Tissue distribution and characterization of drug related material in rats and dogs after repeated oral administration of Casopitant

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RUNNING TITLE

a) Casopitant and its metabolites in rat and dog tissues.

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d) Abbreviations used are:

NK-1 = neurokinin-1

DRM = drug related material

GW679769 = 1-piperidinecarboxamide,4-(4-acetyl-1-piperazinyl)-N-((1R)-1-(3,5-bis(trifluoromethyl)phenyl)-ethyl)-2-(4-fluoro-2-methylphenyl)-N-methyl-(2R,4S)

HPLC = high performance liquid chromatography

HPLC-MS/MS = high performance liquid chromatography tandem mass spectroscopy

NMR = nuclear magnetic resonance

AUC = area under the plasma concentration-time curve

C_max = maximal plasma concentration

T_max = time to reach C_max
ABSTRACT
Casopitant \([1\text{-piperidinecarboxamide,}4\text{-}(4\text{-acetyl-1-piperazinyl})\text{-}N\text{-}((1R)-1\text{-}(3,5\text{-bis(trifluoromethyl)phenyl})\text{-}ethyl)\text{-}2\text{-}(4\text{-fluoro-2-methylphenyl})\text{-}N\text{-}methyl\text{-}(2R,4S)]\) has been shown to be a potent and selective antagonist of the human Neurokinin 1 (NK1) receptor, the primary receptor for Substance P. During long-term toxicity studies conducted in rat and dog, evidence of cardiomyopathy and increased cardiac weight were observed. The distribution and metabolism of casopitant were studied in both species evaluating the accumulation of drug related material (DRM) after repeat dosing and its potential relationship with pathological findings observed in myocardium. Following repeat oral administration of \(^{14}\text{C}\)casopitant to rats (20 days) and dogs (14 days), DRM was quantifiable in all of the tissues examined with lung and liver containing the highest level of radioactivity. The concentration of radioactivity was significantly higher in tissues than in plasma, declining slowly and still quantifiable after a recovery period of 20 days. The principal circulating components identified in both species were casopitant, M12 (oxidized deacetylated), M13 (hydroxylated piperazine), M31 and M134, (two N-dealkylated piperazine). In tissues a similar metabolic pattern was observed, where casopitant, M31, M134, M76 (N-deacetylated) and M200 (N-deacetylated N,N deethylated) were the major components quantified. Following 26 weeks repeat dose study in dog, casopitant and M13 were the major circulating components whilst in myocardium, M200 and M134 were the major ones and their levels increased over the time reaching considerable concentrations (mM magnitude). After a wash out period, all circulating derivatives decreased to undetectable levels whilst M200 was still the major component in myocardium. Overall DRM in plasma did not correlate with respective concentrations in tissues.
Introduction

Neurokinin subtype-1 (NK-1) receptors are widely distributed in the peripheral and central nervous system including areas thought to be involved in the regulation of affective behavior and neurochemical response to stress (Leslie, 1985; Kramer et al., 1998; Holmes et al., 2003). NK-1 receptors are also found in non-neural tissues such as endothelial and inflammatory cells as well as gastrointestinal, respiratory, and genitourinary tissues. Blocking NK-1 neurotransmitter receptors has been demonstrated to be effective for the treatment of Major Depressive Disorder (MDD), one or more anxiety disorders (Kramer et al., 2004; Furmark et al., 2005) and to prevent chemotherapy induced and post operative nausea and vomiting (Warr et al., 2005; Quartara et al., 2009).

Casopitant, also known as GW679769, is a piperidine derivative that has been shown to be a potent and selective antagonist of the human NK-1 receptor, the primary receptor of substance P (SP), both in vitro and in vivo with good brain penetration properties (Minthorn et al., 2008). Based on this mode of action it has been evaluated for the prevention of chemotherapy-induced and post-operative nausea and vomiting (Herrstedt et al., 2009; Khojasteh et al., 2009). In addition, casopitant has been investigated in a number of chronic dosing indications where the NK-1 receptor is believed to play a role, such as anxiety, depression, insomnia, and over-active bladder.

The nonclinical safety package of casopitant included appropriate investigations for both acute and chronic indications. During long term repeat dose toxicity studies in rats (6-month) and dogs (9-month) evidence of cardiomiopathy (namely myocardial necrosis, degeneration and inflammation) and increased heart weight, with no significant sex differences, were recorded,
never detected in the previous shorter toxicity studies. These changes were
accompanied by increases in plasma levels of cardiac Troponin I (cTnI) and
creatine kinase MB-mass (CK-MB) isoenzyme concentrations, biomarkers for
cardiac damage. In addition, transmission electron microscopy (TEM) showed
ultrastructural changes in the heart of both preclinical species, considered
suggestive of phospholipid accumulation. Based on authors’ experience of NK-1
receptor antagonists (unpublished data) and on published preclinical data of this
class of drugs
(http://www.accessdata.fda.gov/drugsatfda_docs/NDA/2008/022023s000TOC.cf
m), it is unlikely that all the above findings could be explained by NK-1 receptor
antagonist mechanism of action.

As a consequence of this cardiac finding, a battery of supplementary
preclinical studies were conducted to determine the time to onset of the
cardiovascular effect, to assess the progression of lesions, to investigate the
reversibility of these effects and to understand if accumulation or high
concentration of DRM in myocardium could be correlated with the observed
toxicological findings.

Following a single oral administration in rats and dogs, casopitant has
been shown to be extensively metabolized, widely distributed with quite long
retention time of DRM in tissues and slow rate of elimination mainly in dog
(Miraglia et al., 2010).

This article provides an overview of a series of disposition experimental
studies conducted to investigate: 1) the distribution and accumulation of DRM in
tissues and plasma after repeat oral administration of casopitant in rats and
dogs; 2) the residual concentration of DRM in tissues after different washing
periods; 3) the nature of metabolites detected and the quantification of the most relevant ones in plasma and selected tissues, myocardium in particular.

Casopitant was administered as its mesylate salt (using either unlabelled or [\(^{14}\text{C}\)] labelled salt) and the doses selected were within the range of cardiotoxic doses.
MATERIALS AND METHODS

Chemicals

Casopitant mesylate (coded as GW679769B), standards of metabolites M12 (coded as GSK631832), M13 (coded as GSK525060), M31 (coded as GSK517142), M15 (coded as GSK1497960), M76 (coded as GW679979), M134 (coded as GSK2110277), M200 (coded as GSK2174599), were all supplied by Chemical Development, GlaxoSmithKline. Metabolite structures are reported in Table 4.

