56092)	14037 (1681)	83750 (22437)
	85	1X	3449 (1366)	1 (1)	34203 (11476)	13795 (5462)	136813 (45903)
		3X	10500 (1008)	1 (0)	102146 (34186)	14000 (1344)	136195 (45582)
		5X	14816 (2767)	2 (1)	170414 (42690)	11853 (2213)	136332 (34152)
	177	1X	3614 (1763)	1 (1)	39164 (17426)	14456 (7052)	156656 (69704)
		3X	8519 (1793)	2 (1)	107832 (42759)	11359 (2390)	143776 (57012)
		5X	14140 (5400)	3 (3)	176723 (40466)	11312 (4320)	141379 (32373)

expected. Similar findings were also noted in previous safety studies in which torasemide was daily administered during 13 weeks with oral dose levels set to 0.8, 2.5 and 8.0 mg/kg (Wada et al., 1994) or during 52 weeks with lower oral doses set to 0.01, 0.08 and 0.4 mg/kg (Okada et al., 1994).

The blood pressure data also correspond with previous studies where torasemide was found to have no effect on blood pressure in healthy dogs (Okada et al., 1994; Wada et al., 1994).

However, no abnormalities were seen in the ECG data collected during previous studies (Okada et al., 1994; Wada et al., 1994), whereas in the current study P waves were found to be significantly higher, and QT intervals significantly longer than controls, predominantly in male animals administered torasemide at the 5X dose level (1.25 mg/kg). Since the differences were mostly confined to the 5X dose group, and as no associated clinical signs were observed, these results are considered to be equivocal. Nevertheless, a causative role of torasemide in plasma electrolyte changes in the current study seems likely.

Diet consumption assessment shows that inappetence was associated with torasemide administration. A reduction of food consumption was also noted by Wada et al. (1994), especially in the first 4 weeks after dose administration. As in the current study, the inappetence in the study of Wada et al. (1994) appeared to be more pronounced in females. Okada et al. (1994) suggested that the decrease in food consumption was related to the slight dehydration caused by the diuretic action of torasemide.

The increased water consumption by treated animals in the current study corresponds with the findings of previous studies (Okada et al., 1994; Wada et al., 1994). This effect is also very likely to be related to the diuretic effect of torasemide.

Although there were no significant differences between control and treated groups for bodyweight throughout the study, there were significant differences for bodyweight gain, especially during the first 3 weeks after daily dosing commenced. The periods during which bodyweight gain was lower in treated animals corresponds to the period during which many instances of inappetence were also noted in the same animals. From Day 36/37 onwards, all animals were offered 100 g of wet diet mixed into their dry diet. From this point onwards until the end of the study, there were no significant differences, for the most part, in bodyweight gain between treatment groups and controls. Significantly lower bodyweight in animals treated with at least 0.8 mg of torasemide/kg was observed by Wada et al. (1994). The effect on bodyweight gain may be partly attributable to the inappetence noted above, but also partly due to the diuretic effect of torasemide, which causes a decrease in bodyweight by reducing body fluid volumes (Vadivelan and Dabhi, 2013). A reduction in bodyweight has also been noted in human subjects administered torasemide (Brater, 1996). Wada et al. (1994) also noted that during the recovery period the bodyweight gain for animals administered higher doses tended to surpass that of control animals.

The torasemide treatment-related increase in red blood cell count (RBC), haemoglobin concentration (HGB) and haematocrit (Hct) is commonly seen with loop diuretics and is considered to be largely due to the haemoconcentration effect of torasemide (Wada et al., 1994). As in the study of Wada et al. (1994), haematology parameters in the current study tended to revert to baseline values following the withdrawal of the drug.

Torasemide treatment-related increases in concentrations were observed during the administration period for serum urea, creatinine, glucose, total protein, albumin and total bilirubin in the current study. Wada et al. (1994) found high serum total protein, albumin, serum urea nitrogen and serum creatinine from week four of daily dosing onwards, compared to controls and pre-dosing values, for torasemide-treated dogs, but noted that levels tended to recover by Weeks 9–13 of administration. With lower torasemide doses, Okada et al. (1994) found increases in serum urea and creatinine (azotemia). Although both authors agreed that these changes could be partly attributed to the haemoconcentration effect of the torasemide (Wada et al., 1994), both agreed that the changes could also be attributable to degenerative changes in kidneys caused by repeated torasemide administration. This could be the case for animals administered overdoses (3X and 5X) in the current study, where changes to the renal tissues were observed, both at the macroscopic and microscopic level at the end of torasemide administration period with some evidence of recovery in the 3X dose group after the 4-week recovery period.

