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Chemical Compatibility and Safety of Imidacloprid/Flumethrin Collar (Seresto®) Concomitantly Used with Imidacloprid/ Moxidectin (Advocate®, Advantage® Multi) and Emodepside/Praziquantel (Profender®) Spot-on Formulations

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Abstract

Safety of concomitant use of veterinary products is of clinical interest. A series of studies was performed to evaluate the chemical compatibility and short term dermal and systemic safety of an imidacloprid/ flumethrin collar (Seresto®/Foresto®, Bayer) used concomitantly with spot-on or tablet formulations.

Chemical compatibility was evaluated *in-vitro* (*study reference A*) on collar pieces, followed by two small, non-controlled clinical studies (*study reference B*) in both, cats and dogs. The studies showed, that certain solvents affected the collar *in-vitro*, but not in their marketed formulations.

Dermal and systemic safety of different spot-on or tablet formulations was first evaluated in a small, non-controlled clinical study (*study reference C*) in cats and dogs, via clinical observations only, followed by controlled clinical safety studies of concomitant use with imidacloprid/ moxidectin (Advocate®/ Advantage® Multi, Bayer) in dogs and cats (*study reference D*) and emodepside/ praziquantel (Profender®, Bayer) in cats (*study reference E*), assessing safety aspects by clinical observations and statistical analyses of hematology and clinical chemistry parameters compared to baseline values and between treated and control groups.

Dermal safety findings over all clinical studies (*study references B to E*) matched those already described for the respective products and included transient cosmetic changes (oily hair and crystal formation) at the site of spot-on application and broken hair, transient alopecia and skin alterations at the site of collar application. There were no indications of these findings aggravating under the conditions of concurrent use. There were no systemic safety findings of clinical significance in any of the clinical safety studies (*study reference C* to *E*). Assessment of blood parameters revealed some deviations from baseline levels and from the reference range in dogs as well as in cats, but no clinical relevance could be deduced. Hematology and clinical chemistry results confirmed the safety of the concomitant treatment. It is concluded that Seresto® is chemically compatible with solvents used in major spot-on formulations on the market and is dermally and systemically safe for adult dogs and cats when used concomitantly with Advocate® and Profender® spot-on formulations.

Introduction

Externally applied antiparasitic drugs used on cats and dogs have "evolved" to often also focus on prevention of transmissible diseases, as well as formulations developed to control both, ecto- and endoparasites (Beugnet and Franc 2012). This is not only the case for externally applied antiparasiticides, but also formulations for which oral efficacy against these parasite types have been evaluated (Snyder and Wiseman 2012). However, there is currently no single product on the market that can treat and prevent the entire range of ecto- and endoparasite taxa affecting companion animals. Despite attempts to broaden the spectrum of parasite species targeted, the use of combinations of antiparisitic drugs is likely to remain the only therapeutic and preventative solution for many years to come (Beugnet and Franc 2012). As a result, concomitant treatment with products targeting different parasites is a fact of life for veterinarians and pet owners. The safety aspect of concomitant use is of interest for any newly introduced formulation. This article presents a series of studies evaluating the chemical compatibility and dermal and systemic safety of a recently introduced ectoparasiticidal imidacloprid 10 %/flumethrin 4.5 % collar (Seresto®/Foresto®, Bayer), when used concomitantly with topical and oral parasiticidal treatments. These are the first published studies which evaluate concomitant use of the Seresto® collar.

The Seresto® collar is designed to provide long-term protection for up to 8 months against fleas and ticks. A long term efficacy and safety study showed that the collar reduced flea counts by at least 95 % and tick counts by at least 90 % for a 7 to 8 month period in both cats and dogs. Safety was confirmed with mainly minor observations localised to the application site (Stanneck et al. 2012a). The collar is licensed for the treatment and prevention of flea infestations (*Ctenocephalides felis*), treatment of tick infestations (*Ixodes ricinus* and *Rhipicephalus turanicus* in cats, *Ixodes ricinus*, *Rhipicephalus sanguineus* and *Dermacentor reticulatus* in dogs) and infestations with chewing lice (*Trichodectes canis*) (Horak et al. 2012; Stanneck et al. 2012b; Stanneck et al. 2012c).

The collar also exhibits repellent (anti feeding) efficacy and has the potential to prevent infections with flea and tick transmitted agents (Fourie et al. 2012; Fourie et al. 2013a; Fourie et al. 2013b; Lappin et al. 2013; Otranto et al. 2013, Reichard et al. 2013; Brianti et al. 2014).

The collar contains two actives, the insecticide imidacloprid active against fleas and lice, and the acaricide flumethrin active against ticks and mites (Stanneck et al. 2012b; Stanneck et al. 2012c; Brianti et al. 2013; Smith et al. 2013). The actives themselves have a relatively long history. Imidacloprid was registered in Europe in 1997 for use as a veterinary medicinal product in cats and dogs, while flumethrin was registered in Europe even earlier in 1986 for use as a veterinary medicinal

product for companion and food producing animals. Imidacloprid is a neonicotinoid insecticide which acts as a strong agonist at nicotinic acetylcholine receptors (nAChRs) on the post-synaptic membrane, causing sustained depolarization of the neuron and death of the flea or louse (Bai et al. 1991). Imidacloprid is highly selective for insect nAChRs, to which it binds up to 1,000 times more strongly than to mammalian nAChRs, so it exhibits extremely low mammalian and environmental toxicity (Casida et al. 1993; Methfessel 1992). In addition to killing adult fleas, imidacloprid also exhibits larvicidal efficacy which controls flea larvae in the pet´s own environment. Flumethrin is a type II pyrethroid with excellent acaricidal efficacy. It kills ticks by binding to voltage gated axonic sodium channels in neural tissue and keeping them open for longer than physiologically normal. This results in membrane depolarization, repetitive discharges and synaptic disturbances, leading to hyperexcitatory symptoms of poisoning (Narahashi 1996). Flumethrin has a simple metabolism and can be excreted directly via bile and faeces without the need for glucuronidation, a phase II conjugation pathway involved in metabolism and excretion of other pyrethroids (Kaneko 2010). Cats have only limited capacity for glucuronidation and thus have an increased sensitivity towards other pyrethroids. However, their sensitivity towards flumethrin is comparable to dogs, as shown in toxicological studies, so flumethrin can be considered to be safe for cats (Stanneck et al. 2012c)

The design of the Seresto® collar is unique. Imidacloprid and flumethrin are blended with skin-compatible neutral oils in a polyvinyl chloride (PVC) matrix of the collar. Neutral oils are used as plasticizers instead of commonly used but potentially harmful phthalate ester plasticizers. Stearic acid is used as the lubricant. The safety of the collar on the animal´s neck is assured by the use of these compounds, as neutral oils are safe enough to be used in the cosmetic industry and stearic acid is so non-toxic that it is used as a hardener in confectionary items. The polymer matrix provides fast and continuous release of the active ingredients which are distributed over the entire body surface within hours of collar application. However, there have been no studies published to date about the chemical compatibility of the collar with spot-on formulations.

This is of particular interest because spot-on formulations are normally applied in the vicinity of the collar and so could potentially damage the collar structure. Twelve widely used solvents from spot-on formulations and one solvent of possible future interest were selected for *in vitro* testing (*study reference A* for ease of discussion), with results confirmed by follow-up *in-vivo* chemical compatability tests (*study reference B* for ease of discussion). All solvents tested are listed in Table 1.

The long term nature of the collar treatment has the advantage that pet owners need apply the collar only once to gain long term protection. However, the long term nature of the collar also means that the question of potential interference of concomitant treatment with other parasiticides may well arise.

The treatment scenarios considered were collar treated dogs or cats, which required an additional spot-on to treat or prevent nematode infections such as heartworm, hookworm, roundworm and whipworm. Collar treated animals might also require concomitant treatment to deal with other ectoparasites such as mites.