Radiolabelled \(^{[14}C\)casopitant mesylate (Figure 1), \(^{[2H_3}^{13}C\)casopitant, \(^{[2H_3}^{13}C\)M13, \(^{[2H_3}^{13}C\)M12 and \(^{[2H_3}^{13}C\)M31 were synthetised by GSK Isotope Chemistry, UK. The specific activities of \(^{[14}C\)casopitant mesylate were 111kBq/mg (radiochemical purity of 99%) for the rat study, 11.1 kBq/mg (radiochemical purity of 99.7%) for the dog study.

Liquid scintillation cocktails were obtained from Perkin Elmer Life Science and Analytical Instruments Inc (Waltham, MA). All other chemicals used were reagent grade or higher and were obtained from approved commercial suppliers.

Animals

Female Sprague Dawley (SD) rats (189-231 g) were obtained from Charles River Ltd. (Margate, UK). Male Beagle dogs (10-16 kg) were obtained from Harlan Ltd. (Blackthorn, UK).

The animals were kept under standard environmental conditions using routine methods of animal husbandry. Water from domestic supply and standard diet was provided to animals. Water was available ad libitum throughout the study period.
Rats were not fasted prior to drug administration whereas dogs were. For dog, food was withheld overnight during collection of samples for clinical pathology and prior to scheduled necropsy.

Rat and dog studies with [14C]casopitant were conducted at Huntingdon Life Sciences (Huntingdon, UK) whilst the dog study with unlabelled version was conducted at GlaxoSmithKline (Verona, IT).

All in life experiments described in this paper complied with national legislation and with the company policy on the care and use of laboratory animals and with related codes of practice.

**Dose preparation**

For distribution and metabolism studies, oral doses were prepared daily, shortly before administration dissolving an appropriate amount of [14C]casopitant mesylate in the appropriate volume of water for injections (with sonication as necessary). The formulation obtained was continually mixed using a magnetic stirrer until completion of dosing. Aliquots of the formulation were analysed by radio-HPLC to determine the radiochemical purity and confirm the stability of [14C]casopitant in the dose formulation during both preparation and over the dosing period. The mean radiochemical purity of [14C]casopitant was always > 99%.

For 26-week oral investigative cardiotoxicity study in dog, an aliquot of unlabelled casopitant mesylate was dissolved in sterile water for injections and stored at room temperature protected from light. The solution obtained was stable for 7 days.

**Repeat dose studies with [14C]casopitant in rats and dogs.**
Rat

Twelve female rats received a single oral administration of $[^{14}\text{C}]$casopitant at a target dose level of 60 mg free base/kg. Blood samples were collected from each rat at 2 h and 24 h (terminal sample) post dose. Other twenty four female rats received the same single daily dose for 20 consecutive days. Twelve of the rats were sacrificed at 24 h after administration on Day 20, following collection from each rat of blood samples at 2 h (non terminal sample obtained via a tail vein) and 24 h (terminal sample obtained via cardiac puncture under isoflurane anaesthesia) post-dose. Six rats were sacrificed after a recovery period, following cessation of dosing, of 7 days, and the remaining six rats after a recovery period of 20 days. On both occasions, a single terminal blood sample was obtained from each rat.

All blood samples were collected into K3EDTA tubes as anticoagulant. After removing aliquots for radioanalysis, the remaining blood samples were centrifuged at 2000 g for 10 min at 4°C to separate plasma samples. The myocardium, liver, skeletal muscle (quadriceps), lungs and kidneys were removed from each of the rats following terminal sampling at 24 h post dose, washed in cold saline, blotted dry, weighed and frozen.

Dog

Four male dogs received repeat oral administrations of $[^{14}\text{C}]$casopitant, as single daily doses on 14 consecutive days, at a target dose level of 40 mg free base/kg/day. Two dogs were sacrificed following collection of blood samples at 2 h and 24 h after the final occasion of dosing (Day 14) and the other two were sacrificed following collection of blood samples after a recovery period of 20 days following cessation of dosing.
Plasma samples were prepared as described above and selected tissues (myocardium, liver, skeletal muscle, single lung and kidney) were removed from the carcasses. All samples were treated as described previously.

**Repeat dose study with unlabelled casopitant in dog.**

In a 26 Week Investigative Cardiotoxicity Study with interim kills, male dogs were administered with casopitant once daily at the dose of 40 mg free base/kg/day.

Designated dogs received a daily oral gavage dose of casopitant (three dogs for each occasions) or vehicle (two dogs for each occasions) for 6, 13, 20 or 26 weeks. In addition, an equal number of animals were similarly treated for 13 weeks followed by a 22 week recovery period and then sacrificed. Plasma samples obtained from blood collected into K3EDTA tubes at the following nominal times: pre-dose, 0.5, 1, 2, 4, 6, 8, 10 and 24 hours after dosing on day prior to each necropsy phase (week 6, 13, 20 and 26) were stored at nominally -20°C until analyzed.

In addition samples of 3-5g (each) of myocardium from all animals were collected during each occasion of necropsy, weighed, briefly washed in ice cold saline and frozen on solid carbon dioxide.

**Assay of total radioactivity**

Aliquots of plasma samples were mixed with Ultima Gold scintillation cocktail.

Tissues samples were weighed, an appropriate amount of water added and the total weight recorded prior to homogenisation on ice. Aliquots of each homogenate and blood samples were combusted using an Automatic Sample
Oxidiser (PerkinElmer Life and Analytical Sciences). The resultant $^{14}$CO$_2$ was collected by absorption in Carbosorb®E to which Permafluor®E+ scintillation fluid was added.

At the end all samples were analysed by using Packard Tricarb 2100 TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences), with automatic quench correction by an external standard method (Botta, 1985).

**Metabolite profiling in plasma and tissue homogenate by Radio-LC-MS/MS**

The radioactivity profiles were determined in plasma and myocardium for both rat and dog; in kidney, lung and skeletal muscle for dog only. Plasma samples as well as tissue homogenate samples from individual animals were pooled to produce a single representative sample per time point.

Sample pools were extracted by mixing aliquots with ca. 3 volumes of acetonitrile. After centrifugation, the supernatants were removed, the pellets re-suspended in the same solvent and volume and the process repeated up to three times. The supernatants were combined and evaporated under nitrogen to be reconstituted in an appropriate volume of water/acetonitrile. Weighed aliquots of each extract together with final residual pellets, previously digested, were radioassayed by liquid scintillation counting (LSC) for the calculation of recovery after extraction and reconstitution prior to radio-HPLC analysis. Three HPLC methods were used as detailed below.