The torasemide treatment-related increases in aldosterone concentrations in the current study are consistent with the findings of previous studies (Hori et al., 2007; Uechi et al., 2003). Uechi et al. (2003) proposed that the increase in aldosterone may occur because the torasemide prevents circulating aldosterone from binding to its receptor. Peddle et al. (2012) also proposed that the greater diuretic effect of torasemide compared to furosemide may be linked to blunting of diuretic resistance due to torasemide's aldosterone antagonist properties. Buggey et al. (2015) cite a study in humans where blocking of the renin–angiotensin–aldosterone system (RAAS) through aldosterone receptor antagonism with spironolactone was shown to increase aldosterone levels. They too speculate that this may be due to the loss of feedback inhibition. Ames and Atkins (2016) also suggested that the dramatic increases in aldosterone levels in dogs caused by torasemide may be related to activation of the RAAS, and Gravez et al (2013) showed that torasemide does not act as a MR antagonist. In the current study, the aldosterone increase observed may be more likely due to the RAAS activation induced at these dosages by torasemide. The data suggest that the effect is reversible, as aldosterone concentrations appeared to revert to baseline during the recovery period. Furthermore, there were no histological abnormalities in the adrenal glands of animals allowed a 28 day recovery period prior to necropsy.

A drug treatment-related increase in concentration was noted for calcium and magnesium. Okada et al. (1994); Wada et al. (1994) reported increases in serum calcium in healthy beagles but did not test for magnesium. However, Peddle et al. (2012) found no significant differences in serum calcium in dogs with CHF that had been treated with torasemide. Wada et al. (1994) proposed that

increases in serum calcium are a common change observed with loop diuretics consequent to the dehydration induced which may eventually lead to an increase of concentration. In contrast, the current study showed a torasemide-related decrease in concentration for phosphorus, chloride, potassium and sodium. Okada et al. (1994) found similar decreases in serum sodium and chloride concentrations in healthy beagles. However, as in the current study, Wada et al. (1994) found equivocal results for some of these parameters. As in the studies of Okada et al. (1994); Wada et al. (1994), the majority of the affected electrolytes in the current study showed a good indication of recovery after torasemide withdrawal.

The decreases in urine specific gravity and increases in urine pH in the current study after torasemide administration are also broadly in line with the results of previous studies (Hori et al., 2007; Okada et al., 1994; Uechi et al., 2003; Wada et al., 1994). These changes are thought to be due to the diuretic action of torasemide itself.

Although a number of studies have been conducted with torasemide in dogs (Hori et al., 2007; Okada et al., 1994; Wada et al., 1994; Uechi et al., 2003), none of these included analysis of plasma torasemide concentrations. Analysis of torasemide plasma concentrations in treated animals, in the current study, revealed largely dose-dependent increases in both plasma concentrations and overall exposure to the drug. The maximal plasma concentrations were observed between 1 and 2 h after dosing, irrespective of the dose level. There was no evidence of accumulation of torasemide plasma concentrations during the current study.

An elevation in kidney weight relative to bodyweight was observed in female beagles in the current study. However, since there was no corresponding change in the kidney weight of male animals, or in the absolute weight, and weight relative to brain weight, of the female kidneys, this finding is not considered to be of clinical significance. This corresponds with the findings of previous studies with torasemide in Beagles where changes in kidney weight were either not found (Okada et al., 1994) or found in males only (Wada et al., 1994).

Similarly, a decrease in heart weight when adjusted to bodyweight was observed in the male animals in the current study. Since there were no corresponding changes in the heart weight of females and the decrease heart weight is not correlated with microscopic changes, this finding was not considered to be of clinical significance. This corresponds with the findings of Wada et al. (1994) who found a decrease of absolute heart weight in both sexes associated with the bodyweight decrease.

Although there appeared to be a dose-dependent elevation in mean adrenal gland weight in the current study, statistical significance was only reached in male animals in the 5X group. This corresponds with the findings of Wada et al. (1994) who found significant increases in adrenal gland weight in Beagles treated with torasemide, but not with the findings of Okada et al. (1994) who found no significant difference between treated animals and controls. However, it should be noted that the dose rates of torasemide used in the study of Okada et al. (1994) were much lower (0.01–0.4 mg/kg) than those administered to either the 5X group in the current study (1.25 mg/kg) or used in the study of Wada et al. (1994) (0.8–8 mg/kg). Therefore, it can be concluded that long-term torasemide administration is likely to increase adrenal weight in Beagles at higher dose rates. The increases in adrenal weight are likely to be related to the hypertrophy and hyperplasia of the adrenal tissues seen during the histological examination (described below), and the increased production of aldosterone seen in treated animals throughout the study.