Dogs fitted with collars might for example need a spot-on for the prevention and treatment of French heartworm, treatment of fox lungworm or demodectic mange. Alternatively cats and dogs protected against fleas or being treated for one of the above conditions with a spot-on formulation might require collar treatment for additional protection against ticks. To test dermal and systemic safety of concomitant treatment with the Seresto® collar, a smaller non-controlled safety study employing Seresto®/Advocate®/Profender® (*study reference C* for ease of discussion) and three larger controlled safety studies (*studies references D* for the first two studies employing Seresto®/Advocate® and *study reference E* for the third employing Seresto®/Profender®) were

performed. In the smaller study (*study reference C*) the following two products for cats were selected as concomitant medication: an imidacloprid/moxidectin spot-on (Advocate®, Bayer) and an emodepside/praziquantel spot-on (Profender®, Bayer). Three products were selected for dogs: an imidacloprid/moxidectin spot-on (Advocate®, Bayer), an imidacloprid/permethrin spot-on (Advantix®, Bayer) as well as an emodepside/praziquantel oral tablet (Profender®, Bayer). In the larger controlled safety studies reported, the imidacloprid/moxidectin spot-on (Advocate®) for both, cats and dogs, were selected for two studies (*study reference D*: Seresto®/Advocate® studies), with Profender® spot-on (emodepside plus praziquantel) for cats in the third (*study reference E*: Seresto®/Profender® study). All of the products chosen in the safety studies are active against either nematodes or mites, and so could be options for use in the treatment scenarios outlined previously. For the scope and purpose of this manuscript, with specific reference to the three larger clinical studies performed, Advocate® and Profender® are of particular interest.

Although both the Seresto® collar and the Advocate® spot-on formulation contain imidacloprid, this was not expected to cause any particular problems due to the very low mammalian toxicity of this active ingredient.

In target animal studies with the imidacloprid mono spot-on product Advantage®, there were no side effects seen in a 20 fold dermal overdose in dogs or a 24 fold overdose in cats, demonstrating imidacloprid´s wide safety margin (Mencke and Jeschke 2002). The other active in Advocate® is moxidectin, a macrocylic lactone of the milbemycin class, which binds to neuronal glutamate-gated chloride channels of nematodes and muscle membranes of arthropods leading to hyperpolarization, paralysis and death (Forrester et al. 2002). Advocate® was licensed in the European Union in 2003 and is a well-known treatment (Hellmann et al. 2003).

Efficacy using this product has been demonstrated against fleas and mites (Krieger et al. 2005; Wenzel et al. 2008; Fourie et al. 2009; Le Sueur et al. 2011), as well as several helminth species (Conboy et al. 2009; Traversa et al. 2009; Fok et al. 2010; Taweethavonsawat et al. 2010; Hellman et al. 2011; Traversa et al. 2012; Austin et al. 2013) in animals as diverse as dogs, cats, ferrets and even reptiles and rodents (Mehlhorn et al. 2005).

Advocate® has also been shown to prevent infection with certain parasites, such as the nematodes *Spirocerca lupi* and *Dirofilaria repens* (Le Sueur et al. 2011; Hellman et al. 2011; Genchi et al. 2013).

Profender® spot-on contains both emodepside and praziquantel. Efficacy against a number of helminths in cats have been demonstrated (Altreuther et al. 2005a; Altreuther et al 2005b; Charles et al. 2005; Reinemeyer et al. 2005; Schaper et al. 2007; Traversa 2009; Wolken 2009; Wolken 2012; Taweethavonsawat et al. 2013), as has efficacy in dogs using the tablet formulation (Altreuther et al. 2009a; Altreuther et al. 2009b; Schimmel et al. 2009a; Schimmel et al. 2009b). Considering scope of activity (nematodes and cestodes), ease of administration (topical spot-on) as well as known contraindications for some of the other anthelmintic actives in reptiles, this product also resolved limitations associated with previous antiparasitic treatments as far as effective anthelmintic treatment in reptiles are concerned (Mans 2013). The product has also been used to effectively treat rodents (Schmahl et al. 2007).

From the discussions above it is clear that concomitant use of the Seresto® collar with any of the two spot-on formulations highlighted (Advocate® or Profender®), will provide therapeutic and preventative efficacy against a very wide range of helminths, ectoparasites and diseases transmitted by the latter. However, safety of concomitant treatment of Seresto® with Advocate® or Profender® has not been tested before and will be of clinical interest to practicing veterinarians.

The studies presented here were designed to answer three main questions.

Firstly, is the new Seresto® collar polymer matrix chemically robust enough to withstand direct contact with a spot-on formulation?

Table 1: Solvents tested *in-vitro* and the spot-on formulations in which they are found

Solvent (Common acronym)	Trial number	Used in
Benzyl alcohol (BA)	1	Advantage®, Advocate®
Propylene carbonate (PC)	1	Advantage®, Advocate®
BA/PC (80:20)	1	Advantage®, Advocate®
N-Methyl-2-pyrrolidone (NMP)*	1	Advantix®, Droncit®
Solketal (Isopropylidenglycerin)	1	Profender®
Diethylene glycol mono ethyl ether	1	Frontline®, Practic®
Isopropyl alcohol	1	Bolfo® Spray
Ethyl alcohol	1	Frontline®
Dimethylsulfoxide (DMSO)*	1	ProMeris®/ProMeris Duo®
Gamma-hexalactone*	2	ProMeris®/ProMeris Duo®
1-Methoxy-2-propyl-acetate*	2	ProMeris®/ProMeris Duo®
Dipropylene glycol mono methyl ether	2	Tiguvon®
Tetrahydro furfuryl alcohol (THFA)	2	Possible future relevance

** The solvents in italics were subjected to additional in-vivo testing*

Secondly, are there any dermal safety implications for cats and dogs, when the collar is used concomitantly with a spot-on formulation?
Thirdly, is the particular combination of the actives (imidacloprid, flumethrin and moxidectin with reference to Seresto® used with Advocate® or imidacloprid, flumethrin, emodepside and praziquantel with reference to Seresto® used with Profender®) systemically safe for cats and dogs?

Materials and Methods

Study reference A: Chemical Compatibility: *In-Vitro* Testing of 13 Solvents

A total of 13 solvents were chosen for *in-vitro* testing. In the first trial 9 commonly occurring solvents in spot-on formulations were chosen. In the second trial 3 more unusual solvents and 1 solvent of possible future relevance were chosen. The Seresto® collars were cut into pieces approximately three cm in length and were placed in small vials, each one containing a different solvent. The pieces were placed so that half of the collar was covered by solvent. The collar pieces were examined visually after 15 minutes and again after 24 hours. The collar pieces were also examined manually for surface changes and texture after 24 hours. The solvents used in the two trials and their spot-on formulations are shown in Table 1.

Study references B to E: General Notes on Animal Husbandry and Other Points Common to All Clinical Studies

All clinical studies presented here were performed in accordance with Good Scientific Practice, with the exception of the Seresto®/Profender® study (*study reference E*) that was performed in accordance with the principles of the Organisation for Economic Co-operation and Development Good Laboratory Practice (OECD GLP).
All animals described in these studies were part of a regularly maintained research colony and were returned either to the colony or transferred to an animal welfare station after study completion.

Table 2: Overview of Chemical Compatibility Cat Study

Study Day (SD)	Activity	Solvents contained in spot-on formulation	Active ingredient in spot-on	Clinical assessment
0	Fitting of collar followed directly by application of ProMeris® spot-on	DMSO, Gamma hexalactone, 1-methoxy-2-propyl-acetate	Metaflumizon	Performed pre-treatment
0, 2 hours	Temporary removal of collar for inspection, then re-fitting			Performed
0, 24 hours	Removal, inspection and disposal of collars			Performed
9	Fitting of collar followed directly by application of Droncit® spot-on	N-methyl-2-pyrrolidone	Praziquantel	Performed pre-treatment
9, 2 hours	Temporary removal of collar for inspection, then re-fitting			Performed
9, 24 hours	Removal, inspection and disposal of collars			Performed

All animals were already acclimatised to the study facilities at the time of enrolment. Housing, light control, humidity control, and feeding were all in accordance with internal standard operating procedures based on the European Commission guidelines for the accommodation of animals used for experimental and other scientific purposes (June 18, 2007/526/EC), as well as applicable local guidelines (The South African National Standard: SANS 10386:2008: The care and use of animals for scientific purposes). Please note that the former guideline had been in use when the studies were designed, but has since been revised in 2013.

The clinical studies all shared animal health as a common inclusion criterion. All animals had to be in good health as determined by a physical examination conducted by a veterinary surgeon on either SD -2 (large safety *study reference D* Seresto®/Advocate® studies performed in Europe) or SD 0 (chemical compatibility *studies B*, small safety *studies C* and safety *study reference E* Seresto®/Profender® study performed in South Africa).

With the exception of the imidacloprid/permethrin formulation (Advantix®) for dogs in the chemical compatibility *study B*, all spot-on products and Profender® tablets were administered at therapeutic doses according to animal body weights and in compliance with manufacturers´ instructions as detailed in the package inserts.