**HPLC method 1 (used for analysis of rat and dog plasma and tissue homogenate samples)**

The chromatographic instrument used consisted of an Agilent 1100 for binary pump, autosampler and column oven (50°C) (Agilent Technologies, Palo Alto, CA) using a Phenomenex (Torrance, CA) Synergi Polar RP column (250 x
4.6 mm, 4 μm). The mobile phase consisted of 5 mM ammonium formate aq. (pH 5 adjusted with formic acid) (solvent A) and 50 mM ammonium formate aq. (pH 5) in acetonitrile (10:90 buffer/acetonitrile) (solvent B) at a flow rate of 1 mL/min. A gradient was used, starting at 37% B with a linear change to 57% B over 60 minutes, followed by further two linear increases to 80% B at 62 minutes, to 100 % B at 80 min. These conditions were maintained for a further 5 minutes.

**HPLC method 2 (used for estimation of relative abundance of metabolites M31, M134, M169 in dog plasma and tissues homogenate samples)**

The chromatographic instrument used consisted of an Agilent 1100 binary pump, autosampler and column oven (35°C) (Agilent Technologies, Palo Alto, CA) using a Phenomenex (Torrance, CA) Synergi Hydro RP column (250 x 4.6 mm, 4 μm). The mobile phase consisted of 2.5 mM ammonium acetate aq. (native pH) (solvent A) and 2.5 mM ammonium acetate aq. (native pH) in acetonitrile (5:95 water/acetonitrile) (solvent B) at a flow rate of 1 mL/min. A gradient was used, starting at 10% B with a linear change to 80% B over 82 minutes, followed by a linear increase to 95% at 82.1 minutes, with these conditions being maintained for a further 5 minutes.

**HPLC method 3 (used for estimation of relative abundance of metabolites M12, M31, M69, M76, M134, M169 and casopitant in rat plasma and tissue homogenate samples)**

The chromatographic instrument used consisted of an Agilent 1100 binary pump, autosampler and column oven (45°C) (Agilent Technologies, Palo Alto, CA) using a Gemini C18 RP column (150 x 4.6 mm, 3 μm). The mobile phase consisted of 10 mM ammonium carbonate aq. (pH 10) (solvent A) and 100 mM ammonium formate aq. (pH 10) in acetonitrile/methanol (10:63:27
buffer/acetonitrile/methanol) (solvent B) at a flow rate of 1 mL/min. A gradient was used, starting at 25% B with a linear change to 80% B over 55 minutes, followed by a linear increase to 100% at 60 minutes, with these conditions being maintained for a further 5 minutes.

For all methods, HPLC column recoveries were determined on selected samples by collecting the total HPLC column eluate for the appropriate run and assaying the radioactivity to assess recovery of injected radioactivity. Recoveries (≥ 85%) of radioactivity were obtained from the HPLC eluate collected.

Radio-HPLC data were captured off line (Bruin, 2006): chromatographic fractions (150 µL each) were collected using a Perkin Elmer fraction collector mod IntekServices FC LH200 onto 96 deep wells LUMAPLATES microtitre plates containing yttrium silicate solid scintillant (PerkinElmer Life and Analytical Sciences). Radioactivity determination was performed by scintillation counting (TopCount NXT counter, PerkinElmer Life and Analytical Sciences).

**Structural identification of metabolites**

Structural characterization was performed on selected samples by Radio-HPLC-Mass Spectrometry using hybrid quadrupole/time-of-flight Q-TOF Ultima (Waters MS Technologies, Manchester, UK) tandem mass spectrometer (Morris, 1996). Electrospray ionization, in positive and/or negative mode, was used. The HPLC flow was split (1:5) between mass spectrometer and a fraction collector model FC 204 (Gilson Inc, Middleton, WI) used for off-line radiodetection.

Metabolites were identified based on charged molecular ions, mass accuracy and their collision induced dissociation fragmentation (Oliveira and
Fully characterized metabolites were designated by the letter M followed by a number; where a synthetic standard was available a GSK code number was assigned.

**Casopitant, M12, M13, M31, M76, M134 and M200 quantification**

Quantification of casopitant and selected metabolites in dog plasma and myocardium homogenate were performed by using two analytical methods (A and B) based on protein precipitation with acetonitrile, followed by HPLC tandem mass spectrometry (HPLC-MS/MS) analysis. The HPLC system used consisted of an Agilent 1100 binary pump (Waldbronn, Germany) and a CTC Analytics HTS PAL (Zwingen, CH) autosampler.

**HPLC-MS/MS validated method A (used for analysis of casopitant, M12, M13 and M31 in dog plasma samples)**

Casopitant, M12, M13 and M31 were extracted from 50 μL dog plasma by protein precipitation using acetonitrile (150 μL) containing \([^{2}H_{3}]^{13}C\)casopitant, \([^{2}H_{3}]^{13}C\)M12, \([^{2}H_{3}]^{13}C\)M13 and \([^{2}H_{3}]^{13}C\)M31 as internal standards, at a concentration of 200 ng/mL each. After vortex mixing, 100 μL of water was added and the deproteinized samples were centrifuged for 10 minutes at approximately 3000 g. The supernatant was separated using a Hypersil Gold column (50 x 3.0 mm, 5.0 μm) and eluted at a flow rate of 0.7 mL/min. The isocratic mobile phase consisted of 35 % 5mM ammonium acetate (native pH) and 65 % acetonitrile. The column eluate was introduced into the
TurboIonSpray source of an API-4000 triple quadrupole mass spectrometer (Applied Biosystem, Ontartio, Canada) operated in positive-ion mode. The temperature of the source heater was maintained at 600°C with a curtain gas setting of 25 and collision gas setting of 5. Casopitant, M13, M12 and M31 were monitored by multiple reaction monitoring of m/z 617 to 167, 633 to 479, 589 to 453, 591 to 184 respectively. \([2\text{H}_3\text{C}]\)casopitant, \([2\text{H}_3\text{C}]\)M13, \([2\text{H}_3\text{C}]\)M12 and \([2\text{H}_3\text{C}]\)M31 were monitored by multiple reaction monitoring of m/z 621 to 171, 637 to 483, 593 to 493 and 595 to 184 respectively.

The concentrations of casopitant and metabolites present in plasma samples were determined from calibration curves constructed from analysis of samples spiked with known concentrations of casopitant, M13, M12, M31 with weighted \(1/x^2\) linear regression applied in each case over the range 15 to 15000 ng/ml.

**HPLC-MS/MS method B (used for analysis of M76, M134 and M200 in dog plasma and for all analytes in myocardium homogenate samples)**

M76, M134 and M200 were extracted from 25 μL dog plasma by protein precipitation as described for method A. The supernatant was analysed using the same HPLC-MS/MS conditions described previously.

Individual dog myocardium samples were weighed after thawing, an appropriate amount of water added [tissue: water 1:3 w/w], total weight recorded prior to homogenization on ice by Ultra turrax (IKA- Werke, Staufen, Germany). Then homogenate samples were analyzed for casopitant, M12, M13, M31, M76, M134 and M200 by using the analytical method B following the same extraction procedures and HPLC conditions employed for plasma. Casopitant, M13, M12, M31, M76, M134 and M200 were monitored by multiple
reactions monitoring of m/z 617 to 167, 633 to 479, 589 to 453, 591 to 184, 575 to 125, 506 to 370, 549 to 141 respectively.