Cortical subcapsular cysts (1–4 mm in diameter) with clear fluid content were observed in the kidneys in both of the overdose groups in the current study. Wada et al. (1994) describes fine granular surface patterns on the kidneys of dogs administered torasemide at either 0.8, 2.5 and 8 mg/kg, daily, over 13 weeks, but does not mention subcapsular cysts. Okada et al. (1994) made similar observations to Wada et al. (1994). However, Greaves (2007) reports on a study in beagle dogs where high doses of the diuretics furosemide and muzolimine were associated with subcapsular cyst formation. Since electrolyte and fluid replacement were seen to alleviate the effects, he maintains that the effects were secondary to excessive electrolyte and water loss rather than a primary cellular toxic effect of the diuretics themselves.

Wada et al. (1994) found degeneration and dilatation of urinary tubules in the kidneys (mainly in the distal and collecting tubules), calcium deposition and cell infiltration. Okada et al. (1994) found evidence of degeneration/dilatation of urinary tubules, cell infiltration, calcium deposition, fibrosis and thickening of the basement membrane in Bowman's capsule. The histological changes seen in renal tissues in the current study are thus consistent with those seen previously in torasemide-treated beagles.

The presence of histological abnormalities in the current study in animals in the 3X and 5X groups that had been allowed a 28-day recovery period prior to necropsy would suggest that the kidneys did not revert to normal after torasemide withdrawal. This is contrary to the view of Greaves (2007), but consistent with the view of Wada et al. (1994) who stated that renal tissues are thought not to recover easily even after drug withdrawal. However, the absence of histological abnormalities in animals administered the maximum therapeutic dose of torasemide (1X) in the current study suggests that histological changes are unlikely to occur when torasemide is used in the therapeutic range.

There was some evidence of recovery in that after the 4-week recovery period the severity of the multifocal interstitial nephritis and the tubular dilatation was only minimal in the 3X dose group. In addition, there was a qualitative difference in the microscopic characteristics of the interstitial nephritis between dogs necropsied immediately after torasemide withdrawal and animals allowed a 28-day recovery period prior to necropsy. While mononuclear inflammatory cells were mainly recorded in the former, a predominantly fibroblast population was noted in the latter, which suggests an ongoing repair process. Some recovery may eventually occur as suggested by Greaves (2007).

Histological changes to the adrenal glands were characterized by a thickening of zona glomerulosa with enlargement (hypertrophy)

and an increased number of mainly diffuse cortical cells (hyperplasia), without atypia or increased mitosis. This was probably associated with the increased production of aldosterone in treated animals throughout the study. The fact that no histopathological changes were noted at necropsy in the adrenal glands of animals allowed a 28-day recovery period, and that aldosterone production appeared to revert to baseline during this period, reinforces the view that the changes to the adrenal glands caused by torasemide administration are reversible.

5 | CONCLUSIONS

The results of this study demonstrate that torasemide is safe when administered once daily to beagle dogs at an initial dose of 0.5 mg/kg for 5 days, followed by a maintenance dose of 0.25 mg/kg for 26 weeks which corresponds to the maximal maintenance dose approved by the EMA for ISEMID® to be used for a long-term treatment with torasemide in cardiac dogs with CHF. Any changes observed at this dose level were consistent with the known diuretic effects of the test item.

At multiples of three and five times this dose, more pronounced effects were noted. Most of the changes appeared to revert to normal after a 28 day recovery period, with the exception of the histopathological findings in the kidney.

This is the first published study looking at the tolerance of an approved drug containing torasemide. Further safety data are warranted from field trials in order to confirm the safety of this drug in the target population (CHF dogs).

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CONFLICT OF INTEREST

Evelyne Coussanes, Emilie Guillot, Reynald Magnier and Anne Geneteau are employees of Ceva Santé Animale who supplied the test item. Jonathan Elliott is preclinical expert of ISEMID® registration and has received consulting fees from Ceva Santé Animale within the past 5 years.

AUTHOR CONTRIBUTIONS

Evelyne Coussanes designed and monitored the study. Emilie Guillot contributed to data interpretation and manuscript content. Reynald Magnier was responsible of the bioanalytical analysis. Anne Geneteau was responsible of the toxicokinetic analysis. Jonathan Elliott contributed to the interpretation of results and manuscript content. All authors contributed to and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data from this study are not publicly available because they contain proprietary information of a recently registered veterinary drug. Requests to access the data sets should be directed to Evelyne Coussanes: evelyne.coussanes@ceva.com.

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SUPPORTING INFORMATION

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