The animals were fitted with Seresto® collars according to their body weight. Dogs with a body weight ≤ 8 kg received a small collar (Seresto® collar for dogs ≤ 8 kg) and dogs with a body weight above 8 kg received a large collar (Seresto® collar for dogs > 8 kg). All treated cats received a small collar (Seresto® collar for cats). Collars were fitted according to label instructions: collars were adjusted around the animal's neck in such a way that a space of two fingers remained between collar and neck. Excess collar was pulled through the collar's buckle and a fixative loop and excess length was clipped off, leaving a 2 cm overlap behind the fixative loop.

Study reference B: Chemical Compatibility: *In-vitro* Clinical Studies

Four solvents found in spot-on formulations were identified as affecting the collars: N-methyl-pyrrolidone (NMP), dimethylsulfoxide (DMSO), gamma-hexalactone and 1-methoxy-2-propyl-acetate. Spot-on formulations containing these four solvents were next tested under conditions of normal collar use in two small scale non-controlled GSP clinical studies, one in cats and one in dogs. In cats the formulations

Table 3: Overview of the Chemical Compatibility Dog Study

Study Day (SD)	Activity	Solvents contained in spot-on formulation	Active ingredient in spot-on	Clinical assessment
0	Fitting of collar followed directly by application of ProMeris® Duo spot-on	DMSO, Gamma hexalactone, 1-methoxy-2-propyl-acetate	Metaflumizon + Amitraz	Performed pre-treatment
0, 2 hours	Temporary removal of collar for inspection, then re-fitting			Performed
0, 24 hours	Removal, inspection and disposal of collars			Performed
9	Fitting of collar followed directly by application of Advantix® spot-on	N-methyl-2-pyrrolidone	Imidacloprid + Permethrin	Performed pre-treatment
9, 2 hours	Temporary removal of collar for inspection, then re-fitting			Performed
9, 24 hours	Removal, inspection and disposal of collars			Performed

tested contained the active ingredients metaflumizon (ProMeris®) and praziquantel (Droncit®). In dogs the formulations tested contained metaflumizon/amitraz (ProMeris Duo®) and imidacloprid/permethrin (Advantix®). The studies were non-randomised and non-blinded as each study contained only 1 study group. On study day zero (SD 0) the animals were weighed and physically examined before being fitted with the Seresto® collar. The first spot-on formulation was applied directly after collar fitting. At the 2 hour time point the collars were temporarily removed, visually and manually inspected and the re-fitted. At the 24 hour time point the collars were removed, visually and manually inspected and then disposed of. On SD 9, the second spot-on formulation was applied to the animals directly after new collars were fitted. At the 2 hour time point the collars were temporarily removed, visually and manually inspected and then re-fitted. At the 24 hour time point the collars were removed, visually and manually inspected and then disposed of.

Clinical assessments were performed pre-treatment and approximately 2 and 24 hours post-treatment. The following were assessed: attitude, behaviour, nutritional status, respiratory system, gastrointestinal system and cardiovascular system. In addition general health observations were performed daily during the whole study period. The following were assessed: eyes, respiration, behavioural attitude, locomotion/musculature, skin/hair, faeces and presence or absence of vomit.

Overview of the Chemical Compatibility Cat Study

A tabulated overview of the cat study is shown in Table 2. Three domestic shorthair cats of different age and the same gender were enrolled onto the study. The first spot-on formulation tested on SD 0 was ProMeris®, containing the solvents DMSO, gamma hexalactone and 1-methoxy-2-propyl-acetate. The second spot-on formulation tested on SD 9 was Droncit®, containing the solvent N-methyl-2-pyrrolidone. The spot-on formulations were applied on the cats´ necks at the base of the skull while the hair was divided with two fingers until the skin was visible. The formulations were applied at therapeutic dose and in accordance with the manufacturers´ instructions.

Overview of the Chemical Compatibility Dog Study

A tabulated overview of the dog study is shown in Table 3. Three beagle dogs of different gender and the same age were enrolled onto the study. The

Table 4: Overview of Cat Part of the Seresto®/Advocate®/Profender® Clinical Safety Study

Study group	No. of cats	Study Day (SD)	Activity	Active ingredient	Volume of spot-on applied	Clinical assessment
1, 2	10	0	Application of Seresto® collar	Imidacloprid 10% / flumethrin 4.5 %	Not applicable	Performed
1	5	4	Application of Advocate® spot-on	Imidacloprid + moxidectin	0.4 ml for cats ≤ 4 kg 0.8 ml for cats > 4–8 kg	Performed
2	5	4	Application of Profender® spot-on	Emodepside + praziquantel	0.75 ml	Performed
1, 2	10	5,6,7	Not applicable	Not applicable	Not applicable	Performed

first spot-on formulation tested on SD 0 was ProMeris Duo® containing the solvents DMSO, gamma hexalactone and 1-methoxy-2-propyl-acetate. The second spot-on formulation tested on SD 9 was Advantix® spot on formulation, containing the solvent N-Methyl-2-pyrrolidone. ProMeris Duo® was applied at therapeutic dose and in accordance with the manufacturers´ instructions. Advantix® was also applied at therapeutic dose, but in order to mimic a worst case scenario the whole volume was applied on one spot between the shoulder blades while the hair was divided between two fingers until the skin was visible. This is contrast to label instructions that the volume should be applied to four spots between the dorsal shoulder blades and the base of the tail.

Study reference C: Seresto®/Advocate®/Profender® study: Small Non-Controlled Clinical Safety Study

This small non-controlled clinical safety study was designed to give preliminary information on dermal and systemic safety of concomitant treatment. The spot-on formulations chosen for use with cats were an imidacloprid/moxidectin formulation (Advocate®) and an emodepside/praziquantel formulation (Profender®). The spot-on formulations chosen for use with dogs were an imidacloprid/moxidectin formulation (Advocate®) and an imidacloprid/permethrin formulation (Advantix®). In addition, dogs in group 2 were also given an oral emodepside/praziquantel tablet (Profender®) at the same time as the spot-on formulation Advantix® was administered.

A total of 16 animals were used, 10 cats and 6 dogs. Additional inclusion criteria for these studies were as follows: (1) no treatments with any of the Investigational veterinary products (IVPs) for at least 30 days before study start and (2) no recent treatments with other drugs which could interfere with the evaluation of IVPs.

The studies were randomised but not controlled. Animals were randomised to study groups based on body weight on SD 0.

The study groups comprised two groups containing three dogs each and two groups containing five cats each. All animals were fitted with collars on SD 0 and concomitant treatments were given on SD 4. The formulations were applied at therapeutic dose and in accordance with the manufacturers´ instructions.

Clinical assessments were performed on SDs 0, 4, 5, 6 and 7 comprising assessments of attitude, behaviour, nutritional status, respiratory system, gastrointestinal system, cardiovascular system and application site. All animals were weighed on SD 0 and abbreviated general health observations were performed daily throughout the study, in which the following were assessed: eyes, respiration, behavioural attitude, locomotion/ musculature, skin/hair-coat, faeces and presence or absence of vomit.

Table 5: Overview of Dog Part of the *Seresto®/Advocate®/Advantix®/Profender®* Clinical Safety Study

Study group	No. of dogs	Study Day (SD)	Activity	Active ingredients	Volume of spot-on applied /No. of tablets given	Clinical assessment
1, 2	6	0	Application of Seresto® collar	Imidacloprid 10 % / flumethrin 4.5 %	Not applicable	Performed
1	3	4	Application of Advocate® spot-on	Imidacloprid + moxidectin	1.0 ml for dogs >4–10 kg	Performed
					2.5 ml for dogs >10–25 kg	
2	3	4	Application of Advantix® spot-on	Imidacloprid + permethrin	1.0 ml for dogs >4–10 kg	Performed
					2.5 ml for dogs >10–25 kg	
			Administration of Profender® tablets for medium sized dogs	Emodepside + praziquantel	1 tablet for dogs >6–10 kg	Not applicable
					1.5 tablets for dogs >10–15 kg	
1, 2	6	5,6,7	Not applicable	Not applicable	Not applicable	Performed

Overview of Cat Part of the Seresto®/Advocate®/Profender® Clinical Safety Study

A total of ten male cats of roughly the same age were included on the study and randomised into two groups each containing five cats. A tabulated overview of this study is given in Table 4.

Overview of Dog Part of the Seresto®/Advocate®/Advantix®/Profender® Clinical Safety Study

Six dogs (five males and 1 female) of different ages were enrolled onto the study**.** A tabulated overview of this study is given in Table 5.