For both analytical methods detailed quality control (QC) samples, prepared in plasma and myocardium homogenate at three different analyte concentrations and stored with study samples, were analyzed with each batch of samples against separately prepared calibration standards in plasma. QC samples and calibration standards were prepared using independently prepared stock solutions of casopitant and metabolites reference materials. For the analysis to be acceptable, no more than one third of the QC results were to deviate from the nominal concentration by more than 15%, and at least 50% of the results from each QC concentration were to be within 15% of nominal.

**Toxicokinetic analysis**

Analysis was performed by noncompartmental pharmacokinetic analysis using WinNonlin™ (version 4.1; Pharsight Corporation, Mountain View, CA). All computations utilized the nominal plasma collection times. The systemic exposure to casopitant and its metabolites M13, M12, M31, M134, M76 and M200 were determined by calculating the area under the plasma concentration-time curve (AUC) from the start of dosing to the last quantifiable timepoint (AUC\(_{0-t}\)) using the linear-logarithmic trapezoidal rule. The maximum observed peak plasma concentration (\(C_{\text{max}}\)) and the time at which it was observed (\(T_{\text{max}}\)) were determined by inspection of the observed data.
RESULTS

Plasma and Tissue distribution of radioactivity

Rat

Following either single or repeated oral administration of [14C]casopitant mesylate to female SD rats for 20 days, at a target dose level of 60 mg free base/kg/day, and after recovery periods of 7 (Day 27) and 20 (Day 40) days following cessation of the repeated administration, radioactivity was quantifiable in all of the tissues examined with the highest levels detected in lung and liver as shown in Table 1 and Figure 2.

DRM accumulated in plasma, blood and in the tissues following repeat oral administration of casopitant. On Day 20 at 24 h post dose, the mean concentrations of radioactivity were 2-3 fold higher for plasma, blood, myocardium, lungs and skeletal muscle and approximately 4-5 fold higher for kidneys and liver than those measured after single dose (Table 1).

Concentrations of radioactivity in all the tissues analysed were higher than concentrations in plasma throughout (Table 1). Specifically on day 20, corresponding tissue:plasma concentration ratio were approximately 6, 33, 21, 44 and 2 for myocardium, lung, kidney, liver and skeletal muscle respectively.

Following cessation of repeat dosing, levels of radioactivity in the tissues declined slowly. On Day 27, mean concentrations of radioactivity in the myocardium, lungs, kidneys, liver and skeletal muscle (quadriceps) had only declined to values of between 25% and 44% those at 24 h following the final dose (Day 20), and on Day 40, further declines were between 38% and 81% of the corresponding values on Day 27. On Day 40, tissue concentrations of radioactivity were between 30- and 400-fold higher than in plasma (Table 1).
Dog

In male Beagle dogs, after repeat oral administration of $[^{14}\text{C}]$casopitant for 14 days at a target dose level of 40 mg free base/kg/day, the concentrations of radioactivity in plasma were higher on Day 14 than at the corresponding times on Day 1, with concentrations at 2 h post-dose being greater than at 24 h post-dose on both occasions (Table 2).

After a recovery period, following cessation of dosing, of 20 days (Day 34), radioactivity in plasma was still quantifiable but had declined to about 24% of the corresponding values at 24 h post-dose (Day 14) (Table 2).

On Day 14, radioactivity was quantifiable in all the tissues examined with the following rank order: liver > lung > kidney > myocardium > skeletal muscle (quadriceps) as shown in Figure 3. On Day 34, tissues levels had declined relatively slowly to between 21% and 58% of the corresponding values on Day 14 (Table 2).

Concentrations of radioactivity in tissues were higher than that in plasma at both sampling times (Day 14 and Day 34) as shown in Figure 3. After 14 days of dosing corresponding tissue:plasma concentration ratio were approximately 5, 18, 6, 21 and 2 for myocardium, lung, kidney, liver and skeletal muscle respectively (Table 2).

Metabolic profile following repeat oral administration of $[^{14}\text{C}]$casopitant

The major metabolites of casopitant were identified and quantified in rat plasma and myocardium samples, dog plasma and myocardium, skeletal muscle, lung and kidney samples, obtained from the distribution studies described previously. Proposed metabolites structures and supporting spectral data for all matrices are shown in Table 4.
Rat

After single oral administration of \([^{14}\text{C}]\text{casopitant}\), metabolic pattern observed in female rat plasma was equivalent to that fully detailed and reported recently (Miraglia et al., 2010), where casopitant, hydroxylated piperazine derivative, M13, and the N-deethylated piperazine, M31 were the major circulating components. On day 20, at 2 h post dose, Casopitant was still the principal component in plasma, accounting for approximately 17 % of plasma radioactivity, but decreased to 1.5 % at 24 h by which time the major metabolites detected were M31 and the N-dealkylated derivative M206 (4-5 % of plasma radioactivity/each). In both recovery phases, the only identified and quantifiable circulating metabolite was M206, representing ca. 13 % of the plasma radioactivity (data not shown). Quantification for some relevant metabolites in plasma after repeat oral administration at 24 h post dose is summarized in Table 3.

In myocardium, metabolite profiles were qualitatively similar in all sampling occasions, with M76 (N-deacetylated), M31, M134, M200 (N-deacetylated N,N-deethylated) and M206 detected as notable metabolites both after repeat dosing and after recovery phase (data not shown). In general, with the exception of M206, which was detected in rat only, rat myocardium homogenates profiles were qualitatively similar to the male dog ones (Figure 4 and Figure 5); therefore only dog results are reported.

Dog

As observed for rat, following 14 days of repeat oral administration, unchanged casopitant was the principal radiolabelled component in male dog plasma, accounting for 16 % and 2 % of plasma radioactivity at 2 h and at 24 h post dose respectively. Other notable circulating metabolites detected at both
time points were M13 (5-7 % of plasma radioactivity), the deacetylated oxidised
derivative M12 (4 % of plasma radioactivity) and M134 (3 - 5 % of plasma
radioactivity). All relevant circulating metabolites have been already identified
as major components in dog plasma following single oral administration
(Miraglia et al., 2010).