Study reference D: Large Controlled Clinical Safety Studies on the concomitant use of the Seresto® collar and Advocate® spot-on in cats and dogs

Two studies were designed to investigate dermal and systemic safety of concomitant treatment with the imidacloprid/flumethrin (Seresto®) collar, imidacloprid/moxidectin (Advocate®) spot-on formulation in dogs, and cats respectively. Safety was assessed by once daily clinical assessments for 14 days and by once weekly blood specimen collection (on SDs 7 and 14) for analyses. Pre-treatment hematological and clinical chemistry blood parameters were compared with post treatment values as determined on SDs 7 and 14 and with those of the non-treated control group.

Statistical analysis of hematological and clinical chemistry was performed on the treated and control group. In addition, the collars were observed daily by qualified personnel and weekly by the investigator or co-investigator to check the appearance, integrity and fit of the collar.

Additional inclusion criteria for both studies were as follows: (1) No treatment with and of the IVP for at least 30 days prior to study start; (2) Clinically healthy based on the results of hematology and clinical chemistry analyses of blood samples taken on SDs -2 or 1.

Due to the large number of animals involved, it was not possible to include all animals at the same time and so the Seresto®/Advocate® studies were conducted in replicates.

Approximately two thirds of the animals were allocated to the treatment group (group 1) and one third of the animals were allocated to the negative control group (group 2). The dog study

Table 6: Study Design – Seresto®/Advocate® clinical safety studies

No. of animals in the treatment group (no. included in statistical analysis)	51 (51)	50 (49)
No. of animals in the control group (no. included in statistical analysis)	26 (21)	24 (19)
Clinical assessments	SD 0: pre-treatment, 2h + 4h post treatment Once daily from SD 1 to SD 14	
Blood sampling	SD -2(-1), SD 7, SD 14	SD -2, SD 7, SD 14

was conducted in seven replicates using a total of 77 Beagle dogs, 31 male and 46 female. The dogs were between 9 months and 3.8 years in age and with a body weight range of 6.1 to 13.6 kg. The number of dogs per study replicate ranged from 6 to 17 animals. . The cat study was conducted in six replicates using a total of 74 European shorthair cats, 37 male and 37 female. The cats were between 1.4 and 3.9 years of age and with body weights ranging from 2.6 to 6.6 kg. The number of cats per study replicate ranged from 8 to 16 animals. Table 6 gives an overview of study design, including the number of animals participating in the study and used for analysis.

Administration of collar and spot-on

On SD 0, treatment group animals were fitted with collars and dosed with spot-on formulation in accordance with the manufacturers' recommended dose and label instructions. Directly after collar application the topical treatment with the spot-on formulation was performed at therapeutic dose in accordance with manufacturers' instructions. For dogs, the spot-on was applied to one spot between the shoulder blades, for cats, the spot-on was applied to one spot at the base of the skull. Negative control group animals were not treated with either collars or spot-on formulations.

Clinical observations

A complete physical examination was performed on all animals before treatment (SD -2). Observations on the systemic and local tolerance of the treatments were performed on SD 0 pre-treatment, two and four hours after treatment and subsequently once daily until study end (SD 14).

These observations included eyes, respiration, systemic signs of irritation or allergy, salivation, vomitus, diarrhea, behavioural changes, CNS system and locomotion system. The application sites were particularly examined for changes of the hair coat and skin, and the collars were checked for their appearance, intactness and placement. If an animal lost its collar during the study and the collar was still intact then it was reapplied; otherwise a new collar was applied.

Sampling

Blood samples for hematology and clinical chemistry were taken from all cats and dogs in both the treated and control groups.

To determine baseline values, samples were taken before treatment (SD -2 in the dog study, SDs -2 or -1 in the cat study). After treatment, sampling was performed on SDs 7 and 14. The samples were collected from the *Vena (V.) jugularis* or *V. cephalica antebrachii*, using a commercially available blood collection system. Tubes for hematology contained EDTA as anticoagulant while for clinical chemistry lithium heparin tubes were used. Samples for clinical chemistry were centrifuged at approximately 4°C and 4000 g for ten minutes. The supernatants were transferred to pre-labeled micro test tubes.

Samples for hematology were analysed with an ADVIA 120 Hematology System (Siemens Healthcare Diagnostics, Deerfield, IL, USA) in accordance with the manufacturers´ instructions. The following parameters were determined: red and white blood cell count, differential cell count, reticulocytes (RETIC), hemoglobin, packed cell volume and platelets. Clinical chemistry parameters were determined with an IDEXX VetTest 8008 Chemistry Analyser (IDEXX Laboratories, Inc., Westbrook,

ME, USA) in accordance with the manufacturers´ instructions. The following parameters were determined: albumin (ALB), alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), creatinine (CREA), gamma-glutamyl-transferase (GGT), globulin (GLOB) and total protein (TP).

Statistical analysis

Descriptive statistics (number of valid cases, arithmetic mean, median, standard deviation, quartiles, minimum and maximum) of all hematology and clinical chemistry values were performed separately for the treatment and control group in both studies. Possible changes over time were evaluated by comparing SD 7 and SD 14 values to the baseline values determined on SDs -2 or -1.

For relative parameters (like %LYMP) the median of change from baseline was used and for all other parameters (units other than %) the median of the percentage change from baseline was used. A more than 10 % change was regarded as potentially relevant. To compare the treatment versus the control group on the different assessment days, the non-parametric Wilcoxon-Mann-Whitney-U-Test (two-sided, alpha=0.05) was used. The analysis was performed using the software Testimate Version 6.5 and Report version 6.6 from IDV Gauting.

It was recognised that single outlying cases might be obscured in a statistical approach. Therefore all individual values that were outside the reference range were additionally carefully checked manually.

Study reference E: Controlled Clinical Safety Study on the concomitant use of the Seresto® collar and Profender® spot-on in cats

This study was designed to investigate dermal and systemic safety of concomitant treatment with the imidacloprid/flumethrin (Seresto®) collar and an emodepside/praziquantel (Profender®) spot-on formulation in cats. For the Seresto®/Profender® study 20 cats included were ranked within sex in descending order of individual body weights (SD -3) and subsequently blocked into ten blocks of two cats each. Since sex distribution was not in multiples of two, the last block of each sex contained only one cat. The female cat at the end of the list of females was allocated to the open slot in the last block of males and followed the assignment for that block. This block thus was a mixed gender block, whereas all other blocks were single gender blocks. Animal IDs were used to break ties. Within blocks, cats were randomly allocated to the two groups using random numbers generated in Microsoft Excel, and sorting these numbers in ascending order. Group 1 (untreated control) consisted of 6 females and 4 males and group 2 (treated) consisted of 7 females and 3 males. The cats were between 15 months and 6.2 years in age and with a body weight range of 3.1 to 5.2 kg. Additional inclusion criteria for the study was as follows: (1) No treatment with and of the IVPs for at least 30 days prior to study start and (2) clinically healthy based on the results of hematology and clinical chemistry analyses of blood samples taken prior to treatment. Table 7 gives an overview of study design, including the number of animals participating in the study and used for analysis.

Table 7: Study Design – Seresto®/Advocate® studies

Variable	Cat Study
No. of animals in the treatment group (no. included in statistical analysis)	10 (10 up to SD 23)*
No. of animals in the control group (no. included in statistical analysis)	10 (10)

* One animal in the Seresto® group was removed on SD 24 whilst the study ended SD 28. Blood analysis, clinical examination and body weight results for SD 28 thus only available for 19 animals.

Administration of collar and spot-on

On SD 0, treatment group animals were fitted with collars and dosed with the Profender® spot-on formulation in accordance with the manufacturers´ recommended dose and label instructions.

Directly after collar application, the topical treatment with the spot-on formulation was performed at therapeutic dose in accordance with

manufacturers´ instructions. The spot-on was applied to one spot at the base of the skull. Negative control group animals were not treated with either collars or spot-on formulations. Collars were observed daily by qualified personnel, investigator or co-investigator to check the appearance, integrity and fit of the collar.

Clinical observations

A complete physical examination was performed on all animals before treatment (SD -14 and SD -3). Observations on the systemic and local tolerance of the treatments were performed on SD 0 pre-treatment, 1, 2, 3 and 4 hours after treatment and twice (morning and afternoon) on SD 1 and SD 2. Daily health observations were performed once daily throughout the study. Observations were similar to that already discussed previously for the Seresto®/ Advocate® study.

Sampling

Blood specimen samples for hematology and clinical chemistry were collected from all cats in both the treated and control groups. To determine baseline values, samples were taken before treatment (SDs -14, -3 and 0). After treatment, sampling was performed on SDs 3, 14 and 28. The samples were collected from the *Vena (V.) jugularis* or *V. cephalica antebrachii*, using a commercially available blood collection system. Blood specimens were collected in collection tubes for clinical chemistry on SDs 14, -3, 0, 3, 14 and on SD 28. Yellow top (SST) collection tubes were used for serum analysis. Grey top (fluoride) collection tubes were used for glucose determination. The serum specimens were centrifuged at 3 000 rpm for 10 minutes and all specimens submitted to the laboratory test site. Parameters measured were total serum protein (TSP) g/L, albumin g/L, globulin g/L, urea mmol/L, creatinine µmol/L, ALP (alkaline phosphatase) units/L, AST (aspartate aminotransferase) units/L, ALT (alanine aminotransferase) units/L, total bilirubin µmol/L, random glucose mmol/L, calcium (total) mmol/L, sodium mmol/L, chloride mmol/L, potassium mmol/L, phosphorus (SIP) mmol/L, magnesium (mmol/L), CK (creatinine kinase) U/L, amylase U/L, LDH (lactic dehydrogenase) U/L, GGT (gamma glutamyltransferase) U/L and random cholesterol mmol/L.

EDTA (purple top) and citrate (blue top) collection tubes were used to collect blood specimens for haematology on SDs 14, -3, 0, 3, 14 and on SD 28. The specimens were submitted to the laboratory test site.

The parameters measured were: full blood count (includes red cell count, haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total white cell count and differential white cell count), platelets, prothrombin time (PT) and activated partial thromboplastin time (aPTT). A blood smear was stained and evaluated microscopically for abnormal cell morphology, and quantitative estimate of platelet numbers. All slides were microscopically reviewed. When platelet clumping was observed, the laboratory staff commented on the severity of platelet aggregation as set out below.

Platelet clumping was reported as:

1+ Scanty platelet clumping was observed (very little impact) increased analyser value by 20

2+ Copious platelet clumping was observed (platelet clumping impacted actual count) increased analyser value by 100

3+ Proliferate platelet clumping was observed (platelet count was compromised and actual count should read within normal to high limits) increased analyser value by 250

The microscopic evaluation was conducted by a person familiar with the microscopic characteristics of normal cat blood smears.

Statistical analysis

The emphasis of the statistical analysis was on the change from baseline values in each of the

haematology and clinical chemistry parameters. The magnitude, and specifically the clinical relevance of such changes, were evaluated and interpreted descriptively from a clinical point of view. The following outlined the statistical approach:

- The individual haematology and clinical chemistry values on all test days were tabulated separately for each variable and each group, together with the following descriptive statistics: mean, standard deviation (StDev), coefficient of variation (CV%), geometric mean, geometric StDev, median, minimum, maximum and the number (N) observations in that group.

- The individual and mean changes and percentage changes from baseline (preadministration values on SD 3 to SD 0) and each of the post-administration days were calculated for each of the two groups. The mean changes from baseline of the groups were compared descriptively.

- The number of SD 0 and post-administration values that fell outside the reference range for a specific laboratory parameter were calculated and listed in tables. The reference range for each laboratory parameter was calculated as the minimum and maximum values at baseline (SD -3) across all groups.

- The SD 0 and post-administration values were compared to the baseline values in a within-administration comparison by means of an ANOVA with an animal and observation time (baseline, SD 0 and post-administration) as effects. Since the aim of the analysis was to statistically evaluate the significance of changes in parameters from baseline in conjunction with relevant clinical changes, a change from baseline that was statistically not significant ($p > 0.05$), did not necessarily indicate that the difference was not clinically relevant. Similarly, a statistical significant change from baseline was not necessarily interpreted as a clinically relevant finding, but was rather considered a finding that necessitated a careful review from a clinical point of view.

- A between-administration comparison (group 2 vs group 1) with respect to the changes from baseline, was also performed by means of an ANOVA with an administration effect. The resulting p-values were interpreted carefully as outlined above.

Approach to the interpretation of statistical results

The strategy outlined below was followed to point out potential differences which would warrant further clinical review:

1. If there were any values on SD 0 or any specific post-administration day in at least one animal in a particular test group, that were outside the reference range, and

2. If the difference in the mean values for that particular parameter between the specific test group and the corresponding control group was statistically significant from the betweengroup comparison, and

3. If the within-group comparison for that particular test group and parameter from baseline to that specific SD 0 or postadministration day was statistical significant, that case was highlighted for further review.

The reason behind condition (1) above was, that a change from baseline which remained within the reference ranges was not of concern, the reason behind condition (2) above was, that the difference in the change from baseline between a test and corresponding control group was of concern, but only if this difference was due to a significant change from baseline in the test group, and not in the control group, as specified by condition (3).

Results

Study reference A: Chemical Compatibility: *In-Vitro* Testing of 13 Solvents

The results are shown in Table 8. The collar was not affected by 8 of the 13 solvents used. Four of the five solvents that affected the collar *in-vitro* were further subjected to *in vivo* testing to find out if they compromised collar use under normal conditions. One solvent (THFA) is not currently used in spot-on formulations and was therefore not tested further.

Study reference B: Chemical Compatibility: Clinical *in–vivo* Study with final formulation

Chemical Compatibility of Collars

There were no changes in texture, form or consistency in any of the collars in either of the studies at any of the timepoints. One collar in the cat study was slightly oily at the 2 hour timepoint after ProMeris® was applied but the oiliness had disappeared by the 24 hour inspection timepoint. In the dog study, the permanent marker was blurred at the 2 hour timepoint in the collar of one dog after ProMeris® was applied. The second collar from the same dog was slightly oily at the 2 hour timepoint after Advantix® was applied.

Clinical Observations and Results

There were no adverse events regarding general health in any of the animals.

Cat study: After treatment with both the metaflumizon (ProMeris®) and praziquantel (Droncit®) spot-on formulations, all cats showed slight to moderate oily hair coat changes at the application site at the 2 hour observation time point. With ProMeris®, a slight to moderate malodour was also noticed at the 2 hour observation time point, and in two cats the hair was still oily 24 hours after product application. With Droncit®, all three cats had very slight to slightly white crystals on the hair tips 24 hours after application.

Dog study: After treatment with the metaflumizon/amitraz (Pro Meris® Duo) formulation at the 2 hour observation point, all three dogs showed moderately oily hair with moderate malodour at the application site. In one dog additionally a moderate amount of whitish crystals at the hair tips was noticed.

At the 24 hour observation point, the hair of two dogs was still very slightly oily and in one dog the amount of crystals was unchanged, slight malodour was still noticed in all dogs. The ProMeris® Duo formulation is in the meantime not marketed any longer in most countries, however, as a formulation using comparable excipients may be marketed again in the future, it was deemed still interesting to report the results.

After treatment with the imidacloprid/permethrin (Advantix®) formulation at the 2 hour observation point, all three dogs showed slightly oily hair and two dogs had a slight amount of crystals at the hair tips. At the 24 hour observation point, very slight to slightly white crystals on the hair tips and slight oily hair could be observed at the application site in two dogs.

Study reference C: Small Non-Controlled Clinical Safety Study

No clinically relevant adverse event related to dermal or systemic safety occurred and no abnormalities concerning general health were noticed during the course of the study. Clinical observations were limited to changes in the hair coat and are given in details below.

Clinical Observations: Cats

Small to medium sized areas of broken hairs under the collar were observed in four out of the ten cats. In one cat broken hairs underneath the collar were observed before concomitant treatment on SD 4. During the observation period from SD 4 to SD 7, three more cats developed broken hairs under the collar that were small to medium in size. These changes remained unchanged during the study and were considered to be treatment

Table 8: Effects of Solvents on Collar (*in-vitro* results)

Solvent (Common acronym)	Results after 15 minutes	Results after 24 hours
Benzyl alcohol (BA)	Unchanged	Unchanged
Propylene carbonate (PC)	Unchanged	Unchanged
BA/PC (80:20)	Unchanged	Unchanged
N-Methyl-pyrrolidone (NMP)*	Collar began to dissolve	Collar completely dissolved
Solketal (Isopropylidenglycerin)	Unchanged	Unchanged
Diethylene glycol mono ethyl ether	Unchanged	Unchanged
Isopropyl alcohol	Unchanged	Unchanged
Ethyl alcohol	Unchanged	Unchanged
Dimethylsulfoxide (DMSO)*	Unchanged	Collar swelled up, maceration occurred
Gamma-hexalactone*	Surface changed (corroded)	Collar began to dissolve
1-Methoxy-2-propyl-acetate*	Unchanged	Collar maybe swelled up
Dipropylene glycol mono methyl ether	Unchanged	Unchanged
Tetrahydro furfuryl alcohol (THFA) **	Unchanged	Collar deformed, surface changed

** These solvents were further tested in-vivo*
** THFA was not tested further as it is not present in commercially available spot-on formulations

related to the collar due to mechanical irritation. All cats displayed temporary local changes in hair coat at the application site 2 hours post-treatment with the spot-on formulations, comprising medium to severe oily hair/skin in all cats. A few crystals were observed in two cats at the application site on SD 5 and 6. The oiliness of the skin/hair had significantly reduced on SD 5 and had completely disappeared by SD 7. The white crystals also disappeared by SD 7. These changes were considered to be treatment related to the spot-on formulation.