In myocardium, skeletal muscle, kidney and lung the metabolic pattern
was similar. The major metabolite was M134, which represented 16 %, 27 %,
20 % and 50 % of myocardium, skeletal muscle, kidney and lung radioactivity
respectively (Table 3). M76 accounted for approximately 3 % and 8 % of
myocardium and skeletal muscle radioactivity respectively; in co-elution with
casopitant it represented 7 % and 12 % of kidney and lung radioactivity
respectively. Following 20 days of recovery from the last dosing (Day 34), a
number of radiometabolites were still detectable in myocardium and skeletal
muscle extracts, among which the piperazine modified derivatives M31, M134,
M200 and M203 were the most relevant ones (data not shown). As reported in
Table 3, all relevant metabolites detected in tissues, with the exception of M203,
were found also circulating but at much higher concentrations than in plasma.

Quantification of casopitant and selected metabolites in dog plasma and
myocardium during an investigative toxicity study.

In male beagle dogs, following a repeat oral dose of 40 mg/kg/day
casopitant for 26 weeks, plasma systemic exposure (in terms of AUC_{0-1} and
C_{\text{max}}) to casopitant and its circulating metabolite M13, M12, M31 and M134
were generally similar between week 6 and week 26 therefore only toxicokinetic
parameters measured on week 26 are reported and discussed (Table 5).
Metabolites M76 and M200 were detected but only occasionally quantified since
their plasma levels, M200 in particular were very close to the quantification limit (50 ng/ml).

As reported previously, the major circulating metabolite was M13, giving a similar exposure to the parent compound. Equivalent exposure (but lower than parent) was observed for M12 and M134 and for M31 and M76 (when quantified). As shown in Figure 6, plasma levels of M12, M134, M31 and M76 remained essentially constant throughout the 24 hour dosing interval on week 26 suggesting an apparently long half life.

Neither casopitant nor its metabolites were detected in the plasma samples taken during recovery phase.

Casopitant and the six selected metabolites were all detected and quantified in dog myocardium samples collected at all occasions of treatment evaluated as reported in Table 6. Among the drug related components detected in the tissue, the most relevant was M200 (major one) and M134, with levels increasing over the time from 6 to 26 weeks of treatment as shown in Figure 7. These two major metabolites were also the only components quantified after 22 weeks of dosing withdrawal following 13 week of treatment (Table 6).

Overall no correlation was observed between myocardium and plasma levels of casopitant and its relevant metabolites.
DISCUSSION

During chronic safety assessment studies with casopitan in rat (6-month) and dog (9-month), cardiomyopathy and cardiovascular changes were the major finding observed in both genders, at the same mean systemic exposure ($\geq 50\mu g.h/ml$).

The incidence of cardiac changes appeared to be dose and time related and were accompanied by an increase in plasma levels of biomarkers for cardiac damage such as cTnI and CK-MBmass. Moreover TEM revealed evidence of ultrastructural changes, in the heart of treated animals, which were considered characteristic of phospholipidosis (PL) (Halliwell, 1997; Anderson and Borlak, 2006). In addition to myocardium, other tissues (i.e. skeletal muscle, lung) showed phospholipidotic features which had also been detected in previous shorter toxicity studies.

PL was not an unexpected finding: casopitan and several notable metabolites are structurally consistent with cationic amphiphilic drugs (CAD) and such agents are known to induce phospholipidosis (Reasor et al., 2006). The phospholipidotic potential of casopitan and active metabolites M13 and M31 was also demonstrated in vitro, reflecting the in vivo observations (data not shown). The peculiarity was that myocardium is not a common PL target tissue; few drugs are known to cause cardiac phospholipidosis which could be associated with the cardiotoxicity observed, although a mechanistic understanding of toxicity linked to PL was not elucidated (Shaikh and Downar, 1987; Roos et al., 2002; Vonderfecht et al., 2004; Rabkin, 2006).

Concomitant to cardiomiopathy, some hemodynamic effects were observed that could be explained by a secondary pharmacologic effect of casopitan or its metabolites on calcium physiology via inhibition of the L-type
and T-type calcium channel on vascular smooth muscle (Waitkus-Edwards et al., 2002; Scragg et al., 2004). This potential effect of DRM was supported by evidence of gingival changes seen in dogs during long term study, a finding characteristic of some classes of calcium channel blocker in animals and humans (Heijl and Sundin, 1989; Ellis et al., 1999; Missouris et al., 2000). In vitro binding studies indicated a low calcium channel blockade by casopitant and M13, M12 and M31. Although activity was low, it could be meaningful if significant accumulation occurred in myocardium.

Based on the findings described and considering that they were manifest only after prolonged daily exposure, it was hypothesized that cardiac effects were likely related to physical chemical characteristics of casopitant or metabolite(s) and to accumulation of DRM with the potential to induce PL. In addition some involvement of altered calcium physiology also seemed possible.

As a consequence of these observations the distribution, accumulation and retention of DRM in selected tissues, after chronic dosing of [14C]casopitant, were investigated. The characterisation and quantification of the most notable metabolites of casopitant in plasma, myocardium, kidney, lung and skeletal muscle (the last three tissues for dog only) were also performed. Selected doses were within the range of cardiotoxic doses previously tested during long term toxicology studies.

Overall, in both preclinical species, the concentration of radioactivity in tissues was much higher than in plasma (Table 1 and Table 2). After the recovery periods considered, radioactivity in plasma and tissues remained detectable and decreased very slowly with the rate of elimination from tissues generally slower than that from plasma in both rat and dog.
These results confirmed the long retention of DRM in tissues and the slow elimination previously reported after acute administration of casopitant (Miraglia et al., 2010). Myocardium was not the tissue with the highest concentration, accumulation or retention of DRM (Figure 2 and Figure 3). Lung and liver contained the highest levels of radioactivity on all occasions (Table 1 and Table 2). However, as reported (Monro, 1990) the presence and retention of a xenobiotic in tissue is not necessarily correlated to a toxicological effect.

In rat and dog the main circulating components after repeat oral dosing were unchanged casopitant and metabolites, M12, M13, M31 and M134. Additionally, in rat only, M206 was notable following repeat oral administration and after the recovery periods.

In rat and dog myocardium the metabolites identified involved multiple oxidations of the piperazine or piperidine rings, loss of the N-acetyl group, piperazine N-deethylation or loss of piperazine ring, N-demethylation and various combinations of these biotransformations.

Furthermore tissues showed a similar metabolite pattern, where M31, M76, M134 and M200 were the major metabolites, still observed after a 20 day recovery period in both species and still quantifiable in the rat (data not shown).