Clinical Observations: Dogs

Five of the six dogs showed local changes of oily hair and crystals in the hair coat at the application site after treatment with the spot-on formulations, but these changes had completely disappeared by SD 7. These changes were considered to be treatment related to the spot-on formulation.

Study reference D: Controlled safety studies – Seresto®/ Advocate®

Clinical observations

Dog study: All dogs tolerated the treatment well. Two out of 51 treated dogs showed transient skin alterations in the region of collar application, namely a crusty spot (approx. 1 cm diameter) on one day, respectively a moderate erythema of small (<1 cm) to medium size (1–4cm) for about two days. Further findings in the treated group that were considered unlikely to be treatment related were conjunctivitis and a congested nasolacrimal duct in one dog, and a hot spot in the tail region of the same dog that showed erythema in the collar region. In the control group one dog also showed wounds and crusts in the neck region. No further clinical signs were present.

Cat study: Four cats showed slight behavioural changes after collar application consisting of licking at the collar and in one cat depression for one day. The local tolerance assessment showed transient skin alterations in the region of collar applications

Table 9: Summary of Parameters with a >10 % change from Baseline and Values outside the Reference Range in the Dog Study

Parameter	Study Day (SD)	Study group (C)ontrol (T)reatment	Median percentage change from baseline	Minimum-maximum values	No. of animals out of reference range above/below	
					on study day (SD)	at baseline
ALT 10–100 U/L	14	C	+22.73 %	10–126 U/L	1/0	1/0
	One dog with a value above the RR (reference range) was already high at baseline.					
AST 0–50 U/L	7	T	-12.82 %	13–111 U/L	18/n.a.	19/n.a
		C	-23.81 %	11–117 U/L	6/n.a	8/n.a
	Ten of 18 treated dogs with values above the RR were already outside the RR at baseline. The control group showed a greater change on the same day. Two of six control dogs with values above the RR were already high at baseline.					
GGT 0–7 U/L	7	T	-16.67 %	0–3 U/L	0/n.a.	0/n.a.
	14	T	-41.67 %	0–3 U/L	0/n.a.	0/n.a.
		C	-75.00 %	0–5 U/L	0/n.a.	0/n.a.
	Highly variable values were seen in both study groups. As none of the dogs showed values outside the RR the median percentage change is of no clinical relevance.					
#MONO 0.2–1.1 x 10^3 /µL	7	T	+11.11 %	0.26–1.28 x 10^3 /µL	1/0	0/0
	14	C	-15.09 %	0.19–0.74 x 10^3 /µL	0/1	0/0
	Deviations from RR were only slight in both dogs and of no clinical relevance. Both dogs showed normal values on the other evaluation dates.					
#BASO 0–0.1 x 10^3 /µL	7	C	-12.50 %	0.01–0.17 x 10^3 /µL	5/n.a.	3/n.a.
	14	T	-12.50 %	0.01–0.19 x 10^3 /µL	8/n.a.	11/n.a.
		C	+20.00 %	0.02–0.20 x 10^3 /µL	5/n.a.	3/n.a.
	Six of eight treated dogs with elevated values on SD 14 were already above the RR at baseline including the dog with the highest value (190/µl). This dog was within the RR on SD 14.					
#RETIC 8.4–129.3 x 10^9/µL	**7**	**C**	**-13.27 %**	18.2–112.9 x 10^9/µL	0/0	1/0
	14	T	-14.40 %	13.3–162.1 x 10^9/µL	1/0	3/0
	Two of three treated dogs with increased values at baseline were within the RR on SD 14.					
#LYMPH 1.3–4.1 x 10^3/µL	**7**	**C**	**+18.62 %**	**1.92–5.43 x 10^3/µL**	0/1	0/1
	One dog with a value above the RR was already high at baseline. On SD 14 the dog was within the RR.					
#EOS 0–0.6 x 10^3/µL	7	C	20.00 %	0.09–0.81 x 10^3/µL	5/n.a.	4/n.a.
	Four of five dogs had values outside the RR already at baseline.					
#LUC 0–0.3 x 10^3/µL	**14**	**C**	**20.00 %**	0.01–0.14 x 10^3/µL	0/n.a.	0/n.a.
	Value variations observed were within RR.					

5 out of 50 treated cats, namely slight to moderate erythema of small (< 1cm) to medium (1–4 cm) size, partially with exudation and crusts, which in one cat was topically treated for three days. Also in 18 of the 50 treated cats collar wearing was visible in the form of broken hair and/or transient alopecia of varying degree. One cat of the treated group had to be excluded from the study on SD 7 due to a painful hip probably related to trauma. No clinical signs were present in the control group.

Table 10: Summary of Statistically Significant Parameters from Treatment Versus Control Comparison in Dog Study

Parameter	Study Day (SD)	Statistical significance	No. of animals out of reference range (above/below)	
			Treatment group	Control group
%RETIC	-2	p=0.0277 higher values in control group	2/0	1/0
	Clinically not relevant. Significant difference at baseline before treatment.			
#NEUT	7	p=0.0216 higher values in treatment group	7/1	2/2
	Slight reduction as well as slight elevation was seen in both study groups, with no clear pattern that could be related to treatment. Six of eight treated dogs with values outside the RR on SD 7 had normal values on SD 14. One treated dog had a high value already at baseline, but constantly decreasing to almost RR on SD 14 (8960/µl). The second dog still showed a slight elevated value on SD 14 (8240/µl).			
#RETIC	7	p=0.0107 higher values in treatment group	2/0	0/0
	Both treated dogs had elevated values already at baseline, constantly decreasing over time. Increase as well as decrease within the RR was present after treatment in both study groups.			
#EOS	7	p=0.0066 higher values in control group	5/n.a.	7/n.a.
	14	p=0.0463 higher values in control group	4/n.a.	5/n.a.
	Four of seven treated dogs with elevated values after treatment were already outside the RR at baseline. Elevations in the other dogs were only slight (maximum 810/µl). In the control group values up to 2220/µl were detected.			
#BASO	14	p=0.0244 higher values in control group	8/n.a.	5/n.a.
	Six of eight treated dogs with elevated values on SD 14 were already outside the RR at baseline. Maximum value in the other two was 120/µl. Values up to 200/µl were detected in the control group.			

In both studies most animals showed cosmetic changes at the site of spot-on application. These were an oily appearance and sometimes crystallization at the tip of the hairs. The changes occurred for a maximum of two days after spot-on treatment and were fully reversible.

Collar observations

The treated group of 51 dogs were fitted with collars and all collars were found to fit correctly, the integrity of the collars remained intact and none of the collars had to be replaced during the study.
The treated group of 50 cats were fitted with collars and collar observations revealed that 33 collars had chewed ends and 5 collars were chewed all over. The integrity of the collar remained nevertheless intact.

Blood hematology and clinical chemistry

No clinically relevant changes were detected for any parameter in the dog or cat study.
All individual hematology and clinical chemistry values determined on SD 7 and SD 14 were statistically evaluated for the change from baseline values on SD -2. More than a 10 % change from baseline was regarded as potentially relevant. All clinical chemistry parameters and some hematology parameters were first measured as numerical values which were then converted into percentages to allow this cut off point to be applied. Increase as well as decrease within the reference range (RR) was found in all parameters in both, treated and control groups on SDs 7 and 14.
The comparison of the treatment versus the control group on the different assessment days with the

non-parametric Wilcoxon-Mann-Whitney-U-Test (two-sided, alpha=0.05) showed statistical significant but clinically non relevant changes.
The significance of individual values can be obscured by statistics, so in addition to all the statistical group work every individual value for each dog and cat was also carefully checked and no clinically relevant changes were detected.
Dog study: Of the relative parameters assessed for hematology, median changes from baseline were less than 10 % in both treated and control groups of dogs for hematocrit (HCT) and percentages of: neutrophil granulocytes, lymphocytes, monocytes, eosinophil granulocytes, basophil granulocytes, leukocytes and reticulocytes.
For clinical chemistry, the medians of the percentage changes from baseline were less than 10 % in both treated and control groups of dogs for albumin (ALB), creatinine (CREA), globulin (GLOB) and total protein (TP).
Parameters with a greater than 10 % change from baseline and values outside the reference range are shown in Table 9. As can be seen from the comments in the table, none of these were considered to be clinically relevant. The comparison of the treatment versus control group on the different assessment days with the non-parametric Wilcoxon-Mann-Whitney-U-Test results showed statistically significant changes for only 5 values, all of which were determined as being not clinically relevant for the reasons shown in Table 10 below.

Cat study: Of the relative parameters assessed for hematology, median changes from baseline were less than 10 % in both treated and control groups of cats for hematocrit (HCT) and percentages of: neutrophil granulocytes, lymphocytes, monocytes, eosinophil granulocytes, basophil granulocytes, leukocytes and reticulocytes.
For clinical chemistry, the medians of the percentage changes from baseline were less than 10 % for albumin (ALB), creatinine (CREA), globulin (GLOB) and total protein (TP).
Parameters with a greater than 10 % change from baseline and values outside the reference range are shown in Table 11. As can be seen from the comments in the table, none of these were considered to be clinically relevant.
The comparison of the treatment versus control group on the different assessment days with the non-parametric Wilcoxon-Mann-Whitney-U-Test results showed statistically significant changes for only 4 values, all of which were determined as being not clinically relevant for the reasons shown in Table 12.

Study reference E: Controlled safety studies – Seresto®/Profender®

Clinical observations

All cats tolerated the treatment well. Three out of ten cats in the untreated control group (group 1) presented with slight scaling during all *local tolerance observations* time points. All cats in the Seresto®/Profender® treated group 2 presented with cosmetic changes (spiking and white deposits on hair tips) recorded on the spot-on application site on SD 0 to SD 2. One cat had slight scaling recorded on SD -3 and one cat on SD 2. One cat had a white deposit on her skin on the spot-on application site recorded on SD 2. No observations were recorded that could be considered as a results of the Seresto collar. The cosmetic changes at the site of spot-on application were fully reversible.
With reference to *daily health observations*, one cat in the Seresto®/Profender® treated group 2 presented with abnormal clinical signs where a relation to treatment could not be ruled out. The cat developed moist dermatitis with superficial skin lesion on her dorsal neck. This was observed for the first time during the clinical examination on SD 14 and a relation to treatment could not be ruled out. As the condition did not improve, it was decided to remove the collar on SD 24 until the symptoms had disappeared. Twenty-five days afterwards, the skin lesion was healed completely without any additional treatment. All other post-treatment

Table 11: Summary of Parameters with a > 10 % Change from Baseline and Values outside the Reference Range in the Cat Study

Parameter	Study Day (SD)	Study group (C)ontrol (T)reatment	Median percentage change from baseline	Minimum-maximum values	No. of animals out of reference range above/below	
					on study day (SD)	at baseline
WBC 6.2–19.6 x 10^3 /µL	7	T	-18.63 %	5.5–17.16 x 10^3 /µL	0/2	2/1
	14	T	-19.89 %	4.04–17.01 x 10^3 /µL	0/7	
	Deviations from RR were only slight and the corresponding differential cell count of no clinical relevance for the individual parameters.					
#NEUT 3.0–13.4 x 10^3 /µL	7	T	-16.98 %	2.38–9.35 x 10^3 /µL	0/5	0/2
		C	-12.93 %	0.23–14.84 x 10^3 /µL	1/5	0/0
	14	T	-26.56 %	2.19–8.11 x 10^3 /µL	0/8	0/2
		C	-12.85 %	2.72–14.85 x 10^3 /µL	1/1	0/0
	One treated cat with values outside the RR, had a low value already at baseline.					
#LYMPH 2.0–7.2 x 10^3 /µL	7	T	-14.01 %	1.26–7.59 x 10^3 /µL	4/3	8/2
	14	T	-19.13 %	1.11–8.16 x 10^3 /µL	4/6	
		C	-14.58 %	1.74–12.98 x 10^3 /µL	5/1	4/0
	Seven treated and four control cats had values outside the RR already at baseline.					
#MONO 0–1.0 x 10^3 /µL	7	C	21.74 %	0.1–24.39 x 10^3 /µL	4/n.a.	0/n.a.
	14	T	-11.11 %	0.06–0.48 x 10^3 /µL	0/n.a.	0/n.a.
		C	-30.77 %	0.13–0.45 x 10^3 /µL	0/n.a.	0/n.a.
#EOS 0.3–1.7 x 10^3/µL	**7**	**T**	**-22.64 %**	**0.18–2.56 x 10^3 /µL**	4/4	8/5
		C	-31.01 %	0.0–1.88 x 10^3 /µL	1/9	2/3
	14	T	-22.64 %	0.06–2.21 x 10^3 /µL	1/8	8/5
	Four treated and three control cats had values outside the RR already at baseline.					
#BASO 0–0.1 x 10^3 /µL	7	C	-37.50 %	0.0–0.16 x 10^3 /µL	4/n.a.	3/n.a.
	14	C	-45.00 %	0.0–0.15 x 10^3 /µL	2/n.a.	
#RETIC 15.0–81.0 x 10^9/µL	7	C	40.91 %	11.9–74.1 x 10^9 /µL	0/2	0/5
	14	C	30.42 %	9.1–54.4 x 10^9 /µL	0/2	
	Two control cats had values outside the RR already at baseline.					

observations in the other animals were considered to be not test item related.

Collar observations

The treated group of 10 cats were fitted with collars and all collars were found to fit correctly, the integrity of the collars remained intact and none of the collars had to be replaced during the study.

Blood hematology and clinical chemistry

With reference to both, clinical chemistry and haematology, no clinically significant changes from baseline were seen during the study. One cat in Seresto®/Profender® treated group 2 showed slightly elevated values outside the reference ranges for ALT and AST on SD 14 and SD 28. However, no other related parameters showed any abnormalities and the cat was clinically healthy.

Table 12: Summary of Statistically Significant Parameters from Treatment Versus Control Comparison in Cat Study

GLOB	-2	p=0.0341 higher values in treatment group	0/0	0/0
	Clinically not relevant. Significant difference at baseline before treatment.			
%EOS	-2	p=0.0126 higher values in treatment group	8/6	2/6
	Clinically not relevant. Significant difference at baseline before treatment.			
WBC	7	p=0.0140 higher values in control group	0/2	3/1
	Increase as well as decrease within the RR was present after treatment in both study groups. Maximum values of up to 24870/µl were detected in the control group. Deviations in the treatment group were only slight and the corresponding differential cell count of no clinical relevance for the individual parameters.			
#RETIC	7	p=0.0166 higher values in control group	0/16	0/2
	14	p=0.0103 higher values in control group	0/17	0/2
	27 treated cats showed a value below the RR on at least one occasion after treatment. 12 of these were below the RR already at baseline. Lowest detected value was 6 x 10^9/µl. Erythrocyte counts, hematocrit and hemoglobin were in the normal to upper range in all affected cats.			

Between group comparison, based on the change from baseline (SD -3) by means of an ANOVA in haematology values at all post-treatment days, indicated significant differences in MCV (SDs 0 and 3), MCHC (SD 0), white cell count (SD 28), neutrophils Abs (SD 28) and eosinophils Abs (SDs 3, 14 and 28). The between group comparison based on the change from baseline (SD -3) by means of an ANOVA in clinical chemistry values at all post-treatment days, indicated significant differences in creatinine (SDs 14 and 28), amylase (SD 14) and glucose random blood (SDs 0 and 14).

The statistical analysis was designed to screen the results in order to point out differences between the test group (group 2) and the control group (group 1) that should be considered more closely from a clinical and safety point of view. The following strategy was followed to point out these differences for further review:

a If there were any values on either SDs 0, 3, 14, and 28 in at least one animal in a particular test group that were outside the reference range, and

b If the difference in the mean values for a particular parameter between the test group (group 2) and the control group (group 1) was statistically significant from the between-group comparison (from the ANOVA), and

c If the within-group comparison for the particular test group from SD -3 (baseline) to the post-administration day was statistical significant.