Overall following repeated administration of [14C]casopitant, a large number of metabolites (about 36) were detected in rat and dog plasma and tissues. Radiolabelled components were present in the tissues at levels significantly higher than those in plasma; after a wash out period following chronic dosing, piperazine modified derivatives were still quantifiable in tissues but decreased to undetectable levels in plasma. A similar metabolic pattern, at least qualitatively, was observed in all tissues examined of both preclinical species and no unique metabolite was detected in the myocardium.
LC-MS assay development for the quantification of all metabolites detected in preclinical species was deemed technically not feasible. The agreed strategy was to focus on those derivatives which were notable, accumulating or showed long retention in both rat and dog tissues. The selected structures included those suggestive of calcium channel blockade or CAD like. Based on these criteria, M13, M12, M31, M76, M134 and M200 were quantified together with casopitant in plasma and myocardium samples collected at different times during a 26 week oral investigative toxicity study in dogs dosed at 40 mg/kg/day with unlabelled casopitant. In all animals treated with casopitant, cardiac changes were initially noted over the first 6 weeks of treatment, progressing in severity with duration of dosing, but with adverse findings at light microscopy appearing only after 20 weeks of treatment. Although signs of reversibility were apparent, full recovery did not occur after the 22 week treatment-free period (Casartelli et al., 2010).

Systemic plasma exposure to casopitant and M12, M13, M31 and M134 was approximately equivalent in weeks 6 and 26 suggesting that steady state was reached (Table 5). In contrast M76 and M200 were only occasionally quantified (plasma levels were very close to the quantification limit) in few animals mainly in week 26. Moreover plasma levels of M12, M31, M134, and M76 remained constant throughout the 24 hour dosing interval suggesting an apparently long half life. 22 weeks after dosing cessation, no detectable levels of casopitant or any of the six metabolites were found, suggesting that their wash out from plasma was largely complete.

In contrast to plasma where M13 was the major metabolite (Table 5), in myocardium M200 was the major component, reaching considerable concentrations (up to 114014 ng/g tissue corresponding to 0.2
mM) and showing a clear time dependent increase from 6 to 26 weeks of treatment (Table 6). Other notable metabolites were M134 and M76 showing increased levels with time. After multiple dosing for 26 weeks, the accumulation of M200 and M134 was clearly evident in myocardium (Figure 7) and in contrast to observations in plasma, the clearance of these metabolites was very slow with both still quantifiable after cessation of casopitant chronic administration. The progression of cardiac changes seen in life, and the continued presence of heart changes during and after the recovery period, correlated with the cardiac concentrations of these metabolites (Casartelli et al., 2010). It is interesting to note that not only elimination from but also accumulation in myocardium was slow: in fact after 14 days M200 was not the major metabolite (Table 3).

In conclusion, these investigative studies showed that plasma concentrations of DRM cannot be considered representative of tissue concentrations and could not be used to predict DRM tissue concentrations. Poor correlation between plasma and tissue concentrations of drug and metabolite(s) is not unusual since several factors influence tissue concentrations including the action of tissue transporters (Lin, 2006; The International Transporter Consortium, 2010). In contrast to plasma where metabolite concentrations generally remained constant at different times, in myocardium major metabolites did not reach the steady state up to 26 weeks and showed a significant tendency to accumulate after chronic administration. This slow trend of tissue accumulation without attaining a steady state following chronic dosing despite stabilization of plasma metabolite levels is characteristic of many CADs phospholipidosis-inducing (Vonderfecht et al., 2004; Gum et al., 2001). After the wash out period, all circulating components decreased to undetectable levels or were completely cleared whilst in myocardium some metabolites were retained longer and still
detectable. Nevertheless, no unique metabolites were detected in myocardium: all relevant drug related components were also present in other tissues. M200 was the major metabolite in dog myocardium with levels increasing over time (39180-114014 ng/g tissue corresponding to 0.07-0.2 mM), remaining relevant (31732 ng/g tissue corresponding to 0.06 mM) after 22 weeks of dosing withdrawal. Accumulation and slow elimination of ethylenediamine derivatives are not unusual (Breyer and Gaertner, 1974; Gaertner et al., 1975). However the presence per se of a xenobiotic in a tissue is not necessarily deleterious (Lafuente et al., 2009; Monro, 1990). Potential M200 toxicity could be related to its chemical structure being suggestive of a potential calcium antagonist (as proven for M31) or chelating activity (not investigated) (Qian and Guo, 2010).

No further investigations were performed to confirm this hypothesis although M200 remains the most intriguing metabolite. Nevertheless even if M200 were proven to be responsible of cardiotoxicity it would not be easily monitored throughout plasma pharmacokinetics to provide a safety margin (Eichler and Muller, 1998).
ACKNOWLEDGMENTS

We thank Patrizia Fizzotti, Maria Vittoria Fogli, Ivan Strepponi for technical and scientific assistance; Roberta Mastropasqua for HPLC-MS/MS quantification assays; Ken Lawrie for synthesizing the labeled compounds; GSK Chemical Development (Verona, IT) particularly Zadeo Cimarosti for synthesizing standards of metabolites; Huntingdon Life Sciences Ltd (UK) and GSK Laboratory Animal Science department (Verona, IT) for conducting experimental activities; GSK General Toxicology, Safety Assessment department (Verona, IT), particularly Marilyn Hill, Rodolfo Comelli and Alessandro Casartelli for coordination of investigative toxicity studies and scientific support.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Pagliarusco, Martinucci, Miraglia and Pellegatti.

Conducted experiments: Miraglia, Bordini and Cufari.

Performed data analysis: Miraglia, Bordini, Pagliarusco, Ferrari and Cufari.

Wrote or contributed to the writing of the manuscript: Pagliarusco, Martinucci, Bordini, Miraglia, Cufari, Ferrari and Pellegatti.
REFERENCES


LEGENDS FOR FIGURES

Figure 1:
Structure of [14C]casopitant mesylate

Figure 2:
Concentration of total radioactivity in female rat plasma and selected tissue after administration of [14C]casopitant at a target dose of 60 mg free base/kg on Day 1, Day 20 and at 7 days (day 27) and 20 days (day 40) following cessation of repeat dosing.

Figure 3:
Concentration of total radioactivity in male dog plasma and selected tissue after administration of [14C]casopitant at a target dose of 40 mg free base/kg on Day 1, Day 14 and at 20 days (day 34) following cessation of repeat dosing.

Figure 4:
A representative reconstructed radiochromatogram of pooled dog myocardium, following a repeat oral administration of [14C]casopitant at a target dose of 40 mg free base/kg for 14 days.

Figure 5:
A representative reconstructed radiochromatogram of pooled rat myocardium, following a repeat oral administration of [14C]casopitant at a target dose of 60 mg free base/kg for 20 days.

Figure 6:
Mean plasma concentrations time profile of casopitant, M12, M13, M31, M76, M134, M200 following oral administration of casopitant at a nominal dose of 40 mg free base /kg/day to male dogs on week 26.

Figure 7:
Mean concentration of casopitant, M12, M13, M31, M76, M134, M200 in
myocardium at different times, following oral administration of casopitant at a nominal dose of 40 mg free base /kg/day to male dogs.
TABLE 1

Concentration of radioactivity in female rat plasma and tissues following single (day 1) and repeat (Day 20) oral administration of \([\text{\textsuperscript{14}}\text{C}]\text{casopitant}\) and at 7 (Day 27) and 20 (Day 40) days following completion of repeat dosing at 60 mg/kg/day.

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time point (h)</th>
<th>Day 1 (N = 12)</th>
<th>Day 20 (N = 12)</th>
<th>Day 27 (recovery phase) (N = 6)</th>
<th>Day 40 (recovery phase) (N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>2</td>
<td>6.06 (+/- 1.26)</td>
<td>18.5 (+/- 2.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.54 (+/- 1.17)</td>
<td>12.4 (+/- 2.1)</td>
<td>1.92 (+/- 0.42)</td>
<td>0.246 (+/- 0.018)</td>
</tr>
<tr>
<td>Blood</td>
<td>24</td>
<td>5.60 (+/- 0.97)</td>
<td>14.5 (+/- 4.7)</td>
<td>7.67 (+/- 1.78)</td>
<td>3.63 (+/- 0.14)</td>
</tr>
<tr>
<td>Myocardium</td>
<td>24</td>
<td>34.6 (+/- 6.5)</td>
<td>79.6 (+/- 16.2)</td>
<td>32.9 (+/- 6.3)</td>
<td>26.7 (+/- 6.0)</td>
</tr>
<tr>
<td>Lungs</td>
<td>24</td>
<td>135 (+/- 55)</td>
<td>405 (+/- 109)</td>
<td>136 (+/- 6.0)</td>
<td>96.8 (+/- 14.2)</td>
</tr>
<tr>
<td>Kidneys</td>
<td>24</td>
<td>59.1 (+/- 6.8)</td>
<td>256 (+/- 91.6)</td>
<td>64.6 (+/- 15.7)</td>
<td>39.2 (+/- 8.4)</td>
</tr>
<tr>
<td>Liver</td>
<td>24</td>
<td>121 (+/- 14.5)</td>
<td>545 (+/- 92.8)</td>
<td>240 (+/- 52.2)</td>
<td>90.4 (+/- 31.7)</td>
</tr>
</tbody>
</table>

<sup>a</sup> μg equivalents of casopitant free base/g.
| Skeletal muscle | 24 | 18.3 (+/- 3.1) | 28.1 (+/- 5.4) | 10.7 (+/- 1.5) | 7.74 (+/- 0.76) |

*a* Results are reported as mean and (+/- SD).

Data are presented to three significant figures or to a maximum of three decimal places and are computer generated and rounded appropriately.
TABLE 2

Concentration of radioactivity in male dog plasma and tissues following single (day 1), repeat (Day 14) oral administration of \[^{14}C\]casopitant and at 20 (Day 34) days following completion of repeat dosing at 40 mg/kg/day.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time point (h)</th>
<th>Day 1 (μg equivalents of casopitant free base/g)</th>
<th>Day 14 (μg equivalents of casopitant free base/g)</th>
<th>Day 34 (recovery phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[ ]</td>
<td>[ ]</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>2</td>
<td>20.8</td>
<td>69.5 (+/- 4.1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>9.68</td>
<td>49.2 (+/- 5.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.6</td>
</tr>
<tr>
<td>Blood</td>
<td>2</td>
<td>11.3</td>
<td>47.4 (+/- 2.5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>9.40</td>
<td>35.3 (+/- 3.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.95</td>
</tr>
<tr>
<td>Myocardium</td>
<td>24</td>
<td>ND</td>
<td>243</td>
<td>96.2</td>
</tr>
<tr>
<td>Lungs</td>
<td>24</td>
<td>ND</td>
<td>905</td>
<td>345</td>
</tr>
<tr>
<td>Kidneys</td>
<td>24</td>
<td>ND</td>
<td>308</td>
<td>102</td>
</tr>
<tr>
<td>Liver</td>
<td>24</td>
<td>ND</td>
<td>1050</td>
<td>612</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>24</td>
<td>ND</td>
<td>99.2</td>
<td>21.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are reported as mean (N = 2 unless stated otherwise) and standard deviation (+/- SD) if applicable.

<sup>ND</sup> not determined

<sup>b</sup> N = 4
Data are presented to three significant figures and are computer generated and rounded appropriately.
TABLE 3

Percentage of radioactivity of casopitant and its relevant metabolites in female rat plasma (day20, 24h) and male dog plasma and tissues (day 14, 24h) following repeat oral administration of [14C]casopitant at the dose of 60 and 40 mg /kg/day, respectively.

<table>
<thead>
<tr>
<th>Peak ID</th>
<th>RAT Plasma</th>
<th>Plasma</th>
<th>Myocardium</th>
<th>Skeletal Muscle</th>
<th>Kidney</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casopitant (P)</td>
<td>1.5 (+M76+M111)</td>
<td>1.9</td>
<td>3.7 (+M200)</td>
<td>2.4 (+M111+M200)</td>
<td>7.0 (+M76)</td>
<td>11.9 (+M76)</td>
</tr>
<tr>
<td></td>
<td>(0.176)</td>
<td>(0.960)</td>
<td>(9.05)</td>
<td>(2.33)</td>
<td>(21.7)</td>
<td>(108)</td>
</tr>
<tr>
<td>M12</td>
<td>1.2</td>
<td>4.1 (+M69)</td>
<td>1.6 (+M69)</td>
<td>3.5 (+M69)</td>
<td>2.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(0.135)</td>
<td>(2.03)</td>
<td>(3.46)</td>
<td>(6.87)</td>
<td>(10.9)</td>
<td></td>
</tr>
<tr>
<td>M13</td>
<td>0.3</td>
<td>4.8</td>
<td>2.1</td>
<td>4.2</td>
<td>2.9</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>(0.037)</td>
<td>(2.41)</td>
<td>(5.12)</td>
<td>(4.15)</td>
<td>(9.02)</td>
<td>(17.2)</td>
</tr>
<tr>
<td></td>
<td>M15</td>
<td>0.2</td>
<td>0.9</td>
<td>0.5</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>-----</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>(0.021)</td>
<td>(0.424)</td>
<td>(1.19)</td>
<td>(1.30)</td>
<td>(3.44)</td>
<td>(5.43)</td>
</tr>
<tr>
<td></td>
<td>M31+M134+M169</td>
<td>4.1</td>
<td>4.8</td>
<td>16.3</td>
<td>26.7</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td>(0.469)</td>
<td>(2.41)</td>
<td>(39.7)</td>
<td>(26.50)</td>
<td>(60.3)</td>
<td>(452)</td>
</tr>
<tr>
<td></td>
<td>M57+M155</td>
<td>ND</td>
<td>1.0</td>
<td>0.9</td>
<td>2.9</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>(0.514)</td>
<td>(2.14)</td>
<td>(2.85)</td>
<td>(5.37)</td>
<td>(24.4)</td>
<td></td>
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<tr>
<td></td>
<td>M76</td>
<td>1.5(+M11+P)</td>
<td>0.8</td>
<td>3.4</td>
<td>7.6</td>
<td>7.0(+P)</td>
</tr>
<tr>
<td></td>
<td>(0.176)</td>
<td>(0.380)</td>
<td>(8.22)</td>
<td>(7.52)</td>
<td>(21.7)</td>
<td>(108)</td>
</tr>
<tr>
<td></td>
<td>M200</td>
<td>ND</td>
<td>BQL</td>
<td>3.7(+P)</td>
<td>2.4(+M1+P)</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>(9.05)</td>
<td>(2.33)</td>
<td>(14.8)</td>
<td>(20.8)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>M201</td>
<td>ND</td>
<td>✓</td>
<td>0.7</td>
<td>1.6</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>(1.79)</td>
<td>(1.56)</td>
<td>(6.44)</td>
<td>(9.96)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M202</td>
<td>ND</td>
<td>✓</td>
<td>0.7</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td>(1.79)</td>
<td>(1.21)</td>
<td>(3.44)</td>
<td>(20.8)</td>
</tr>
<tr>
<td>-------</td>
<td>----</td>
<td>----</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>M203</td>
<td>ND</td>
<td>ND</td>
<td>2.0</td>
<td>3.6</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4.76)</td>
<td>(3.54)</td>
<td>(9.02)</td>
<td>(25.3)</td>
</tr>
<tr>
<td>M206</td>
<td>4.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td></td>
<td></td>
<td></td>
<td>(0.527)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantification Limit</td>
<td>0.2</td>
<td>0.7</td>
<td>0.4</td>
<td>1.0</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>(0.021)</td>
<td>(0.345)</td>
<td>(0.877)</td>
<td>(1.03)</td>
<td>(2.39)</td>
<td>(4.74)</td>
</tr>
</tbody>
</table>