The reason behind condition (a) above is, that a change from baseline which remains within the reference ranges should not be of concern, the reason behind condition (b) above is, that the difference in the change from baseline between the test and the untreated group is of concern, but only if this difference is due to a significant change from baseline in the test group, and not in the untreated group, as specified by condition (c).

Based on these conditions (bullets a, b and c above), a number of cases were detected to comply with all three of them. Table 13 summarises the haematology and clinical chemistry parameters that showed a statistically significant change from baseline for five cats. All five cats were from group 2. Five of the eight end values were higher than the normal reference ranges and three were lower. None of these cats had abnormalities recorded from related parameters for the corresponding days and all cats

Table 13: Summary of Parameters with a Statistically Significant Change from Baseline and Values outside the Reference Range in the Seresto®/Profender® Cat Study

Parameter	Study Day (SD)	Study group (C) ontrol (T) reatment	Animal ID	Reference range	Base value	End value	Change from baseline
Creatinine-S (µmol/L)	14	T	CDB CDD	84–147 µmol/L	142 µmol/L	169 µmol/L	27 µmol/L
	28				142 µmol/L	155 µmol/L	13 µmol/L
Eosinophils Abs (x 10^9/L)	28	T	CC2 EE2	0.18–1.56 x 109/L	1.41 x 109/L	1.71 x 109/L	0.3 x 109/L
			DF8 811		0.87 x 109/L	0.06 x 109/L	-0.81 x 109/L
MCHC (g/dL)	0	T	DF8 811	32–34 g/dL	32 g/dL	31 g/dL	-1 g/dL
MCV (fL)	0	T	CD5 135	39–51 fL	51 fL	52 fL	1 fL
	3						
Neutrophils Abs (x 10^9/L)	28	T	DF6 952	2.66–18.87 fL	4.35 fL	2.40 fL	-1.95 fL

were clinically healthy. None of these changes had any clinical significance or influence on the health of the cats. Body weight and food consumption as measured during the study could be regarded as normal for clinically healthy adult cats and were not influenced by the use of the test items.

Discussion

Chemical Compatibility

The results from the *in-vitro* study (*study reference A*) showed that only certain solvents found in spot-on formulations could potentially damage the Seresto® collar. Under real-life conditions small cut-up sections of collar would never come into contact with such large amounts of solvent, but the study conditions were made deliberately extreme in order to identify potentially problematic solvents. The four spot-on solvents identified as potentially damaging were then tested further in the chemical compatibility clinical studies (*study references B and C*), under real, yet stringent conditions, with the spot-on formulations being applied directly after collar fitting without any time interval between. No visible or palpable changes occurred, and the integrity of the collars was uncompromised. In the controlled Seresto®/Advocate® safety studies (*study reference D* Seresto®/Advocate® studies) the collars of the 51 treated dogs and the 50 treated cats were observed daily by an animal caretaker or lab technician and weekly by the investigator or co-investigator, and no chemical compatibility issues arose. The same results were obtained after daily observation of 10 treated cats in the Seresto®/Profender® safety study (*study reference E* Seresto®/Profender® study).

Dermal Safety

There were no clinically significant dermal safety findings in the chemical compatibility clinical studies (*study reference B*). The slight to moderate oily hair, malodour and slightly white crystals, that were observed at the 2 and 24 hour timepoints, are symptomatic of spot-on formulations, were of no clinical significance and had no impact on the dermal health of the animals.

There were no clinically significant dermal findings in the non-controlled safety studies (*study reference C Seresto®/Advocate®/Profender® study*). In these studies the animals were assessed for 7 days following collar and spot-on application and by day 7 the oily hair and crystals observed in most of the animals had disappeared. The oily hair and crystals were obviously transient in nature and corrected themselves within a few days of spot-on

application. Such cosmetic changes at the spot-on application site are well described for this product class and are of low clinical significance. The alterations are transient in nature and unavoidable with this kind of formulation.

In the large controlled Seresto®/Advocate® safety studies (*study reference D* Seresto®/Advocate® studies) only 2 out of 51 treated dogs, and also 5 out of 50 treated cats, showed skin alterations in the region of the collar that could have been either concomitant treatment related or a result of mechanical irritation. In the Seresto®/Profender® safety study (*study reference E* Seresto®/Profender® study) there was only one cat that presented with abnormal clinical signs (moist dermatitis with superficial skin lesion on her dorsal neck) where a relation to treatment could not be ruled out. Additionally in the large controlled Seresto®/Advocate® safety studies (*study reference D* Seresto®/Advocate® studies) broken hair and/or transient alopecia was visible in 18 cats. Experiences from previous studies had shown hair or skin alterations to occur after collar application, more frequently in cats than in dogs. They were also more frequently observed in the situation of laboratory studies with the necessity of single housing, indicating the possible impact of increased occupation with the collar in those specific housing conditions. The number of hair alterations at application site seen in this laboratory study exceeded those seen in a large multicentre field study conducted for the approval of the Seresto® collar, in which changes in the skin around and under the collar were observed to the same degree between cats wearing the product collar and cats wearing a placebo collar (Stanneck et al. 2012). The study was negatively controlled, so changes around the collar area did not arise in the control group, but the uneven distribution of affected cats over study replicates made it clear that in cases where alterations occurred some level of manipulation of the collar by the affected cats was involved. Many of the collar ends were chewed and some cats lost their collars, while only in five cats with skin/hair alterations the collars appeared untouched. We assume, that the local reactions to the collar strongly depend on the individual character of the cat and the social interaction and playing behaviour expressed under the respective housing conditions. Intense interaction with tearing and yanking of the collar obviously increases the risk of hair coat and skin changes. Although the cat study presented here was rather short, improvement was already seen in some animals towards study end, in all but one case without additional treatment or collar removal. Dermal safety findings over all clinical studies (*study references B to E*) matched those already described for the respective products, there were no indications of these findings aggravating under the conditions of concurrent use.

Systemic Safety

The behavioural changes seen in cats at the beginning of the *study reference D* Seresto®/Advocate® studies (licking at the collar and transient depression in one cat) are of low clinical significance. Similar observations have been made in previous trials and reflect the response of this mentally sensitive species to an unknown and new situation.

There were no systemic safety findings of clinical significance in either the non-controlled (*study reference C* Seresto®/Advocate®/Profender® study) or the controlled safety studies (*study reference D* Seresto®/Advocate® studies). In the controlled safety Seresto®/Advocate® studies (*study reference D* Seresto®/Advocate® studies) both formulations contained imidacloprid as an active ingredient, however it was not expected that the simultaneous treatment would lead to accumulative problems and no systemic clinical effects were seen in the studies presented here. The high dermal safety of imidacloprid and the fact that the active is released steadily in low amounts from the collar without peak concentrations makes it very unlikely that a buildup of drug would occur with concomitant treatment. In the controlled safety Seresto®/Profender® study (*study reference E*) the formulations contained different active ingredients with no known contra-indications. As a result no adverse effects were anticipated.

The assessment of a range of blood parameters revealed some alterations during the study course, in dogs as well as in cats. Deviations from baseline levels and from the reference range were present, some with statistical significant difference in the group comparison, but no clinical relevance could be deduced from these results. Hematology and clinical chemistry results of the current study confirmed the safety of the concomitant treatment.

Conclusions

The answers to the three questions posed are as follows. Firstly, the imidacloprid/flumethrin Seresto® collar is chemically robust enough to withstand direct contact with any spot-on formulation containing any of the solvents tested. Secondly, there are no dermal safety implications for adult cats and dogs when the collar is used concomitantly with the spot-on formulations tested, with specific reference to Advocate® and Profender®. Thirdly, there are neither dermal nor systemic safety issues with the particular combination of the three actives imidacloprid, flumethrin and moxidectin in adult cats and dogs, or imidacloprid, flumethrin, emodepside and praziquantel in adult cats. The Seresto® collar is safe to use concomitantly with Advocate® and Profender® spot-on formulations, when used according to labeling instructions.

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Conflict of interests

The studies reported here were funded by Bayer Animal Health GmbH, of whom Eva Maria Krüdewagen, Carolin Remer, Katrin Deuster, Bettina Schunack and Dorothee Stanneck are employees. The other authors are employees of ClinVet International, an independent Contract Development Organisation, contracted to manage the conduct of one of the studies reported. Sonja Wolken and Dionne Crafford contributed to the preparation of this manuscript. All authors voluntarily publish this article and have no personal interest in this study other than publishing the scientific findings that they have been involved in via planning, setting-up, conducting and compiling and analysing the results.

Disclaimer

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