BQL = below quantification limit, set to 25 cpm as peak area

ND = not detected

✓ = Observed by HPLC mass spectrometry only.

# The quantification of M31, M134 and M169 in all matrices was grouped together due to co-eluting chromatography with the HPLC method 1. Further analyses performed with HPLC method 2 and/or 3 revealed that the majority of this radio-peak corresponded to M134 for all dog matrices and M31 for rat ones.
### TABLE 4

**Relevant metabolites of \([^{14}C]\)casopitant**

<table>
<thead>
<tr>
<th>Proposed Structure</th>
<th>Positive [M+H]^+ or Negative [M+H]^- ion &amp; Major Fragment Ions (m/z)</th>
</tr>
</thead>
</table>
| ![Casopitant](image) | 617 [M+H]^+  
489, 481, 346, 327, 298, 277, 241, 210, 167 |
| ![GSK631832](image) | 589 [M+H]^+  
489, 453, 249, 241, 182, 139 |
| ![GW525060](image) | 633 [M+H]^+  
615, 497, 489, 479, 461, 275, 183, 165 |
<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight</th>
<th>Mass Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK1497960 (M13)</td>
<td>631 [M+H]⁺</td>
<td>495, 489, 353, 327, 241, 181, 175</td>
</tr>
<tr>
<td>GSK517142 (M15)</td>
<td>591 [M+H]⁺</td>
<td>489, 455, 327, 320, 251, 241, 184, 175, 141</td>
</tr>
<tr>
<td>M57</td>
<td>607 [M+H]⁺</td>
<td>548, 489, 412, 327, 298, 241, 141</td>
</tr>
<tr>
<td>GW679979 (M76)</td>
<td>575 [M+H]⁺</td>
<td>489, 439, 241 168, 125</td>
</tr>
<tr>
<td>Structure</td>
<td>Formula</td>
<td>Masses</td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>GSK2110277 (M134)</td>
<td>506 [M+H]^+</td>
<td>370, 315, 298, 241, 175</td>
</tr>
<tr>
<td>or</td>
<td>607 [M+H]^+</td>
<td>489, 241, 200, 157</td>
</tr>
<tr>
<td>M155</td>
<td>550 [M+H]^+</td>
<td>414, 327, 298, 279, 272, 241, 210, 184, 175, 143, 100</td>
</tr>
<tr>
<td>M169</td>
<td>549 [M+H]^+</td>
<td>489, 413, 315, 241, 175, 142</td>
</tr>
<tr>
<td>GSK2174599 (M200)</td>
<td>619 [M+H]^+</td>
<td>315, 241, 184</td>
</tr>
</tbody>
</table>
The structure of underlined metabolites in the table have been confirmed with synthetic standards.
TABLE 5

Toxicokinetic parameters for casopitant, M12, M13, M31, M76, M134 and M200 following repeat oral administration of casopitant mesylate to male Beagle dogs at 40 mg/kg/day on 26 week.

<table>
<thead>
<tr>
<th>Period</th>
<th>Parameter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Toxicokinetic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Casopitant</td>
<td>M12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ng.h/ml)</td>
</tr>
<tr>
<td></td>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt;</td>
<td>53599</td>
</tr>
<tr>
<td></td>
<td>(ng.h/ml)</td>
<td>[27722-84585]</td>
</tr>
<tr>
<td>Week 26</td>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>7167</td>
</tr>
<tr>
<td></td>
<td>(ng/ml)</td>
<td>[4800-10193]</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>(h)</td>
<td>[1.00-1.00]</td>
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<sup>a</sup> Results are reported as mean (when n=3) for AUC and C<sub>max</sub> or median for T<sub>max</sub> and [Range].

<sup>b</sup> Mean was not calculated where n< 3 therefore individual toxicokinetic parameters are reported.
NA = toxicokinetic parameters not available since plasma levels were below the limit of quantification (50 ng/mL) at all time-points considered.
**TABLE 6**

*Individual quantification of casopitant, M12, M13, M31, M76, M134 and M200 in myocardium following repeat oral administration of casopitant mesylate to male Beagle dogs at 40 mg/kg/day.*

<table>
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<tr>
<th>Period</th>
<th>Animal No.</th>
<th>Concentration (ng/g of tissue)</th>
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<td>3286</td>
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<tr>
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</table>

NQ = not quantifiable (below the limit of quantification of 50 ng/mL).
**fig 6**

Graph showing plasma concentration (ng/mL) over time (h) for different compounds:
- **Casopitant**
- M12
- M13
- M31
- M76
- M134
- M200

Time range from 0 to 24 hours, with plasma concentration ranging from 0 to 7000 ng/mL.
fig 7